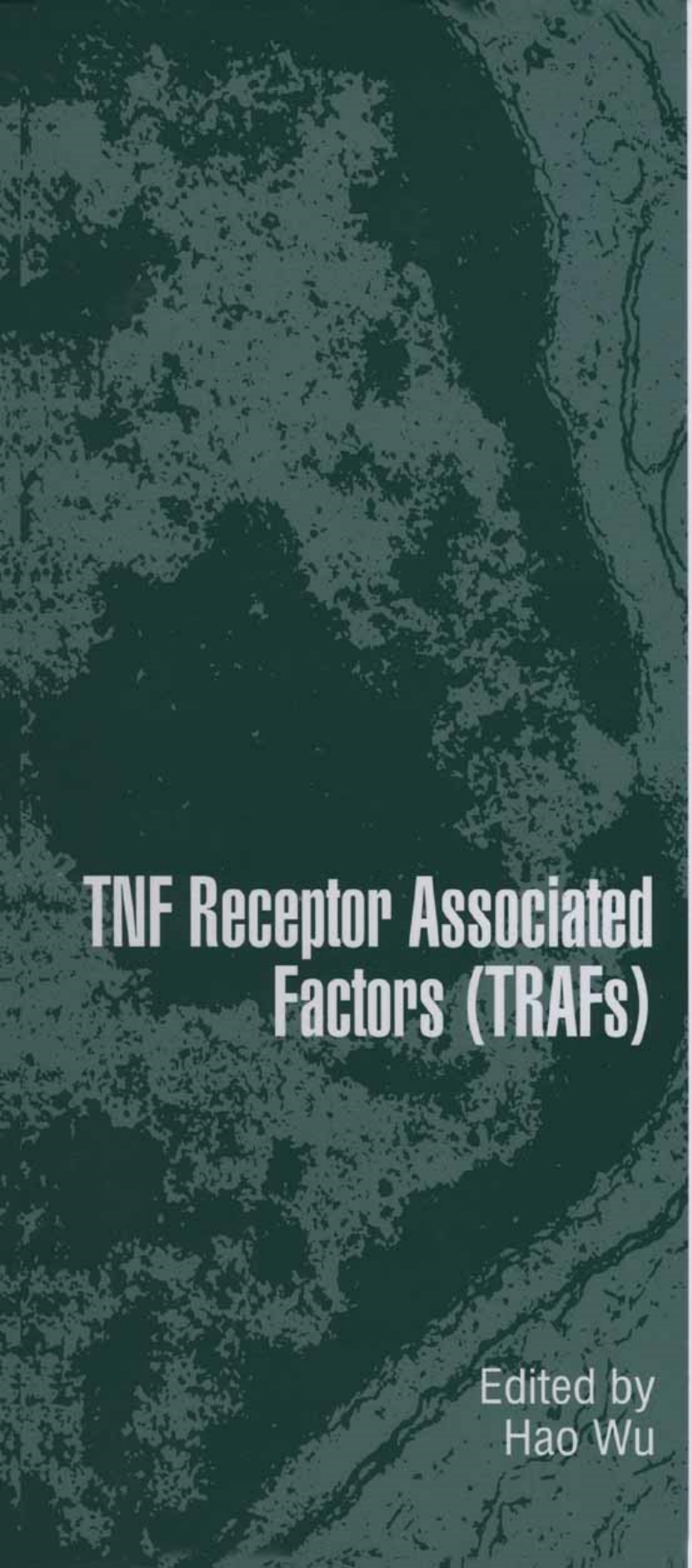

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 597

TNF Receptor Associated Factors (TRAFs)

Edited by
Hao Wu

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FOREWORD

The discovery of the TRAF molecules in the early nineties as TNF-receptor associated factors (TRAF) provided a long sought after link between pro-inflammatory cell surface receptors and the distal signaling machinery resident in the cell's interior. When first studied they appeared to be mysterious molecules indeed: a veritable hodgepodge of domains including RING and Zn finger, coiled coil, and the mystifying TRAF domain that somehow docked with other components of the signaling complex. Studies by a number of laboratories have added considerably to our knowledge base and illuminated the central role that the TRAF adapters play as a signaling conduit for a number of receptors, including TNFRs and members of the TLR/IL-1R superfamily that play a critical role in innate immunity.

Dr. Hao Wu, as Editor, has done an outstanding job in getting the primary contributors in this fast moving and burgeoning field to contribute lucid, up-to-date chapters that in great depth, but with considerable clarity, tackle the various aspects of this multi-faceted topic. Since Dr. Wu elucidated the all important structure of the TRAF domain in a landmark series of papers, she is eminently qualified to oversee as ambitious a task as producing a definitive text on arguably the most important signaling adaptors in innate immunity.

In closing, I would urge any serious student of this topic to study this text with the greatest of attention as there are many gems to be discovered within its covers that will surely propel one's research to new heights. Dr. Wu is to be congratulated for a job well done and for which her community of peers owes her a great debt.

Vishva M. Dixit
Genentech

ABOUT THE EDITOR...

HAO WU is a Professor of Biochemistry and Immunology at Weill Medical College of Cornell University, New York, U.S.A. Her main research activities include structural and functional studies of cell death and cell survival signaling. In particular, she has a major interest in elucidating the molecular mechanisms of TRAF functions. She is the recipient of many academic awards and fellowships including the Howard Hughes Medical Institute Pre-doctoral Fellowship, the Aaron Diamond Post-doctoral Fellowship, the Pew Scholar Award, the Rita Allen Scholar Award, the Margaret Dayhoff Memorial Award from the Biophysical Society and the New York Mayor's Award for Excellence in Science and Technology.

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CONTENTS

CHAPTER 1

Phylogeny of the TRAF/MATH Domain

Juan M. Zapata,* Vanesa Martínez-García and Sophie Lefebvre

Abstract

The TNF-receptor associated factor (TRAF) domain (TD), also known as the meprin and TRAF-C homology (MATH) domain is a fold of seven anti-parallel β-helices that participates in protein-protein interactions. This fold is broadly represented among eukaryotes, where it is found associated with a discrete set of protein-domains. Virtually all protein families encompassing a TRAF/MATH domain seem to be involved in the regulation of protein processing and ubiquitination, strongly suggesting a parallel evolution of the TRAF/MATH domain and certain proteolysis pathways in eukaryotes.

The restricted number of living organisms for which we have information of their genetic and protein make-up limits the scope and analysis of the MATH domain in evolution. However, the available information allows us to get a glimpse on the origins, distribution and evolution of the TRAF/MATH domain, which will be overviewed in this chapter.

Introduction

TNF-receptor associated factors are a family of proteins that were initially identified for their capability of interacting with and regulating different members of the TNFR family.[1-4] TRAFs are characterized by a C-terminal region encompassing about 180 aminoacids, forming a 7-8 anti-parallel β-sheets fold (TRAF-C domain) (Fig. 1), which is preceded by a coiled coil (TRAF-N) domain.[1] Meprins, a family of extracellular metalloproteases, also contain a C-terminal domain with high sequence homology to the TRAF-C domain[5] which is predicted to form a similar anti-parallel β-sheets fold.[6] Therefore, the TRAF-C domain is also known as the meprin and TRAF Homology (MATH) domain.

MATH domain (MATHd) encompassing proteins are found associated to a discrete set of other protein domains, including peptidases, filamin and RluA domains, broad-complex, tramtrack and bric a brac (BTB) domain, tripartite motif (TRIM), astacin domain and RING and Zinc finger domains (Fig. 2).

Remarkably, all different MATHd encompassing protein subfamilies seem to have a role in the regulation of protein processing. In this regard, the members of the MATHd ubiquitin proteases (UBPs) and meprins have intrinsic protease activity, while TRAF, TRIM/MATHd and BTB/MATHd proteins, as well as the MATHd structural homolog SIAH, appear to function as E3 ubiquitin ligases (Fig. 3).

The MATHd is found in a variety of proteins in humans,[7] and blast searches using as bait the MATHd of TRAF2 readily produced hundreds of hits widely distributed among *eukaryota*. Thus, MATHd encompassing proteins are currently found in 72 species of eukaryotes and 3 iridoviruses (Fig. 4), but this number will grow as we gather more information of the genomes of new organisms.

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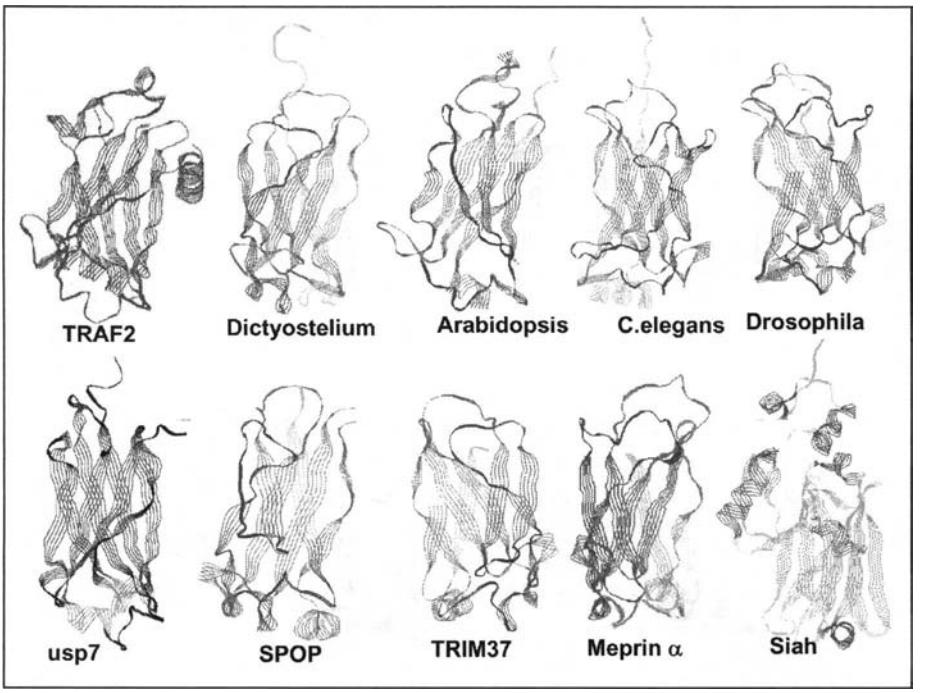


Figure 1. Models of the MATHd of human TRAF2 (1CZY), USP7 (2FOP) and SIAH (1K2F) were modeled using protein explorer. The MATHd of DTRAF-1, TRIM37, SPOP, Meprin and examples of *Dictyostelium*, *Arabidopsis* and *C.elegans* MATHds were constructed by threading on the structure of the MATHd of TRAF2,[16,17] using FFAS and MODELER.[112,113]

Interestingly, genes encoding MATHd proteins have been found in lower eukaryotes such as protozoa and unicellular fungi, but not in any of the *prokaryota* and *archaea* species for which the full genome sequence is known, suggesting that the MATHd might have appeared early in the evolution of eukaryotes.

Next we will overview the different types of MATHd encompassing proteins according to the other protein domains they are found associated to.

Ubiquitin Proteases (UBPs)

Ubiquitin proteases (UBPs), also known as deubiquitinating enzymes, are a family of cysteine proteases involved in the removal of ubiquitin from proteins. There are five UBP subclasses, each of them encompassing a different type of deubiquitinase domain: (1) ubiquitin specific proteases (USP), (2) ubiquitin C-terminal hydrolases (UCH), (3) ovarian tumor proteases (OTU), (4) Machado-Joseph disease protein domain proteases (MJDP) and JAMM motif proteases.[8,9] Some UBPs are found associated to the proteasome where they remove the poly-ubiquitin chains from the proteins that are being degraded by the proteasome, allowing for ubiquitin recycling. In contrast, other UBPs remove the poly-ubiquitin chains from proteins to prevent their degradation by the proteasome.[8] This is the case of ubiquitin specific proteases (USP), which account for the majority of UBPs found in mammalian genomes. USPs seem to counterbalance E3 ubiquitin ligases by removing ubiquitins from proteins, thus preventing their degradation. Indeed, a coevolution of both types of enzymes has been suggested.[10]

MATHd encompassing UBPs are found in unicellular organisms, such as *Mycetozoa* (*Dictyostelium*), *Alveolata* (*Cryptosporidium*) and *Fungi* of the *Ascomycota* and *Basidiomycota* phyla. They are also found in plants (*Arabidopsis*, *Oryza*) and in *Metazoa* (Fig. 4). Interestingly, there is a

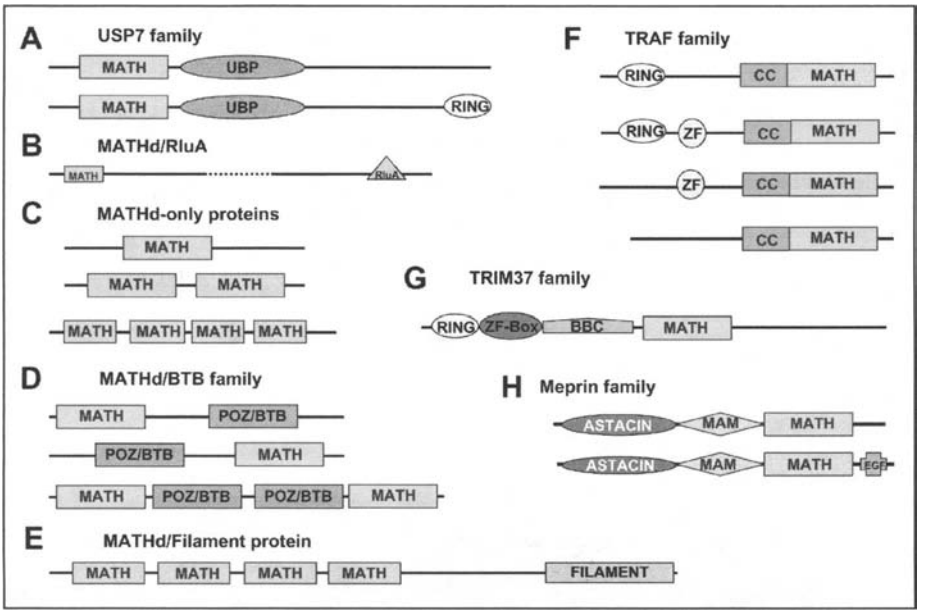


Figure 2. Proteins encompassing MATH domains and their association with other protein domains were identified using the Conserved Domain Search service (CD-Search),[114] the Conserved Domain Database for protein classification (CDD)[115] and the Conserved Domain Architecture Retrieval Tool (CDART),[116] three NCBI web-based tools for detection of structural and functional domains in protein sequences. A) MATHd encompassing ubiquitin proteases (USP7 family), B) MATHd/Rlu proteins. C) MATHd-only proteins. D) MATHd encompassing broad-complex, tramtrack and bric a brac (BTB) domain protein family. E) MATHd/filament protein. F) TRAF family. G) TRIM37 family. H) Meprin family. MATH: meprin and TRAF homologous domain (cd00121); UBP: ubiquitin protease domain(peptidase C19C, cd02659); RING: Really Interesting New Gene finger domain (cd00162); Rlu; pseudo-uridine synthase Rlu (cd02869); POZ/BTB Pox virus and Zinc finger/broad-complex, tramtrack and bric a brac domain (smart00225); Filament: intermediate filament protein domain (pfam00038); ZF: TRAF-type zinc finger domain (pfam02176); CC: coiled coil, TRAF-N domain; ZF-Box: B box zinc finger (pfam00643); BBC: B-box C-terminal domain, coiled coil (smart00502); Astacin: astacin (peptidase family M12A) domain (pfam01400); MAM: Meprin and mu domain (smart00137); EGF: epidermal growth factor-like domain (smart00181).

remarkable conservation among organisms of both, the size of the protein, which contains over 1000 amino-acids, and its domain organization, with the MATHd located at the N-terminus of the protein followed by the ubiquitin protease domain. Theoretical phylogenetic analysis based on the homologies of all MATHd protein sequences available showed that all UBP/MATHd proteins form a distinctive cluster, and that fungi, metazoa and plant UBP/MATHd proteins were distributed in three well defined subgroups (Fig. 5). One exception corresponds to the UBP/MATHds from *Cryptosporidium* (*Alveolata*), which according to the protein sequence comparison, contain the most divergent MATHds of all analyzed (Fig. 6, bottom).

The analysis of the MATHd sequences shows a unique conservation among UBPs of the residues ExDWGF in the β-sheet 7 (Fig. 6), which correspond to residues ^{162}ENDWGF^{167} of human USP7.[11] Furthermore, in all cases the protease domain is a peptidase C19C, a subgroup of the peptidase 19 group of ubiquitin carboxyl-terminal hydrolases that removes ubiquitin molecules from polyubiquinated peptides by hydrolyzing bonds involving the carboxyl group of the C-terminal Gly residue of ubiquitin. It is also noteworthy that 35 out of 37 species included in our UBP/MATHd analysis contain only one UBP/MATHd. Only two species (*O. sativa and C. elegans*) contain two different UBP/MATHd genes. Altogether, these results illustrate the strong

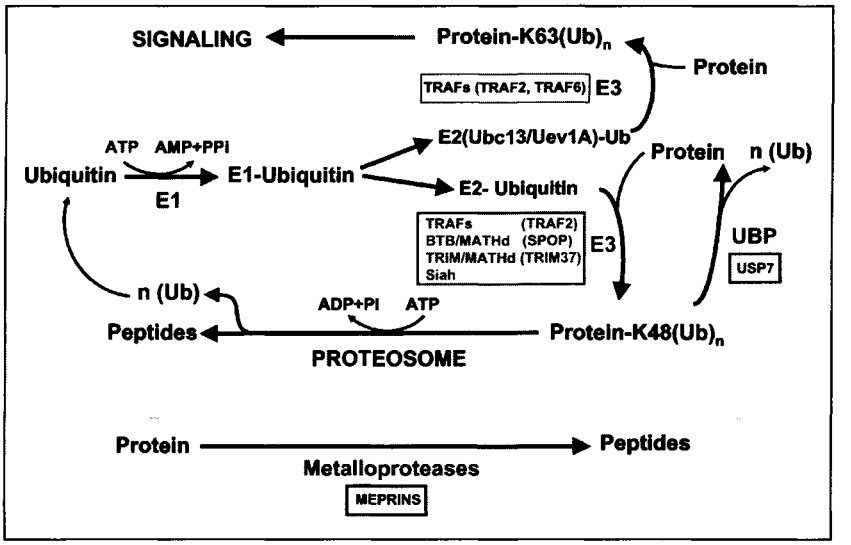


Figure 3. MATHd proteins regulate protein processing. The selective degradation of many proteins in eukaryotic cells is carried out by the ubiquitin system.[117] In this pathway, proteins are targeted for degradation by covalent ligation at lysine (K)48 to ubiquitin, one of the most phylogenetically well-conserved proteins in eukaryota.[118] Ubiquitin-mediated protein degradation by the proteasome is key for the control of numerous biological processes, including the control of cell-cycle, transcriptional regulation, and signal transduction, to mention just a few (reviewed in ref. 117). A different type of ubiquitination (K63) does not result in degradation of the targeted proteins, but instead is necessary for the activation of and signaling from these proteins.[53] UBP (USP) can counteract the effect of E3 ligases by removing ubiquitin chains from proteins. Meprins are a family of extracellular metalloproteases that are involved in cleaving growth factors, extracellular matrix proteins, and biologically active peptides.[46,47]

conservation of UBP/MATHd genes in eukaryotes, suggesting that UBP/MATHd proteins in different species might be orthologs.

The only UBP/MATHd protein found in the human and mouse genomes is USP7. The crystal structure of the MATHd of USP7 has been recently solved.[11] It is a fold of eight anti-parallel β-sheets very similar to the TRAF-C domain of TRAFs (Fig. 1). The USP7's MATHd has been implicated in substrate recognition. Indeed, USP7 was originally identified by its interaction with ICP0 protein from herpes simplex virus.[12] USP7 also interacts with p53 and Mdm2,[13] and Epstein-Barr virus nuclear antigen 1 (EBNA1) protein.[11]

Interestingly, USP7's MATHd accommodate the MATHd-binding-motifs of p53, Mdm2 and EBNA1 in a shallow surface groove in the middle of the β-sandwich which is much alike the TNFR-peptide binding crevice located across the edge of the β-strands of TRAFs.[14-17] However, the mode of peptide binding and the adopted conformation of the bound peptide differ significantly from previously observed TRAF-peptide interactions.[11,13,18] Most interestingly, the key aminoacids in USP7's MATHd interacting with all these different substrates are ^{164}DWGF^{167}, which shape the peptide-binding pocket and, as indicated above, are distinctively conserved among UBPs. Other aminoacids participating in interaction might account for the differences in affinity of the different substrates, but interactions with the DWGF, in particular with Trp165, are critical for the specificity of the binding.[11,13,18]

Structural and competition data support that Mdm2 has a higher affinity for binding USP7 than has p53.[13,18] Mdm2 is a ubiquitin ligase that regulates p53 activity, traffic and degradation.[19] It has been shown that USP7 might stabilize Mdm2 and promote p53 degradation.[20,21] However, functional[22] and structural[13,18] data shows that USP7 also interacts with and deubiquitinates p53, thus

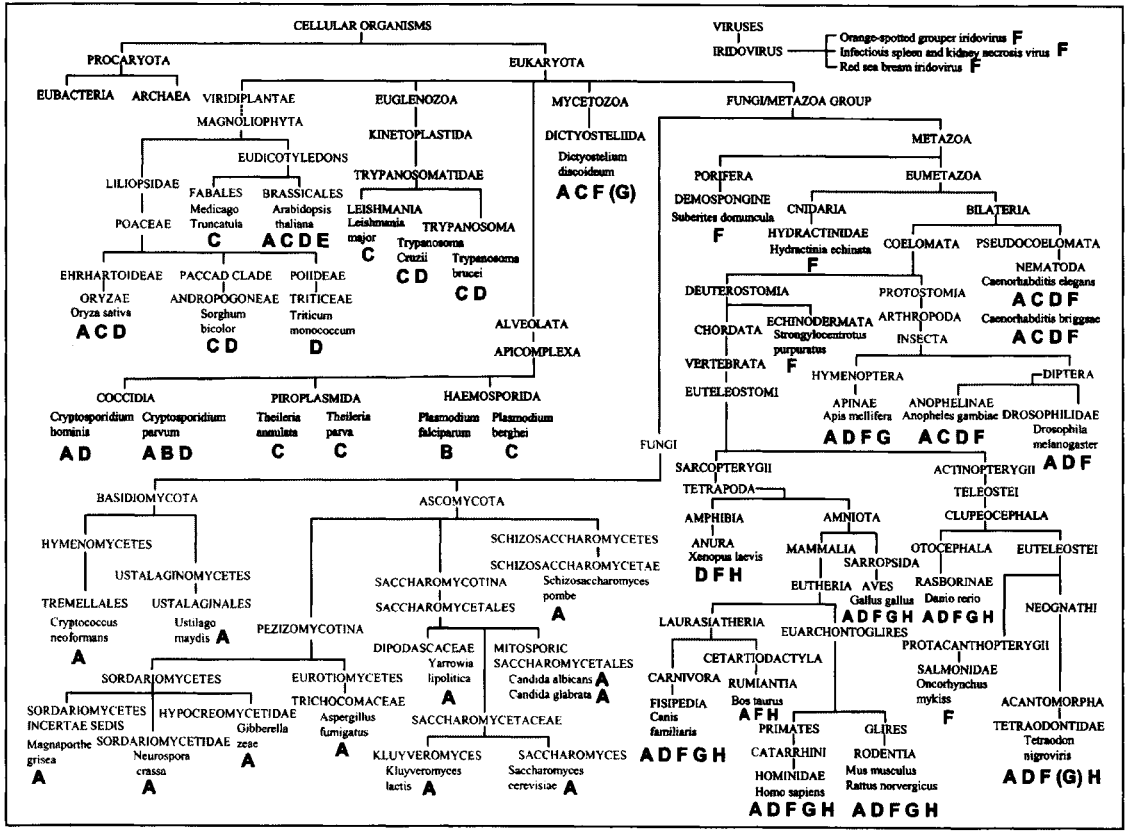
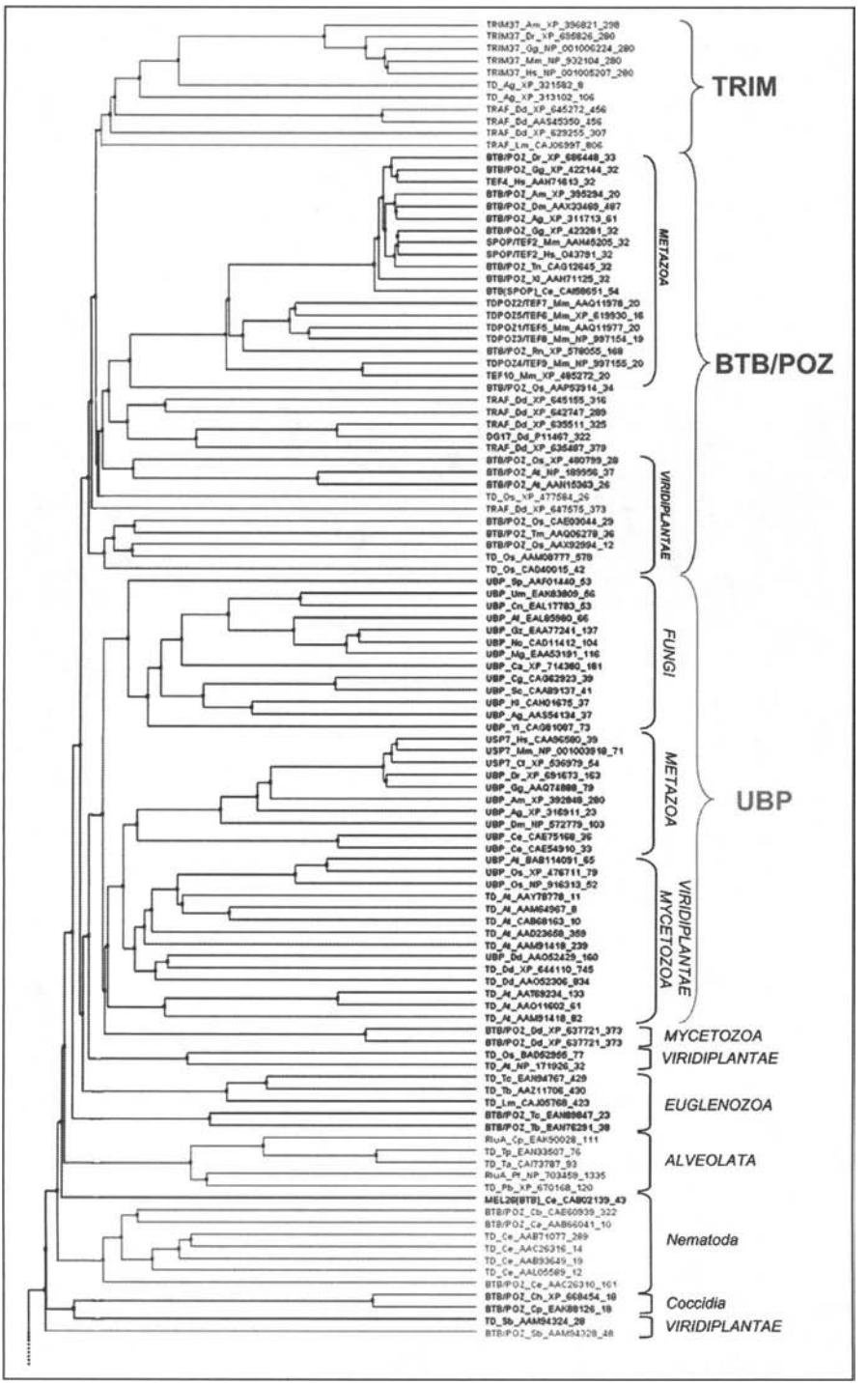


Figure 4. Phylogenetic tree of MATHd in living organisms and viruses. The nomenclature and phylogenetic relations of the NCBI Taxonomy browser (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy) were followed. Only those branches with species containing MATHd encompassing gene(s) are represented. The letters refer to the different subclasses of MATHd encompassing protein families described in Figure 2.



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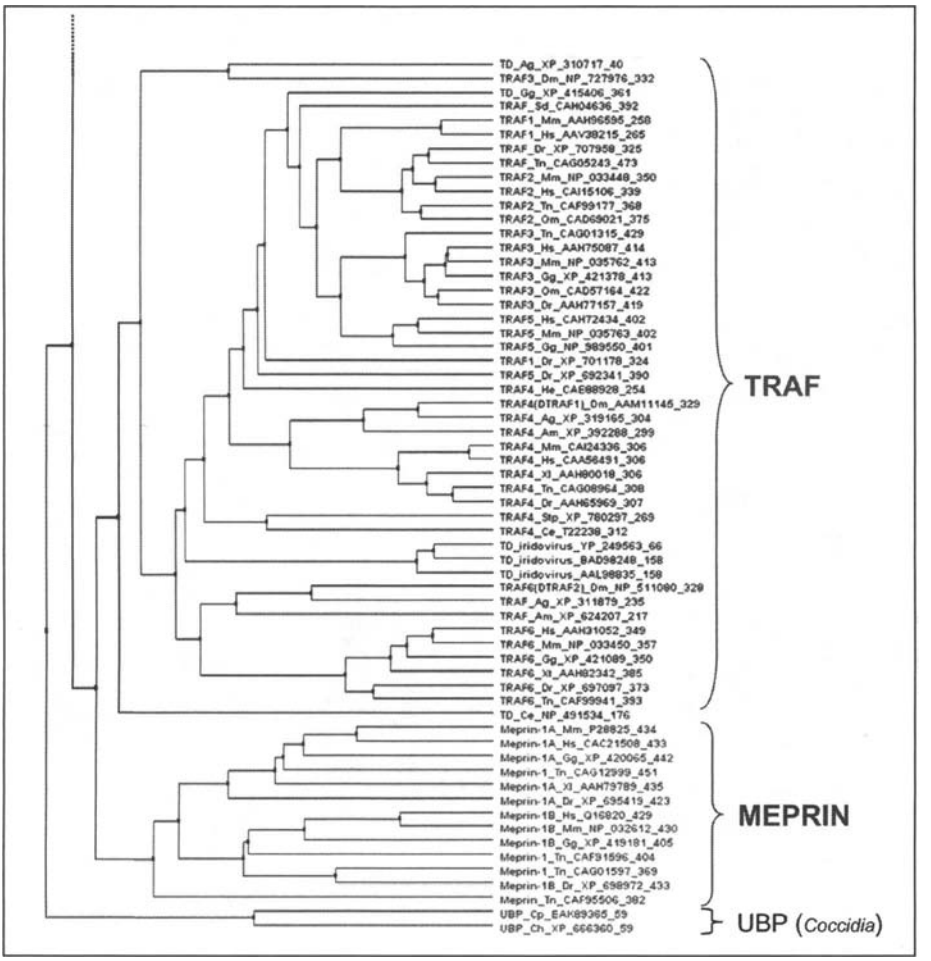

Figure 5, continued. Rooted tree representing the theoretical phylogenetic relations of MATHd encompassing proteins. Sequence similarities are determined using MUSCLE,[119,120] and a BLOSUM62 matrix that is based on the likelihood method estimating the occurrence of each possible pairwise substitution.[121] Species listed (by alphabetical order): A.f.: *Aspergillus fumigatus*; A.g.: *Anopheles gambiae*; A.m.: *Apis melifera*; A.t.: *Arabidopsis thaliana*; C.a.: *Candida albicans*; C.b.: *Caenorhabditis brigsae*; C.e.: *Caenorhabditis elegans*; C.f.: *Canis familiaris*; C.g.: *Candida glabrata*; C.h.: *Cryptosporidium hominis*; C.n.: *Cryptococcus neoformans*; C.p.: *Cryptosporidium parvum*; D.d.: *Dictyostelium discoideum*; D.m.: *Drosophila melanogaster*; D.r.: *Danio rerio*; G.g.: *Gallus gallus*; G.z.: *Gibberella zeae*; H.e.: *Hydractinia echinata*; H.s.: *Homo sapiens*; K.l.: *Kluyveromyces lactis*; L.m.: *Leishmania major*; M.g.: *Magnaporthe grisea*; M.m.: *Mus musculus*; N.c.: *Neurospora crassa*; O.m.: *Oncorhynchus mykiss*; O.s.: *Oryza sativa*; P.b.: *Plasmodium berghei*; P.f.: *Plasmodium falciparum*; R.n.: *Rattus norvegicus*; S.b.: *Sorghum bicolor*; S.c.: *Saccharomyces cerevisiae*; S.d.: *Suberites domuncula*; S.p.: *Schizosaccharomyces pombe*; St.p.: *Strongylocentrotus purpuratus*; T.a.: *Theileria annulata*; T.b.: *Trypanosoma brucei*; T.c.: *Trypanosoma cruzi*; T.m.: *Triticum monococcum*; T.n.: *Tetraodon nigroviridis*; T.p.: *Theileria parva*; U.m.: *Ustilago maydis*; X.l.: *Xenopus laevis*; X.p.: *Xenopus tropicales*; Y.l.: *Yarrowia lipolytica*.

preventing its degradation by the proteasome. Therefore, USP7 appears to play multiple roles in regulating the p53-Mdm2 pathway.

Interestingly, EBNA1 seems to have the higher affinity for USP7 of all known substrates, and functional studies have shown that interaction of EBNA1 with USP7 protects cells from apoptotic

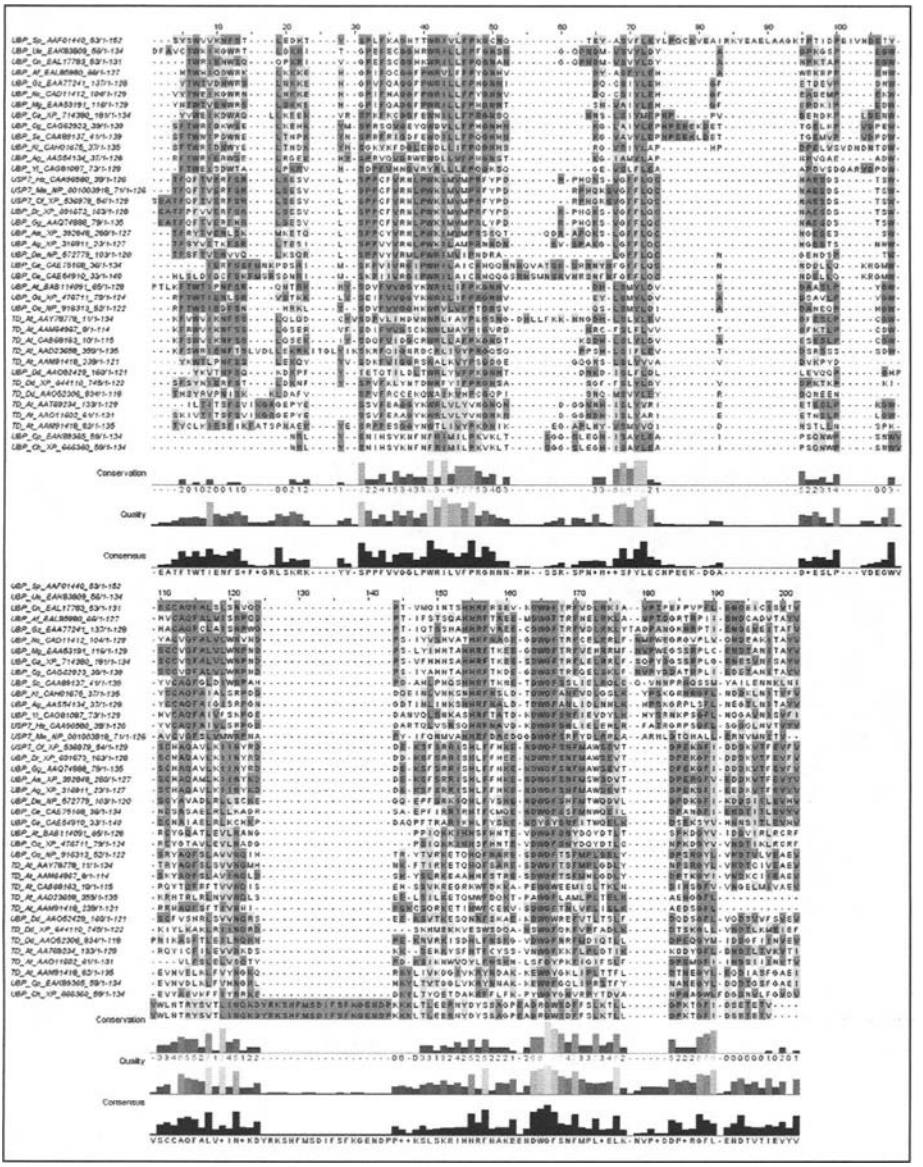

Figure 6. Protein sequence alignment of the MATHd of UBPs. Multiple alignment was performed using MUSCLE.[119,120]

insults by preventing USP7-mediated deubiquitination of p53 in vivo, resulting in p53 degradation. Indeed, it has been proposed that this is the mechanism by which EBNA1 contributes to the survival of Epstein-Barr virus-infected cells.[11] However, Mdm2 activity would also be affected by EBNA1 association to USP7, which as a result might prevent p53 degradation. Additional work is necessary to clarify the role of USP7 and their substrates in the control of p53-mediated activities.

It is noteworthy that p53, Mdm2 and related proteins are only found in vertebrates. In addition, herpes viruses only infect vertebrates. It seems likely that Epstein Barr virus EBNA1 and herpes

simplex virus ICP0 have coevolved along with p53 and Mdm2 to efficiently out-compete these two proteins from binding USP7, thus efficiently hick-jacking p53-mediated pathways. Consequently, none of these proteins could be the original substrate(s) for the putative using USP7 orthologs in lower eukaryotes and plants. Considering the high conservation of the MATHd/UBPs in evolution, it should be expected that this still unidentified primordial protein substrate(s) should play an essential role for all these organisms and consequently, it should also have remained well conserved during evolution.

There is an additional set of MATHd sequences from *Arabidopsis thaliana* and *Dictyostelium discoideum* sharing a high homology with UBP/MATHd but lacking the ubiquitin protease domain. Those sequences still have the ExDWGF motif characteristic of USP7 orthologs or close variations of it, further suggesting that they arose from a common ancestor (Fig. 6).

RluA and Filament Domains

MATHd encompassing proteins that also contain a pseudo-uridine synthase Rlu domain are found in *Alveolata*. One of these putative proteins is encoded by the genome of *Cryptosporidium parvum* (CAD98470), and the other one is found in *Plasmodium falciparum* (NP703459). The Rlu domain is involved in the conversion of uracil bases to pseudo-uridine.[23]

The filament domain represents the N-terminal head region of intermediate filaments that bind DNA[24] and is found associated to MATHd in one putative protein from Arabidopsis thaliana (AAD23659). The function of this MATHd encompassing protein in *Arabidopsis* is not known.

BTB/POZ Domains

The BTB (for broad-complex, tramtrack and bric a brac) domain, also know as POZ (for Pox virus and Zinc finger) domain is an evolutionarily conserved domain broadly distributed in eukaryotes.[25,26] The crystal structure of the POZ domain of the human promyelomonocytic leukemia zinc finger (PLZF) protein consists of a cluster of alpha-helices flanked by short beta-sheets at both the top and bottom of the molecule, that tightly homodimerizes by intertwining both domains producing an extensive hydrophobic interface.[26,27]

Proteins encompassing a MATH domain and a BTB/POZ domain are broadly represented among eukaryotes (Fig. 4). Thus, proteins of this group are found in lower eukaryotes, such as *Trypanosomatidae* (*Euglenozoa*), and *Coccidia* (*Alveolata*), plants (*Viridiplantae*) including both eudicotyledons and monocotyledons (*liliopsidae*), and in *metazoa*. However, MATH/BTB proteins have not yet been found in *fungi*. The vast majority of BTB/MATHd proteins have an N-terminal MATHd and a C-terminal BTB domain, but there are also examples of genes encoding proteins with a N-terminal BTB domain and a C-terminal MATHd. Also, some proteins contain MATHd/BTB domains in tandem (Fig. 2).

A large cohort of putative genes encoding MATH/BTB proteins (about 49) are found in *Caenorhabditis elegans*. These genes would encode proteins with an N-terminal MATHd and a C-terminal BTB/POZ domain. One of these proteins, the maternal effect lethal (MEL)-26, has been shown to regulate the transition from meiotic to mitotic chromosome segregation.[28] MEL-26 is part of the CULLIN (CUL)-3 ubiquitin ligase complex.[29-31] CUL-3 is an E2 ubiquitin conjugating enzyme. MEL-26 interacts with CUL-3 through its BTB/POZ domain and acts as an E3 ubiquitin ligase, which is required for ubiquitination and degradation of the meiosis defective (MEI)-1 protein.[29,30] Three other MATH/BTB proteins that interact with CUL-3 were identified in *C. elegans*.[29]

SPOP (also known as TEF2)[7,32] and its close relative TEF4[33] deserve special mention among the MATHd/BTB proteins. These two genes are strikingly conserved in metazoans, including *nematoda, arthropoda* and *chordata* (Fig. 7). SPOP (TEF2) has been shown to form an E3 ubiquitin ligase complex with CUL-3. SPOP (TEF2) regulates the ubiquitination and subsequent proteasome-mediated degradation of several proteins, including the polycomb group protein BMI1, which regulates survival and senescence of stem cells and cancer cells,[34-36] the variant histone MACROH2A, which is required for the silencing of one of the two X chromosomes in XX female somatic cells[37] and DAXX, a multifunctional protein involved in the regulation of cell death (ref.

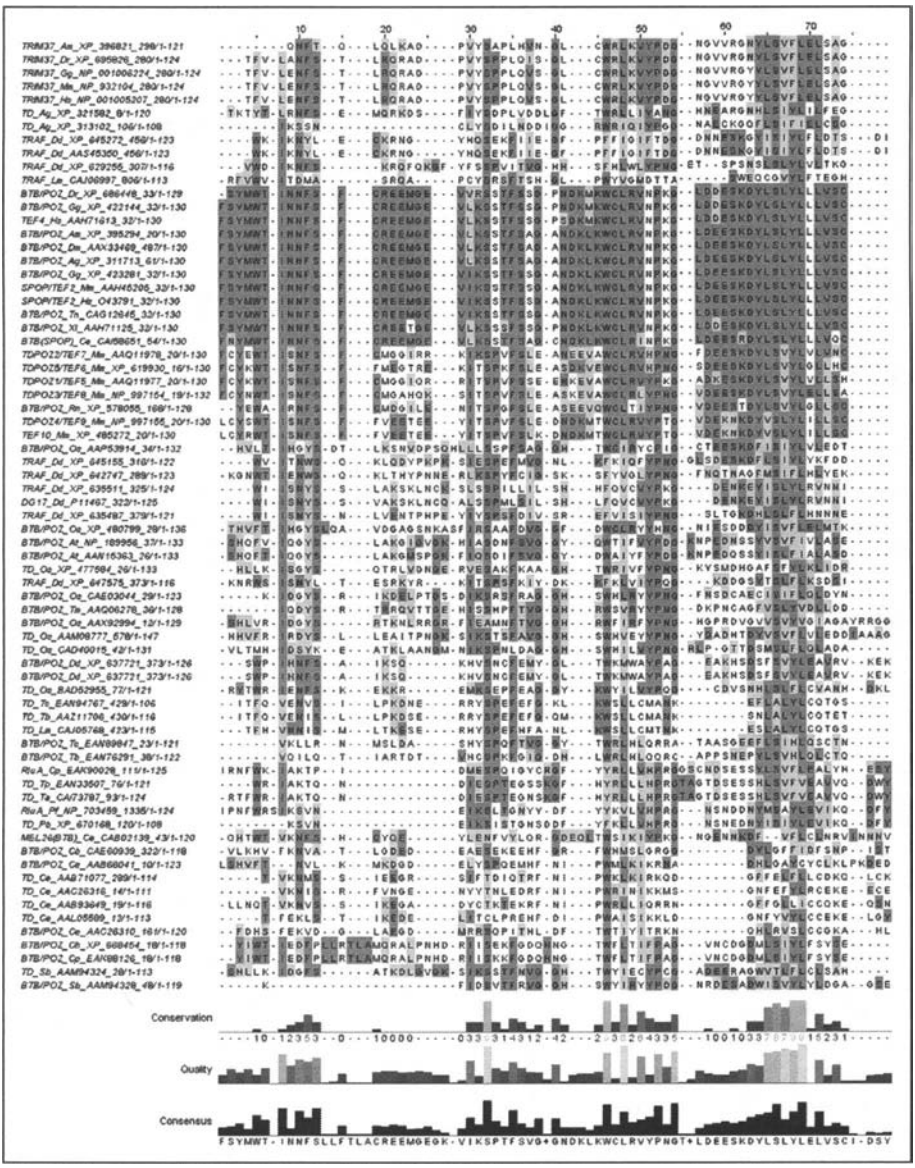

Figure 7. Protein sequence alignment of the MATHd of TRIM and BTB proteins. Multiple alignment was initially performed using MUSCLE.[119,120] Figure continued on next page.

38 and refs. therein). These examples further extend the role of MATHd/BTB proteins as E3 ubiquitin ligases. It is worth noting, that the MATHds of SPOP and relatives are more conserved than their corresponding BTB domains. For instance, the percentage of identity between the BTB domains of *C.elegans* and *homo sapiens* is 56%, while the percentage of identity of their MATHds is 89%, thus suggesting a strong evolutionary pressure to maintain the MATHd of SPOP and relatives virtually unchanged from worms to humans.

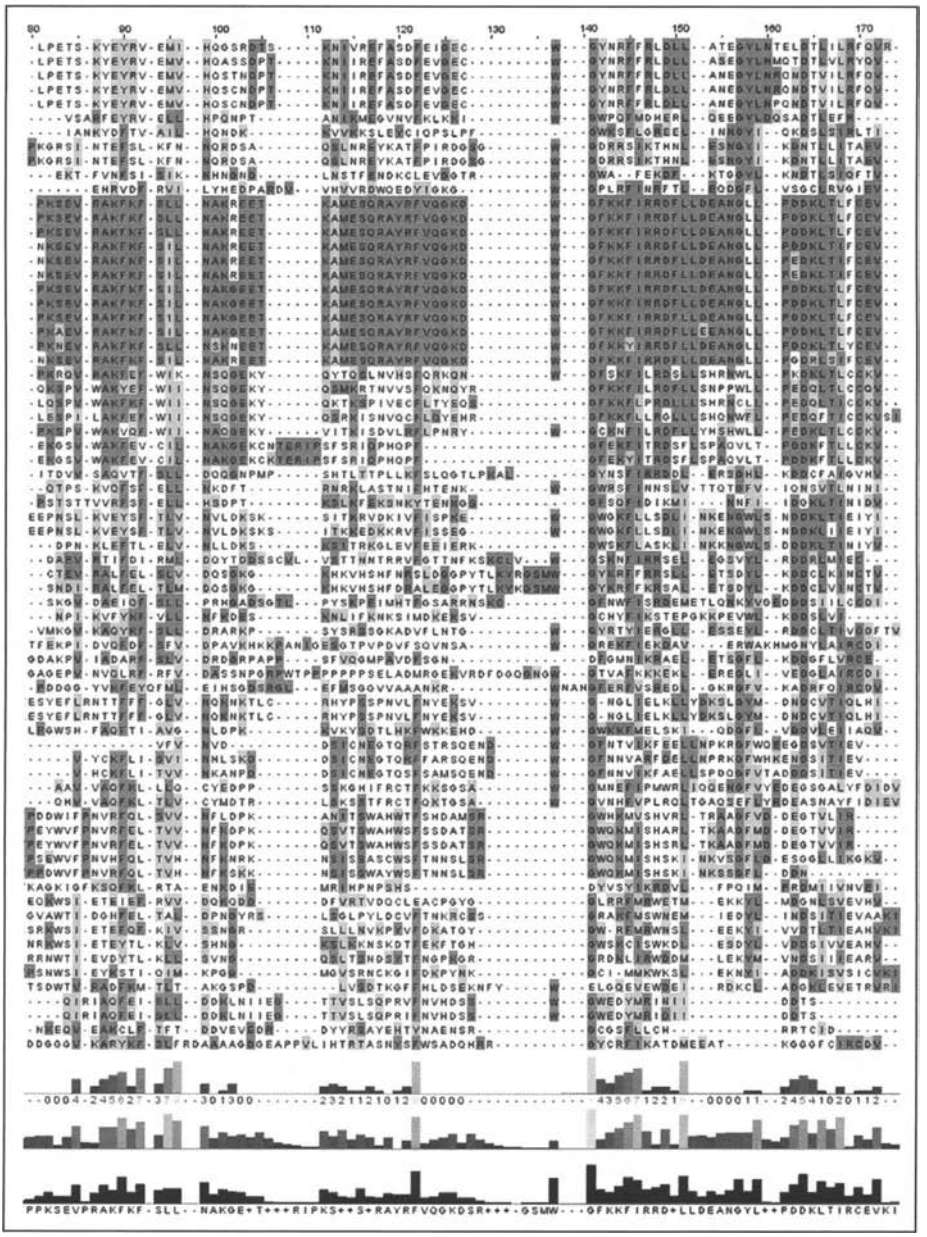

Figure 7, continued. Protein sequence alignment of the MATHd of TRIM and BTB proteins.

It is worth mentioning that rice (*Oryza sativa*) contains the larger number of MATHd/BTB proteins (about 70) of all the species studied so far, including other monocotyledons (*liliopsidae*) such as wheat (*Triticum monococcum*) and sorghum (*Sorghum bicolor*). The vast majority of these rice's MATHd/BTB proteins have the MATHd at the N-terminus and the BTB/POZ domain at the C-terminus.

In summary, proteins combining the MATH domain with the BTB domain are found very early in the evolution of eukaryotes. The available evidence suggests that they might link specific protein substrates to ubiquitin ligase complexes. Additional research will be required to identify these putative substrates and to determine whether all the MATHd/BTB proteins found in diverse organisms have this function, particularly in rice and *C.elegans* that contain the larger number of *MATHd/BTB* genes identified so far. These studies will also help to elucidate whether each of these proteins is specific for a particular substrate or whether a functional redundancy exists, with different MATHd/BTB proteins targeting the same substrates.

MATH Domain-Only Proteins

There is a large number of hypothetical proteins containing one or multiple MATH-domains in tandem and lacking of any other distinguishable associated protein domain (Fig. 3). These proteins are found in plants (*Arabidopsis, Medicago, Oryza,* and *Sorghum*), lower *eukaryota* (*Trypanosoma, Dyctiostelium, Theileria* and *Plasmodium*), and in lower *metazoa* such as nematodes (*Caenorhabditis* sp) (Fig. 4). Many of these proteins are identified by conceptual translation of the genome and therefore some of them could be part of larger genes that might encompass other protein domains. However, it is noteworthy that these MATHd-only sequences represent the majority of the MATHd-encompassing proteins identified in *Arabidopsis thaliana* and *Caenorhabditis* sp, but are not very common in other species, including some whose genome have been fully sequenced. The MATHd of these MATHd-only proteins is in general more similar to that of MATHd/BTB proteins (Fig. 7), but some of them have more similarity to the MATHd of UBP proteins (see above and Fig. 6). The function of these MATHd-encompassing proteins as well as the significance of their elevated number in *Arabidopsis* and *Caenorhabditis* are not yet known.

It is noteworthy that TRAF1 could be considered as a MATH-domain only protein. However, TRAF1 is readily identified as a member of the TRAF family by the high homology of its MATH domain to those of other members of the TRAF family.

TRIM37

A group of MATHd encompassing proteins appears associated to a combination of protein domains known as the tripartite motif (TRIM). The tripartite motif is composed by a RING finger domain, followed by a special type of zinc finger domain coined the ZF-B box and a coiled coil (Fig. 2). In humans, there are 37 genes encoding proteins encompassing a tripartite domain[39] of which only one (*trim37*) contains a MATHd.[7]

Mutations in the *Trim37* gene in humans are causative of Mulibrey Nanism, an autosomal recessive growth disorder that affects several tissues of mesodermal origin.[40] Mulibrey Nanism is characterized by severe growth failure of prenatal onset, constrictive pericardium with consequent hepatomegaly, hypoplasia of several endocrine glands, fibrodysplasia of bones and muscle and sterility.[41-43] Similar to other members of the MATHd and TRIM[44] families, TRIM37 seems to function as a E3 ubiquitin ligase, although its physiological substrate(s) is still unknown.[45]

TRIM/MATHd proteins form a distinctive cluster in the tree (Fig. 5) and have a high degree of conservation (Fig. 7). Overall, the TRIM's MATHd is more similar to those of BTB/MATHd proteins than to any other MATHd-encompassing protein subclass. Orthologs of TRIM37 are only found in *Coelomata* (*Metazoa*) (Fig. 4). Indeed, TRIM37 is found in some insects such as bees (*Apis melifera, hymenoptera*) but not in others such as *Drosophila* or *Anopheles (both Diptera)*. However, there are two genes encoding a MATHd-only protein in *Anopheles gambiae* that have high similarities to the MATHds of the TRIM37 orthologs. TRIM37 seems to be very well conserved in *deuterostomia*, including the echinoderm *Strongylocentrotus purpuratus* (sea urchin) (XP791708; not shown) and *vertebrata*, including mammals, birds and fishes. Interestingly, although TRIM37 is found in zebra fish (*Danio rerio*) and spotted green pufferfish (*Tetraodon nigroviridis*), the latter seems to contain a *trim37* gene with a truncated MATHd. It is interesting to mention the high level of conservation between different TRIM37 orthologs. For instance, the MATHd of *Apis melifera* TRIM37 is 69% identical to rodent's TRIM37, 74% to chicken's, 71% to zebra fish's and 74% to

human's. Future research will determine the function of TRIM37 in insects and nonmammalian vertebrates and whether it also has a role in development, as it does in humans.

Meprins

Meprins are a family of extracellular metalloproteases, which are anchored to the plasmatic membrane and are involved in cleaving growth factors, extracellular matrix proteins, and biologically active peptides.[46,47] Meprins have two subunits, α and β, that form hetero- and homo-dimers covalently linked by disulfide bridges. Two of these dimers associate as a tetramer forming the functional meprin complex.[46] The MATHd of meprins is located at the C-terminus and the catalytic astacin-like protease domain at the N-terminus (Fig. 2). This MATHd seems to be involved in the oligomeric association of meprin subunits, similar to other MATHds,[6] but it might be also implicated in the recognition, folding and activation of zymogens. The MATHd of meprins form a distinctive cluster in the phylogenetic tree (Fig. 5), and seems to be closely related to the MATHd of TRAFs.

MATHd encompassing meprins appeared late in evolution, as they are only found in vertebrates, including fishes, *amphibia*, birds and mammals.

TRAFs

TNF-Receptors associated Factors (TRAFs) constitute a family of adapter proteins that were initially identified in humans and rodents by their association with different members of the TNF-Receptor (TNFR) family. Humans and other mammals contain seven TRAFs (TRAF1 to 7). The TRAF domain (TD) of TRAFs is always located at the C-terminus (Fig. 2). The crystal structures of the TD of human TRAF2 and TRAF3 showed that it is composed by the 7-8 anti-parallel β-sheet fold (the MATHd, also known as TRAF-C domain) (Fig. 1), followed by a coiled coil (TRAF-N domain). These crystal structures also showed that mammalian TRAFs associate in trimers, with the intertwining coiled coils helping to stabilize the complex.[1] TRAF family-members, with the sole exception of TRAF1, also have a distinctive N-terminal RING finger domain followed by a variable number of zinc finger domains (Fig. 2).[1-4,48] Although TRAF7 does not contain a TRAF domain, it was arguably included in the TRAF family because it contains a RING finger domain and zinc finger domains that are similar to those found in other TRAF family members and because it seems to participate in the control of TNF-family signaling as do other TRAFs.[49]

Similarly to the other members of the MATHd superfamily, TRAFs have been implicated in the control of proteolysis. Indeed, TRAF2 seems to function as a E3 ubiquitin ligase that regulates TRAF2 and TRAF3 ubiquitination and proteolysis.[50,51] TRAF3 has been shown to regulate NIK proteolysis.[52] However, TRAFs (in particular TRAF2 and TRAF6), working in conjunction with the E2 ligase complex Ubc13/Uev1A, have the ability to self-ubiquitinate or ubiquitinate other proteins with K63-linked poly-ubiquitin chains (Fig. 3). Contrary to K48 ubiquitination, K63 ubiquitination does not target TRAFs or other proteins for degradation, but instead confers TRAF2 and TRAF6 the ability to activate other components of the pathway.[53]

TRAFs are key components of the Toll Receptor (TLR) family and Tumor Necrosis Receptor (TNFR) family signal transduction. TRAFs regulate the recruitment of kinases and other effector proteins to the activated receptor and other signaling complexes. TRAFs also mediate the activation of downstream components of these pathways, control the subcellular relocalization of the receptor-ligand complexes, and modulate the extent of the response by controlling the degradation of key proteins in the pathway.

Distribution of TRAFs

TRAFs are broadly represented in metazoans (Fig. 4). The older members of the family seem to be TRAF4 and TRAF6. Indeed, in a theoretical phylogenetic tree based on MATHd similarities (Fig. 5), all TRAF6 and TRAF4 orthologs form two distinctive clusters. Interestingly, a putative TRAF4 ortholog is already found in *Hydractinia echinata* (snail fur), a member of the phylum *cnidaria* that includes corals and jellyfishes. Other TRAF4 orthologs are found in *nematoda* (*C. elegans*), insects, such as the fruit fly *Drosophila* (where is known as DTRAF1),[54,55] mosquito (*Anopheles*

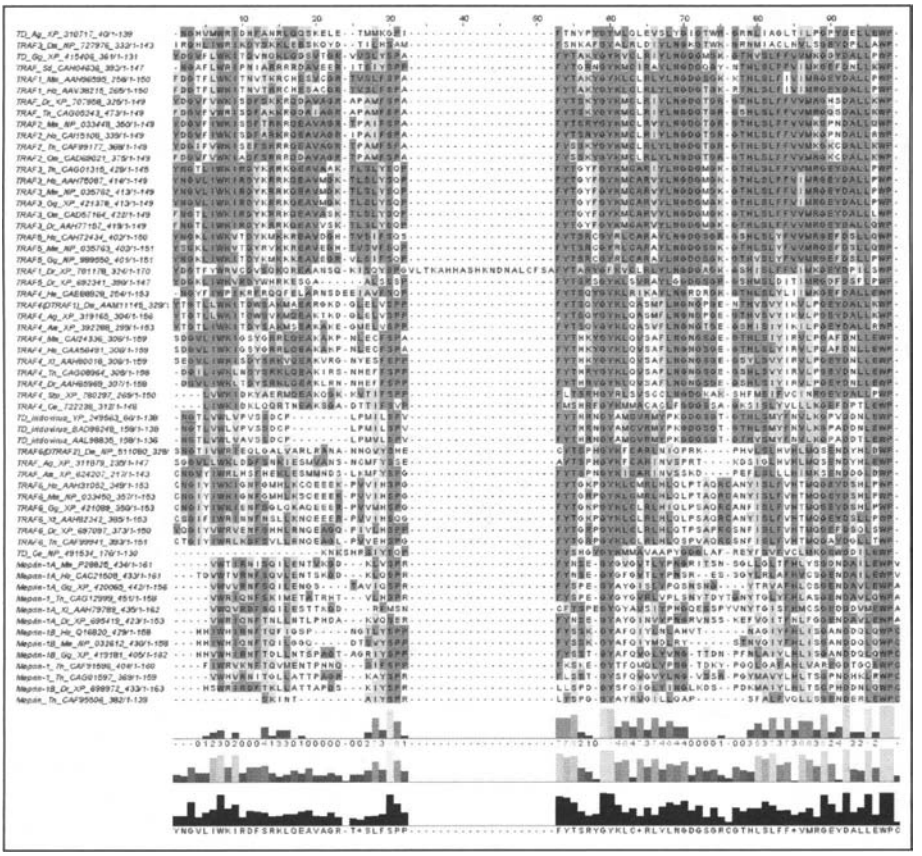

Figure 8. Protein sequence alignment of the MATHd of TRAFs and Meprins. Multiple alignment was initially performed using MUSCLE.[119-120] Figure continued on next page.

gambiae) and bee (*Apis melifera*), as well as in sea urchin (*Strongylocentrotus purpuratus*), early chordates, such as *Ciona intestinalis* (BAE93281, not shown in Fig. 4) and vertebrates.

TRAF6 orthologs have been found so far in insects, such as *Drosophila* (where is known as DTRAF2),[56] mosquitoes and bees, as well as in vertebrates.

As suggested by the analyses shown in Figure 5, TRAF1, TRAF2, TRAF3 and TRAF5 seem to be the members of the TRAF family that appeared later in evolution. These analyses also suggest that these TRAF family members might have originally diversified from a common TRAF4 ancestor. However, there is a TRAF family member (CAH04636) in the sponge *Suberites domuncula*, a member of the older metazoan taxon still extant.[57] Interestingly, this sponge's TRAF shows closer similarities to TRAF3 than to any other TRAF family member when the sequence comparison is performed with the complete protein sequence. Indeed, this TRAF contains a RING and zinc finger domains in its N-terminus and the MATHd at the C-terminus. When only the MATHd is compared, this TRAF still shows higher similarities (about 30% identities) to both TRAF2 and TRAF3 than to any other member of the TRAF family (Fig. 8). Although it is unclear whether this *Suberites domuncula's* TRAF is a bona fide TRAF3 ortholog, this evidence suggests that TRAF3 might have arisen very early in the evolution of metazoans. TRAF3 is also found in *Drosophila*,[58] where it is known as DTRAF3,[58] and in vertebrates.

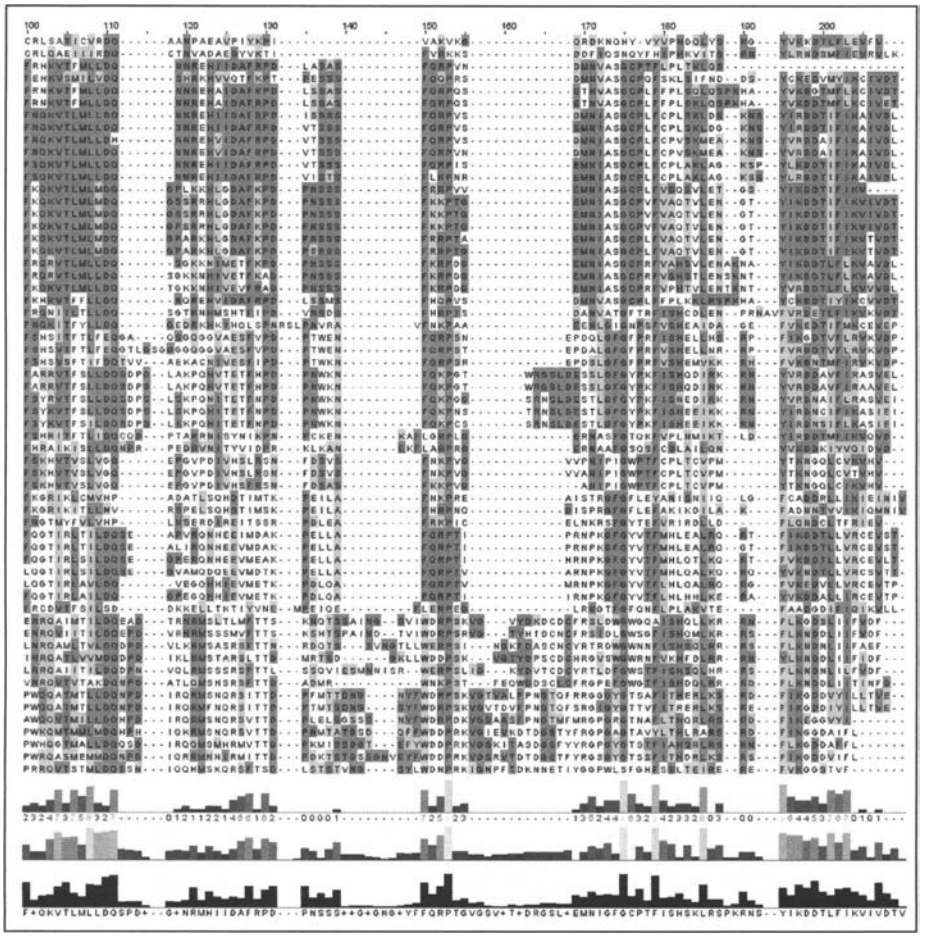

Figure 8, continued. Protein sequence alignment of the MATHd of TRAFs and Meprins.

In contrast, TRAF1, TRAF2 and TRAF5 have been so far only found in vertebrates. Furthermore, TRAF1 and TRAF2's MATHds share a high percentage of homology, suggesting that they arose not long ago from a common ancestor, probably by gene duplication. A similar scenario might have taken place between TRAF3 and TRAF5, which are closely related to each other. Considering also that TRAF1, TRAF2 and TRAF5 are functionally closer to TRAF3 (see below), it seems likely that these TRAFs diversified from a common TRAF3 ancestor early in vertebrate evolution. In contrast TRAF3 might have split from TRAF4 much earlier in metazoan evolution. However, a much larger representation of TRAF sequences from early metazoans would be necessary to obtain a clearer picture of the phylogeny of TRAFs in this *phylum*.

Interestingly, several MATHd encompassing genes are encoded by the genome of the slime mold *Dictyostelium discoideum* (*Mycetozoa*) and among those, some of these proteins have striking similarities to TRAFs (see below). *Dictyostelium* is an unicellular amoebae that grows as independent, separate cells that aggregate under adverse conditions, forming colonies of up to 100,000 cells. These colonies are a true multicellular organism, organizing different tissues composed by differentiated cell types and having the ability of regulating its proportions and morphogenesis.[59] Although

the mechanism of formation of the multicellular organism is radically different to that of metazoans, many of the underlying molecular and cellular processes, such as differential cell sorting, pattern formation, stimulus-dependent gene expression, and cell-type regulation seem to be common to *Dictyostelium* and metazoans, thus suggesting that they have arisen from common primitive precursor cells.[60] Genome and proteome based phylogeny analysis supported that *mycetozoa* are a true sister group of the *fungi/metazoa phylum*.[59]

Remarkably, a subset of *Dictyostelium*'s MATHd encompassing proteins contains a N-terminal RING and zinc finger domains similar to those found in TRAFs. Furthermore, the number of aminoacids encoded by these TRAF-like genes is also strikingly similar to that of TRAFs. However, based on the homology of their MATHd, *Dictyostelium* TRAF-like genes are more similar to MATHd/BTB (the case, for instance of DG17, EA61916, EAL61981) and MATHd/TRIM (EAS66947) proteins (Fig. 7). Although still merely speculative, these results suggest that the association of MATHd from BTB proteins to TRAF-like RING (and zinc finger) domains might have been the starting point in TRAFs evolution, an event that might have happened after the branch leading to plants separated from the branch leading to *mycetozoa* and *metazoa*.[59]

Evolutionary Aspects of TRAFs Function

The analyses on the function of TRAF family members also has provided valuable insights into the evolutionary pathways followed by this protein family.

The older member of the TRAF family for which we have functional information is the cnidarian *Hydractinia echinata* HyTRAF1.[61] HyTRAF1 has a N-terminal RING finger and 5 zinc finger domains and its TD is more similar to TRAF4 than to any other TRAF. One isoform of this TRAF family member lacking the RING and one of the zinc finger domains is exclusively found at the larval and early metamorphic stages, and seems to regulate apoptosis mediated by c-jun N-terminal kinase (JNK) signaling. Additional functional information is provided by *Drosophila*'s DTRAF1 (TRAF4 ortholog), which has 7 zinc finger domains but no RING finger domain.[54,55] DTRAF1 seems to control apoptosis and imaginal discs and photosensory neurons development by regulating the activation of JNK and its upstream kinases Hep and DTAK1.[62] The role of TRAF4 in development is also supported by studies in zebra fish (*Danio rerio*)[63] and mouse,[64] where it has been shown to participate in neurulation in vivo. TRAF4 involvement in JNK activation has been also demonstrated in mammals.[65,66] Indeed, TRAF4 seems to regulate JNK activation by binding to MEKK4 and promoting MEKK4 oligomerization.[66] Most interestingly, mice deficient in MEKK4 develop strikingly similar neurulation and skeletal patterning defects to those observed in TRAF4 deficient mice.[67,68]

TRAF6 is also considered one of the older members of the TRAF family. *Drosophila*'s, TRAF6 ortholog, DTRAF2 participates in the control of gene expression by regulating the activation of the NFκB pathways.[56,62,69] Indeed, DTRAF2 is necessary for the expression of the anti-microbial peptides diptericin, diptericin-like protein and drosomycin in response to fungal and gram-positive bacteria infection, thus supporting a role for DTRAF2 (TRAF6) in innate immunity.[62]

The involvement of TRAF6 in the control of innate immune responses has been also demonstrated in mammalians. TRAF6 is a common mediator of the Toll-like Receptors (TLR)/ Interleukin-1 Receptor (IL1R) superfamily. Eleven TLR family members have been identified in mice and ten in humans, which function as receptors for pathogen-associated molecular patterns (PAMPs) controlling host defense responses as part of innate immunity.[70-73] Nine members of this family are already present in *Drosophila melanogaster*[74] and 11 in *Anopheles gambiae* (mosquito).[75] However, only 4 *Drosophila* Toll family members have unambiguous orthologs in *Anopheles*, thus reflecting a very dynamic evolution of this family. The majority of the Toll family members in *Drosophila* are involved in the control of developmental patterning, and only two of them are also involved in the control of innate immune responses to fungal and gram-positive bacteria infections. However, these Drosophila Toll Receptors do not recognize PAMPs, as do their mammalian counterparts,[76,77] thus strongly suggesting that the Toll-Receptor family evolved to recognize specific types of PAMPs after the diversification of *protostomia* and *deutorostomia*.

TRAF6 does not directly interact with the members of the TLR/IL1R family. Instead, receptor activation recruits different adaptors, such as the myeloid differentiation factor 88 (MyD88), TIRAP, and TRAM. Receptor activated MyD88 will induce the formation of complexes containing TRAF6, IRAK family proteins, and IRF-5. In contrast, receptor activated TRAM will induce the formation of complexes containing TRIF, TRAF6 and RIP-1. In both types of complexes, TRAF6 will then associate with the ubiquitin conjugating enzymes Ubc13 and Uev1A. TRAF6 functions as a ubiquitin ligase (E3) that catalyzes the assembly of K63-linked polyubiquitin chains on itself and on IKKγ/NFκB essential modulator (NEMO) (Fig. 3).[78] K63-ubiquitinated-TRAF6 is required for the activation of the MAP3K transforming growth factor β activated kinase-1 (TAK-1), and TAK-1 binding proteins (TAB)-1 and 2.[79,80] Activated TAK1 will phosphorylate IKK and MKK6, leading to the activation of NFκB, JNK, p38 and ERK (reviewed in ref. 73).

As indicated above, the response to fungal and gram-positive bacteria infections in *Drosophila* is mediated by Toll Receptors and by MyD88 which is recruited to the receptors upon activation. Similar to what is observed with their mammalian counterparts, MyD88-dependent recruitment of DTRAF2 (TRAF6) to the complex is essential for signaling.[77] However, *Drosophila*'s response to gram-negative bacteria infection involves the protein IMD, the *Drosophila* RIP-1 ortholog, and requires the *Drosophila* E2 ubiquitin ligase Bendless (Uev1A) that catalizes K63-ubiquitination (ref. 81 and refs. therein).

Interestingly, RIP-1 is required for TRIF-mediated NFκB induction in response to TLR-3 and -4 activation in mammalian cells.[82] In this regard, two recent reports have shown that TRIF utilizes TRAF3 for signaling.[83,84] Indeed, similar to TRAF6, TLRs also recruit TRAF3 through MyD88 and IRAK1 and 4, but rather than activating MAP3K and IKK, which induce pro-inflammatory cytokines, TRAF3 engages TRIF-dependent signaling pathways leading to activation of TBK-1 and IKK-ε, inducing the expression of type I interferons and the anti-inflammatory IL-10.[83,84] Thus, TRAF3 may play important roles in interferon-dependent responses to viral pathogens, as well as in down-regulating innate immune responses via its effects on IL-10 production. The function of DTRAF3, the *Drosophila* TRAF3 ortholog[58] is still unknown. It will be specially interesting to determine whether *Drosophila* TRAF3 plays also a role in pathogen protection.

As mentioned above, TRAFs were initially identified by their ability to interact with and regulate TNFRs. There are two subclasses of TNFRs. The first subclass contains a cytosolic death domain (DD) and does not normally engage TRAFs for signaling, with the exception of TNFR1, that recruits TRAF1 and TRAF2 through their interaction with TRADD. These DD-encompassing TNFRs seem to be the older members of the family. Indeed, the older bona fide TNFR currently on record is Eiger, a *Drosophila* TNFR family member which encompasses a death domain (DD). Eiger regulates apoptosis by engaging similar signal transduction pathways than those used by other mammalian DD-encompassing TNFRs.[85] The second subclass of TNFRs does not encompass any recognizable protein domain in the cytosolic tail, but its members contain peptide sequences that support the specific interaction of TRAFs. All available information suggests that this TNFR subclass has appeared during vertebrate evolution.

A total of 20 mammalian TNFR family members that utilize TRAFs for signaling have been described so far.[2] TRAF6 regulates some of these TNFRs by specifically interacting with the hexapeptide motif PxExx(Ar/Ac), where the last amino-acid residue is either aromatic or acidic, in the cytosolic tail of TNFRs.[86] In contrast, TRAF1, TRAF2, TRAF3 and TRAF5 recognize the tetrapeptide sequence (P/S/A/T)x(Q/E)E. TRAF4 has been proposed to interact with and regulate the Nerve Growth Factor Receptor (NGFR),[87,88] which seems to be one of the older members of the TNFR family, and the glucocorticoid-induced TNFR (GITR),[89] but its role as a TNFR regulator remains controversial.

The fact that TRAF1, TRAF2, TRAF3 and TRAF5 recognize the same peptide motif in the cytosolic tail of various TNFR family members further support the idea that these TRAFs have diverged recently, most likely from a common TRAF3 ancestor (see above). Interestingly, TRAF1 and TRAF2 are closer to each other than to any other TRAF family member, and functional evidence suggests that TRAF1 regulates TRAF2 activities.[90-92] Similarly, TRAF3 and TRAF5 are more

similar to each other than to any other TRAF. However, TRAF5 is able to activate NFκB, whereas TRAF3 has an inhibitory role on the activation of NFκB mediated by TRAF2 and TRAF5.[93] Altogether, these results suggest that TRAF2 might have split from TRAF3 first, and later TRAF1 and TRAF5 diverged from TRAF2 and TRAF3, respectively. Interestingly, TRAF1, TRAF2 and TRAF5 are only found in vertebrates. Given the fast diversification of the TNFR family during vertebrate evolution, these members of the TRAF family might have emerged to increase the functional versatility of the members of the TNFR family.

The role of TRAFs in regulating TNFRs function has been more studied in the immune system, where they regulate differentiation, proliferation, and apoptosis, and are essential for adaptive immune responses. However, there are also some developmental functions associated to TRAFs that seem to be also related to their role in the regulation of TNFR activities. TRAF6 has been shown to be involved in the control of bone formation and hair follicular development through its role in regulating RANK and XEDAR.[94-98] TRAF4 is also involved in development by controlling neurulation,[64] although whether it is through the regulation of a TNFR family member is not know.

In summary, the available evidence suggests that TRAF4 and TRAF6 are the older members of the TRAF family, with TRAF3 probably diversifying from TRAF4 early in metazoan evolution. TRAF2, TRAF1 and TRAF5 appeared later during vertebrate evolution, most likely splitting from a TRAF3 ancestor. However, as new information on new genomes is gathered, the phylogenetic relations between the different TRAF family members should became more evident.

Viral Immune Evasion Strategies Interfering with MATH-Dependent Pathways

Protein sequence comparison analyses also identified the existence of three TRAF proteins in fish infecting iridoviruses. These viral TRAFs form a distinctive cluster in the tree shown in Figure 5. These viral TRAFs seem to be more related to TRAF2, sharing a 56% homology with zebra fish TRAF2 and 43% homology with human TRAF2. Two of these genes also encode a zinc finger domain at the N-terminus of the molecule (AAL98835 and BAD98248), while the other one (YP249563) is a MATHd-only protein. The function of these iridovirus TRAFs is yet not known, but they are likely to interfere with the signaling of the host TRAF family proteins.

Other viral proteins have been described to interfere with the function of various MATHd proteins of the host, although they are not MATHd proteins themselves. Two of these proteins (LMP-1 and EBNA-1) are encoded by the genome of Epstein-Barr virus (EBV), a member of the herpes virus family that infects over 90% of the world adult population and can cause several immunological disorders and cancer in immunosuppressed individuals.[99] The latent membrane protein (LMP)-1 mimics an activated CD40 and recruits various members of the TRAF family for signaling, but in a seemingly deregulated manner, leading to amplified and sustained B cell activation.[100-104] The Epstein-Barr nuclear antigen 1 (EBNA1) protein interacts with USP7 and contributes to the survival of the virus-infected cells by out-competing p53 from binding to USP7 and enforcing p53 degradation.[11]

Herpes simplex virus type 1 immediate-early regulatory protein ICP0 also interfere with USP7 function. ICP0 stimulates lytic infection and reactivation from latency. ICP0 functions as a ubiquitin E3 ligase that ubiquitinate itself and is subsequently degraded by the proteasome. USP7 removes the ubiquitin chains from ICP0, thus preventing its degradation and preserving its function.[12,105]

Kaposi's sarcoma herpesvirus[106] and human molluscipox virus[107] encode homologs of the Flice/caspase 8 inhibitory protein (v-Flip) that interact with TRAFs to interfere with the pro-apoptotic pathways of the host cell.

SIAH

Drosophila seven in absentia (sina) protein and several orthologous genes in other species deserve special mention. Sina proteins are found already in plants and have remain very well

conserved in evolution. Humans have two members of this family known as *seven in absentia* homologous (SIAH)-1 and 2. SIAHs are E3 ubiquitin ligases, acting either as single proteins or as part of a multiprotein complex that is analogous to the Skp1-cullin-1-F-box (SCF) complex.[108] SIAH and TRAFs have only 10% sequence homology in their C-terminal domains based on a three-dimensional structure-based sequence alignment. However, the crystal structure of SIAH1a shows that its C-terminal domain forms an eight-stranded antiparallel β-sandwich, a fold that is virtually identical to a MATH domain (Fig. 1).[109] SIAH also have a N-terminal RING finger domains followed by 2 Zinc finger domains, besides the C-terminal TD-like domain,[109] which further extents the similarities between SIAHs and TRAFs. However, the RING and zinc finger domains found in SIAH and in TRAFs are of different subtypes, further suggesting that these two protein families are not related.

Similar to TRAFs and other MATHd encompassing proteins, SIAHs are E3 ubiquitin ligases that regulate ubiquitination and degradation of proteins implicated in a variety of physiological processes, including cell growth, differentiation, angiogenesis, oncogenesis, inflammation and stress (ref. 110 and refs. therein). Interestingly, one of these proteins targeted by SIAH is TRAF2. Indeed, SIAH interacts with TRAF2 through its TD and catalyzes its ubiquitination and degradation under stress conditions.[111]

There are two possibilities to explain the similarities between sina C-terminus fold and the MATHd. First, it would be possible that Sina and MATHd genes were originally related, sharing a common ancestor very early in eukaryotes evolution, but have since then diversified at sequence level beyond recognition while preserving the 8 anti-parallel β-sheet fold characteristic of the MATHd. A second possibility would be that these genes are an unparallel example of convergent evolution, having evolved to develop similar protein structure and function. The answer to this question awaits further research.

Concluding Remarks

In this chapter we have overviewed different aspects of the evolution of the MATHd. This fold appeared early in the evolution of eukaryotes and has remained very well preserved. Indeed, MATHd encompassing proteins from distant species can still associate both physically and functionally.[55] Furthermore, MATHd encompassing proteins from different families can also interact with each other in vitro.[7] Functional diversification has been achieved by association of the MATHd with other protein domains. However, the overall function of the vast majority of MATHd encompassing protein families involves protein processing by participating in different aspects of protein ubiquitination and protein degradation.

Current efforts on the sequencing of genomes from different organisms, including low eukaryotes with biomedical relevance, will provide new information on the origins of the MATHd. Furthermore, in addition to the large pool of functional data already available on TRAFs, recent reports have provided the first insights into the function of other MATHd encompassing protein families, such as UBPs (USP7) and MATHd/BTB (SPOP, MEL26) proteins. These studies illustrate the seminal roles of MATHd proteins in different aspects of physiology and predict new exciting discoveries to come.

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Dedication

This chapter is dedicated to the memory of José Zapata Ruiz, my beloved and admired father and strongest supporter.

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CHAPTER 2

TRAF1 and Its Biological Functions

Soo Young Lee and Yongwon Choi*

Abstract

Tumor necrosis factor (TNF) receptor-associated factor (TRAF)1 was originally identified based on its ability to interact with the cytosolic domain of TNF receptor type 2 (TNFR2). TRAF1 is unique among TRAF proteins in that it lacks RING domain found in the N-terminal regions of other TRAFs. TRAF1 can associate with multiple TNFR family members and can also bind several protein kinases and adaptor proteins suggesting that this protein likely possesses multiple functions in cytokine signaling networks. Although our understanding of TRAF1 functions and the underlying mechanisms at molecular and cellular levels has been advanced in recent years, much still needs to be learned before we have a full grasp of TRAF1 biology.

Introduction

The TNF receptor-associated factor (TRAF) family[1-6] is a group of structurally similar scaffold proteins that link members of the TNF receptor (TNFR) superfamily[7] to signaling cascades. Downstream signaling components of these cascades include the IκB kinase and mitogen-activated protein kinases, which in turn control gene expression through trascription factors such as NF-κB and AP-1. Analyses of knockout mice demonstrated critical roles of TRAF2,[8] TRAF5,[9] and TRAF6[10] in TNF receptor- and interleukin (IL)-1R/Toll receptor-induced activation of IκB kinase complex and Jun N-terminal kinase (JNK). Although TRAF1's function has been implicated in signaling by multiple receptors, the molecular modes of TRAF1 action are far from being fully understood. This chapter outlines what is known about TRAF1 so far and reviews recent data on the biological role of TRAF1 in regulating cytokine signaling networks.

Discovery of TRAF1

In 1984, TNF-α and LT-α (lymphotoxin, TNF-β), closely related two forms of TNF, were isolated from activated macrophages and T cells, respectively. These proteins have become representatives of a novel and unique superfamily of ligands called TNF ligand superfamily which currently includes at least nineteen family members all of which are identified in both mouse and human. Together with their corresponding family receptors (TNFR) containing 32 members, they are believed to play pivotal roles in many biological processes in mammalian cells, such as host defense, inflammation, apoptosis, autoimmunity, and development and organogenesis of the immune system (for a review see refs. 7, 11).

TNF-α is the prototype of TNF ligand superfamily members. Mammalian TNF-α signals through two distinct cell surface receptors: TNFR1, the primary receptor for soluble TNFs, and TNFR2, the main receptor for membrane-bound TNFs.[12] Although the study of receptor interacting proteins of

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TNFR1 has been one of the productive research areas in the field of cellular signaling, the receptor binding partners of the less extensively studied TNFR2 were first to be identified. The term "TRAF" was originally coined in 1994 by Rothe and coworkers.[13] Using biochemical purification techniques and yeast two-hybrid screening aimed at the identification of signal transducers of TNFR2, they identified two TNF receptor-assoicated factors which they termed TRAF1 and TRAF2. The hallmark feature of TRAFs is the C-terminal TRAF domain of approximately 230 amino acids.

One year later, Mosialos and coworkers[14] identified the human homolog of TRAF1 as Epstein-Barr virus (EBV)-induced mRNA 6 (EBI6), which means that it is more abundant in EBV-infected B lymphocytes than in uninfected control cells. The predicted 416 amino acid human protein is 86% identical to mouse TRAF1. Direct interaction of TRAF1 with CD30, a member of TNFR superfamily and a surface marker for Hodgkin's lymphoma, was first demonstrated using yeast two-hybrid screening in 1996. Deletion analyses of CD30 showed that the same C-terminal part of CD30 necessary for cell death induction was sufficient for interaction with TRAF1.[15] Since 1995, five additional members of the TRAF family, TRAF3-7, have been identified in both mouse and huma in a rapid succession. Over the last ten years it has been firmly established that the TRAFs are a group of structurally similar adaptor proteins for a wide variety of cell surface receptors and play important roles in regulating not only stress responses but also apoptosis (see refs. 1-6 for an extensive review).

Structural Domain of TRAF1

As mentioned above, the structural hallmark of TRAF proteins is a novel C-terminal homology region of 230 amino acids designated as the TRAF domain.[13] The domain can be subdivided into the more divergent N-proximal (TRAF-N) and the highly conserved C-proximal (TRAF-C) sub-domains. The TRAF domain is responsible for homo- and heterodimerization of the TRAF proteins as well as for their direct and indirect interactions with cognate surface receptors. In fact, amino acid sequence differences in the TRAF domain influence the range of receptors, heterodimerization partners, adaptor molecules and downstream signal transducers with which each TRAF interacts (for a review see ref. 6). Another important structural element is the N-terminal RING finger domain. It is found in TRAF2-7 and displays a highly conserved domain at the amino acids level. TRAF1 is unique among TRAFs in that it lacks the N-terminal RING finger domain. The deletion of the N-terminal RING domain of TRAF2, TRAF5, or TRAF6 proteins leads to the generation of dominant-negative TRAF mutants, suggesting RING domain of TRAF proteins is critical for relaying the signal to downstream effectors such as NF-κB or JNK.[16,17]

Expression and Regulation of TRAF1

The expression patterns of some of the TRAF proteins are strikingly specific and dynamic indicating the importance of proper spatio-temporal regulation of each TRAFs. Although other TRAFs including TRAF2, TRAF3, and TRAF6 are expressed ubiquitously,[2,18] TRAF1 mRNA expression can only be detected in a limited number of tissues such as spleen, lung, and testis. Furthermore TRAF1 can be dramatically induced by several distinct types stimuli such as interleukin (IL)-1, TNF-α, CD40 ligand[19] or EBV infection.[20] This is well demonstrated by in vitro and in vivo activation of T cells and B cells using a variety of stimuli including anti-CD3, anti-IgM, anti-CD40, LPS and PMA/ionomycin which results in a strong up-regulation of TRAF1.[15,21,22] To understand how the TRAF1 is transcriptionally regulated, Schwenzer and coworkers[19] performed in vitro DNA binding assays, promoter-driven reporter assays, and RNase protection assays with the human TRAF1 gene. Importantly, binding of NF-κB to three of five putative binding sites within the human TRAF1 promoter was found in electrophoretic mobility shift assay studies, and functional analyses of TRAF1 gene promoter luciferase constructs confirmed the importance of these *cis*-elements in TRAF1 expression. They found that the promoter of TRAF1 gene containing several functional κB sites is highly inducible by NF-κB. These results explain why various stimuli that activate NF-κB also induce TRAF1 expression.

Zapata and coworkers[18] performed a series of immunohistochemcal staining experiments to examine how the expression of TRAFs change in association with malignant transformation.

Interestingly, they found that TRAF1 is overexpressed in B cell lymphoma, including nonHodgkin lymphoma (NHL) and circulating chronic lymphocytic leukemias (CLL). This implies that TRAF1 overexpression does not require unique microenvironments found in lymphoid organs in vivo and further indicates that autonomous deregulated expression of TRAF1 may be associated with the development of B cell lymphomas. While the molecular explanation for the aberrant expression of TRAF1 in these cases is still elusive, it is proposed that deregulation of signaling pathways that regulate NF-κB causes TRAF1 overexpression in the lymphomas.

TRAF1-Binding Receptors and Intracellular Proteins

Many researchers have contributed to our knowledge of the plethora of TRAF1-interacting partners. A variety of binding proteins of TRAF1 including receptors, kinases, adaptors and regulator proteins have been identified by yeast-two hybrid screening, in vitro binding and overexpression experiments. As has been pointed in insightful reviews,[5,23] TRAF1 directly interacts with the cytoplasmic domains of distinct members of the TNFR superfamily such as CD30, 4-1BB, OX40, ATAR/HVEM, TRANCE-R/RANK, AITR, EDAR, TAJ/TROY. TRAF1 can also be recruited to the TNFR1 and TNFR2 through its interaction with TNFR-associated death domain protein (TRADD)[24] and TRAF2[13] respectively. From the biochemical and structural analyses, two consensus sequences recognized by the TRAF domain have been defined: the major one, (P/S/A/T)x(Q/E)E and the minor one, PxQxxD.[6,25-27] Furthermore, other motifs such as ExGKE[28] and the VxxSxxEE[29] could also mediate binding to the TRAF proteins. TRAF1 also binds several intracellular proteins, including protein kinases such as NF-κB-inducing kinase (NIK), apoptosis signal-regulating kinase 1 (ASK1), receptor-interacting protein (RIP) 1 and RIP2; adaptor and regulator proteins such as TRADD, TRAF-associated and NF-κB activator (TANK), TRAF-interacting protein (TRIP), the NF-κB inhibitory protein A20, apoptosis-suppressors inhibitor of apoptosis 1 (cIAP1), cIAP2, and FADD-like interleukin-1ß converting enzyme (FLICE)-like inhibitory protein (FLIP). The specific domains and motifs of TRAF1 that mediate the interaction with these proteins are yet to be clarified.

Antiapoptotic Role of TRAF1

Although TARF1 can be recruited to a number of distinct members of the TNFR superfamily and bind to several intracellular proteins, the biochemical and cellular functions of TRAF1 in these receptor signaling are less characterized. For example, the role of TRAF1 in regulation of NF-κB seems to be obscure in signaling by several TNFRs. Some investigators observed a stimulatory effect of TRAF1 on NF-κB activation mediated by TRAF2[30] whereas others did not.[31] Although TRAF1 itself does not activate NF-κB, it is believed that TRAF1 is involved in the regulation of NF-κB, possibly through its heterodimer formation with TRAF2. Arron and coworkers[32] recently proposed a novel stimulatory mechanism of TRAF1 in TRAF2-mediated signaling. According to this model, TRAF1, when it is up-regulated in response to TRAF2-mediated signal, regulates the removal of CD40 and TRAF2 from the membrane rafts and modulates the ability of TRAF2 to mediate sustained activation of NF-κB and JNK. In an apparent contradiction to this idea, Fortin-Mleczek and coworkers[33] found that NF-κB-induced TRAF1 expression counteracts CD40-mediated NF-κB activation. They thus proposed that, as TRAF1 itself is a target gene of the NF-κB pathway, it might serve as a feedback regulator of this pathway that interferes with NF-κB activation by selected range of NF-κB-inducing receptors. The inhibitory role of TRAF1 in CD40 signaling is clearly contradictory to the observations of Arron and coworkers.[32] We will further discuss the positive effect of TRAF1 on NF-κB pathway deduced from the analyses of TRAF1 knockout mice (see section "Role of TRAF1: Lessons from Knockout Mice").

The main function of TRAF1 is believed to be suppression of TNF-α- or T cell receptor (TCR)-mediated apoptosis (Fig. 1). Upon binding of TNF-α to TNFR1, TRADD recruits secondary adaptors RIP1, TRAF2, or TRAF5 (for a review see ref. 34). This causes activation of the IKK complex which in turn activates NF-κB.[35] TNFR1 also recruits the adaptor protein FADD and caspase-8 upon binding of the ligand TNF-α to initiate apoptosis. In the other interaction, TNFR1, when in complex with TRADD, TNFR1-TRADD complex recruits ancillary proteins such as TRAF1,

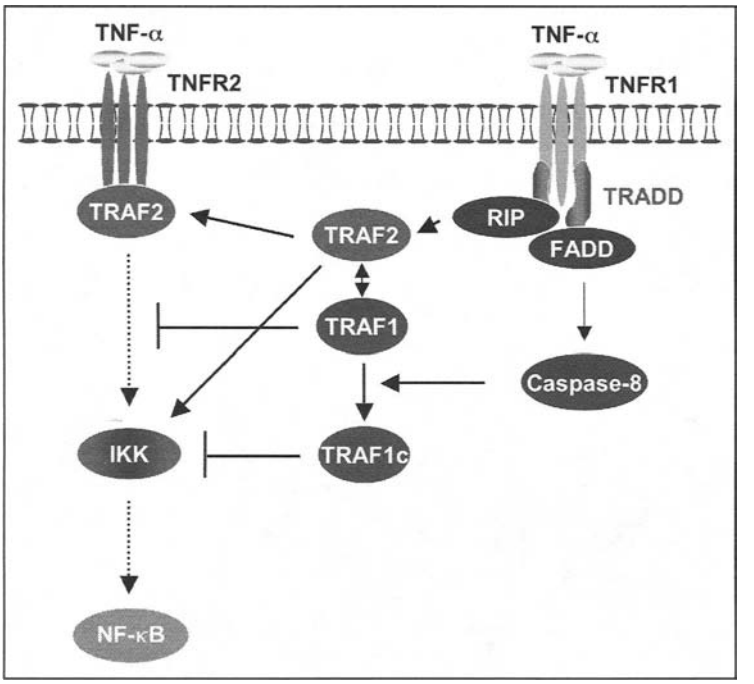

Figure 1. Proposed model of the regulatory role of TRAF1 in TNFR1 and TNFR2 signaling. Left) Upon TNF-α ligand engagement, TNFR2 recruits TRAF2 to activate transcription factors such as NF-κB. TNFR2 also recruits TRAF1 via its interaction with TRAF2. TRAF2-TRAF1 heterodimers inhibit TNFR2-mediated signal, possibly due to interfering with the recruitment or activation of the protein kinases such as IKK complex. Right) With regard to TNFR1 signaling, TRAF1 can be cleaved by caspase-8 which generates two fragments. The C-terminal TRAF1 fragment (TRAF1c) inhibits NF-κB activation through a physical interaction with IKK.

TRAF2, cIAP1 and cIAP2,[36] which can suppress TNF-α-dependent caspase-8 activation, thereby preventing apoptosis.[37]

Speiser and coworkers[38] demonstrated first in vivo role of TRAF family member using TRAF1 overexpressing transgenic approach. TRAF1 overexpression inhibits the antigen-induced deletion of activated $CD8^+$ T cells in vivo and in vitro. The precise mechanism by which TRAF1 overexpression inhibits the induction of apoptosis is not clear. Since TRAF1 (and also other TRAF proteins) does not contain any known catalytic domain and TCR-induced apoptosis of $CD8^+$ T cells is mediated by the TNFR2 signaling complex, it is most likely that TRAF1 overexpression inhibits the TNFR2-mediated apoptosis by altering the constituents of the TNFR2 signaling complex. It is also possible that TRAF1 may mediate an as-yet-to-be determined antiapoptotic signal during antigen-induced cell death of mature T cells.

Interestingly, several investigators showed a proapoptotic role of caspase-cleaved TRAF1 fragment.[39-41] TRAF1 can be converted into a proapoptotic version after cleavage by an upstream initiator caspase-8 during Fas ligand- or TNF-α-induced apoptosis. Caspase-8 cleaves TRAF1 into two fragments. Overexpression of the C-terminal TRAF1 fragment but not N-terminal fragment enhances TNFR1- and Fas-mediated apoptosis. Recently, Henkler and coworkers[42] showed that TRAF1 and its cleavage product selectively interfere with the recruitment of TRAF2 to some members of TNFR family. They also demonstrated that the C-terminal fragment of TRAF1 but not full-length version blocks IKK activation through a physical interaction with IKK. It would be important to determine whether the relative amount of full-length TRAF1 and caspase-cleaved TRAF1 accumulated in cells may dictate cytoprotective versus cytotoxic role of TRAF1 when cells

are exposed to stimuli such as TNF-α. Unknown factors that alter the ratio of these two versions of the TRAF1 protein presumably would drive the signaling process either toward or away from an apoptotic result. The generation of knock-in mice expressing cleavage-resistant but signaling competent TRAF1 mutant protein would be required to address the in vivo functions of TRAF1.

Role of TRAF1: Lessons from Knockout Mice

Tsitsikov and coworkers[43] generated TRAF1 knockout mice. They found that TRAF1-/- mice are born normal and grossly normal in appearance. TRAF5-/- mice,[44] like TRAF1-/- mice, showed no apparent phenotype. In contrast, TRAF2-[8] and TRAF3-deficient mice[45] exhibit lymphopenia and die prematurely, and TRAF4-/- mice[46] exhibit tracheal malformations. TRAF6-/- mice[10] display severe osteopetrosis, become runted, and die at the age of 17-19 days. Although the T and B cell development in lymphoid organs of TRAF1-/- mice and the function of TRAF1-/- B cells appeared to be normal, TRAF1-/- T cells exhibited a hyperproliferative response when stimulated through TCR complex with anti-CD3. TNF-α caused marked proliferation of anti-CD3 stimulated T cells from TRAF1-/- mice but not wild type T cells. Since activated T cells selectively express TNFR2 but not TNFR1, TRAF1 likely inhibits activation signals delivered via TNFR2. They also showed that TRAF1-/- T cells had enhanced NF-κB and JNK activation in response to TNF-α. These findings together suggest that TRAF1 is a negative regulator of TNFR2 signaling in T cells. Tsitsikov and coworkers suggested several possible scenarios of how TRAF1 inhibits TNF signaling. One possible mechanism is that TRAF1 competes with TRAF2 for interaction with TNFR2. Another possibility is that TRAF1 binds to TRAF2 to form an inactive heterodimer. Yet another possibility is that TRAF1 may regulate molecules other than TRAF2 that are required for efficient TRAF2 signaling. Finally, TRAF1 may recruit other molecules that negatively regulate TNF-α signaling.

Arron and coworkers[32] also generated TRAF1 knockout mice and examined their physiological roles in dendritic cells (DCs). Consistent with their findings from overexpression experiments, they showed that maturation induced by CD40 ligand (CD40L) leads to a loss of soluble TRAF2 and a concomitant reduction in TNF-α and CD40L-mediated survival, revealing a physiological role for TRAF1 in the regulation of TRAF2-dependent signaling. Given that TRAF1 is highly expressed in DCs[18] and that it regulates the availability of TRAF2 for antiapoptotic signaling, they proposed that the balance between caspase activation and pro-survival signals is regulated to some extent by TRAF1 in DCs. This hypothesis is consistent with their finding in TRAF1-/- DCs that maturation of DCs by CD40L tilts the balance of TNF-α signaling from survival to apoptosis due to a depletion of soluble TRAF2.

Concluding Remarks

The TRAF1 story began in 1994 with the connection to signal transduction pathways of TNFR2. Despite significant progresses, challenges remain to be overcome before we completely understand the biological roles of TRAF1. Chief among these is understanding why TRAF1 protein is typically overexpressed in B cell lymphomas. Could it be that TRAF1 overexpression is causally linked to B cell lymphoma development? Further work is needed to determine the precise biochemical basis of TRAF1's function in neoplastic B cells. It will also be important to define precisely inhibitory or stimulatory role of TRAF1 in TNFR superfamily signaling through a careful analyses of TRAF1 knockout mice. Given that TRAF1 can be recruited to a variety of distinct members of TNFR family, it will be of importance to determine whether these different roles of TRAF1 are dependent on the distinct receptors binding to TRAF1.

Understanding the mechanisms underlying TRAF1's action in cytokine signaling networks may lead to improved diagnostic techniques and development of new therapy for pathophysiological conditions associated with malfunctions of TNF-lelated cytokines.

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CHAPTER 3

Physiological Roles and Mechanisms of Signaling by TRAF2 and TRAF5

Ping-Yee Billie Au and Wen-Chen Yeh*

Abstract

TRAF2 and TRAF5 are closely related members of the TRAF family of proteins. They are important signal transducers for a wide range of TNF receptor superfamily members, including TNFR1, TNFR2, CD40 and other lymphocyte costimulatory receptors, RANK/TRANCE-R, EDAR, LTβR, LMP-1 and IRE1. TRAF2 and TRAF5 therefore regulate diverse physiological roles, ranging from T and B cell signaling and inflammatory responses to organogenesis and cell survival. The major pathways mediated by TRAF2 and TRAF5 are the classical and alternative pathways of NF-κB activation, and MAPK and JNK activation. TRAF2 is heavily regulated by ubiquitin signals, and many of the signaling functions of TRAF2 are mediated through its RING domain and likely its own role as an E3 ubiquitin ligase.

Introduction

The focus of this chapter will be on TRAF2 and TRAF5, which are closely related in both structure and function. Since they play important roles in mediating signals induced by the TNF receptor superfamily, the physiological roles of TRAF2 and TRAF5 will be discussed in the context of the receptors that they associate with. In addition, TRAF2 has been reported to play roles in LMP-1 signaling and endoplasmic reticulum (ER) stress responses. These two signaling contexts will be discussed at the end of this chapter.

TNF-associated factor 2 (TRAF2), a 56kD protein, was discovered through yeast two-hybrid screening for proteins interacting with the c-terminal region of human TNF receptor 2.[1] Along with TRAF1, TRAF2 was one of the first members of the TRAF protein family to be identified. TRAF5 was later discovered through yeast two hybrid interaction, while screening for proteins binding to the cytoplasmic tail of CD40.[2] Furthermore, TRAF5 was independently identified as a protein interacting with the lymphotoxin β receptor (LTβR).[3]

Like all TRAF family members, TRAF2 and TRAF5 are characterized by a highly conserved carboxy-terminal TRAF domain, which can be further subdivided into TRAF-N and TRAF-C domains. The TRAF domain mediates receptor binding, interactions with a number of adapter and signaling molecules, self association, and interactions with other TRAF proteins. TRAF2 can oligomerize with itself or with TRAF1 or TRAF6.[1,4] TRAF5 also associates with itself, but is also known to hetero-oligermize with TRAF3.[5] In addition to the conserved TRAF domain, TRAF2 and TRAF5 each contain an N-terminal ring finger domain followed by five zinc fingers and a coiled-coil domain.

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TRAF5 is highly similar to TRAF2 both structurally and functionally. However, whereas TRAF2 is expressed ubiquitously, TRAF5 expression is only found at significant levels in lung, thymus, spleen, and kidney and at lower levels in brain and liver.[1,2,6] This more restricted expression pattern may explain to some extent why deletion of TRAF2 leads to perinatal lethality whereas deletion of TRAF5 only leads to more specific defects in CD40 and CD27 mediated lymphocyte activation. On the other hand, double knockouts of TRAF2 and TRAF5 suggest some functional redundancy between these two molecules in the context of TNF induced NF-κB activation.[6-8]

Mechanisms of TRAF2/5-Mediated Signal Transduction

There have been many studies over the years that have examined TRAF2 signaling and regulation. TRAF5 has also been examined, albeit to a lesser extent, therefore, the focus of this section will be on TRAF2.

TRAF2/5 and NF-κB Activation

NF-κB is one of the primary pathways activated by TRAF2 and TRAF5. Since NF-κB activation is not significantly impaired in mouse embryonic fibroblasts derived from TRAF2 and TRAF5 single knockouts, but is significantly reduced in TRAF2/TRAF5 double knockouts, there is some functional redundancy between the two molecules in this context.[8]

Activation of most receptors, including TNFR1, result in the activation of the canonical NF-κB pathway. The canonical pathway generally depends on the activation of IKKβ and IKKγ/NEMO by upstream kinases, including the involvement of the TAK1 kinase complex.[9] The IKK complex, consisting of IKKα, IKKβ and IKKγ/NEMO, then goes on to phosphorylate IκB, which targets the molecule for ubiquitination and proteasome-mediated degradation. As IκB normally binds and sequesters NF-κB in the cytoplasm, its degradation results in release and translocation of NF-κB to the nucleus. The canonical pathway results in the formation of primarily p65/RelA-p50 heterodimers.[10-14]

Early studies have indicated that an intact RING domain is important for TRAF2 functions, including activation of NF-κB and JNK. The RING presence of the domain also suggested a role for ubiquitination in TRAF2 function and regulation. Interestingly mutational analyses also indicated that the RING domain and fourth zinc finger are necessary for TRAF2 ubiquitination.[15] Mutational analyses have also identified that the amino-terminal ring finger and two adjacent zinc fingers of TRAF2 are required for NF-κB activation.[16] Like TRAF2, TRAF5 contains a similar RING domain.[2]

TRAF2 associates with the E2 ligase complex Ubc13-Uev1A to catalyze the synthesis of polyubiquitin chains through a lysine-63 (K63) linkage.[15] K63 linkage poly-ubiquitin chains are found an TRAF2, TRAF6, RIP1 and NEMO, and are therefore important for signalling in TNFR family pathways. In TRAF2-deficient cells, K63 polyubiquitination of RIP1 is defective, indicating that TRAF2 is likely the E3 ligase involved in RIP1 ubiquitination.[17] Alternatively, TRAF2 may be required for recruiting other E3 ligases, such as A20, to help processing and turnover (see Fig. 1).[18,19] Since recruitment of the TAK1 kinase complex is dependent on ubiquitinated RIP1, TRAF2 mediated ubiquitination is likely critical in activating the canonical NF-κB pathway.[17] It as also been shown that TNFR1 activation of the IKK complex and NF-κB activation requires both RIP1 and TRAF2, where RIP1 is responsible for IKK activation and TRAF2 is necessary for recruitment of IKK to the complex.[11,20] Interestingly, while overexpression of TRAF2 RING domain mutants incapable of auto-ubiquitination suggested that the RING domain is not necessary for IKK activation, complete deletion the RING domain prevented IKK activation.[15] Furthermore, TRAF2 is also known to complex with proteins such as TANK and the kinase T2K/TBK1, which have also been shown to play a role in NF-κB activation.[21,22]

Activation of NF-κB may also occur through an alternative pathway. This pathway is primarily found in B cells; however, it can be present in other cell types as well. The noncanonical or alternative pathway depends on activation of NIK and IKKα. IKKα activation leads to NFκB2/p100 processing to p52 and the formation of p52/RelB-p50 heterodimers.[23-27]

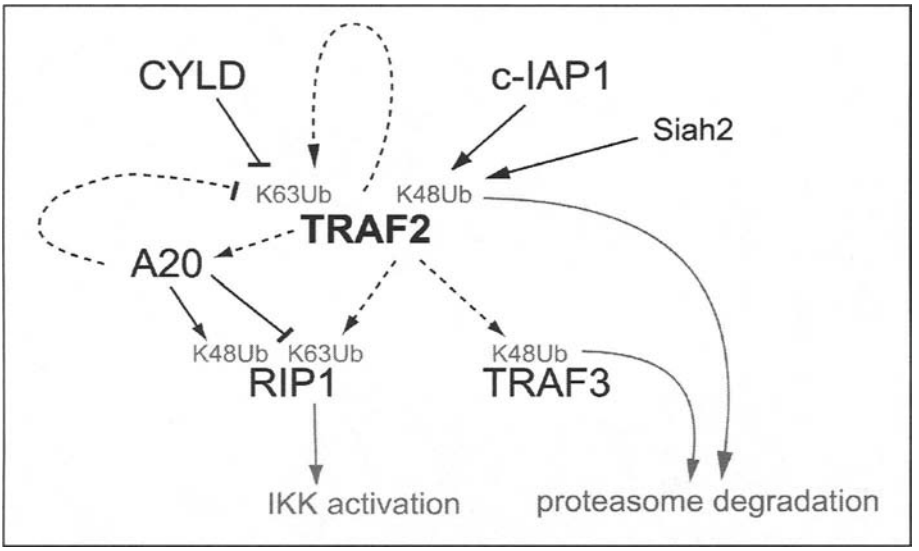

Figure 1. TRAF2 and regulation by ubiquitin. TRAF2 is regulated by both K63 and K48 type ubiquitin chains. K63 linkage may be mediated by TRAF2 auto-ubiquitination, and is required for TRAF2 activation. A20 and CYLD can remove K63 ubiquitin chains to inhibit TRAF2 activity. c-IAP1 and Siah2 are known E3 ligases that can K48-ubiquitinate TRAF2 to target it for proteasome-dependent degradation. TRAF2 may also act as an E3 ligase itself to modulate the activity of downstream molecules. RIP1 may be a direct target for TRAF2 mediated K63-ubiquitination. K48-ubiquitination, leading to degradation of RIP1, may be mediated by TRAF2 recruitment of A20. TRAF3 may be a target for TRAF2 K48-ubiquitination as well.

TRAF2 has been implicated in both activation and negative regulation of the noncanonical NF-κB pathway. As mentioned later in the discussion of the role of TRAF2 in CD40 signaling, conditional knockout of TRAF2 in B-cells results in high levels of alternative NF-κB pathway activation, suggesting that TRAF2 can inhibit p100/p52 processing.[25] On the other hand, mutation of the TRAF2/5 binding site on CD40 abolished p52/RelB translocation to the nucleus, suggesting that TRAF2 and TRAF5 may be required for noncanonical NF-κB pathway activation as well.[28]

Regulation of the alternative pathway by TRAF2 is complicated further by the involvement of TRAF3. In the context of CD40 signaling, TRAF3 overexpression has been recently found to inhibit of TRAF2/TRAF5 mediated activation of the alternative pathway, but not TRAF6 dependent activation of the canonical pathway.[28] Interestingly, TRAF2-deficient B cells appear to have increased levels of TRAF3, indicating that TRAF2 helps target TRAF3 for ubiquitinantion and degradation(see Fig. 1).[29]

TRAF2 and JNK and MAPK Activation

TRAF2 and TRAF5 also signal through MAPK induction, primarily through activation of JNK. TRAF2 has been found to interact with a variety of upstream MAP3Ks, including MEKK1 and ASK-1, germinal center kinase and germinal center kinase kinase.[30-33] TRAF5-deficient cells do not, however, demonstrate detectable impairment in JNK activation in response to TNF.[7]

K63 ubiquitination of TRAF2 appears to be critical for activation of JNK. TRAF2 is able to bind ASK1, GCK and GCKR through its RING domain, however, siRNA knockdown of Ubc13 has shown that activation of GCKR and the SAPK/JNK pathway also depends on the presence of Ubc13 E2 ligase complex. Activation of ASK1, in contrast, only marginally depends on Ubc13, and neither p38 MAPK nor IKKβ activation is affected by knockdown of Ubc13.[15,34]

Regulation of TRAF2

TRAF2 signaling also appears to be regulated by translocation. Recruitment of TNFR1, TRADD, RIP and TRAF2 to plasma membrane lipid rafts is important for signalling NF-κB activation.[35] Furthermore, TRAF2 ubiquitination appears to coincide with the translocation of TRAF2 to the insoluble membrane/cytoskeletal fraction, and appears to have a role in regulating TRAF2 levels.

Studies have demonstrated that translocation to lipid rafts precedes ubiquitination, and have also suggested that compartments such as the endoplasmic reticulum may play roles in modulating TNFR signalling.[35,36] Upon TNFR2 engagement, c-IAP1, an E3 ligase, can ubiquitinate TRAF2.[37] Recent research has shown that c-IAP1 associates with the E2 ligase Ubc6, which is a an ER transmembrane protein. c-IAP1 and Ubc6 are responsible for synthesis of K48 type ubiquitin chains on TRAF2 that target it for degradation in a proteasome-dependent manner.[36,37] In addition to c-IAP1, the E3 ligase Siah2 has also been found to regulate TRAF2 levels through ubiquitination. Using Siah2-deficient cells from knockout mice, it was found that Siah2 targeted TRAF2 for degradation under stress conditions, including TNF stimulation with cyclohexamide and UV irradiation.[38]

TRAF2 is also regulated by de-ubiquitinating enzymes. The tumour suppressor CYLD has be found to inhibit NF-κB activation. CYLD appears to regulate NF-κB by binding and removing ubiquitin chains on TRAF2, therefore preventing TRAF2 activation of the IKK complex.[39-41] A20, a TNF-inducible gene, has also been found to interact with TRAF2 and inhibit NF-κB activation.[18] A20 has been found to possess both ubiquitin ligase and de-ubiquitination activity, and is known to downregulate NF-κB activity by removing K63 ubiquitin chains from RIP1 and by adding K48 ubiquitin. It is probable that A20 is also involved in de-ubiquitinating and de-activating TRAF2.[19]

Finally, it is also important to note that TRAF2 can target itself for degradation through K48 ubiquitin chain synthesis. CD40 induced TRAF2 degradation, for example, requires an intact TRAF2 RING domain.[42] The duality of the TRAF2 E3 ligase, in that it is able to generate ubiquitin chains that lead to both activation (such as RIP1) or inactivation (such as TRAF3), gives this molecule a unique role depending what it interacts with (see Fig. 1).

Receptors and Pathway Anchor Proteins That Utilize TRAF2 and TRAF5

TNFR1

The role of TRAF2 is perhaps best characterized for TNF signaling through TNF receptor 1 (TNFR1). TNF is a major proinflammatory mediator, and can induce apoptosis under certain circumstances. It is responsible for not only immune response, but also development and tissue regeneration, and has been found to have pathophysiological roles in septic shock, autoimmune disease, and cancer. TNFR1 appears to be the key mediator of TNF signalling in the majority of cells.

Upon TNF binding, TNFR1 recruits several signaling proteins to its cytoplasmic death domain. TNFR1-associated death domain protein (TRADD) is first recruited via its death domain to the death domain of TNFR1, and acts as an adaptor molecule. Fas-associated death domain protein (FADD) interacts with the carboxy-terminal death domain of TRADD, which exposes the death effector domain of FADD, allowing FADD to recruit caspase 8/FLICE, which leads to the activation of the apoptotic cysteine protease cascade.[43-47] RIP1 is a serine-threonine kinase that binds the carboxy-terminal death domain of TRADD in a TNF-dependent manner. TRADD also directly interacts with TRAF2 via its amino-terminal half[43,46-49] Recent studies have suggested that a complex involving TNFR1, TRADD, RIP1 and TRAF2 at the plasma membrane is formed first, and rapidly signals NF-κB activation and cell survival. A second complex consisting of TRADD, TRAF2, RIP1, FADD and caspase-8 is formed later in the cytoplasm to signal cell death under certain contexts.[50] (see Fig. 2)

Half of mice deficient in TRAF2 die at E14.5 with a similar phenotype to RelA deficient mice, whereas the rest are born normal but are runted and die prematurely with atrophy of the thymus and spleen, and show elevated serum TNF levels. Thymocytes and other hematopoietic cells also show extreme sensitivity to TNF induced cell death. These phenotypes suggest that TRAF2 plays an important physiological role in regulating cell survival, particularly in response to TNF, since TRAF2

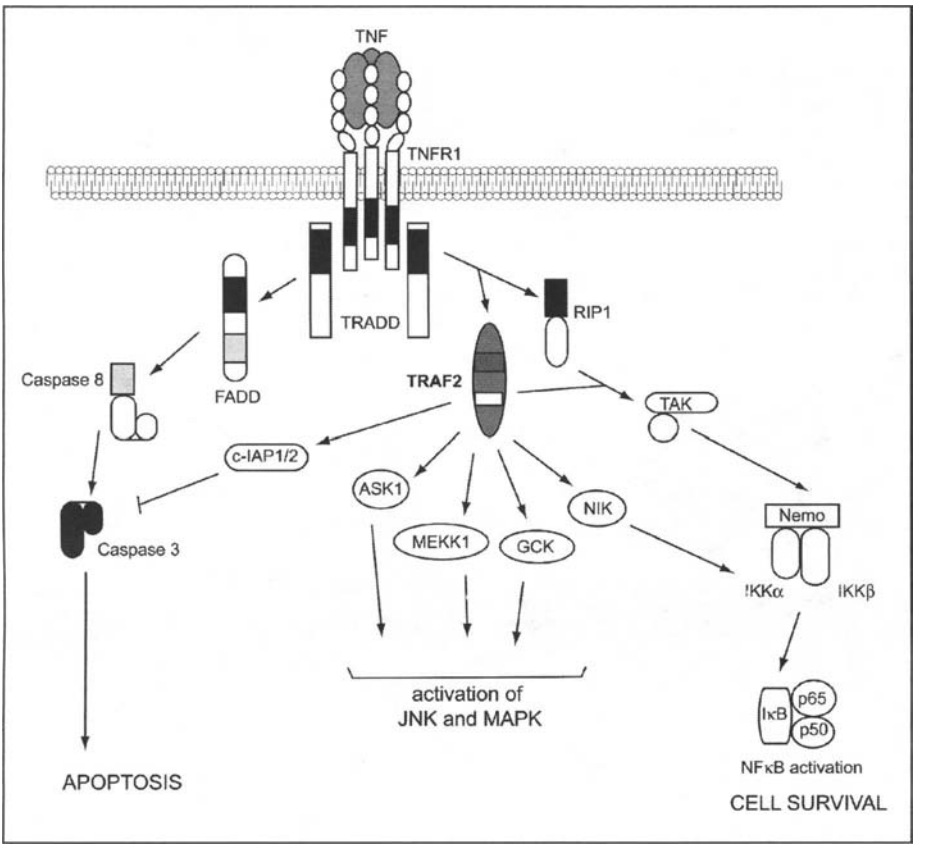

Figure 2. The function of TRAF2 in TNFR1 signaling. TRAF2 is a key molecule in TNFR1 signaling. Upon receptor activation, TRAF2 is recruited via the adapter TRADD. TRAF2 can then go on to activate a variety of downstream MAPKs and JNK. Together with RIP1, TRAF2 is also important in activating the IKK complex. IKK phosphorylates and targets IκB for proteasome-dependent degradation to allow the release and nuclear translocation of the NF-κB. Transactivation of NF-κB target genes isimportant for cell survival and inflammatory signals. TRAF2 is also able to recruit c-IAP1 and c-IAP2, which inhibit caspase activation and apoptosis. TRAF5 appears to play a similar and redundant role in this pathway.

knockout mice can be rescued by crossing with TNF or TNFR1 knockout.[6,51] On the other hand, targeted disruption of TRAF5 in mice does not lead to perinatal lethality, suggesting that it has a more minor role in TNF cytoprotection. Furthermore, hyperactivity of certain TNF responses, including increase NO and TNF production by macrophages, has also been observed in TRAF2 knockout mice, indicating that TRAF2 also has an important role in regulating TNF mediated immune responses.[51]

Activation of NF-κB and JNK/SAPK may be important pathways through which TRAF2 mediates cytoprotection against TNF. From knockout studies, it is known that RIP1 is essential for NF-κB activation induced by TNF, and that NF-κB activation is essential for cell survival in response to TNF.[13,52] However, as mentioned previously, TRAF2-deficient cells are not significantly defective in NF-κB activation[6,52,53] even though overexpression of TRAF2 or TRAF5 can activate NF-κB in cells.[2,3,54] TRAF2 and TRAF5 double knockout cells, however, do demonstrate more impaired NF-κB activation than single knockouts, suggesting that TRAF5 may compensate for the lack of TRAF2 in this signalling pathway.[8] The TNFR1-TRADD-RIP1-TRAF2 signaling complex

primarily leads to induction of the classical NF-κB signalling pathway. As discussed in the previous section, this depends on the activation of IKKβ and IKKγ/NEMO, and results in the formation of primarily p65/RelA-p50 heterodimers.

TRAF2 and TRAF5 have also been implicated in MAPK activation and regulation of the AP-1 transcription factor, as cells lacking TRAF2 also demonstrate severe impairment in JNK/SAPK activation upon TNFR1 stimulation.[6] Cells deficient in both TRAF2 and TRAF5 have been found in some cases to demonstrate a delayed but prolonged MAPK activation in response to TNF, which has been linked to increased TNF-induced reactive oxygen species signalling and induction of cell death.[6,55]

TRAF2 also promotes survival in response to TNF by recruiting c-IAP1 and c-IAP2 to the TNFR1 complex. c-IAP1 and c-IAP2, both typical members of the BIR domain containing inhibitors of apoptosis family, are able to prevent caspase-3 activation and apoptosis.[48,56,57]

TNFR2

Unlike TNFR1, TNFR2 does not possess a Carboxy-terminal death domain, and TRAF2 directly binds to the cytoplasmic tail of TNFR2. While TRAF1 cannot bind directly to TNFR2, TRAF1 can be recruited to complex indirectly via interaction with TRAF2, and may act as a negative regulator of TNFR2 signaling through TRAF2.[1,58] TRAF5 has not been found to bind to the cytoplasmic tail of TNFR2.[2] Signaling downstream of TNFR2 and TRAF2 is relatively similar to TNFR1. As mentioned previously, RIP1 can bind to TRAF2, and also associates with TNFR2. TNFR2 recruitment of TRAF2 is also involved in both NF-κB and MAPK activation, indicating that TRAF2 is important in the crosstalk between TNFR2 and TNFR1.[59,60]

As mentioned previously, TRAF2 interacts with both c-IAP1 and c-IAP2. This interaction was initially identified as part of TNFR2 complex.[61] More recent studies looking at the TNFR2-TRAF2 complex have demonstrated that the carboxy-terminal of c-IAP1 acts as an E3 ubiquitin ligase that is able to ubiquitinate TRAF2 and target it for proteasomal degradation.[37] As TRAF2 typically signals cell survival through NF-κB and JNK activation, this suggests a mechanism through which proteins recruited by TRAF2 can enhance TNF induced apoptosis, and that TNFR2 activation can help regulate TNFR1 signals.

CD40

CD40 is a TNFR family member that is expressed constitutively by antigen presenting cells, such as B-lymphocytes, macrophages and dendritic cells. Activation by its ligand, CD40L/CD154, induces a variety of effector functions, including upregulation of molecules involved in antigen presentation and B and T cell interactions, antibody production, isotype switching, cytokine secretion, and protection from apoptosis.[62,63]

Both TRAF2 and TRAF5 have been implicated in CD40 signaling. Although TRAF5 was originally identified as a protein binding to the cytoplasmic domain of CD40, subsequent studies have shown that TRAF5 recruitment to CD40 is indirect through hetero-oligomerization with TRAF3. TRAF2, on the other hand, is able to directly associate with CD40.[5] TRAF1, TRAF2, and TRAF3 associate with CD40 via a PVQET motif, wherease TRAF6 associates in a different region. Competition and different combinations of TRAF recruitment to CD40 may therefore contribute to modulating receptor signals across different cell types.[64]

While CD40 can induce p65RelA-p50 NF-κB activation, CD40 is also known to induce NFκB2/p100 processing and the alternative NF-κB pathway.[27] Dominant negative TRAF2, which lacks the amino-terminal RING finger domain, inhibits CD40 mediated NF-κB activation.[54] Studies using TRAF2-deficient B cell lines expressing mutant CD40 defective in TRAF6 binding have also shown that NF-κB pathway activation, as demonstrated through IκB phosphorylation and degradation, is impaired when both TRAF2 and TRAF6 binding are absent. However, neither TRAF2 nor TRAF6 binding alone are indispensable for CD40-induced NF-κB activation.[29]

More recent studies looking at conditional knockout of TRAF2 in B-cells have shown that while TRAF2 is necessary for canonical activation of NF-κB in response to CD40, deficiency in TRAF2 actually results in hyperactivity of the alternative NF-κB pathway. TRAF2-deficient B-cells demonstrated a survival advantage and upregulation of CD21/35. TRAF2 can therefore act as a

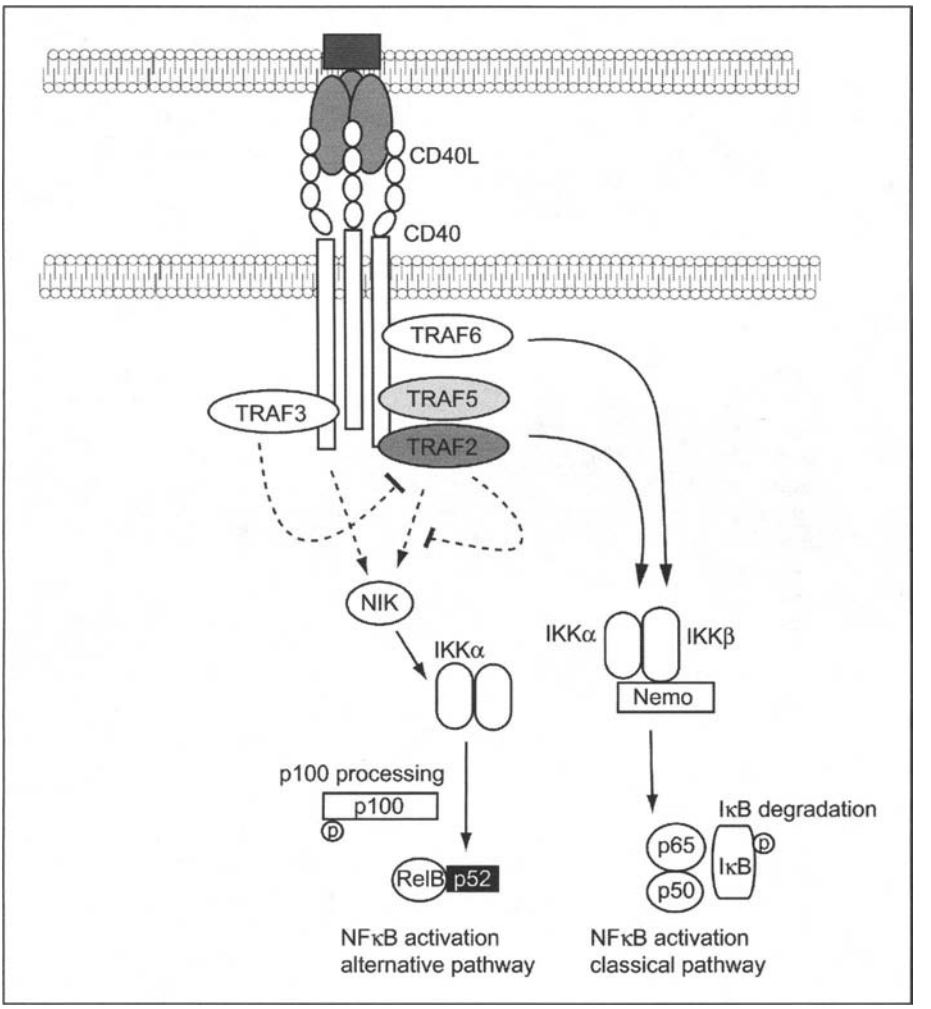

Figure 3. The function of TRAF2 and TRAF5 in CD40 signaling. Unlike TNFR1, but similar to the majority of TNF superfamily receptors, CD40 is able to recruit TRAFs directly to its cytoplasmic domain. TRAF2, TRAF5 and TRAF6 have all been implicated in NF-κB activation. Interestingly, TRAF2 has been found to activate and inhibit the alternative NF-κB pathway mediated through NIK and IKKα, resulting in p100 processing to p52. TRAF3 may compete with and inhibit TRAF2.

negative regulator of p100/p52 processing.[25] In contrast to TRAF2, deficiency in TRAF5 does not affect NF-κB or JNK signalling in response to CD40.[7] (see Fig. 3)

TRAF2 has also been found to be important in B-cell receptor (BCR) and CD40 synergy. Antigen stimulation of BCR leads to activation of a variety of downstream signalling molecules and second messengers, including members of the protein kinase C family (PKC) and protein kinase D (PKD). Pharmacological inhibition of PKD in B-lymphocytes was found to prevent CD40 and BCR synergy. B cells expressing a mutant CD40 defective in TRAF2 binding also demonstrate a BCR-CD40 synergy defect, however, overexpression of constitutively active PKD in these cells is unable to overcome the defect observed, indicating that TRAF2 is required for PKD-mediated enhancement of BCR-CD40 signals.[29,65,66]

Deficiencies in either TRAF2 or TRAF5, however, demonstrated CD40 signaling defects in vivo. Crossing with TNF or TNFR1-knockout mice aids the survival of TRAF2-knockout mice and has allowed the investigation of TRAF2-deficiency on CD40 responses in lymphocytes. TRAF2-deficiency results in impaired isotype switching and failure to mount IgG responses induced by vesicular stomatitis viral infection. TRAF2-deficiency also leads to defective CD40 mediated proliferation and NF-κB activation in splenocytes.[51] TRAF5-deficient mice reveal impairment of CD40 stimulated B-cell proliferation and upregulation of surface markers, and also show mild defects in affinity maturation of IgG antibodies.[7]

TACI, BCMA

BCMA (B-cell maturation antigen) and TACI (transmembrane activator and CAML interactor) are TNF receptor superfamily members that share the ligands BAFF (B-cell activating factor) and APRIL. Both receptors are expressed primarily on B-lymphocytes. TRAF2, TRAF5 and TRAF6 have been shown to associate with TACI. TRAF2 and TRAF5 share a binding motif, but the majority of positive clones from yeast-two hybrid were TRAF2-TACI interactions, suggesting that TRAF2 may play a more prominent role. Like other TNF receptor superfamily members interacting with TRAFs, activation of TACI also results in NF-κB and JNK activation.[67]

TRAF1, TRAF2 and TRAF3 interact with the cytoplasmic region of BCMA. Analyses of deletion mutants of the TRAF binding domain in BCMA demonstrated that TRAF association is required for NF-κB, Elk-1 and JNK activation in response to BCMA.[67,68] However, although BAFF stimulation is important for B cell survival and proliferation, the phenotypes of mice deficient in TACI and BCMA indicated that these receptors are not responsible for the survival signal. There is no obvious phenotype for the BCMA knockout,[69,70] and TACI-deficient mice actually show increased numbers of B-cells.[71] The survival signal was actually found to be mediated primarily through TRAF3 by BAFF activation of BAFF-R. Furthermore, while activation of BAFF-R leads to alternative NF-κB pathway activation, which has been implicated in B-cell survival, activation of BCMA or TACI does not.[71,72] Therefore, signalling through BCMA or TACI through TRAF2 appears to mediate signals other than BAFF-R signals.

CD30

CD30 is a cell surface receptor characteristic of activated T-lymphocytes. CD30 stimulation can lead to cell proliferation, survival, differention, or cell death, depending on cell type and costimulation. CD30 is also an important marker for Hodgkin's and other lymphomas, and is upregulated in several virally transformed cell lines.[73]

Like many other TNFR family members, signalling through CD30 is transduced through TRAFs and can lead to activation of NF-κB and MAPKs.[74-76] Two different regions in the c-terminal tail of CD30 are capable of binding TRAFs. The more N-terminal domain in the tail can bind TRAF3, TRAF2, and TRAF5, whereas the more C-terminal domain can bind TRAF1, TRAF2 and TRAF5. Expression of a dominant negative TRAF2 or TRAF5 resulted in impaired CD30 mediated NF-κB activation. While TRAF2 and TRAF5 are both implicated in NF-κB activation in response to CD30 stimulation, mutation of a more membrane proximal domain that is not known to bind TRAFs can also abrogate CD30 induced NF-κB activation.[74,75,77,78]

CD27

CD27 is a receptor expressed on T, B, and NK cells. CD27 plays an important role in T cell interactions and T and B cell interactions, and provides an important costimulatory signal for proliferation.[73] Both TRAF2 and TRAF5 interact with the cytoplasmic tail of CD27. Deletion analysis of the cytoplasmic domain identified a critical motif that is necessary for CD27 mediated NF-κB and JNK activation, and that this motif coincides with the binding site for TRAF2 and TRAF5. Overexpression of dominant negative TRAF2 or TRAF5 was also found to block NF-κB activation.[79,80] In vivo, thymocytes from TRAF5-deficient mice demonstrate defects in CD27 costimulation of CD3-induced T cell proliferation. However, NF-κB and JNK activation are not noticeably altered in these cells, which may be due to either compensation from other TRAFs or a

role for TRAF5 in CD27 signaling that does not require NF-κB or JNK.[7] Furthermore, since CD27 has been implicated in regulation of humoral responses, the effect of TRAF deficiency on both CD40 and CD27 responses may contribute to observed lymphocyte phenotypes.

Ox40

Ox40 is another TNFR superfamily member involved in costimulation, and is expressed on activated T cells. Studies from Ox40-deficient mice demonstrate important roles for this receptor in regulating the number of effector T cells during primary immune response, and the number of memory T cells that develop and remain.[73]

TRAF1, TRAF2, TRAF3 and TRAF5 have all been found to associate with the cytoplasmic domain of Ox40. NF-κB activation in response to Ox40 stimulation appears to depend on TRAF2 and TRAF5 as deletion of the TRAF binding site in Ox40 or overexpression of dominant negative TRAF2 or TRAF5 can block NF-κB activation. In contrast, TRAF3 appears to act as a negative modulator.[81,82] In vivo, TRAF2 has been implicated in Ox40-mediated memory T cell expansion. T cells from OVA-specific TCR transgenic mice crossed with dominant negative TRAF2 mice were adoptively transferred to naïve BALB/c recipients, and stimulated with antibody to Ox40. The increase in antigen-specific T cells after Ox40 engagement was reduced with TRAF2 deficiency, and Ox40 engagement only enhanced the survival of antigen specific cells in wildtype but not mutant cells expressing dominant negative TRAF2.[83] TRAF5 has also recently been implicated in regulating T cell differentiation to Th1 and Th2 lineages by modulating Ox40 stimulation. Immunization of TRAF5-deficient mice with protein in adjuvant plus anti-Ox40 antibody leads to increased Th2 development.[84]

4-1BB

4-1BB, like CD27 and OX40, is another T cell costimulatory molecule, and is thought be be involved in antigen presentation, generation and long term survival of cytotoxic T lymphocytes, and induction of helper T cell anergy.[73]

TRAF1, TRAF2 and TRAF3 are known to bind to the cytoplasmic domain of 4-1BB. Like many other TNF receptor superfamily members, activation of 4-1BB leads to NF-κB activation. However, expression of dominant negative TRAF2 can inhibit 4-1BB induction of NF-κB.[81,85] Furthermore, while 4-1BB engagement results in activation of NF-κB and IL-2 production in wild-type T-lymphocytes, TRAF2-deficient lymphocytes are defective in this response.[86] 4-1BB induced TRAF2 dependent IL-2 production, however, appears to be mediated primarily through JNK activation through ASK-1.[87] TRAF2 has also been shown to be required for p38 MAPK activation in response to 4-1BB, which is thought to be critical for the development of Th1 and Th2 reponses.[88]

LTβR

Lymphotoxin (LT) α and β can heterotrimerize to form three distinct ligands for lymphotoxin β receptor (LTβR). LIGHT is another ligand for LTβR, but also interacts with HVEM. The signalling pathways controlled by these receptors and ligands are involved in lymphoid tissue development and organization, adaptive and innate immune responses, and central tolerance.[89] LTs can also bind TNFR1 and TNFR2. While LTβR and TNFR1/2 activation elicit distinct downstream signals, they also have complementary and overlapping functions, and employ shared mechanisms of signal propagation, including TRAF2 and TRAF5. LTβR is also known to bind TRAF3.[3,90,91]

TRAF2 is able to interact directly with the cytoplasmic domain of LTβR.[92] Recent studies have shown that LTβR stimulation is able to activate the alternative NF-κB pathway.[93,94] NF-κB2/p100 knockout and LTβR knockout have similar phenotypes—both showing aberrant development of peripheral lymphoid organs—indicating that alternative pathway activation significantly contributes to the physiological role of LTβR signalling.[95-97] TRAF2 appears to participate directly in LTβR mediated induction of both classical and alternative NF-κB pathways. However, TRAF2-deficient animals do not show defects in lympho-organogenesis. JNK activation induced by LIGHT stimulation of LTβR is also absent in TRAF2-deficient cells. Interestingly, unlike TNF signalling, LIGHT induced NF-κB and JNK activation are normal in both TRAF5-deficient and RIP1-deficient cells.[90]

HVEM/ATAR

The other receptor bound by LIGHT and LTα is the herpes virus entry mediator HVEM, which is expressed on lymphocytes. HVEM activation generally confers anti-apoptotic and proliferative signals to cells, and is thought to be important in T-cell costimulation, activation and modulation.[73,89]

Yeast two hybrid analyses have shown that HVEM interacts directly with TRAF2 and TRAF5 but not TRAF3.[98] Like other TNF receptor super family members discussed so far, recruitment of TRAF2 and TRAF5 to HVEM leads to NF-κB, JNK and AP-1 activation.[98,99] Remarkably, coexpression of HVEM with TRAF5, but not TRAF2, leads to synergistic NF-κB activation,[98] suggesting that TRAF2 and TRAF5 may play different roles downstream of HVEM.

RANK/TRANCER

TRANCE/RANKL/OPGL, a survival factor for activated dendritic cells, binds TRANCE-R/RANK. More importantly, RANK signalling is crucial for osteoclast activation and differentiation and therefore critical for maintaining bone homeostasis.[100,101]

TRAF2 and TRAF5, in addition to TRAF1 and TRAF3, can interact with the cytoplasmic tail of RANK via two different motifs.[102] TRAF6 also binds RANK, but in a distinct region more proximal to the membrane.[103] RANK signalling leads to NF-κB activation and JNK activation that is mediated through TRAFs. Dominant negative forms of TRAF2, TRAF5 and TRAF6 are all able to inhibit NF-κB activation induced through RANK.[104] TRAF6, however, is likely the key adapter for TRANCE-R, as TRAF6-deficient mice are phenotypically similar to TRANCE-R-deficient mice. Unlike TRAF6-deficient mice, however, neither TRAF2 nor TRAF5-deficient mice exhibit osteopetrosis, suggesting a more minor role for these TRAFs in osteoclastogenesis induced by RANK signalling.[105,106]

EDAR

Mutation of the ectodysplasin-A (Eda) receptor (EDAR) or the X-linked Eda receptor (XEDAR) leads to hypohidrotic ectodermal dysplasia (HED), a disease characterized by loss of hair, sweat glands and teeth.[107]

Unlike XEDAR, which can asscociate directly with TRAF3 and TRAF6, EDAR is similar to TNFR1 and unable to bind to TRAFs directly. EDAR utilizes the adaptor EDARADD, which associates via its death domain to the cytoplasmic death domain of EDAR. EDARADD is then able to recruit TRAF1, TRAF2 and TRAF3, and possibly with TRAF5 and TRAF6 as well.[108,109] There is considerable evidence suggesting that NF-κB is important for EDAR signalling. Hypomorphic mutations that inhibit IKKγ/NEMO activity result in defects similar to those seen in HED.[110,111] Although it is likely that TRAF2 and TRAF5 are involved in EDAR induced NF-κB activation, it is currently unknown whether these TRAFs play a role in ectodermal organ development.

$p75^{NTR}$

The common neurotrophin receptor $p75^{NTR}$ is unusual as it binds dimeric neurotrophins, unlike the majority of TNF receptor superfamily members which bind trimeric ligands. Signaling through this receptor controls apoptosis in neurons under conditions such as neurotrophin withdrawal or exposure to inappropriate neurotrophins.[112]

All six TRAF proteins have been shown to bind $p75^{NTR}$ in vitro. Curiously, TRAF2 appears to bind preferentially to the monomeric form of the receptor, unlike TRAF4 or TRAF6. Interactions with different TRAFs also have different effects—whereas coexpression of $p75^{NTR}$ with TRAF2 appears to enhance cell death, coexpression with TRAF6 is cytoprotective. Both TRAF2 and TRAF6 are able to induce NF-κB activation, albeit to a lesser extent by TRAF2.[113]

TAJ/TROY

TAJ/TROY is a TNF superfamily orphan receptor, recently identified to have a role in regulation of axonal regeneration, via association with Nogo-66 receptor 1.[114] Earlier coimmunoprecipitation

experiments have shown that TAJ/TROY is capable of binding TRAFs 1, 2, 3 and 5 in vitro. TAJ was also shown to activate the JNK pathway, however, dominant negative TRAF2 or TRAF5 is unable to block TAJ mediated JNK activation,[115] so these TRAFs may be involved in other TAJ signalling pathways.

GITR

Glucocorticoid-induced TNFR-related receptor (GITR) is a TNFR superfamily member expressed on T lymphocytes, and is activated by GITRL, which is expressed mainly on endothelial and antigen presenting cells. GITR is thought to have a role in augmenting T cell responses and a pathophysiological role in autoimmune disease.[73,116]

TRAF1, TRAF2, TRAF3, and TRAF4 have been found to interact with the cytoplasmic domain of GITR in a ligand-dependent manner. GITR stimulation also leads to activation of NF-κB, and this was found to require TRAF recruitment. However, recent studies have also shown that TRAF2 can have an inhibitory effect on NF-κB activation in response to GITR signalling.[117,118]

LMP-1

Epstein Barr Virus is an etiological factor in many lymphomas, including Burkitt's lymphoma and Hodgkin's disease. The latent membrane protein 1 (LMP-1) of Epstein Barr Virus is crucial for B-lymphocyte transformation, and is known to have transforming effects on nonlymphoid cells as well.[119] It was found that LMP-1 essentially a constitutively active TNF receptor family member, and able to associate with TRAF1, TRAF2 and TRAF3. LMP-1 induction of NF-κB appears to partially depend on TRAF1 and TRAF2, since dominant negative TRAF2 is able to block NF-κB activation.[120,121]

ER stress and IRE1

TRAF2 also has a unique role in endoplasmic reticulum (ER) stress pathways. Misfolded proteins in the ER, induced by stress conditions such as starvation or hypoxia, can induce cellular stress responses. These responses are mediated by IRE1s, which are ER membrane receptors that sense stress through their lumenal domains and transduce the signal across the ER via their cytoplasmic domains, leading to JNK activation. IRE1 was originally identified in yeast as the inositol auxotrophy gene, and mammalian homologs have been recently identified.[122-124]

Induction of IRE1 leads to JNK activation that is dependent on TRAF2. TRAF2 was found to bind the cytoplasmic region of IRE1, a dominant negative TRAF2 is able to inhibit IRE1 induction of JNK.[125] Additional studies have shown that JNK inhibitory kinase (JIK) also associates with IRE1 and TRAF2 in a complex to modulate IRE1-TRAF2 activation of the JNK pathway. Furthermore, in this pathway, TRAF2 is capable of binding and inducing oligomerization of caspase-12 and therefore its cleavage and activation. Activation of caspase-12 then promotes an apoptosis in response to ER stress.[126]

Conclusion

TRAF2, and to a lesser extent TRAF5, play critical roles in the signalling of many TNF receptor superfamily members. As these pathways share TRAF2 and TRAF5, these proteins are likely critical for signal integration and crosstalk. TRAF2 has a particularly diverse set of functions, as it is able to act as an activator or as an inhibitor under different contexts, in addition to its role as an adaptor protein.

The diverse set of receptors that rely on TRAF2 and TRAF5 for signal transduction also highlights their importance in the regulation of a wide range of physiological processes, including adaptive and innate immunity, inflammation, development, and cell survival. Dysregulation of these signalling pathways can result in pathophysiological states such as autoimmune disease and cancer. Since effective strategies for therapy may be derived from targeting molecules in these pathways, an understanding of the key roles played by TRAF2 and TRAF5 is critical.

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CHAPTER 4

TRAF3 and Its Biological Function

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Abstract

Tumor necrosis factor receptor associated factor 3 (TRAF3) is one of the most enigmatic members in the TRAF family that consists of six members, TRAF1 to 6. Despite its similarities with other TRAFs in terms of structure and protein-protein association, overexpression of TRAF3 does not induce activation of the commonly known TRAF-inducible signaling pathways, namely NF-κB and JNK. This lack of a simple functional assay in combination with the mysterious early lethality of the TRAF3-deficient mice has made the study of the biological function of TRAF3 challenging for almost ten years. Excitingly, TRAF3 has been identified recently to perform two seemingly distinct roles. Namely, TRAF3 functions as a negative regulator of the NF-κB pathway and separately, as a positive regulator of type I IFN production, placing itself as a critical regulator of both innate and adaptive immune responses.

Introduction

Beginning in the 1990s, many studies were emerging reporting the discovery of a diverse family of surface receptors which have collectively been come to known as the tumor necrosis factor superfamily of receptors (TNFRs).[1] Currently, more than 29 members have been identified. These receptors are grouped together based on the similarity of their extracellular domains which contain cysteine-rich regions. Each of these TNFRs plays a significant and unique role in fundamental biological processes and, importantly, deregulation of signaling pathways downstream of these TNFRs are believed to be causative factors in many immune and inflammatory diseases.[2] Consequently, the scientific and medical communities possess a tremendous interest in the characterization of signaling mediators downstream of these receptors in the hope of identifying therapeutic targets for the treatment of related diseases.

In 1994, TRAF1 and TRAF2 were the first molecules identified as associating factors to TNFR II.[3] Accordingly, these molecules were given the name tumor necrosis factor receptor associated factors (TRAFs). At a similar time, TRAF3 was identified through its association with the cytoplasmic tails of CD40 and the Epstein-Barr virus latent membrane protein (LMP-1).[4-6] Given the fact that TRAF proteins shared significant sequence homology and the emerging studies showing TRAFs association with multiple TNFRs, researchers speculated that members of the TNFR superfamily may initiate their specific signal transduction cascades by recruitment of specific TRAF proteins. To date, six TRAFs have been identified and are grouped as a family of intracellular adaptors which transmit signals downstream of most if not all of the TNFRs as well as other non-TNF receptors such as the toll-like receptors. As such, TRAF proteins mediate a plethora of biological functions; the most well studied involving the initiation of innate and adaptive immune responses against pathogen infections.

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Characterization of TRAF3

TRAF3 is a ubiquitously expressed protein, suggesting that it may perform significant physiological and cellular functions in multiple organs. TRAF3 expression has been observed in many murine tissues, including brain, heart, lung, liver, spleen and thymus.[6] It is also expressed in several human cell types including myeloid progenitor cells, monocytes, and plasma cells.[7]

The TRAF3 protein is evolutionarily conserved between human and mouse, with 96% of their amino acid sequence being identical.[6] The human TRAF3 protein is composed of 568 amino acids with a molecular weight of approximately 64kDa. Similar to all the other TRAF members, TRAF3 possesses a signature TRAF domain at the carboxyl terminus. At the N-terminus, TRAF3 contains a typical C3HC4 RING finger domain, followed by five zinc-binding fingers, and an isoleucine zipper. While the TRAF domain has been shown to be important for binding to the cytoplasmic domain of tumor necrosis factor receptor (TNFR) family members, intracellular signaling mediators, and for forming homo- or hetero-dimers with other TRAFs, the function of the other TRAF3 domains has yet to be characterized.[8-11]

Structure-Function Study of TRAF3

Although TRAF3 has similar secondary structures as other TRAFs, over-expression of TRAF3, in contrast to TRAFs 2, 5 and 6, fails to activate the JNK or NF-κB pathways.[12-16] To understand the structural basis for the functional differences between TRAF3 and its family members, various domains of TRAF3 were replaced with domains from TRAF5, its closest TRAF member in terms of amino acid identity. Results indicated that the first zinc finger and 10 residues of the second zinc finger of TRAF5 are sufficient to convert TRAF3 into an activator of both JNK and NF-κB pathways.[17] This suggests that the zinc fingers of TRAF3 contribute to its inability to activate both JNK and NF-κB. Interestingly, the cellular localization of TRAF3 seems to differentiate it from the other TRAF family members as TRAF3, unlike TRAF2, 5 and 6, is not preferentially localized to the insoluble cell pellet fraction.[18] This may partly explain the differences in pathway activation potential exhibited by TRAF3 in comparison to other TRAFs. In support of this, myristoylation of TRAF3, which forces TRAF3 to the insoluble membrane fraction, converts TRAF3 into an activator of the JNK pathway.[18]

TRAF3 Association with Surface Membrane Receptors

Following the initial biochemical identification of TRAF3 as a CD40 associating factor, a tremendous amount of effort was put forth to uncover its role in CD40 signaling and biology. However, this endeavor was complicated by the observation that multiple TRAFs, including TRAF2, 3, 5, and 6, can bind to CD40 and that TRAF2 and 3 even bind to an overlapping region. Nevertheless, initial studies indicated an inhibitory role for TRAF3 in CD40 biology as overexpression of this protein inhibits CD40-induced CD23 expression and antibody secretion in B cells.[6,19] However, overexpression of a CD40 mutant which abolishes TRAF3 but not TRAF2 binding, had no effect on CD40-mediated NF-κB and JNK activation which suggests a neutral role for TRAF3 in CD40 signaling transduction.[20] In agreement with the latter finding, CD40-induction of CD23 expression and NF-κB activity were normal in a TRAF3-deficient cell line and antibody secretion and JNK activity were only slightly increased.[21] Consequently, it remained unclear if TRAF3 plays a significant role in CD40 signaling.

The role of TRAF3 in LMP-1 signaling has also been extensively investigated. LMP-1 is a transforming protein from Epstein Barr virus which mimics the signaling characteristics of constitutively active CD40. Like CD40, LMP-1 can associate with TRAF 2 and 3 and activate the NF-κB and JNK pathways.[22,23] Analogous to CD40, LMP-1 induces expression of B cell markers ICAM-1, LFA and CD23.[24] In contrast to its unidentified role in CD40 signaling, TRAF3 appears to function as an important mediator of LMP-1 signal transduction. In one study, using a TRAF3-deficient B cell line that stably expresses LMP-1, results indicated that TRAF3 served a positive role in LMP-1 activation of NF-κB and JNK.[21] This observation may be explained by the innate differences between these two receptors. For example, LMP-1 appears to have a higher affinity for TRAF3 than CD40 and unlike CD40, LMP-1 does not induce the degradation of TRAF3.[25,26] Still, how these differences actually contribute to the differential roles of TRAF3 in CD40 and LMP-1 signal transduction remains unclear.

Following the identification of TRAF3 association with CD40 and LMP-1, an increasing number of TNF receptors have been shown to bind to TRAF3.[10] All these receptors share a domain called the TRAF interacting motif (TIM). This TIM sequence can vary between receptor to receptor, but can be generally described as (P/S/A/T)X(Q/E)E which is found in CD40, CD30, HVEM, OX40, p75NGFR, and RANK.[12,27-29] Intriguingly, for some receptors, TRAF2 and 3 seem to be able to bind to the same TIM. For instance, both TRAF2 and 3 bind to PVQET on CD40.[30] This suggests that TRAF3 may compete with TRAF2 for binding to the receptor and/or that TRAF2 and TRAF3 may form a signalosome when receptors are oligomerized. Indeed, TRAF2 and 3 form heterodimers though the importance of this partnership remains to be determined.[31] In addition, crystal structures of the TRAF domain of TRAF2 and 3 and a CD40 peptide encompassing the TRAF2/3 binding motif showed that CD40 assumed different conformations depending on which of these two TRAFs it binds.[32,33]This provides a possible scenario where CD40 may elicit unique and specific signaling outcomes depending on the TRAF complex bound to its cytoplasmic tail.

TRAF3 Interacting Molecules

In addition to characterizing TRAF3 association with surface receptors, extensive effort was focused on identifying TRAF3 associating molecules in an attempt to uncover its function. This approach yielded a number of TRAF3-associating molecules including Act 1, ASK1, c-src, MIP-T3, NIK, p62 nucleoporin, p85 subunit of PI-3K, $p40^{phox}$, RIP1, RIP4, TANK, T3JAM, TNAP and TTRAP.[15,34-46] Among all these molecules, many of them can bind to the other TRAF members as well, whereas MIP-T3, p62 nucleoporin, and T3JAM appear to specifically bind to TRAF3.[35,36,40] Further studies are required to establish the physiological roles of these associated proteins in TRAF-mediated biological events.

Phenotype of TRAF3-Deficient Mice

Besides using a biochemical approach to study the function of TRAF3, a genetic approach was also employed. TRAF3-deficient mice were generated in 1996. Despite a relatively normal gestation period, Traf3 knockout mice rapidly degenerated after birth with symptoms including stunted growth and progressive hypoglycemia, hypercortisolemia, and leucopenia resulting in a premature death within two weeks of age.[48] Despite numerous efforts, the instigating factor in this perinatal lethality remained undetermined for many years.

Because TRAF3 was identified as a CD40-associating molecule, the role of TRAF3 in the CD40 pathway was assessed in TRAF3 null cells.[48] In vitro stimulation of *Traf3*$^{-/-}$ B cells with anti-IgM and CD40L showed no difference in proliferation compared to wild-type cells. Furthermore, *Traf3*$^{-/-}$ B cells showed no defect in upregulating B7.1 and CD23 upon CD40 ligation. Therefore, TRAF3 is not required for CD40-induced B cell proliferation and activation. However, TRAF3 was involved in generating an immune response to T-dependent antigen. Mice reconstituted with *Traf3*$^{-/-}$ fetal liver cells could not mount a proper immune response to a T-dependent antigen. In addition, in vivo primed *Traf3*$^{-/-}$ T cells were defective in proliferative responses to antigen presentation. It remains to be determined whether this defect is in result of problems with *Traf3*$^{-/-}$ antigen presenting cells or in T helper cell functions. Due to the promiscuity of TRAF3 in binding to at least twenty TNF receptors and the ubiquitous expression of TRAF3, generation of cell-type specific or tissue-specific disruption of the TRAF3 gene is necessary to tease out the role of TRAF3 in different cell types and organs.

Breakthrough in Identification of TRAF3 Function: The Noncanonical NF-κB Pathway

As mentioned above, unraveling the mystery of TRAF3 function had proven difficult due to the early post-natal lethality of *Traf3*$^{-/-}$ mice and the failure of traditional biochemical studies to establish a link between TRAF3 and known signal transduction pathways. Five years after the targeted disruption of TRAF3, however, studies began to emerge about a second, evolutionary conserved NF-κB activation pathway, and pointed in a new direction for the study of TRAF3 function.

In brief review, the NF-κB family of transcription factors plays pivotal roles in the propagation of innate and adaptive immune responses through the activation of multiple gene targets including

those involved in cell growth, survival, apoptosis, and inflammation.[49,50] Five NF-κB family members exist in mammals: NF-κB1 (encoding p105 which is constitutively processed to p50), RelA (p65), cRel, NF-κB2 (encoding p100 which is processed to p52), and Rel B. Under normal conditions, inactive Rel dimers are retained in the cytoplasm through interaction with one of a family of inhibitory molecules, termed inhibitors of κB (IκBs).[51] Signal-dependent phosphorylation of IκBs on key serine residues results in IκB degradation and the translocation of Rel dimers capable of binding DNA in the nucleus.[52]

Classical or canonical NF-κB activation requires the IκB kinase (IKK) complex which consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (NEMO/IKKα). IKK activation results in the degradation of IκBα and -β which release p50:RelA and p50:cRel dimers.[53,54] Activation of the 'alternative' or noncanonical pathway involves activation of NF-κB inducing kinase (NIK) which associates with two molecules of IKKα.[55,56] Together, NIK and IKKα, bind to the C-terminal portion of p100 (also termed IκBδ) leading to the processing of p100 to p52 and the release of p52:RelB dimers.[57-59] Another important distinction between these two NF-κB activation pathways involves the kinetics/pattern of activation and the requirement for new protein synthesis. Here, canonical NF-κB activation occurs within minutes post-stimulation and does not require new protein synthesis. In addition, canonical NF-κB activation leads to the induction of IκBs which results in strong negative feedback. As a consequence, canonical NF-κB activation is characterized by an oscillatory function with decreasing amplitude over time.[60] In contrast, activation of the noncanonical NF-κB pathway requires several hours, new protein synthesis and does not decrease in strength over time.[61] While targeted disruption of Rel family members has identified overlapping functions in cellular proliferation and survival, they have also identified specific and unique biological roles for individual Rel proteins.[53] Importantly, disruption of signaling components of the noncanonical NF-κB pathway present highly similar phenotypes characterized by severely disorganized splenic and lymph node architecture, reduced B-cell numbers in the bone marrow and periphery, and defective T-dependent and independent immunologic responses.[62,63] At the same time, mice deficient in LTβR, CD40, or BAFFR, all of which strongly bind TRAF3 and activate the noncanonical NF-κB pathway, present with similar phenotypes suggesting a connection between TRAF3 receptor binding and noncanonical NF-κB activation.[59,61,64-67]

The first study that clearly establishes a link between TRAF3 and noncanonical NF-κB activation was performed by Liao et al.[68] Here, under overexpression in 293T cells, the authors showed via coimmunoprecipitation, a strong interaction between NIK and TRAF3. The authors further demonstrated that overexpression of TRAF3 resulted in a marked decrease in NIK levels and that siRNA-mediated suppression of endogenous TRAF3 resulted in accumulation of NIK and increased processing of p100 to p52. Finally, the authors showed that inhibition of the proteasome resulted in the accumulation of ubiquitinated NIK, and strikingly, that a NIK mutant lacking a short sequence which mediates TRAF3 binding, was protected from ubiquitination in this assay. This study therefore suggests that TRAF3 plays a crucial role in the suppression of NIK activity. Importantly, the authors were unable to see TRAF3 mediated ubiquitination of NIK in a standard 293T cell assay using an exogenous tagged form of ubiquitin, which strongly suggests that while TRAF3 is necessary for the negative regulation of NIK, it is also not sufficient. While the Liao et. al. study was compelling, the history of TRAF3 study suggested that the field should wait for a corroborative study before embarking on this new path of examination of TRAF3 biology. Conveniently, this condition was soon met by Hauer et. al. in a study showing that overexpression of any TNFR family member capable of binding TRAF3 led to nuclear accumulation of p52 and that dual overexpression of TRAF3 prevented this event.[69] Together, these studies strongly suggest that TRAF3 negatively regulates the processing of p100 to p52 through suppression of NIK. How might this occur? One possibility involves the observation that ligation of TRAF3-binding TNFR receptors results in TRAF3 degradation.[70] This suggests a simple model of noncanonical NF-κB activation wherein p100 processing is constitutively inhibited by TRAF3 mediated degradation of NIK. Upon appropriate receptor ligation, TRAF3 is recruited and degraded allowing for accumulation of NIK and activation of IKKα thus explaining the delayed kinetics and protein synthesis-dependent nature of noncanonical NF-κB activation. Can it be this simple? Probably not. First, it was recently reported that loss of TRAF2 also results in constitutive activation of

the noncanonical NF-κB pathway indicating that TRAF2 and TRAF3 (and possibly additional molecules) cooperate in the negative regulation of NIK.[71] Second, TRAF proteins have only been shown to have ubiquitin ligase activity for Lys-63 linkages which are not associated with protein degradation but rather the promotion of complexes and signal activation (similar to the role of tyrosine phosphorylation in signal transduction).[72] As such, it remains to be seen whether or not TRAF3 contributes to the negative regulation of NIK through Lys-63 or Lys-48 (proteasome targeting) ubiquitin linkages or simply as an adaptor molecule which recruits enzymatic components that regulate NIK stability. In depth analysis of the domains of TRAF2 and TRAF3 required for the negative regulation of NIK will be required to elucidate the complex mechanism of noncanonical NF-κB activation.

Previous genetic studies involving constitutive activation of the canonical (by deletion of IκBα) and noncanonical (by deletion of the p100 C-terminus) NF-κB activation pathways have show the critical importance of proper regulation of NF-κB activity (Fig. 1).[73,74] In consideration of this and these recent biochemical studies indicating that TRAF3 functions as a critical negative regulator of noncanonical NF-κB activity, one wonders how this may relate to the cause of the TRAF3-null phenotype. Indeed, it was recently reported that the TRAF3 null phenotype can be rescued by the compound deletion of the p100 gene.[75] So, 10 years after its discovery, the scientific community now has a much better understanding of why so many TNFR family members critical to the propagation of adaptive immune response recruit the enigmatic adaptor molecule, TRAF3.

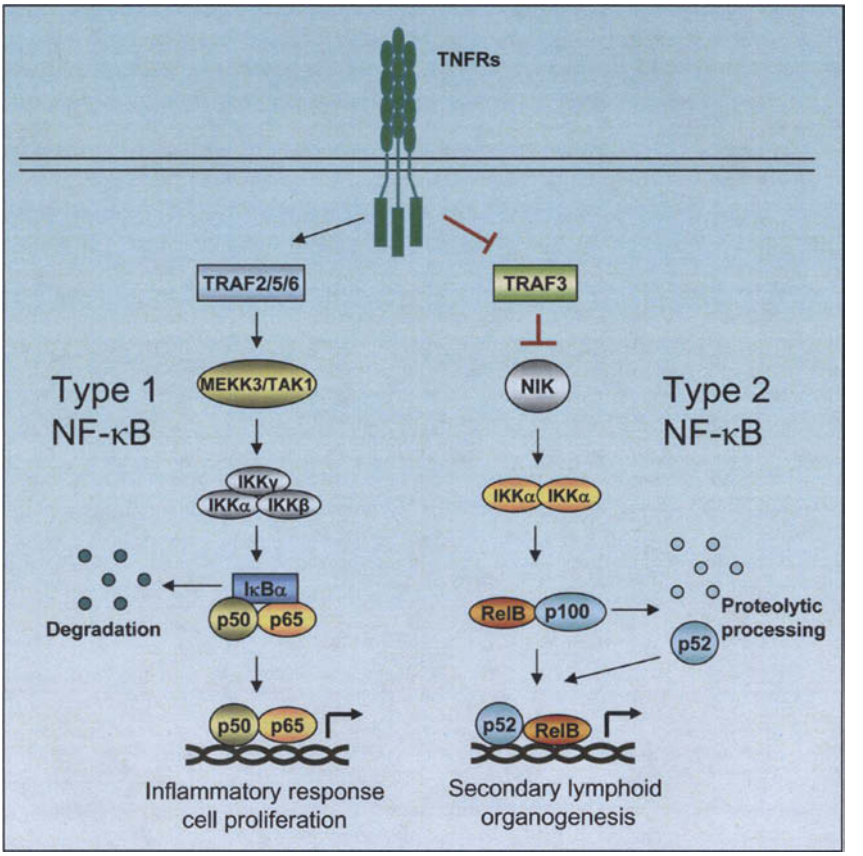

Figure 1. A schematic illustrating how TRAF3 may be involved in the activation of the noncanonical NF-κB pathway by TNFR family members such as BAFFR, CD40, and LTβR.

TRAF3 in Innate Immunity

At this point, the function of this mysterious TRAF family member might seem straightforward. TRAF3 acts as a powerful negative regulator of the noncanonical NF-κB pathway and this function is somehow inhibited through direct interaction with certain members of the TNFR superfamily, such as CD40 and BAFFR. However, two different lines of evidence began to emerge that hinted at another highly unexpected role for this molecule.

The type I IFN family of cytokines, composed of multiple IFNα's, IFNβ, and a few other subtypes, make up the most vital component of our innate immune response against viral infection. In addition, they play a major role in enhancing adaptive immunity and have been closely linked to autoimmune diseases such as System Lupus Erythematosus.[31,76] Thus, the mechanisms by which type I IFNs are produced by both leukocytes and stromal cells following viral infection or Toll-like receptor (TLR) ligation has been a major focus of attention in recent years. In addition to bacterial products such as LPS or flagellin, certain TLRs that localize to endosomes can recognize viral products such as dsRNA, ssRNA, and unmethylated CpG motifs (CpG) in DNA. In macrophages and plasmacytoid dendritic cells (pDCs), recognition of these products by TLRs 3, 7, and 9, respectively, results in the potent induction of type I IFNs.[77]

TLRs are a family of transmembrane receptors that represent an evolutionarily conserved recognition system for pathogen associated molecular patterns (PAMPs) found in microbial pathogens. Like the TNFR superfamily, the TLR family can potently activate NF-κB; however, TLRs can also induce antiviral responses through a family of cytokines called type I interferons (IFNs). Also like TNFR family members, TLRs require a member of the TRAF family to activate NF-κB, specifically TRAF6. Rather than directly binding the cytoplasmic receptor tail, as is the case in TNFR recruitment of TRAFs, TRAF6 is activated by TLRs through a signaling complex involving MyD88, IRAK4, and IRAK1. TLR3 is unlike most other TLRs by virtue of its potent activation of the antiviral response in macrophages and its predominant utilization of the adapter TRIF rather than MyD88.[78] The additional recent finding that TRAF6 is not required for TLR3 signaling left open the possibility that another TRAF family member may take its place in the TRIF-dependent pathway.[79]

Not long after its discovery, TRAF3 was used as bait in a yeast-two hybrid screen to identify novel interacting molecules. One of the strongest TRAF3 interacting molecules by yeast two hybrid screen was an adapter protein with unknown function later termed TANK for TRAF-associated NF-κB activator.[34,45] TANK was subsequently used in a yeast-two hybrid screen to identify an IKK-related molecule coined TBK1 for TANK-binding kinase1.[80,81] While TBK1 is homologous to IKKα and IKKβ, TBK1 is not involved in NF-κB activation. Instead, TBK1 and its close relative IKKε were later shown to be critical kinases of IRF3, one of the major transcription factors for type I IFNs.[82] For example, *Tbk1*$^{-/-}$ cells are defective in the antiviral response to TLR activation.[83,84] Thus, several lines of evidence suggested the possibility that TRAF3 may be involved in the regulation of antiviral responses.

When TRAF3-deficient macrophages were stimulated with the TLR3 ligand, polyI:C, the surprising possibility was confirmed. *Traf3*$^{-/-}$ macrophages treated with a synthetic form of dsRNA produced far less type I IFNs than their wild-type counterparts. Further study traced this phenotype to a failure of TRAF3-deficient macrophages to activate the type I IFN transcription factor IRF3. In contrast, TRAF3 was not required for activation of NF-κB by any of the TLRs tested. The fact that TRAF3 could also associate with both TRIF and TBK1 in coimmunoprecipitation studies suggested that TRAF3 may be linking TRIF to downstream IRF3 phosphorylation by TBK1.[75]

Plasmacytoid dendritic cells, the most potent known producers of type I IFNs, have demonstrated the ability to recognize different viruses through distinct TLR receptors based on the structure of the viral genome.[85,86] For instance, TLR7 is required for the recognition of the ssRNA viruses such as influenza and VSV, whereas TLR9 is required for recognition of DNA viruses including HSV-1, HSV-2, and MCMV.[87-89] This recognition event, which can be mimicked by synthetic TLR7 and TLR9 ligands, R848 and CpG, results in the secretion of high levels IFNα by the pDCs in a manner that depends on both MyD88 and IRAK1.[78] Because TLRs 7 and 9 utilize MyD88 rather than TRIF, it was an additional surprise when it was found that *Traf3*$^{-/-}$ pDCs are

also greatly defective in the antiviral response to ligation of TLRs 7 and 9. However, further study suggested that TRAF3 may actually interact with IRAK1 to activate the transcription factor for IFNα, IRF7.[75] Thus two distinct pathways appeared to converge on TRAF3 to induce a specific antiviral response.

In contrast to pDCs, nonimmune cells do not appear to recognize viral infection via TLRs or other known surface receptors. Instead, cytoplasmic protein receptors are thought to directly bind viral components such as dsRNA and subsequently activate an appropriate cellular response, including the induction of type I IFNs. Recently, RIG-I and MDA5 (Helicard) have been implicated as potential receptors for the detection of intracellular viral infection in nonimmune cells such as murine embryonic fibroblasts (MEFs).[78] Interestingly, Cardif, a critical adapter for signaling by Helicard and RIG-I, contains TRAF binding motifs (TBMs) similar to those found in the CD40 receptor.[90-93] Thus, it is not too surprising that TRAF3-deficient MEFs failed to induce type I IFNs following direct viral infection. In fact, *Traf3*$^{-/-}$ MEFs were several fold more susceptible to viral infection.[75] Although TRAF3 was previously only thought to be involved in adaptive immunity due to its association with CD40, BAFF, and LTβ receptors, it now seems apparent that this molecule plays a major role in innate immunity as well (Fig. 2).

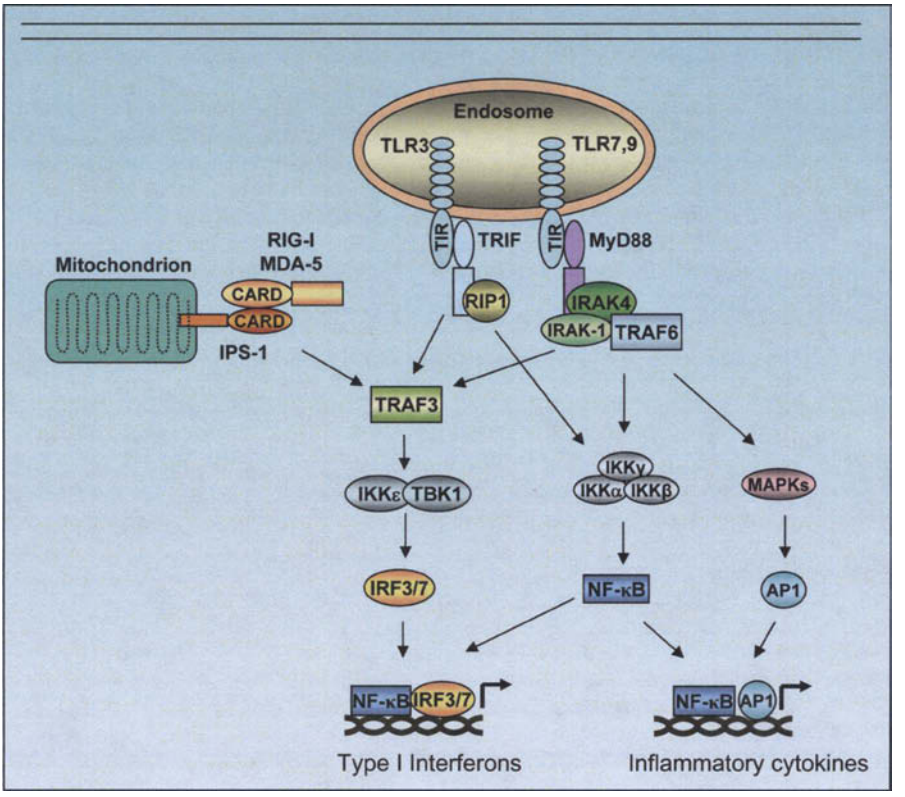

Figure 2. A schematic illustrating how TRAF3 may be involved in the activation of type I interferons by TLR-dependent and TLR-independent viral recognition pathways.

Concluding Remarks

How can TRAF3 simultaneously control two seemingly unrelated pathways, acting as a negative regulator in one and a positive regulator in another? A complete mechanistic understanding of how TRAFs function has remained elusive. TRAF6 is thought to activate NF-κB through an auto-ubiquitination event. The RING finger domain of TRAF6 acts as an E3 ligase for itself, resulting in the polyubiquitination of TRAF6. These ubiquitin chains then recruit a complex including TAK1, TAB1 and TAB2, which then results in the phosphorylation and activation of the IKK complex.[94] When proposing a model for TRAF3 function, one cannot ignore the striking homology between the TRAF3 and TRAF6 pathways. TAK1 and NIK, both MAP kinase kinase kinase family members, are thought to phosphorylate homologous residues on an activation loop, or "T-loop," of IKKβ or IKKα, respectively.[95] Interestingly, this same activation loop is present in both TBK1 and IKKε and required for their ability to activate IRF3. Thus, it would appear likely that similar mechanisms are governing TRAF6-mediated activation of the IKK family and TRAF3-mediated activation of TBK1/IKKε.

As mentioned above, biochemical studies have suggested that TRAF3 suppresses NF-κB by constantly mediating the degradation NIK.[68] Presumably, TRAF3 acts as an E3 ubiquitin ligase for NIK through its N-terminal RING finger domain. However, this has not yet been formerly proven. Is the same E3 ligase activity of TRAF3 involved in both NIK degradation and regulation of IRF transcription factors? Like other TRAFs, TRAF3 is composed of multiple domains capable of mediating numerous protein-protein interactions, including zinc fingers, an isoleucine zipper, and a common TRAF domain. Extensive structure function studies may one day reveal the relative contributions of these TRAF3 domains to its multiple distinct functions.

The recent progress toward understanding the functional role of TRAF3 now creates a more complete picture of the specificity involved in signaling by TNFR and TLR family members. While TRAF family members have homologous structures, the early lethality caused by loss of TRAF6, TRAF3, and TRAF2 expression is testament to their nonredundant and distinct roles. A detailed mechanistic understanding of how TRAFs are activated and translate that activation event to downstream pathways may therefore provide researchers with novel specific targets for therapeutic manipulation of numerous biological processes.

Although in vitro studies have implicated a role for TRAF3 in both adaptive immunity and innate antiviral responses, future in vivo functional analysis through the use of tissue-specific genetic disruption of the *traf3* locus will likely provide a more complete assessment for the potential of therapeutically targeting TRAF3-related pathways. Interestingly, Epstein Barr virus may have already discovered this potential as evidenced by the EBV-encoded transmembrane protein LMP-1, which specifically targets and binds TRAF3.[22,23] This sequestration of TRAF3 may serve the dual purpose of preventing antiviral responses resulting from the EBV infection in addition to simultaneously triggering constitutive noncanonical NF-κB activation, thereby extending the lifespan of EBV-infected B cells. Although this has yet to be demonstrated, it is likely that loss of TRAF3 function in B cells would result in the survival of autoantibody-producing B cells through this constitutive noncanonical NF-κB activity. On the other hand, TRAF3 is the only molecule known to be generally required for type I interferon production following both TLR ligation and viral infection in macrophages, pDCs, as well as fibroblasts.[75] The strong correlation between excessive type I interferon production, enhanced survival of autoantibody-producing B cells and autoimmune diseases such as Systemic Lupus Erythematosus (SLE) may place TRAF3 in the rare and delicate position of both suppressor and enhancer of autoimmune diseases. Thus, our current understanding of the biological importance of TRAF3 in both physiological and pathophysiological processes may just be the tip of the iceberg.

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CHAPTER 5

TRAF4, the Unique Family Member

Valérie Kedinger and Marie-Christine Rio*

Abstract

The fourth member of the TRAF protein family (TRAF4) presents several characteristics that distinguish it from the other members of the family. These characteristics concern the primary sequence of the protein, a strong evolutionary conservation, and a tightly regulated physiological expression during development. The subcellular localization of TRAF4 is controversial as it has been detected at the cell membrane, in the cytoplasm and in the nucleus. Using mouse and fly models, it has been established that TRAF4 is a key molecule in diverse ontogenic processes, particularly in the nervous system. However, the molecular mechanisms of action of TRAF4 remain evasive as it was found to interact with diverse types of proteins, leading either to pro-apoptotic or anti-apoptotic functions. Finally, few studies implicated TRAF4 in human diseases.

The Fourth Member of the TRAF Protein Family

The Tumor Necrosis Factor Receptor-Associated Factor 4 (TRAF4) belongs to the canonical TRAF protein family that contains six members. They are defined by the presence of a carboxy- (C-) terminal TRAF domain composed of two parts, N-TRAF and C-TRAF, the second exhibiting a higher level of conservation. A seventh member, TRAF7, has recently been added although it is devoid of TRAF domains (for general reviews see refs. 1-4).

TRAF4 is unique in several aspects (Table 1, Fig. 1). Although all TRAFs (with the exception of TRAF1) contain an N-terminal RING finger motif, TRAF4 (as well as TRAF5 and TRAF6) contains the C3HC3D motif instead of the classical C3HC4 RING motif, and it is the only one that contains a nuclear localization signal (NLS).[5] The core of TRAF4 is composed of 3 HC3HC3 cysteine-rich domains, defined by Regnier et al as CART domains (Cystein-Rich domain Associated with RING and TRAF domain); each CART domain contains 2 putative zinc fingers.[5] While several groups have interpreted the numerous C and H residues present in this region to suggest the presence of seven zinc fingers instead of six in TRAF4,[1,6] the fact that each CART domain is encoded by distinct exons (exons 4, 5, and 6 for TRAF4) in all TRAFs strongly supports a HC3HC3 structure, and suggests that each is derived from an ancestral exon. Indeed, TRAF4 was first named CART1 because of this domain.[5] TRAF4 is the only member to possess three CART domains; the other TRAFs have two. Furthermore, the first TRAF4 CART domain exhibits a second putative NLS. In the N-TRAF domain, the coiled-coil domain of TRAF4 is short compared with the other TRAFs, with only three heptad repeats while others have more than ten. This might explain the low capacity of TRAF4 to form heterotypic associations. Finally, The three residues R, Y and S, present in TRAF1, 2, 3 and 5, that are involved in the recognition of the cytoplasmic TRAF member interacting motif (TIM) of the TNF-receptors (TNF-R),[7] are not conserved in TRAF4 but replaced by S, F and F, respectively. These substitutions could explain the reduced interaction of TRAF4 with

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Table 1. Summary of TRAF4 characteristics

Gene	
Chromosomal localization	17q11-12 (human) ; 11B5-11C (mouse)
organization	7 exons
regulation	weak kozak sequence; no TATA box; p53; PMA; CD40 ligand; TNF
Protein	
primary sequence	RING C3HC3D; 3 CARTs; 2 NLS; TIM: S, F, F
structure	Short coiled-coil N-TRAF domain; 3 heptads
Expression pattern	
develoment	early and widespread; CNS, PNS, postmitotic undifferenciated neurons
adult	ubiquitous basal expression; regulated in some tissues
Subcellular localization	membrane; cytoplasm; nucleus
KO phenotype	high in utero lethality; CNS, PNS and skeletal alterations
Human diseases	
malignant	breast cancer, Hodgkin
benign	schizophrenia
Putative function	nervous system; pro-apoptosis; anti-apoptosis; cell cycle progression; oxidant production

the members of TNF-R family and suggest that TRAF4 might interact with other types of transmembrane proteins.

Thus, the primary sequence of TRAF4 suggests that it is a particular TRAF member that might be implicated in particular function(s).

TRAF4 Is Highly Conserved during Evolution

TRAF4 protein orthologues have been reported for several species (Fig. 2). The mouse TRAF4 primary protein sequence shows 97% identity with its human counterpart. Databases also contain a rat TRAF4 sequence that shows 97% identity with human TRAF4. The Drosophila genome contains three TRAFs, DTRAF1 corresponds to TRAF4 (45% identity with the human protein), DTRAF2 corresponds to TRAF6, and DTRAF3 corresponds to TRAF1, 2, 3 and 5.[6] Two zebrafish orthologues, TRAF4a (77% identity with the human protein), and TRAF4b (68% identity with the human protein), have also been identified.[8] To date, they are the only TRAFs described in zebrafish. Since the Caenorhabditis elegans genome contains only one TRAF (37% identity with the human TRAF4), the question of the existence of other TRAFs in fish remains open. Searches in the Public Dictybase reveal the existence of a related TRAF gene, zfaA, in Dictyostelium discoideum. This gene

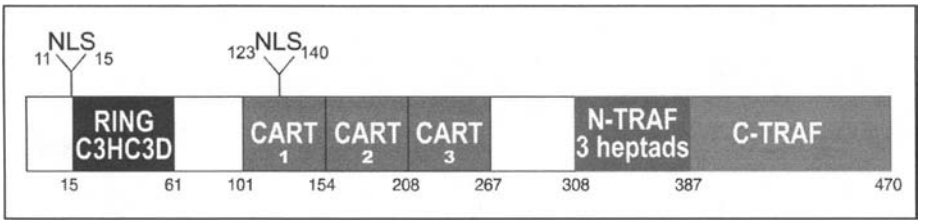

Figure 1. Schematic representation of TRAF4 protein.

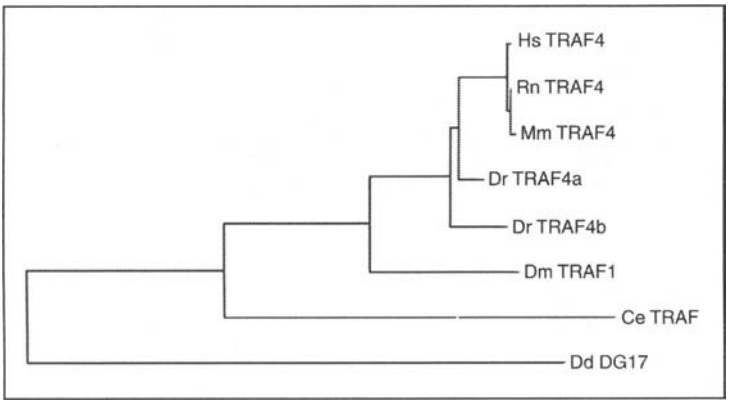

Figure 2. Phylogenic tree built from the multi-alignment comparing the human (Hs), Rat (Rn), mouse (Mm), zebrafish (Dr), and fly (Dm) amino acid sequence of the TRAF4 protein, the TRAF protein present in worm (Ce) and DG17 protein of Dictyostelium discoideum (Dd).

encodes the protein DG17, a presumed zinc ion binding protein expressed during Dictyostelium discoideum aggregation.[5]

At the molecular level, regardless of species, the promoter region of TRAF4 does not have a consensus TATA box and contains a relatively weak Kozak sequence, two characteristics often observed in ubiquitously expressed genes. The various TRAF4 genes also share a similar gene organization; each gene is composed of 7 exons, exons 1 and 2 encode the RING domain, exons 4, 5 and 6 encode the three CART domains, and exon 7 encodes the TRAF domain. Moreover, a syntenic linkage conservation has been reported between mouse and man; the human TRAF4 gene localizes to chromosome 17q11-q12 and the mouse gene lies in the corresponding 11B5-11C region.[9]

The strong evolutionary conservation reinforces the idea that TRAF4 exerts an important biological function. Accordingly, it has been shown that, as TRAF6, TRAF4 precursor gene has arisen early during evolution whereas the other TRAFs have diverged more recently.[6]

Physiological Expression

In all species studied (human, mouse, zebrafish and drosophila), TRAF4 expression during embryogenesis is highly dynamic and complex (Fig. 3). In human fetal tissues at 12-18 weeks of gestation, immunohistochemistry experiments show a strong cytosolic TRAF4 staining that is mostly restricted to the basal epithelial cells.[10] In the mouse embryo, TRAF4 is widely expressed. TRAF4 mRNA is observed in 3.5 day post coitum (dpc) embryonic stem (ES) cells, and reaches maximum expression by 8.5 to 13.5 dpc.[9] Depending on the developmental stage, TRAF4 expression is observed in various organs including neural crest cells, the first, second and third branchial arches, intestine, thymus, salivary gland and the epithelium of the trachea.[9,11] During mouse odontogenesis, TRAF4 is detected in the dental papilla mesenchyme and in both the internal and external enamel epithelium.[12] During zebrafish embryogenesis, TRAF4b is weakly expressed in a ubiquitous manner, but TRAF4a is strongly expressed in a specific and regulated fashion in the sensorial and neural cells, the somites and the blood vessels, suggesting that TRAF4a is responsible for all TRAF4 function.[8] Similarly, the highest levels of expression in the mouse[9] are observed during the ontogenesis of the central (CNS) and peripheral (PNS) nervous systems, and in the nervous tissues of sensory organs. TRAF4 is preferentially expressed by post-mitotic undifferentiated neurons and in oligodendrocytes. Moreover, TRAF4 is developmentally regulated in the mouse CNS, as it is down-regulated between neonates and recently weaned 4-week-old mice.[13] In drosophila, DTRAF1 accumulates in mesodermal cells and neural precursors and is correlated with the onset of morphogenetic and cellular movements. It is largely absent in terminally differentiated cells.[14]

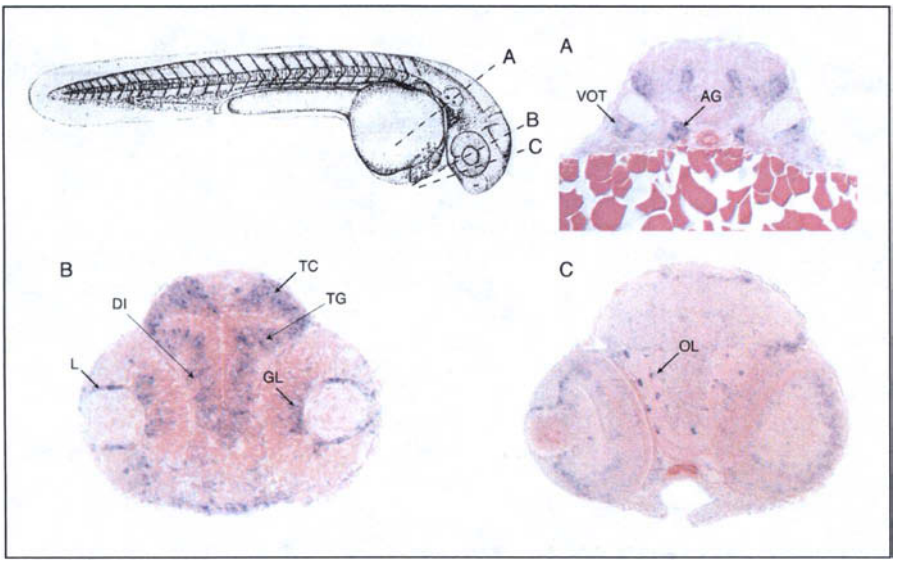

Figure 3. Histological sections highlighting the expression pattern of TRAF4a in sensorial and nervous system during zebrafish development. A) At 36 hours post fecondation (hpf), TRAF4a is detected in ventral otic vesicle (VOT) and statoacoustic ganglia (AG). B) At 48 hpf, TRAF4a is expressed in the tectum (TC), the tegmentum (TG) and the diencephalon (DI). In the eye, expression of TRAF4a is present in the lens (L), and in the ganglion cell layer (GL). C) At 60 hpf, TRAF4a is expressed in the oligodendrocytes (OL).

At the RNA level, no expression was detected in human breast, heart, brain, skin, lung, stomach, colon, liver, kidney and placenta.[5] However, TRAF4 EST have been reported in 27/31 human adult tissues (NCBI Unigene database). This near ubiquitous expression was confirmed at the protein level in a survey of normal adult human tissues that showed strong TRAF4 positivity in the basal cell layer lining the basement membrane of complex epithelia throughout much of the body.[10] Accordingly, in situ hybridization[9] indicates basal levels of TRAF4 expression in most adult mouse tissues. Interestingly, in addition to this constitutive expression, strong TRAF4 expression is observed in some tissues such as adult CNS where TRAF4 is highly expressed in the hippocampus and the olfactive bulb, and in the Purkinje cells of the cerebellum.[9]

Widespread TRAF4 expression at the basal level suggests a generic function in shared biological processes. However, in distinct tissues, high TRAF4 expression is tightly regulated, indicating that it might exert additional tissue-specific function(s). In this context, whereas the other TRAF functions are mostly related to the immune system, that of TRAF4 is related to the nervous system.

Subcellular Localization: A Matter of Debate

Since its discovery, the subcellular localization of TRAF4 has been controversial. Indeed, TRAF4 has been detected at the cell membrane, in the cytoplasm and in the nucleus.

Several studies have shown that TRAF4 preferentially associates with the insoluble fractions of cell extracts. TRAF4 is abundant in the insoluble pellet fraction of human embryonic kidney epithelial cells (HEK293T) transfected with HA-tagged-TRAF4, whereas little is seen in the soluble fraction.[15] In addition, Xu and colleagues recovered TRAF4 largely from the cytoskeleton/membrane fraction that also contains p47phox.[16] In immunofluorescence experiments, Glauner et al noticed a significant local increase of TRAF4 at points of cell-cell contact that is dependent on the C-TRAF domain of the protein.[17] Moreover, a recent study reported a perinuclear distribution of TRAF4 in unstimulated HMEC-1 cells and clear cell surface membrane labeling after exposure to TNFα.[18]

On the other hand, TRAF4 has been detected within cytosolic vesicles or organelles of TRAF4-tranfected HEK293T cells.[10] These authors also reported a cytoplasmic localization in vivo in human breast cancer sections. Furthermore, Sax and El-Deiry showed TRAF4 cytoplasmic localization, even after induced cell damage at the DNA level.[19] In the same way TRAF4-GFP has mainly been found in the cytoplasm and excluded from the nucleus in HeLa cells.[20]

In human breast cancer sections, TRAF4 has been seen in the nuclei of cancer cells by immunohistochemistry.[5] Consistent with a nuclear localization, Glauner et al has shown that full-length TRAF4-GFP chimeric proteins localize to the cytoplasm of HeLa cells while C-terminal TRAF4(259-470)-GFP proteins localize predominantly to the nucleus. Moreover, TRAF4(259-470) can translocate a full-length TRAF4 molecule to the nucleus by forming TRAF4-TRAF4(259-470) heteromeric complexes. A truncated form of TRAF4 lacking the C-terminal end but containing the 2 NLS also goes to the nucleus.[17] However, it remains to be seen if such truncated TRAF4 forms exist in vivo.

Collectively, these data are consistent with the characteristics of TRAF4. Thus, it can be hypothesized that TRAF4 shuttles between different cellular compartments. TRAF4 can be present in the cytoplasm and recruited to the membrane via its association with transmembrane or membrane-related proteins. It can also translocate to the nucleus since it contains 2 putative NLS. However, since nuclear localization has only been observed under pathological conditions, this purported function may also be nonphysiological.

In Vivo Evidence That TRAF4 Is Biologically Relevant

TRAF4-deficient mice, on a mixed 129/Svj X C57BL/6 genetic background, have a localized developmental defect in the upper respiratory tract, with a constricted upper trachea at the site of the tracheal junction with the larynx, showing that TRAF4 is required for anastomosis of the upper and lower respiratory systems during development.[11] This restricted phenotype was strange since TRAF4 is widely expressed during embryogenesis. However, on a pure 129/Svj genetic background, TRAF4 deficiency is embryonic lethal in approximately one third of the homozygote mutants, suggesting that TRAF4 is crucial for early embryogenesis. Surviving animals manifest numerous alterations. Tracheal disruption and respiratory disorders affect 100% of the survivors although other alterations are not fully penetrant. The most frequent and important malformations concern the axial skeleton (ribs, sternum, tail), and defect of the neural tube closure giving rise to spina bifida phenotypes.[21] The phenotypic discrepancies between the two strains of TRAF4-null mice point to the impact of genetic background on gene deficiency studies.

In drosophila, homozygous mutants with a P-element insertion (EP(2)578) in the first exon of DTRAF1, which leads to markedly reduced expression of this gene, show a higher number of adult dorsal bristles, a typical structure of the drosophila peripheral nervous system.[22] Moreover, a null allele for DTRAF1 ($DTRAF1^{ex1}$) is lethal and these mutants fail to develop into the pupal stage.[23] Mutant larvae contain small-sized imaginal discs, especially eye discs, and photoreceptor axons form few axonal bundles and fail to defasciculate in the brain hemisphere. Thus, DTRAF1 is indispensable for the development of imaginal eye discs and the formation of a correct photosensory neuronal array in the brain hemisphere. Heterozygous $DTRAF1^{ex1}$ mutants also exhibit defects of the thorax closure, a phenomenon tightly controlled by the drosophila c-Jun amino terminal kinase (JNK) signaling pathway.

Accordingly, depletion of TRAF4a expression in zebrafish using antisense morpholino oligonucleotides also leads to dramatic abnormalities during embryonic development, with particular defects in the sensory organs of the ear and the eye (our unpublished results).

Thus, TRAF4 is a key molecule in diverse ontogenic processes, particularly in the nervous system. This is a particular role for a member of the TRAF protein family. In fact, the other TRAF-deficient mice with the exception of TRAF6 that also presents nervous alterations, show alteration of their immune system.

Table 2. TRAF4 interacting-proteins, TRAF4 domain involved and function

Protein	Domain	Signaling*	Function*	Reference
TNF-receptor signaling				
p75-NGFR	TRAF	NFκB ↓	cell apoptosis ↑	24
LT-βR	TRAF	ND	ND	10
GITR	ND	NFκB ↑	cell survival ↑	27
Msn	TRAF	JNK ↑	cell apoptosis ↑	28
Pelle	TRAF	NFκB ↑	ND	26
Membrane-related proteins				
p70S6K	ND	S6 phosphorylation ↑	cell cycle ↑	30
p47phox	TRAF	JNK ↑	cell apoptosis ↑ oxidant production ↑	16
		ERK1/2; p38 ↑	ND	18
TFAF2/SNX6	ND	TGFβ-R ?	GF traffiking ?	32
Apoptosis-related proteins				
DIAP1/c-IAP-1; c-IAP-2	TRAF	JNK ↓	cell apoptosis ↓	22
Miscellaneous proteins				
TRAF4	TRAF	ND	ND	26
MUL	TRAF	ND	ND	40
USP7/HAUSP	TRAF	ND	ubiquitination ?	40
TFAF1	ND	ND	ND	32
Hic 5	ND	RAFTK/Pyk2	scaffolding ?	16

* ↑ = activation; ↓ = inhibition; ? = putative

Can TRAF4 Transduce Extracellular TNF Signals?

Although in vivo studies show that TRAF4 is involved in important biological functions, how it works at the molecular level remains evasive. Because transient expression of some TRAFs induces nuclear factor kappa B (NFκB) activity, we and others have tested the effects of TRAF4 overexpression on the activation of a NFκB reporter plasmid on transient transfection assays in HEK293T cells. However, no NFκB activity has ever been detected. DTRAF1 does not interact with NFκB signaling either.[23] In order to investigate the signaling pathway(s) involving TRAF4, numerous experiments have been performed to identify its protein partners. Interestingly, upstream TNF-receptors and downstream kinase partners have been found that might engage TRAF4.

TRAF4-Interacting TNF-Rs

Numerous members of the TNF-R family were tested for their interaction with TRAF4. In contrast to other TRAF family members, very few interacted with TRAF4 and only under certain conditions (Table 2).

TRAF4 interacts weakly with the human p75 neurotrophin receptor (p75-NGFR), a member of the TNF-R present in the nervous system, and with the lymphotoxin-beta receptor (LTβ-R).[10,24] The latter receptor mediates an essential signaling system for the development, organization and differentiation of lymphoid tissues.[25] Paradoxically, LTβ-R also induces apoptosis of some epithelial tumors. Moreover, Zapata et al were also able to show an interaction between DTRAF1 and these two receptors.[26]

Recently, it has been shown that TRAF4 increases NFκB activation through the glucocorticoid-induced TNF-R (GITR), a receptor expressed on T cells, B cells and macrophages. This effect is mediated via a TRAF-binding site located in the cytoplasmic domain of GITR, and

is inhibited by the cytoplasmic protein A20, a TNF-inducible zinc finger protein that interacts with TRAF1. This was the first indication that TRAF4 induces GITR signaling, which is presumed to inhibit the suppressive function of regulatory T cells (Treg cells) and to promote the activation of T cells.[27]

TRAF4-Interacting Kinases

TNF-induced signal transduction pathways usually involve kinase cascades (ie : serine/threonine kinases for NFκB and JNK). Knowledge of the TRAF4-interacting kinases is therefore of importance to determine the pathway in which it could be involved.

Misshapen (Msn), a member of the SPS1 protein kinase family, has been shown to probably act as a mitogen-activated protein (MAP)KKKK (MAP4K) in drosophila by activating the JNK pathway. DTRAF1 appears to interact with Msn via its TRAF domain.[28] Moreover, the TRAF domain from DTRAF1 but not DTRAF2 is sufficient to activate JNK. Thus, TRAF4 is a good candidate for an upstream molecule that regulates JNK pathway via interaction and activation of Msn, suggesting that TRAF4 might be involved in regulating Ste20 kinases in mammals. Interestingly, the Trp/Lys/Ile sequence present in the N-TRAF domain, which is responsible for the recruitment of Nck-interacting kinase (NIK), the mammalian homologue of Msn, is conserved in TRAF4. NIK belongs to the germinal center kinase (GCK) subfamily of Ste20 kinases that couples cell surface receptors (ie: Ephrine) to the JNK pathways.[29] Interestingly, it has been shown that interaction of Msn with the Frizzled receptor (Wnt receptor) regulates dorsal closure via JNK pathway.

Another specific association was reported between DTRAF1 and the regulatory N-terminal domain of Pelle, a fly homologue of the mammalian kinase interleukin-1 receptor-associated kinase (IRAK). Individually, Pelle and DTRAF1 are unable to induce NFκB in HEK293T cells, but their coexpression results in significant NFκB activity. Pelle mediates signaling by the cytoplasmic tail of Toll (Interleukin-1 receptor).[26] Whether IRAK can physiologically interact with mammalian TRAF4 has not yet been tested.

Despite intensive research, the function of TRAF4 in signaling pathways triggered by TNF-R-related proteins remains enigmatic. Since the expression pattern of most identified interacting receptors and kinases can be superimposed with that of TRAF4, it is likely that these molecules interact in vivo and lead to functional pathways.

Can TRAF4 Transduce Extracellular Signals via Membrane-Related Partners?

Aside from transmembrane proteins, various membrane-related proteins located more downstream are also implicated in signal transduction. Some have been shown to interact with TRAF4.

p70S6K is a ser/thr kinase localized in the cytosol which, after cytokine stimulation, is also found in the nucleus. The phosphoinositide 3 kinase (PI3K)/p70S6K signaling pathway regulates the translation of key mRNAs of proteins required for cell cycle progression via phosphorylation of the ribosomal S6 protein. Fleckenstein et al identified TRAF4 as a new partner of this kinase,[30] after screening a HeLa cDNA expression library with p70S6K as bait. This interaction was confirmed by several experiments, and complexes were observed in both cytoplasmic and nuclear fractions. These authors also showed that p70S6K/TRAF4 interaction can be induced through activation of LTβ-R in the human TF-1 erythroleukemic cell line. Moreover, in wild-type HEK-293 cells, which do not express endogenous TRAF4, TNFα did not induce p70S6K while cells transfected with TRAF4 showed a strong increase in S6 phosphorylation upon stimulation, suggesting a role for TRAF4 in the activation of this kinase.

Xu et al found TRAF4 in a screen of lung and endothelial libraries for partners of p47phox. p47phox is an adapter subunit of the NAD(P)H oxidase that participates in TNFα signaling, and is associated with the cytoskeleton.[16] p47phox interacts with TRAF4 via a tail-to-tail interaction between the C-terminus of p47phox and the conserved TRAF domain of TRAF4. While these proteins alone have minimal effect, together they constitutively activate JNK and increase oxidant production. The authors postulate that TRAF4 might function to couple p47phox to upstream signaling events.[16] This hypothesis was recently confirmed by Li et al who demonstrated that the

acute response to TNFα involves a rapid PKC-dependent phosphorylation of p47phox, an increase in p47phox-TRAF4 association, translocation of p47phox-TRAF4 to the cell membrane, and activation of the NAD(P)H oxidase, ERK1/2 and p38 MAPK.[18]

Lastly, TRAF4-associated factor 2 (TFAF-2)/sorting nexin 6 (SNX6)[31,32] is a peripheral membrane protein that exhibits a characteristic membrane and cytosolic distribution.[33] It is also localized in endosomal compartments, predominantly in the early endosomes. SNX6 interacts with cargo and is thought to participate in the intracellular trafficking of plasma membrane receptors. SNX6 has been shown to bind to TGFβ-R. Furthermore, the oncogenic serine/threonine kinase Pim1 can phosphorylate TFAF2/SNX6 and induce its translocation from cytoplasm to nucleus.[32]

These membrane-associated TRAF4 partners might determine the subcellular localization of TRAF4, by regulating the proximity of individual signaling complexes to TRAF4, and therefore the activation of specific downstream signals via TRAF4.

Does TRAF4 Regulate Cell Life and Death?

Many TRAF family members negatively regulate apoptotic pathways by increasing the expression of genes which promote cell survival.[2,34] Several groups have hypothesized that TRAF4 might also be involved in apoptosis. However, depending on the study, some authors have proposed a pro-apoptotic function while others an anti-apoptotic function.

TRAF4 as a Pro-Apoptotic Factor

JNK is known to mediate a physiological stress signal that leads to cell death. Two studies[22,23] have reported that DTRAF1 overexpression, notably in S2 cells, can activate the Hep/JNK signaling pathway leading to an increase in JNK phosphorylation, and subsequent apoptosis (30% increase). This activity is independent of the RING finger, as DTRAF1 does not contain a RING finger domain. These authors placed DTRAF1 activity upstream of DTAK1 (drosophila TGFβ-activated kinase). Moreover, DTRAF1 directly interacts with the inhibitor of apoptosis, DIAP1, and its human homologue cIAP-1. c-IAP-1 is normally predominantly localized in the nucleus, but apoptotic stimuli induces its export from the nucleus. Finally, c-IAP-1 associates with mid-bodies in dividing cells.[35] Increased amounts of DIAP1 lower the amount of DTRAF1 in cells. Indeed, DIAP1 (as c-IAP1) contains ubiquitin ligase activity and can stimulate DTRAF1 degradation through ubiquitination. Thus, DIAP1 can prevent DTRAF1-induced activation of JNK as well as cell death.

TRAF4 was also found to be the only TRAF member that is regulated by the tumor suppressor p53, in a microarray analysis of p53-regulated genes.[19] The TRAF4 promoter contains a functional p53 DNA-binding site approximately 1 kb upstream of the initiating methionine residue, and overexpression of TRAF4 induces apoptosis. Since this apoptosis occurs at a slow rate, these authors proposed that TRAF4 is not directly involved but may be a late mediator in a pro-apoptotic signaling pathway. Thus, TRAF4 might play a role in p53-mediated pro-apoptotic signaling in response to cellular stress. Furthermore, TRAF4 suppresses colony formation in 4 cell lines in this study regardless of p53 activity, an activity that is dependent on the TRAF domain.

Finally TRAF4 has been shown to suppress the ability of the common neurotrophin receptor $p75^{NTR}$ dimers to block cell death induced by $p75^{NTR}$ monomers, also suggesting a pro-apoptotic role for TRAF4.[24]

TRAF4 as an Anti-Apoptotic Factor

In Jurkat leukemic T cells expressing IκB-alpha delta N, a super repressor of NFκB activation, treatment by the survival agent and tumor promoter PMA strongly induces apoptosis, indicating that NFκB promotes cell survival. Interestingly, while TRAF4, c-IAP-1 and c-IAP-2 expression is usually induced by PMA, it is not in these cells lacking NFκB activity. This suggests that TRAF4 might be anti-apoptotic like c-IAPs. Among TRAF1-4, TRAF4 is upregulated the most and the fastest to PMA treatment (2h, 3.2X).[17,36]

CD40 has a 62 amino acid long cytoplasmic domain comprising 2 distinct TRAF binding sites.[20] All TRAF proteins except TRAF4 have been reported to associate directly or indirectly with CD40.

However, human multiple myeloma (MM) cells treated with soluble CD40 ligand (gp39) show lower TRAF4 and TRAF6 expression (38% and 32% decrease, respectively) while expression of the other TRAFs remains stable.[37] This was accompanied by inhibition of MM cell growth and apoptosis. These results suggest that TRAF4 is affected downstream of this signaling pathway and not involved in CD40 function. Accordingly, Craxton and colleagues found that TRAF4 mRNA levels are up-regulated following CD40 signaling in B cells.[38] This effect might be cell-specific since it was not found in CD40+ human monocytes or in THP1, a human promonocytic leukemia cell line.[39]

Fleckenstein et al also postulated an anti-apoptotic function for TRAF4 when they found that the anti-Fas antibody, CH-11, induces apoptosis in HEK293 cells, but not when these cells are stably transfected with TRAF4. Thus, TRAF4 confers unresponsiveness to apoptotic stimuli.[30]

Although seemingly paradoxical, these data could all be correct depending on the cells examined. Further experiments are clearly needed to determine the function of TRAF4 in cell life and death.

Miscellaneous TRAF4 Partners

Like all TRAFs, TRAF4 has been shown to homodimerize in fly, fish, mouse and man. Moreover, various TRAF4-interacting proteins have also been reported.

Zapata et al have identified a new family of TRAF-domain containing proteins called TEF, for TRAF domain (TD)-encompassing factors.[40] In vitro, two of these proteins, human MUL/TEF3 and USP7/TEF1 bind to TRAF4 (and five other TRAFs) via their TRAF domains, located at the N-terminal or in the central region, respectively. MUL localizes to cytosolic bodies of unknown nature; USP7/HAUSP is an ubiquitin-specific protease (USP) that localizes in the nucleus in structures positive for promyelocytic leukemia (PML). Such proteins exist in diverse eukaryotic plant and animal species. For example, the TDPOZ subfamily includes more than 30 proteins that associate a TD domain with POZ/BTB domains, and are presumably nuclear scaffold proteins.[41]

Glutathione transferases (GSTs) catalyze the conjugation of glutathione with reactive compounds and is involved in the cellular protection against oxidative stress. They are also proposed to modulate kinases, and GST P1-1 interacts with JNK. GST A1-1 has been shown to interact with TFAF1 (TRAF4-associated factor 1),[42] a protein which has recently been shown to be transcriptionally regulated by nitric oxide.[43]

Xu et al also found a dozen TRAF4-interacting partners among endothelial proteins, which are notably involved in cell proliferation and apoptosis. For example, Hic 5 is a tyrosine kinase scaffold protein that is the paralogue of paxillin, which binds to, and is a substrate for related adhesion focal tyrosine kinase (RAFTK, Pyk2). Such pathways are activated by oxidants.[16]

The in vivo existence and meaning of all these interactions remain to be studied at the functional level.

Is TRAF4 Implicated in Human Diseases?

Very few experiments have been performed to test the involvement of TRAF4 in human diseases.

Malignant Diseases

TRAF4 corresponds to clone MLN62 first identified by differential screening in a human metastatic lymph node from a breast cancer cDNA library.[44] About 17.5% of breast tumors overexpress TRAF4 due to gene amplification, alone or in association with the erbB2/HER oncogene.[45] The positive cells are malignant epithelial cells. This expression suggests that TRAF4 might be involved in the formation and/or progression of primary breast cancers and metastases. In support of this hypothesis, TRAF4 is overexpressed in some human breast tumors, as shown by microarray analysis in one study.[46] However, no extensive study has yet been performed to establish the significance of TRAF4 overexpression in terms of diagnosis or prognosis. On the contrary, a second study has shown that TRAF4 is downregulated in many breast tumors, with less than 10% of the primary tumors expressing TRAF4, while strong TRAF4 expression was observed in normal ducts.[10] These discrepancies might derive from the difference in the differentiation grade of the tumors studied or

from the antibodies used, which might recognize various TRAF4 forms. It has also been reported that Hodgkin disease cell lines L428, KMH2 and HS445 expressed moderately TRAF4.[47]

The implication of TRAF4 in malignant processes has been tested using animal models. It has been shown that TRAF4 is not an oncogene or a tumor suppressor gene. In fact, both gain-of-function (transgenic mice expressing high amounts of TRAF4 under the control of either the ubiquitous promoter CMV or the mammary gland specific promoter MMTV) (our unpublished results) and loss-of-function (TRAF4-deficient mice[11,21]) mouse models for TRAF4 do not result in tumor development.

Neuronal Benign Diseases

Microarray analyses (12000 genes studied) of postmortem temporal cortexes from patients with schizophrenia show that the expression of 38 genes is altered. Notably, decreased expression of myelination related genes (among them erbB3), TRAF4, Neurod1, and histone deacetylase 3 (HDAC3) was observed.[48] The authors hypothesized that TRAF4 decrease might have an important effect on this disease.

To date, no relationship between TRAF4 expression and diagnosis or prognosis has been established in cancers. Moreover, TRAF4 alteration seems to be involved in benign diseases of the CNS. More experiments are needed to determine the clinical significance of TRAF4 alteration in both benign and malignant human diseases.

Conclusion and Perspective

Collectively, data show a strong structural homology among species, suggesting a functional conservation of TRAF4 throughout Metazoan evolution. This conservation strengthens the idea that TRAF4 exerts crucial biological function(s) distinct from those previously assigned to the other TRAF proteins. Various and sometimes opposing functions have been proposed for TRAF4 (ie: pro-apoptotic and anti-apoptotic). Consistent with its wide expression pattern, it might be hypothesized that TRAF4 is pleiotropic and exerts several functions depending on the nature of the cell/organ concerned or even on the cell compartment, each of them driving specific signaling pathways. Accordingly, a great variety of TRAF4 partners have been identified including cytoplasmic adaptors, membrane-related proteins, membrane receptors, apoptosis inhibitors, nuclear proteases. Most of the data suggest that TRAF4 may be preferentially involved in stress-related events. However, to date, TRAF4 has not been placed in a clear signaling cascade(s) and future studies will aim to determine these molecular mechanisms.

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CHAPTER 6

Characteristics and Biological Functions of TRAF6

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Abstract

TRAF6 is divergent from other members of the TRAF family. Therefore, TRAF6 was expected to play physiological roles distinct from those of other TRAFs. In this chapter, we focused on the physiological functions specific to TRAF6 but not other TRAFs in immune system, formation of skin appendices, and nervous system development by describing abnormal phenotypes observed in *TRAF6*-deficient mice. The role of TRAF6 in osteoclastogenesis and the molecular mechanisms of TRAF6-mediated signal transduction are described in other chapters.

Introduction

TRAF6 was first identified by yeast two-hybrid screening with the cytoplasmic tail of CD40 as bait[1] and independently by screening of an expressed sequence tag (EST).[2] Of the members of the TRAF family, TRAF6 has the most divergent TRAF-C domain, which binds to the cytoplasmic tails of receptors and other upstream molecules. The TRAF-C domain of TRAF6 recognizes amino acid sequences that are different from those recognized by other TRAFs. TRAF6 binds to the X-X-P-X-E-X-X-Acidic or Aromatic consensus-binding site, whereas TRAF2, TRAF3, and TRAF5 bind to the P-X-Q-X-T motif.[3] As a consequence of this difference, TRAF6 was expected to have physiological functions distinct from those of other TRAFs. Generation of *TRAF6*-deficient mice revealed that TRAF6 plays crucial roles in several important processes and that other TRAFs cannot compensate for loss of TRAF6 (Fig. 1).[4,5]

Role of TRAF6 in Immune System

Interleukin-1 (IL-1) Receptor Signaling

IL-1 is a key inflammatory cytokine that has profound effects in various organ systems.[6] IL-1 activates the innate immune response and is also a potent stimulator of the adaptive immune system. The receptor that triggers intracellular signaling in response to IL-1 is the type I IL-1 receptor (IL-1RI). The cytoplasmic portion of IL-1RI is homologous to the cytoplasmic domains of the other members of the IL-1R family as well as the cytoplasmic domains of Toll family members. This conserved cytoplasmic domain is called the Toll/IL-1R (TIR) domain. A homolog of IL-1RI, IL-1R accessory protein (AcP), is also required for IL-1 signaling. AcP does not bind IL-1, but when IL-1 binds IL-1RI, AcP is recruited to the ligand-receptor complex to form the high-affinity receptor complex that is necessary and sufficient for signal transduction. Ligand binding also induces association of IL-1RI with a TIR domain-containing adaptor protein, MyD88, through a TIR-TIR domain interaction.[7]

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

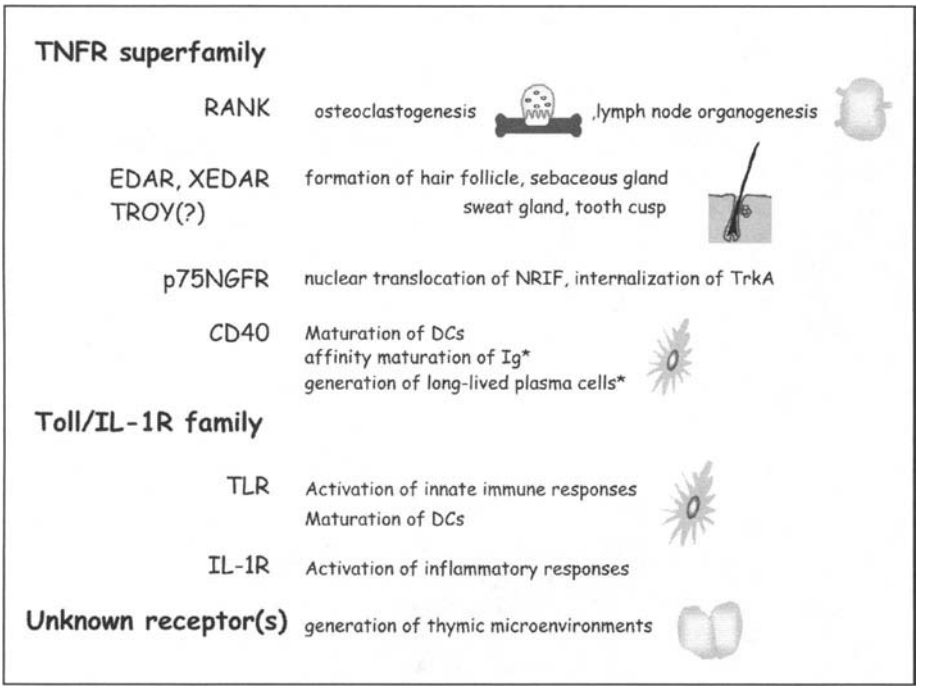

Figure 1. Physiological roles of TRAF6. See text for details. Asterisks denote functions that have not been confirmed to be impaired in *TRAF6*$^{-/-}$ mice.

Subsequently, IL-IR -associated kinase-4 (IRAK-4) and IRAK-1 then associate with MyD88.[8] Cao et al[2] identified TRAF6 as a stimulation-dependent IRAK-1 binding protein appears to be involved in IL-1 signaling. To determine whether TRAF6 is essential for IL-1 signaling, we generated mouse embryonic fibroblast (MEF) cell lines from wild-type and *TRAF6*$^{-/-}$ mice.[9] IL-1-induced activation of NFκB, JNK, and p38 was abrogated in *TRAF6*$^{-/-}$ MEF cells. In contrast, activation of the NFκB and MAPK pathways by tumor necrosis factor α (TNFα) or sorbitol in *TRAF6*$^{-/-}$ MEF cells was similar to that in wild-type MEF cells. Furthermore, expression of exogenous TRAF6 in *TRAF6*$^{-/-}$ MEF cells restored activation of NFκB, JNK, and p38 in response to IL-1. These results indicate that TRAF6 is essential for IL-1 signaling linked to NFκB and MAPK activation.

Toll-Like Receptor Signaling

Toll-like receptors (TLRs) recognize specific structural motifs expressed by various pathogens. Those motifs are known as pathogen-associated microbial patterns (PAMPs). TLRs are critical for innate immune responses.[10,11] The intracellular signaling pathways utilized by various TLRs differ, which may provide a molecular basis for differences in the immune-related genes induced by distinct TLRs. Similar to IL-1RI, the cytoplasmic regions of TLRs contain a TIR domain that meditates homo- and heteromeric associations between TLRs and TIR-containing adaptor proteins, including MyD88, Mal/TIRAP, TRIF/TICAM-1, and TRAM.[12] Members of the TLR family can be classified into at least four groups on the basis of differential usage of TIR-containing adaptors. The first group contains TLR5, TLR7, and TLR9, which have only MyD88 in their pathways, whereas the second group, comprising TLR1, TLR2, and TLR6, utilizes both MyD88 and Mal/TIRAP. The third group, which contains TLR3, has a pathway mediated only by TRIF, and the fourth group, which contains TLR4, uses both MyD88-MAL/TIRAP-mediated and TRAM-TRIF-mediated pathways. By analogy with IL-1 signaling, it was originally speculated

that all TLR pathways were mediated by TRAF6, although there was no genetic evidence to support this idea. In addition, two groups reported that TRAF6 binds to TRIF and is involved in TRIF-mediated activation of NFκB.[13,14] These findings suggest that TRAF6 may be involved in both MyD88-dependent and -independent pathways. However, there are discrepancies between the findings in these studies, and the roles of TRAF6 in TLR signaling under physiological conditions were not adequately addressed.

To clarify the physiological role of TRAF6 in TLR signaling, macrophages generated from *TRAF6*$^{-/-}$ and *TRAF6*$^{+/-}$ mice were analyzed and the following results were obtained.[15] We examined the effects of *TRAF6* deficiency on signaling from members of the first and the second TLR groups. MALP-2 (a ligand of the TLR2/TLR6 heterodimer), bacterial lipopeptide (BLP) (a ligand of the TLR1/TLR2 heterodimer), flagellin (a ligand of TLR5), imidazoquinoline (R848) (a ligand of TLR7), and CpG-DNA (a ligand of TLR9) could not induce activation of NFκB and MAPKs in *TRAF6*$^{-/-}$ macrophages. *TRAF6*$^{+/-}$ macrophages produced TNFα or IL-6 in response to MALP-2, R848, and CpG-DNA, whereas *TRAF6*$^{-/-}$ macrophages did not produce these cytokines in response to any of the listed ligands. These results indicate that TRAF6 is essential for signaling from TLR2, TLR5, TLR7, and TLR9. Poly(I:C), a ligand of TLR3, induced activation of MAPKs and NFκB to a similar extent and with similar kinetics in both *TRAF6*$^{+/-}$ and *TRAF6*$^{-/-}$ macrophages. In addition, production of TNFα and IL-6, induction of interferon-β (IFN-β) and several IFN-β-inducible genes, including IP10, MCP-1, and RANTES, and up-regulation of CD86 in response to poly(I:C) stimulation was not affected by *TRAF6* deficiency. These findings indicate that TRAF6 is not required for TLR3 signaling. When *TRAF6*$^{-/-}$ macrophages were stimulated with LPS, a ligand of TLR4, activation of NFκB and MAPKs was observed, however, the kinetics were somewhat slower than those in *TRAF6*$^{+/-}$ macrophages. It has been shown that early-phase NFκB activation is dependent on MyD88, whereas late-phase NFκB activation is dependent on TRIF and TRAM and that both phases of NFκB activation are required for cytokine production.[16,17] LPS-induced production of TNFα or IL-6 was not detected in *TRAF6*$^{-/-}$ macrophages. These results indicate that TRAF6 is involved in MyD88-mediated but not TRIF-mediated NFκB activation. In addition, LPS-induced expression of IP10 and enhanced expression of CD86, which is TRIF-dependent, in both *TRAF6*$^{+/-}$ and *TRAF6*$^{-/-}$ macrophages, indicating that the TLR4-TRIF pathway is independent of TRAF6. Therefore, TRAF6 is required for the MyD88-dependent pathway but not the TRIF-dependent pathway of TLR4. Consistent with this idea, a recent report by Covert et al[18] suggested that activation of NFκB by the TRIF-dependent pathway results from a secondary response to secreted TNFα induced by IFN regulatory factor-3 (IRF-3). which is activated by TRIF signaling. Recently, IRF-5[19] and IRF-7[20,21] were shown to form signaling complexes with MyD88 and TRAF6. IRF-5 mediates MyD88-dependent induction of proinflammatory cytokine genes, while IRF-7 is required for MyD88-dependent IFN-α induction.

Development and Maturation of Dendritic Cells

Dendritic cells (DCs) play a crucial role in acquired immunity by capturing, processing, and presenting antigens to T cells.[22] In addition to presenting antigenic peptides bound to major histocompatibility complex (MHC) molecules, DCs provide costimulatory signals to trigger efficient T cell responses. Although TLR and CD40 signals are known to induce maturation of DCs, the signal transducers for these receptors critical for DC maturation remained unknown. Kobayashi et al[23] tested the maturation and development of DCs in the absence of TRAF6, which was thought to be involved in TLR and CD40 signaling. Cultured DCs induced from splenocytes by incubating with granulocyte-macrophage colony-stimulating factor (GM-CSF) were treated with various PAMPs or CD40L and then upregulation of MHC class II and B7.2, which is indicative of DC maturation, was assessed. Upregulation of MHC class II and B7.2 was observed in wild-type DCs but not in *TRAF6*$^{-/-}$ DCs. These results indicate that optimal DC maturation in vitro in response to TLR and CD40 stimulation requires TRAF6. Furthermore, levels of MHC class II and B7.2 were increased significantly in splenic DCs of wild-type mice after injection of LPS or anti-CD40 antibody, whereas expression of these markers was not increased in response to LPS or

anti-CD40 antibody in splenic DCs from *TRAF6*$^{-/-}$ mice. Therefore, TRAF6 is required for DC maturation both in vitro and in vivo. Splenic DCs were then analyzed for surface markers without culture or stimulation to understand the role of TRAF6 in DC development in vivo. In the absence of TRAF6, the number of DCs in 14 day-week-old *TRAF6*$^{-/-}$ mice was significantly lower than that in control littermates. To address whether the defect in splenic DC development in *TRAF6*$^{-/-}$ mice was due to *TRAF6*-deficiency in hematopoietic cells, bone marrow chimeras reconstituted with fetal liver cells from *TRAF6*$^{-/-}$ mice or wild-type littermates were generated. *TRAF6*$^{-/-}$ bone marrow chimeras exhibited a markedly reduced frequency and total number of DCs, indicating that TRAF6 in DCs is essential for DC development in vivo.

Lymph Node Organogenesis

Lymph nodes are the major peripheral lymphoid organs in which distinct cell lineages interact to initiate immune defenses. Molecules involved in lymphotoxin (LT) signaling, including LTα, LTβ, LTβ-receptor (LTβR), NFκB-inducing kinase (NIK), and NFκB, are required for formation of lymph nodes.[24,25] The fact that both RANK- and *TRAF6*-deficient mice lack lymph nodes strongly suggested that both the RANK-TRAF6 pathway and the lymphotoxin pathway play crucial roles in lymph node organogenesis.[5,26] Histological analysis of the early phase of lymph node organogenesis suggested that the IL-7Rα^{+} cells accumulating in the lymph node anlagen are the Lτα1β2-expressing cells. These Lτα1β2-expressing cells stimulate VCAM-1 expression by surrounding mesenchymal cells, which then become lymph node anlagen. IL-7Rα^{+} cells in the mesenteric lymph node express cell surface Lτα1β2 in response to RANK ligand (RANKL).[27] To determine if RANK signaling requires TRAF6 for induction of Lτα1β2 expression, IL-7Rα^{+} cells isolated from the mesentery of *TRAF6*$^{-/-}$ or wild-type embryos were stimulated with RANKL, and induction of Lτα1β2 was measured. IL-7Rα^{+} cells from *TRAF6*$^{-/-}$ embryos did not express Lτα1β2 in respond to RANKL, whereas cells from control embryos did, indicating that the RANK-TRAF6 pathway is required for Lτα1β2 induction in IL-7Rα expressing cells during lymph node organogenesis.[27]

Central Tolerance of T Cells

Thymic microenvironments are essential for generation of a T cell repertoire.[28] Cortical thymic epithelial cells (cTECs) are involved in selection of thymocytes capable of recognizing self-MHC, whereas medullary TECs (mTECs) play a crucial role in self-tolerance by eliminating self-reactive T cells. In *TRAF6*$^{-/-}$ mice, size of the thymic medulla was reduced, and the corticomedullary junction was ill-defined.[29] Immunohistochemical analysis revealed an abnormal distribution and impaired maturation of mTECs in *TRAF6*$^{-/-}$ thymus. Aire protein promotes ectopic expression of peripheral tissue-specific antigens (TSAs), thereby establishing central tolerance to TSAs.[30,31] Expression of *aire* and TSAs are reduced significantly in 14-day-old *TRAF6*$^{-/-}$ thymus.[29] The altered thymic organization and reduced aire expression strongly suggest that *TRAF6*$^{-/-}$ mice may possess an autoimmune phenotype. This speculation was supported by observations of inflammatory infiltrates in lung, liver, pancreas, and kidney of *TRAF6*$^{-/-}$ mice. Furthermore, the number of regulatory T cells (regT) was dramatically reduced in 14-day-old *TRAF6*$^{-/-}$ mice. To determine whether the autoimmune-like phenotypes of *TRAF6*$^{-/-}$ mice are related to altered thymic stroma, fetal thymi isolated from embryonic day 14 *TRAF6*$^{-/-}$ and control mice were grafted under the renal capsules of nude mice. Eight weeks after grafting, normal generation of thymocytes and distribution of mature T cells in spleen and lymph nodes were observed in recipients grafted with *TRAF6*$^{-/-}$ thymus (KO/nu) or control thymus (WT/nu). KO/nu mice had inflammatory infiltrates in lung, liver, pancreas, and kidney similar to those in *TRAF6*$^{-/-}$ mice. Furthermore, sera from KO/nu mice contained autoantibodies against whole liver, islets in Langerhans of the pancreas, and blood vessel walls in the lung. Taken together, these data indicate that the altered thymic stroma in *TRAF6-/-* mice is sufficient to induce autoimmunity.

A similarly altered thymic structure and multi-organ inflammation were reported as abnormalities in RelB-deficient mice[32,33] and in alymphoplasia (*aly*) mice, which carry a mutation in NIK.[34] RelB and its transcripts were not detected in *TRAF6*$^{-/-}$ fetal thymic stroma, indicating that

RelB expression requires TRAF6 signaling.[29] RelB forms heterodimers with p52, a processed product of p100. Processing of p100 is triggered by phosphorylation pf p100 catalyzed by IKKα, which is activated by NIK.[35] In *aly* mice, RelB induction and p100 processing are impaired,[34] while the ratio of p52 to p100, which is indicative of p100 processing, was not affected by *TRAF6*-deficiency.[29] Therefore, at least two critical NFκB-related events may be essential for the initial stage of mTEC differentiation: induction of RelB expression, which requires both TRAF6 and NIK, and optimal processing of p100 to p52, which requires NIK.

Autoimmunity induced by *TRAF6*$^{-/-}$ thymic stroma may be due to a defect in *aire* gene expression or a defect in the production of regT. Reduced regT production is also observed in *aly* mice.[34] These results suggests that normal development of regT requires thymic microenvironments whose formation is directed by both TRAF6 and NIK-mediated signals.

Role of TRAF6 in Development of Skin Appendices

Hypohidrotic ectodermal dysplasia (HED) is a congenital disorder of ectodermal differentiation in which the individuals have no sweat glands, sparse scalp hair, and abnormal teeth.[36] This disorder is caused by altered signaling from a member of the TNFR superfamily, ectodysplasin receptor (EDAR).[37] Thus, mutations in ectodysplasin (EDA, a ligand of EDAR) or EDARADD (a signal transducer that binds cytoplasmic tail of EDAR) cause HED. Mouse models of HED, Tabby (*Ta*), downless (*dl*), and crinkled (*cr*), are caused by mutations in the murine EDA, EDAR, and EDARADD genes, respectively. Recently, two additional EDAR-related members of the TNFR superfamily, X-linked ectodysplasin-A2 receptor (XEDAR)[38] and TROY[39] were described. Signals from these three receptors activate transcription factor NFκB, and specific missense mutations in NEMO (IKKγ) result in HED and immunodeficiency. Thus, HED may result from impaired NFκB signaling, possibly triggered by members of the TNFR superfamily expressed in skin and hair follicles. *TRAF6*$^{-/-}$ mice have focal alopecia behind their ears and alopecia of the tail.[36] They also have a distinctive kink near the tip of the tail. Identical phenotypes have been reported for *Ta*, *dl*, and *cr*, which led us to speculate that *TRAF6*$^{-/-}$ mice display HED. Although many hair follicles are developed in *TRAF6*$^{-/-}$ mice, guard hair follicles were absent, although these follicles are present in heterozygous mouse skin. Absence of guard hair follicles is also observed in *dl* mice. When the fine structure of hair shafts was examined, *TRAF6*$^{-/-}$ mice have a single type of pelage hairs that contains two or three rows of air cells and many constrictions. Although the pelage hairs of *dl* mice also have two or three rows of air cells, they have no constrictions. *TRAF6*$^{-/-}$ mice, like *Ta*, *dl*, and *cr*, mice, lack sweat glands. In addition, formation of sebaceous glands was severely impaired in *TRAF6*$^{-/-}$ mice, whereas sebaceous gland development was not altered in *Ta*, *dl*, and *cr* mice. Similar to *dl* mice, formation of modified sebaceous glands, such as meibomian glands, anal glands, and preputial glands, are severely impaired in *TRAF6*$^{-/-}$ mice. Examination of *TRAF6*$^{-/-}$ mice revealed abnormalities in molar teeth that are similar but more severe than those produced by mutations in EDA signaling molecules.[40] This finding indicates that, in addition to the EDA-EDAR signaling, TRAF6 is involved in molar tooth cusp formation. Taken together, these data indicate that *TRAF6*$^{-/-}$ mice have a form of HED that slightly differs from the HED observed in *Ta*, *dl*, and *cr*.

Three members of the TNFR superfamily, DL/EDAR, TROY, and XEDAR are expressed in skin and hair follicles. Because some phenotypes of *TRAF6*$^{-/-}$ mice are different from those of *Ta*, *dl* and *cr*, mice, it is interesting to investigate roles of *TRAF6* in signaling from these three receptors. TRAF6 binds with high affinity to the cytoplasmic tail of XEDAR and to lower affinity to that of TROY. TRAF6 does not bind DL. Involvement of TRAF6 in EDAR-mediated NFκB activation has been reported.[41] We also found that TRAF6 is an essential signal transducer for XEDAR.[36] Therefore, it is possible that EDAR, XEDAR and TROY act cooperatively in development of epithelial appendices, although no apparent defect in formation of the skin appendices was reported in XEDAR- or TROY-deficient mice.[42,43]

Role of TRAF6 in Nervous System

The neutrophin receptor p75 is a member of the TNF superfamily. Depending on the context of the cell, p75 either promotes survival or induces apoptosis after neutrophin stimulation.[44] The survival signal emanating from p75 is mediated by NFκB, whereas the apoptotic signal is mediated by JNK activation. Although the consensus TRAF6 binding site is not present in the cytoplasmic tail of p75, TRAF6 is thought to be involved in the p75 signaling because p75-mediated NFκB activation is inhibited by expression of a dominant-negative mutant of TRAF6.[45] To confirm the involvement of TRAF6 in p75 signaling, this signaling pathway was analyzed in *TRAF6*$^{-/-}$ mice.[46] In Schwann cells isolated from wild-type mice, nerve growth factor (NGF) stimulation caused a 2-fold increase in transcription of an NFκB reporter gene, whereas the response to NGF was absent in *TRAF6*$^{-/-}$ mice. Furthermore, NGF activation of JNK was scarcely observed in Schwann cells from *TRAF6*$^{-/-}$ mice. In sympathetic neurons cultured from the superior cervical ganglia (SCG), stimulation of p75 by brain-derived neurotrophic factor (BDNF) resulted in JNK activation and apoptosis, whereas *TRAF6*$^{-/-}$ SCG did not respond to BDNF. Consistent with this observation, there was a significant reduction in the number of TUNEL-positive cells in the SCG of postnatal day 4 *TRAF6*$^{-/-}$ mice during naturally occurring cell death in vivo when compared with wild-type littermates. These results indicate that TRAF6 plays an essential role in p75 signaling.

It was recently reported that TRAF6 participates in protein translocation in response to NGF. Upon NGF stimulation, TRAF6 mediates K63-linked polyubiquitination of neutrophin receptor interacting factor (NRIF) and TrkA. Ubiquitinated NRIF translocates to the nucleus and is required for p75-induced apoptosis. Nuclear translocation of NRIF is abrogated in the absence of TRAF6.[47] In addition, NGF stimulates TrkA polyubiquitination only in the presence of p75 because NGF induces association of p75 with TrkA through p62, which then recruits TRAF6. Blockade of TRAF6-mediated polyubiquitination of TrkA resulted in retention of the receptor at the membrane and failure to activate specific signaling pathways.[48] These data indicate that TRAF6 is required for NGF-dependent internalization of TrkA and signaling.

Future Studies of TRAF6

Accumulating evidence indicates that the TRAF proteins are key molecules that mediate signaling from members of the TNFR superfamily and the Toll/IL-1R family. Among members of the TRAF family, TRAF6 plays critical but distinct roles in regulating various biological processes as described above. In many cases, impairment of TRAF6 signaling results in abnormalities that are similar to those observed in human diseases related to bone metabolism, skin appendices, neuron development, inflammation, and immune response. Therefore, strategies to inhibit or activate TRAF6 may be useful for treatment of such diseases. To develop TRAF6-based therapies, the molecular mechanisms by which a number of molecules regulate TRAF6 need to be clarified.

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CHAPTER 7

Ubiquitination and TRAF Signaling

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Introduction

The Ubiquitin-Proteasome Pathway

Ubiquitin (Ub) is a highly conserved small polypeptide that is ubiquitously expressed in all eukaryotic cells. The best-known function of ubiquitin is to target protein degradation through covalent attachment of this polypeptide on protein substrates.[1-3] This covalent modification, known as ubiquitination, is carried out via a three-step enzymatic cascade. In the first step, Ub is activated by the Ub-activating enzyme (E1) in an ATP-dependent reaction to form an E1-Ub thioester. In the second step, the activated Ub is transferred to a cysteine residue in the active site of a Ub-conjugating enzyme (Ubc or E2) to form an E2-Ub thioester. Finally, in the presence of a Ub-protein ligase (E3), ubiquitin is conjugated to a protein substrate by forming an isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the protein target. After Ub is conjugated to a protein substrate, Ub itself can be conjugated by another Ub through one of its seven lysines, typically lysine-48. This process reiterates itself in a highly processive manner to form a polyubiquitin chain, which is then recruited to a large ATP-dependent protease complex called the 26S proteasome. The polyubiquitinated protein substrates are degraded inside the proteasome, whereas the polyubiquitin chains are cleaved to monomeric ubiquitin, which is recycled.

The 26S proteasome is composed of the 20S catalytic core and 19S regulatory particle.[4] The 20S proteasome is a cylinder-like structure formed by four rings, each containing seven subunits. These subunits form an enclosed proteolytic chamber within which the catalytic residues reside. This chamber is impermeable to proteins, except for a narrow channel that connects to the 19S proteasome, which gates the entry of protein substrates. The 19S complex can be further separated into a base and a lid. The base contains multiple ATPase subunits, which presumably function to unfold ubiquitinated protein substrates and propel the unfolded polypeptides through the narrow channel into the catalytic chamber of the proteasome. The lid contains nonATPase subunits, some of which bind to polyubiquitin chains and recruit polyubiquitinated proteins to the proteasome.

The substrate specificity of ubiquitination is dictated by a large family of E2s (more than 40 members in human) and a very large family of E3s (more than 700 members in human). All E2s contain a highly conserved domain called the Ubc domain, which has an invariant cysteine residue in the active site. The vast majority of E3s contain either a RING (Really INteresting Gene) or HECT domain (Homology to E6AP C-Terminus).[5-9] The RING domain E3s function either as a single polypeptide, such as TRAF (TNF Receptor Associated Factor; see below) and IAP (Inhibitor of Apoptosis Protein), or as a subunit of multi-protein complexes. The classical examples of multi-subunit E3s include APC/C (Anaphase Promoting Complex/Cyclosome), which ubiquitinates cell cycle proteins such as cyclins,[10,11] and SCF (Skp1-Cul1-F-box), which ubiquitinates many cellular proteins

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

such as the NF-κB inhibitor IκB and the cyclin-dependent kinase inhibitor p27.[12-15] APC/C contains the RING domain protein APC11, whereas SCF contains the RING domain protein Rbx1 (also known as Roc1 or Hrt1) as the catalytic core.[5] The RING domain of E3s interacts with E2s to facilitate polyubiquitination, but the detailed mechanism by which RING E3s facilitate polyubiquitination is not understood. In contrast, the catalytic mechanism of HECT domain E3s is better understood. The HECT domain contains a catalytic cysteine which accepts Ub from an E2 in a thioester relay, and transfers the Ub directly to a lysine residue of the target protein.[16] Examples of the HECT domain E3s include E6AP, which ubiquitinates p53 and targets p53 for degradation in cells expressing the human papillomavirus (HPV) protein E6,[17-19] and NEDD4, which ubiquitinates several cell surface proteins and targets these proteins for endocytosis.[20]

Like other reversible covalent modification such as phosphorylation, ubiquitination can also be reversed by a large family of deubiquitination enzymes (DUBs, also known as isopeptidase).[21] The majority of DUBs are cysteine proteases, which can be classified into four subfamilies based on the following related but distinct domains: UBP (ubiquitin-specific protease), UCH (ubiquitin carboxyl-terminal hydrolase), OTU (ovarian tumor related), and Ataxin-3/Josephin. The fifth subfamily of DUBs are metalloproteases that contain a unique JAMM/MPN+ domain, which was first discovered in a subunit (Rpn11) of the 19S regulatory particle of the proteasome and a subunit (JAB1/CSN5) of the proteasome-like particle termed COP9/Signalosome (CSN).

The NF-κB Pathway

The NF-κB/Rel family of transcription factors controls many physiological processes including inflammation, immunity and apoptosis.[22-24] Members of this family include Rel-A (p65), Rel-B, c-Rel, p50 and p52. These proteins form homo- or hetero-dimers that bind to a consensus DNA sequence known as the κB site, which is present in a large variety of genes. All members of the NF-κB family contain a highly conserved Rel-homology domain (RHD), which is responsible for DNA binding, dimerization, nuclear translocation, and interaction with the NF-κB inhibitor IκB. IκB binds to the nuclear localization sequence of NF-κB, thus sequestering NF-κB in the cytoplasm. IκB is also a multi-member family, which includes IκBα, IκBβ and IκBε. All of these IκB proteins contain 6-7 repeats of ankyrin motifs, which bind to the RHD domain of NF-κB. The ankyrin repeats are also present at the C-termini of the NF-κB precursors p105 and p100, which are processed to the mature subunits p50 and p52, respectively.

The NF-κB activation pathway is broadly classified into the canonical and noncanonical pathways, depending on whether the pathway involves the degradation of IκB or processing of the NF-κB precursors, especially p100.[25] In the canonical pathway, stimulation of cells with an NF-κB agonist, such as tumor necrosis factor-α (TNFα) or interleukin-1β (IL-1β), leads to the activation of a large kinase complex consisting of IKKα, IKKβ and the essential regulatory protein NEMO (also known as IKKγ). This IKK complex, in particular IKKβ, phosphorylates IκB proteins at two N-terminal serine residues, thereby targeting IκB for ubiquitination and subsequent degradation by the proteasome. NF-κB is then liberated to enter the nucleus to carry out its nuclear functions. In the noncanonical pathway, which usually occurs in B cells, stimulation of certain subsets of the TNF receptor superfamily, such as CD40 and BAFF receptor, leads to activation of the protein kinase NIK. NIK in turn phosphorylates and activates IKKα, which then phosphorylates p100 and targets this precursor for polyubiquitination. Unlike IκB, polyubiquitinated p100 is not completely degraded by the proteasome. Rather, the polyubiquitin chain recruits the proteasome to degrade only the C-terminal domain of p100, while leaving the N-terminal RHD domain intact, thus generating the mature p52 subunit. p52 forms a heterodimer with Rel-B, and this dimeric complex translocates to the nucleus to activate target genes involved in B cell maturation. p105 can also be processed to p50 cotranslationally or post-translationally, both requiring the proteasome.[26,27] The cotranslational processing is a constitutive process that may not require phosphorylation or ubiquitination, whereas post-translational processing requires phosphorylation and ubiquitination of p105, which is induced by some agents such as the bacterial lipopolysaccharides (LPS). LPS can also induce the complete degradation of p105, leading to the activation of the p105-associated kinase Tpl2, a MAP3K required for ERK activation.[28]

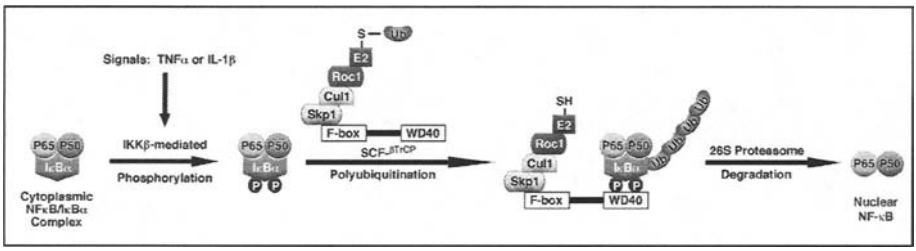

Figure 1. The biochemical pathway of IκBα ubiquitination and degradation. In response to NF-κB stimuli, IκBα is phosphorylated by IKK at two specific N-terminal serine residues. The phosphorylated IκBα is recruited to the $SCF^{\beta TrCP}$ ubiquitin ligase complex, which is composed of Skp1, Cul1, Roc1, and the F-box protein βTrCP. βTrCP contains seven WD40 repeats that bind specifically to the phosphorylated form of IκBα. The RING domain protein Roc1 recruits the E2 Ubc5, and facilitates the transfer of ubiquitin from the E2 to two N-terminal lysine residues of IκBα. After IκBα is polyubiquitinated, it remains bound to NF-κB (shown as the p50/p65 heterodimer), but is selectively degraded by the 26S proteasome. NF-κB then enters the nucleus to regulate the expression of target genes that mediate inflammation, immunity and cell survival.

Both IκB degradation and NF-κB processing require the SCF E3 complex containing Skp1, Cul1, the F-box protein βTrCP, and the RING domain protein Roc1 (Fig. 1).[29] βTrCP contains seven WD40 repeats, which bind specifically to the phosphorylated form of IκB, p100 and p105. The F-box of βTrCP binds to Skp1, which in turn binds to Cul1. Cul1 interacts with Rbx1, which recruits the E2 Ubch5 to ubiquitinate the phosphorylated substrates. This model is verified by the elegant crystal structure of the SCF-βTrCP complex bound to a phosphorylated peptide that contains the destruction motif DpSGΨXpS (where Ψ denotes hydrophobic residue, X any amino acid, and pS phosphoserine).[30,31] This motif is present in several βTrCP targets including IκB, p100 and β-catenin, a transcriptional coactivator in the Wnt pathway.

Roles of Ubiquitination in IKK Activation by TRAF Proteins

Structure and Function of TRAF Proteins

TRAF proteins are crucial signal transducers that mediate the activation of NF-κB and mitogen-activated protein kinases (MAPKs) by TNF receptors (TNFRs), IL-1 receptor (IL-1R) and Toll-like receptors (TLRs).[32,33] The founding members of the TRAF family, TRAF1 and TRAF2, were identified as proteins that associate with the type-2 TNFR (TNF-R2).[34] This family has now expanded to seven members. Except for TRAF7, all TRAF proteins contain a conserved C-terminal TRAF domain, which mediates interaction with cell surface receptors as well as other upstream signaling proteins. The N-terminal segment of the TRAF domain contains a coiled-coil structure that mediates the oligomerization of TRAF proteins. All TRAF proteins except TRAF1 also contain a conserved N-terminal RING domain followed by several zinc finger domains. These N-terminal domains are responsible for downstream signaling to NF-κB and MAPKs such as JNK and p38.

Among TRAF proteins, TRAF2 and TRAF6 have been most extensively studied. TRAF2 mediates the TNFR signaling cascade, whereas TRAF6 is essential for signaling from IL-1R and TLRs. In the TNFR pathway, the binding of the trimeric TNFα ligand leads to the trimerization of the type-I TNF receptor (TNF-R1), which recruits the death domain adaptor protein TRADD.[35] TRADD interacts with TRAF2 as well as the receptor-interacting kinase-1 (RIP1). The formation of these receptor-associated protein complex results in the activation of IKK and JNK, ultimately leading to the activation of NF-κB and AP1, respectively. Genetic ablation of RIP1 abolishes NF-κB activation by TNFα; however, reconstitution experiments show that the kinase activity of RIP1 is not required for NF-κB activation.[36,37] Deletion of TRAF2 in mouse embryonic fibroblasts (MEF) blocks JNK but not NF-κB activation by TNFα.[38] The normal NF-κB activation in TRAF2-deficient cells is likely due to the compensatory function of TRAF5, as the double knockout of TRAF2 and TRAF5 eliminates TNFα-induced NF-κB activation.[39] In the TRAF6 pathways, stimulation of

IL-1R or TLR with a cognate ligand leads to the sequential recruitment of adaptor proteins—MyD88, IRAK4, IRAK1 and TRAF6—to the receptor complex.[40] The kinase IRAK4 phosphorylates IRAK1, resulting in the release of IRAK1 and TRAF6 into the cytoplasm, where they activate the IKK and JNK pathways. Genetic experiments show that TRAF6-deficiency not only prevents NF-κB and JNK activation by IL-1R and the majority of TLRs, but also abolishes signaling by several receptors of the TNFR superfamily, including CD40, lymphotoxin-β receptor, and the latent membrane protein 1 (LMP1) of Epstein-Barr virus.[41-43] Recent studies have also shown that TRAF6 is essential for the development of regulatory T cells that suppress autoimmunity.[44]

TRAF Proteins Are Ubiquitin Ligases

Recent biochemical studies have begun to unravel the signaling mechanism of TRAF proteins. In the course of studying how TRAF6 activates IKK, two intermediary factors that link TRAF6 to IKK activation were identified. The first factor, termed TRIKA1 (TRAF6-regulated IKK activator 1), is a Ub-conjugating enzyme (E2) complex comprised of Ubc13 and a Ubc-like protein Uev1A.[45] The second factor, termed TRIKA2, is a ternary complex consisting of the protein kinase TAK1 and two adaptor proteins TAB1 and TAB2.[46] The identification of Ubc13/Uev1A as an activator of IKK was particularly interesting, and it led to the discovery of TRAF6 as a RING domain ubiquitin ligase (E3) that functions together with Ubc13/Uev1A to synthesize a unique lysine 63 (K63)-linked polyubiquitin chain.[45] Subsequent studies have identified several targets of K63-linked polyubiquitination, including NEMO and TRAF6 itself.[46-56] Through a proteasome-independent mechanism, the K63 polyubiquitination of TRAF6 leads to the activation of TAK1, which subsequently phosphorylates IKKβ at two serine residues in the activation loop, resulting in IKK activation (Fig. 2). TAK1 also phosphorylates an MKK such as MKK6, which activates the JNK and p38 kinase pathways.[46]

Like TRAF6, TRAF2 is also a RING domain protein that catalyzes K63-linked polyubiquitin chain synthesis in conjunction with Ubc13/Uev1A.[45,57] A dominant negative mutant of Ubc13 inhibits NF-κB activation by TRAF2, suggesting that TRAF2 activates NF-κB through a ubiquitination-dependent mechanism.[45] Ubc13 and TRAF2 polyubiquitination have also been shown to mediate the activation of germinal center kinase-related (GCKR) and JNK by TNFα.[54] A recent study confirmed the importance of TRAF2 ubiquitination in JNK activation, but found that TRAF2 ubiquitination is not required for the activation of p38 kinase and NF-κB.[55] This finding is consistent with the phenotypes of TRAF2-deficient MEF cells, which are defective in JNK activation but have normal NF-κB function.[38] Thus, ubiquitination of other proteins such as TRAF5 or RIP may also be important for NF-κB activation in the TNFα pathway.[39,57-59] The ubiquitin ligase activity of TRAF2 may have both positive and negative effects on the NF-κB signaling pathways.[60] For example, while TRAF2 is an activator of the canonical NF-κB pathway, it functions as an inhibitor of the noncanonical pathway, perhaps by targeting certain signaling proteins in this pathway for degradation by the proteasome.[61] Indeed, TRAF2 has been shown to target TRAF3 for ubiquitination and degradation in B cells following CD40 stimulation.[62] TRAF2 itself can also be degraded in certain B cell lines after stimulation with CD40 ligand.[63] Furthermore, stimulation of TNFR2 in T cells by TNFα leads to the polyubiquitination of TRAF2 by another RING domain protein c-IAP1 (cellular inhibitor of apoptosis 1), resulting in TRAF2 degradation by the proteasome.[64] Thus, polyubiquitination of TRAF2 may lead to the activation of downstream kinases or result in proteasomal degradation, perhaps depending on the configuration of the polyubiquitin chains.

The discovery of the role of TRAF ubiquitination in IKK activation provides an explanation for the earlier observations that the RING domains of TRAF2 and TRAF6 are the effector domains in downstream signaling. Removal of the RING domains of TRAF2 and TRAF6 converts these proteins into dominant negative mutants that inhibit the TNFα and IL-1β pathways, respectively.[65,66] Conversely, when the C-terminal TRAF domains of TRAF2 and TRAF6 were replaced with an inducible dimerization domain, it was found that dimerization of the chimeric TRAF proteins was sufficient to activate IKK and JNK.[46,67] Consistent with an essential role of ubiquitination in TAK1 and JNK activation, TRAF6-deficient MEF cells complemented with a TRAF6 mutant lacking the RING domain failed to activate TAK1 or JNK.[68] Surprisingly, these

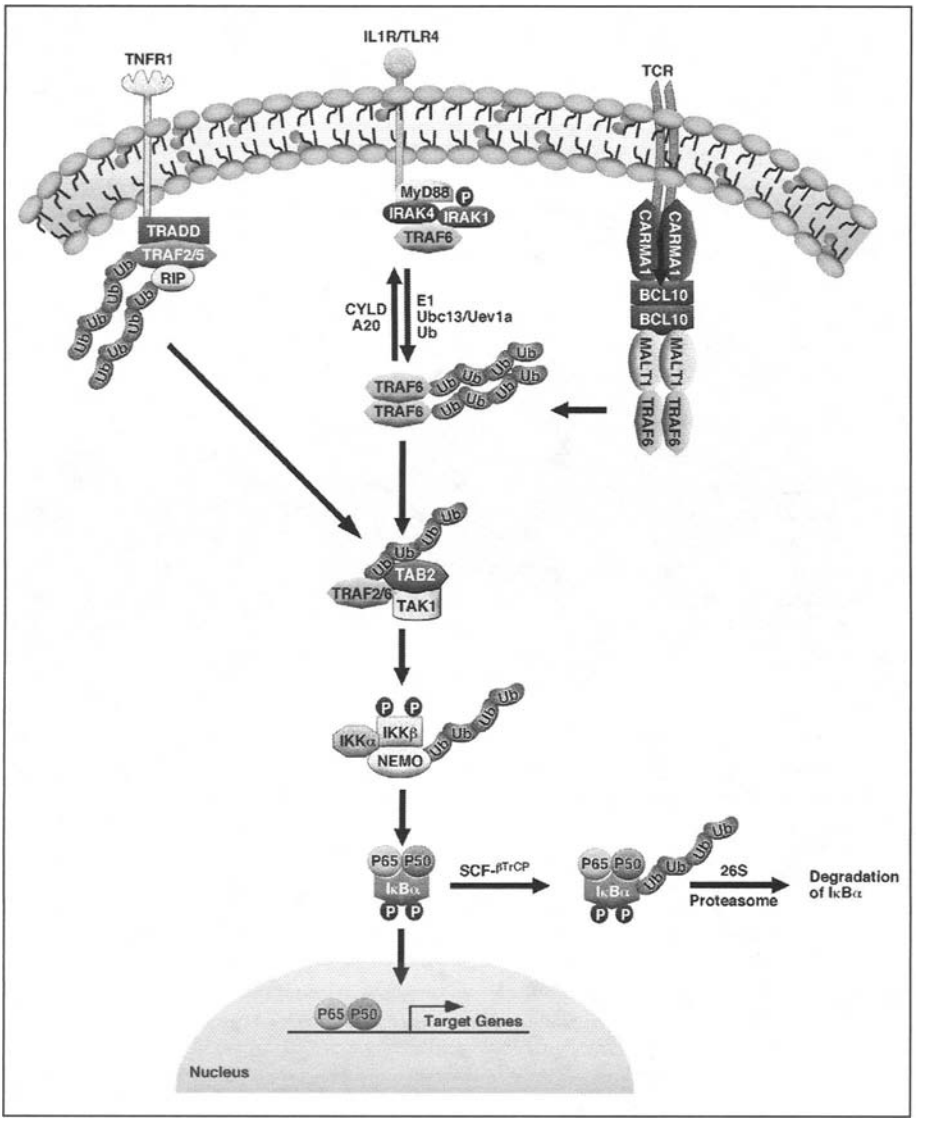

Figure 2. Ubiquitin-mediated activation of TAK1 and IKK by TRAF proteins. In response to proinflammatory cytokines or pathogens, TNF receptors (TNFR), IL-1 receptor (IL-1R), or Toll-like receptors (TLR) bind to their cognate ligands and activate a signaling cascade leading to the activation of TRAF ubiquitin ligases, including TRAF2 and TRAF6. Similarly, in the adaptive immunity pathway, stimulation of the T cell receptors (TCR) with antigenic peptides leads to the membrane recruitment of a protein complex consisting of CARMA1, BCL10 and MALT1. These proteins regulate TRAF2 and TRAF6 by promoting their oligomerization, resulting in the activation of TRAF ubiquitin ligase activity. Activated TRAF proteins catalyze the K63-linked polyubiquitination of target proteins including RIP, NEMO and the TRAF proteins themselves. This polyubiquitination requires E1, Ubc13/Uev1A (E2), and ubiquitin, and can be reversed by deubiquitination enzymes CYLD or A20. The K63-linked polyubiquitin chains facilitate the recruitment of the TAK1/TAB2 complex through interacting with the novel zinc finger (NZF) domain of TAB2. The recruitment of TAK1/TAB2 to ubiquitinated TRAF proteins leads to the activation of TAK1, which in turn activates IKK through direct phosphorylation of IKKβ within the activation loop. IKKβ then phosphorylates IκB and targets this inhibitor for degradation by the ubiquitin-proteasome pathway.

cells were still capable of activating NF-κB in response to IL-1 or LPS, suggesting the existence of a TAK1-independent pathway of NF-κB activation in MEF cells (see below).

Regulation of TRAF Ubiquitin Ligase Activity

As discussed above, chemical-induced dimerization of TRAF6 is sufficient to activate the NF-κB pathway. Interestingly, forced dimerization of TRAF6 also leads to polyubiquitination of TRAF6 itself, suggesting that the ubiquitin ligase activity of TRAF6 is activated by dimerization or oligomerization.[46] Recently, several cellular proteins in the NF-κB pathways have been found to promote the oligomerization of TRAF6. One of these proteins, TIFA [TRAF interacting protein with forkhead associated (FHA) domain], has been identified as a protein that connects IRAK1 to TRAF6 in the IL-1 pathway.[69] Biochemical experiments show that TIFA binds to TRAF6 and induces TRAF6 oligomerization and polyubiquitination, thereby activating IKK.[70]

Another example of TRAF6 regulation by oligomerization is provided from the study of the T cell receptor (TCR) signaling pathway.[48] Stimulation of TCR with an MHC (major histocompatibility complex)-bound antigenic peptide leads to the activation of a tyrosine phosphorylation cascade that in turn activates the serine/threonine kinase PKCθ.[71] PKCθ then facilitates the formation of a complex containing the CARD domain proteins CARMA1 and BCL10, and the paracaspase MALT1.[72,73] This complex is recruited to the lipid rafts where activated TCR and other signaling proteins are localized. The environment within the lipid rafts may promote the oligomerization of BCL10 and MALT1. Two recent studies show that BCL10 and MALT1 activate IKK by inducing K63-linked polyubiquitination of NEMO.[48,49] In one study, it was shown that MALT1 is a ubiquitin ligase that functions together with Ubc13/Uev1A to mediate the polyubiquitination of NEMO at a specific lysine (K399).[49] In the other study, it was found that MALT1 binds to TRAF6 through a C-terminal TRAF6-binding site.[48] Through this binding, the oligomerized forms of MALT1 induce TRAF6 oligomerization and the activation of TRAF6 ubiquitin ligase, which catalyzes the polyubiquitination of NEMO as well as TRAF6 itself. The latter study also showed that the TAK1 kinase complex is involved in IKK activation in T cells, and that the T cell signaling pathway from BCL10 to IκB phosphorylation can be reconstituted in vitro using purified proteins. In any case, these studies show that oligomerization of ubiquitin ligases may be an important mechanism of ligase activation.

Deubiquitination Enzymes Downregulate IKK Activation

The activation of NF-κB by proinflammatory cytokines is a rapid and transient process. For example, in most cells TNFα induces the activation of IKK and nuclear translocation of NF-κB within a few minutes. After NF-κB enters the nucleus, it turns on many genes involved in immune and inflammatory responses, as well as some genes that shut down the NF-κB pathway. One of the immediate early target genes of NF-κB is IκBα, which can enter the nucleus to displace NF-κB from the DNA, and transport it back to the cytoplasm.[74-76] To prevent the newly synthesized IκBα from being degraded, IKK activation must also be turned off. While the mechanisms of IKK down regulation are not fully understood, recent studies suggest that deubiquitination is a key mechanism. Two inhibitors of IKK activation have recently been shown to function as deubiquitination enzymes to disassemble K63-linked polyubiquitin chains from signaling proteins that are required for IKK activation. One of these inhibitors is the cylindromatosis protein CYLD, a tumor suppressor found in human patients with a type of skin tumor called cylindroma.[77] CYLD contains a C-terminal UBP domain that is frequently mutated in cylindroma patients. CYLD binds to TRAF2 and NEMO, and inhibits IKK activation by cleaving K63-linked polyubiquitin chains on TRAF2, TRAF6 and NEMO.[51,52,56] The UBP domain mutations found in the cylindroma patients abrogate the ability of CYLD to inhibit IKK and NF-κB, resulting in hyperactivation of NF-κB, which may contribute to tumorigenesis. However, it is not known why the loss of CYLD function only leads to skin tumor. CYLD is one of the target genes of NF-κB, indicating that the NF-κB pathway has a built-in negative feedback loop to regulate its own activation. CYLD is also regulated by IKK-dependent phosphorylation, which inactivates the ability of CYLD to prevent TRAF2 polyubiquitination.[78] CYLD also inhibits JNK activation by multiple proinflammatory cytokines that signal through

TNFRs, IL-1R and TLRs.[79] As NEMO is not required for JNK activation, the targets of CYLD in the JNK pathway are likely to be TRAF2 and TRAF6, not NEMO.

The other NF-κB inhibitor, A20, is also a well-known target gene of NF-κB and its expression is rapidly induced by TNFα.[80,81] Mice lacking A20 develop severe inflammation in multiple organs, owing to prolonged activation of IKK.[82] A20 contains an N-terminal OTU deubiquitination enzyme domain, and seven zinc finger domains at the carboxyl terminus. Recent studies show that both the N- and C-terminal domains of A20 are utilized to inhibit IKK.[59,83,84] The OTU domain first disassembles K63-linked polyubiquitin chains on RIP1 in the TNFα pathway,[59] and TRAF6 in the LPS pathway,[83] thereby inhibiting IKK. Subsequently, the C-terminal zinc finger domains function as a ubiquitin ligase to synthesize K48-linked polyubiquitin chains on RIP1, thus targeting RIP1 for degradation by the proteasome.[59] Interestingly, it was shown that the K63 polyubiquitin chains on RIP1 must be removed before RIP1 can be conjugated by the K48 chains. This coupling of deubiquitination and ubiquitination by A20 results in the potent suppression of IKK.

Signaling Pathways Downstream of TRAF Proteins

TAK1 and Its Associated Proteins

TAK1 was initially identified as a TGFβ-activated kinase.[85] Subsequent experiments show that TAK1 mediates NF-κB and JNK activation by IL-1β and TNFα.[46,57,86] Biochemical experiments provide the direct evidence that TAK1 is an IKK kinase that phosphorylates IKKβ at key serine residues in the activation loop.[46] Numerous experiments employing different technologies including RNAi and chemical inhibition of TAK1 have now provided strong evidence that TAK1 is required for IKK and JNK activation by IL-1β and TNFα in mammalian cells.[87-89] However, it remains to be seen whether genetic knockout of TAK1 in higher organisms affects NF-κB or JNK activation in vivo. In *Drosophila*, the essential role of TAK1 in IKK and JNK activation in vivo has been demonstrated.[90] *Drosophila* mutants lacking dTAK1 is severely defective in producing antimicrobial peptides in response to bacterial infection, which activates an NF-κB-like (Relish) pathway in *Drosophila*.[22] In addition, RNAi of dTAK1 in *Drosophila Schneider* cells abolishes IKK and JNK activation by bacterial peptidylglycans.[91,92] Thus, the role of TAK1 in NF-κB activation and innate immunity is evolutionarily conserved.

TAK1 forms a complex with TAB1 and TAB2.[93,94] The recently identified TAB2-associated protein, TAB3, can also associate with TAK1 and TAB1.[57,95,96] TAB2 and TAB3 may have redundant functions, as the TAB2-deficient MEF cells have normal activation of NF-κB and JNK in response to TNFα or IL-1β.[97] Indeed, RNAi of both TAB2 and TAB3 markedly reduced IKK and JNK activation by TNFα or IL-1β[57,95,96] TAB2 and TAB3 contain two highly conserved domains, an N-terminal CUE domain, and a C-terminal domain NZF (novel zinc finger) domain. While both domains are Ub-binding domains, the CUE domain appears to be dispensable for NF-κB activation.[57] In contrast, removal or mutation of the NZF domain abolishes the ability of TAB2 and TAB3 to activate TAK1 and IKK. The NZF domain binds preferentially to K63 polyubiquitin chains, and the replacement of the NZF domain with different classes of Ub-binding domains from unrelated proteins restores the signaling function of TAB2 and TAB3.[57] Thus, polyubiquitination may facilitate the interaction between TRAF6 and TAB2 (or TAB3), resulting in the activation of the TAB2-associated kinase TAK1. The mechanism of Ub-mediated activation of TAK1 and IKK by TAB2 and TAB3 is evolutionarily conserved. *Drosophila* has a TAB2-like molecule (dTAB2), which also has the conserved CUE and NZF domains. Remarkably, *Drosophila* harboring mutations in the NZF domain of dTAB2 are defective in antibacterial responses (D. Ferrandon, personal communication). Further supporting the role of ubiquitination in IKK activation in *Drosophila*, RNAi of the *Drosophila* homologues of Ubc13 and Uev1A leads to impaired IKK activation and reduced antibacterial peptide expression.[96a] *Drosophila* also has a TRAF homologue (dTRAF2) that contains the RING domain. The role of dTRAF2 in the immunity pathway is not clear, as RNAi of dTRAF2 in *Schneider* cells has no apparent effect on antibacterial peptide expression.[96a] However, a recent report shows that dTRAF2 mutant larvae are partially defective in the expression of some antimicrobial peptides following *E. coli* challenge.[98]

TAB1 is a potent activator of TAK1, even in the absence of ubiquitination.[93,99,100] However, the endogenous TAK1 complex is inactive, even though it contains TAB1 and TAB2. In vitro reconstitution experiments showed that TRAF6-dependent activation of IKK requires TAK1 and TAB2, but not TAB1.[46] Thus, the role of TAB1 in IKK activation is not clear. In fact, there is no apparent TAB1 homologue in *Drosophila*. Mice devoid of TAB1 are embryonic lethal, and the mutant embryos exhibit abnormal cardiac phenotypes that resemble those of TGF-β2 knockout mice.[101] It is possible that TAB1 is important for TGF-β rather than NF-κB signaling.

TAK1-Independent Signaling Pathways Downstream of TRAF Proteins

Several lines of evidence suggest that TAK1 is not the only mediator of TRAF signaling. First, although the *Drosophila* mutants lacking functional TAK1 or TAB2 are severely defective in antibacterial immunity, these mutants are nevertheless more resistant to bacterial killing than those mutants lacking dIKK or other essential signaling components (e.g, IMD, a RIP1 homologue).[90] Second, in mammalian cells, knockdown of TAK1 expression by RNAi, or chemical inhibition of TAK1 activity, blocks JNK activation, but does not completely inhibit IKK activation by TNFα or IL-1β.[57,87,88] Third, in TAB2-deficient MEF cells,[97] or in MEF cells expressing a TRAF6 mutant lacking the RING domain,[68] IL-1-induced activation of TAK1 is impaired, but NF-κB activation appears to be largely normal. Thus, it is likely that TRAF protein can activate IKK through some pathways that are independent of, or redundant with, the TAK1 pathway. One of these pathways may be mediated through MEKK3, as MEKK3-deficient cells are partially defective in IKK activation in response to TNFα, IL-1β or LPS.[102,103] MEKK3 binds to TRAF2, TRAF6, TRAF7 and RIP, and may link these proteins directly to the IKK complex.[102-104] However, the role of MEKK3 in IKK activation may depend on cell types, as we found that effective silencing of MEKK3 expression in several human cell lines did not inhibit IKK activation by TNFα or IL-1β, whereas silencing of TAK1 expression in the same cell lines markedly reduced IKK activation (C-K.Ea, M. Hong, Z. Chen, unpublished). Furthermore, simultaneous knockdown of both TAK1 and MEKK3 by RNAi did not further inhibit IKK activation beyond what was achieved with TAK1 RNAi alone.

Several other kinases may also be the downstream targets of TRAF proteins. One of these kinases is GCKR, a MAP3K that can be activated by TNFα or TRAF2. It has been shown that TRAF2 and Ubc13/Uev1A promote GCKR polyubiquitination and activation, resulting in the activation of JNK.[54] Another TRAF-interacting MAP3K, apoptosis signal-regulating kinase 1 (ASK1), is required for sustained activations of JNK, p38 and apoptosis.[105] ASK1 interacts with and is activated by several TRAF proteins, including TRAF2 and TRAF6.[106] Interestingly, a TRAF2 mutant lacking the RING domain inhibits the TNFα-dependent activation of ASK1. A recent study shows that the binding of LPS to TLR4 induces the production of intracellular reactive oxygen species, which leads to the formation of a complex containing TRAF6 and ASK1.[107] Through an unknown mechanism, TRAF6 activates ASK1, which in turn activates the p38 kinase required for innate immune responses against bacteria. Another example of TRAF6 activating a downstream kinase in innate immunity is provided from the study of interferon-α induction by TLRs that bind to viral RNA (TLR7-8) and bacterial DNA (TLR9).[108-110] The induction of interferon-α requires MyD88, TRAF6 and the transcription factor IRF7. Following the activation of TLRs by viral RNA or bacterial DNA, IRF7 forms a complex with MyD88, IRAK1, IRAK4 and TRAF6. TRAF6 then activates a putative IRF7 kinase that phosphorylates IRF7, allowing IRF7 to dimerize and translocate to the nucleus to turn on interferon-α. Interestingly, Ubc13 and the RING domain of TRAF6 are required for IRF7 activation, suggesting that K63-linked polyubiquitination may play a role in the activation of an IRF7 kinase.

Conclusions and Perspectives

Research in the past few years has firmly established the central role of TRAF proteins in inflammation and immunity. The discovery of TRAF proteins as ubiquitin ligases and the in vitro reconstitution of TRAF6 signaling pathways have set the stage for a detailed study of the TRAF signaling mechanism. This mechanism involves, at least in part, the lysine-63 polyubiquitination of several proteins in the NF-κB pathway, including RIP, NEMO, and TRAF proteins themselves. However,

the roles of polyubiquitination of these proteins in the NF-κB pathway have not been fully investigated. In addition, the mechanism by which polyubiquitination activates TAK1 and IKK requires further studies. In this regard, the identification of TAB2 and TAB3 as polyubiquitin chain binding proteins provides some clues to the mechanism of TAK1 activation, but more work employing modern biophysical techniques is clearly needed in order to understand how the binding of a polyubiquitin ligand to the receptors (TAB2 and TAB3) activates the receptor-associated kinase (TAK1). Future research should also address the in vivo functions of TAK1 in higher organisms, and to investigate other mechanisms of IKK activation that may be independent of TAK1. Finally, it will be of enormous interest to explore the possibility that the ubiquitin signaling mechanism learnt from TRAF proteins may be applicable to other signaling pathways.

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CHAPTER 8

Structural Revelations of TRAF2 Function in TNF Receptor Signaling Pathway

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Abstract

The tumor necrosis factor (TNF) receptor (TNFR) superfamily consists of over 20 type-I transmembrane proteins with conserved N-terminal cysteine-rich domains (CRDs) in the extracellular ligand binding region, which are specifically activated by the corresponding superfamily of TNF-like ligands. Members of this receptor superfamily have wide tissue distribution and play important roles in biological processes such as lymphoid and neuronal development, innate and adaptive immune response, and cellular homeostasis. A remarkable feature of the TNFR superfamily is the ability of these receptors to induce effects either for cell survival or apoptotic cell death. The downstream intracellular mediators of cell survival signal are a group of proteins known as TNFR associated factors (TRAFs). There are currently six canonical mammalian TRAFs. This review will focus on the unique structural features of TRAF2 protein and its role in cell survival signaling.

Identification of TNF and Its Role in Death and Survival Signaling

The tumor necrosis factor (TNF) receptor and ligand superfamily are widely distributed and are important for the proper function of the immune system. Currently, over 20 receptors have been identified including TNF-R1, TNF-R2, Fas, CD30 and CD40.[1] Agents that can manipulate the signaling of these receptors are currently being used and are showing promise towards the treatment and prevention of many human diseases.[2-4]

An interesting dichotomy of the TNFR superfamily is the ability of these receptors to induce both cell survival (proliferation and differentiation) and apoptotic cell death.[1,5,6] The fate of the cell depends on the intracellular region of TNFR members of the superfamily, specifically those with or without death domains (DD). Receptors that contain DD, such as Fas, DR4 and DR5, are mostly pro-apoptotic whereas receptors without DDs, such as TNF-R2, CD40, CD30, Ox40, 4-1BB, LTβR and TRANCE-R (also known as RANK), induce mostly survival effects. The functional divergence within the receptor superfamily is a consequence of the recruitment and assembly of different signaling proteins to the intracellular portion of the receptors (Fig. 1).

One of the most thoroughly studied member of the TNF-ligand superfamily is TNF-alpha. Many anecdotal but persuasive observations of tumor necrosis or regression by TNF-alpha were in cancer patients who had concurrent bacterial infections. Such stories have been noted throughout history and all over the world. In particular, pioneering clinicians in the late 19th century began treating various kinds of tumors including sarcomas, cancers of the bone and connective tissues, breast cancer, ovarian cancer, Hodgkin's disease, and melanoma by inducing acute skin infections,

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

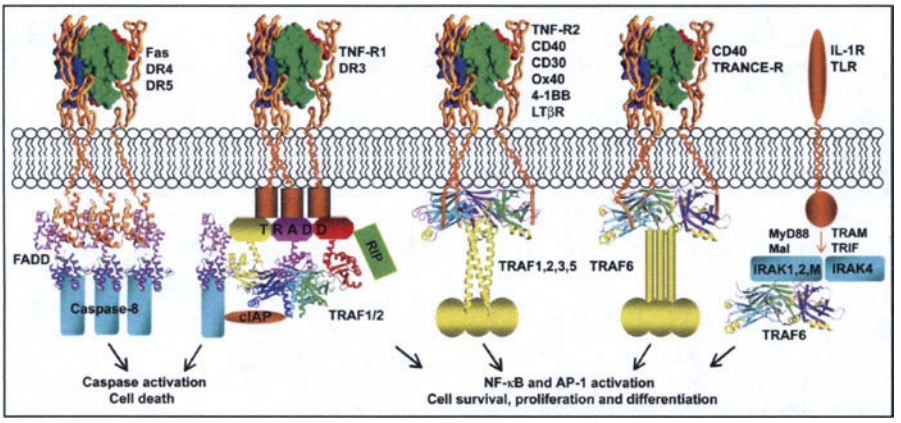

Figure 1. Intracellular signaling pathways for the TNFR superfamily and the IL-1R/TLR superfamily. Proteins with known structures are shown as ribbon drawings. Hypothetical transmembrane helices are built to connect extracellular and intracellular domains of these receptors (shown in orange). The amino terminal domains of TRAFs are shown as yellow spheres. Reproduced from: Wu H. Adv Protein Chem 2004; 68:225-79; ©2004 with permission from Elsevier.[44]

such as erysipelas.[7] The underlying mechanism of this novel "toxin" cancer therapy was attributed to a factor that could be produced and released by immune cells such as macrophages that have been stimulated by bacterial endotoxins.[8] The promise of TNF as a cancer cure led to the molecular identity of TNF through purification, characterization, and cloning.[9-12]

Further research with TNF for its anti-tumor activity led to the realization that TNF is a pleiotropic cytokine important in host defense against pathogens and capable of inducing cell survival, proliferation, and differentiation, as well as cell death, mediated by two TNF receptors, TNF-R1 and TNF-R2.[13-15]

Identification of TRAFs as Major Signal Transducers of the TNFR Superfamily

The TNFR superfamily members that promote survival signaling are those without DD in the intracellular region leading to the direct recruitment of adapter proteins called TNF receptor associated factors (TRAFs).[16-18] Currently, there are six canonical mammalian TRAFs (TRAF1-6) identified, of which all but TRAF4 are involved in the signal transduction of the TNFR superfamily,[16,19-29] and a recently identified "noncanonical" member, TRAF7.[28,29] Among the TRAF proteins, TRAF1, 2, 3 and 5 are considered TRAF2-like because they recognize and associate with TNF receptor family members through a conserved sequence motif on these receptors. In contrast, TRAF6 has a unique sequence requirement for its binding sites that does not overlap with TRAF2.[17] TRAF7 is also implicated in a map kinase signal transduction pathway, similar to the functions of other TRAFs.[29] However, not much is currently known about the upstream TRAF7 activation mechanism.

TRAF signaling activates transcription factors in the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) family,[30,31] which can turn on numerous genes involved in cellular proliferation, differentiation, and regulation of immune response. Most TRAF proteins can be divided into two domains, the N- terminal RING and Zinc finger downstream signaling domain and the C-terminal TRAF domain. The TRAF domain can be further divided into a TRAF-N domain and a TRAF-C domain, which are important for self-association and receptor interaction, respectively.[16] TRAF7 does not conform to the canonical TRAF domain organization. It also consists of N-terminal RING and zinc finger domains, but instead of the TRAF domain, it consists of seven WD40 repeats.[29] Recent studies on the activation mechanism of TRAF2 downstream signal transduction has shown the involvement of a unique lysine-63 linked nondegradative polyubiquitination event, as shown by

the negative regulation of NF-κB activity by a TRAF2-interacting deubiquitination enzyme that was specific for nondegradative polyubiquitin chains.[32-34]

Interestingly, TNF-R1 and TNF-R1-like receptors possess the intrinsic capability to induce either cell death or cell survival. The mechanism by which these opposite cellular fates can coexist within one receptor lies on the recruitment of a multifunctional protein, TNF receptor-associated DD (TRADD), which can interact with both the DD within the receptor, as well as TRAF2.[35] The amino terminal domain of TRADD (TRADD-N) can recruit TRAF2,[35] while the carboxyl terminal DD of TRADD can recruit a death effector signaling protein called Fas associated DD protein (FADD) and a DD-containing Ser/Thr kinase called receptor-interacting protein (RIP), via DD-DD interactions.[35-37] Therefore, the fate of the cell depends on which proteins associate with TRADD, since both TRAF2 and RIP contribute to survival signaling,[38,39] whereas FADD recruitment activates caspases to induce apoptosis. However, the regulation of survival and death pathways from TNF-R1 is likely to be more complex and may involve cellular inhibitors of apoptosis (cIAPs), FLICE-inhibitory proteins (FLIPs) and c-Jun N-terminal kinase (JNK).[40-43]

Over the last six years, several TRAF protein structures, including TRAF2, TRAF3, and TRAF6 both alone and in complex with receptor peptides, have been determined[44] (Table 1). In addition, thermodynamic studies on TRAF2-receptor interactions were conducted and the results are summarized in Table 2. This chapter will focus on the structure and signaling mechanism of TRAF2. For detailed analyses of TRAF3 and TRAF6 structures, please refer to their respective chapters in the book.

Domain and Oligomeric Structures of TRAF2

Unique Anti-Parallel β-Sandwich Topology of TRAF2 C-Domain

The unique topology of the TRAF-C domain was first revealed from the crystal structure of the TRAF2 TRAF domain (Fig. 2A,B), alone and in complex with a receptor peptide from TNF-R2.[45] The main structural architecture of the TRAF-C domain features an eight-stranded anti-parallel β-sandwich. The first sheet of the anti-parallel β-sandwich consists of β1, β8, β5 and β6 strands and β2, β3, β4 and β7 strands make up the second sheet. The results from the Structure Classification Of Protein (SCOP) database[46] and the automatic structural similarity search engine, Dali program[47] showed that TRAF-C domain represents a novel fold for an eight stranded anti-parallel β-sandwich.

A more detailed inspection of the TRAF-C domain reveals additional structural features of the β strands. The β strands, β2 and β7 of the second sheet, present a bulge due to its highly twisted state. Preceding the β1 strand, there is a short stretch of residues (348-350) labeled β0, which runs parallel to β2, immediately after the β-bulge in this strand. The side chains of β0 residues partly cover one edge of the β-sandwich. Therefore, the twisting of β2 appears to play a structural role in the TRAF-C domain. The structure of the TRAF-C domain in complex with TNFR-2 peptide revealed that β7 strand contains the primary receptor peptide interaction site, thus the β-bulge and the twist in this strand may also play important structural and biological roles. A three-turn helix is present in the crossover connection between strands β1 and β2. Comparison and superposition of the 48 independent copies of the TRAF-C domain of TRAF2 in different crystal forms[45,48,49] showed that the structures are highly conserved with r.m.s.d of around 0.3-0.6 Å, with the exception of the flexible β7-β8 loop (up to 3-4Å in Cα distance). Structural comparison of TRAF-C domain structures in the absence and presence of receptor peptide interactions shows little conformational change, which indicates that its overall architecture is fairly rigid.

Sequence analysis of the TRAF-C domain showed that a diverse set of proteins with unrelated functions to TRAFs also possesses the TRAF-C domain. These proteins include meprins, a family of extracellular metalloproteases,[50] MUL, a protein involved in Mulibrey Nanism syndrome, USP7 (HAUSP), an ubiquitin protease, and SPOP, a POZ (poxvirus and zinc finger) domain-containing protein.[51] Because of its similarities with meprins, TRAF-C domain is also known in literature as meprin- and TRAF-homology (MATH) domain.[50]

A recent protein crystal structure of seven in absentia homolog (Siah), revealed that its substrate-binding domain (SBD) adopts an eight stranded anti-parallel β strand structure similar to the TRAF-C domain structure, despite a lack of significant sequence homology[52] (Fig. 2B,C). In

Table 1. Experimental structures of TRAFs and their complexes

Protein	Binding Partner and Sequence[a]	Method	Resolution	#Protein, #Partner[b]	PDB Code, Reference
TRAF2 (327-501)			2.2Å	6	1CA4 [45]
TRAF2 (310-501)	TNF-R2 (420-428) QVPFSKEEC	Co-crystallization	2.3Å	6, 2	1CA9 [45]
TRAF2 (310-501)	CD40 (250-266) PVQETLHGCQPVTQEDG	Co-crystallization	2.7Å	3, 2	1CZZ [49]
TRAF2 (327-501)	CD40 (250-254) PVQET	Co-crystallization	2.0Å	8, 8	1D00 [49]
TRAF2 (327-501)	CD40 V251I mutant (249-254) YPIQET	Co-crystallization	2.0Å	8, 8	1QSC [48]
TRAF2 (327-501)	CD30 (576-583) MLSVEEEG	Co-crystallization	2.0Å	6, 3	1D01 [49]
TRAF2 (327-501)	Ox40 (262-266) PIQEE	Co-crystallization	2.0Å	6, 6	1D0A [49]
TRAF2 (327-501)	m4-1BB (231-236) GAAQEE	Co-crystallization	2.5Å	6, 5	1D0J [49]
TRAF2 (327-501)	LMP1 (204-210) PQQATDD	Co-crystallization	2.0Å	3, 2	1CZY [49]

continued on next page

Table 1. Continued

Protein	Binding Partner and Sequence[a]	Method	Resolution	#Protein, #Partner[b]	PDB Code, Reference
TRAF3 (341-568)			2.8Å	2	1FLK [78]
TRAF3 (341-568)	CD40 (247-266) TAAPVQETLHGCQPVTQEDG	Soaking	3.5Å	2, 2	1FLL [78]
TRAF3 (377-568)	TANK (178-195) SVPIQCTDKTDKQEALFK	Soaking	2.9Å	1, 1	1L0A [91]
TRAF3 (377-568)	TANK (171-191) IATDTQCSVPIQCTDKTDKQE	Soaking	3.5Å	1, 1	1KZZ [91]
TRAF3 (377-568)	LTbR (385-408) PYPIPEEGDPGPPGLSTPHQEDGK	Soaking	3.5Å	1, 1	1RF3 [92]
TRAF6 (346-504)			2.4Å	1	1LB4 [93]
TRAF6 (346-504)	CD40 (230-238) KQEPQEIDF	Co-crystallization	1.8Å	1,1	1LB6 [93]
TRAF6 (346-504)	TRANCE-R (342-349) QMPTEDEY	Co-crystallization	2.0Å	1,1	1LB5 [93]
TRAF2 (327-501)	TRADD-N (7-163)	Co-crystallization	2.0Å	1,1	1F3V [75]
TRADD-N (1-169)		NMR			1F2H [76]

[a] m: mouse; otherwise from human. [b] number of protein and partner molecules per crystallographic asymmetric unit. This table was taken from Wu H.[44]

Table 2. Thermodynamic characterizations of TRAF2-receptor interactions

TRAF	*Receptor/Adapter and Sequence* [a]	K_d [b]	ΔH	-TΔS	ΔC_P	Method [c]	Reference
TRAF2 (310-501)	CD30 (573-583) SDVMLSVEEEG	40 μM	-14.0±0.8 kcal/mol	8.03 kcal/mol	-245 cal/mol•K	ITC	Ye H et al[73]
	CD40 (250-266) PVQETLHGCQPVTQEDG	60 μM	-9.5±1.0 kcal/mol	3.87 kcal/mol	N.D.		
	Ox40 (262-266) PIQEE	50 μM	-13.0±0.9 kcal/mol	7.22 kcal /mol	N.D.		
	TNF-R2 (420-428) QVPFSKEEC	0.5 μM	N.D.	N.D.	N.D.		
	m4-1BB (231-236) GAAQEE	1.0 μM	N.D.	N.D.	N.D.		
	LMP1 (204-210) PQQATDD	1.9 μM	N.D.	N.D.	N.D.		
TRAF2 (327-501)	TRADD (7-163)	7.8 μM	N.D.	N.D.	N.D.	SPR	Park YC et al[75]

[a] m: mouse; otherwise from human. [b] K_d: dissociation constant; DCp: heat capacity change with temperature. [c] ITC: isothermal titration calorimetry; SPR: surface plasmon resonance. N.D.: Not determined.

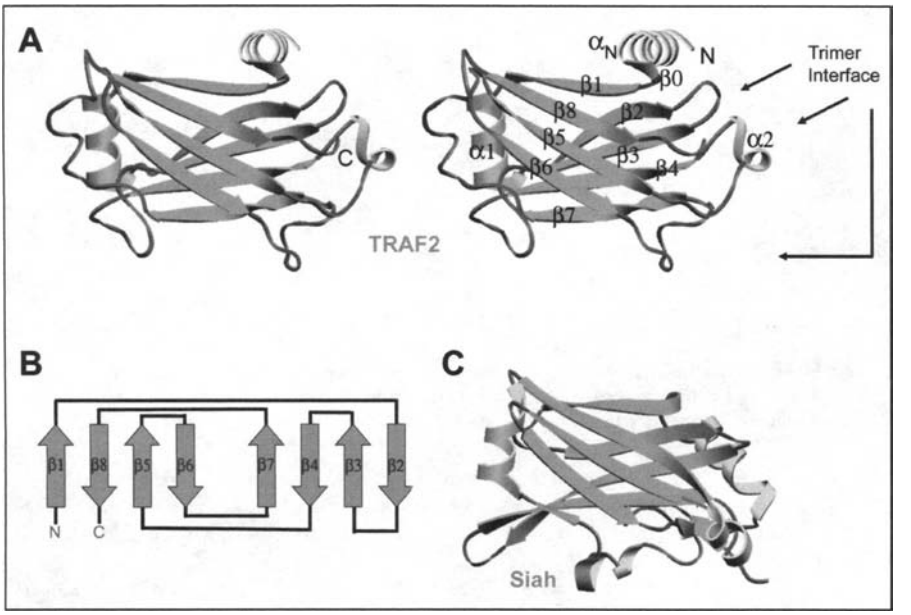

Figure 2. TRAF2 TRAF domain structure. A) Stereo drawing of the TRAF domain of TRAF2 with labeled secondary structures. B) Topology of TRAF-C domain. C) Ribbon drawing of Siah. Modified from: Wu H. Adv Protein Chem 2004; 68:225-79; ©2004 with permission from Elsevier.[44]

addition, the SBD is dimeric rather than a trimer. Interestingly, Siah is a member of the E3 ubiquitin ligase RING domain proteins and does have sequence similarity in this region to TRAFs. Furthermore, it appears that the SBD of Siah enhances TNF-mediated NF-κB activation, which suggests a potential functional similarity between Siah and TRAFs.

The Energetics and Specificity of the Trimeric TRAF Domain

The TRAF domain, consisting of the coiled-coil region and the TRAF-C anti-parallel β sandwich domain resembles the shape of a mushroom, in which the TRAF-C forms the cap and the coiled-coil region forms the stalk[45,48,49] (Fig. 3A,B). TRAF domain trimer portrays a perfect or near perfect three-fold symmetry. The diameter of the mushroom cap ranges between 50 to 80 Å while the stalk is approximately 50 Å long. The stalk consists of 5 characteristic coiled-coil heptad repeats (residues 311-347), which are seven amino acid residues denoted as abcdefg, in which the core residue positions of a and d are usually occupied by hydrophobic residues,[53] as is the case in this three-stranded parallel coiled coil structure. Both the coiled-coil domain and the TRAF-C domain contribute to TRAF domain trimerization.

The trimeric interface of the TRAF-C domain is formed by packing one end of the β-sandwich (the β2-β3, β4-β5 and β6-β7 connections) of one protomer against an edge and a face of the β-sandwich (β0, β1, and β8 strands, β5-β6 and β7-β8 connections) of the neighboring protomer (Fig. 3C). Both hydrophobic and hydrophilic residues are involved at the interface of the protomers, such as residues I355, Y386, A420, L421 and F491 of one protomer and K357, R385, R458, and D487 of the neighboring protomer.

The calculation of surface area burial upon TRAF domain trimerization reveals the importance of coiled-coil region in stabilizing the trimer formation. Roughly 640 Å^2 surface area is buried upon TRAF-C domain trimerization,[45] which is considered small compared to other stable protein-protein interactions.[54] This implies that the TRAF-C domain alone may not be sufficient for trimerization. In support of this analysis, biochemical studies on several TRAF

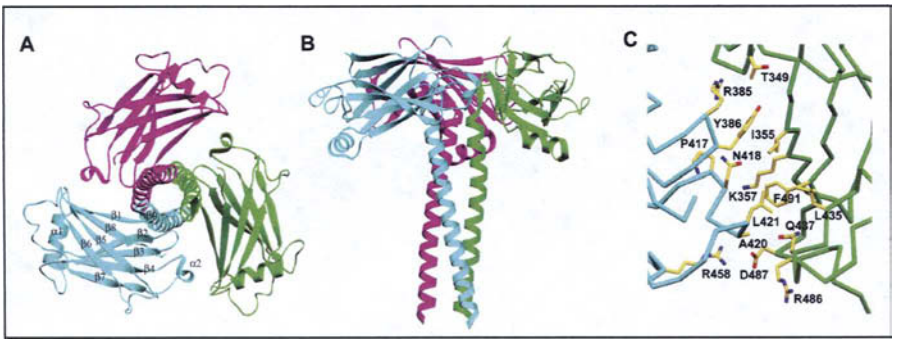

Figure 3. TRAF trimerization. A,B) Trimeric structure of the TRAF domain of TRAF2, shown with the threefold axis into the page and vertical, respectively. C) Detailed interaction between the TRAF-C domains in the trimer. Modified from: Wu H. Adv Protein Chem 2004; 68:225-79; ©2004 with permission from Elsevier.[44]

domain constructs of TRAF2 showed that at minimum, three heptad repeats (residues 327-347) which increases the surface area burial to 1060 Å,^{2} are required for trimer formation.[45] The coiled-coil domain of TRAF2 appears to contain up to 14 heptad repeats, which could stretch to 140Å long and indicates a strong interaction.

Structural and computational analyses suggest that the major specificity determinant for TRAF domain trimerization lies in the TRAF-C domain residues. The analysis showed that the amino acid residues contributing to trimerization of the TRAF domain of TRAF2 are largely conserved among the TRAF family members.[45] This sequence conservation among the different TRAFs suggests that they may also be able to form similar homotrimers as well. On the other hand, the coiled-coil domains do not contain conserved sequences characteristic of trimeric coiled-coil, in fact TRAF2 coiled-coil was predicted by the Multi-coil program to form a dimeric rather than a trimeric coiled-coil.[55,56] Therefore, it appears that the TRAF-C domain, rather than the coiled coil domain, determines the observed specificity of TRAF trimerization, whereas the coiled coil is the major stability determinant for trimerization.

TRAF2-Receptor Interactions

Conserved Recognition of Diverse Receptors

The first glimpse of a TRAF2-receptor interaction provided by the crystal structure of the TRAF domain of TRAF2 in complex with a receptor peptide from TNF-R2[45] shows a different mode of interaction than that of TNF ligand to its receptor TNF-R1. Each peptide binds symmetrically to a shallow surface depression on the side of the mushroom-shaped trimer, extending from the top to the bottom rim of the mushroom cap (Fig. 4A,B). The peptide contacts only one protomer of the TRAF domain trimer. Therefore, this type of interaction does not rely structurally on TRAF2 trimerization, but relies energetically on avidity-mediated affinity enhancement for the receptor afforded by TRAF2 and receptor trimerization.

A major structural question is how TRAF2 can interact with a diverse group of receptors in the TNF receptor superfamily.[17] To go about answering this question, a total of eight crystal structures of the TRAF domain of TRAF2 in complex with several receptor peptides have been determined,[45,48,49] of which three structures are with CD40 receptor peptides (two are not shown) (Fig. 4C). These different complex structures include the three TRAF2 binding motifs proposed previously from biochemical and functional studies, the PxQx(T/S/D) (x = any amino acid) motif in LMP1, CD30, CD40, and CD27,[57-64] the ϕSxEE (ϕ = large hydrophobe) sequence in TNF-R2 and CD30,[16,59] and the QEE motif in 4-1BB and Ox40.[65]

Despite the high degree of sequence variability in the receptor peptides, the peptides contain a conserved binding mode at a common site on the TRAF domain. Superposition of seven different

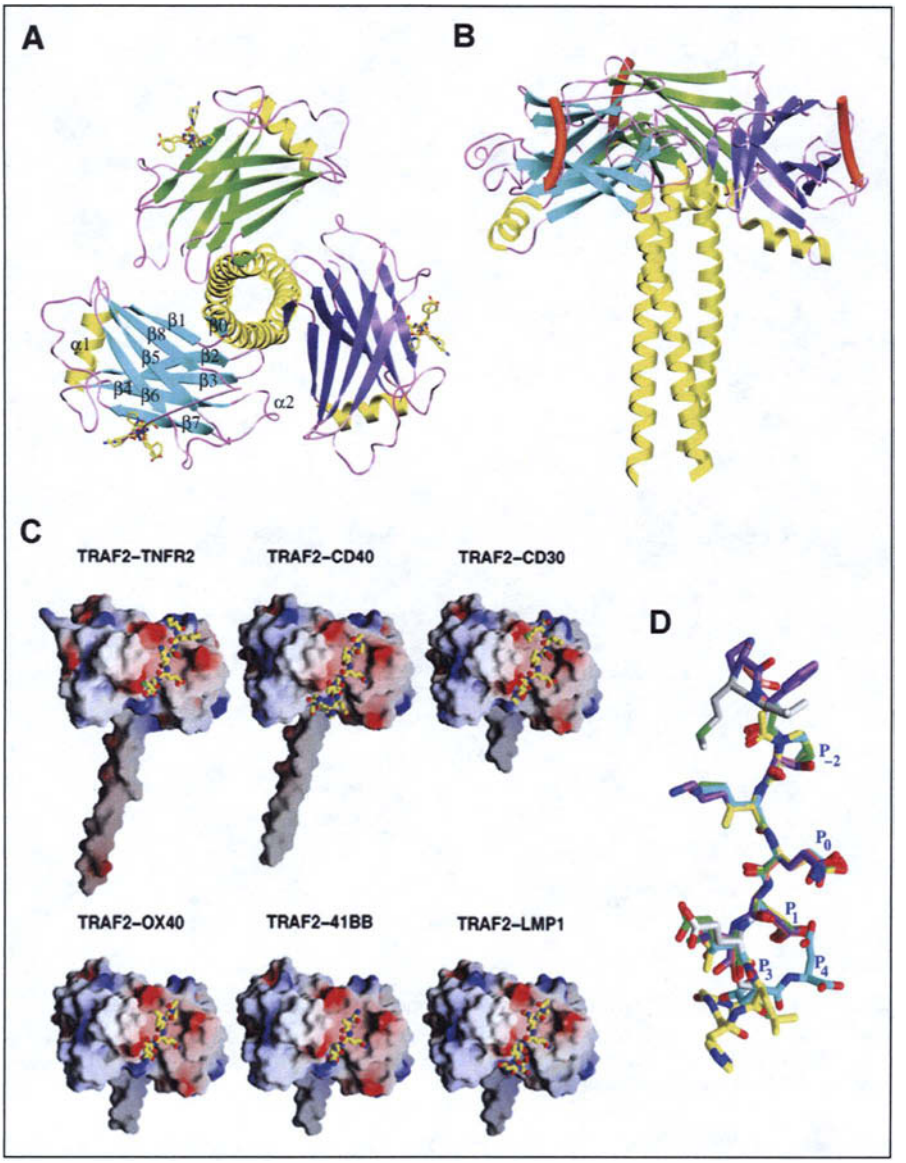

Figure 4. TRAF2-receptor interactions. A) Ribbon diagram of a TRAF2-receptor complex, looking down the threefold axis. The bound receptor chains are shown as stick models. B) Ribbon diagram of a TRAF2-receptor complex with the threefold axis vertical. The bound receptor chains are shown as arrows. C) Surface electrostatic representation of TRAF2-peptide complexes. D) Superposition of bound receptor peptides, showing the structural conservation of the main chain conformations and the side chain conformations at P_{-2}, P_0 and P_1 positions. Parts of this figure were modified from: Ye H et al, Mol Cell 1999, 4(3):321-330, ©1999;[49] and, Wu H, Adv Protein Chem 2004, 68:225-79, ©2004, with permission from Elsevier.[44]

structures of receptor peptide complexes showed four highly conserved residues with r.m.s.d of less than 0.1Å along the main chain atoms of these residues (Fig. 4D). The third residue of this four

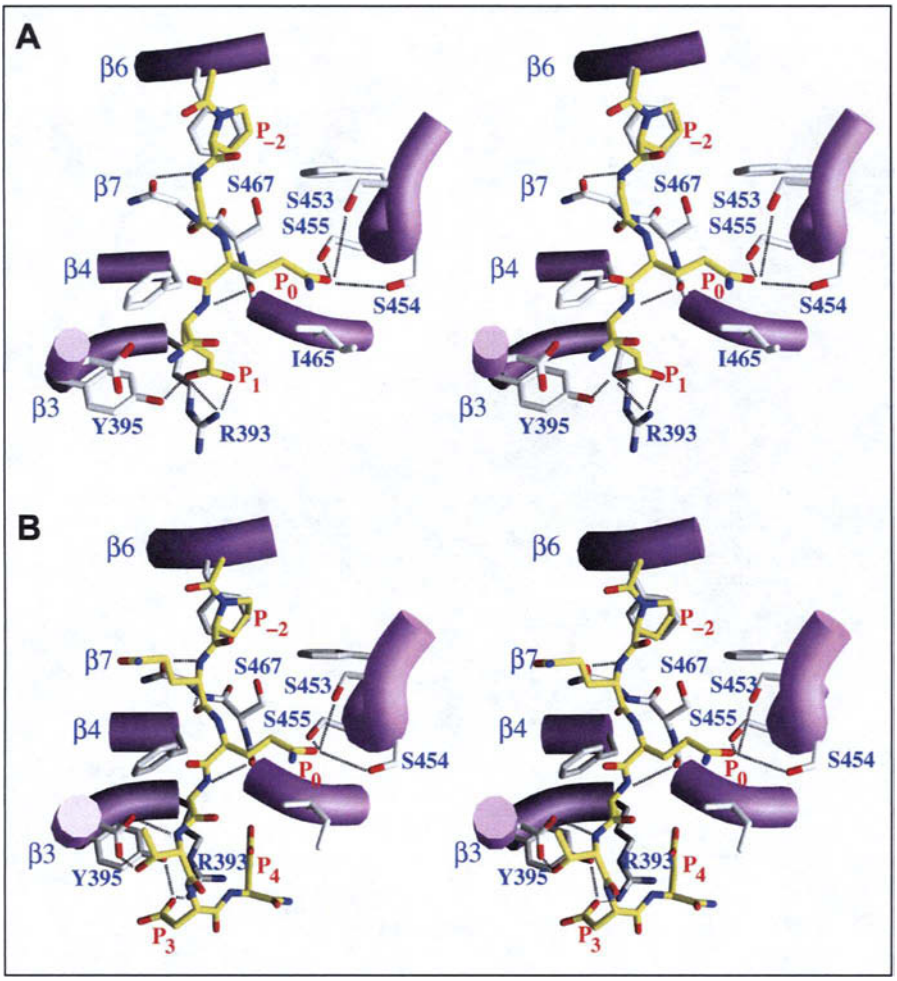

Figure 5. Detailed TRAF2-receptor interactions. A) Interactions seen in the major TRAF2-binding motif. B) Interactions seen in the minor TRAF2-binding motif. TRAF2 structures are shown as magenta worms and white stick models. The bound receptors are shown as yellow stick models. Modified from: Ye H et al. Mol Cell 1999; 4(3):321-330; ©1999 with permission from Elsevier.[49]

residue core is invariably a Gln or a Glu. This position has the highest degree of conservation and is denoted P_0 or the zero position of the TRAF binding motif. Hence, the labeling scheme for this four residue core is P_{-2}, P_{-1}, P_0, and P_1.

The receptor peptides are extended across four β-strands (β6 of the first sheet, β7, β4, and β3 of the second sheet) on one side of the β-sandwich structure of TRAF-C domain. Residues in position P_{-1} to P_1 runs anti-parallel and adjacent to the latter half of β7 strand (residues 466-468), which is immediately after the β-bulge. This leads to three anti-parallel β-edge main chain hydrogen bond formation between the peptide and the β7 of TRAF2 and creates an extra β-strand within the second sheet (Fig. 5A).

The formation of an extra β-strand by the extension of the peptide on the surface of a protein has been frequently observed in peptide-protein interactions.[66,67] Careful analysis of the peptide core

position (P_{-2}, P_0, and P_1) revealed a highly twisted β-strand which can also qualify as a polyproline II (PPII) helix conformation structure. PPII structure has also been observed in peptide-protein interactions such as in peptide recognition by SH3 domains[68] and class II MHC molecules.[69] The advantage of a PPII conformation in the peptides is that it maximizes side chain interactions with the protein surface. This is apparent in the peptide-TRAF2 structures where the twisting of the peptide allows for the side chains of P_{-2}, P_0, and P_1 to become buried at the TRAF2 interface. Therefore, the PPII conformation of the receptor peptides on TRAF2 maximizes both main chain and side chain interactions with the TRAF2 surface.

Key Residues of the Universal Major TRAF2 Binding Motif

The side chains of residues P_{-2}, P_0, and P_1 constitute the major structural determinant for peptide interaction with TRAF2 (Fig. 5A). The residues at these positions occupy distinct pockets within the TRAF2 surface. The P_{-2} residues are frequently Pro or Ser, which make extensive van der Waals contacts with TRAF2. In fact, the side chains of residues at P_{-2} are completely buried by the TRAF domain surface. In the case of Ser at P_{-2}, additional interaction is observed by hydrogen bond formation between the hydroxyl group and the side chain of S467 in TRAF2. The size and enclosure of P_{-2} binding pocket indicates only medium sized and nonpolar residues such as Thr, Cys, and Ile, can occupy this space. For example, residues such as Glu or Ala would not fit as well due to its charge and its small size, respectively. As predicted by the structural study of P_{-2} binding pocket, the Ala in P_{-2} position of 4-1BB receptor results in a weaker interaction with TRAF2, as evidenced by weaker binding affinity and electron density in this region of the complex structure.

The major structural determinants of Gln and Glu at P_0 position is the shape and hydrogen bonding interactions afforded by these particular residues. The aliphatic part of these residues pack against I485 while the hydrophilic region is surrounded by three hydroxyl groups of S453, S454, and S455 in TRAF2. Between the two residues, Gln is in the position to form hydrogen bonds with all three Ser residues of TRAF2, whereas Glu can only form one hydrogen bond. Due to the absence of charged residues near the vicinity of the P_0 site, there appears to be a need for the negative charge in Glu to be more heavily solvated than in Gln.

The P_1 position in most TRAF2 binding peptides is occupied by Glu. The carboxylate moiety of the Glu residue forms a bi-dentate ion-pair interaction with the side chain guanidinium group of R393 and a hydrogen bond with Y395 in TRAF2. The size of the P_1 binding pocket predicts a substitution with a smaller residue such as Asp residue, will not be sufficient to form the hydrogen bond that is observed with Glu.

The sequence and structural conservations at the P_{-2}, P_0, and P_1 positions define the major TRAF2 binding motif. These positions are occupied by the consensus sequence px(Q/E)E, where proline is in lower case because it can be substituted by other medium size nonpolar residues (Fig. 6). The major TRAF2 binding motif can also be found on receptors which interact with TRAF1, 3, and 5, which explains the overlapping receptor-binding specificity of these TRAFs.

The Minor TRAF2 Binding Motif

The crystal structure of TRAF2 with LMP1[49] revealed a second TRAF2 binding motif that utilizes the residue at P_3 position rather than at P_1. The P_1 position is occupied by Ala in LMP1 and cannot make the same interactions as a Glu residue (Fig. 5B). However, the Asp residue of LMP1 at P_3 makes the same ion-pair hydrogen bonds with R393 and Y395 that the Glu makes at the P_1 of the major TRAF2 binding motif. This structural information along with sequence analysis shows the existence of a minor TRAF2 binding motif, px(Q/E)xxD (Fig. 6). In addition to LMP1, the intracellular protein, TANK (also known as I-TRAF)[70,71] possesses the minor TRAF2 binding consensus motif (Fig. 6) and may interact with TRAFs similarly as seen in the TRAF2-LMP1 complex.

Extent and Variations of TRAF2 Binding Motif

The next highest degree of structural conservation outside of P_{-2} to P_1 lies at P_2 and P_{-3} positions of TRAF2 interacting receptor peptides. Beyond P_2 and P_{-3} positions, there are large conformational differences among the various peptides (Fig. 4D). Therefore, the TRAF2 binding motif

	P_{-4}	P_{-3}	P_{-2}	P_{-1}	P_0	P_1	P_2	P_3	P_4	P_5	P_6
hTNF-R2 (422-432)	P	F	S	K	E	E	C	A	F	R	S
hCD40 (248-258)	A	A	P	V	Q	E	T	L	H	G	C
hCD30 (576-586)	M	L	S	V	E	E	E	G	K	E	D
hCD30 (559-569)	H	Y	P	E	Q	E	T	E	P	P	L
hCD27 (244-254)	T	I	P	I	Q	E	D	Y	R	K	P
hLTβR (386-410)	Y	P	I	P	E	E	G	D	P	G	P
hLTβR (400-410)	S	T	P	H	Q	E	D	G	K	A	W
hATAR (266-276)	T	V	A	V	E	E	T	I	P	S	F
hOx40 (260-270)	R	T	P	I	Q	E	E	Q	A	D	A
m4-1BB (230-240)	T	G	A	A	Q	E	E	D	A	C	S
m4-1BB (242-252)	R	C	P	Q	E	E	E	G	G	G	G
h4-1BB (232-242)	V	Q	T	T	Q	E	E	D	G	C	S
h4-1BB (244-254)	R	F	P	E	E	E	E	G	G	C	E
bLMP1 (204-214)	R	T	P	V	Q	E	S	G	Y	P	D
bLMP1 (219-229)	R	P	P	V	Q	E	T	G	G	G	G
bLMP1 (243-253)	H	P	P	V	Q	E	T	G	G	G	G
bLMP1 (315-325)	H	P	P	V	Q	E	T	G	E	G	G
bLMP1 (359-369)	H	P	P	I	Q	E	T	G	N	G	G
hTANK (178-188)	S	V	P	I	Q	C	T	D	K	T	D
hLMP1 (202-212)	P	H	P	Q	Q	A	T	D	D	S	S
rLMP1 (315-325)	P	Y	P	I	Q	A	T	D	G	G	N
rLMP1 (377-387)	P	H	P	I	Q	A	T	D	G	A	N
rLMP1 (425-435)	P	H	P	V	Q	A	S	D	G	G	D
Major Motif			p	x	Q/E	E					
Minor Motif			p	x	Q/E	x	x	D			

Figure 6. Sequence alignment of TRAF2 binding sequences, illustrating the two TRAF2-binding motifs. h: human; m: mouse; b: bovine; r: rat. Modified from: Ye H et al. Mol Cell 1999; 4(3):321-330; ©1999 with permission from Elsevier.[49]

can incorporate up to 6 residues (P_{-3} to P_2). However, it should be noted that additional N- and C-terminal contacts are made with the TRAF domain by the receptors. For example, the TNFR-2 peptide-TRAF2 interaction shows ordered residues starting at P_{-4} position and the CD40-TRAF2 structure shows ordered residues up to P_6. Incorporating these ordered residues may indicate that a complete TRAF2 binding sequence may contain up to eleven residues (P_{-4} to P_6). These additional residues outside of the core binding region are most likely exposed on the surface of the TRAF domain, which makes them tolerant to substitutions by other amino acids. As a final note, the actual TRAF binding region may vary from receptor to receptor since the conformations of end residues appear highly dependent on their side chain chemistry.

It should be pointed out that the presence of these motifs is often necessary but may not be sufficient for the receptor-TRAF2 interactions, and that other residues at different positions may also be important. For example, the P_2 residue may also contribute to TRAF2-receptor interaction since it is in close proximity to D399 of TRAF2 to allow hydrogen bond formations. In the CD40 receptor peptide, the P_2 is occupied by a Thr and the mutation of this residue to all but Ser eliminated association with TRAF2.[72] Therefore, it may not be too uncommon to find variations in the TRAF2 binding motifs.

Thermodynamics of TRAF-Receptor Interactions

Weak Affinity and Avidity

Several quantitative studies using isothermal titration calorimetry (ITC) and surface plasma resonance (SPR) on receptor peptide interactions with TRAF2 showed weak affinities between receptor

peptides and TRAF in the absence of ligand. For example, the dissociation constants of CD40, CD30, and Ox40 peptides with TRAF2 range between 40-60 μM, and 0.5 to 1.9 mM for TNFR-2, 4-1BB, and LMP1 (Table 2).[73] These quantitative measurements of receptor peptides to TRAF2 likely represent the interaction of TRAF2 with actual full length intracellular receptor tails, since structural studies showed that only four core residues within receptor peptides act as the major determinant for TRAF2 interaction.[45,49]

The measured binding affinities for TRAF2-receptor interaction is relatively lower than most observed protein-protein and protein-peptide interactions.[67] This observation indicates that TRAF recruitment is entirely dependent on affinity enhancement through avidity by receptor trimerization. The exact magnitude of affinity enhancement is difficult to quantify and most likely depends on the conformational state of the trimerized or oligomerized receptors.

Favorable Enthalpy, Unfavorable Entropy and Induced Fit

ITC experiments on TRAF2-receptor peptide interactions consistently showed favorable enthalpy gain and unfavorable entropy loss, which indicate that these interactions are energetically driven by an exothermic mechanism. The enthalpy of TRAF2-receptor peptide interaction showed a large negative linear dependence with increase in temperature, as measured for TRAF2-CD30 interaction at 10, 20, and 30°C.[49] This enthalpy dependence on temperature is indicative of specific interactions, rather than nonspecific, as shown from other thermodynamic studies involving protein-DNA interactions.[74]

The observed unfavorable entropy despite the burial of significant hydrophobic surfaces upon peptide binding is likely due to conformational restraints on the receptor peptide by TRAF2 interaction. Secondary structure prediction of the intracellular domains of most TNFR superfamily members shows a lack of preformed well-ordered three-dimensional structures. Therefore, this suggests that conformational changes and induced fit occur between TRAF2 and receptors.

TRAF2-TRADD Interaction: A Novel Mode of TRAF Signaling

The TRADD-N Domain

The interaction between TRAF2 and TRADD occurs through the TRAF domain of TRAF2 and the N-terminal domain of TRADD (TRADD-N). The structure of TRADD-N domain shows a α-β sandwich fold with a four-stranded anti-parallel β-sheet and six α helices[75,76] (Fig. 7A). There are two helices involved at each crossover between β-strands, β1-β2 (helices A and B) and β3-β4 (helices C and D). A hairpin-like turn is formed between β2-β3 strands. The remaining E and F helices are near the carboxy-terminus of the domain. The EF loop partially covers the exposed face of the β-sheet.

The α-β sandwich of TRADD-N is most similar to the family of ferredoxin-like α-β sandwiches.[77] Similar α-β sandwich topology has been observed in the structures of the palm domain of polymerases and the dimerization domain of carboxypeptidases. However, the extra helices in the β1-β2 and β3-β4 connections as well as the additional E and F helices makes TRADD-N a more elaborate structure.

Interactions and Energetics at the TRADD-TRAF2 Interface

The trimeric structure of the TRAF domain enforces the threefold symmetry to the stoichiometrically bound TRADD-N (Fig. 7B,C). The side view of the TRADD-TRAF2 complex shows TRADD bound to the upper rim of the mushroom cap, which adds a wing-like appearance to the complex structure. The carboxyl terminus projects upwards towards the membrane bound receptor direction. The orientation allows TRADD to interact with TNFR1 via the death domains and acts as a platform for other proteins to associate, such as TRAF2, FADD, and RIP (Fig. 7D).

The TRADD-TRAF2 interface partially overlaps with the site of TRAF2-receptor interaction. This indicates a competitive nature of TRAF2-TRADD and TRAF2-receptor interactions. Each TRADD-N molecule contacts one protomer of TRAF2, much like the receptor peptides. The interaction buries a surface area of 1500Å,^{2} which leads to small conformational changes in the Cα positions of TRAF2 (0.5-1.0Å) within or immediately adjacent to the TRADD binding site.

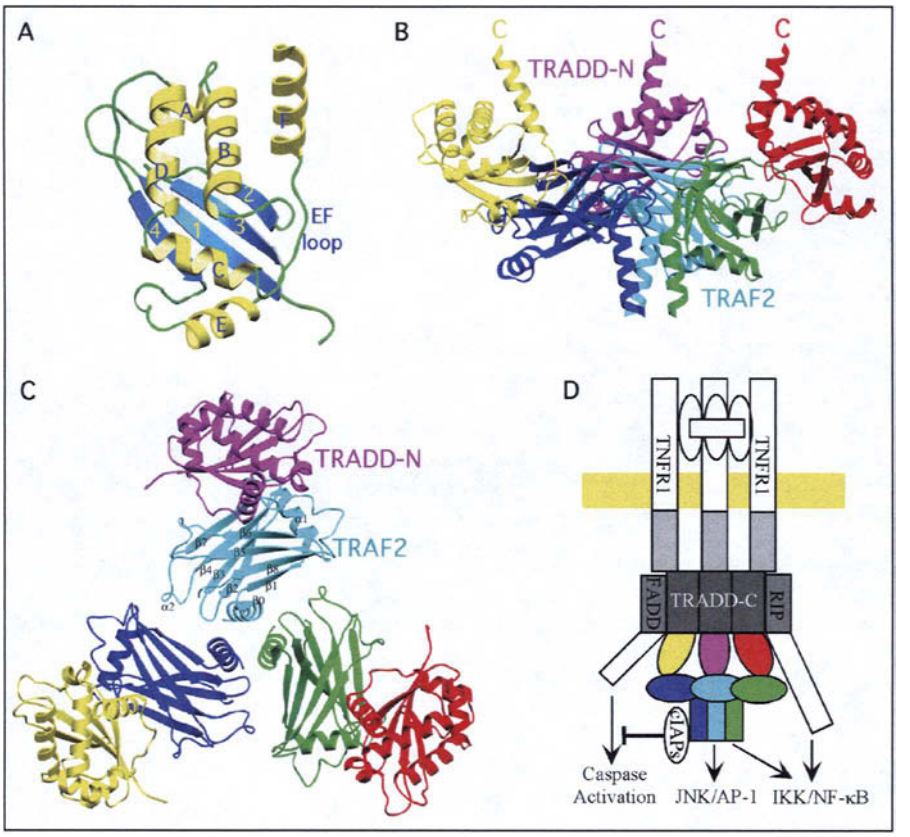

Figure 7. TRAF2-TRADD interaction. A) Ribbon drawing of the TRADD-N domain. B,C) Ribbon diagrams of the TRAF2-TRADD complex. D) Schematic representation of the TNF-R1 signaling complex. Reproduced from: Park YC et al. Cell 2000; 101(7):777-787; ©2000 with permission from Elsevier.[75]

The interface between TRADD-TRAF2 resembles a "ridge into groove" type of contacts, exemplified by complementary elevations and depressions on the surfaces of TRADD-N and TRAF2 TRAF domain (Fig. 8A). The interface can be separated into two distinct and adjacent regions (Fig. 8B). Region I consists of the shallow β-sheet face of TRADD-N and a surface protrusion of TRAF2 β7 strand, the following loop, and the loop between β3 and β4 strands. The specific residues that contributes to the TRADD-TRAF2 interaction in Region I are, Y16, F18, H65, S67, and I72 of TRADD and T401, H406, L471, and P474 of TRAF2 (Fig. 8C). The interaction Region II consists of a highly charged ridge formed by TRADD residues 143-149 in the EF loop and a surface depression formed between TRAF2 β6 and the following loop. Many hydrogen bond interactions are made by the residues in Region II, including anti-parallel main chain hydrogen bonds between the TRADD residues 145-147 and 448-450 of TRAF2, as well as side chain hydrogen bonds and salt bridges between R146 of TRADD with D445 of TRAF2, between R76 of TRADD and D450 of TRAF2, between Q143 of TRADD and S454 of TRAF2, and between D145 of TRADD with the main chain of G468 of TRAF2 (Fig. 8D). Many water molecules are also present at the Region II and at the boundary between the two regions (Fig. 8E).

Mutational studies of residues involved in TRADD-TRAF2 interactions in both Region I and Region II showed differential effects on the binding affinity irrespective of the surface area burial contributions. Alanine substitutions of residues in Region I (Y16, F18, H65, and S67 of TRADD)

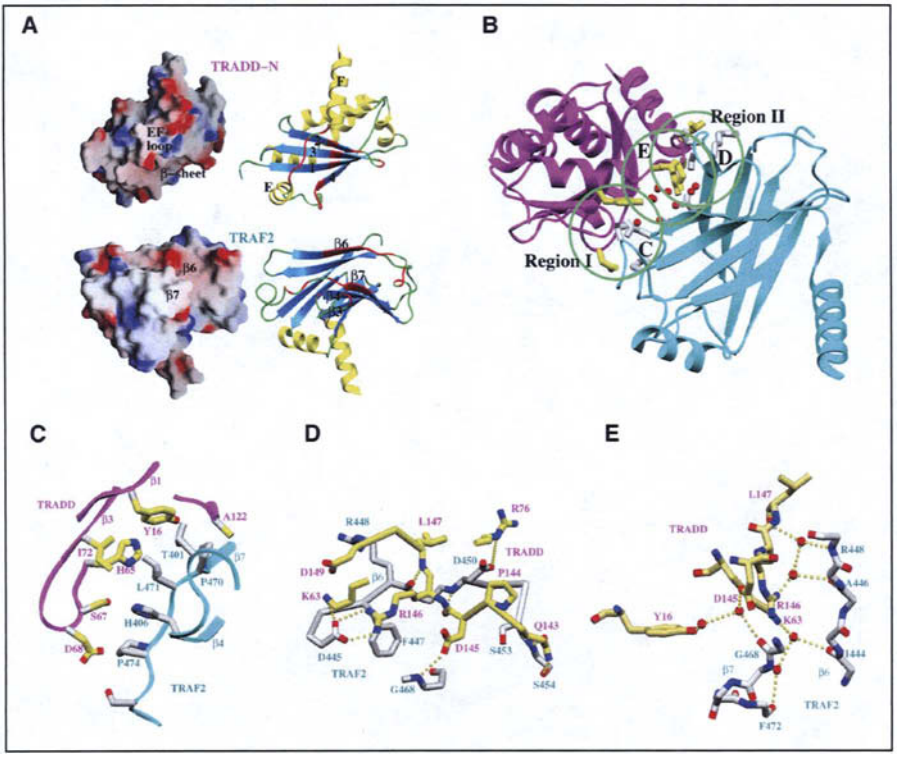

Figure 8. Detailed TRAF2-TRADD interaction. A) Interaction surfaces and their locations on the individual structures (in red). B) Molecular interactions at the two regions of the interactions. C,D,E) Details of region I, region II and water-mediated interactions, respectively. Modified from: Park YC et al. Cell 2000; 101(7):777-787; ©2000 with permission from Elsevier.[75]

had a much more detrimental effect on binding affinity than residues in Region II. This result indicates that despite the larger surface area burial of Region II compared to Region I, the largely hydrophobic interaction in Region I plays the dominant role in the energetics of the interaction.

Higher Affinity and Distinct Specificity of TRADD-TRAF2 Interaction

Surface plasma resonance experiments on TRAF2-TRADD interaction revealed a higher binding affinity (K_d = 7.8 μM) compared to TRAF2-receptor interactions (K_d = 40 μM - 1.9 mM).[73] The higher affinity between TRADD-TRAF2 suggests that TRADD may be a stronger inducer of TRAF2 signaling. This hypothesis was examined in cells expressing exclusively TNF-R1, which signals through TRADD, and cells that only expressed TNF-R2, which signals through direct TRAF2 recruitment. The strength of TRAF2 recruitment was measured by the activation of a major TRAF2 downstream effector, JNK protein kinase.[38] As predicted from the in vitro binding affinity studies, the JNK activation was much stronger for TNF-R1 than for TNF-R2 expressing cells.

The TRADD interaction with TRAF proteins appear to be limited to only TRAF2 and TRAF1 (Table 2). This selectivity by TRADD is not observed by TNF superfamily receptors lacking the intracellular death domain, since these receptors show similar binding specificities for TRAF1, 2, 3, and 5.[45,49,78] The ability of TRADD to associate with both TRAF1 and TRAF2 may have significance in the prevention of apoptosis by TNF-R1 activation (Fig. 9). Rothe et al has shown in TNF-R2 signaling complex, both TRAF1 and TRAF2 are constitutively associated with cellular inhibitors of apoptosis proteins (cIAPs), cIAP1 and cIAP2, and that this association requires the presence of both

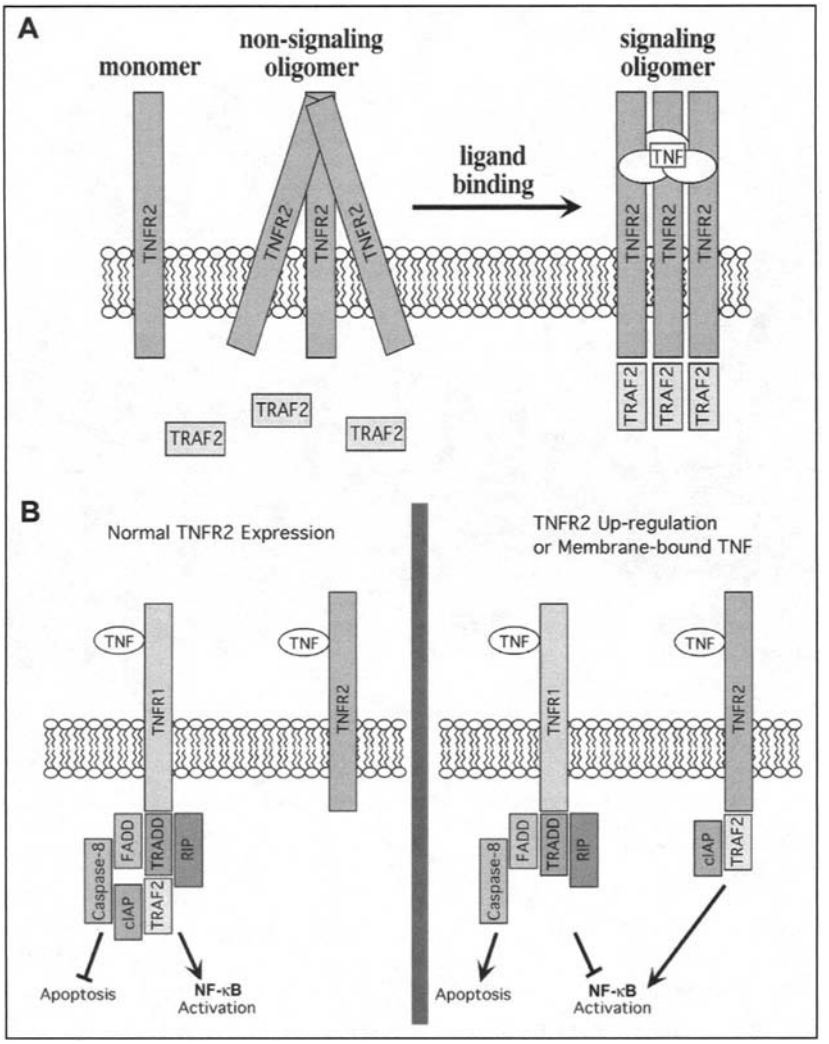

Figure 9. Principles of post-receptor signal transduction. A) Receptor activation and TRAF recruitment. B) Competitive TRAF recruitments and regulation of cell survival and death. Reproduced from: Wu H. Adv Protein Chem 2004; 68:225-79; ©2004 with permission from Elsevier.[44]

TRAF1 and TRAF2.[79] Therefore, as a consequence of the specificity of TRADD for TRAF1 and TRAF2, the cIAPs are brought to TNF-R1 and likely play an important role in blocking the apoptosis pathway.[40]

The predominant outcome of TNF-R1 activation is not apoptosis, as is the case for Fas receptor activation, but rather cell survival or proliferation. The evidence that TRADD binds specifically and selectively to TRAF1 and TRAF2 strongly supports the survival phenomenon. TRAF2 signaling has been shown in TRAF2 knockout studies to protect cells from apoptosis induced by TNF.[38] In addition, mutational studies on TRADD which resulted in reduced affinity for TRAF2 greatly sensitized cells to cell death.[75] These observations implicate TRAF2 as a critical determinant of cellular survival in the TNF-R1 pathway.

Based on these observations, a natural question arises as to when or in what situation does TNF-R1 activation lead to apoptosis? One possible answer may be through the mitochondrial release of Smac protein through JNK activation.[43] Smac may interact with cIAPs and remove them from TRAF1 and TRAF2. Another possible answer may lie on the NF-κB-inducible protein c-FLIP. In the absence of NF-κB activation and c-FLIP, TNF-R1 can induce cell death through a cytoplasmic complex containing TRADD, RIP1, FADD, and caspase-8 activation.[42]

Summary: Emerging Principles of Post-Receptor Signal Transduction

Increased Affinity through Avidity

Structural and biophysical studies on TRAF2-receptor and TRAF2-TRADD interactions showed that receptor peptides and TRADD contact one protomer of the TRAF domain trimer and that they interact with TRAF2 at low affinity. This suggests that receptor oligomerization and affinity enhancement through avidity is required for TRAF recruitment (Fig. 9). However, because a wide range of affinities between TRAF2 and receptors or TRADD have been observed (Table 2), the issue of whether different receptors would require different avidity contributions for TRAF2 recruitment is raised.

Interestingly, many TNF-like cytokine ligands, including TNF, are membrane-bound and therefore may be able to create a higher order of receptor aggregation through membrane-patching or clustering. This membrane receptor aggregation would then increase avidity and thereby enhance affinity for TRAF2. In support of this avidity induced affinity hypothesis, both soluble forms of CD40L and TNF have been shown to be weak inducers of TRAF2 signaling via CD40 and TNF-R2, respectively.[80,81] However, this is not the case for TNF-R1 activation by soluble TNF due to a much stronger TRADD-TRAF2 interaction and recruitment to the receptor.

Based on the TRAF2-receptor structures and the biophysical measurements of binding affinities, the need for receptor aggregation for efficient TRAF2 signaling corresponds well to what is evident in biology. Many other members of the TNF receptor superfamily such as CD30, Ox40, and 4-1BB ligands are membrane bound and mediate signaling in this state. The soluble ligand forms of these TNF receptor superfamily members are reported to be inefficient in activating the intracellular signal transduction pathway. In fact, such soluble ligands have been implicated in the role as decoys to down-regulate receptor activity.[82,83]

Competition Based Regulation of Survival and Death by TRAF2

TRAF2 plays a central role in the regulation of cell death and cell survival by TNF receptors, TNF-R1 and TNF-R2. Studies have shown that overexpression of the survival receptor TNF-R2 sensitizes cells to TNF induced apoptosis.[84-88] This contradictory outcome can be explained by the competitive recruitment hypothesis (Fig. 9). It may be that abundant TNF-R2 levels on the cell membrane draws all the TRAF2 as well as TRAF1 to its intracellular domain, which then depletes cIAPs from TNF-R1 to block caspase activation. Thus, cell survival or death is dependent on intracellular pool of cIAPs associated with TRAF1 and TRAF2.

Similar type of TNF-R1 and TNF-R2 interplay may exist among the other members of the TNF receptor superfamily, such as CD40, CD30, LTβR and CD27. These receptors have also shown the ability to induce apoptosis in certain circumstances.[89] Activation of any of these receptors could lead to sequestration and or degradation of TRAF1, TRAF2, and cIAP proteins.[90] This will then make the cells vulnerable to TNF induced apoptosis via TNF-R1 signaling.

Remaining Questions

Significant amount of structural information on TRAF2 interaction with receptor peptides and TRADD-N has provided an elegant explanation and agreement with biological observations of TRAF2 function. However, many questions are left unanswered regarding the molecular mechanism of TRAF2 signaling. For example, is TRAF2 in monomeric or in a constitutive trimeric state before recruitment to receptors? Is the activation of downstream effectors dependent on oligomerization or on conformational changes induced by receptor interaction? What is the

exact molecular basis for this activation? Finally, the ultimate challenge will be in translating the structural and functional studies into potential therapies for many important diseases involving TNF receptor superfamily members.

Acknowledgement

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CHAPTER 9

Protein-Protein Interactions in TRAF3

Kathryn R. Ely,* Ramadurgam Kodandapani and ShuangDing Wu

Abstract

TNF-receptor-associated factors (TRAFs) are intracellular proteins that bind to the cytoplasmic portion of TNF receptors and mediate downstream signaling. The six known TRAF proteins play overlapping yet distinct roles in controlling immune responses as well as cellular processes such as activation of NF-κB and JNK signaling pathways. For example, CD40 binds to TRAF2, TRAF3 and TRAF6 to control B cell differentiation, proliferation and growth.[1] In contrast, binding of lymphotoxin-β receptor (LTβR) to TRAF2 and TRAF5 propagates signals leading to activation of NF-κB,[2] while binding to TRAF3 induces negative regulation of this pathway and leads to apoptosis in tumor cells.[3,4] Binding recognition is mediated by specific contacts of a consensus recognition sequence in the partner with residues in a hydrophobic crevice on the TRAF molecule. Since each of these protein-protein interactions occurs within this same binding crevice, it appears that TRAF-mediated cellular mechanisms may be regulated, in part, by the level of expression or recruitment of the adaptor proteins or receptors that are competing for the crevice.

The specific contacts of CD40, LTβR and BAFF-R have been defined in crystal structures of the complex with TRAF3.[5-7] In addition, the downstream regulator TANK and the viral oncogenic protein LMP1 from the Epstein Barr virus also bind to the same TRAF crevice and these contacts have also been described crystallographically.[8,9] Comparison of these five crystal structures has revealed that the recognition motifs in each of these proteins are accommodated in one TRAF3 binding crevice and that the binding interface is structurally and functionally adaptive.[10] In this chapter, the molecular details of the interactions will be described and correlated with the functional implications for multiple TRAF3 roles in cellular regulation.

TRAF3 Is a Trimeric Assembly

TRAF proteins share a common folding pattern (TRAF2;[11,12] TRAF3;[7] TRAF6,[13]) that is a trimeric assembly stabilized by coiled-coil interactions of elongated N-terminal α-helices (see Fig. 1A,B). A conserved C-terminal TRAF domain follows the helix and is independently folded as an eight-stranded β-sandwich formed by two layers of β-sheet that each contain four antiparallel strands enclosing a hydrophobic core (see Fig. 1A,B). In TRAF3, the three subunits are structurally identical and related by strict crystallographic three-fold symmetry.[14] The N-terminal α-helix extends from residues 277 to 347 and this long amphipathic helix forms a coiled-coil when the trimer forms. The coiled-coil remains stabilized by nine heptad repeats of hydrophobic residues, even when proteolytically shortened by 36 residues.[14] The C-terminal segment of the molecule, called the TRAF domain, which is composed of residues 348-504, folds into an elongated β-sandwich and the long axis of this domain is approximately at right angles to the helical stalk of the mushroom-shaped trimer (Fig. 1 A,B and ref. 7). The protein-binding interface is in a crevice that extends across the edge of the β-sandwich on each subunit. Thus, there are three identical binding sites that can accommodate three binding partners for

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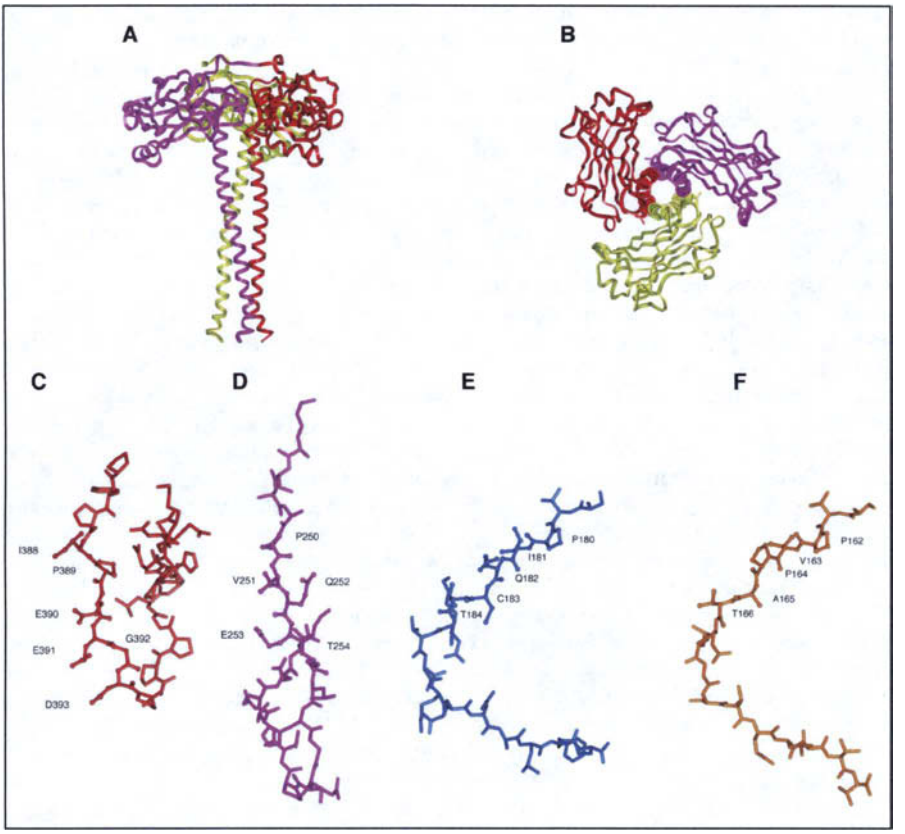

Figure 1. TRAF3 is a symmetric trimer. In this schematic representation, the TRAF3 trimer is shown as a ribbon diagram with each subunit colored separately. Panel A) The view on the left illustrates the 'mushroom' shape of the trimer with the N-terminal elongated helices forming the stalk and the conserved TRAF domains, which fold as a β-sandwich, at the top. The subunits associate and are stabilized by coiled-coil interactions between the long helices.[7] In this orientation, the cell membrane would be located at the top of the image. Panel B) The trimer is viewed from the top. TRAF3 has three identical binding crevices, located in each subunit at the edge of the β-sandwich. A recognition motif in each binding partner is bound in this crevice and can be presented in several structural frameworks. In Panels C and D, the binding regions from two TNF receptors that bind to TRAF3 in a 'hairpin' or 'reverse-turn' configuration are shown. For direct comparison, the recognition motifs are shown as ball-and-stick models in the same orientation, with LTβR in Panel C and CD40 in Panel D. Residues that provide specific TRAF3 recognition in each of the receptors are labeled, with the labels positioned near the β-carbon of each amino acid. In Panels E and F, two binding partners that bind to TRAF3 in a 'boomerang' configuration are shown as ball and stick models. Panel E shows the binding region of TANK as it is bound to TRAF3 and can be compared with the binding region from BAFF-R in Panel F. As in Panels C and D, the two binding regions are oriented to optimize direct comparison, with the residues in the recognition motifs positioned the same, and labeled.

the TRAF3 trimer. Binding recognition in the crevice requires a consensus motif PxQxT or [P/S/T/A)x(Q/E)E] which can be presented to the TRAF3 site within several structural frameworks.

Structurally and Functionally Adaptive Binding Crevice in TRAF3

The protein-protein contact region on TRAF3 can bind to several diverse molecules with differing function and thus represents a binding interface that is structurally and functionally adaptive.[10] The functional consequence of TRAF3 binding to several TNF receptors and down-

stream regulators provides a mechanism for competitive binding and has important implications for TRAF3 modulation of NF-κB activation. The conformational adjustments of side chains in the binding crevice are adaptive changes that occur on interaction with the individual binding partners. In a series of crystal structures of complexes of TRAF3 with different binding partners,[5-9] we have been able to identify residues that we define as 'hot spots' in the TRAF3 protein-interaction interface, since these residues provide the same principal contacts for each of the different binding partners. The experiments were facilitated by the fact that a large solvent 'cave' exists in the TRAF3 crystal lattice, and long peptides representing the binding motifs of individual partners can be soaked into the crystals. These soaking experiments avoid the need for cocrystallization with the peptides which carries a risk of change of crystallization conditions, and space group or conformational adjustments due to new packing interactions. Using the existing TRAF3 crystals, peptides can diffuse through large solvent channels that are found primarily around the extended α-helical coiled-coil regions of the mushroom-shaped trimer. The long peptides bind with the recognition pentapeptide motif located in the binding crevice, where the primary intermolecular contacts are made. Flanking residues are accommodated in a restricted solvent 'cave' that is immediately adjacent to the binding crevice. The space in this portion of the crystal lattice (15 x 19 x 22 Å) is sufficient so that peptide conformations observed in the complexes reflect actual conformations and are not an artifact of crystal packing.

The binding surface on TRAF3 is accessible and nonpolar and is essentially large enough to accommodate 5-6 residues, corresponding to the length of the recognition motifs carried by proteins that bind to TRAFs.[10,15] Within this crevice, there are three 'hot spots' that provide the critical contact sites with each of the partners in each of the five TRAF3 complexes we have studied.[5-9] These residues have been shown by mutagenesis to be critical or essential for binding (as documented in detail in each of the references), similar to what has been shown in other studies that characterize protein-protein recognition sites.[16-18] The character of each of the 'hot spots' in TRAF3 is distinct. In the first, five residues line a hydrophobic pocket (Leu432, Phe411, Phe448, Phe457 and Cys470). This hydrophobic pocket accommodates the N-terminal residue in each recognition motif. The second 'hot spot' is hydrophilic and involves three serines (Ser454, Ser455, and Ser 456) clustered as a group that was described as 'serine tongs' in TRAF2.[11] These serines form hydrogen bonds with residues in the third position in the recognition motif of the binding partner. The third 'hot spot' is polar in nature. Here, three residues (Arg393, Tyr395, and Asp399) make direct contacts with the fifth or last residue in the binding motif. Binding of different proteins to TRAF3 is enabled by conformational changes in the side chains of the amino acids at the 'hot spots' without extensive changes of the polypeptide backbones. At the hydrophilic 'hot spot', adjustments are minimal and involve only small reorientation of the serine hydroxyl groups. In the polar 'hot spot', major movements of the side chains are observed (2-3 Å) permitted by the flexible nature of the longer side chains. Residues in this 'hot spot' make direct contact with the recognition motifs through hydrogen bonds and the pattern of these hydrogen bonds is adaptable for each partner. Even in the hydrophobic 'hot spot', substantial side chain movements are observed so that the shape of this pocket is remodeled for each of the binding interactions.

Structural Framework for TRAF3 Recognition

Two structural frameworks were observed in the partner molecules in the TRAF3 complexes studied to date. The recognition motif is imbedded in a 'hairpin' configuration or a more open 'boomerang', as shown in Figure 1. Cytoplasmic portions the TNF receptors CD40 and LTβR present the recognition motifs in 'hairpin' or reverse turn configurations,[5,7] while the motif in TNF receptor BAFF-R and the downstream regulator TANK is embedded in an open 'boomerang' structure.[6,8] When the recognition motif is presented from the closed reverse-turn conformation, primarily residues in the consensus motif make intermolecular contacts with TRAF3. In contrast, when the motif is presented in the open 'boomerang' configuration, direct contacts are made not only by residues in the motif, but also by residues that are distal and toward the C-terminus of the peptide fragment. The second contact is made with a β-strand at the edge of one layer of β-sheet in the TRAF3 domain. Here, hydrophobic contacts are made with Trp356 in TRAF3 and hydrogen bonds are formed with Gln379 in TRAF3, to form a stable complex.

As with residues in the TRAF3 binding crevice, there also is molecular adaptation at the site of the second contact, depending on whether the binding partner is in a closed or open framework. In comparing the complexes of TRAF3 with CD40 and LTβR (closed configuration) with the structures of BAFF-R and TANK bound in the open configuration, we observed that Tyr377 in TRAF3 undergoes a striking conformational shift between the two structures. In unliganded TRAF3 and TRAF3 bound to CD40 or LTβR, this tyrosine is engaged in a hydrogen bond with Arg393, but when bound to BAFF-R or TANK, this hydrogen bond is not formed. Instead the phenyl ring of Tyr377 rotates away from the domain and makes a new van der Waal's contact with leucine in the C-terminal contact region of the binding partner, stabilizing the formed complex. Such conformational adjustments may be essential for binding affinity in the context of multiple protein-protein interactions that modulate TNF receptor signaling.

Specific Recognition at the TRAF3 Site

Proteins that bind to TRAFs bear a consensus motif that is key to recognition because the residues in this motif bind in the binding crevice on the TRAF domain. The binding sequences in four of the five proteins we have studied in complex with TRAF3 are closely similar: ^{250}PVQET^{254} in CD40, ^{180}PIQCT^{184} in TANK, ^{162}PVPAT^{166} in BAFF-R and ^{204}PQQAT^{208} in LMP1.[6-9] For LTβR, the motif ^{388}IPEEGD^{393} is not as similar although the TRAF3 contacts are the same.[5] The complete description of the intermolecular interactions is reported in the primary references to these studies, but a brief discussion and comparison (see Fig. 2), of the complexes is presented in the following sections.

TRAF3/CD40

The recognition motif in CD40 ^{250}PVQET^{254} is presented to TRAF3 in a hairpin configuration[7] that is stabilized by an intramolecular hydrogen bond involving the last residue in the motif, Thr254. This threonine does not make direct contact with TRAF3, yet is essential for binding because of its role in maintaining the hairpin, and substitution of any residue other than serine abolishes binding to TRAF3 (or TRAF2;[19]). Pro250 lies in the hydrophobic 'hot spot' pocket on TRAF3 and Glu252 is within hydrogen binding range of the serines in the hydrophilic 'hot spot'. In CD40, one consequence of the hairpin is formation of a TRAF3-specific hydrogen bond between Gln263, on the opposite arm of CD40 from the consensus motif, with Tyr395 in TRAF3.

TRAF3/LTβR

Like CD40, LTβR also assumes a reverse turn configuration for presentation of the recognition motif to TRAF3.[5] However, as shown in Figure 1A,B, the conformations of the structures in the cytoplasmic domains of these two TNF receptors is not closely similar. Moreover, unlike the CD40 hairpin, there were no intramolecular interactions observed in the LTβR fragment studied in the complex. The structure revealed an unexpected recognition motif in LTβR ^{388}IPEEGD^{393} for TRAF3 binding. Within the LTβR sequence ^{385}PYPIPEEGDGPPGLSTPHQEDGK^{408} which had been implicated in NF-κB activation and apoptosis,[20] there are actually three segments that loosely resemble the consensus PxQxT TRAF3 binding motif: PIPEE, PEEGD and PHQED. Only after the structure of the complex was determined did it become clear that LTβR binds to TRAF3 with a distinct binding motif IPEEGD. Despite the differences, the motif is accommodated in the same binding crevice where the consensus motif PxQxT binds, involving intermolecular contacts with the same 'hot spots' in the site, and these are the only intermolecular contacts between the two proteins. First, the N-terminal residue in LTβR, isoleucine, binds in the hydrophobic 'hot spot' where proline binds in other TRAF3 binding partners. The side chain of Glu390 forms a hydrogen bond with Ser456 in the hydrophilic TRAF3 'hot spot'. A salt bridge is formed between Glu391 in LTβR and Arg393 in the polar TRAF3 'hot spot'. The final intermolecular interaction is made as a critical hydrogen bond between Asp393 and TRAF3 Tyr395. In this case, the TRAF3 binding motif is six residues in length, contrasted with five residues seen in the other complexes studied. When the three consecutive carboxylate residues were mutated to alanine, binding was completely abolished, while substitution of alanine for the two glutamates alone merely reduced binding. Thus, for stable binding of LTβR to TRAF3, the key hydrogen bond involving the sixth residue in the motif, Asp393 must be formed.

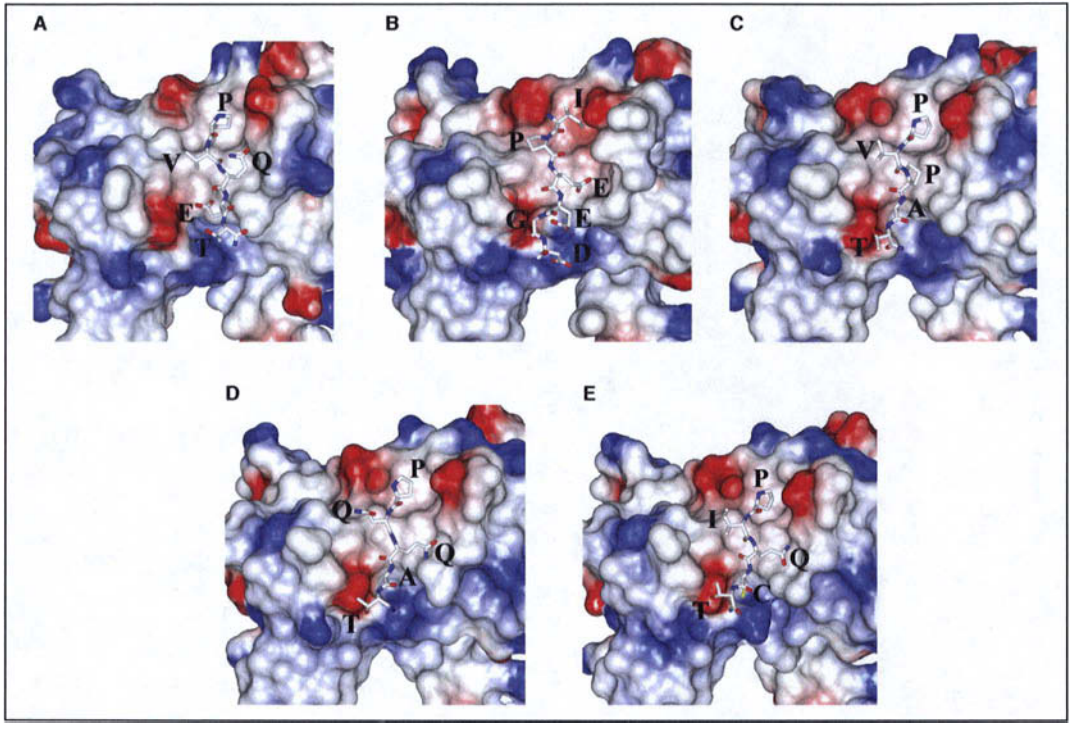

Figure 2. Binding crevice in TRAF3 is structurally adaptive. Short recognition motifs in proteins that bind to TRAF3 are bound in a surface crevice that contains three 'hot spots'. Residues in these 'hot spots' undergo molecular adaptation to permit specific binding to each partner. The binding partners that are shown are: Panel A, CD40; Panel B, LTβR; Panel C, BAFF-R; Panel D, LMP1; Panel E, TANK. In each image, the view into the TRAF3 binding crevice is shown in the same orientation for direct comparison. The recognition motifs are labeled and shown as ball-and-stick models in their bound configurations. The TRAF3 crevice is presented as a molecular surface colored according to electrostatic characteristics, with blue corresponding to negative regions, red representing positive regions and white as the neutral regions. The effect of molecular adaptation of the TRAF3 residues when each of the binding partners makes contact with TRAF3 can be seen in the comparison of the electrostatic patterns.

TRAF3/BAFF-R

The third TNF receptor studied in the series, BAFF-R, binds only to TRAF3, and not to other TRAFs.[21] The motif ^{162}PVPAT^{166} binds in the TRAF3 binding crevice,[6] but unlike the other two TNF receptors, the cytoplasmic portion of BAFF-R is embedded in an open configuration for recognition by TRAF3. N-terminal Pro162 makes hydrophobic contacts in the hydrophobic 'hot spot' in the TRAF3 crevice, while the C-terminal Thr166 participates in a hydrogen-bonded network with TRAF3 Tyr395 and Asp399 in the polar 'hot spot'. Glu167 in BAFF-R is in position to form a salt bridge with Arg393 in TRAF3. In contrast to the other receptors, BAFF-R has a proline in the third position in the motif and so no hydrogen bonds are possible with the three serines in the hydrophilic 'hot spot' on TRAF3. Although BAFF-R makes fewer direct contacts through residues in the recognition motif, other intermolecular interactions are made well away from the binding crevice by residues in the extended arm of the 'boomerang' configuration. Backbone interactions between this strand and a β-strand on TRAF3 stabilize the docking in typical parallel β-sheet topology so that a salt bridge can be formed between Glu172 in BAFF-R and Arg364 in TRAF3. In addition, a critical hydrogen bond is formed engaging BAFF-R Thr175 and Gln379 in TRAF3. When alanine is substituted for Thr175 by mutagenesis, binding of BAFF-R to TRAF3 is abolished. In TRAF2, the equivalent of Gln379 is proline, so the critical hydrogen bond with Thr175 would not be possible, and this fact may explain why BAFF-R does not bind to TRAF2. Next to the threonine, Val174 in BAFF-R participates in a hydrophobic interaction involving TRAF3 Trp356. One other hydrophobic interaction in this region demonstrates the dynamic nature of complex formation when BAFF-R binds to TRAF3. In this case, TRAF3 Tyr377 undergoes a dramatic conformational shift, from an internally hydrogen-bonded configuration before the complex is formed, to swing out toward the partner. The phenyl ring of this tyrosine is in van der Waal's contact with Leu173 in BAFF-R, producing a new hydrophobic stabilizing contact.

TRAF3/TANK

TANK is a downstream modulator of TRAF-mediated signaling, and is associated with activation as well as inhibition of the NF-κB pathway, depending on the level of expression.[22] TANK binds to TRAF1, -2 and -3 in a 21 residue region located in the middle of the molecule that contains a sequence ^{180}PIQCT^{184} that resembles the TRAF recognition sequence found in TNF receptors. This sequence binds to the same binding crevice used by the TNF receptors and is presented in an open 'boomerang' configuration similar to that seen in BAFF-R when it binds to TRAF3.[8] In this manner, TANK grips the TRAF domain of TRAF3 around the outer edge of the β-sandwich and makes intermolecular contacts with residues located in each extended arm of the binding segment. Pro180 is accommodated in the hydrophobic 'hot spot', while Gln182 contacts TRAF3 through a hydrogen-bonded network with the cluster of serines in the TRAF3 hydrophilic 'hot spot'. Unlike CD40, the last residue in the pentapetide motif, Thr184 does not participate in intramolecular interactions, but instead is hydrogen-bonded to the side chain of Asp399 in TRAF3 polar 'hot spot'. From the C-terminal extended strand, TANK makes two intermolecular contacts that are similar to those observed in the BAFF-R complex with TRAF3: a hydrophobic interaction between TANK Phe194 and TRAF3 Trp356, and a hydrogen bond between TANK Lys195 and TRAF3 Gln379. Like the interactions in the TRAF3/BAFF-R complex, these C-terminal contacts serve as stabilizing interactions while the principal recognition is mediated by the intermolecular hydrogen bonds formed in the binding crevice. Similar to the dynamic changes that occur when the TRAF3/BAFF-R complex forms, when TANK binds, Tyr377 at the TRAF3 surface flips down to form an additional intermolecular complex with Leu193 in TANK, making a hydrophobic contact. The secondary contacts involving the C-terminal strand may contribute to stronger binding affinity of TANK for TRAF3 than for CD40. When peptides bearing the recognition motif from TANK and CD40 were soaked into TRAF3 crystals in competitive binding experiments, only TANK bound to TRAF3.[8] Stronger binding of the downstream regulator could provide a mechanism for release of TRAFs from CD40, influencing TANK-mediated inhibition of NF-κB activation by CD40.

TRAF3/LMP-1

In another situation, competition for TRAF3 influences malignant transformation of B lymphocytes. The latent membrane protein1 (LMP1) encoded by the Epstein-Barr virus causes lymphoproliferative malignancies and acts as a constitutively active mimic of CD40. LMP1 bears a sequence ^{204}PQQATDD^{210} that binds to TRAF3, and closely mimics signaling events and effector functions of CD40 in B lymphocytes, including activation of NF-κB and JNK.[23,24] LMP1 binds to TRAFs 1, 2, 3 and 5 by recognition of the PQQAT sequence which conforms to the motif for TRAF recognition. In TRAF3, this motif binds to the TRAF3 binding crevice in a mode that is quite similar to that seen in the complex of TANK with TRAF3.[9] Proline 204 binds in the hydrophobic 'hot spot', Gln206 forms hydrogen bonds in the hydrophilic 'hot spot' with one or more serines, and Thr208 participates in a hydrogen-bonded network with Asp399 in the TRAF3 polar 'hot spot', as well as an intramolecular hydrogen bond with the side chain of Asp210. In addition, Asp210, two residues away from the recognition motif in LMP1, forms two hydrogen bonds with Tyr395 and Arg393 in the polar 'hot spot'. This is in contrast to TANK where Asp185 which is immediately adjacent to the pentapeptide motif forms hydrogen bonds with the same TRAF3 residues. Substitution of alanine for Asp210 in LMP1 diminishes binding, while mutation of Asp209 to alanine does not affect binding. Overall, a comparison of the LMP1 motif with the consensus motif in CD40 reveals structural similarity, however the differences in intermolecular contacts provide some insight that stronger binding of LMP1 than CD40 to TRAF3 may be critical for LMP1 to transform B lymphocytes. In LMP1, more intermolecular hydrogen bonds are formed to strengthen binding, involving Thr208 and Asp210. Like TANK, LMP1 may compete with CD40 for the TRAF3 binding crevice, docking as a molecular decoy for CD40.

Summary

TRAF3 contains a protein-protein interaction crevice on the surface of the conserved TRAF domain that acts as a docking site for proteins bearing a consensus motif PxQxT. This crevice contains three 'hot spots' for intermolecular contacts that differ in character (i.e., hydrophobic, hydrophilic and polar) and residues at these 'hot spots' are structurally adaptive to permit binding to multiple binding partners. This molecular adaptation, as well as dynamic conformational adjustments of a key tyrosine away from the crevice, provide the structural basis for recognition of individual partners, affecting binding affinity and functional outcome of the binding event. The crevice serves as a binding site for several TNF receptors as well as a downstream regulator and a viral protein that binds as a structural decoy. A series of crystallographic studies of the complexes of five of these binding partners with TRAF3 has identified the precise protein-protein interactions made in each complex, and proposed detailed insights into the molecular events that permit specific recognition of these diverse proteins. The results are the first steps to understand the complex mechanisms for structural discrimination of competing proteins that influence signaling pathways mediated by TRAF3.

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CHAPTER 10

Molecular Basis for the Unique Specificity of TRAF6

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Abstract

Tumor necrosis factor (TNF) receptor (TNFR) associated factor 6 (TRAF6) is a unique member of the TRAF family of adaptor proteins that is involved in both the TNF receptor superfamily and the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily signal transduction pathways. The ability to mediate signals from both families of receptors implicates TRAF6 as an important regulator of a diverse range of physiological processes such as innate and adaptive immunity, bone metabolism, and the development of lymph nodes, mammary glands, skin, and the central nervous system. This chapter will highlight the structural and biochemical studies of TRAF6 in receptor interactions and discuss the potential for peptidomimetic drug application based on TRAF6 receptor binding motif.

Introduction

TRAF6 was first identified in the signal transduction pathways of CD40 and IL-1R,[1,2] which makes it the only member of the TRAF family of adaptor proteins to mediate signals from both the TNFR and the IL-1R/TLR superfamily. Gene deletion studies of TRAF6 confirmed the role of TRAF6 in innate and adaptive immunity, bone metabolism, and the development of lymph nodes, mammary glands, skin, and the central nervous system.[3-7]

TRAF6 has a unique sequence specificity for receptor interaction that does not overlap with other TRAF family members.[8,9] Although TRAF6 interacts directly with TNFR family members, CD40 and TRANCE-R (also known as RANK), TRAF6 does not directly bind to IL-1R/TLR superfamily members. TRAF6 is coupled to IL-1R/TLR activation by interacting with Ser/Thr kinases IRAK1, IRAK2, IRAK-M and possibly IRAK-4.[2,10-12] IRAKs are recruited to activated receptors through interaction with adaptor proteins containing the Toll and IL-1R (TIR) domain such as MyD88, Mal/TIRAP, TRIF, TRAM, Tollip, and SARM,[13,14] which then interacts with TIR-domain of the receptors.

The downstream signaling events of TRAF proteins converge on the activation of transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1),[15,16] that transcribe genes involved in numerous cellular and immune regulation. Interestingly, the activation pathways for NF-κB and AP-1 by TRAF2 and TRAF6 may both utilize a nondegradative lysine-63 linked polyubiquitin chains for downstream signaling. In vitro reconstitution assay has shown that the RING domain of TRAF6 functions as a ubiquitin ligase to synthesize lysine-63 linked polyubiquitin in the presence of the ubiquitin conjugation enzyme system, Ubc13 and Uev1A.[17,18] These nondegradative polyubiquitin chains have been shown to be important in the activation of protein kinase complex

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called IκB kinase (IKK), which directly activates NF-κB.[17] Similarly, the inhibition of NF-κB activation was observed by the deubiquitination of TRAF2 and RIP (receptor interacting protein) by the nondegradative deubiquitinating enzymes, CYLD[19-21] and A20,[22] respectively.

Expression and Crystallization of TRAF6

The domain organization of TRAF6 is consistent with other TRAF family members. The N-terminal domain is comprised of a RING and five zinc finger regions followed by a coiled-coil TRAF-N domain and a conserved TRAF-C domain.[23] The N-terminus of TRAF6 mediates downstream signaling, whereas the C-terminus is involved in self-association and receptor interaction.[24]

Structural studies on the TRAF domain of TRAF6 were initiated to determine the receptor interaction specificity by TRAF6. Extensive TRAF6 construct variations were utilized to produce soluble protein that led to the successful crystallization of TRAF6.[25] Mapping studies of the TRAF-C domain defined residues 351-522 to be the region responsible for receptor interaction.[2,26] Initial construct designs were based on the above domain definitions and sequence alignments.[2,24,26] These early constructs were mostly insoluble or had a tendency to aggregate. Based on the successful TRAF2 TRAF-domain crystallization,[27] similar constructs were made for TRAF6 (residues 333-508 and residues 333-512). These TRAF2 based constructs contained a small portion of the coiled-coil TRAF-N domain along with the TRAF-C domain. These new constructs were partially soluble.

At high TRAF6 protein concentrations, TRAF6 exists in trimer form, which is consistent with the structure of TRAF2.[27] However, TRAF6 333-508 construct was only able to crystallize at low protein concentrations of 1-2 mg/ml. The X-ray diffraction of this TRAF6 construct was weak, nevertheless, a dataset was collected and the structure was solved by molecular replacement.

Analysis of the structure showed one TRAF6 monomer per crystallographic asymmetric unit. Interestingly, the coiled-coil region of the TRAF6 was situated in a position that would clash sterically with another symmetry related molecule. The location of the coiled-coil region therefore explains why TRAF6 was only able to crystallize at low protein concentrations and as a monomer. Based on this information, further constructs starting at 343, 346, and 349 were made, which deleted the short coiled-coil region. These proteins were partially soluble and the construct with residues 346-504 was readily crystallized both alone and in complex with CD40 and TRANCE-R peptides.[28]

Molecular Basis for the Distinct Specificity of TRAF6

The TRAF-C domain of TRAF6 shows the highest degree of difference compared to other TRAF protein structures, when compared to TRAF2 TRAF-C domain (Fig. 1). The TRAF2 TRAF-C domain is comprised of an eight-stranded anti-parallel β-sandwich, with strands β1, β8, β5 and β6 in one sheet and β2, β3, β4 and β7 in the other[27] (Fig. 1A). Although the overall architecture is the same, superposition of TRAF6 with TRAF2 shows an r.m.s.d of 1.1-1.2 Å for 127 aligned Cα positions within 3.0 Å (Fig. 1C). This TRAF6-TRAF2 structural difference is larger than for TRAF3 TRAF-C domain (Fig. 1B).

There are numerous residue insertions or deletions within the loop regions of TRAF6 structure. Specifically, β3-β4 loop contains one residue insertion, β5-β6 loop contains three residue insertions, and β7-β8 loop contains one residue deletion. The β3-β4 loop of TRAF6 exhibit a movement of up to 12 Å in Cα positions, relative to TRAF2. Therefore, TRAF6 no longer interacts with receptor peptides in this region. The remaining loop regions show on average 2-5 Å Cα movement in comparison to TRAF2. In the absence of a receptor peptide the β6-β7 loop is disordered. Although TRAF6 crystallized as a monomer, the TRAF trimerization loops, β2-β3 and β4-β5, are conserved in TRAF6. This shows that on a structural level TRAF6 can form trimers.

In agreement with the distinct receptor specificity and function of TRAF6, crystals of TRAF6 in complex with CD40 or TRANCE-R peptides revealed novel binding modes.[28] The receptor chain binds across the TRAF domain of TRAF6 that exhibits a trajectory which is 40° away from the receptor peptide position on TRAF2 (Fig. 2A,B). This mode of receptor peptide association on TRAF6 results in a completely different receptor side-chain interactions compared to TRAF2 (Fig. 2C).

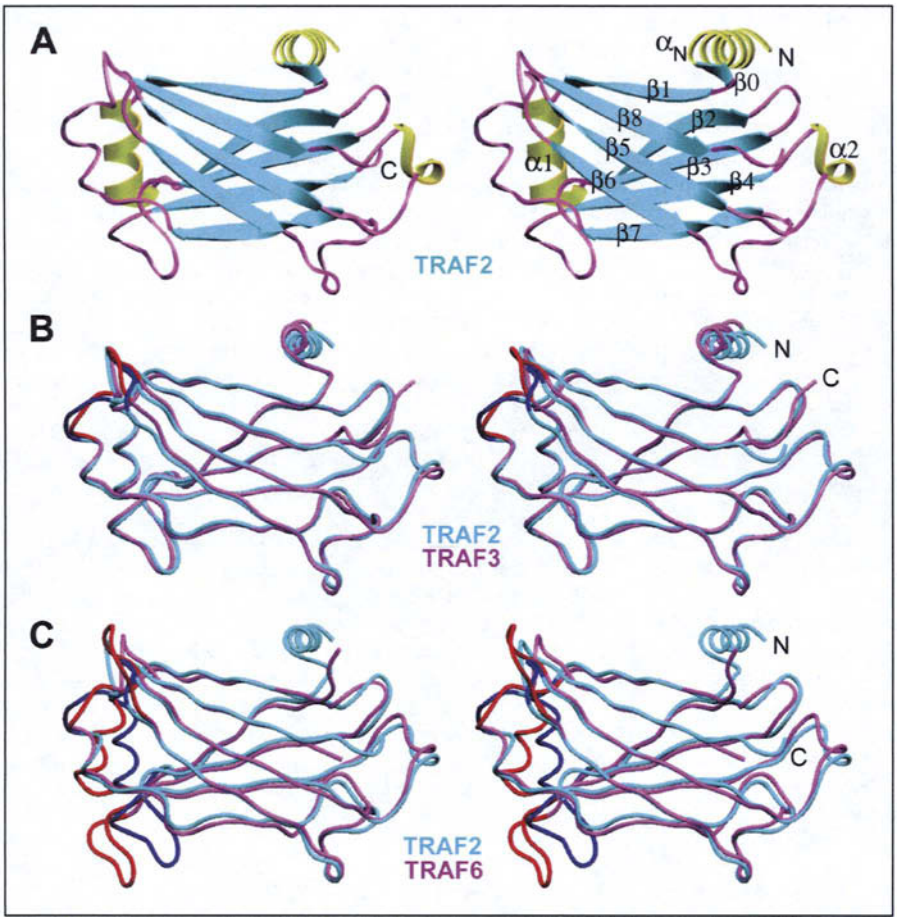

Figure 1. TRAF domain structures. A) Stereo drawing of the TRAF domain of TRAF2 with labeled secondary structures. B) Superposition of the TRAF domain of TRAF2 (cyan) and TRAF3 (magenta). Regions with large differences between the two structures are shown in blue for TRAF2 and red for TRAF3. C) Superposition of the TRAF domain of TRAF2 (cyan) and TRAF6 (magenta). Regions with large differences between the two structures are shown in blue for TRAF2 and red for TRAF6. Modified from: Wu H. Adv Protein Chem 2004; 68:225-79; ©2004 with permission from Elsevier.[29]

One of the major structural differences between TRAF6 and TRAF2 is the insertion of a proline residue in the β-bulge of the β7 strand (P468). The P468 insertion allows a more extensive main chain hydrogen bond formations to occur between the receptor peptides and the TRAF-C domain (residues 234-238 of CD40 and 344-349 of TRANCE-R with residues P468-G472 of TRAF6 TRAF-C domain) (Fig. 2D). The CD40 and TRANCE-R peptides assume a typical β conformation rather than a highly twisted polyproline II helix-type conformation observed in TRAF2 binding peptides.

A similar nomenclature of peptide positions as TRAF2 is used for TRAF6 binding peptides. The residues E235 of CD40 and E346 of TRANCE-R were designated as the P_0 position of TRAF6 binding peptides. These residues occupy a similar, although not an identical position as P_0 residue (Q/E) in the TRAF2 binding motif. The peptide residues corresponding to P_{-4} to P_3 of both CD40 and TRANCE-R directly interact with TRAF6. Based on the surface area burial and

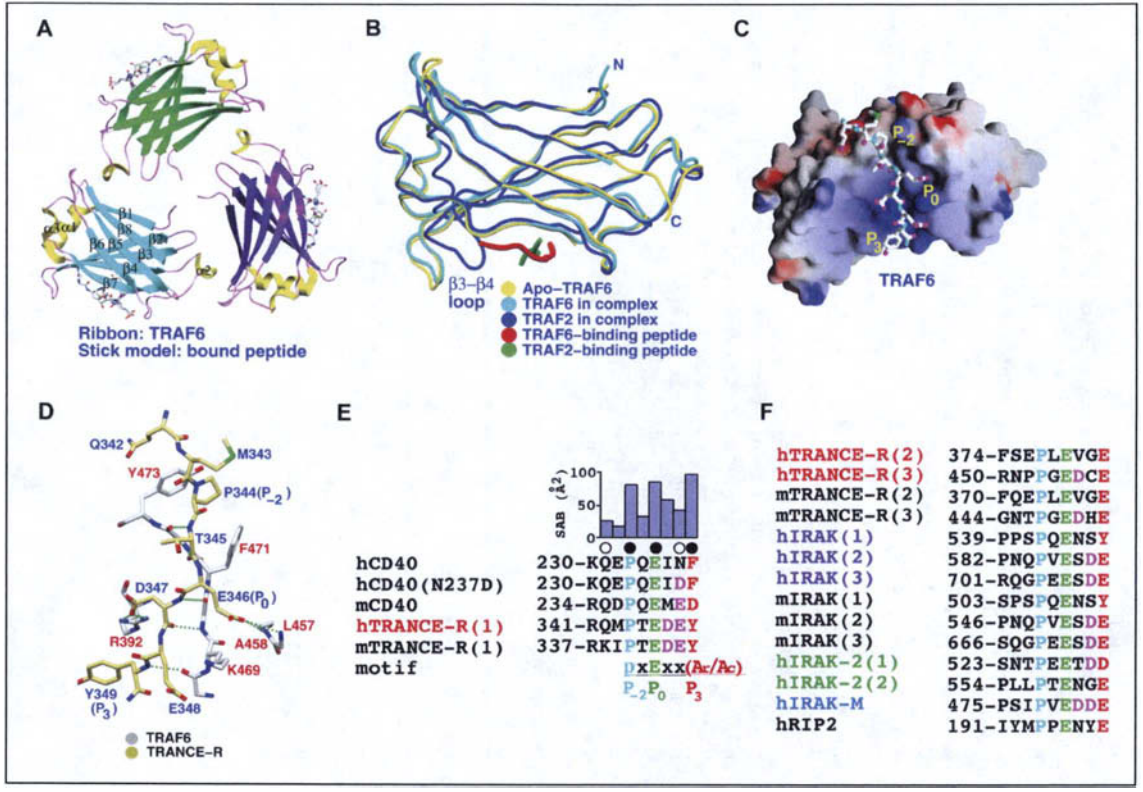

Figure 2. TRAF6-receptor interactions. A) TRAF6 trimeric model. B) Superposition of TRAF6 with TRAF2, with bound receptor peptides. C) Electrostatic surface of TRAF6 in complex with the TRANCE-R peptide. D) Detailed interactions between TRAF6 and TRANCE-R. E) Sequence alignment of CD40 and TRANCE-R and the corresponding surface area burial (SAB) and the TRAF6-binding motif. F) TRAF6-binding sequences identified based on the structurally defined motif. Modified from Ye et al.[28]

specific side chain interactions of residues at P_{-2}, P_0 and P_3, these residues contribute the most to the interactions (Fig. 2D).

The Pro at P_{-2} position interacts with the hydrophobic pocket created by the residues F471 and Y473 of TRAF6. The carboxylate of the P_0 Glu residue forms hydrogen bonds with the main chain amide nitrogen atoms of L457 and A458, while the aliphatic portion of the side chain aligns nicely along the TRAF6 surface. In addition, the carboxylate of the P_0 Glu may form a favorable charge-charge interaction with the side chain of K469. The P_3 residue in CD40 (F238) and TRANCE-R (Y349) is among several aromatic and basic residues of TRAF6, including H376, R392, H394, and R466. There is an amino-aromatic interaction observed between Y349 of TRANCE-R and R392 of TRAF6. A similar amino-aromatic interaction is possible for F238 of CD40.

Despite the differences between TRAF6 and TRAF2, the peptide interaction sites on TRAF6 are quite analogous to those of TRAF2. The residues forming the P_{-2} pocket of TRAF6 function similarly to the Ser467 and Cys469 of TRAF2. The corresponding TRAF6 P_{-2} pocket is about 3 Å away and consists of Phe471 and Tyr473. The residues forming the pocket for P_0, Leu457 and Ala458, are analogous to Ser454 and Ser455 of TRAF2. In addition, the residues R392 and H394 of TRAF6 are the structural correspondents of R393 and Y395 of TRAF2, which are two critical residues forming the P_1 pocket of TRAF2. Similarities between TRAF6 and TRAF2 indicate an evolutionary mechanism in which the same mutations result in the formation of new interaction specificity for TRAF2 while at the same time abolish interactions for TRAF6.

A consensus sequence for TRAF6 binding motif was derived from the structure-based sequence alignment of TRAF6 binding sites in human and mouse CD40 and TRANCE-R. The motif representing the positions P_{-2} to P_3 consists of pxExx(Ar/Ac), where p is written in lowercase to represent tolerance for other small to medium sized residues, x can by any residues, Ar represents any aromatic residues, and Ac represents any acidic residues (Fig. 2E,F). Mutational studies have shown that similar to what is observed in TRAF2 binding peptides, the proline at P_{-2} can accommodate changes to small residues such as Ala without loss of binding affinity to TRAF6 (Table 1). The P_0 position can also accommodate a Gln substitution from Glu, but not to Ala. Also, the side chain at P_3 position is necessary for proper receptor peptide interaction with TRAF6. Furthermore, the residues

Table 1. Structure-based mutational studies

TRAF6	Receptor/Adapter, Motif Position	Effects[a]	Method	Ref.
WT TRAF6	CD40 (P237A) P_{-2}	+	GST-pulldown and NF-κB activation	28
	CD40 (P237Q) P_{-2}	--		
	CD40 (E239Q) P_0	--		
	CD40 (D242A) P_3	-		
	CD40 (Q235A) P_{-4}	+		
	TRANCE-R (E342A, E375A, E449A) $P_0/P_0/P_0$	--	NF-κB activation	
	TRANCE-R (E342A, E375A) P_0/P_0	+		
	TRANCE-R (E342A, E449A) P_0/P_0	+		
	TRANCE-R (E375A, E449A) P_0/P_0	+		
	IRAK (E706A) P_0	-	NF-κB activation	
	IRAK (E587A, E706A) P_0/P_0	--		
	IRAK (E544A, E587A, E706A) $P_0/P_0/P_0$	---		
TRAF6 (R392A)	IRAK	--	TRAF6 dominant negative effect on NF-κB activation	
TRAF6 (F471A)		---		
TRAF6 (Y473A)		---		

[a] +: no effect; -: decreased; --: greatly decreased; ---: drastically decreased.

Table 2. Characterizations of TRAF6-receptor interactions using isothermal titration calorimetry

TRAF6	Receptor/Adapter and Sequence [a]	K_d [b]	Ref.
TRAF6 (333-508)	CD40 (216-245) KKVAKKPTNKAPHPKQEPQEINFPDDLPGS	59.9 μM	28
	CD40 (230-238) KQEPQEIDF	84.0 μM	
	mTRANCE-R (337-345) RKIPTEDEY	78.0 μM	
	mTRANCE-R (370-378) FQEPLEVGE	770.0 μM	
	mTRANCE-R (444-452) GNTPGEDHE	763.0 μM	
	IRAK (539-548) PPSPQENSYV	518.1 μM	
	IRAK (582-590) PNQPVESDE	79.0 μM	
	IRAK (701-710) RQGPEESDEF	54.3 μM	
	IRAK-2 (523-532) SNTPEETDDV	66.2 μM	
	IRAK-M (475-483) PSIPVEDDE	142.2 μM	

[a] m: mouse; otherwise from human. [b] K_d: dissociation constant.

at P_1 and P_2 may have a preference for acidic residues to compliment the basic TRAF6 surface formed by the side chains of R392 and K469. Isothermal titration calorimetry (ITC) measurements confirmed this hypothesis by showing much higher binding affinities to TRAF6 by peptides with acidic residues at P_1 and P_2 (Table 2).

Inhibitors of TRAF6 Signaling

TRAF proteins are known to play a critical role in regulating inflammatory responses as well as cell survival and proliferation. The down-regulation of TRAFs may be therapeutically beneficial since it has been implicated in many disease processes involving inflammation and tumorigenesis. One method of inhibiting TRAF signaling is to block TRAF-receptor interaction with short peptides or small molecules.

Based on the crystal structure of TRAF6-TRANCE-R complex, cell permeable TRAF6-interacting decoy peptides were constructed by fusing the TRAF6 interacting sequences from TRANCE-R with the hydrophobic signal peptide of the Kaposi fibroblast growth factor.[12] The effectiveness of the decoy peptides in blocking TRANCE-R mediated signaling was examined by measuring NF-κB activation in RAW264.7 cells. Pretreatment of RAW264.7 cells with the decoy peptides led to a dose-dependent inhibition of NF-κB activation (Fig. 3A). In addition, TRANCE-induced osteoclast differentiation in RAW264.7 and primary mouse monocytes was blocked by cotreatment with the decoy peptides (Fig. 3B,C). These cell-based assays demonstrate the potential of TRAF6-binding motif peptides to inhibit specifically TRAF6 mediated signal transduction.

The success of the TRAF6 binding motif decoy peptides in cell culture studies indicates two possible modes of action. The first mechanism may rely on the low level of endogenous receptors that may be competed out by the higher decoy peptide concentration. The second mechanism may involve the hydrophobic signal peptide sequence of the decoy peptide which can allow association with cellular membranes, thereby achieving high local concentrations of the decoy peptides to compete out the receptor TRAF6 interaction. These mechanisms describe how it may be possible to compete with oligomeric endogenous interactions.

Structural and thermodynamic studies indicate several features of TRAF-receptor interactions that can be manipulated to design high affinity TRAF binding inhibitors. The first feature is the inherent low affinity interaction between the receptor and TRAFs, which indicates a nonideal steric or chemical complementation. Secondly, surface pockets such as the hydrophobic P_{-2} pocket, can be

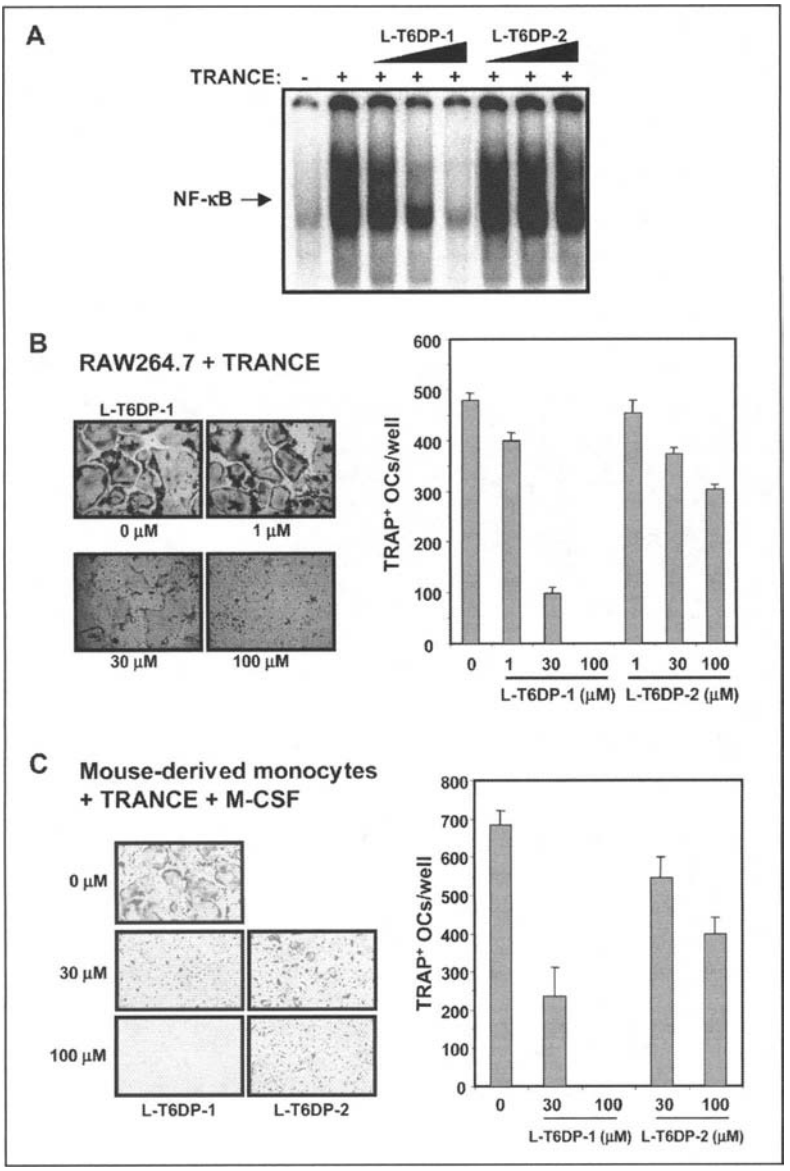

Figure 3. Inhibitory effects of TRAF6 decoy peptides (L-T6DP-1 and L-T6DP-2) in TRANCE-mediated signal transduction and osteoclast differentiation. A) Inhibition of TRANCE-mediated NF-κB activation by TRAF6 decoy peptides, as shown by EMSA. B,C) Inhibition of TRANCE-mediated osteoclast differentiation in RAW264.7 cells (B) and primary monocytes (C) by TRAF6 decoy peptides. Cells were stained for TRAP. Modified from Ye et al.[28]

ideal targets for small molecule inhibitors. Finally, an increase in decoy peptide affinity for TRAFs may be achieved by rigidifying the TRAF binding moieties, since reduction of conformational entropy can lead to a negative contribution to the interaction.

Remaining Questions

The structural and functional studies of TRAF6 have revealed both the similarities as well as the differences between TRAF6- and TRAF2-receptor signaling. There are still many more studies to be conducted to elucidate specific TRAF6 activation mechanisms. For example, we still do not know whether TRAF6 is monomeric before recruitment to the receptors and whether oligomerization per se or oligomerization-induced conformational changes govern TRAF6 activation. These questions and others such as the role of ubiquitination in TRAF6 activation remain to be answered.

Acknowledgment

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CHAPTER 11

TRAF Proteins in CD40 Signaling

Gail A. Bishop,* Carissa R. Moore, Ping Xie, Laura L. Stunz and Zachary J. Kraus

Abstract

The tumor necrosis factor receptor (TNFR) superfamily molecule CD40 is expressed by a wide variety of cell types following activation signals, and constitutively on B lymphocytes, macrophages, and dendritic cells. CD40 signals to cells stimulate kinase activation, gene expression, production of a antibody and a variety of cytokines, expression or upregulation of surface molecules, and protection or promotion of apoptosis. Initial steps in CD40-mediated signal cascades involve the interactions of CD40 with various members of the TNFR-associated factor (TRAF) family of cytoplasmic proteins. This review summarizes current understanding of the nature of these interactions, and how they induce and regulate CD40 functions.

Introduction

CD40 in T Cell-B Cell Collaboration and B Cell Activation

The functions of CD40 in immune responses were first revealed by the discovery that the natural ligand for CD40, CD154, is encoded on the X chromosome, and defects in CD154 are responsible for the human immunodeficiency disease, X-linked hyper-IgM syndrome (HIGM).[1-3] The phenotype of both CD40 and CD154-deficient mice is very similar to that of HIGM patients,[4-6] indicating that CD154 is the major ligand for CD40 in mice and humans. These and various model experimental systems revealed that CD40 signals are of major importance to successful T-dependent B cell activation, promoting B cell proliferation, antibody production, isotype switching, and germinal center (GC) formation together with the development of B cell memory (reviewed in refs. 7-11). While each of these events is also promoted by alternative receptors and ligands, the striking defects in humoral memory, GC development, and production of 'switched' Ig isotypes that are manifested by HIGM patients and CD40 or CD154-deficienct mice suggest that CD40's roles cannot be completely compensated by other molecules. Thus, CD40 plays unique roles in the activation of a specific cell type, the B lymphocyte.

Activation of Antigen Presenting Cells (APC) by CD40

CD40 is expressed not only by B lymphocytes, but also cells of the monocyte/macrophage lineage, and dendritic cells (DC).[12,13] CD40 signals to all of these potential APC serve to upregulate cell surface molecules that play important roles in the process of antigen presentation, including class II MHC, adhesion, and T cell costimulatory molecules.[14-17] Additionally, CD40 signals stimulate production of a variety of cytokines by different types of APC, including IL-6, TNF, and IL-12, among others.[15,18-22] Thus, CD40 signaling can promote T cell activation by enhancing the function of APC, and it is not surprising that a lack of these signals also results in impaired cell-mediated immune responses.[16,23-27]

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

CD40-Mediated Activation of Nonhematopoietic Cells

Although of clear importance to the function of B lymphocytes, macrophages, and DC, the CD40 molecule was first identified expressed on cells of a bladder carcinoma,[28] and has since been shown to be expressed on a variety of epithelial cell types,[29-32] as well as fibroblasts,[12,33-35] and vascular endothelium.[36-38] CD40 signals to these cell types can also upregulate molecules involved in antigen presentation,[34,35,37,38] as well as induce the production of a variety of cytokines and chemokines.[39-43] Thus, while CD40 expression on tumor cells could potentially make them more effective APC, and hence stimulate T cells to kill them,[44,45] CD40 expression can also result in the promotion of inflammation,[39,42,46-48] as well as enhanced cell survival,[31,47,49-51] which could exacerbate autoimmune and malignant diseases.[52]

CD40 Association with TRAF Molecules

The cytoplasmic domain of CD40 lacks direct kinase activity, so must associate with other intracellular proteins to initiate signaling cascades. Members of the TRAF family play a key role in this process. The first cytoplasmic signaling protein shown to associate with CD40 was TRAF3,[53,54] although the function of TRAF3 in CD40 signaling is only recently becoming clear, 20 years later. TRAF2 was identified in 1994 to associate with TNF receptors,[55] and subsequently shown to bind CD40.[56] TRAF1 was initially identified at the same time as TRAF2,[55] and later also shown to associate with CD40.[57] The initial report identifying TRAF6 also demonstrated its ability to bind CD40.[58] A more complex situation exists for TRAF5. The first identification of TRAF5 was made by two groups of investigators, both using yeast two-hybrid analysis. Although the approaches were the same, one group reported that CD40 binds TRAF5,[59] while the other, reporting at the same time, saw no direct CD40-TRAF5 association.[60] It has not been subsequently reported whether or not CD40, expressed at normal levels in vivo, directly binds TRAF5, so this remains an unanswered question. TRAF4 has not been reported to bind to CD40, and neither the normal expression pattern nor receptor associations are known for a proposed TRAF7 molecule.[61]

Model Systems for Studying CD40-TRAF Interactions

With the realization that CD40 binds at least 4 distinct TRAF molecules (1, 2, 3, and 6), came great interest in discovering the specific functions of each TRAF in regulating particular CD40 signaling pathways. This has proven a complex undertaking, due to technical challenges in experimental design and data interpretation. Figures 1-3 illustrate the relative CD40 binding positions of TRAFs 1, 2, 3 and 6 to CD40. It can be appreciated that while crystal structure analysis has revealed the binding of TRAFs 2 and 3 to CD40 to be distinct,[62,63] their binding sites have considerable overlap. Although TRAF1 can bind weakly to CD40 alone,[57] the majority of TRAF1 association with CD40 occurs via heterotrimerization with TRAF2. Thus, it is difficult to alter the binding of one type of TRAF to CD40 without also affecting the association of other TRAF molecules.

One of the simplest and most straightforward approaches to study the interaction of CD40 with TRAFs is the use of in vitro protein-protein or protein-peptide binding systems. This approach provides quantitative binding data in the absence of other cellular proteins. However, such additional cellular proteins could significantly alter the binding of specific TRAFs to CD40 in vivo, as is certainly the case for members of the group with overlapping binding, TRAFs 1, 2, and 3. Thus, the high artificiality of these systems is a limitation to data interpretation.

Perhaps the most popular approach for studying TRAF interactions with CD40 to date has been the exogenous, usually transient overexpression of CD40 and TRAF constructs in a cell type that is easily transiently transfected with high efficiency, such as transformed cell lines of epithelial or fibroblast origin. The advantages of this approach are its relative ease, and that actual cells are examined. Certain early signaling functions, such as kinase and transcription factor activation, can be measured. However, expression is typically several orders of magnitude higher than expression of endogenous TRAF molecules. Thus, the stoichiometry of the TRAF-receptor complex is markedly abnormal, and results may not always be valid for the functions of TRAFs and receptors expressed at endogenous levels. For example, using this approach, it was concluded that CD40-mediated NF-κB

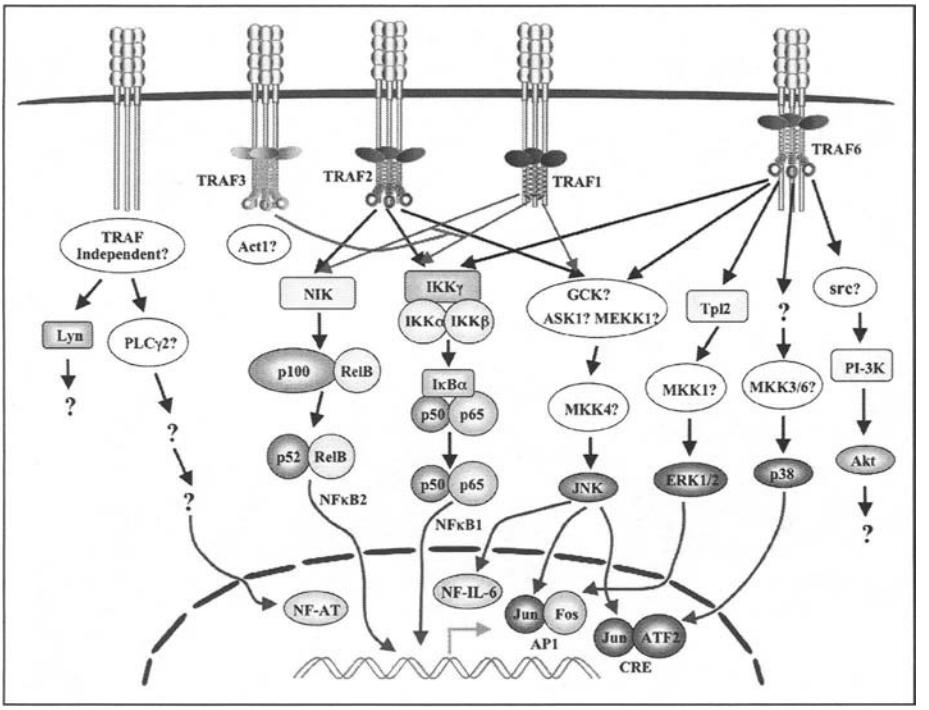

Figure 1. Activation of kinases and transcription factors by CD40 signaling. A summary scheme depicting both TRAF-dependent and TRAF-independent activation of kinases and transcription factors in response to CD40 signals is shown. Upon CD40 engagement, trimeric TRAF molecules are recruited by CD40 trimers to membrane microdomains (rafts). Following this recruitment, they associate with downstream signaling molecules, such as NIK, IKK and Tpl2, to activate several kinase cascades, leading to activation of transcription factors, including NF-κB, AP1, CRE, and NF-IL6. These transcription factors regulate the expression of a wide variety of target genes. Intermediate kinases shown are either established (boxes) or tentative (question marks). TRAF2 and TRAF6 activate both unique and overlapping kinase cascades. TRAF3 inhibits TRAF2-mediated signals, while TRAF1 cooperates with TRAF2 to activate JNK and NFκB. See text for details.

activation is absolutely dependent upon TRAF2,[56] but subsequent studies of TRAF-deficient mice and B lymphocytes demonstrated that a lack of TRAF2 reduces, but does not eliminate, CD40-mediated NF-κB activity.[64-69] Additionally, there is strong evidence that CD40 binding to TRAFs, and downstream functional roles, are likely to be cell-type specific. A point mutation in the TRAF binding motif PXQXT in CD40's cytoplasmic (CY) domain substantially reduces TRAF3 binding to CD40 in the transformed epithelial cell line 293, but TRAF3 binds normally to this CD40 mutant in B lymphocytes.[70] Thus, this approach cannot be reliably used to discover the normal associations and functions of TRAFs associated with CD40 in cells of the immune system that express CD40.

It has been shown that removal of the Zn-binding domains of TRAFs 2, 3, and 6 create a TRAF that can associate with CD40, but no longer initiate downstream signals, and so function as a "dominant negative" molecule. CD40 functions altered by the expression of such mutants can provide hints about the biologic roles of their normal WT TRAF counterparts in CD40 signaling.[56,58,71,72] However, as illustrated in Figure 1, unambiguous data interpretation is not possible at least for TRAFs 2 and 3, because DN versions of each can also alter the association of other TRAFs with CD40. An alternative is to create mutants in the CY domain of CD40 itself, and correlate their

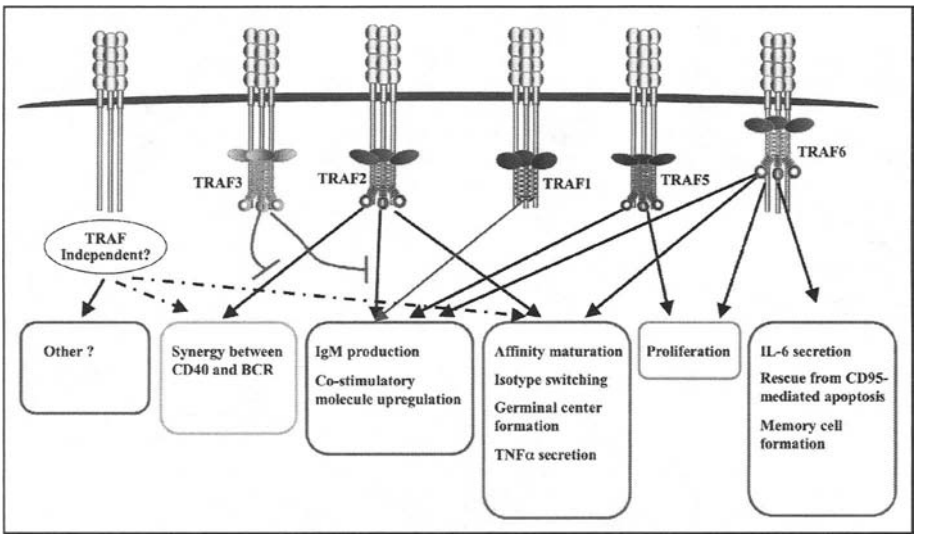

Figure 2. TRAF roles in CD40-mediated effector functions in B lymphocytes. CD40 engagement triggers a variety of events critical to an efficient humoral immune response. B cell proliferation is initiated, surface molecules involved in antigen presentation and T cell interaction are upregulated, antibodies and inflammatory cytokines are produced, and developmental programs leading to isotype switching and B cell memory are initiated. TRAF2, TRAF5 and TRAF6 mediate distinct but overlapping effector functions of CD40. TRAF1 cooperates with TRAF2 to induce IgM secretion in response to CD40 signals, while TRAF3 inhibits the synergy between CD40 and B cell antigen receptor (BCR), and other TRAF2-dependent signals.

function with their TRAF-binding properties. This approach has been successfully applied in both cell lines and transgenic mice, and has yielded considerable information about the relationship between TRAF association and CD40 functions.[65,72-80] When using this approach, it is important to verify that the CD40 mutant used actually has the TRAF binding properties expected, in the cell types being analyzed. The discussion above emphasizes that results obtained using in vitro approaches or binding data from disparate cell types and/or overexpression studies cannot be assumed to be universally valid. While this caveat can be overcome by careful experimental design, another limitation cannot; the possibility that any mutation made in CD40 alters not only the verifiable binding of specific TRAFs, but also the binding of additional unknown molecules. This is a limitation of all receptor mutant approaches and does not invalidate their usefulness, but should be considered in data interpretation.

Widespread application of the technology of gene targeting by homologous recombination has led to the creation of mice deficient in most known signaling proteins. The importance of many signal transduction proteins to multiple pathways involved in normal development and physiology, however, has resulted in early lethality of many of these strains. This has been the case for mice made deficient in TRAFs 2, 3, or 6,[81-83] limiting their usefulness in studies of receptor signaling in mature cell types expressing CD40. At the other end of the spectrum, "knockout" mice for a given signaling protein may have a very subtle or minimal phenotype, because the protein in question overlaps in function with other proteins, and compensation occurs, particularly when the deficiency is manifest from the earliest developmental stages. In this regard, TRAF1-deficient mice have few abnormalities, and their B cells can proliferate in response to CD40 signals;[84] further studies of CD40 function in these mice have not been reported. The great advantage of gene-targeted mice is that a single type of TRAF can be eliminated completely without disrupting the binding of other TRAFs (unless they require the missing TRAF to associate with CD40), and a range of physiologic functions can be examined in vivo. However, early lethality and developmental effects limit data interpretation for

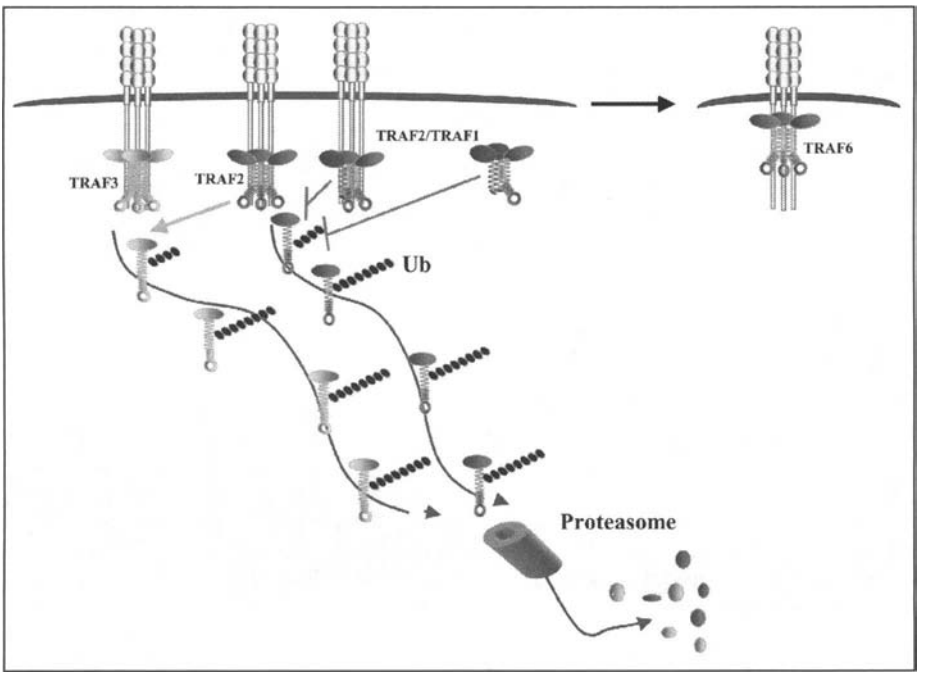

Figure 3. TRAF degradation induced by CD40 signaling. Ligation of CD40 leads to recruitment followed by rapid polyubiquitination of TRAF2 and TRAF3, which are then degraded via the proteasome. This polyubiquitination is mediated by the Zn domains of TRAF2. It is not clear if this is due to the E3 ubiquitin ligase activity of TRAF2 or TRAF2 recruitment of another E3 ubiquitin ligase. The removal of TRAF2 and TRAF3 could enhance the lower affinity binding of TRAF6 to CD40. TRAF1 is expressed at low levels in resting B cells, but is rapidly upregulated upon CD40 stimulation. TRAF1 can block the degradation of TRAF2 and TRAF3. This could be caused by competition with TRAF2 for CD40 binding, or by forming heterotrimers with TRAF2 that either block the degradation physically at CD40, Alternatively, TRAF1/TRAF2 heterotrimers could retain TRAF2 in membrane rafts, sequestered from CD40.

questions of CD40-TRAF interaction and function, and also precludes production of mice deficient in multiple members of the TRAF family. Advances in gene targeting techniques permit production of 'conditional' knockout mouse strains, in which a gene flanked by bacterial recombinase recognition sequences is removed from specific cell types in the targeted mouse by breeding with a strain expressing the recombinase behind a promoter specific for expression in the desired cell type(s).[85] This technology has recently been applied to the removal of the TRAF2 gene in mice.[68] Complete removal of TRAFs is difficult and dependent upon the efficiency of expression of the Cre transgene, and developmental effects are still a concern of this new technology. However, it holds great promise for gaining additional insights into TRAF function.

A complementary model that is considerably less time, cost, and labor-intensive than the production of conditional gene-targeted mice is a recently described approach of gene targeting by homologous recombination in somatic cell lines. The combination of various features of vector design have recently been applied to produce B lymphocyte cell lines specifically and completely deficient in TRAF2,[66] TRAF3,[86] TRAF1,[87] and TRAF6 (B. Hostager et al, manuscript in preparation). While such cell lines can never allow assessment of the variety of in vivo functions provided by whole animal models, they offer distinct advantages. All cells of the subclone are totally deficient in the desired TRAF, (which is difficult to achieve with inhibitory RNA transfection), but the cells reached a mature phenotype prior to TRAF removal, so their phenotype is independent of

developmental effects. Also in contrast to inhibitory RNA approaches, TRAF-deficient subclones can be transfected with the WT TRAF or specific TRAF mutants, which is very helpful for addressing questions of how TRAF structure relates to function. Additionally, multiple TRAF molecules can be removed from a single cell, to address questions of overlap and cooperation in function; this has recently been shown to occur for TRAFs 1 and 2.[87]

It is clear that no single approach to investigating how CD40 or other receptors use TRAF molecules is sufficient to obtain a complete understanding of this complex question, as each approach has both strengths and limitations. In the succeeding sections, we shall discuss how each of the known TRAF molecules is known to impact CD40 function, in the context of the approach(es) used to gain this information. Future unanswered questions will also be discussed.

Roles of TRAF Molecules in CD40 Function

TRAF2

Early studies into the functions of TRAF2 in the CD40 signal cascade used overexpressed TRAF2, dominant negative or mutant TRAF2, and/or CD40 mutants that disrupt TRAF binding. These studies indicated that TRAF2 is an adaptor protein with no kinase functions of its own, that links CD40 to downstream effector pathways, such as activation of JNK and NF-κB.[88] Although these techniques shed some light on the functions of TRAF2,[76] they were often difficult to interpret as TRAFs1, 2 and 3 have overlapping binding sites on CD40. Additionally, high level overexpression sometimes led to results that differed from what was seen studying TRAFs expressed at endogenous levels.[89] Another difficulty in understanding TRAF2 mediated signaling is the large variety of reported TRAF2 binding kinases, including germinal center kinases (GCK),[90] NF-κB inducing kinase (NIK),[91] PNK1,[92] apoptosis signal-regulating kinase 1 (ASK1),[93] sphingosine kinase,[94] and receptor interacting protein (RIP)[88] (Fig. 1). Unfortunately, due to techniques, reagents, and cell type differences these findings are often difficult to reproduce in response to CD40 stimulation in B cells. Therefore, it is still unclear which kinases TRAF2 utilizes to mediate signaling in response to CD40 in B cells. To better understand what CD40 signals TRAF2 mediates in hematopoietic cells and how, TRAF2 deficient mice and cell lines were created.

TRAF2$^{-/-}$ mice were created in order to understand the physiologic role of TRAF2 in vivo. These mice have very low viability, with only a small number of TRAF2$^{-/-}$ live births.[82] Those TRAF2$^{-/-}$ mice that do survive show increasing runting over time and early lethality with only a small percentage living past 3 weeks. These mice are lymphopenic and fail to develop secondary germinal centers, although there is no obvious block in B and T cell development. This phenotype is partially due to TNF toxicity as TRAF2$^{-/-}$/TNFRI$^{-/-}$ mice show increased viability, although the doubly-deficient mice have defective isotype switching, CD40 induced proliferation, and NF-κB activation.[95] However, the phenotype of TRAF2$^{-/-}$/TNFRI$^{-/-}$ mice is difficult to interpret, as CD40 induces TNFα production[96] and TNFRI uses TRAF2 to mediate some downstream signals.[97] Signaling studies in TRAF2$^{-/-}$ embryonic fibroblasts revealed a defect in JNK activation and delayed NF-κB activation in response to TNFα treatment.[82] These in vivo studies expanded but did not complete the understanding of TRAF2 in CD40 signaling.

To avoid the lethality problems of TRAF2$^{-/-}$ mice, subsequent studies used mice transgenic for the TRAF binding mutant CD40T234A, which had been reported to fail to bind TRAF2 or TRAF3.[76] However, several studies of mice transgenic for mutant CD40 molecules obtained divergent findings about the roles of TRAF2 in CD40 function.[78-80] In addition to differences in transgene expression and structure in the different mouse strains, it was also found that the CD40 T234A mutant can bind normal amounts of TRAF3 at the PxQxA mutant motif and small amounts of TRAF2 through a second, lower affinity TRAF2 binding site on CD40.[70,98] A new complementary approach to understanding TRAF2 mediated CD40 signaling came with the development of TRAF2$^{-/-}$ B cell lines.[66] These B-cells deficient in TRAF2 show a defect in phosphorylation and subsequent degradation of the inhibitory IκBα subunit of NF-κB in response to CD40. However, this defect is not complete, as clearly detectable phosphorylation and degradation of NF-κB does occur. It

appears that the binding of both TRAF6 and TRAF1 to CD40 also contribute to NF-κB1 activation.[66,87] TRAF2 deficient B-cells show a marked defect in CD40 induced Rel-B and p52 nuclear translocation,[67] hallmarks of the NF-κB2 pathway. TRAF2 also plays an important, albeit indirect, role in CD40-mediated IgM secretion, principally through its interaction with TNFR2/CD120b.[67,96] The most striking defects in TRAF2 deficient B cells are the inability to activate JNK or induce TRAF3 degradation in response to agonistic anti-CD40 mAb[66] (Figs. 1, 3).

A recent development in understanding CD40-TRAF2 interactions came with the creation of mice conditionally deficient in TRAF2 in B cells.[68] These mice have do not have the gross morphological defects and reduced survival of $TRAF2^{-/-}$ mice,[68] allowing considerably more questions to be addressed. The $TRAF2^{-/-}$ B cells in these mice show increased survival, and there is selective expansion of marginal zone B (MZB) cells. The TRAF2-deficient B cells have defective CD40-induced proliferation, and increased c-Rel expression.[68] In agreement with findings using TRAF2 deficient B cell lines, $TRAF^{-/-}$ B cells from these mice have high levels of constitutive NF-κB2 activity, decreased CD40-induced NF-κB1 activity, and defective CD40-induced TRAF3 degradation.[66,68]

Most signaling activity ascribed to TRAF2 is mapped to its Zn-binding domains[88] which consist of a Zn RING domain, that has been reported to act as an E3 ubiquitin ligase,[99] and five Zn FINGER domains, that most likely mediate protein-protein interactions. Based on recent reports and our own observations, it seems likely that TRAF2 potentially undergoes several ubiquitination events that affect its signaling.[100-102] Ubiquitination unrelated to degradation includes a monoubiquitination event[102] and a K63 linkage mediated by its own Zn RING that is required for downstream activation of JNK but not IκBα. There is also a degradation-promoting K48 ubiquitin linkage mediated by c-IAP1, c-IAP2, or another E3 ubiquitin ligase.[100] These ubiquitination events may be independent or linked. TRAF2 appears to control not only K63 linkages of addition of ubiquitin to itself but also to other proteins, such as germinal center kinase related kinase (GCKR).[101] TRAF2 is also implicated in mediating the degradation of receptor interacting protein (RIP),[103] although this may be due to the recruitment of another E3 ubiquitin ligase, such as c-IAP1or c-IAP2.[104] Further work is needed to determine the true role(s) of TRAF2 ubiquitination in CD40 signaling.

One of the most interesting aspects of TRAF2 is its ability to mediate rapid ubiquitination and proteasome-mediated degradation of both itself and TRAF3, following CD40 stimulation of B cells[66,100] (Fig. 3). This degradation requires the presence of intact Zn RING and FINGER domains of TRAF2, although it is not clear if this is directly mediated by TRAF2 or by a TRAF2-associated protein.[66,100,105] The degradation of TRAF2 alters the CD40 signal cascade by terminating JNK activation, delaying IκBα degradation and increasing ERK1/2 phosphorylation in response to a second CD40 signal.[100,105] Blocking TRAF2 degradation in response to CD40 increases the JNK response in B cells.[100,105] Additionally, TRAF3 binding to CD40 blocks CD40-BCR synergy,[106,107] so TRAF2-mediated TRAF3 degradation is important in the successful cooperation between CD40 and the BCR. CD40 clearly uses TRAF2 both to induce signaling cascades, and in their regulation.

TRAF1

TRAFs 1 and 2 were the first two TRAF molecules described, and were found to interact when associating with the 75 kDa TNF receptor, CD120b/TNFR2.[55] TRAF2 binds CD120b directly, while TRAF1 associates primarily via heterotrimerization with TRAF2.[55] Similarly, most of the TRAF1 that associates with CD40 does so indirectly through formation of mixed oligomers with TRAF2.[76] However, we have noticed that in B cells lacking TRAF2, a small amount of TRAF1 still associates with CD40,[87] which may indicate direct binding or the ability to associate with other CD40-binding proteins. Whether TRAF1 associates directly or indirectly with CD40, its binding site overlaps with that of TRAF2[76] (Figs. 1-3), creating the potential for inter-TRAF interactions, either cooperative or competitive. In dendritic cells, evidence suggests that TRAF1 may promote enhancement of TRAF2-mediated CD40 signals by prolonging membrane localization of TRAF2.[108] Our recent findings on the roles played by TRAF1 in B cells are consistent with this positive and cooperative role for TRAF1 with TRAF2 in CD40 signaling. $TRAF1^{-/-}$ B cell lines show decreases in CD40-mediated

IgM production, and TRAF1$^{-/-}$ TRAF2$^{-/-}$ B cells show functional compromise in NF-κB activation, JNK activation, and IgM production much more marked than seen in cells deficient in either TRAF alone. Further, TRAF1 inhibits the CD40-induced degradation of TRAF2[87] (Figs. 2, 3).

TRAF3

TRAF3 was first identified as a protein associated with CD40 by three studies with the yeast two-hybrid system using the CD40 cytoplasmic tail as bait.[53,54,109] Another independent study identified the same protein as a factor that binds to the cytoplasmic domain of latent membrane protein 1 (LMP1), the oncoprotein encoded by Epstein-Barr virus (EBV).[110] This unexpected finding provided the first connection between the signal transduction pathways of CD40 and LMP1. Subsequently, it was found that TRAF3 also interacts with a number of other receptors of the TNF-R superfamily, including CD27, CD30, lymphotoxin-β receptor, OX40, 4-1BB, RANK, HVEM, GITR, EDAR, XEDAR, BCMA, TACI, and BAFF-R.[111-114]

TRAF3 is expressed ubiquitously in various tissues and cell types.[111,115,116] Initial studies overexpressed wild type (WT) or dominant negative mutants (DN) of TRAF3 to explore the functional roles of TRAF3. Unlike TRAF2, 5, or 6, overexpression of TRAF3 alone does not activate NF-κB or JNK in the model system of 293T epithelial cells. However, cooverexpression of TRAF3 with TRAF5 enhances TRAF5-mediated activation of NF-κB in 293T cells.[117-120] Overexpression of either WT (full-length) or DN (truncated) TRAF3 inhibits CD40-mediated antibody secretion in B cells, and this inhibitory effect of TRAF3 is dependent on an intact TRAF3 binding site on CD40.[71] Overexpression of a DN TRAF3 suppresses CD40-induced production of reactive oxygen species (ROS) in WEHI 231 B cells.[121] Similarly, overexpression of either WT or DN TRAF3 inhibits LMP1-mediated NF-κB and JNK activation in 293T cells and B cell lines.[122-124] The Ramos B cell line stably transfected with a DN TRAF3 expressed at normal to below-normal levels shows decreased CD40-mediated activation of JNK and p38, cytokine secretion and Ig production.[125] However, interpretation of all these data is complicated by the fact that the binding sites for TRAFs 1, 2, 3 and 5 on CD40 or LMP1 overlap.[115,122,126] It was found that TRAF5 cannot directly bind to CD40, but may associate with CD40 through heterotrimerization with TRAF3.[119] Thus, the inhibitory effect of exogenous expression in cells of either WT or DN TRAF3 may result from an inhibition of the association of other TRAF molecules (TRAFs 1, 2, or 5) with CD40 or LMP1. Interestingly, mRNA expression of several splice deletion variants of TRAF3, generated through mRNA alternative splicing, have been identified in human T and B cell lines, and exogenous overexpression of some of these splice variants induces NF-κB activation in 293T cells and BJAB B cells.[127,128] However, whether and how these TRAF3 splice variants participate in CD40 and LMP1 signaling is unclear, as their expression at the protein level has not been demonstrated. Use of antisense oligodeoxynucleotide (ODN) specific for TRAF molecules to inhibit the protein expression of TRAFs, showed that antisense ODN for TRAF3 decreases CD40-mediated activation of MEK1-ERK and enhancement of IL-4-driven germline Cε transcription in DG75 B cells.[129]

A complementary approach employed to study TRAF function was the generation of CD40 or LMP1 mutants, in which TRAF binding sites on CD40 or LMP1 were mutated or deleted.[89,119,122] However, because the binding sites for TRAFs 1, 2, 3 and 5 on CD40 or LMP1 closely overlap, CD40 or LMP1 mutants specifically defective only in TRAF3 binding could not be created.[89,119,122] Thus, none of the signaling defects observed with CD40 or LMP1 mutants can be specifically assigned to TRAF3.

The physiological role of TRAF3 was investigated by generation of TRAF3$^{-/-}$ mice.[81] TRAF3$^{-/-}$ mice die by day 10 after birth with severe progressive runting and massive loss of splenic cellularity. Histologically, however, the structure of the spleen or thymus of TRAF3$^{-/-}$ mice appears normal. TRAF3$^{-/-}$ mice also display a progressive depletion of all lineages of white cells in the periphery, a decrease in CD4^{+}CD8^{+} double positive T cells in the thymus, and a reduced percentage of B220^{+}IgM^{-} B lineage precursor cells in the bone marrow. Fetal liver cells from day 14 TRAF3$^{-/-}$ embryos can reconstitute T cell, B cell, granulocytic, and erythroid lineages in lethally irradiated mice. However, these TRAF3$^{-/-}$ reconstituted mice show a partial reduction of B lineage precursors in the bone

marrow, suggesting that TRAF3 may play a role in B cell development. TRAF3$^{-/-}$ B cells purified from spleens of these chimeric mice upregulate CD23 and CD80 in response to CD154 in vitro.[81] Interestingly, the immune response to a T-dependent antigen is defective in the TRAF3$^{-/-}$ reconstituted mice, although in vitro B cell responses to CD40 were not decreased.[81]

The early lethality of TRAF3$^{-/-}$ mice limited their use as a model to delineate the detailed functional roles of TRAF3 in signaling by CD40 and LMP1. To circumvent this limitation, two TRAF3$^{-/-}$ mouse B cell lines were recently generated by employing a novel somatic cell gene targeting strategy through homologous recombination.[86] Characterization of these TRAF3$^{-/-}$ B cells revealed that CD40-induced JNK activation and antibody secretion are enhanced in the absence of TRAF3. CD40-mediated activation p38, ERK, Akt and NF-κB, upregulation of surface molecules, and secretion of cytokines are intact in TRAF3$^{-/-}$ B cells (Fig. 2). Interestingly, the amount of TRAF2 that associates with CD40 in membrane rafts is increased in the absence of TRAF3, suggesting that TRAF3 may exert its inhibitory effects on CD40 signaling by competing with TRAF2 for CD40 association.[86] Another possibility is that TRAF3 may form heterotrimers with TRAF2 and thus inhibit its ability to transduce CD40 signals.[130] In sharp contrast, LMP1 signaling is defective in TRAF3$^{-/-}$ B cells.[86] LMP1-induced activation of JNK, p38 and NFκB, upregulation of CD23 and CD80, as well as antibody secretion induced by LMP1 are severely impaired by TRAF3 deficiency. The association between TRAF2 and LMP1 is unaffected by the absence of TRAF3. As in WT B cells, TRAF2 does not undergo degradation in response to LMP1 signaling. In addition, LMP1-mediated signaling events are normal in TRAF2$^{-/-}$ B cells.[86] Thus, the functional defects in LMP1 signaling observed in TRAF3$^{-/-}$ B cells are directly related to the loss of TRAF3. Reconstitution of TRAF3 expression decreases CD40-induced JNK activation and antibody secretion, and restores LMP1 signaling.[86] Further analyses of TRAF3$^{-/-}$ B cells revealed that TRAF3 mediates LMP1 signaling both through direct interactions with the carboxyl-terminal activating region 1 (CTAR1) of LMP1 and through indirect interactions with the CTAR2 region of LMP1 in B cells.[131] Taken together, these findings indicate that although CD40 and LMP1 provide highly similar signals to B cells, they utilize TRAF3 in remarkably different ways.

A negative role for TRAF3 in the synergy between CD40 and the B cell antigen receptor (BCR) has been described.[70,107] Interestingly, it was found that TRAF2 plays a positive role in such synergy and functions to block TRAF3 from exerting its negative effects, and TRAF2 is not required for CD40-BCR synergy in the absence of TRAF3.[70,107] BCR-induced activation of protein kinase C-μ and Bruton's tyrosine kinase (Btk) are also required for the synergy between CD40 and BCR but only if TRAF3 binds CD40. This suggests that these kinases are required for the as yet unidentified modification of TRAF2 that is necessary for counteracting the negative effects of TRAF3.[106,107] In marked contrast, TRAF3 may play a positive role in the synergy between CD40 and LMP1, as suggested by the finding that TRAF3 deficiency dramatically affects the synergistic effects of CD40 and LMP1 on activation of JNK and NF-κB, as well as Ig secretion in B cells[86] (Figs. 1, 2).

In addition to B cells, other cell types, including dendritic cells, monocytes/macrophages, activated T cells, eosinophils, epithelial cells and endothelial cells, also express both CD40 and TRAF3.[111,115,116,132] Although little is known about the functional roles of endogenous TRAF3 in CD40 signaling in cell types other than B cells, available information suggests that TRAF3 may have cell type-specific functions (Table 1). In airway epithelial cells, overexpression of WT TRAF3 enhances CD40-mediated NF-κB activation and expression of the chemokine RANTES, while overexpression of DN TRAF3 inhibits these events. This suggests that, in contrast to B cells, TRAF3 is required for CD40-mediated NF-κB activation in certain epithelia.[133] In vascular endothelial cells, shear stress specifically upregulates TRAF3 mRNA and protein expression.[134] Overexpression of TRAF3 in endothelial cells prevents CD40-induced activation of the transcription factor AP-1, and expression of proinflammatory cytokines and tissue factor.[134] Interestingly, it was found that in the absence of receptor engagement, TRAF3 preferentially localizes in the nucleus in endothelial cells as determined by both immunohistochemistry and biochemical fractionation,[134] which is markedly different from the predominant cytoplasmic localization of TRAF3 in B cells, T cells, NIH 3T3 fibroblasts and HeLa epithelial cells.[134-137] Consistent with this finding, TRAF3 does not bind to

Table 1. TRAF functions in CD40 signaling

TRAF	B Lymphocyte	Monocyte/Macrophage	Dendritic Cell	Non-Immune Cell Types
TRAF1	↓TRAF2 degradation Cooperates with TRAF2 to enhance JNK and NF-κB activation, and IgM production	Unclear	Prolongs CD40 signals ↑Duration of TRAF2 at membrane	↓NF-κB activation (kidney epithelium)
TRAF2	↑JNK activation; other kinases ↑NF-κB1 and 2 activation ↑Costimulatory molecule upregulation Induces TRAF3 degradation Blocks negative effect of TRAF3 on CD40-BCR synergy	Unclear	Unclear	↑JNK activation; other kinases ↑NF-κB1 and 2 activation (kidney epithelium, fibroblasts)
TRAF3	↓JNK activation ↓IgM production ↓CD40-BCR synergy ↓Costimulatory molecule upregulation	Unclear	Unclear	↑NF-κB activation, RANTES production (lung epithelium) ↓NF-κB activation (kidney epithelium) ↓AP-1 activation, cytokine production (vascular endothelium)
TRAF5	↑Costimulatory molecule upregulation, Ig production, proliferation	Unclear	Unclear	↑NF-κB2 activation (kidney epithelium)
TRAF6	↑JNK, ERK activation ↑NF-κB activation ↑IL-6 production ↑Isotype switching, GC formation ↑Costimulatory molecule upregulation	↑ERK activation ↑NF-κB activation ↑TNF, IL-6 production	↑Surface MHC class II ↑IL-6, IL-12 production	↑JNK, ERK activation ↑NF-κB activation (kidney epithelium)

CD40 in these endothelial cells. Thus, TRAF3 may exert its inhibitory roles on CD40 signaling in endothelial cells through a mechanism very different from that observed in B cells.[86,134] Furthermore, TRAF3 is cleaved by caspases during CD95- or CD3- triggered apoptosis in Jurkat T cells.[137] Although the potential function of the cleavage products of TRAF3 remains to be determined, it was found that the amino-terminal fragment of TRAF3 shows a different intracellular localization from the full-length TRAF3, with preferential distribution to particulate fractions and the nucleus.[137] Collectively, these findings suggest that TRAF3 function may be cell type-specific. Future efforts should be directed to better understanding the role of TRAF3 in different physiological contexts, and tissue-specific or cell lineage-specific TRAF3 knockout mouse models using the Cre/*lox*P system will be required to serve as powerful analytical tools to achieve this goal.

How TRAF3 negatively regulates CD40 signaling but positively transduces LMP1 signaling is another important area for future research. Although TRAF3 has a domain structure similar to that of TRAF2, no E3 ubiquitin ligase activity of TRAF3 has been described. CD40- or BAFF- induced degradation of TRAF3 is dependent on the presence of WT TRAF2.[66,68,100,138] A variety of intra-cellular proteins have been suggested to interact with TRAF3, including Act1, MIP-T3 (microtubule interacting protein that associates with TRAF3), p62 nucleoporin, T3-JAM (TRAF3-interacting JNK-activating modulator), RIP, RIP4, c-Src, NIK, ASK1, p85 of PI-3K, $p40^{phox}$, TANK (TRAF-associated NFκB activator), TNAP (TRAFs and NIK-associated protein), and TTRAP[111,121,139-145] (Fig. 1). Most of these proteins were originally identified by yeast 2-hybrid analysis using TRAF3 as bait, and the interactions were subsequently verified by coimmunoprecipitation assays using highly overexpressed proteins in 293T epithelial cells. Among the 6 characterized TRAF molecules, only TRAF3 specifically interacts with $p40^{phox}$, p62 nucleoporin, MIP-T3, and T3JAM.[121,140-142] In contrast, NIK, ASK1, c-Src, RIP, RIP4, TANK, TNAP and TTRAP are able to interact with multiple TRAF molecules in yeast 2-hybrid or in protein overexpression analysis in 293T cells.[91,93,143-149] For example, ASK1 coimmunoprecipitates with TRAF1, 2, 3, 5, or 6 when both are overexpressed in 293T cells.[93] Although TNFα-induced ASK1 activation is impaired in $TRAF2^{-/-}$ mouse embryonic fibroblasts (MEF), LIGHT-induced activation of ASK1 is diminished in $TRAF3^{-/-}$ or $TRAF5^{-/-}$ but not $TRAF2^{-/-}$ MEF, suggesting that ASK1 activation induced by different receptors may be dependent on different TRAF molecules.[150] However, for most of the above TRAF3-interacting proteins, it remains to be determined whether their interactions with TRAF3 can occur between endogenous proteins in physiologically relevant cell types, and whether the endogenous interactions are constitutive or only inducible upon receptor engagement. Notably, the interaction between endogenous Act1 and TRAF3 has been demonstrated in human IM9 B cells only upon stimulation with CD154 or BAFF.[151] $Act1^{-/-}$ mice exhibit a dramatic increase in peripheral B cells, which culminates in lymphadenopathy and splenomegaly, hypergammaglobulinemia, and autoantibodies. This pathology is greatly diminished in CD40-Act1 and BAFF-Act1 double knockout mice, suggesting that Act1 plays negative roles in both CD40- and BAFF- mediated B cell activation or survival.[151] In light of the evidence that TRAF3 is a negative regulator of CD40 signaling in B cells,[86,106,107] the physiological significance and molecular mechanisms of Act1/TRAF3 interaction in B cell activation and survival warrants further investigation. Furthermore, additional novel TRAF3-interacting proteins may be identified through new proteomic approaches.

TRAF5

The role of TRAF5 in CD40 mediated signal transduction remains controversial. Early studies in 293T and Jurkat cell lines demonstrated that TRAF5 could bind to CD40 in in vitro fusion protein assays and coimmunoprecipitation.[59,152] Other groups found that TRAF5 was capable of binding the TNF receptor family member LT-βR, but not CD40 in coimmunoprecipitation experiments in COS7 cells.[60] Studies in B cell lines showed that stimulation of CD40 results in TRAF5 movement into lipid rafts, however, coimmunoprecipitation assays have been unable to confirm TRAF5-CD40 interactions (Xie and Bishop, unpublished data). It remains unclear whether TRAF5 can bind directly to CD40 or is recruited to CD40 indirectly through interactions with other TRAFs. TRAF5 has been shown to heterotrimerize with TRAF3 by fusion protein association assays, coIP and FRET, and may be responsible for bringing TRAF5 to the receptor complex.[57,130,153] Alterna-

tively, CD40 may utilize TRAF5 as a cytoplasmic protein, without requiring CD40-TRAF5 physical association for TRAF5 to participate in CD40 signaling (Figs. 1, 2, Table 1).

Early studies in the mouse B cell line WEHI-231 showed that disruption of the N-terminal domain of TRAF5 caused defective CD23 surface expression in response to CD40 stimulation.[59] However, the effect of this truncated TRAF5 on NF-κB and JNK activation was not investigated. Decrease of TRAF5 expression by siRNA reducesCD40-induced nuclear levels of p52 and p65, suggesting that TRAF5 may play a role in CD40 mediated activation of NF-κB1 and NF-κB2 pathways.[154] Studies of TRAF5 deficient mice demonstrated that TRAF5 deficient B cells have partially decreased costimulatory molecule expression, immunoglobulin production, and proliferation in response to CD40 stimulation.[155] Many reported functions of TRAF5 are shared by TRAF2, giving rise to the notion that the role of TRAF5 in CD40 signaling is a redundant one. Data from the TRAF5 knockout mice suggest that TRAF5 is necessary for CD40 to optimally activate signal transduction pathways. While TRAF5 and TRAF2 appear to share many properties, there is growing evidence that TRAF function is context-dependent (reviewed in ref. 89). This may be due to the differential recruitment of other proteins including TRAFs to particular receptors. Further investigation is needed to determine the role(s) and importance of TRAF5 in CD40 signaling.

TRAF6

The interaction of TRAF6 with CD40 was first proposed on the basis of yeast-two hybrid experiments performed using the CY tail of human CD40 as bait.[58] These studies identified a 15 amino acid region that contained the binding site for TRAF6, a site that was more membrane proximal and distinct from the previously identified region implicated in CD40's interaction with TRAFs 1,2,3, and 5. The binding site was further characterized in vitro, using peptide interactions with TRAF6 produced in insect cells to identify QEPQEINF in human CD40 as the TRAF6 binding site.[156] On the basis of comparison to RANK, IRAK1 and IRAK2, the putative consensus TRAF6 binding site was refined to be: basic-QXPXEX-acidic.[157] In mouse CD40 the analogous TRAF6 binding site is RQDPQEME.[158]

CD40 ligation initiates multiple signal cascades. Some absolutely require TRAF6, TRAF6 is a partial contributor to others, and some signal pathways are TRAF6 independent (Figs. 1, 2). Because CD40 is expressed on a number of different cell types, the signaling pathways are likely to differ in part, depending on the system described. Initial studies exploring the requirement for TRAF6 in CD40 signaling were performed in 293T kidney epithelial cells or Jurkat T cells using transfected CD40 and TRAF molecules, employing CD40 molecules with mutated TRAF binding sites as well as TRAF6 molecules mutated in the Zn RING domain ("dominant negative", DN).[58,117,152,153,159] The results supported roles for TRAF6 in promoting CD40-mediated NF-κB, JNK and ERK activation. A DN form of the serine-threonine kinase NIK had little effect on TRAF6-dependent CD40-induced NF-κB reporter gene activity, while TRAF2-dependent NF-κB reporter gene activity was more easily inhibited, suggesting that there are different NF-κB activation pathways mediated by the different TRAFs.[152] The NF-κB2 pathway was also studied using CD40 mutants overexpressed in 293T cells. The results in this model system suggest that NF-κB2 activation by CD40 is TRAF6 independent.[160,161] Recently, a role for the serine/threonine protein kinase Tpl2 has been demonstrated in TRAF6-dependent CD40 stimulation of ERK, in mouse embryo fibroblasts and keratinocytes[162] (Fig. 1).

Findings in 293T cells contrasted with B cell studies, in which little TRAF6-dependent NF-κB activation was detected by gel mobility shift or reporter gene assays in response to signals through CD40.[65,72,163] In contrast to the TRAF6-dependent CD40 pathway initially described in 293T cells,[159] ERK1 and 2 are not activated in human B cells after CD40 signaling,[164,165] though mouse splenic B cells activate ERK downstream of CD40.[162] TRAF6 binding to CD40 appears unnecessary for JNK activation,[66,72,158,163] and is not required for IkBα or p38 phosphorylation, although there is a partial decrease in these events if TRAF6 binding is inhibited.[158,163] TRAF6 has been shown to function as an E3-ubiquitin ligase in a number of systems.[99,166] Interestingly, while TRAFs 2 and 3 become ubiquitinated and degraded after CD40 ligation in B cells,[138] TRAF6 does not[105] (Fig. 3).

There are problems in the interpretation of studies that use CD40 molecules with mutations of the TRAF1,2,3,5 binding site to draw conclusions about the requirement for TRAF6 in many of these pathways. Although the commonly used T235A mutation binds almost no TRAF2 or TRAF3 when overexpressed in epithelial cells, or used in in vitro binding assays,[76] this mutant binds detectable amounts of TRAF2 and normal levels of TRAF3 when expressed at normal levels in B cells.[70] Thus, in many studies it has been assumed that B cell signaling carried out by this molecule must be initiated by TRAF6, but the participation of TRAF3 in such signals cannot be formally excluded. The requirement for CD40-TRAF6 binding in B cell NF-κB activation was clarified in experiments in which CD40 molecules with mutated TRAF6 binding sites were expressed in B cell lines deficient in TRAF2. These studies established that TRAF2 and TRAF6 can each activate NF-κB in response to CD40 signaling, but if neither TRAFs 2 nor 6 can bind CD40, the NF-κB response is abrogated[66] (Figs. 1, 2).

CD40 signaling is involved in a number of distal events that can only be studied in relevant cell types, including the up-regulation of costimulatory molecules, immunoglobulin class switching, and cytokine production. CD40 dependent antibody secretion in the mouse B cell line CH12.LX was shown to be dependent upon TRAF6 binding,[66,71,72,75] as was CD40 stimulated IL-6 secretion in B cells.[22,72] Studies of costimulatory molecule upregulation through CD40 stimulation suggest that CD80 upregulation is partially dependent on TRAF6 binding,[72,163] and in the absence of TRAF2, TRAF6 binding is required for this response.[66] TRAF6 is also implicated in CD40 dependent activation of the IgCγ1 and IgCε1 promoters[167] (Fig. 2).

Studies of the TRAF6 dependence of distal CD40-driven events were extended by three groups who produced mice transgenic for CD40 molecules, with or without TRAF binding site mutations. Details of transgene design differed: in one study, human CD40 and mutants were used,[78] in the second, chimeric molecules with human extracellular domains and mouse intracellular domains were used,[168] and in the third study, full length mouse CD40 was used.[158] The CD40 binding site mutations differed as well. Yasui, et al used deletions of the TRAF1,2,3,5 binding site, or both TRAF6 and TRAF1,2,3,5 sites, and also examined transgenes with the PXQXT→A mutation in the TRAF1,2,3,5 binding site, and a CD40 construct containing only the TRAF1,2,3,5 binding site.[78] Ahonen, et al used point mutations of the TRAF1,2,3,5 and TRAF6 binding sites either alone or in combination.[168] Jabara, et al used a deletion of the TRAF6 site with or without the PXQXT→A point mutation of the TRAF1,2,3,5 site.[158] In all cases the transgenic mice were bred to CD40 deficient mice. Given the caveat noted above, about the binding properties of the CD40 PXQXT→A mutant in B cells, the Yasui experiments may allow the most accurate testing of the role of TRAF6 binding to CD40, in the complete absence of TRAFs that bind at the PXQXT site.[78] These results indicate that TRAF6 binding alone is sufficient to drive B cell proliferation in response to CD154, and IgM and IgG1 production in response to CD154 + IL-4. CD40 molecules able to bind only TRAF6 can stimulate upregulation of a number of surface molecules, but are somewhat defective in promoting T-dependent antigen-specific IgG1 responses. B cell responses in these mice show affinity maturation but GC formation is not normal.[78] Ahonen et al obtained similar results, but the mice expressing CD40 molecules that bind TRAF6, TRAF3, and a small amount of TRAF2 have better antigen specific responses, and form GC.[168] Jabara et al confirmed that TRAF6 binding is sufficient for GC formation, but they also saw an impaired IgG1 antigen-specific response, and defects in several molecular events in isotype switching, in mice expressing CD40 mutants which bound TRAF6 but had TRAF1,2,3,5 binding site mutations.[158] Results of all the studies showed that mutation or deletion of both TRAF6 and TRAF2,3,5 binding sites resulted in a CD40 molecule unable to effectively induce most distal events, consistent with a model of partial redundancy of TRAF2 and TRAF6 signals in CD40-mediated signaling.

Three groups have characterized TRAF6 deficient mice. These mice have a phenotype of poor viability and osteopetrosis due to the dependence of osteoclast development on signaling through RANK, a TRAF6 dependent process.[83,169,170] They also display a number of immune system anomalies, including thymic aplasia, splenomegaly, disorganized spleen structure, and lymph node deficiency.[83,169,170] The $CD4^+CD8^-$ DC population is missing from the spleens of $TRAF6^{-/-}$ mice.[170]

Splenic B cells purified from TRAF6$^{-/-}$ mice show profound defects in proliferation and NF-κB activation in response to anti-CD40 stimulation.[83] This result is seemingly inconsistent with the studies in B cell lines showing only a minor role for TRAF6 binding in NF-κB activation through CD40. However, the TRAF6$^{-/-}$ B cells may not be comparable to normal mature B cells, due to the young age of the mice studied (necessitated by their early lethality) and a reported defect in the normal production of peripheral B cells in at least one of these transgenic models.[169]

An alternative explanation for the profound effect of the absence of TRAF6 in B cells, versus the effects of preventing CD40-TRAF6 binding, could be that TRAF6 plays a role in CD40 signaling that is independent of receptor binding. TRAF6 overexpression can result in the activation of NF-κB and AP-1 without any apparent receptor engagement.[171-173] The expression of TRAF6 lacking the TRAF domain, and therefore unable to bind to CD40, can partially reconstitute CD80 upregulation in TRAF6$^{-/-}$ B cell lines (B. Hostager et al, manuscript in preparation). There is strong precedence for an important role of CY TRAF6. The innate immune Toll-like receptors and IL-1 receptor employ TRAF6 as an important component of their signaling pathways, but do not themselves directly bind TRAF6 (reviewed in ref. 174).

CD40 molecules with mutated TRAF binding sites have also been used to investigate TRAF dependence of proximal signaling in immortalized mouse macrophage lines.[175] Like 293T cells, but in contrast to B cells, ERK1,2 phosphorylation and NF-κB activation are completely dependent upon TRAF6 binding in macrophages. The production of inflammatory cytokines induced through CD40, including TNFα and IL-6, could be inhibited by the introduction of either a TRAF6 binding protein, or DN TRAF6.[175] DCs derived from TRAF6 knockout mice are unable to upregulate MHC class II in response to CD154, and are also impaired in their ability to produce IL-6 and IL-12[170] (Table 1).

CD40 ligation inhibits apoptosis of B cells. In the WEHI-231 mouse B cell line, cells treated with anti-Ig undergo death unless a rescue signal such as CD40 ligation is provided.[176-178] In this system, a CD40 molecule with an intact TRAF6 binding site but no TRAF 2,3,5 site was unable to rescue WEHI-231 from anti-Ig induced death, while the isolated TRAF1,2,3,5 site was sufficient to rescue the cells.[77,179] Our laboratory has found that CD40 can rescue mouse splenic B cells and B cell lines from anti-CD95 induced apoptosis. In this case, rescue through CD40 signaling is dependent upon TRAF6, as TRAF6$^{-/-}$ B cell lines cannot be rescued by CD40 signaling. However, CD40 rescue from CD95, in contrast to anti-Ig-induced apoptosis, does not require the binding of TRAFs 1, 2, or 3.[180] It is clear that TRAF6 plays an important and distinct role in CD40-mediated signal transduction. Future studies will clarify the reasons for differences in TRAF6 dependence of downstream pathways in different cell types. Given the unique status of TRAF6 in transduction of signals through TLR family members and IL-1 receptors, we expect that this TRAF may be important in integrating the responses to multiple cellular signals.

Conclusions

Multiple studies of TRAF functions in CD40 signaling to various cell types from mice and humans reveals that they have multiple roles, both unique and overlapping, as illustrated in Figures 1 and 2 and Table 1. Early effects include activation of kinases and transcription factors, and interactions with other signaling proteins. Downstream biological effects are many and diverse, depending upon the cell type studied. Functions common to many cell types include production of cytokines, upregulation of various surface receptors, and induction of a wide variety of genes. These various pathways can culminate in either the induction or inhibition of biological functions. The ultimate outcome depends upon the cell type expressing CD40, as well as other signals that the cell is receiving at any given time. Future questions include understanding exactly how CD40-TRAF signaling complexes assemble in different situations, precisely how TRAFs initiate various CD40-induced signaling cascades, and the influence of relative amounts and modifications of each TRAF in determining the outcome of CD40 signals. Bringing a variety of current and future approaches to bear upon these questions should yield new information about the varied ways in which CD40 uses the family of TRAF molecules.

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CHAPTER 12

TRAFs in RANK Signaling

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Abstract

Members of the tumor necrosis factor (TNF) family govern many diverse physiological and cellular responses including cellular proliferation, differentiation, and apoptosis. Ligands of this family interact through a distinct set of specific receptors that lack enzymatic activity and therefore are dependent on the association of adaptor molecules. One receptor/ligand pair known as receptor activator of nuclear factor-kappa B (RANK) and RANK ligand (RANKL) regulates bone remodeling, mammary gland development, and lymph node organogenesis. RANK interacts with five members of the TNF receptor-associated factor (TRAF) family, of which TRAF6 is indispensable for its signaling capability. An accumulation of evidence from various research laboratories indicates TRAFs, but more importantly TRAF6, is the key to understanding how RANKL links cytoplasmic signaling to the nuclear transcriptional program.

Introduction

Bone remodeling is a dynamic and continuing process of degradation of old bone by the resorption activity of the osteoclast and deposition of new bone by the osteoblast. The osteoclast is a fully differentiated, multi-nucleated cell originating from the hematopoietic monocyte-macrophage linage. The physiological importance of maintaining a balance of the osteoclast and osteoblast is underscored by diseases related to increased osteoclast activity such as postmenopausal osteoporosis, Paget's disease, rheumatoid arthritis, and tumor-induced osteolytic bone destruction.[1-4] Recent evidence has indicated that RANKL, a member of the tumor necrosis factor family, and its receptor RANK are essential regulators of osteoclast differentiation and activation.[1-4]

In mice, targeted disruption of the genes for *RANKL* or *RANK* leads to a severe defect in bone resorption due to the lack of multi-nucleated osteoclasts, which is indicated by severe osteopetrosis.[5-9] In contrast, mice lacking osteoprotegerin (OPG), a soluble decoy receptor for RANKL, develop severe osteoporosis. Thus, the regulation of differentiation and activation of this specialized cell by RANKL, OPG, and RANK emphasizes the physiological significance of these molecules in bone homeostasis. Therefore, the precise identification of the regulatory network controlled by the signaling of RANK is essential to understanding the molecular mechanism of osteoclast differentiation and may lead to the development of novel therapeutic agents to treat bone diseases. We understand that RANKL functions in other cellular and biological systems such as mammary gland development and dendritic-cell/T-cell communication, but most the signaling by RANKL and RANK has been uncovered in the context of the osteoclast. Thus, we will focus this review to signal transduction identified through the efforts of many investigators in the area of bone biology.

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

TRAF Interaction Motifs in the Cytoplasmic Domain of RANK

TRAFs constitute a family of seven known adaptor proteins and most of them participate in activation of the transcription factor NF-κB and members of the mitogen-activated protein (MAP) kinase family including MAPK, c-Jun N-terminal kinase (JNK), and p38.[10-12] Several TRAF proteins interact directly with the intracellular regions of various members of the TNF receptor family, including CD27, CD30, CD40, TNFR2, lymphotoxin beta-receptor, and the Herpes virus entry mediator. All TRAF proteins have a highly conserved motif at the C terminus, termed the TRAF domain, which mediates its interaction with the receptor. In contrast, the N-terminal domain of the TRAFs is less well conserved, but consists of Zn-finger motifs and in some TRAFs a RING (Really Interesting New Gene) domain, which has E3 ubiquitin ligase activity as discussed below.

Like most other members of the TNF receptor family transient expression of RANK in mammalian cells or stimulation of RANK expressing cells with RANKL leads to the activation of signaling pathways including NF-κB, JNK, p38, and MAPK. Since these pathways are most likely regulated by RANK interacting with TRAF adaptor molecules, various groups attempted to determine which TRAFs interact with RANK and which regions of RANK are responsible for binding to the TRAFs by using a variety of experimental approaches.[13-19] We first reported the interaction of TRAF2, TRAF5, and TRAF6 with RANK and demonstrated that RANK could activate both the NF-κB and JNK pathways.[14] Following our initial report others also demonstrated the interaction of TRAF2, TRAF5, and TRAF6 and additionally TRAF1 and TRAF3 with RANK.[16-19] Subsequently, through a detailed deletion analysis approach, we identified a novel TRAF6-binding motif in RANK that is distinct from that of the binding sites for TRAF2 and TRAF5.[15] In addition, an identical TRAF6-binding motif in CD40 was described using a combinatorial peptide library approach.[20] Taken together, the cytoplasmic domain of RANK could interact with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6, however, only TRAF2, TRAF5, and TRAF6 are functionally competent to activate signaling pathways.

Crystallographic analysis of TRAF6 in a complex with the TRAF6-binding peptide derived from either RANK or CD40[21] revealed the molecular basis for recognition of the TRAF6-binding peptide. Interestingly, these results suggested that TRAF6 recognizes a consensus motif consisting of Pro-Xaa-Glu-Xaa-Xaa-Ar/Ac (where Xaa represents any amino acid and Ar is an aromatic and Ac an acidic residue)[21] that was different from the known TRAF2 consensus motif of Pro/Ser/Thr/Ala-Xaa-Gln/Glu-Glu.[22,23] Furthermore, the structure of TRAF2 in complex with its binding peptide indicated that similar surface residues of TRAF2 are conserved in TRAF1, TRAF3, and TRAF5 implying that these TRAFs also recognize the same TRAF2 consensus binding domain.[23] Therefore, further inspection of the entire cytoplasmic domain of RANK indicated nine potential TRAF binding sites, six TRAF2-like binding sites (PTM1-6)[19,24] and three TRAF6-like binding sites (BSI-III)[21] (Fig. 1). Taken collectively with all of the published data, PTM5 and PTM6 most likely contribute to the interaction of TRAF2 and TRAF5 with RANK, while sites depicted by PTM1-4 presumably represent nonfunctional TRAF binding sites. Although not experimentally confirmed, TRAF1 and TRAF3 could potential interact with PTM5 and PTM6 because they also recognize the same motif as TRAF2. In contrast, all three TRAF6-binding sites in RANK appear to interact with TRAF6, but BSI seems to be the major site.

This structure based analysis of TRAF6 confirmed that there are distinct differences in peptide binding to TRAF6 and to the other TRAFs, which may provide the specificity of TRAF6 and its biological function. As stated above, inspection of the sequence of RANK indicated two more additional TRAF6-binding motifs denoted by BSII and BSIII (Fig. 1). While TRAF6 apparently binds to BSI with the highest affinity, TRAF6 also interacts with BSII and BSIII albeit with a 10-fold lower affinity.[21,25] The binding specificity of TRAF6 provided the means to determine whether the RANK-TRAF6 interaction is required for RANKL signaling. We employed a novel approach by constructing a cell-permeable TRAF6 decoy peptide (T6DP) derived from BSI and BSII to investigate the effects of blocking the RANK-TRAF6 interaction. Due to the difference in peptide binding to the TRAFs, we could specifically block the RANK-TRAF6 interaction without hindering the interaction of the other TRAFs to RANK. Results from these studies indicated

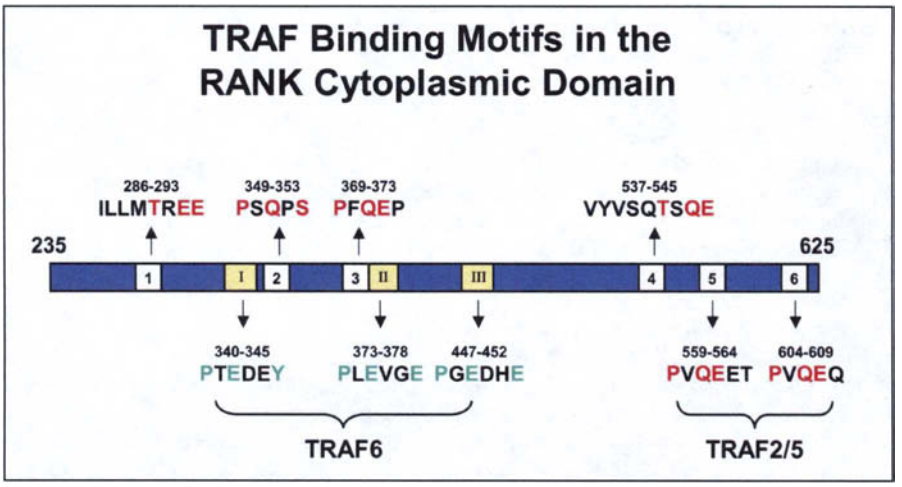

Figure 1. TRAF binding motifs in the RANK cytoplasmic domain. The cytoplasmic domain of mouse RANK from residues 235-625 is shown. The putative TRAF binding sites (PTM) indicated as numbered boxes 1-6 are identical to those described previously.[14,15,19,24] The TRAF6-binding sites indicated by roman numerals I-III are identical to those described previously.[15,21,25,26] The red and green letters indicate the consensus binding sites for TRAF2 and TRAF6, respectively.

that T6DPs significantly blocked RANKL-mediated activation of NF-κB, JNK, and osteoclast differentiation, however T6DP-I was more effective than T6DP-II.[21] In support of our hypothesis, Gohda et al[25] constructed a CD40-RANK chimeric receptor with different combinations of mutants in the three TRAF6-binding sites. Their data corroborated our hypothesis that TRAF6 binds with highest affinity to BSI and that this site alone is sufficient for osteoclast differentiation. Although BSIII is capable of binding to TRAF6, a mutant chimeric receptor having only intact BSIII failed to cause calcium oscillations and activation of nuclear factor of activated T cells (NFAT) c1, which are critical for osteoclast differentiation. Furthermore, this hypothesis was supported by a similar report indicating that at least two of the three TRAF6-binding sites have the potential to induce osteoclast differentiation.[26] So, why does RANK have more than one TRAF6-binding site? The occurrence of several TRAF6-binding sites may indicate a need of cooperation in order to amplify the TRAF6 signal more efficiently than having only a single TRAF6-binding site. This type of amplification of the TRAF6 signal appears to be required for efficient osteoclastogenesis and for the establishment of normal bone remodeling in vivo.

Whereas the significance of TRAF6 in RANK signaling is clear based on the phenotype of the TRAF6 deficient mice,[27,28] the functional significance of RANK interacting with TRAF1, TRAF2, TRAF3, and TRAF5 remains elusive. In order to potentially identify a function of these TRAFs in RANK signaling, we used a similar approach described earlier[21] with cell-permeable decoy peptides derived from the TRAF2 and TRAF5 binding sites in RANK. To date, we have failed to identify a function of TRAF2 and TRAF5 in RANK signaling by this approach, since incubation of these decoy peptides with cells appears not to interfere with any RANKL signaling pathway (A. T. P. and B. G. D., unpublished observations). Nonetheless, further studies are required to unravel the significance of RANK interacting with TRAF molecules other than TRAF6.

TRAF6—The Critical Adaptor for RANK Signaling

Many RING domain proteins have been shown to function as E3 ubiquitin ligases that mediate polyubiquitination of target proteins, which are subsequently degraded by the 26S proteasome.[29,30] The N-terminus of TRAF6 contains a conserved RING domain that is common to many ubiquitin

E3 ligases. Indeed, the copurification of a dimeric E2 ubiquitin-conjugating enzyme consisting of Ubc13 and Uev1A with TRAF6 provided the initial proof that TRAF6 may function as an E3 ubiquitin ligase.[31] Unlike most E3 ligases, the primary function of TRAF6 is not to target proteins for degradation, but to activate downstream kinase cascades. In fact, the TRAF6-Ubc13-Uev1A complex catalyses lysine 63 (Lys63)-linked polyubiquitin chains to mediate the activation of TGF-β-activated kinase 1 (TAK1), that further activates the MAPK kinase 6 (MKK6) and Inhibitor of NF-κB alpha (IκBα) kinase (IKK) complexes.[31,32] A single point mutation of the highly conserved cysteine residue in the RING domain of TRAF6 abolishes the ubiquitin-conjugating activity of TRAF6 and its ability to activate NF-κB, suggesting that the NF-κB-inducing activity of TRAF6 is linked to its ubiquitin-conjugating activity[31] (B. L. and B. G. D., unpublished observations). Notably, the auto-ubiquitination of TRAF6 via a Lys63-linked polyubiquitin chain mediates the recruitment of the Zn-finger domain of TAK1 binding protein 2 (TAB2), resulting in the activation of TAK1 and in turn the phosphorylation of the active-site loop in MKK6 and IKK.[33] While Mizukami et al[34] demonstrated that RANKL induces the formation of a complex consisting of RANK-TRAF6-TAB2-TAK1-TAB1, the authors did not explore the biochemical mechanism for this complex interaction. Nonetheless, these results support the importance of a TRAF6-TAB2-TAK1-TAB1 complex in mediating RANKL signaling.

The functional role of the E3 ubiquitin ligase activity of TRAF6 remains largely unknown in RANKL and RANK signaling. Indeed, preliminary evidence from our laboratory indicates RANKL stimulates the Lys63-linked polyubiquitination of TRAF6 and that the E3 ubiquitin ligase activity is required for RANK signaling and osteoclast formation (B. L. and B. G. D., unpublished observations). In somewhat contrast to our data, Kobayashi et al[35] suggested that the RING domain is not required for the formation of multinucleated, tartrate resistant acid phosphatase (TRAP) positive osteoclasts, but is required for the formation of the actin ring and maturation of osteoclasts. The discrepancy between these results could reflect the differences in experimental design and future studies are required to resolve these inconsistencies.

Importantly, not only is ubiquitination necessary for signaling by TRAF6, but also its de-ubiquitination is essential for controlling its activity. A20, a molecule that was discovered more than 10 years ago and shown to interact with TRAF2 and TRAF6, negatively regulates NF-κB activation induced by TNF, IL-1, and LPS but its mechanism of action remained largely unknown.[36,37] However, recent evidence suggests that A20 might be related to the ubiquitin pathway because reexamination of its sequence has revealed an N-terminal ovarian tumor (OTU) domain that has de-ubiquitinating activity.[38-41] In confirmation of its de-ubiquitinating activity, A20 is capable of de-ubiquitinating Lys63-linked polyubiquitinated TRAF6[40] (B. L. and B. G. D., unpublished observations). Additionally, A20 is able to negatively regulate RANKL-mediated signaling (B. L. and B. G. D., unpublished observations), which suggests that ubiquitin editing functions of TRAF6 and A20 may in fact regulate RANKL signaling. Thus, further investigation of the E3 ligase activity of TRAF6 and the role of de-ubiquitinating enzymes is needed to understand how the RING domain of TRAF6 influences signaling by RANK.

Recent evidence indicates that the activation of NFATc1 is critical for RANKL-mediated osteoclast differentiation.[42,43] NFATc1 expression is dependent on both the TRAF6 and c-Fos pathways,[43] but how RANKL induces the expression of c-Fos still remains unclear.[44] RANKL-induced recruitment of TRAF6 mobilizes intracellular calcium, by an unknown mechanism, which results in the activation of calcineurin that directly de-phosphorylates NFATc1 allowing for its rapid translocation into the nucleus. While activated NFATc1 induces a number of genes involved in cell differentiation, NFATc1 also regulates itself to amplify the transcriptional program for terminal osteoclast differentiation.[1] Furthermore, RANKL also regulates cytoskeleton reorganization during osteoclast differentiation, which has been hypothesized to originate from the activation of phosphatidylinositol-3-kinase (PI3K) through a TRAF6-Src complex.[45] Collectively, RANK induces a series of signals initiated by the E3 ubiquitin ligase activity of TRAF6 acting through a unique Lys63-linked polyubiquitin chain to activate kinases and phosphatases, which subsequently trigger a distinct set of genes required for osteoclast differentiation and function (Fig. 2).

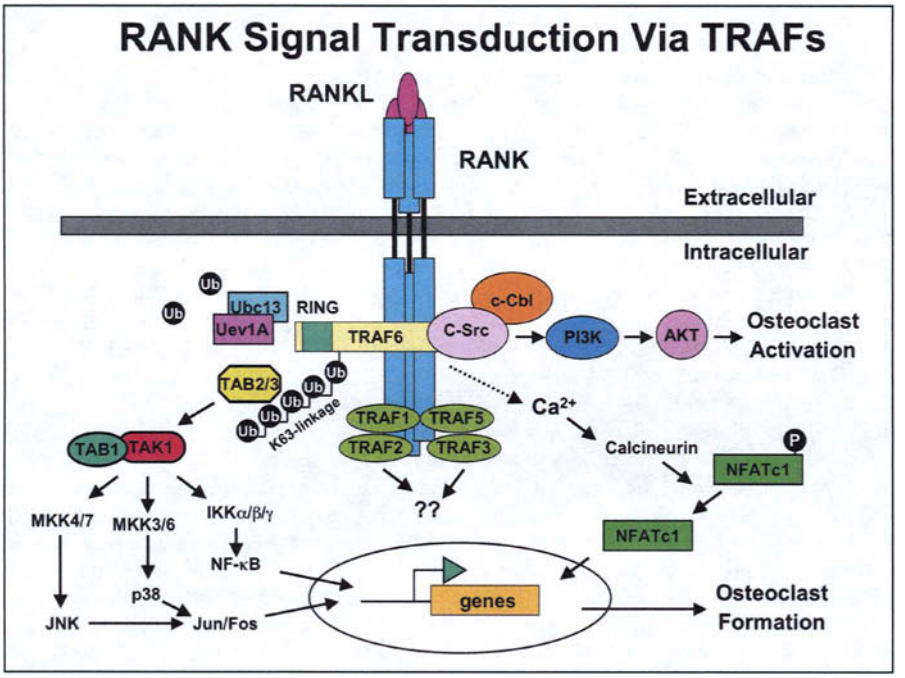

Figure 2. RANK Signal Transduction Via TRAFs. Shown is a schematic diagram depicting the role of TRAF6 in regulating RANK-signaling events. RANKL stimulates the Lys63-linked polyubiquitination of TRAF6 in the presence of the dimeric E2 enzyme consisting of Ubc13 and Uev1A. The Lys63-linked polyubiquitin chain on TRAF6 recruits the Zn-finger domain of TAB2 to the complex, which results in the auto-activation of TAK1 by an unknown mechanism. Subsequently, TAK1 activates downstream kinases to activate the transcription factors NF-κB and AP1. By an unknown mechanism, TRAF6 also mobilizes intracellular calcium to activate calcineurin causing the de-phosphorylation of NFATc1 and its translocation into the nucleus, where it cooperates with NF-κB and AP1 to drive the transcription program for osteoclast differentiation. RANKL also stimulates the activity of the Src kinase pathway through TRAF6 to initiate cytoskeleton rearrangements and actin ring formation for the bone resorbing activity of the osteoclast. The role of the other TRAFs in RANKL signaling is not well characterized.

TRAFs in Osteoclast Differentiation

Signaling through RANK is essential for osteoclast formation, maturation and survival. A lack of RANK or its ligand RANKL in mice leads to osteopetrosis due to a complete absence of osteoclasts.[6,8,46] Since the deletion of TRAF6 in mice leads to severe osteopetrosis with defects in bone remodeling and tooth eruption, TRAF6 appears to have a dominant, nonredundant role in osteoclast function. These phenotypes are attributed to a lack of osteoclast function, but conflicting results have been reported from two independent groups that have generated TRAF6 knockout mice. Naito et al[28] showed a significant reduction in osteoclast numbers in bone sections of TRAF6 deficient mice and the inability of TRAF6$^{-/-}$ splenocytes to differentiate into functional osteoclasts. In contrast, Lomega et al[27] observed a comparable number of osteoclasts in wild-type and TRAF6 deficient mice; however, the osteoclasts lacked contact with bone surfaces and were unable to resorb bone suggesting that TRAF6 is important for osteoclast activation rather than differentiation. The discrepancy between these two reports has yet to be resolved, which might have been caused by different deletion strategies. Nonetheless, in vitro differentiation of osteoclast progenitors with monocyte/macrophage-colony stimulating factor (M-CSF) and RANKL is completely abolished in the absence of TRAF6. Although TRAF6 interaction with RANK is essential for osteoclast cytoskeletal

organization and resorption,[13,21,45] overexpression of TRAF6 in osteoclast progenitor cells leads to RANKL-independent osteoclast formation.[25,26] (B. L. and B. G. D., unpublished observations), underlying an important role of TRAF6 in early osteoclast differentiation.

The role of TRAF5 in osteoclastogenesis is not as clear as for TRAF6. Mice lacking TRAF5 are healthy and do not show any obvious bone phenotype under physiological conditions,[47] arguing against an important function of TRAF5 in osteoclast function. However, Kanazawa et al[48] reported impaired acute osteoclastogenesis in TRAF5 deficient mice after parathyroid hormone (PTH)-induced hypercalcemia and a decrease in osteoclast differentiation from bone marrow-derived monocytes, suggesting that TRAF5 is at least important for acute, stress-induced osteoclastogenesis. An explanation for the mild phenotype could be a redundancy between TRAF5 and TRAF2, therefore masking the importance of TRAF5 in osteoclastogenesis.

Unveiling the role of TRAF2 in osteoclastogenesis is hindered by the fact that loss of TRAF2 leads to embryonic and neonatal lethality.[49] Interestingly, RANKL treatment of fetal liver-derived monocytes from TRAF2 knockout mice only indicated a 20% decrease in osteoclast formation, but osteoclast formation by TNF was completely ablated, suggesting only a minor role of TRAF2 in RANKL induced osteoclastogenesis.[50]

Conclusions and Future Directions

The discovery of RANKL and RANK has provided insights into normal physiological bone homeostasis and novel therapeutic targets for the treatment of diseases associated with increased bone resorption. Targeted deletion of each TRAF molecule in the mouse has provided a link to their physiological function and importantly to the role of TRAF6 in bone maintenance and RANK signaling. The identification of the TRAF interaction motifs in the RANK cytoplasmic domain appear to have been well described, however the functional significance of RANK interacting with TRAF1, TRAF2, TRAF3, and TRAF5 remains elusive at this time. Do these TRAFs serve an alterative function in RANK signaling in other tissues beside bone? RANKL stimulation also induces the phosphorylation of MAPK most likely through TRAF6, but the mechanism is not clear. The evidence supporting the E3 ubiquitin ligase activity of TRAF6 in IL-1 and Toll receptor signaling is strong, but little is known about its function in RANK signaling. Specifically what are the molecular targets for Lys63-linked polyubiquitination that is facilitated by TRAF6 in RANK signaling? What roles do de-ubiquitinating enzymes play in terminating RANKL signaling? TRAF6 recruits Src to the receptor complex, but how does TRAF6 induce the activity of Src in the context of osteoclast differentiation and function? What is the molecular mechanism by which TRAF6 regulates calcium oscillations during osteoclast differentiation? The importance of research in bone diseases will continue due to the high prevalence of osteoporosis and other metabolic bone disorders. Thus, the elucidation of how TRAF molecules regulate the impact of RANKL signaling will provide a further understanding of normal physiological bone homeostasis.

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CHAPTER 13

The LTβR Signaling Pathway

Paula S. Norris and Carl F. Ware*

Abstract

The lymphotoxin-β receptor (LTβR, TNFRSF3) signaling pathway activates gene transcription programs and cell death important in immune development and host defense. The TNF receptor associated factors (TRAF)-2, 3 and 5 function as adaptors linking LTβR signaling targets. Interestingly, TRAF deficient mice do not phenocopy mice deficient in components of the LTβR pathway, presenting a conundrum. Here, an update of our understanding and models of the LTβR signaling pathway are reviewed, with a focus on this conundrum.

Introduction

The lymphotoxin-β receptor (LTβR, TNFRSF3) signaling pathway activates responses controlling cellular differentiation, growth and death manifested in the formation and organizations of peripheral lymphoid organs, dendritic cell homeostasis, hepatic regeneration, interferon responses to pathogens, and death of mucosal derived carcinomas.[1-3] The LTβR signaling activates gene transcription programs controlled in part by nuclear factor κB (NFκB) and others, which help orchestrate these diverse processes. Biochemical and genetic evidence supports a model of signal propagation from the LTβR to kinase complexes that activate distinct forms of nuclear factor κB (NFκB). The TNF receptor associated factor (TRAF)-2 and 5 appear to function as adaptors linking LTβR to transcriptional programs for NFκB, and TRAF3 to cell death, however, this is not always apparent from genetically defined phenotypes, presenting an interesting conundrum.

The LTβ Receptor

Gene

The lymphotoxin β receptor (LTβR) was first identified as a transcript containing a cysteine-rich tumor necrosis factor receptor (TNFR)-like domain in somatic cell hybrids.[4] The *LTβR* gene resides on chromosome 12p13 forming a tripartite locus with *TNFRI* and *CD27*. Interestingly, this chromosomal region is thought to have duplicated giving rise to the cluster of TNFR genes located on Chromosome 1p36. The mRNA encodes a 435 amino acid protein sharing 41% and 46% homology with TNFRI and TNFRII, respectively. Mouse *LTβR* maps to chromosome 6 in a region in conserved synteny with human chromosome 12p13[5] and the encoded protein is highly homologous to the human version with 68% amino acid sequence identity.

Protein Structure and Expression

The LTβR is a type 1 single transmembrane protein with a theoretical mass of 46.7 kDa, however, the observed mass is 61 kDa suggesting that the two potential N-glycosylation sites are utilized. LTβR has a ligand-binding ectodomain containing four cysteine-rich pseudo repeats characteristic

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TNF Receptor Associated Factors (TRAFs), edited by Hao Wu.

of the TNF motif. The cytoplasmic domain is 175 residues, containing a proline-rich membrane proximal region, grouping it with TNFR family members that bind directly to TRAF proteins, including CD40, CD30, HVEM and CD27. This is in contrast to those TNFR with death domains such as TNFRI, Fas and TRAIL Receptors 1 and 2 that require an intermediate adaptor (e.g., TRADD) to engage TRAF. Like other TNFR, LTβR has no intrinsic kinase or other enzymatic activities encoded by its cytosolic domain.

Expression of the LTβR is constitutive on most cells with a promoter region more like a typical house keeping gene. LTβR is expressed on stromal cells in lymphoid tissue[6] but is also expressed on myeloid lineage cells,[5,7] blood monocytes, alveolar macrophages,[8] mast cells[9] and dendritic cells.[10,11] Most adherent primary cells and cell lines including normal diploid fibroblasts, bronchial airway epithelial cells, the follicular dendritic cell line FDC-1, U937 promyelomonocytic cell line, HT-29 colon adenocarcinoma line, HeLa cervical carcinoma line and HEK 293 embryonic kidney cells express LTβR.[6] A prominent feature of LTβR expression is the conspicuous absence on T and B lymphocytes and NK cells. By contrast, the ligands for LTβR are often expressed by T and B cells. This expression pattern indicates that signaling may be unidirectional, and for LTαβ, which is not cleaved into a soluble form, requires cell to cell contact between the lymphocyte to the LTβR-bearing cell to transmit signals.

Ligands

LTβR binds two members of the TNF superfamily (Fig. 1), the LTαβ heterotrimers, and LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; TNFSF14). Two distinct lymphotoxin heterotrimers, $LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$, can be formed between LTα and LTβ, which are membrane

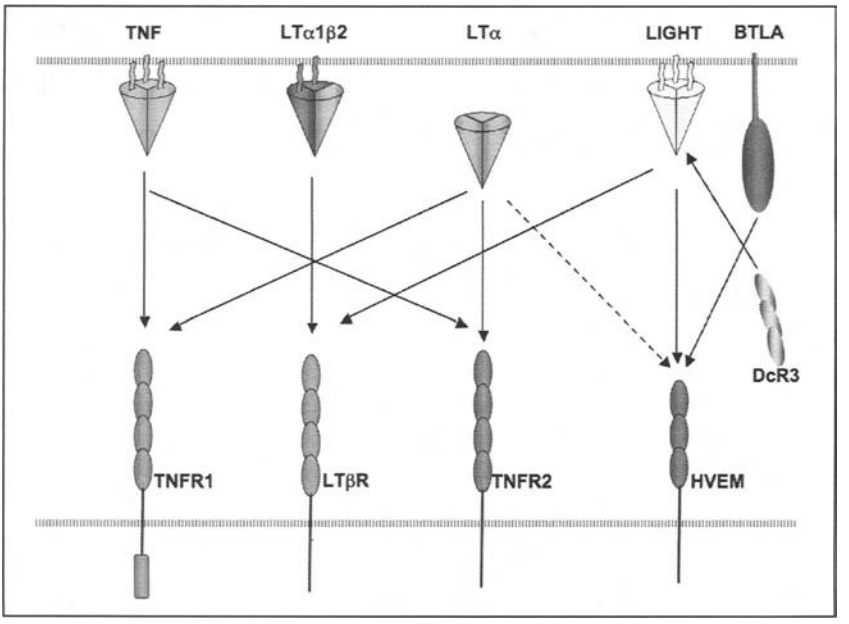

Figure 1. The Immediate TNF/LT family. The TNF ligands are type II transmembrane proteins that form trimers that interact with their corresponding TNF receptors. The arrowed lines indicate the specific binding interactions between ligands and receptors. TNFR1 contains a death domain (DD), the other receptors contain TRAF binding motifs. BTLA is an Ig family member that is activated by HVEM, initiating inhibitory cosignaling via recruitment of tyrosine phosphatases that attenuate signaling by the T cell antigen receptor; DcR3 is a soluble protein that binds LIGHT, Fas Ligand and TL1A (not pictured).

bound via the LTβ subunit (TNFSF3).[12] LTβR also binds another closely related ligand, LIGHT.[13] The binding of $LT\alpha_1\beta_2$ is specific for LTβR, whereas $LT\alpha_2\beta_1$ also binds TNFRI and TNFRII, and LIGHT also engages the herpes virus entry mediator (HVEM, TNFRSF14). The LTβR binds the membrane forms of these ligands, the recombinant soluble forms of $LT\alpha_1\beta_2$ and LIGHT with high affinity, but relatively weakly to soluble $LT\alpha_2\beta_1$. DcR3 is a soluble protein, which can compete with LIGHT for binding HVEM or LTβR.[14] A novel inhibitory cosignaling membrane protein, B and T lymphocyte attenuator (BTLA) was identified as a receptor for HVEM.[15,16] The importance of this pathway is underscored by the finding that two evolutionarily distinct herpesviruses target this pathway.[17,18]

LTβR Signaling Pathway

Binding of LTβR by its trivalent ligands induces an ordered aggregation or "clustering" initiating signal transduction pathways. Receptor signaling can also be initiated by anti-LTβR antibodies that mimic receptor "clustering" or by overexpression of the receptor in cell lines. Ligation of the LTβR activates gene transcription via nuclear factor κB (NFκB) and c-Jun N-terminal kinase (JNK) pathways, and, in some cell lines, can activate apoptosis (Fig. 2). Although it can induce death, LTβR does not contain a death domain. The LTβR interacts directly with members of the TNF receptor-associated factors (TRAF) family of zinc RING finger proteins.[19] Ligation of the LTβR by $LT\alpha_1\beta_2$ or LIGHT rapidly recruits TRAF to the cytosolic domain.[20] TRAF 2, 3 and 5, but not TRAF1 or 6 have been shown to bind directly to its cytosolic domain.[20-22] Interestingly, the hepatitis C virus core protein also directly binds to this region.[23] TRAF4 has been reported to bind LTβR in in vitro studies, but this has not been confirmed in vivo.[24]

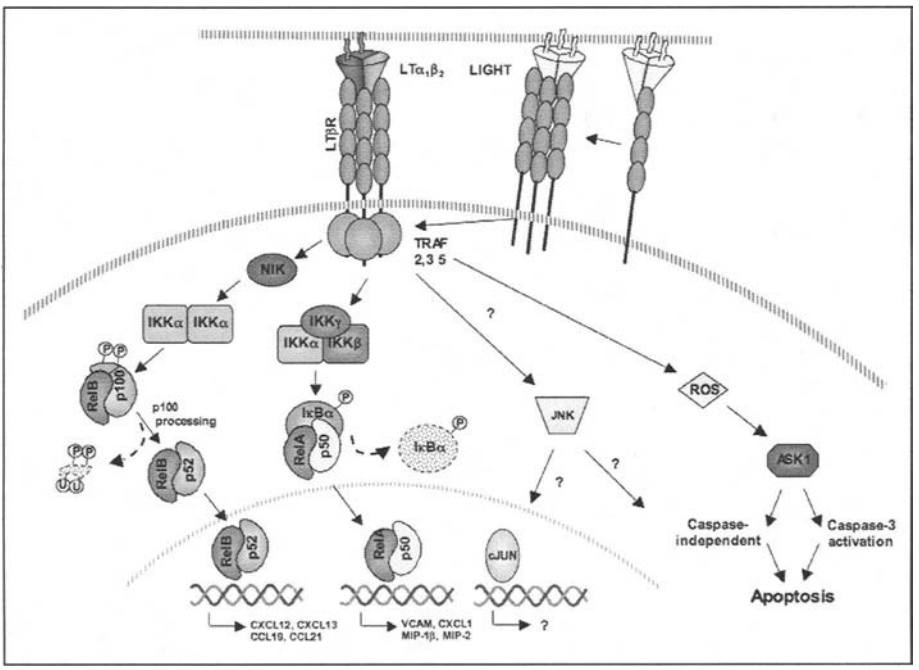

Figure 2. The LTβR Signaling Pathway. LTαβ2 or LIGHT both engage the LTβR initiating receptor clustering, and recruitment of TRAF2, 3 or 5. LTβR activation leads to activation of both the RelA/p50 and RelB/p52, which activate different sets of genes. LTβR can also activate JNK leading to activated AP1 as well as induction of reactive oxygen radicals that contribute to cell death by as yet unknown mechanisms.

Studies of LTβR cytoplasmic tail mutants suggest that the regulation of LTβR signaling is complex with discrete regions controlling different aspects of signaling.[25] A truncation mutant which removes the TRAF binding region (Δ389) greatly diminished receptor-induced NFκB activation and cell death, however, further truncation revealed an adjacent region (Δ379) that negatively regulated signaling, and a cryptic NFκB-activation region (345-359) that is independent of TRAF binding. Additional regions mediated trafficking and self-association.

LTβR-Mediated NFκB Activation

In mammalian cells, the NFκB family of transcription factors consists of five members: RelA, RelB, c-Rel, p50/NFκB1 and p52/NFκB2.[26,27] p50 and p52 are the products of the proteolytic processing of p105 and p100, respectively. Homo and hetero-dimers of NFκB family members are held inactive in the cytosol by inhibitors of κB (IκBs), such as IκBα, masking nuclear localization motifs. p105 and p100 also contain C-terminal IκB-homologous inhibitory regions and retain some NFκB dimers within the cytosol. The phosphorylation and ubiquitin-dependent degradation of the IκBs and subsequent nuclear translocation of NFκB occurs in response to a wide variety of stimuli. These stimuli trigger the activation of the IκB kinase (IKK) complex, which consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit IKKγ/NEMO, and is responsible for initiation the degradation of IκB.[28]

Two separate, yet related mechanisms activate distinct forms of NFκB: RelA/p50 and RelB/p52.[29] The first defined (or classical) pathway is activated by IL-1R and TNFR1, and is characterized by the activation of the NFκB dimer Rel A (p65)-p50 in a process occurring within minutes that is initiated through IKKβ-mediated phosphorylation of IκB, ubiquitination and proteosome-dependent degradation. The more recently identified noncanonical (or alternative) pathway involves degradation of p100 forming the active p52 component, which often associates with RelB. This process of RelB/p52 accumulation in the nucleus is optimal several hours after the stimulus. Activation of p100 is independent of IKKβ and γ, but dependent upon IKKα and the NFκB-inducing kinase (NIK).[30] Overexpression of NIK has been shown to trigger the processing of p100 to p52 by site-specific phosphorylation and subsequent ubiquitination and degradation of the IκB-like C-terminus of p100.[31]

NIK, a critical component in the activation of the p100 processing NFκB pathway by LTβR, was identified as a TRAF2-interacting protein that contained a serine/threonine protein kinase motif resembling MAP3K proteins.[32] Overexpression of NIK leads to activation of NFκB, but not JNK,[33] and NIK is required for activation of the alternative pathway characterized by p100 processing.[30,34-37] More recent studies demonstrate that depletion of NIK by siRNA blocked the activation of both the classical and alternative NFκB pathways by CD27, CD40 and BAFF-R, but not by TNFRI, which is restricted to activating RelA/p50 complex through the classical pathway.[38] These findings suggest a role for NIK in facilitating the activation of both NFκB pathways by receptors that harbor that capacity, but not in triggering the classical pathway by single NFκB-inducers like TNFRI.

The binding of NIK with TRAF1, 2, 3, 5 and 6 has been demonstrated in overexpression studies,[32,33] and in the case of TRAF2, the interaction requires the carboxy-terminus of NIK (residues 624-947). Residues 735-947 of NIK are also required for interaction with IKKα.[39] Mutation of glycine 855 to arginine in the C-terminus of murine NIK (G860R in human) causes alymphoplasia (*aly*) in mice. NIK in the *aly* mouse has an intact kinase domain and binds to TRAF2, 3 and 5, however, it fails to bind IKKα.[40,41] The N-terminus of NIK contains a basic region (residues 127-146) and a proline rich region (250-317) shown to be a cis-acting negative regulatory domain by interacting with the C-terminal region of NIK and interfering with binding to IKKα.[42] Liao et al reported that TRAF3 binds at the N-terminus of NIK within residues 78-84 (ISIIAQA), and targets NIK for degradation in the proteasome resulting in an inhibition of NIK-induced p100 processing.[43] Treatment of cells with agonistic anti-CD40 antibody or soluble BAFF, but not TNF, lead to decreased TRAF3 levels, an increase in NIK levels and p100 processing, suggesting that TRAF3 acts as a negative regulator of NIK. Unfortunately, many of these studies have yielded conflicting data on the regions responsible for TRAF-NIK interactions perhaps because of an

over-reliance on large deletion mutations and forced overexpression systems. Alternatively, NIK may interact with different TRAF molecules at more than one region.

The kinase domain of human NIK resides between residues 366 and 624, and is required for activation of NFκB.[32] Autophosphorylation of NIK on threonine 559[39] in a manner dependent upon the active-site lysine and an adjacent lysine (KK429/30AA)[32] is required for subsequent phosphorylation of IKKα upon its binding to NIK. NIK is thought to serve as a docking molecule recruiting IKKα to p100. The NIK *aly* mutant exhibits reduced activity in promoting the IKKα/p100 association, however recruitment of IKKα to p100 by NIK is not dependent upon kinase activity of IKKα. Similarly, kinase inactive NIK promotes binding of IKKα to p100. After recruitment to p100, IKKα phosphorylates serine residues at the N- and C-terminus of the protein (S99, 108, 115, 123 and 872). This phosphorylation is a prerequisite for ubiquitination and degradation of p100 mediated by the beta-TrCP ubiquitin ligase and 26S proteosome.[44]

In addition to NIK being essential for LTβR-induced NFκB activation, TRAF2 was recently shown to play a key, nonredundant role in LIGHT-LTβR signaling.[45] Murine fibroblast lacking TRAF2 failed to activate both NFκB pathways, as well as JNK, in response to treatment with LIGHT. Defects in NFκB or JNK activation were not observed in cells deficient in TRAF5 or RIP, a death domain kinase known to associate with TNFRI. Moreover, following LIGHT treatment, TRAF2 was recruited to the LTβR complex along with IKKα and IKKβ at early times, while only TRAF2 and IKKα were present at 8 hours post treatment. These observations suggest that TRAF2 may recruit different downstream targets to initiate distinct pathways: IKKβγ for activation of the classical NFκB pathway and IKKα for activation of the noncanonical pathway. That TRAF5 does not appear to be essential for LTβR-mediated NFκB or JNK activation may be explained by tissue-restricted expression of these adaptor molecules since these studies were limited to fibroblasts.

JNK Activation

JNK (c-Jun N-terminal kinase or stress-activated kinase) is activated by many apoptosis-inducing stimuli and is thought to be an important mediator of this process.[46,47] JNK activation is involved in pathways needed during embryogenesis, cell proliferation and immunological responses. Most members of the TNF superfamily are strong inducers of JNK activation: TNF treatment dramatically elevates JNK activity, as does overexpression of HVEM[48] and related receptors. LTβR-mediated activation of JNK has been reported in 293HEK that overexpress the receptor and in HeLa cells and mouse embryo fibroblasts treated with LIGHT.[45,49] LIGHT-initiated JNK activation in HeLa cells and fibroblasts was shown to be dependent on TRAF2 but did not require TRAF5. What role, if any, TRAF3 may play in JNK activation initiated by LTβR has yet to be determined.

Cell Death

Treatment of some human adenocarcinoma cell lines with recombinant $LT\alpha_1\beta_2$ or agonistic antibody against LTβR in combination with interferon γ (IFN-γ) results in cell death.[20,50] Cell death and growth arrest of tumor cells is also observed with CD40 and CD30, indicating nondeath domain TNFR can impinge signals on cell survival. Similarly, treatment of the adenocarcinoma line HT29 with LIGHT and IFN-γ induces death in an LTβR-dependent, but HVEM-independent, manner even though the cells express both receptors.[51] TRAF3 is important for LTβR-mediated apoptosis, as dominant negative mutants of TRAF3 abrogate cell death induced by treatment with $LT\alpha1\beta2$[25] or LIGHT[51] but do not alter NFκB activation. Overexpression of the self-association domain of LTβR (324-377) in HT-29 or HeLa cells results in IFN-γ-independent cell death that also is blocked by dominant negative TRAF3 or by caspase inhibitors.[52]

The death signaling mediated by death domain-containing receptors, such as TNFRI and Fas, can be inhibited efficiently by caspase inhibitors, whereas, LIGHT-LTβR-induced cell death is only partially affected.[53,54] In contrast, free radical scavenger carboxyfullerenes can completely inhibit LIGHT-LTβR-induced death indicating the important role for reactive oxygen species (ROS) in this process. ASK1 (apoptosis signal-regulating kinase 1/ MEKK5) can be activated in response to various stress signals, including ROS, and in response to LTβR signaling and the

subsequent production of ROS.[55] LTβR-mediated activation of ASK1 is dependent on TRAF 3 and 5, but not TRAF2 as it is in the case of TNFRI-activation of ASK1.

Analysis of endogenous LIGHT-LTβR complexes from U937 and HEK293 cells revealed the association of TRAF2, cIAP1 and Smac, in addition to TRAF3, with the receptor. cIAP1, a cellular member of the inhibitor of apoptosis (IAP) family, was first identified as part of the TNFRI complex via its association with TRAF2.[56] Smac, whose function is to antagonize the inhibition of caspases by IAP and thus promote apoptosis, is recruited to LTβR in a cIAP-dependent manner and potentiates receptor-induced apoptosis.[57] Nonetheless, the intermediates connecting LTβR signaling to death pathways involving either caspases or radicals remain elusive.

The LTβR-TRAF3 Complex

The cytoplasmic domain of LTβR contains a large proline-rich region (~60 residues) near the C terminus that is responsible for initiating NF-κB activation and apoptosis.[25] A series of deletion mutants localized the binding site for TRAF2, TRAF3 and TRAF5 to a minimal region (^{389}PEEGDPG^{395}) with limited homology to the TRAF-binding motifs (PXQXT/S) similar but not identical to other TNFR family members including CD40, HVEM, CD27 and BAFF-R. Structural studies of TRAF3 in complex with a fragment of the cytoplasmic domain of LTβR refined the TRAF3 binding motif to be ^{388}IPEEGD^{393}, (Fig. 3)[58] and demonstrated that the primary intermolecular contacts are made in the same surface binding crevice on TRAF3 that accommodates CD40[59] and TANK (TRAF-associated NF-κB activator or I-TRAF),[60] as well as BAFF-R (B cell-activating factor belonging to the TNF family receptor).[61]

Mutational analysis of the residues within the binding motif of LTβR and the binding crevice on TRAF3 revealed interesting distinctions between the interactions of LTβR with TRAFs 2, 3 and 5 and those of TRAF3 with LTβR, CD40 and TANK.[58] Mutation of Pro^{387} to alanine led to a loss of TRAF5 binding, but not TRAF2 or TRAF3. Similarly, mutation of the two adjacent glutamates, Glu^{390}-Glu^{391}, resulted in a loss of TRAF5 binding and reduced TRAF3 binding but did not affect TRAF2 binding. Only when Glu^{390}-Glu^{391} and Asp^{393} were simultaneously mutated was binding by each of the three TRAF affected. These studies suggest that the molecular contacts of these TRAF with LTβR are not identical. The contact residues with LTβR in the TRAF3 binding crevice were compared to those with CD40 and TANK. Binding of each molecule was disrupted upon mutation of either of the phenylalanines (Phe^{448} and Phe^{457}) in the hydrophobic pocket of the TRAF3 binding crevice to glutamic acid. Mutation of Tyr^{395} to alanine in TRAF3 reduces its binding to CD40 and TANK while abolishing binding to LTβR. An arginine to alanine substitution at residue 393 reduced binding to each protein. Other residues in TRAF3 proved to be important for CD40 and TANK binding but not LTβR, most notably some of the serine residues in the serine tong (Ser$^{454\text{-}456}$) whose mutation affects binding to CD40 and TANK but not LTβR.

The LTβR, CD40 and the downstream regulator TANK each bind to the same crevice on TRAF3. This observation stimulates an important question regarding the specificity of binding recognition and the trigger of the LTβR signaling process. These three molecules bind at the same surface pocket, and similarly, peptides bearing the motif PxQxT from a number of TNFRs bind to the homologous crevice on TRAF2. TRAF3 appears to contain 'hot spots' corresponding to residues that provide the same principal contacts for each of several different binding partners mediated by adjustments of side chains. 'Plasticity' or 'flexibility' of residues is apparent in the molecular interactions[62,63] and may influence binding affinity. In the case of LTβR, signaling through two different NFκB pathways[30] may involve similar adaptations at the interface, with distinct responses from the adaptable TRAF molecule. At least 18 different receptors in the TNFR family can engage TRAFs, and others in the IL-1R family, suggesting that selection of an adaptable binding crevice in TRAF3 may be a parallel evolutionary event to compensate for gene duplicative mechanisms driving expansion of the TNFR and IL-1R family. The flexibility of the TRAF binding site may also represent an advantageous evolutionary adaptation, serving as a defense against more rapidly mutating viral pathogens that target TRAF components of the TNFR pathway such as EBV LMP1 protein.[21,37]

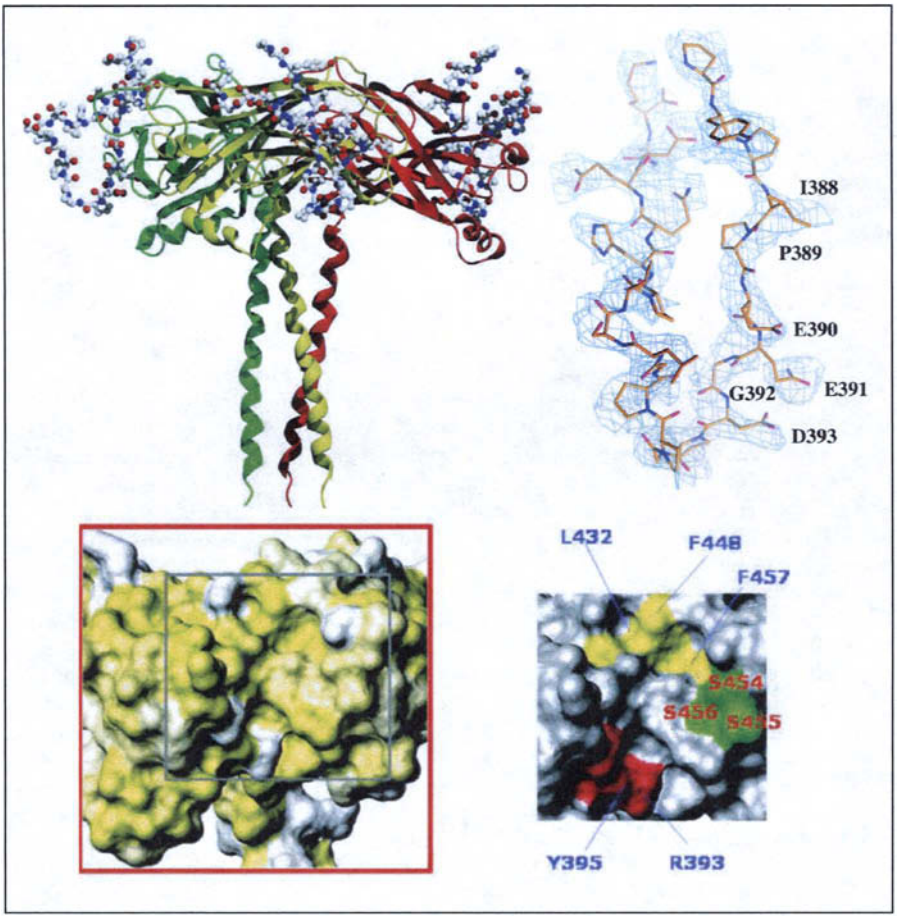

Figure 3. Structure of the LTβR/TRAF3 complex. Upper left panel) Schematic representation of the TRAF3 trimer is shown as a ribbon diagram with each subunit colored separately. Each subunit binds one LTβR molecule, which is represented as a ball-and-stick model. LTβR binds to a crevice at the edge of the TRAF3 β-sandwich domain. In this view, the cell membrane is located at the top of the image. Upper right panel) Model of LTβR displayed in electron density map. Lower panels) Hot spots in the TRAF3 receptor interaction binding crevice. The solvent-accessible surface of the TRAF β-sandwich domain is shown in the left panel. The view in this orientation has the cell membrane located at the top of the image, and the N-terminal helix that participates in the coiled-coil interactions upon trimer formation is at the bottom of the image (not visible). The binding crevice is at the edge of the β-sandwich, and the view here is directly into the crevice. The area that contains the contact residues for TNFR or TANK is enclosed in a box and the surface is colored yellow according with intensity proportional to increasing hydrophobic character. The right panel is colored to highlight three separate hot spots for protein-protein interactions; each is colored separately. The first hot spot is polar in nature with two charged residues (red); the second hot spot is hydrophilic (green) with a serine cluster and the third is hydrophobic (yellow). Reproduced with permission from Li, Norris et al. 2003, J Biological Chemistry.[58]

Genes Induced by LTβR

Through its activation of NFκB and JNK, the LTβR plays critical roles in inflammation and lymphoid organogenesis. LTβR signaling induces IKKα-dependent expression of the chemokines CCL19 (ELC), CCL21 (SLC), CXCL3 (BLC) and CXCL12 (SDF-1a) and the cytokine BAFF.[30]

ELC and SLC are thought to play important roles in the organization of lymphoid organs, while SDF-1 is important in the early stages of B cell development.[64,65] Stimulation of the LTβR also increases expression of CCL4 (MIP-1β) and CXCL2 (MIP-2), inflammatory chemokines, and their expression is enhanced in the absence of IKKα, suggesting that the IKKα-dependent pathway suppresses LTβR-mediated induction of MIP-1β and MIP-2.[30] Another in vitro study demonstrated that LTβR activation in HEK293 cells increased IL-8 promoter activity and lead to IL-8 release in a manner requiring NFκB and AP-1 binding sites located in the IL-8 promoter.[49] LTβR or TNFR1 signaling can activate gene expression of interferon β in diploid fibroblasts but only in the context of virus infection. Recent studies have focused on genes induced by LTbR in vivo in specific tissue systems. In the follicle-associated epithelium (FAE) that overlies Peyer's patches, expression of the chemokine CCL20 (6Ckine) is mediated, at least in part, by LTβR signaling in an NFkB-dependent manner.[66] Additionally, Huber et al[67] recently reported a set of LTαβ -responsive transcripts in FDC-enriched cell clusters. This set included transcripts for the cell adhesion related proteins GlyCAM-1 and MFG-1, the chemokine CXCL13, the ECM component cochlin, the apoptosis related protein clusterin and the proteolysis protein serpin a1a.

Genetic Phenotypes of LT and TRAF-Deficient Mice

Deletion of LTα in the mouse was the first identified specific deficiency in lymph node formation.[68,69] Both LTβR and LTβ, but neither TNF nor its two receptors, exhibited this phenotype providing genetic evidence that LTαβ and LTβR are involved in a common signaling pathway. We now appreciate that at least two cell types are necessary for lymph node formation, the LTαβ expressing lymphoid tissue inducer cell (a CD4+ IL7Rα+ non T non B lymphocyte) and embryonic stromal organizer cell expressing the LTβR (reviewed in ref. 70). The lymph node deficient phenotype is found in several other knockout mice delineating the framework of a signaling pathway involved in mammalian organ development. Ikaros, ID2 and RORγt are transcriptional regulators essential for lymphocyte progenitors or lymph node inducer cells to develop.[71-75] Mice deficient in certain members of the NFκB activation pathway, including NIK, IKKα and Rel B are missing lymph nodes. Potential target genes activated by the LTαβ-LTβR-NFκB pathway includes the lymphocyte organizing chemokines, such as CXCL13, CCL19 and CCL21, and their receptors CXCR5 and CCR7 whose knockouts also show defective lymphoid organogenesis.

We now appreciate the formation of lymph nodes (lymph node) is a complex process that is illustrative of the multiple components associated with the LT signaling pathway. An effective immune response requires transient interactions between multiple cell types that are facilitated in secondary lymphoid tissue (spleen, lymph nodes and Peyer's patches) by a specialized microarchitecture that positions populations of cells in discrete regions. The development and homeostasis of secondary lymphoid tissue microenvironments require signaling by the LT/TNF related cytokines (reviewed in refs. 70,76).

TRAF2-deficient mice display premature death due in part to severe runting.[77] In addition, TRAF2-deficient cells are highly sensitive to TNF-induced cell death. TNF-mediated toxicity through TNFRI contributes significantly to the survival defects in TRAF2 deficient mice because mice lacking both TRAF2 and TNFRI have increased survival.[78] Restriction of the TRAF2 deletion to B cells revealed that TRAF2 acts as a positive mediator of canonical NFκB activation while also serving as a negative regulator of the noncanonical pathway. While this role for TRAF2 was demonstrated in the context of CD40 signaling in B cells, it may also translate to the function of the adaptor in LTβR signaling in other cell types.

Mice lacking TRAF3 have poor perinatal and neonatal survival,[79] and, similar to TRAF2-deficient mice, exhibit severe runting and hypotrophy of the spleen and thymus. TRAF3-deficient mice have normal lymph nodes, and the immune system is compromised in T-cell-dependent antigen responses.[79]

TRAF5 is a close functional and structural homologue of TRAF2, with a more restricted expression pattern compared to the widely expressed TRAF2. Deletion of TRAF5 in mice did not cause perinatal lethality, rather led to more specific defects in CD40- and CD27-mediated

lymphocyte activation. As was seen in TRAF2-deficient animals, TNF-mediated NFκB activation was only modestly affected in mice lacking TRAF5.[80] The finding that TRAF2 and 5 double knockout animals did exhibit severe defects in NFκB activation suggests that their roles are partially redundant.[81]

The LTβR-TRAF Conundrum

An interesting but perplexing discordance between genetic and biochemical evidence arises with the TRAF adaptors. LTβR does not engage TRAF6, yet unexpectedly a lymph node deficiency was found in TRAF6-/- mice.[82] Moreover, the TRANCE/RANK Ligand-RANK system, which utilizes TRAF6 as an adaptor, when genetically deleted, also revealed a lymph node-deficient phenotype.[83] However, Yoshida and colleagues revealed that the RANK/TRAF6 pathway is required for the induction of LTαβ on the lymphoid tissue inducer cell, thus accounting for the discordance with the biochemical data (LTβR does not bind TRAF6).[84]

Ligation of LTβR recruits TRAF3 to the receptor under normal cellular physiological conditions,[20] which is the earliest step identified in signaling. From these types of biochemical studies, TRAF2, 3 and 5 are implicated in the LTβR signaling mechanism, yet deletion of these TRAF genes in mice (including double KO of TRAF2 and 5) failed to disrupt lymph node development (however other dramatic phenotypes are present).[77,79,80] TRAF5-/- mice do not phenocopy LT deficient mice, but do with Ox40 and CD27.[85] TRAF2 and 3 deficient mice are both neonatal lethal, which has provided technical limits to resolving this question. TRAF2-/- deficiency results in increased TNF production and liver failure, and is partially restored by crossing onto TNFR1-/- mice; TRAF3-/- is not. Thus, LTβR, NIK, IKKα and RelB are clearly linked phenotypically, but TRAF2 and 3 are not, at least to the most obvious LTβR associated phenotypes. The situation remains an intriguing puzzle.

What then is the role of TRAF2 and 3 in LTβR signaling and how can the lymph node phenotype be TRAF2-independent if TRAF2 is required to activate p100 processing and Rel B? Biochemical and genetic evidence supports a model in which LTβR ligation activates NFκB2 via a stepwise event involving the serine kinases NIK and IKKα, which control the proteolytic processing of p100→p52.[30] What remains mysterious is how signaling is propagated to NIK and what role TRAF2 and 3 play in this process, if any. One possibility is that TRAF proteins may not function directly as adaptors that propagate signals but rather as regulators restraining the activity of key enzymes or regulators. TRAF3 and NIK are preassociated in an inactive complex and not with LTβR. In this scenario the ligated LTβR cytoplasmic domain binds to the TRAF3 crevice acting to competitively displace NIK, releasing those molecules to interact with their partners or substrates to propagate the signaling event, e.g., liberating NIK to phosphorylate protein substrates, such as IKKα kinase, setting in motion the conversion of p100→52. Alternatively, TRAF independent mechanisms that activate NFκB may account for these developmental phenotypes, which is supported by LTβR deletion mutants that activate NFκB reporter but fail to bind TRAF2, 3 or 5.[25] The phenotypes of the TRAF deficient mice are complex in part because TRAF family is utilized by multiple TNFR and IL1-R and phenotypes controlled by these other pathways may be accumulatively displayed in the TRAF deficient mice.

The cell survival and proliferation phenotype displayed by TRAF2 and 3 deficient mice may have some counterpart in LT deficient mice. In addition to the selective death induced signaling by the LTβR in carcinomas, LTβR has recently been shown to provide positive cellular proliferation signals in the context of adult organs. LTβR is needed to maintain the homeostasis of CD4+ myeloid dendritic cells within lymphoid organs,[11] a phenotype also seen in RelB deficient mice. The proliferation of hepatocytes may also require LTαβ-LTβR signaling specifically during hepatic regeneration, but not hepatic organogenesis.[86] In addition, LTβR signaling is needed to protect T and B cells from death during herpesvirus infection, but through an indirect mechanism suggested to involve interferon β signaling.[87] These cell death and proliferation phenotypes associated with the LTβR present an intriguing correlation, however the link between the LTβR and the TRAF molecules remains elusive.

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CHAPTER 14

LMP1 TRAFficking Activates Growth and Survival Pathways

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Abstract

Epstein-Barr Virus (EBV) Latent Infection Membrane Protein 1 (LMP1) is expressed in all the EBV related malignancies. LMP1 expression is critical for transformation of human B-cells by EBV. LMP1 expression in human B cells induces activation and adhesion molecule expression and cell clumping, which are characteristic of CD40 activated B lymphocytes. In immortalized fibroblasts, LMP1 mimics aspects of activated ras in enabling serum, contact, and anchorage independent growth. Reverse genetic analyses implicate six transmembrane domains (TM), TM1-6, and two C-terminal cytosolic domains, transformation effector sites 1 and 2 (TES1 and 2) or C-terminal activation regions 1 and 2 (CTAR1 and 2) as the essential domains for LMP1 effects. The 6 transmembrane domains cause intermolecular interaction, whereas the C-terminal domains signal through tumor necrosis factor receptor (TNFR) associated factors (TRAFs) or TNFR associated death domain proteins (TRADD) and activate NF-κB, JNK, and p38. LMP1 TES1/CTAR1 directly recruits TRAFs 1, 2, 3 and 5 whereas LMP1 TES2/CTAR2 indirectly recruits TRAF6 via BS69. LMP1 TES1/CTAR1 activates TRAF2, NIK, IKKα and p52 mediated noncanonical NF-κB pathway and LMP1 TES2/CTAR2 activates TRAF6, TAB1, TAK1, IKKα/ IKKβ/ IKKγ mediated canonical NF-κB pathway. Interestingly, TRAF3 is a negative regulator of noncanonical NF-κB activation, although a positive role in LMP1 signaling has also been described. LMP1 mediated JNK activation is predominantly TES2/CTAR2 dependent and requires TRAF6. LMP1 specifically increases TRAF3 partitioning into lipid rafts and interestingly does not induce degradation of any of the TRAFs upon NF-κB activation. Studies of the chemistry and biology of LMP1-TRAF interaction mediated activation of signaling pathways are important for controlling EBV infected cell survival and growth.

Introduction

Epstein-Barr Virus (EBV) is an oncogenic herpes virus that causes significant human diseases including lymphoproliferative diseases in immune-compromised people (LPD), Hodgkin's disease (HD), and Naso-Pharyngeal Carcinoma (NPC) (for review see refs. 1,2). EBV infection is the most common cause of lymphoma in AIDS patients. In vitro, EBV efficiently converts B lymphocytes into lymphoblastoid cell lines (LCLs).

Latent Membrane Protein 1 (LMP1) is the major EBV oncogene. LMP1 expression is a common feature of most EBV-associated malignancies. Furthermore, LMP1 is required for LCL outgrowth in vitro. LMP1 phenotypically transforms rodent fibroblasts enabling growth in reduced serum, loss of contact inhibition, adhering without actin cables, anchorage independence and

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tumor formation in nude mice.[3-6] The oncogenic potential of LMP1 has also been demonstrated in transgenic mice. In transgenic mice, Ig promoter and enhancer driven LMP1 expression in B lymphoyctes results in clonal B cell proliferations[7] while polyoma promoter driven expression in the epidermis results in hypertrophy.[8]

In human lymphocytes, LMP1 activates signaling cascades that result in changes in cell gene expression which are important for cell growth or survival. LMP1 induces activation markers, adhesion proteins and anti-apoptotic effects.[9,10] LMP1 strongly activates NF-κB and JNK/p38 pathways.[11-15] LMP1 mutations that affect NF-κB activation are defective for LCL outgrowth.[16] Furthermore, interruption of NF-κB activation causes abrupt LCL apoptosis.[17] LMP1 is likely to have a similar survival role in EBV induced NPC, and NF-κB activation is implicated in the survival of other hematological malignancies.

Substantial experimental evidence indicates that LMP1's effects are due to constitutive activation of Tumor Necrosis Factor Receptor (TNFR) intracellular signaling pathways.[18-21] The key LMP1 functional elements are: (i) Six TransMembrane domains (TM), which mediate raft association, constitutive aggregation, and constitutive signaling,[10,22-30] and (ii) Two C-terminal cytosolic domains, Transformation Effecter Sites (TES) 1 and 2, which interact with signaling molecules that mediate TNFR cytoplasmic domain signaling (Fig. 1). TES1 and TES2 engage TRAFs and TRADD, respectively.[3,10,18-23,31-38] Recruitment of TRAFs and TRADD results in NF-κB, JNK, and p38 activation.[11,13,32,39-58]

The TRAF proteins comprise a family (TRAFs 1-6) of signal transducers that interact with members of the TNF receptor superfamily. TRAF1, TRAF2, and TRAF3 were isolated based on their interaction with the cytoplasmic tails of LMP1, TNF-R2 and CD40.[18,59-63] TRAF4 was isolated by screening a cDNA library of lymph nodes that contained metastatic tumor cells.[64] TRAF5 was identified by utilization of degenerate PCR primers against a highly conserved carboxy-terminal end of TRAF proteins (TRAF-C domain) and independently in a yeast two-hybrid screen.[65,66] TRAF6 was isolated by screening an EST expression library and by utilizing CD40 as bait for a yeast two-hybrid screen.[67,68]

Each member of TNFR superfamily distinctly recruit specific TRAFs to mediate specific downstream signaling.[69] The recruitment of TRAFs onto TNFR superfamily can be direct or indirect. Members of the TNF receptor superfamily such as TNFR2 and CD40, recruit TRAFs directly via short sequences, TRAF binding motif, in their intracellular tails.[59,61,70] Those that contain an intracellular death domain, such as TNFR1, first recruit a death domain containing adapter protein, TRADD, via a death domain-death domain interaction.[71] TRADD then recruits TRAF2[72] and RIP[73,74] for survival signaling, and FADD and caspase-8 for the induction of apoptosis.[72] Members of the IL-1R/TLR superfamily also recruit TRAFs indirectly through their TIR domain,[75] which recruits a TIR and death domain containing protein, MyD88.[76] MyD88 recruits death domain containing Ser/Thr kinases IRAKs,[76-79] which recruit TRAF6 to elicit signaling.[67,80-82]

The TRAF proteins are characterized by the presence of a novel TRAF domain at the C-terminus, which consists of a TRAF-N coiled-coil domain followed by a conserved TRAF-C domain (Fig. 2). The TRAF domain is responsible for homo- and heterodimerization of the TRAF proteins, as well as for their direct and indirect interactions with cognate surface receptors.[59,61,83,84] All the TRAFs except TRAF1 have at their N-terminus a RING finger and several zinc finger motifs, which are important for downstream signaling events.[83,85] The TRAFs have been shown to induce a wide array of functions from cell differentiation, proliferation and survival to cell death. The biological effects of TRAF signaling appear to be mediated through the activation of transcription factors of the NF-κB and AP-1 family. NF-κB promotes the expression of genes involved in inflammatory and anti-apoptotic responses.[86-89] AP-1 activity is stimulated by mitogen-activated protein (MAP) kinases such as JNK, ERK and p38 through either direct phosphorylation or transcription of AP-1 components.[88] The stimulation of AP-1 activity by MAP kinases may elicit stress responses and promote both cell survival and cell death.[90]

NF-κB is a dimeric transcription factor composed of homo or hetero-dimers of p50, p52, RelA, RelB, or c-Rel that are held inactive in the cytosol by IκB family protein. In response to microbial

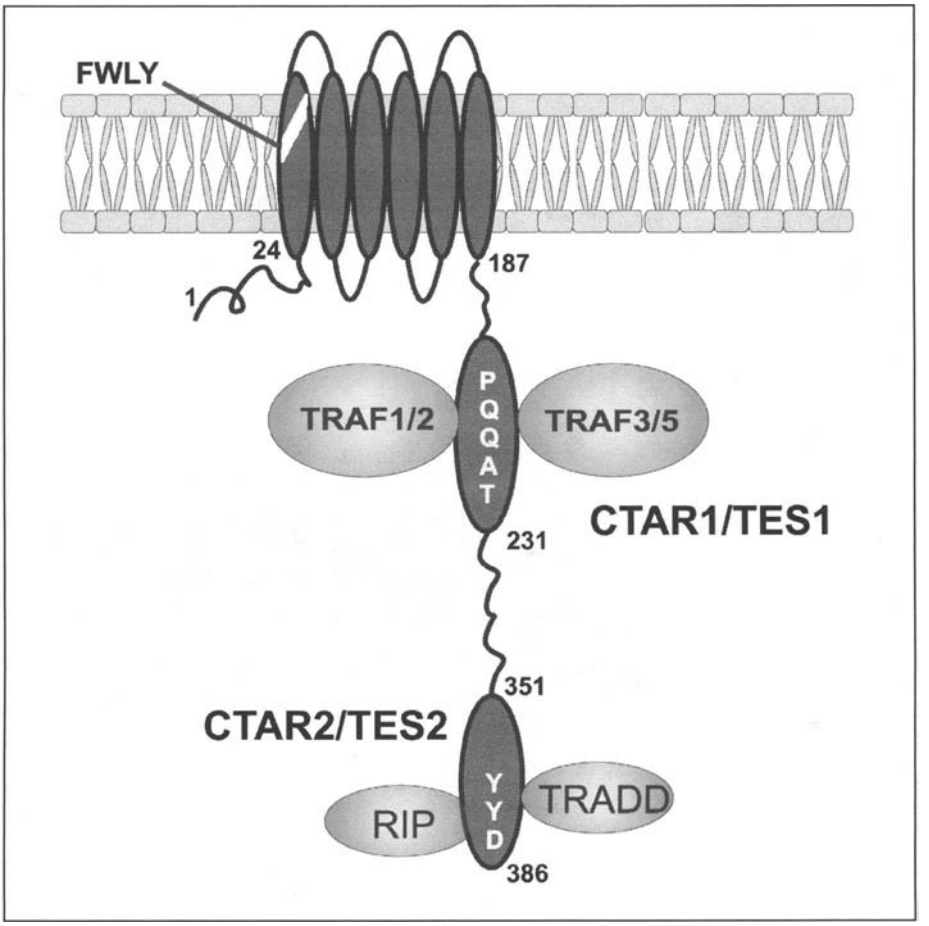

Figure 1. Schematic diagram of LMP1. Various adaptor proteins directly recruited by TES1 and TES2 are also shown.

infection or cytokine induction, IκB is phosphorylated by IκB kinase (IKK) complex which consists of three core members; IKKα, IKKβ and IKKγ. The phosphorylated IκB is a target for the ubiquitylation and degradation by proteasome-dependent pathway which leads to translocation of the dimeric NF-κB complex to the nucleus for transcriptional activation. Two distinct NF-κB pathways have been recognized:[91-93] in one pathway, termed as canonical pathway, IKKβ and IKKγ are critical for nuclear translocation of heteromeric p65 DNA-binding complexes whereas IKKα is nonessential;[94-96] and second, termed as noncanonical/alternate pathway, where IKKα is essential for p100 processing into p52 by BAFF, CD40, lipopolysaccharide (LPS) and LTβ. NF-κB Inducing Kinase (NIK), a member of MAP3K superfamily, is critical for phosphorylation and activation of IKKα in noncanonical pathway.[97-103]

The role of TRAFs in LMP1 mediated activation of NF-κB and AP-1 family factors came into limelight with the discovery of interaction between TRAF1, TRAF3 and LMP1 C-terminus.[18] TRAFs in LMP1 signaling has been a topic of considerable interest since then. Continuous and dedicated efforts of various groups worldwide have shed some light on some of the important players and their probable role in LMP1 signaling.

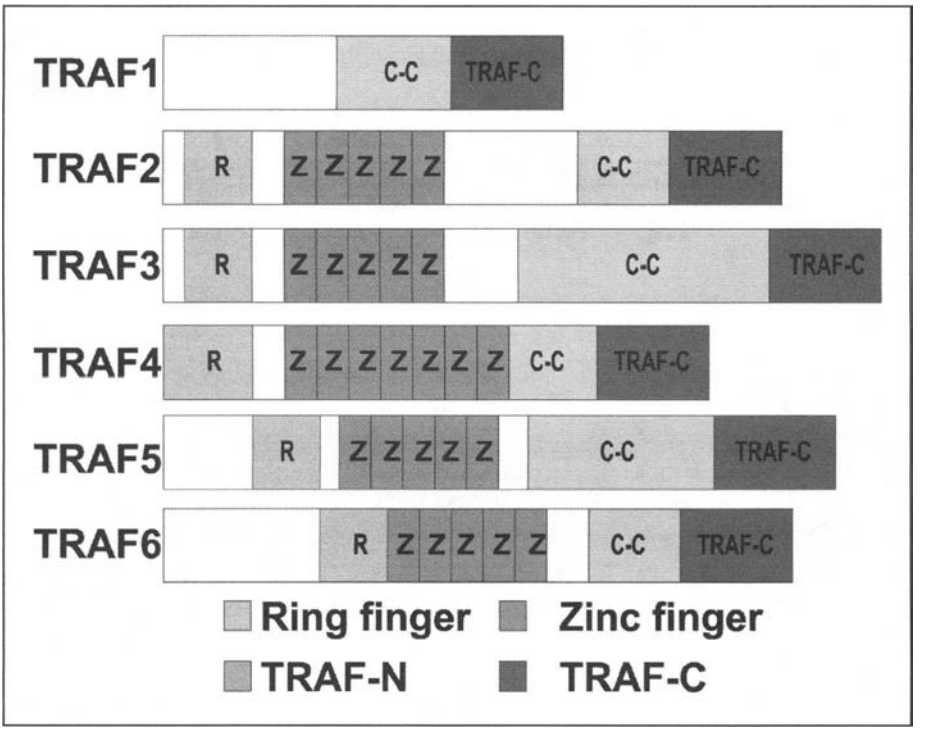

Figure 2. Box diagram of mammalian TRAFs.

LMP1 TES1 and TES2 Recruit TRAFs

The effects of LMP1 in activation of expression of ICAM-1, LFA-1, Bcl-2, CD23, A20, and HIV led to the discovery that LMP1 activates NF-κB.[15,104] Coincident with the identification and localization of TES1 and TES2 to the CT first and last 36aa,[20,35] NF-κB activation was similarly localized to C-Terminal Activation Regions, CTAR1 and 2.[105,106] LMP1 C-terminus was simultaneously discovered to engage TRAF1 and TRAF3, establishing LMP1 signaling to be through TNF Receptor pathways and to mimic CD40 in engaging TRAF3 in TES1/CTAR1.[18]

LMP1 activates various signaling pathways thorough its two effecter sites; TES1/CTAR1 and TES2/CTAR2 at the C-terminus (Fig. 1). TES1/CTAR1 has a PXQXT TRAF biding motif whereas TES2/CTAR2 has a YYD motif which recruits Death Domain containing proteins such a TRADD, Receptor Interacting Protein 1 (RIP1).[18-21,32,35,40,41,105-113]

LMP1 TES1 Directly Recruits TRAFs 1, 2, 3 and 5

LMP1 TES1 directly associates with a substantial fraction of the cellular TRAF1 and TRAF3 and lower levels of TRAF2 and TRAF5.[39,40,112] TRAF1 immunoprecipitates from LCLs specifically coprecipitates TRAF2 and LMP1 whereas TRAF3 immunoprecipitates specifically coprecipitate TRAF5 and LMP1. Thus, LMP1 recruits TRAF1/2 and/or TRAF3/5 heterodimers and this heterodimer binding is mutually exclusive. LMP1 also stabilizes soluble TRAF1 heterodimers with TRAF2 and TRAF3 heterodimers with TRAF5.[40] LMP1 interacts with TRAFs through a PXQXT motif that is conserved in rhesus and baboon LMP1 and other TNFR family members such as CD40 and CD30.[107,112,114,115] Mutation of either P to A or Q to A in LMP1 disrupts TRAF1 and TRAF2 binding with almost no effect on TRAF3 association. In contrast, double mutation of P and Q to A disrupts binding of all the TRAFs.

Consistent with the mutational studies, cocrystalization of a peptide encompassing residues P_{204}QQATDD$_{210}$ of LMP1 with the TRAF domain of TRAF2 shows that LMP1 P_{204}QQATDD$_{210}$ interacts with the outer beta sheet surface of a canyon in each TRAF2 monomer in the trimeric TRAF2 structure.[113] The canyon would significantly hinder interaction of adjacent LMP1 residues with other proteins. LMP1 interaction with TRAF3 appears to be slightly different in that LMP1 D_{210} interacts with TRAF3 Y_{395} and R_{393} forming a more stable complex.[116] The LMP1 TRAF binding site is almost quantitatively associated with TRAF molecules in EBV transformed B lymphocytes, leaving little opportunity for the TRAF binding site to participate in other interactions.[40]

LMP1 TES2 Indirectly Recruits TRAF6

TRADD and RIP binding to LMP1 TES2 likely function to recruit TRAF6.[20,117] TRAF6 does not bind to PXQXT motif instead it binds to PXEXX (aromatic/acidic residue) motif[118] which is not present on LMP1 C-terminus. Consistent with this, any effort to show direct binding of TRAF6 with LMP1 has failed (unpublished observation). Using chemical crosslinking agents which allow evaluation of weak or indirect interactions, LMP1 successfully coimmuneprecipitates TRAF6 through BS69.[119,120]

LMP1 Alters TRAF Partitioning

LMP1 Partitions into Various Cellular Fractions

A significant fraction of LMP1 is associated with a cholesterol rich membrane microdomain termed rafts.[25,27] Biochemical and reverse genetic analyses indicate that raft localization is mediated by the LMP1 trans-membrane domains; the cytosolic C-terminus does not appear to have any role.[27,28] Raft localization is critical for signaling by CD40, TNFR1, RANK, Fas (CD95) and many other signaling molecules.[121-126] Whether LMP1 association with lipid rafts is critical for signaling is not very clear. LMP1 mutated for aromatic amino acids F_{38}WLY$_{41}$ in TM1 does not partition to lipid rafts and does not signal.[28] However, experiments that show LMP1 signaling is diminished by cholesterol depletion and disruption of the raft have not been reported.

LMP1 is significantly associated with the cytoskeleton. After treating B cells with nonionic detergents, a significant portion of LMP1 is found in the pellet fraction and presumed to be cytoskeltal associated.[29] The C-terminal cytoplasmic domain is required for cytoskeletal association. LMP1 strongly associates with vimentin and relocalization of vimentin during mitosis can relocalize LMP1.[29] However, vimentin, per se, is not required for LMP1 effects on lymphocyte activation or adhesion; activation is similar in a lymphoblast that lacks vimentin.[30] Likely, other intermediate filament proteins can substitute.

TRAF binding to LMP1 TES1 likely mediates LMP1 association with the cytoskeleton. TRAF3 can stably associate with microtubules through MIP-T3.[127] CD40 activation detaches TRAF3 from MIP-T3.[127] Since LMP1 is similar to a constitutively active CD40 in biochemical and cell signaling effects, LMP1 might be expected to constitutively detach TRAF3 from microtubules. TRAF2 associates with filamin.[118] TRAF2 association with filamin has been implicated in enabling downstream signaling effects.

LMP1 appears to be stabilized by association with the cell cytoskeletal. In pulse labeling experiments, LMP1 moves from the nonionic detergent soluble cell fraction to the insoluble cytoskeleton associated fraction, where the LMP1 half life is somewhat extended.[29,38,128-130] The temporal sequence of LMP1 association with rafts, cytoskeleton, and downstream signaling molecules has not as yet been analyzed.

TRAF Redistribution by LMP1

LMP1 expression leads to redistribution of TRAFs into various cellular fractions. Stimulation of a chimera composed of the extracellular and transmembrane domains of human CD40 and the C-terminus of LMP1 (hCD40LMP1) expressed in a murine B lymphoma redistributes TRAFs 1, 2 and 3 from 1% Brij58 soluble to insoluble complex. TRAF binding motif of TES1/CTAR1 is

critical for this redistribution. Interestingly, TRAFs 5 and 6 partioning into the soluble and insoluble fractions does not change with stimulation of hCD40LMP1.[131-133] Wildtype LMP1 expression in HeLa or BJAB also partitions TRAF3 into rafts and the insoluble fraction whereas partitioning of TRAF1 and TRAF2 is not effected much.[27,134]

LMP1 Carboxy Terminal Signaling and TRAFs

The LMP1 C-terminal cytoplasmic domain has two signaling components that are critical for LMP1 effects in latency III mediated LCL outgrowth; aa187-231 (TES1), which engage TRAFs and aa351-386 (TES2), which engage death domain proteins, such as TRADD and RIP.[19,20,40,104,105,135,136] These two domains have also been referred to as C-Terminal Activation Regions (CTAR1 and 2) (Fig. 1). Both sites independently activate NF-κB and are critical for LCL outgrowth. LMP1 CTAR1/TES1 $PQQAT_{208}$ binds to TRAFs 3, 5 and 1, 2.[40] CTAR1/TES1 mutation $AQAAT_{208}$ abrogates TRAF binding and NF-κB activation from this domain.[40] EBV mutants that harbor LMP1 mutations that delete the TRAF binding site fail to transform B-lymphocytes into LCLs.[19] Mutations $Y_{384}YD$ to ID or Y_{384} to G abrogate CTAR2/TES2 interaction with TRADD or RIP and NF-κB activation by this domain.[20,111,135] LMP1 $Y_{384}YD$ to ID or C-terminally truncated at aa232 can initially transform B cells but are severly impaired for outgrowth.[34] Cocultivation with fibroblast feeders can partially complement for the absence of either TRAF or TRADD binding.[35,137] CTAR1/TES1 and CTAR2/TES2 are also sufficient for B cell transformation in vitro. LMP1 deleted for aa 232-351 can fully enable LCL outgrowth, indicating that aa 232-351 (a potential JAK3 binding site) is not critical for LMP1 growth or survival effects as measures in LCL outgrowth.[135,138]

Since both CTAR1/TES1 and CTAR2/TES2 are required for efficient long term LCL outgrowth, both contribute a unique signal necessary for cell growth. LMP1 CTAR2/TES2 weakly binds TRADD and RIP,[20,117] without propagating a death signal, and substantially activates IKKβ, phosphorylation of IκBα resulting in canonical NF-κB activation.[45,139] CTAR1/TES1 strongly binds TRAFs at a very high level resulting in strong activation of NIK and IKKα phosphorylation of p100 NF-κB2, leading to p52/Rel B nuclear translocation.[45,52,139,140] CTAR1/TES1 may also induce canonical NF-κB activation as LCLs transformed with virus expressing LMP1 1-231 have the same complement of nuclear NF-κB complexes as wild type LCLs. Nuclear NF-κB complexes include p52/RelA and /RelB heterodimers, p50/RelA, /RelB, and /c-Rel heterodimers, and p50/p50 homodimers.[34]

TRAF2 Is Important for LMP1 TES1/CTAR1 Mediated Noncanonical NF-κB Activation

Although TRAF1 and TRAF3 heterodimerize with TRAF2 and TRAF5, respectively, and TRAF 2 and 5 can activate NF-κB, LMP1 does not require TRAF2 or TRAF5 for RelA nuclear localization in MEFs.[51] LMP1 activates RelA nuclear localization to similar levels in TRAF2/5 double knockout and normal mice. However, LMP1 TES1/CTAR1 mediated NF-κB activation is dependent on TRAF2 (Soni V. et al unpublished). In TRAF2 KO MEFs only TES2/CTAR2 mediated NF-κB activation can be observed. LMP1 TES1/CTAR1 mediated NF-κB activation critically requires a functional NIK, IKKα and p52 (Soni V. et al unpublished). Since TRAF2 can interact with NIK and IKKα (68-70), TRAF2 likely functions as an adaptor for LMP1 TES1/CTAR1 mediated TRAF2/NIK/IKKα/p52 noncanonical NF-κB pathway (Fig. 3).

The ability of LMP1 CTAR1/TES1 activation of p52 complexes may account for requirement for initial transformation, mediated preferential induction of TRAF1, EBI3 or EGFR expression and fibroblast independent initial LCL outgrowth. In epithelial cells, LMP1 induces p52/RelA and p52/RelB heterodimers, p50/p52 heterodimers and p50/p50 homodimers. CTAR1/TES1 null mutants induce p52/RelA and weakly induce p50/p50 homodimers, but do not induce p50/p52 or p52/RelB. The failure of CTAR1/TES1 mutants to induce p52/RelB and p50/p52 correlates with an inability of CTAR1/TES1 mutants to induce EGFR expression in these cells.[110,141] In B cells, p52/RelB activation by CTAR1/ TES1 may be critical for TRAF1 induction and fibroblast independent initial LCL outgrowth.[19,39,52,139,140]

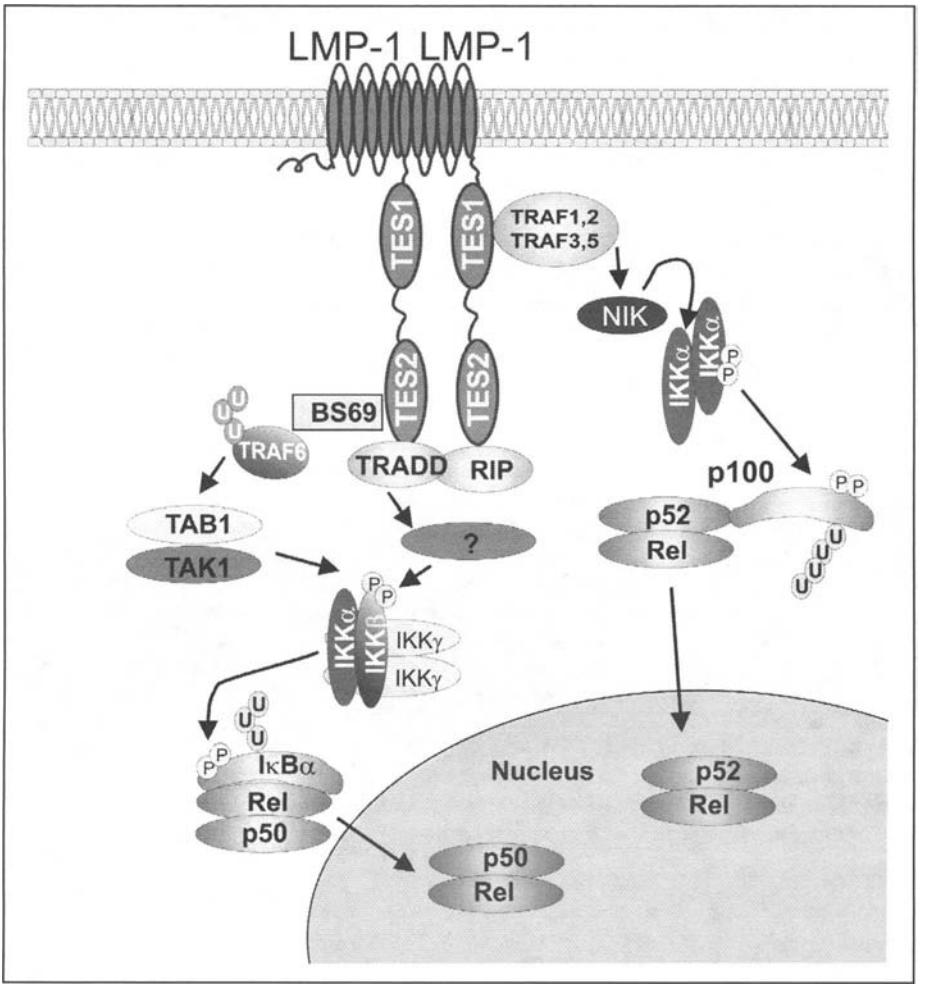

Figure 3. LMP1 mediated NF-κB activation pathways.

TRAF6 Is Important for LMP1 TES2/CTAR2 Mediated Canonical NF-κB Activation

TRAF6 binds to LMP1 C-terminus indirectly and is, along with IRAK1, essential for LMP1 mediated NF-κB and JNK activation in MEFs.[51,57,119] However MEFs also do not express TRAF1 and this can increase LMP1 dependence on CTAR2/TES2 mediated signaling.[11,40,50] A careful and detailed analysis of LMP1 mediated NF-κB activation in TRAF6 KO MEFs shows that TES2/CTAR2 critically require TRAF6 whereas TES1/CTAR1 mediated NF-κB activation is independent of TRAF6 (Soni V. unpublished data and see ref. 142). LMP1 also mediates K48 and K63 ubiquitination of TRAF6 (Soni V. unpublished). In IL-1R or TLR signaling pathway K63 ubiquitination of TRAF6 recruits TAB2 (TAK1 binding protein 2) and activates TAK1 (TGFβ-activated kinase) which further phosphorylate and activates IKK.[82,143,144] TAK1 is important for LMP1 TES2/CTAR2 mediated JNK and NF-κB activation (Soni V. unpublished and see refs. 119,142). Interestingly, TAK1 expression plasmid cotransfection into TAK1 KO MEFs shows only a partial dependence of TES2/CTAR2 mediated NF-κB activation on TAK1. LMP1 TES2/

CTAR2 mediated NF-κB activation is independent of MyD88 and IRAK4 (Soni V. unpublished and see refs.51,142) which differentiates LMP1 TES2/CTAR2 signaling from IL-1R/TLR signaling pathway. TAK1 can lead to phosphorylation of IκBα[142] which suggests TAK1 can activate IKKα/IKKβ complex thereby suggesting canonical NF-κB activation from TES2/CTAR2 wherein TRAF6 upon polyubiquitinated at K63 recruit TAB1, TAK1 and probably some other kinase, which leads to activation of IKKs which ultimately leads to IκBα degradation and subsequent nuclear localization of p50/RelA or p50/c-rel and activation of NF-κB responsive genes (Fig. 3).

TRAF3 May Be Central to LMP1 Signaling through TES1/CTAR1 and TES2/CTAR2 in Mouse B-Cells

LMP1 activation of NF-κB, JNK and p38 has been noted to be substantially deficient in a TRAF3 knock out mouse lymphoma cell line. This places TRAF3 in a central role for signaling from both domains.[132,133] However in 293, human epithelial cells, TRAF3 over expression inhibits LMP1 and LMP1 TES1 mediated NF-κB activation.[40] Analyses of LMP1 induced CD23, CD80, and CD95 in $TRAF3^{(-/-)}$ B cells indicate a more complicated dependency on TRAF3 with CD80 induction being almost nil, whereas CD23 induction is ~40%, and CD95 induction is equivalent to that observed in $TRAF3^{(+/+)}$ B cells.[133] Further, LMP1 stimulated IgM secretion through CTAR1 is almost completely TRAF3 dependent, whereas CTAR2 is variably TRAF3 dependent.[132] Moreover, TRAFs 1 and 2 are not necessary for LMP1 mediated JNK activation in $TRAF3^{(+/+)}$ mouse B cells.[133]

TRAF3 has a particularly significant role in directly binding NIK and causing NIK degradation.[143] CD40 or BAFF, which activate NIK in a ligand dependent fashion, cause TRAF3 degradation, releasing and stabilizing NIK to bind to p100, recruit and activate IKKα, and cause NF-κB2/RelB nuclear translocation.[143] LMP1 CTAR1 has the unique ability to aggregate much of the cell TRAF3, but does not mediate its degradation. Possibly, LMP1 sequesters TRAF3 and enables NIK to assemble p100/NIK/IKKα complexes and activate NF-κB2/RelB nuclear translocation.

TRAFs and LMP1 Activation of JNK and p38 Pathways

LMP1 mediated JNK and p38 activation is more CTAR2 dependent in epithelial cells but may be mediated by both CTAR1 and CTAR2 in lymphocytes.[11,119] Deletion of CTAR2 or mutation of CTAR2 Y to G, which abrogate TRADD binding, render LMP1 unable to activate JNK in epithelial cells.[11] However, epithelial cells do not express TRAF1 and TRAF1 expression enables CTAR1 to active JNK and NF-κB in epithelial cells.[50] In B cells and some epithelial cells, CTAR1 can induce TRAF1 and more effectively activate JNK and NF-κB.[40,50] CTAR1 mediated JNK activation in B cells may be mediated by TRAF3 binding to T3JAM, a TRAF3 interacting protein that activates JNK after CD40 stimulation.[145]

Like NF-κB activation, LMP1 mediated JNK activation is dependent on TRAF6 and is independent of IRAK4.[119] IRAK1 is required for LMP1 mediated NF-κB activation but is dispensable for JNK activity.[51,119] LMP1 mediated JNK activation also requires TAB1 and TAK1 and does not require MyD88, TRADD, RIP or TRAF2.[119] Since TRADD binding is essential for TES2 NF-κB activation, another adapter molecule, BS69,[146] must interact with TES2 to recruit TRAF6 to activate the JNK pathway. These data indicate that LMP1 signaling can not be simply modeled after a TNF receptor since most TNFRs require TRAF2 for JNK activation or an IL-1/TLR receptor, since IL-1/TLRs require MyD88 and IRAK4 for signaling.[119] Rather LMP1 seems to function in a unique way that maximizes survival and growth signals, without the propensity to induce apoptosis.

TRAF Induction or Degradation and LMP1

Various TNFR superfamily such as CD40, CD30, TNFRII/CD120b, B cell-activating factor receptor, and receptor activator of NF-κB (RANK) members control the extent and duration of signaling by activating proteasome mediated degradation of TRAFs 2 and 3.[131,143,147-150] Interestingly, LMP1 does not induce TRAF2 and TRAF3 degradation in B- lymphocytes and epithelial cells.[131,134] TRAF degradation is a method for down-regulating a receptor signal cascade, and that inability to initiate TRAF degradation could contribute to the transforming properties of latent membrane protein 1, an oncogenic viral mimic of CD40 produced by the EBV.[131,147,151]

LMP1 Induces TRAF1 Expression

TRAF1 is expressed at a low level in resting B lymphocytes and is strongly up-regulated by NF-κB activation.[39] In epithelial cells and fibroblasts, TRAF1 is frequently not expressed and may be NF-κB inducible. LMP1 TES1/CTAR1 induces TRAF1 expression TRAF1 expression enables TES1/CTAR1 to active JNK and NF-κB in epithelial cells.[50] In B cells and some epithelial cells, TES1/CTAR1 can induce TRAF1 and more effectively activate JNK and NF-κB.[40,50]

Summary

TRAFs play a central role in EBV mediated growth transformation in B-cells. LMP1 TRAF interactions lead to NF-κB activation which leads to increased transcription of various pro-inflammatory and proliferation inducing cytokines and chemokines.[52] LMP1 TRAF interactions and NF-κB activation are critical for LCL survival; NF-κB inhibition induces LCL apoptosis without additional pro-apoptotic stimuli.[17,152] LMP1 mediated TRAF activations also protect LCLs from apoptosis and can protect BL cells from growth factor withdrawal.[106,153] Indeed, virtually all LMP1 effects in LCL growth transformation are mediated through TRAF1, TRAF2, TRAF3, TRAF5 or TRAF6.

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CHAPTER 15

Targeting TRAFs for Therapeutic Intervention

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Abstract

TNF-receptor associated factors (TRAFs) are the molecules that upon engagement of the TNF-receptor (TNFR) by a TNF-family ligand come first in contact with the activated TNFR, initially acting as docking molecules for kinases and other effector proteins that are recruited to the activated receptor. TRAFs later regulate the subcellular relocalization of the receptor-ligand complex and finally they modulate the extent of the response by controlling the degradation of key proteins in the pathway.

In this chapter, we review the involvement of different TRAF family members in the etiology of a variety of pathologies and address the question of whether the use of TNFR-mimic-peptides or small molecule modulators targeting TRAFs might be suitable for therapeutic intervention, discussing the advantages and disadvantages of this strategy.

TNF-Receptor Associated Factors (TRAFs)

A total of seven TRAF-family members participate in the regulation of as many as 20 TNFRs. TRAF3 and TRAF6 are also involved in the regulation of different members of the Toll-like Receptor (TLR) and interleukin-1 receptor (IL-1R) family. Furthermore, TNFR-family members generally utilize more than one TRAF family member for signaling, often activating similar pathways and even the same downstream effectors. Therefore, the levels of expression of the different TRAF-family members and downstream effectors will likely play an important role in the outcome of the response.

The consensus amino-acid motif supporting binding of TRAF1, TRAF2, TRAF3 and TRAF5 to TNFR-family proteins is (P/S/A/T)x(Q/E)E,[1,2] implying that TRAF1, 2, 3 and 5 potentially interact with the same TNFR family members and that they might compete among themselves for the binding. In contrast, the consensus sequence for TRAF6 is PxExx(Ar/Ac) (where the last amino-acid residue is aromatic or acidic).[3] The binding motif for TRAF4 is yet to be identified. TRAF7 lacks a TRAF domain and does not directly interact with TNFRs.[4]

The crystal structures of TRAFs bound to different TNFR family members have confirmed that the peptide core motif provides the specificity of the binding. However, the actual composition of the core motif as well as other amino-acids adjacent to this core can affect the interaction, by establishing molecular interactions with residues in the TRAF-domain, by decreasing the binding affinity by steric impediments or electrostatic repulsions, or by intramolecular interactions that affect the conformation of the TRAF-binding peptidyl motifs. These results provide a molecular explanation for the differences in binding specificity and affinity of the members of the TRAF family for the different TNFR family members.[3,5-9] These results also imply that it would be

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conceivable to design peptides that could act as agonist or antagonist of the function of different TNFR family members, either by modulating the binding of particular TRAF proteins to those receptors; or by activating TRAF-signaling pathways independently of the activation of the TNFR. In this regard, it has been shown that 11-residue linear peptides bearing the intracellular CD40/TRAF binding motif were sufficient to induce NF-κB activation in WEHI-231 lymphoma cells,[10] thus indicating that small peptides can mimic TNFR signaling. Also, Ye et al[3] using RANK peptides mimicking its TRAF6 docking site could block osteoclast differentiation in vitro in both primary cells and cell lines, without affecting cell viability. These results support the suitability of using peptides mimicking TRAF-binding motifs to modulate TRAF-family signaling and associated biological functions.

No nonpeptidyl small molecules that bind TRAFs have been described to date, though it conceivably should be possible to generate such molecules. Nonpeptidyl molecules could afford the advantage of superior cell permeability compared to peptides, and also probably better pharmacological properties in terms of half-life, bioavailability and biodistribution. Structural studies however reveal that the pocket on the surface of TRAFs responsible for binding peptidyl motifs found in the cytosolic tails of TNF-family receptors is somewhat shallows,[3,5-9] which may hinder the ability to generate high affinity antagonists. In this regard, peptides representing core motifs of the TRAF-binding sites of TNFRs typically bind to TRAFs with low affinity. For instance, the interaction between TRAF2 and monomeric receptors is relatively weak (K_d = 0.04-1.5 mM) which ensures that TRAFs do not interact with nonactivated receptors and implyes that multivalency of TRAFs (note that TRAFs and TNFRs are functional as trimeric molecules) may play a large role in generating sufficient free energy to account for binding in vivo.[1]

TRAFs and Disease

TRAFs are emerging as essential components of the TNFR-family signaling, acting as coordinators of the downstream signaling pathways and consequently having a key role in the outcome of the response. Not surprisingly, growing evidence is pointing out a direct involvement of TRAFs in different pathologies. An overview of some of the pathologies where manipulation of TRAF activities might have therapeutic interest is discussed below.

TRAF2 and Chronic Lymphocytic Leukemia

Recent results from our laboratory have revealed a tumor suppressor role for TRAF2 in B lymphocytes. Transgenic mice with B cells lacking functional TRAF2 and overexpressing Bcl-2 developed Small B cell lymphoma/Chronic Lymphocytic leukemia (SBL/CLL) with high incidence.[11] The mechanism underlying the tumor suppressor function of TRAF2 might involve its role in the control of apoptosis in B cells. In this regard, we and others have shown that TRAF2-deficient B cells are more resistant to various apoptotic stimuli[11,12] and accordingly, the absence of functional TRAF2 increases B cells numbers in vivo.[13] These results support an important role for TRAF2 in B cell homeostasis. In our transgenic mouse model of SBL/CLL, deregulation of TRAF2 might increase the resistance of subsets of B cells to apoptosis induced by TNF-family members, while overexpression of Bcl-2 increases the resistance of these cells to stimuli involving the mitochondrial pathway of apoptosis, ultimately resulting in the development of malignancies.

Interestingly, TRAF2 is overexpressed in Reed-Sternberg cells from Hodgkin lymphoma patients[14,15] where it is located in cytosolic aggregates.[16] However, TNF failed to induce both TRAF2 translocation to the insoluble fraction and JNK activation in Hodgkin Reed-Sternberg L-428 cells,[17] strongly suggesting that TRAF2 is not fully functional in these cells. In contrast, Reed-Sternberg cells have aberrant constitutive activation of both the canonical and noncanonical NFκB pathways,[17-19] which is also similar to what has been observed in TRAF2-deficient B cells.[12]

Altogether, these results suggest a role for TRAF2 in controlling B cell homeostasis and indicate that inhibition of TRAF2 increases development of B cell malignancies. Consequently, devising strategies aimed to restore TRAF2 expression or function might prove useful for the treatment of certain types of cancer.

TRAF1 in B Cell Leukemia and Lymphoma

Among the members of the TRAF family, TRAF1 shows the most striking deregulation of its expression in B cell malignancies. In normal physiological conditions, expression of TRAF1 has a very restricted pattern. It is only found in some epithelia, dendritic cells and activated lymphocytes.[20,21] In contrast, TRAF1 expression is upregulated in a variety of hematopoietic malignancies, such as chronic lymphocytic leukemias (CLL),[20] nonHodgkin lymphomas (NHL),[22] Reed-Sternberg cells of Hodgkin disease,[14,15] LMP-1 positive post-transplant lymphoproliferative disease and HIV-associated lymphoma,[23] strongly suggesting a possible role for TRAF1 in the etiology of these B cell malignancies.

In this regard, the known functions of TRAF1 are consistent with a role in tumorigenesis. First, TRAF1 protects against apoptosis. TRAF1's anti-apoptotic role might be mediated by its interaction with various anti-apoptotic proteins that it helps to recruit to the activated TNFRs, including the NF-κB inhibitory protein A20, the inhibitor of apoptosis proteins (cIAP-) 1 and 2, and FADD-like interleukin-1β converting enzyme (FLICE)-like inhibitory protein (FLIP).[24] Indeed, TRAF1 recruitment of cIAP1 and cIAP2 to TNFR1 seems to inhibit receptor-mediated caspase-8 activation.[25] Consistent with the anti-apoptotic function of TRAF1, epithelial cells lacking TRAF1 were more sensitive to apoptosis induced by TNF,[26] and TRAF1-deficient dendritic cells displayed severely impaired survival in response to TNF and CD40L.[27] Furthermore, enforced expression of TRAF1 in T cells blocks apoptosis of reactive T cells thus preventing antigen-induced tolerance.[28] TRAF1 overexpression was also able to partially protect TRAF2 -/- MEF cells from TNF-mediated apoptosis.[29]

Second, considerable evidence supports a role for TRAF1 in the regulation of TRAF2 activities, with TRAF1 primarily operating as an antagonist of TRAF2. In this regard, TNF was able to induce NF-κB and JNK activation more efficiently in *TRAF1*-deficient T cells than in normal T cells, an effect that was dependent on TNFR2[26] and would likely involve a more efficient TRAF2 recruitment to the activated receptor in the absence of TRAF1. Furthermore, an excess of TRAF1 abrogated the interaction of TRAF2 and CD40, with the consequent inhibition of CD40-dependent NF-κB activation.[30] Conversely, down-regulation of TRAF1 with small interfering RNAs enhanced CD40/CD40L-induced NF-κB activation. Interestingly, TRAF1 expression disrupted the subcellular relocalization of TRAF2 and its association to cytoskeleton in CD40-activated cells.[27]

In summary, TRAF1's upregulation in leukemia and lymphoma, its anti-apoptotic functions, and its role as a TRAF2 inhibitor make it a likely candidate to be implicated in the etiology of B cell malignancies. Therefore, development of peptidomimetics or small molecule inhibitors that interfere with TRAF1 functions might be useful for treating those leukemias where upregulation of TRAF1 is a hallmark, although additional research is needed to elucidate the actual role of TRAF1 in the etiology of these diseases.

Caveats of Targeting TRAF1 and TRAF2

As indicated above, interfering with TRAF1 function in B cell malignancies could hypothetically improve the outcome of the disease by, for instance, sensitizing these malignancies to apoptosis-inducing cytokines and possibly other types of apoptosis inducers. However, mice deficient in TRAF1 are hyper-responsive to TNF and, as a result, they display hyper-proliferation of T cells and suffer from skin epithelium apoptosis,[26] as well as TNF-mediated acute liver injury.[31] Interfering with TRAF1 function might consequently enhance TNFR1 and TNFR2 responses and thus predispose to autoimmunity and chronic inflammation. In this regard, increased TNF produced by reactive leukocytes is a common feature of several autoimmune diseases, including rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis and other chronic inflammatory diseases. For example, excessive production of TNF can drive synovial inflammation and degradation of articular cartilage and bone, which are common features of RA (reviewed in ref. 32). In Crohn's disease, high levels of TNF cause inflammation of the digestive track.[33] Thus, even if TNF levels remain normal, targeting TRAF1 might increase the responsiveness of T lymphocytes (and maybe other cell types) to this lymphokine, causing autoimmunity. These potential side-effects of TRAF1

antagonists might be counteracted by treating patients with commercial biological anti-TNF agents, such as etanercept, infliximab and adalimumab, but are nevertheless worrisome.

TRAF2-deficient and TRAF2-dominant-negative (DN) mice have severe defects in T cell function, and fail to mount a cytotoxic response in mixed lymphocyte reaction assays,[34-36] thus highlighting an important role for TRAF2 in the control of cytotoxic T-cell responses. Therefore, it is conceivable that blocking TRAF2 function might have positive implications for transplantation, ameliorating host versus graft disease.[36] However, TRAF2-deficient macrophages produce increased amounts of nitric oxide and TNF in response to TNF stimulation[35] and mice lacking TRAF2 also develop cachexia as a result of the increased levels of TNF.[34,35] Thus, enhancing the pro-inflammatory effects of macrophages by targeting TRAF2 would not be an acceptable outcome.

In addition, it is important to mention that the mechanism by which TRAF2 operates as a tumor suppressor in B cells is unknown, but could be related to its role as a regulator of TNFR-mediated apoptosis.[2,37] However, the role of TRAF2 in controlling apoptosis might be cell dependent and/or TNFR dependent. In this regard, there is evidence supporting an anti-apoptotic function for TRAF2 in thymocytes,[34] muscle[38] and fibroblasts,[39,40] further cautioning about the use of TRAF2 modulators in therapy.

TRAF3 and EBV-Mediated Diseases

Epstein Barr virus (EBV) is a member of the herpes virus family that infects over 90% of the world adult population. It persistently infects B lymphocytes, although rarely causing disease. However, immunosuppressed carriers infected with EBV might be prone to develop different pathologies of lymphoid origin, such as infectious mononucleosis, X-linked lymphoproliferative disease, B lymphoproliferative disease, Burkitt's lymphoma Hodgkin's disease and nasopharyngeal carcinoma, among others.[41] Different proteins encoded by the EBV genome are involved in the control of proliferation and survival of the infected cell, and therefore are essential for the persistence of the infection and eventually for the development of the overt pathology. However, latent membrane protein (LMP)-1 is the only EBV-encoded protein that seems to be sufficient to induce oncogenic transformation of mammalian cells[42,43] and to sustain the development of lymphoma in at least one transgenic mouse model.[44,45] Furthermore, ample evidence exists supporting a key role for LMP-1 in the etiology of EBV-associated lymphoproliferative disease and lymphomas.[41,43,46,47]

Several reports demonstrate a role for TRAF-family members in LMP-1 signaling. TRAFs associate with LMP-1 through its C-terminal activating region (CTAR)-1, encompassing amino-acids 194 to 232.[48-50] It has been suggested that LMP-1 mimics CD40 and utilizes similar signal transduction pathways (reviewed in refs. 47,51,52). However, LMP signals in a seemingly deregulated manner, leading to amplified and sustained B cell activation.[53,54] Both CD40 and LMP-1 recruit TRAFs to lipid rafts, a class of nonionic detergent-insoluble, sphingolipid-enriched membrane microdomains.[54-57] However, recent investigations have highlighted significant differences in the usage of TRAFs by CD40 and LMP-1. Thus, TRAF3 is more efficiently recruited to LMP-1 than to CD40, while TRAF2 seems the opposite.[49,57] Furthermore, the crystal structure of the LMP-1 peptide ^{204}PQQATDD^{210} encompassing the CTAR-1 bound to TRAF3[58] shows that both LMP-1 and CD40 bind the same TRAF3 crevice.[7] However, CTAR-1 also forms additional hydrogen bonds that stabilize its interaction with TRAF3. Thus, LMP-1 has a higher affinity for TRAF3 than CD40. These observations suggest that LMP-1 mimicking peptides might be more potent as competitive antagonists of TRAF3, compared to peptidyl inhibitors based on the sequence of various TNF-family receptors.

TRAF1[59] and TRAF6[60-62] have been also implicated in LMP-1 signaling, but additional in vivo data are necessary to determine the actual roles of these two TRAFs in LMP-1 signaling under physiological conditions. Overall, the available data are consistent with the critical role played by TRAF3 in LMP-1 signaling, as illustrated by the abrogation of LMP-1 signaling in TRAF3 deficient cells.[63-65] If the essential role of TRAF3 in LMP-1 signaling is confirmed, targeting TRAF3 binding to LMP-1 would be a reasonable strategy for treating EBV-related diseases.

TRAF3 and Mantle Cell Lymphoma

It has been recently reported that TRAF3 and TRAF5 are upregulated in splenic marginal zone lymphoma (MZL).[66] TRAF3 has been shown to be an inhibitor of TNFR-family mediated NF-κB activation.[67] However, TRAF3 can form heterotrimers with TRAF5,[68] and TRAF5 is able to induce NF-κB activation.[69,70] Therefore, since both TRAF3 and TRAF5 are upregulated in MZL, the formation of these heterotrimers might be favored and support the induction of NF-κB activity. Also, it is important to note that TRAF3 seems to work as an inhibitor of various TRAF2-mediated functions[71] and in some context, it might have functions similar to TRAF1.

TRAF3 and Autoimmunity

Immune tolerance ensures an inability of the cellular components of the immune system to react to self-antigens while preserving defenses against pathogens. Several safeguard mechanisms are in place to protect the organism from autoreactive lymphocytes and autoantibodies, and their failure results in autoimmune diseases. One of these control mechanisms is the elimination of autoreactive B and T cells by apoptosis. Blockage of cell death pathways in the immune cells can therefore result in autoimmunity and/or cancer.

The autoimmune pathologies caused by BAFF deregulation deserve special mention. BAFF (TNFSF13B) is a TNF-family member required for survival of transitional and mature B cells[72,73] and which is essential for later stages of B cell maturation and for Mantle Zone (MZ) B cell differentiation (reviewed in ref. 74). BAFF expression is deregulated in several autoimmune diseases and other pathologies. For instance, BAFF levels are elevated in sera from patients with severe B cell autoimmune disorders, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome.[74] Higher levels of BAFF are also found in the sera of human immunodeficiency virus (HIV) patients, which are prone to develop SLE.[75] Furthermore, BAFF and BAFF-R (TNFRSF13C) overexpression has been also described in several B cell malignancies, such as multiple myeloma,[76,77] nonHodgkin's lymphoma[78,79] and B-cell chronic lymphocytic leukemia (B-CLL). Indeed, most B-CLL cells express BAFF-R mRNA and a subset display BAFF on the surface, suggesting that BAFF might operate as an autocrine survival factor for B-CLL,[80-82] in addition to promoting autoimmune manifestations observed in B-CLL patients (review in ref. 83). Furthermore, chronic infection may also lead to the sustained release of BAFF and thus the emergence of autoimmunity. Consistent with these results, BAFF-transgenic mice developed immunoglobulin-based autoimmune disorders similar to systemic lupus erythematosus (SLE) and Sjögren's syndrome,[84-87] thus proving the direct involvement of BAFF in the development of autoimmunity.

BAFF-mediated autoimmunity seems to be result of the preservation of maturing autoreactive T2 B cells which colonize forbidden follicular and marginal zone microenvironments.[88,89] Survival of these cells causes a dramatic alteration of peripheral tolerance and the development of autoimmunity. Several lines of evidence indicate that among the different TNF-family receptors that can interact with BAFF, the BAFF-R (TNFRSF13C) protein is the one primarily responsible for increasing B cell survival (reviewed in ref. 74).

Little is known about the signal transduction pathways utilized by BAFF-R. TRAF3 might be the only member of the TRAF family that interacts with BAFF-R.[90,91] The specificity of this interaction seems to be mediated by the sequence motif ^{162}PVPAT^{166}, which is different from the canonical TRAF1/2/3/5-binding motif. Furthermore, other amino-acids in the cytosolic tail of BAFF-R participate in the stabilization of the complex.[9,90,91] It is well established that BAFF-R signaling induces the activation of the noncanonical NF-κB pathway.[92,93] However, the role of TRAF3 in this process is conflicting. Experiments involving TRAF3 overexpression indicate that it inhibits BAFF-R-mediated NF-κB activation and IL-10 production, thus supporting a role for TRAF3 as a negative regulator of at least some of the signaling events mediated by BAFF-R.[90] Conversely, mutations in the ^{162}PVPAT^{166} motif that abolished TRAF3 interaction with BAFF-R abrogated BAFF-R ability to activate the noncanonical NF-κB pathway.[91] These seemingly opposite results could be explained if the activation of the noncanonical NF-κB pathway by BAFF-R requires receptor-mediated degradation of TRAF3.[94]

It is worth noting that Hauer and coworkers[67] have recently shown that TRAF3 is a general inhibitor of TNFR-mediated noncanonical NF-κB activation, which may preclude its use as a drug target. However, if TRAF3 is indeed the only member of the TRAF family that regulates BAFF-R signaling, then TRAF3 would be a worthy target for therapeutic intervention against SLE and Sjögren's syndrome. Resolution of the question of whether TRAF3 is the only TRAF-family member capable of binding BAFF-R thus is required to direct future possible therapeutic strategies.

Caveats of Targeting TRAF3

Mice lacking TRAF3 have hypoglycemia and high glucocorticoid levels in serum, which results in depletion of peripheral white cells. These mice also develop cachexia and die by day 10 after birth. TRAF3 is prominently expressed in adrenocorticotropin hormone (ACTH)-secreting cells in the hypophysis.[95] Altogether, these results strongly support a role for TRAF3 in the regulation of ACTH production. Consequently, targeting TRAF3 might result in severe alterations in the metabolism of glucocorticoids.

TRAF3 and TRAF6 in Infections and Septic Shock

Toll Receptors (TLR) are key players in the regulation of innate immune responses.[96-98] Ten TLR family members have been identified in humans. These receptors recognize pathogen-associated molecular patters (PAMPs), triggering host defense responses as part of innate immunity. Different TLRs recognize distinct PAMPs. Thus, bacterial lipoproteins are recognized by TLR2, double stranded DNA by TLR3, bacteria lipopolysaccharide by TLR4, flagellin by TLR5, single-stranded viral RNA by TLR7, and unmethylated CpG DNA of bacteria and viruses by TLR9 (reviewed in ref. 97). Important for the host responses against pathogens are also the members of the IL-1R family, which regulate inflammation responses.[99,100]

Alterations in TLR structure, expression, and function have been implicated in several diseases. In this regard, polymorphisms of proteins in the TLR pathways are related to anomalous responses against pathogens, and have been correlated with immunoinsufficiency (i.e., chronic infection), atherosclerosis, cancer, and asthma.[101]

TRAF6 is a common and critical mediator of signal transduction by the TLR/IL-1R family.[96,102] This is well illustrated in *traf6* deficient mice, which have severely impaired TLR-mediated responses to various PAMPs[103,104] and fail to properly respond to IL-1 stimulation.[103,105]

TRAF6 does not directly interact with either TLRs or IL-Rs. Instead, TIR domain adaptors, like MyD88, TIRAP, TRIF and TRAM, as well as IRAK-family proteins, mediate its recruitment to the receptors. Then, IRAKs and TRAF6 dissociate from the complex, allowing TRAF6 to interact with ubiquitin conjugating enzymes Ubc13 and Uev1A. These enzymes covalently attach noncanonical poly-ubiquitin chains to TRAF6, in which the isopeptide bond occurs at the lysine 63 residue in ubiquitin, instead of lysine 48. This form of polyubiquitin does not target TRAF6 for degradation, but rather induces TRAF6 to associate with a complex composed by TAB1, TAB2 and transforming growth factor β activating kinase (TAK)-1, resulting in TAK1 phosphorylation and activation. Activated TAK-1 then activates the IκB kinase kinase (IKK) complex and also activates MAP kinase kinase (MKK)-6, resulting in NF-κB and c-JUN (AP-1) activation, and the induction of expression of multiple proinflammatory genes.[106,107]

TRAF6's role as a mediator of TLR/IL1R family signaling, makes it an attractive drug target for possible use in treatment of a wide variety of acute and chronic inflammatory conditions. Septic shock provides a good example. Studies in traf6-/- mice have shown profound impairment of TLR-mediated responses to different PAMPs[103,104] supporting the notion that TRAF6 might be a suitable target in severe cases of infection. In this regard, the lethal consequence of systemic bacterial invasion have been linked to overstimulation of the TLR pathways, resulting in massive production of pro-inflammatory cytokines, causing severe systemic inflammation that may progress to multiple organ failure and death even after the bacterial infection has been clinically controlled.[108-111] Septic shock is associated with a 30-50% death rate in severe cases,[109,110] accounting for over 100,000 deaths annually in the United States alone.[101]

Interestingly, recently it was reported that TRAF3 deficient cells fail to induce type I interferons and anti-inflammatory cytokines in response to TLR activation, which has led to the identification of a new TRAF3 dependent pathway involved in the control of innate immunity.[112,113] Similar to TRAF6, the TRAF3 protein could be recruited to the TLRs through MyD88 and IRAK1 and 4, but rather than activating MAP3K and IKK, which induce pro-inflammatory cytokines, TRAF3 engages TRIF-dependent signaling pathways leading to activation of TBK-1 and IKK-ε, inducing the expression of type I interferons and the anti-inflammatory IL-10.[112,113] Thus, TRAF3 may play important roles both in interferon-dependent responses to viral pathogens, as well as in down-regulating innate immune responses via its effects on IL-10 production. Therefore, by pharmacologically modulating the recruitment of either TRAF3 or TRAF6 to the activated TLR, or by interfering with their downstream functions, it may be possible to manipulate the type of response emanating from TLRs, depending on the pathogen, stage of infection, or other scenarios.

TRAF4 might also function as a silencer of TLR-signal transduction through its association to TRAF6 and TRIF,[114] but additional in vivo data using TRAF4 deficient cells or TRAF4 knock-out mice would be required to ascertain the role of TRAF4 in innate immunity.

TRAF6 and Other Diseases

The analysis of the phenotype developed by TRAF6-deficient mice has highlighted a seminal role of TRAF6 in the regulation of signaling by various TNFR-family members. These results suggest additional avenues for the usage of TRAF6 agonists and/or antagonists as therapeutics. For instance, TRAF6 is a critical regulator of RANK. This TNFR family member is essential for the differentiation and activation of osteoclasts, the cells responsible for bone resorption.[115,116] This is demonstrated by the phenotype developed by mice deficient in RANK or its ligand (RANKL), which are osteopetrotic as the result of lack of bone resorption and remodeling caused by functionally deficient osteoclasts.[116] TRAF6 is essential for RANK signaling and consequently it is required for osteoclast cytoskeletal organization and resorptive function.[117] Accordingly, TRAF6 deficient mice lack functional osteoclasts and develop severe osteopetrosis.[103,105]

X-linked hypohidrotic ectodermal dysplasia is a genetic disorder characterized by lack or anomalous formation of hair follicles, teeth and sweat and sebaceous glands. Affected children have a reduced ability to sweat, which can result in life-threatening high fever.[118,119] This disease is caused by mutations of the ectodysplasin A gene (Eda) encoding the TNF family ligands EDA-1 and EDA-2, which interact with the TNFR-family members EDAR and XEDAR, respectively.[120-122] Besides EDAR and XEDAR, the TNFR family member TROY might also regulate the development of these epidermal appendages.[123] TRAF6 deficient mice also develop a phenotype similar to hypohidrotic ectodermal dysplasia.[124] In this regard, TRAF6 interaction and regulation of XEDAR and TROY has been reported.[123,125] However, given the total absence of sweat glands in *traf6* -/- mice, it is suspected that TRAF6 might also participate in the control of EDAR activities.[124]

Caveats of Targeting TRAF6

In summary, the key role of TRAF6 in innate immune responses, as well as in bone formation and resorption, and hair follicle formation opens the possibility of using TRAF6 modulators for treating diseases such as septic shock, osteoporosis, arthritis, periodontal disease, cancer-induced bone lesions and even alopecia.[126] However, blocking TRAF6-mediated signaling would increase the risk of opportunistic bacterial infections, which might preclude the use of drugs targeting TRAF6 for chronic diseases and immunosuppressed patients. On the other hand, as a short-term treatment, it might prove helpful for reducing the mortality associated with septic shock by shutting down TLR-mediated induction of pro-inflammatory cytokines.

Perspectives

The various phenotypes of the TRAF-specific knock-out and TRAF-transgenic mice have brought to light the pleiotropic roles of TRAFs in cell physiology and have warned of the adverse effects of dysregulating their expression and function. Studies of genetically engineered mice, however, have

also uncovered the participation of TRAFs in processes relevant to several human diseases for which new therapeutic approaches are desperately needed (Fig. 1).

Despite the difficulty in identifying small molecule modulators that can either disrupt or enhance specific protein-protein interactions, the development of new screening and structure-based drug design technologies raises optimism. Thus, the application of high throughput screening technologies to test large synthetic and natural chemical compound libraries, as well as structured-based drug design will likely identify compounds capable of interfering with the functions of specific members of the TRAF family or other proteins in the pathways that are dependent on TRAFs. In this regard, recent articles have shown the potential of these technologies for modulating the activity of TNF-family proteins. Thus, Takasaki and coworkers[127] have identified exocyclic small peptidomimetics corresponding to critical binding sites in the TNFR1 that prevent TNF-mediated apoptosis. He and coworkers[128] have identified a small-molecule inhibitor of TNF that binds trimeric TNF and promotes subunit disassembly and its functional inhibition. Also, Fournel and coworkers[129] have reported the structure-based design of small molecules with C3 symmetry that mimic CD40L and act as agonist of CD40 functions. Altogether, these results provide proof of concept that similar approaches could result in the identification of compounds that modulate TRAF-trimerization or their association with TNFRs and other proteins in the pathway.

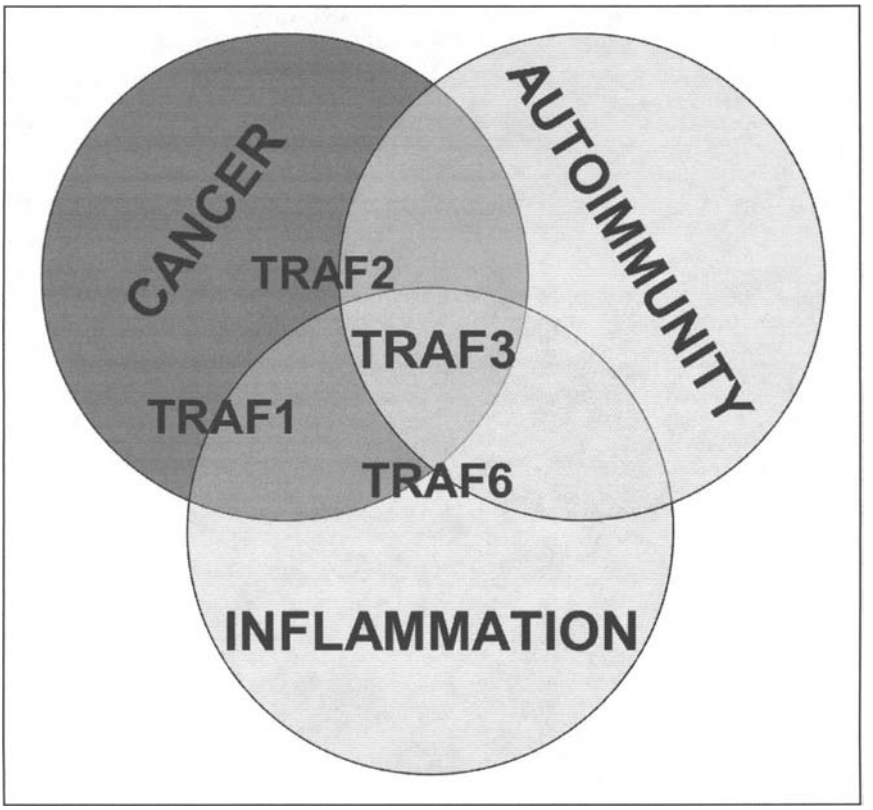

Figure 1. TRAFs regulate both the acquired and innate immune systems, as well as certain additional physiological processes. Deregulation of these immune pathways is causative of cancer, autoimmunity and inflammation. Targeting the function of specific TRAF family members could provide novel approaches to restoring normal immune system function, but caution must be taken to avoid unwanted side-effects.

Development of TNFR-mimic peptides that target the function of specific members of the TRAF-family is a complementary approach that might yield significant success. Indeed, the suitability of TNFR-mimic peptides to interfere with TRAF activities has been already shown in cell cultures.[3,10] The crystal structures of different TRAF-family members bound to TRAF-binding peptides from several members of the TNFR family support the notion that development of peptidomimetics that preferentially interact with and modulate the function of particular members of the TRAF family is feasible and worth exploring for therapeutic purposes. Recent advances in cell permeable peptide technology, improving cellular penetration and stability[130-132] also raises optimism that peptidomimetics could be eventually translated to the clinic.

Alternatively, enzymes that associate with TRAFs may be attractive and more pharmaceutically tractable targets for drug discovery. For instance, inhibitors of Ubc13, the unique E2 that associates with the RING domains of TRAF, would be predicted to short-circuit signal transduction mediated by many of these adapter proteins. Similarly, the protein kinases recruited to TRAFs could also be targeted. The relative advantages and disadvantages of these various targets from the perspective of efficacy and toxicity, however, are beyond the scope of this review.

While the pleiotropic effects of TRAF-family proteins and the partner proteins with which they associate caution against the use of pharmacological TRAF modulators, at least for chronic diseases, rapidly evolving new drug delivery systems and nanodevices that restrict drugs to sites of disease forecast emerging opportunities to consider therapeutic approaches for either enhancing or inhibiting the activities of TRAFs for future drug development.

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