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Modulation of Protein Kinase C in Antitumor Treatment

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1 Introduction

Protein kinase C (PKC) is a family of serine/threonine specific protein kinases. The PKC isoenzymes can be classified into three groups: i) the conventional (cPKCs) α, βI, βII, and γ (require negatively charged phospholipids, diacylglycerol or phorbol ester, and calcium for optimal activation), ii) the novel (nPKCs) δ, ε, θ, η/L (mouse/human) and μ (require negatively charged phospholipids, diacylglycerol or phorbol ester, but no calcium), and iii) the atypical (aPKCs) λ/ι (mouse/human) and ζ (do not require calcium, diacylglycerol or phorbol ester, but only negatively charged phospholipids for optimal activity) (Nishizuka, 1995; Newton and Johnson, 1998). The PKC isoenzymes (Fig. 1) are characterized by four conserved (C1–C4) and five variable (V1–V5) domains (Stabel and Parker, 1991; Azzi et al., 1992; Hug and Sarre, 1993; Stabel, 1994). The regulatory domain consists of the C1 and the C2 region. C1 contains the pseudosubstrate region that can inhibit the enzyme by binding to the catalytic site (C4). In PKCμ, the pseudosubstrate domain is lacking. C1 also contains tandemly repeated cysteine-rich regions to which DAG (diacylglycerol), phorbol esters and bryostatins can bind. cPKCs and nPKCs contain two zinc fingers in the phorbol ester binding site, aPKCs are characterized by a single zinc finger. C2 contains the calcium binding region present only in cPKCs but not in nPKCs and aPKCs. Between the C2 and the C3 region the so called hinge region is situated which serves as cleavage site for calpain and trypsin during degradation. The C3 region is believed to be the ATP binding site and the C4 region the catalytic site.

PKC isoenzymes seem to play an important role in activation of signal transduction pathways leading to synaptic transmissions, the activation of ion fluxes, secretion, proliferation, cell cycle control, differentiation or tumorigenesis. PKC has become of major interest as target for therapeutic

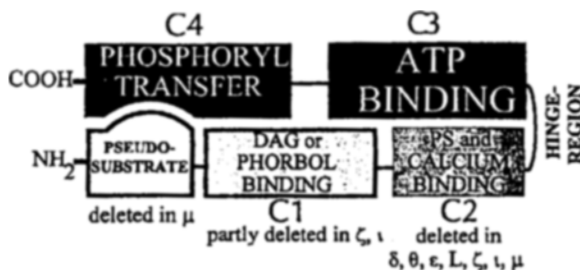

Fig. 1. Schematic representation of protein kinase C. The cartoon shows the different domains of PKC in the inactive form of the enzyme

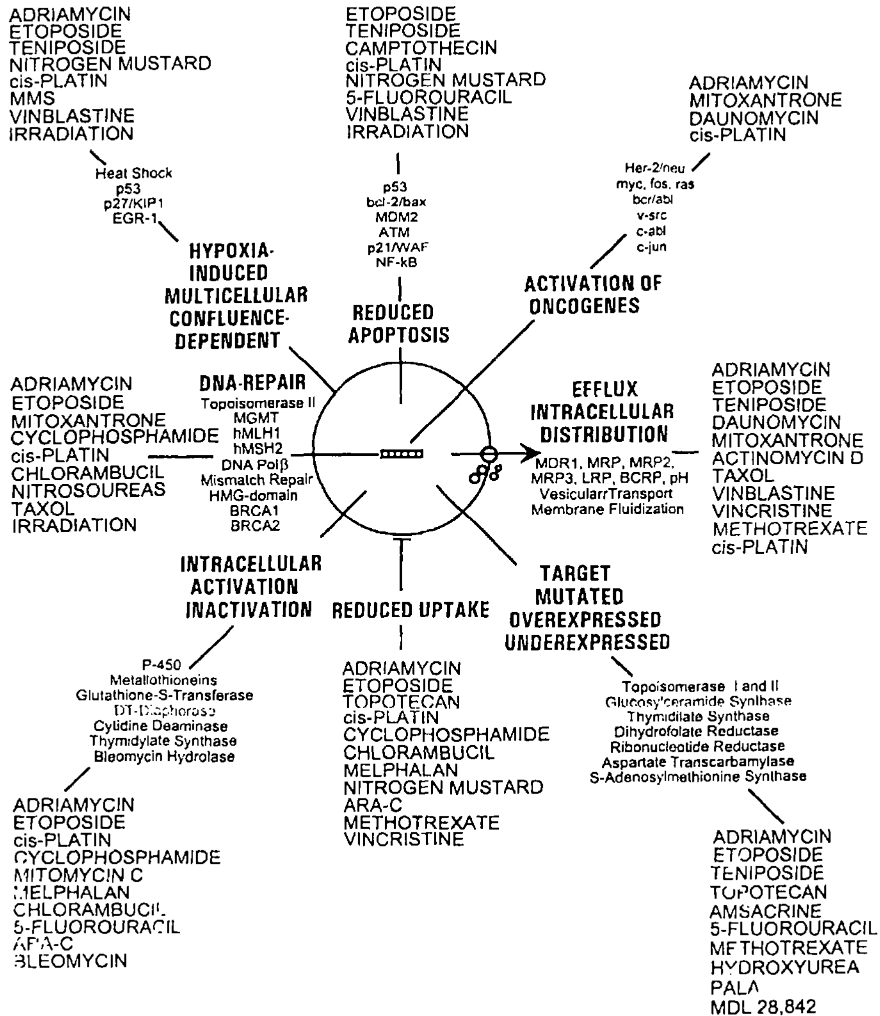

Fig. 2. Mechanisms causing resistance to antitumor treatment. ATM, ataxia telangiectasia gene, (Westphal et al., 1998; Xu and Baltimore, 1996), bcl-2/bax (Farrow and Brown, 1996, Zunino et al., 1997; Haq and Zanke, 1998), bcr/abl (McGahon et al., 1994), BCRP, breast cancer resistance protein (Doyle et al., 1998; Ross et al, 1999); bleomycin hydrolase (El-Deiry, 1997), BRCA1 (Husain et al., 1998; Chen et al., 1998), BRCA2 (Chen et al., 1998; Chen et al., 1999), c-abl (White and Prives, 1999), c-jun (Sanchez-Perez and Perona, 1999), cytidine deaminase (El-Deiry, 1997), DNA polβ, DNA polymerase β (Ochs et al., 1999), dihydrofolate reductase (Schimke, 1986), DT-diaphorase (Riley and Workman, 1992; Fitzsimmons et al., 1996; El-Deiry, 1997), EGR-1 (Ahmed et al., 1996), fos (Niimi et al., 1991), glucosylceramide synthase

intervention in a range of different diseases (Gescher et al., 1992; Bradshaw et al., 1993; Basu 1993; Deacon et al., 1997; Nixon, 1997; Goekjian and Jirousek, 1999). PKC may be involved in chronic granulomatous disease, allergy, asthma, rheumatoid arthritis (Westmacott et al, 1991), transplantation (Woodley et al., 1991), AIDS (Kinter et al., 1990; Accornero et al., 1998), Alzheimer's disease (Chauhan et al., 1991), multiple sclerosis (Defranco, 1991), hypertension (Ek et al., 1989), cardiac hypertrophy (Kwiatkowska-Patzer and Domanska-Janik, 1991), atherosclerosis (Kariya et al., 1987), diabetes (Inoguchi et al., 1992; Ishii et al., 1996) and cancer (Basu, 1993; Blobe et al., 1994, Gescher, 1998). PKC is the intracellular receptor for tumor promoting phorbol esters (Castagna et al., 1982; Niedel et al., 1983; Leach et al., 1983). Short term exposure of intact cells with phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate, TPA) activates PKC, long term exposure down-regulates PKC activity. Phorbol esters are able to promote tumor formation. Therefore, it was presumed that activation of PKC by TPA induces tumors and inhibition may reduce carcinogenesis or inhibit tumor growth. However, investigations revealed that the situation is more complicated. For example, bryostatin 1, another PKC

(Lavie et al., 1997; Liu et al., 1999), glutathione-S-transferase (Ozols et al., 1990, Tew,1994), heat shock (Ciocca et al., 1993); her-2/neu (Tsai et al., 1996), HMG-domains (Huang et al., 1994), hMLH1 (De las Alas et al. 1997), hMSH2 (Aebi et al., 1996), hypoxia (Bush et al., 1978; Vaupel et al., 1989; Sakata et al., 1991; Höckel et al., 1996), LRP, lung resistance-related protein (Scheffer et al., 1995), MDL 28,842, (*Z*)-5′-fluoro-4′,5′-didehydro-5′-deoxyadenosine (Dwivedi et al., 1999), mdm2 (Kondo et al., 1995a), MDR1 (Juliano and Ling, 1976; Gottesman and Pastan, 1993), membrane fluidization (Regev et al., 1999), metallothioneins (Kelley et al., 1988; Kaina et al., 1990; Kondo et al., 1995b), MGMT, O^6-methylguanine-DNA methyltransferase (Erickson, 1991; Mattern et al., 1998), methyl methanesulfonate (MMS; Chen et al., 1998), mismatch repair (Moreland et al., 1999, White and Prives, 1999), MRP, multidrug resistance-associated protein (Cole et al., 1994), MRP2 (Cui et al., 1999; Hooijberg et al., 1999), MRP3 (Kool et al., 1999), multicellular resistance (Sutherland, 1988; Kobayashi et al., 1993; Pizao et al., 1993; Graham et al., 1994; StCroix et al., 1996), myc (Sklar and Prochownik, 1991), NF-κB (Wang et al., 1999b), P-450 (Doehmer et al., 1993), p53 (Lowe et al., 1993; Levine, 1997; Zunino et al., 1997; Piovesan et al., 1998); p27/KIP (StCroix et al., 1996); p21/WAF1 (Wang and Walsh, 1996; McDonald et al., 1996; Fan et al., 1997), pH (Martinez-Zaguilan et al, 1999; Williams et al., 1999), ras (Scanlon et al., 1991; Isonishi et al., 1991; El-Deiry, 1997), reduced uptake (Fry and Jackson, 1986; Perez et al., 1990; Slapak et al., 1990; Chu, 1994; Ma et al., 1998a; Moscow, 1998; Ma et al., 1998b), repair (Masumoto et al., 1999; Chen et al., 1998; Husain et al., 1998, Chen et al., 1999), ribonucleotide reductase (Ask et al, 1993; Yen et al., 1994), thymidilate synthase (Kinsella et al., 1997), topoisomerase I and II (Yarbro, 1992; Robert and Larsen, 1998), vesicular transport (Dietel et al., 1990), v-src (Masumoto et al., 1999)

modulator with properties similar to those of TPA (Blumberg, 1991; Kennedy et al., 1992; Szallasi et al., 1994) does not induce tumor formation. The compound exhibits potent antitumor activity and is currently undergoing phase I (Jayson et al., 1995, Grant et al., 1998) and phase II (Propper et al., 1998) clinical evaluation as an anticancer drug.

In addition to be a direct target of antitumor treatment, PKC has been shown to be involved in the resistance to antitumor treatment and in the modulation of apoptosis. Resistance to cancer chemotherapy is a major

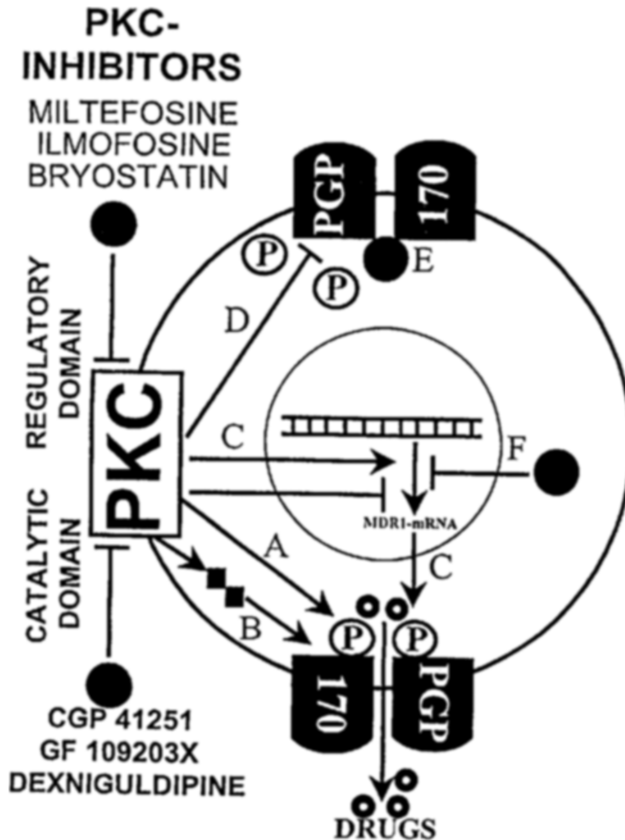

Fig. 3. Possible interactions between PKC and MDR1-mediated drug resistance. Activation of PKC might activate the drug efflux by phosphorylation of PGP (A), induce or activate proteins which modulate PGP (B, Castro et al., 1999), or induce the transcription and translation of MDR1-mRNA (C). Inhibitors of PKC might prevent phosphorylation of PGP leading to a decrease the drug efflux (D), inhibit the efflux of drugs by direct interaction with the drug binding site(s) or the ATP-binding sites of PGP (E), or prevent the expression of MDR1-mRNA (F)

problem in the treatment of cancer (Goldie and Coldman, 1984). A variety of mechanisms causing resistance have been observed (Fig. 2), among them: enhanced DNA-repair, reduced drug uptake, intracellular inactivation of drugs, reduced prodrug activation, mutated, overexpressed or not expressed targets, hypoxia, cell-cell interactions (multicellular resistance), increased drug efflux by overexpression of multidrug resistance gene 1 (MDR1), multidrug resistance-associated gene (MRP) or lung resistance protein (LRP), export of drugs by vesicles, intracellular compartimentalization of drugs, and altered expression or mutation of genes involved in apoptosis. Members of the PKC family have been reported be involved in multidrug resistance (MDR) and in apoptosis. Figure 3 shows an overview of the possible mechanisms by which PKC might interfere with MDR1-mediated drug resistance. However, the results of investigations concerning PKC and resistance are contradicting. This review summarizes data indicating a role of PKC in antitumor treatment and apoptosis, and on the other hand, data that argue against an involvement of PKC. The association of experimental results with pro or contra of a contribution of PKC is not always unambiguous.

2 PKC in Cell Proliferation and Tumor Growth

2.1 PKC Isoenzymes and Cell Proliferation

Investigations into the expression of distinct PKC isoenzymes in various tissues revealed a highly variable tissue distribution. PKCα and PKCζ are ubiquitously expressed. Brain contains all isoenzymes, whereas others such as skin and skeletal muscle contain only a few (Blobe et al., 1994). Such a different pattern of expression suggests that the PKC isoenzymes play different roles in the tissue of expression and do not suggest a general role of all isoenzymes in cell proliferation. In many publications an influence of PKC on cell proliferation has been reported.

Deletion or mutation of PKC1, the only member of the PKC family expressed in Saccharomyces cerevisiae (Mellor and Parker, 1998) led to osmotic instability, an arrest of protein synthesis and cell proliferation (Levin et al., 1990; Levin and Bartrett-Heubusch, 1992). NIH3T3 cells overexpressing PKCα exhibited an altered, "transformed-like" morphology, an increased growth rate, a higher saturation density and were able to grow in soft agar after treatment with TPA. These effects could be reversed by the unspecific PKC inhibitor staurosporine (Finkenzeller et al., 1992). With respect to untreated control liver, an activation and increased expression of PKCα was observed in diethylnitrosamine-induced liver tumors and lung metastases (La Porta et al., 1997). Early events in the transformation of keratinocytes

have been found to be the mutation and activation of ras, activation of the epidermal growth factor receptor, upregulation of PKCα, inactivation of through tyrosine phosphorylation (Yuspa, 1998), and downregulation of the expression of PKCδ (Geiges et al., 1995). In cultured myoblasts PKCα was found to have an important role in maintaining proliferation (Capiati et al., 1999). PKCα has been shown to activate telomerase in human breast cancer cells which may represent an essential step in the maintenance of proliferation in human cancers (Li et al., 1998). On the other hand, increased expression of PKCα led to cessation of growth, induction of differentiation in B16 melanoma cells and to gene dose-dependent inhibition of proliferation in K562 cells (Gruber et al., 1992). Recombinant chimaeras with the regulatory domain of PKCα and catalytic domains of other PKC isoenzymes inhibited cell growth (Acs et al., 1997). PKCα overexpressing bovine aortic endothelial cells exhibited reduced proliferation and increased accumulation in the G2/M phase of the cell cycle (Rosales et al., 1998). Overexpression of PKCα in MCF10A cells suppressed proliferation endowing cells with properties consistent with a metastatic phenotype (Sun and Rotenberg, 1999).

PKCβ overexpressing cells were more susceptible to transformation with the H-ras oncogene (Hsiao et al., 1989). Overexpression of PKCβI in rat fibroblasts led to disordered growth (Housey et al., 1988). The human erythroleukemia K562 cell line expresses PKCα, βII and ζ. The cells undergo megakaryocytic differentiation and cessation of proliferation when treated with TPA. K562 cells overexpressing human PKCα grew more slowly and were more sensitive to the cytostatic effects of TPA than control cells, whereas cells overexpressing βII were less sensitive to TPA. Antisense experiments demonstrated that PKCβII is required for K562 cell proliferation, whereas PKCα is involved in megakaryocytic differentiation (Murray et al., 1993). In F9 embryonal carcinoma PKCα seems to play an active role in differentiation and PKCβ activity is incompatible with differentiation (Cho et al., 1998). PKCβI overexpressing bovine aortic endothelial cells promoted growth and shortened the doubling time, whereas PKCα exhibited reduced proliferation (Rosales et al., 1998). However, in human HL-60 promyelocytes activation of PKCβ was found to be necessary and sufficient for TPA-induced differentiation (MacFarlane and Manzel, 1994). In murine keratinocytes overexpression of βI led to growth inhibition and Ca^{2+}-induced differentiation (O'Driscoll et al., 1994). PKCβI expression was higher in a well-differentiated SKUT-1-B mixed mesodermal uterine cell line compared to the moderately differentiated endometrial HEC-1-B adenocarcinoma cell line (Bamberger et al., 1996). As shown in mice homozygous for a targeted disruption of the PKCβII gene, this isoenzyme seems to play an important role in B-cell activation (Leitges et al., 1996).

When PKCγ was overexpressed in NIH 3T3 cells, reduced growth factor requirements, growth to higher saturation density and formation of tumors in nude mice were observed (Persons et al., 1988). Human U251 MG glioma cells overexpressing PKCγ showed an increased rate of growth in monolayer culture, increased colony-forming efficiency on soft agar, and increased DNA synthesis in response to epidermal growth factor and basic fibroblast growth factor (Mishima et al., 1994).

PKCδ seems to be involved in growth inhibition, differentiation, apoptosis and tumor suppression (Gschwendt, 1999). TPA stimulation of PKCδ overexpressing CHO cells led to a cell division arrest (Watanabe et al., 1992). PKC δ overexpressing glioma cells showed a decreased rate of growth and decreased colony-forming efficiency (Mishima et al, 1994). However, overexpression of PKCδ in rat HT mammary adenocarcinoma cell lines significantly increased anchorage-independent growth, although it had no effect on growth of adherent cells. PKCδ seems to be involved in regulating attachment and anchorage-independence, which may be related to increased metastatic potential in this system (Kiley et al., 1999). Recombinant chimaeras with the regulatory domain of PKCδ and catalytic domains of other PKC isoenzymes inhibited cell growth (Acs et al., 1997). Proteolytic cleavage of PKCδ was found to activate this isoenzyme during apoptosis (Emoto et al., 1995; Ghayur et al., 1996). The antitumor agents taxol, vinblastine and vincristine specifically activated PKCδ (Das et al., 1998). Transgenic mice overexpressing PKCδ in the epidermis were found to be resistant to skin tumor promotion by TPA (Reddig et al., 1999). In NIH 3T3 cells which normally express only PKCα, overexpression of PKCδ by transfection induced significant changes in morphology and caused the cells to grow more slowly and to a decreased cell density in confluent cultures. These changes were accentuated by treatment with TPA. (Mischak et al., 1993). Overexpression of PKCε did not lead to morphological changes, but caused increased growth rates and higher cell densities in monolayers. None of the PKCδ overexpressers grew in soft agar with or without TPA, but all the cell lines that overexpressed PKCε grew in soft agar in the absence of TPA, but not in its presence. NIH 3T3 cells that overexpressed PKCε also formed tumors in nude mice with 100% incidence, indicating that high expression of PKCε contributes to neoplastic transformation (Mischak et al., 1993). Overexpression of PKCε in Rat 6 embryo fibroblasts led to a 7–13-fold increase in Ca^{2+}-independent PKC activity, to formation of dense foci in monolayer culture, decreased doubling time, increased saturation density, decreased serum requirement, growth in soft agar, and tumor formation in nude mice (Cacace et al., 1993). In nontumorigenic rat colonic epithelial cells overexpression of PKCε caused marked morphological changes in two transfected

clones, which were accompanied by increased saturation densities and anchorage-independent colony formation in semisolid agar. These growth effects were attenuated or reversed by chronic incubation with TPA (Perletti et al., 1996). A small cell lung cancer cell line that exhibited rapid growth compared to other small cell lung cancer cell lines overexpressed a constitutively active catalytic fragment of PKCε (Baxter et al., 1992). Dominant negative PKCε inhibited the proliferation of NIH3T3 cells. Constitutively active PKCα or PKCε overcame this inhibitory effect (Cai et al., 1997). Recombinant chimaeras between the regulatory domains of PKCα, PKCδ, PKCε and the catalytic domains of these isoenzymes were transfected into NIH 3T3 cells. All chimaeras containing a regulatory or a catalytic domain of PKCε exhibited growth-promoting activity (Acs et al., 1997). These data indicate that PKCε seems to have oncogenic properties. However, it has also been reported, that inhibition of cell proliferation by tamoxifen is associated with activation of PKCε (Lavie et al., 1998). In neuronal cells PKCε seems to be important for differentiation (Ohmichi et al., 1993; Hundle et al., 1995; Fagerstrom et al., 1996).

Overexpression of PKCζ has been reported to be required for mitogenic maturation of Xenopus oocytes and led to deregulation of growth control in mouse fibroblasts (Berra et al., 1993). However, these effects of PKCζ in Xenopus oocytes seem not to be clear (Carnero et al., 1995). In U937 monocytic leukemia cells PKCζ overexpression decreased proliferation rate and saturation density, indicating the induction of differentiation (Ways et al., 1994). In normal NIH3T3 fibroblasts (Montaner et al., 1995; Crespo et al., 1995) and K562 cells (Murray et al., 1997) no effects of PKCζ overexpression on cell proliferation or oncogenic transformation were observed. PKCζ overexpression in v-raf-transformed NIH-3T3 cells drastically retarded proliferation, abolished anchorage-independent growth, and reverted the morphological transformation (Kieser et al., 1996). Activation of PKCζ by ceramide in acute lymphoblastic leukemia MOLT-4 cells induced apoptosis. However, it was also shown that ceramide treatment, in addition to activation of PKCζ, inactivated PKCα (Lee et al, 1996a). Exposure of cells to a genotoxic stimulus that induced apoptosis, led to an inhibition of PKCζ (Berra et al, 1997). The product of the par-4 gene interacted with PKCζ and inhibited its enzymatic activity. The expression of par-4 correlated with growth inhibition and apoptosis (Diaz-Meco et al, 1996). In Cos-7 cells conventional and novel PKCs activated the ERK/MAPK cascade via raf-1, whereas PKCζ stimulated this pathway without raf-1 activation (Schönwasser et al., 1998). N-myc acts to increase the malignancy of neuroblastoma cells. Overexpression of N-myc in these cells caused suppression of PKCδ and induction of PKCζ (Bernards, 1991).

PKCμ seems to be involved in murine keratinocyte proliferation. A correlation between PKCμ expression and enhanced cell proliferation was also observed for NIH3T3 mouse fibroblasts overexpressing human PKCμ (Rennecke et al., 1999).

2.2 PKC Expression in Tumor Cells and Tumors

Overexpression of PKC seems to be involved in breast cancers. Elevated levels of PKC activity in breast tumors relative to normal breast tissue was found by O'Brian et al. (1989a). TPA inhibited the growth of mammary carcinoma MCF-7, BT-20, MDA-MB-231, ZR-75-1, and HBL-100, but not that of T-47-D cells. TPA-non-responsive T-47-D cells exhibited the lowest PKC activity. A rapid TPA-dependent translocation of cytosolic PKC to membranes was found in the five TPA-sensitive cell lines without affecting cell growth. However, TPA-treatment for more than 10 hours inhibited reversibly the growth of TPA-responsive cells. This effect coincided with the complete loss of cellular PKC activity due to the proteolysis of the translocated membrane-bound PKC. Resumption of cell growth after TPA-removal was closely related to the specific reappearance of the PKC activity in the TPA-responsive human mammary tumor cell lines suggesting an involvement of PKC in growth regulation (Fabbro et al., 1986). During a 4-day culture period, various phorbol ester derivatives inhibited the proliferation of MCF-7 breast carcinoma cells in a dose-dependent manner. A correlation between the relative potencies of the various phorbol ester derivatives for inhibiting both phorbol-12,13-dibutyrate (PdBu) binding and cell proliferation was found (Darbon et al., 1986). However, it was also reported that PKC activation by TPA and DAG inhibited MCF-7 cell proliferation (Issandou and Darbon, 1988; Issandou et al., 1988). PKC activity was described to be higher in estrogen receptor negative human mammary tumor cells compared to estrogen-receptor-containing counterparts (Borner et al., 1987; Ways et al., 1995; Morse-Gaudio et al., 1998). MCF-7 cells transfected with PKCα displayed an enhanced proliferative rate, anchorage-independent growth, dramatic morphologic alterations including loss of an epithelioid appearance, and increased tumorigenicity in nude mice. PKCα overexpressing MCF-7 cells exhibited a significant reduction in estrogen receptor expression and decreases in estrogen-dependent gene expression (Ways et al., 1995). Phorbol esters were found to down-regulate the expression of estrogen receptors in breast cancer cell lines (Hähnel and Gschwendt, 1995). PKCα was found to be activated in situ in a significant number of human breast tumors (Ng et al., 1999). MCF-7 breast cancer cells transfected with PKCα led to a more aggressive phenotype compared to untransfected cells (Ways et al., 1995).

Differential display between the non-metastatic and the PKCα overexpressing metastatic MCF-7 cells showed that a homologue to a putative glialblastoma cell differentiation-related protein is upregulated. Histone 3B, integrins 3α and 6α were downregulated (Carey and Noti, 1999). In contrast to the publication by Ways et al. (1995), another report showed that overexpression of PKCα in MCF-7 cells caused upregulation of PKCβ and this led to a less aggressive phenotype, which was characterized by reduced in vitro invasiveness and markedly diminished tumor formation and growth in nude mice. These findings were explained by the down-regulation of estrogen receptor levels observed in tumors derived from PKCα-infected MCF-7 cells (Manni et al., 1996). TPA and bryostatin 1 inhibited the growth of MCF-7 breast cancer cells. TPA induced rapid translocation of PKCα protein and PKC activity to the membrane fraction of MCF-7 cells. In contrast, bryostatin 1 treatment resulted in the loss of the PKCα activity from both cytosolic and membrane compartments within 10 minutes of treatment. These results suggested that PKCα may specifically play a role in inhibiting growth of human breast cancer cells by bryostatin (Kennedy et al., 1992).

PKCα expression in human astrocytomas was found to be highest in well-differentiated (grade 1) tumors, intermediate in anaplastic (grade 2) astrocytomas, and low or nondetectable in dedifferentiated glioblastomas (grade 3 astrocytomas) and normal controls (Benzil et al., 1992). It was also found that the levels of PKCα in eight glioblastoma cells lines were similar to those in normal glial cells (Misra-Press et al., 1992). PKC activity was significantly higher in glioma cell lines compared to bladder, colorectal, rhabdomyosarcoma-oligodendrocyte hybrid, melanoma, cervix, and fibroblast cells, even though 3 of 8 of the non-glioma lines had higher proliferation rates than A172 glioma cells. In non-glioma cell lines, no correlation was found between the PKC activity and proliferation rates (Baltuch et al., 1993). In a comparison between rat C6 glioma cells and non-malignant rat astrocytes, both C6 glioma cells and astrocytes were found to express PKCα, β, δ, ε and ζ, but not γ. Enzyme activity measurements revealed that the elevated PKC activity of glioma cells was due to overexpression of PKCα (Baltuch et al., 1995). In another investigation all human glioma lines examined, and the rat glioma C6, displayed high PKC activity relative to nonmalignant glial cells, which correlated with their proliferation rates over their respective growth phase. Frozen surgical human malignant glioma specimens also displayed high PKC activity (Couldwell et al., 1992). The administration of PdBu or TPA resulted in a dose-related inhibition of growth of human glioma cell lines in vitro. The synthetic nonphorbol PKC activator SC-9 produced an even more pronounced decrease in cell proliferation. Conversely, the administration of 4-αTPA, a phorbol ester that binds but does not activate

PKC, had no effect on the proliferation rate. In contrast to the response of glioma cells, nonmalignant human adult astrocytes treated with the PKC activators responded by increasing their proliferation rate. The opposed effects of PKC activators on nonmalignant astrocytes versus glioma growth may be due to a high intrinsic PKC activity in glioma cells, with resultant down-regulation of enzyme activity following the administration of the pharmacological activators (Couldwell et al., 1990). Cell lines derived from high-grade gliomas expressed higher levels of PKCα than did cell lines derived from low-grade gliomas. In glioblastoma-derived cell lines PKCα was mainly expressed in the cytosolic fraction, indicating an inactive state of the enzyme. Bryostatin 1 specifically down-regulated PKCα in glioblastoma-derived cell lines. However, this was not associated with significant growth inhibition illustrating that PKCα seems not to be essential for proliferation (Zellner et al., 1998). Compared to two normal glial cell lines, in eight glioblastoma cell lines PKCα and PKCγ were similar. PKCε was elevated three to thirty times in six of the eight tumors. PKCζ was elevated twofold in all of the tumors (Xiao et al., 1994).

Elevated levels of PKC were also found in thyroid cancers if compared to normal thyroid tissue (Hagiwara et al., 1990; Hatada et al., 1992). TPA enhanced, tamoxifen and staurosporine inhibited invasion and growth of estrogen receptor-negative follicular thyroid cancer cells (Hoelting et al., 1996). In human thyroid cancers (Prevostel et al., 1995; Prevostel et al., 1997) and in pituitary cancers (Alvaro et al., 1997; Alvaro et al., 1993) point mutations in PKCα were detected. However, in 11 human pituitary tumours cDNA was subcloned and up to ten individual clones were sequenced from each tumour, resulting in 85 clones analyzed in total. All of the pituitary adenomas showed a normal wild-type sequence of PKCα DNA. Even if the tumor was invasive (infiltration of the dura mater) no mutation was found. Moreover, Western blot analyses did not show any differences in PKCα protein expression in invasive as compared with noninvasive pituitary adenomas. These data argue against suggestions that mutated PKCα is a feature of invasive pituitary tumours (Schiemann et al., 1997). PKC activity and expression were higher in adenomatous pituitaries than in normal human or rat pituitaries. PKC expression in growth hormone-secreting and non-secreting tumors was significantly higher than that in prolactin-secreting tumors. PKC activity was significantly higher in invasive tumors than in non-invasive tumors. In 3 adenomas which were obtained from patients treated with bromocriptine or octreotide, particulate- and soluble-PKC activities were significantly lower than those measured in non-treated adenomas (Alvaro et al., 1992). In human pituitary tumors predominantly PKCα in all adenomas, and variable expression of PKCβ and PKCγ in some tumors

was found. Normal and neoplastic pituitaries expressed abundant mRNA for PKCε, whereas some tumors and one normal pituitary had a few cells positive for PKCζ (Jin et al., 1993).

A murine UV-induced fibrosarcoma cell line had an unusual PKC subcellular distribution with 87% of the PKC activity associated with the membrane. Sequencing of PKCα DNA from ultraviolet-induced-fibrosarcoma cells showed four point mutations in the fibrosarcoma PKC, of which three are in the highly conserved regulatory domain and one is in the conserved region of the catalytic domain. Expression of this mutant PKCα gene in normal Balb/c 3T3 fibroblasts resulted in a fibrosarcoma-like PKC membrane localization and in cell transformation, as judged by their formation of dense foci, anchorage-independent growth and ability to induce solid tumours when inoculated into nude mice. By contrast, transfectants expressing the normal PKCα cDNA did not display a morphology typical of malignant transformed cells and failed to induce tumours in vivo. These findings seemed to demonstrate that point mutations in the primary structure of PKC modulate enzyme function and are responsible for inducing oncogenicity (Megidish and Mazurek, 1989). However, these results could not be reproduced (Borner et al., 1991). The nontumorigenic, immortal line of murine melanocytes, Mel-ab, required the continual presence of biologically active phorbol esters for growth. Comparable treatments of murine B16 melanoma cells resulted in partial inhibition of cell proliferation. Significant levels of PKC were present in quiescent Mel-ab cells, whereas no immunoreactive protein was detected in cell extracts from either proliferating Mel-ab or B16 cells. These data showed that PKC down-regulation, and not activation, correlates with the growth of melanocytes in culture (Wilson et al., 1989; Brooks et al., 1991). It has been shown that TPA stimulated the proliferation of normal human melanocytes, whereas it inhibited the growth of human melanoma cell lines. PKCδ, ε and ζ were detected in both normal melanocytes and in four melanoma cell lines. In contrast, both PKCα and β were expressed in normal melanocytes, whereas only either PKCα or β was detected in melanoma cells. TPA inhibited the growth of cells lacking PKCα more efficiently than the other melanoma cell lines which lacked PKCβ. It was further shown that PKCβ was not detected in freshly isolated human primary or metastatic melanoma tissues. (Oka et al., 1996). This may be a consequence of lack of induction of terminal differentiation by PKCβ. In human early prostatic adenocarcinomas an increase of PKCα, PKCε, PKCζ and a decrease of PKCβ was consistently observed during the genesis and progression of prostate cancer compared with nonneoplastic prostate tissues (Cornford et al., 1999). An increased nuclear PKCβ activity was observed in lung metastases compared to the parental liver tumor induced by diethylni-

trosamine (La Porta et al., 1997). Spontaneously and chemically transformed mouse pulmonary epithelial cells exhibited reduced levels of PKC (Morris and Smith, 1992).

Underexpression of PKC seems to be involved in colon cancer. PKC has been shown to be important for growth arrest and differentiation of intestinal cells (Saxon et al., 1994, Assert et al., 1993). Human Vaco 10 MS colon cancer cells were growth-inhibited by activation of PKC (McBain et al., 1990). Significantly higher Ca^{2+}-dependent PKC activities were observed in both the cytosolic and particulate fractions of the normal mucosa relative to the corresponding values obtained with the human colon carcinoma fractions. The average specific activity ratios were 5.1 (normal cytosolic/carcinoma cytosolic) and 3.7 (normal particulate/carcinoma particulate) for PKC (Guillem et al., 1987). In patients with colonic adenomas and colonic carcinomas, total PKC activity was found to be significantly reduced as compared to adjacent mucosa (Kopp et al., 1991). In 15 of 15 primary human colon tumors there was a decrease of approximately 40% in the levels of diacylglycerol when compared to paired adjacent normal mucosa samples. Assays on the same samples indicated that this decrease was seen both in tumors that did and did not display mutations in codon 12 of c-K-ras. These results suggested that the PKC signal transduction pathway is suppressed in human colon cancer (Phan et al., 1991). In another investigation the mean value for cellular PKC enzyme activity in the colon tumors from 39 patients was approximately 60 percent of that found in the paired adjacent normal mucosa samples (Levy et al., 1993). In 18 human colonic adenomas and carcinomas a significant decrease in particulate PKC activity compared with the adjacent normal mucosa was observed. Decreased PKC activity correlated with increased adenoma size (Kusunoki et al., 1992). Five of six PKC isoenzymes present in normal mucosa showed reduced protein levels during tumor development in the human colon (Kahl-Rainer et al., 1994). Decrease of PKCα seems to be of major importance for development of colorectal cancers (Suga et al., 1998). DNAs for PKCα in sense or antisense orientations were transfected into human colonic adenocarcinoma CaCo-2 cells. Sense transfected clones exhibited 3-fold increases and antisense transfectants approximately 95% decreases in PKCα expression with no significant alterations in other PKC isoforms. Transfection of CaCo-2 cells with PKCα in the antisense orientation resulted in enhanced proliferation and decreased differentiation, as well as in a more aggressive transformed phenotype compared with empty vector-transfected control cells. In contrast, cells transfected with PKCα cDNA in the sense orientation demonstrated decreased proliferation, enhanced differentiation, and an attenuated tumor phenotype compared with these control cells. (Scaglione-Sewell et al., 1998). Human

HT-29M6 colon cancer cells transfected with an activated form of PKCα showed decreased proliferation and increased invasion due to alterations in cell adhesion. When these cells were xenografted into athymic mice, higher expression of activated PKCα led to a reduction in tumor size (Batlle et al., 1998). The abundance of PKCβ mRNAs was decreased in 30 of 39 colon tumors (Levy et al., 1993). HT-29 colon cancer cells transfected with rat PKCβI displayed increased doubling time, decreased saturation density, loss of anchorage-independent growth in soft agar, and decreased tumorigenicity in nude mice following exposure to TPA (Choi et al., 1990). Overexpression of PKCβI in SW480 colon cancer cells caused growth suppression (Goldstein et al., 1995). In small adenomas of 18 patients a significant increase of cytosolic PKCα and decrease of membrane PKCβII compared to normal neighboring mucosa was found. In 7 patients PKCδ was reduced (Assert et al., 1999). In human colon cancer tissue a significant decrease in PKCε was found (Pongracz et al., 1995a). Unsaturated free fatty acids and bile acids which are present in the colon are activators of PKC (Weinstein, 1991). It was reported that activation of PKC by unsaturated fatty acids was associated with proliferation of rat colonic epithelium (Craven and de Rubertis, 1988; Craven and de Rubertis, 1992).

2.3 Antiproliferative and Antitumor Effects of PKC-Modulation

2.3.1 Bryostatins

PKC is a phorbol ester receptor (Castagna et al., 1982, Niedel et al., 1983). Phorbol esters such as TPA or PdBu modulate PKC activity and, therefore, have been used for investigations into PKC effects on apoptosis and antitumor drug resistance. However, the use of TPA or PdBu can give apparently contradictory findings because they can activate several PKC isoenzymes. Unlike the physiological activator diacylglycerol, TPA is stable and induces a persistent activation leading to the downregulation of PKC (Blumberg, 1991). So short term exposure of intact cells to phorbol esters activates PKC, prolonged exposure depletes cells of the enzyme, most likely due to proteolytic degradation (Chida et al., 1986). In many cases it is difficult to distinguish whether the effects of phorbol esters are due to activation or inactivation of PKC. In addition to PKC, TPA also interacts with other proteins (Ahmed et al., 1993; Valverde et al., 1994; Caloca et al., 1997). Gnidimacrin is another PKC activator which leads to downmodulation of the enzyme following long term exposure. The compound exhibits antitumor activity in vivo (Yoshida et al., 1996).

Bryostatins are a group of macrocyclic lactones which are isolated from marine bryozoans (Pettit et al., 1970). These compounds exhibit a remarkable affinity for PKC where they compete with phorbol esters for the same binding site (Berkow and Kraft, 1989; Blumberg, 1991). The majority of investigations with bryostatins were carried out with bryostatin 1. In intact cells bryostatins, like phorbol esters, activate PKC and cause a translocation from the cytosol to the membrane (Hocevar and Fields, 1991). Following prolonged exposure, PKC is proteolytically degraded (Berkow and Kraft, 1989; Blumberg, 1991; Kennedy et al., 1992). Several differences between bryostatins and TPA have been observed. Like phorbol esters, bryostatins stimulate growth in some systems and are growth-inhibitory in others (Smith et al., 1985; Gschwendt et al., 1988; Kraft et al., 1988; Stone et al., 1988). However, in contrast to phorbol esters, bryostatins do not act as tumor promoters. In many systems bryostatin 1 induced only a subset of the responses to TPA and sometimes blocked those which it did not induce (Blumberg, 1991).

In NIH 3T3 fibroblasts bryostatin 1 showed similar potency to TPA for translocating PKCα to the cell membrane but was a much more potent downregulator of PKCα activity than TPA. It was also a much more potent translocator and downregulator of PKCδ and PKCε than TPA (Szallasi et al., 1994). The compound inhibited the proliferation of human A549 lung carcinoma, human MCF-7 breast cancer, murine renal adenocarcinoma, B16 melanoma, M5076 reticulum cell sarcoma, L10A B-cell lymphoma cells and exhibited antitumor activity in the murine P388 leukemia screening system (Dale and Gescher, 1989; Kennedy et al., 1992; Hornung et al., 1992). Another investigation showed that TPA and bryostatins 1 and 2 inhibited the growth of A549 cells. At high concentrations the bryostatins did not affect cell growth. Incubation with bryostatins 1 or 2 also led to PKC translocation, which was, however, much weaker than that observed with TPA. Exposure of cells to TPA or the bryostatins for longer than 30 minutes caused the gradual disappearance of total cellular PKC activity. PKC downregulation was concentration-dependent and complete after 24 hours. The bryostatins were potent inhibitors of the binding of [^{3}H]PdBu to its receptors in intact cells, and the inhibition was dependent on bryostatin concentration. (Dale et al., 1989). A P388 subline resistant to bryostatin was developed. The ability of the cytosol of these cells to phosphorylate PKC-specific substrate, phorbol ester binding and PKC isoenzyme expression was decreased. The resistant subline was also resistant to the activation by TPA (Prendiville et al., 1994). TPA inhibited growth of four out of six cell lines by up to 75% in 5-day cultures. Bryostatin 1 inhibited growth of MCF-7 cells only at a high dose. However, bryostatin 1 completely antagonized the growth inhibition and mor-

phological changes induced by TPA in MCF-7 cells. The divergent effects of these two agents were associated with differing effects on PKC activity and isoform expression in MCF-7 cells. TPA induced rapid translocation of PKCα to the membrane fraction of MCF-7 cells. In contrast, bryostatin treatment resulted in the loss of PKCα from both cytosolic and membrane compartments within 10 minutes of treatment. In coincubation assays the bryostatin effect was dominant over that of TPA. Similar effects on PKCα were seen in the MDA-MB-468 cell line whose growth was inhibited by TPA but not by bryostatin 1. In contrast, in the T-47-D cell line, TPA was not growth inhibitory and failed to induce translocation of PKCα to the cell membrane. Bryostatin, however, still caused loss of PKCα and PKC activity from cytosolic and membrane fractions. Thus, differential actions of bryostatin 1 and TPA on PKC activity and the PKCα isoform level in the membrane-associated fraction of MCF-7 and MDA-MB-468 cells may account for the divergent effects of these two agents on cell growth and morphology (Kennedy et al., 1992). Bryostatin 1 produced faster than TPA, an inactive, dephosphorylated form of PKCα in the LLC-MK2 line of renal epithelial cells which predisposes the enzyme to proteolysis. This explains a more efficient downregulation of PKCα by bryostatin (Lee et al., 1996b). However, several experimental approaches suggested that the growth inhibition by bryostatin is independent of PKC. 26-epi-bryostatin 1 showed 60-fold reduced affinity for PKC and 30–60-fold reduced potency to translocate and downregulate PKC isozymes compared with bryostatin 1, but was similar potent in inhibiting the growth of B16/F10 mouse melanoma cells. 26-epi-bryostatin 1 was found to exhibit a 10-fold reduced toxicity in C57BL/6 mice compared with bryostatin 1. From these results it was concluded that the growth inhibition of the bryostatins, at least in this system, did not result from interaction with PKC (Szallasi et al., 1996).

In clinical phase I trials with bryostatin 1, myalgia appeared as the dose-limiting side effect. Conventional toxicities included fever, anemia, fatigue, phlebitis, headache and thrombopenia (Rea et al., 1992; Prendiville et al., 1993; Jayson et al., 1995; Grant et al., 1998). From 29 patients, 11 with relapsed non-Hodgkin's lymphoma and chronic lymphocytic leukemia achieved stable disease for 2 to 19 months. An in vitro assay for total PKC evaluation in the patient's peripheral-blood samples demonstrated activation within the first 2 hours with subsequent downregulation by 24 hours, which was maintained throughout the duration of the 72-hour infusion. (Varterasian et al., 1998). Of 15 patients with malignant melanoma pretreated with chemotherapy only one developed stable disease for 9 month, 14 developed progressive disease (Propper et al., 1998).

2.3.2 Phospholipid Analogues

Phospholipid analogues are a new class of drugs which exhibit broad antineoplastic activity (Berdel, 1991; Hilgard et al., 1993; Brachwitz and Vollgraf, 1995). Typical representatives from this group are 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine (edelfosine, ET-18-OCH_3), ilmofosine (BM 41440) and hexadecylphosphocholine (miltefosine). These compounds exhibited remarkable antitumor activity against various experimental tumors in vitro as well as in vivo (Berdel, 1991; Workman, 1991). Miltefosine is the first of these compounds used in the clinic. In a phase II trial 8 (35%) partial remissions out of a total of 23 patients with metastatic breast cancer were observed (Clavel et al., 1992). The drug is approved in several countries for the topical treatment of skin metastases resulting from breast cancers (Hilgard et al., 1993). In the clinic an overall complete and partial response rate of 33%, and if clinically relevant minor responses were added, a total response rate of 53% could be observed (Klenner et al., 1998). The molecular mechanism responsible for the cytotoxic effect of these compounds is not quite clear. Interference with mitogenic signalling in the cell membrane seems to be the target of these compounds (Workman, 1991; Brachwitz and Vollgraf, 1995). The compounds inhibit PKC in cell free extracts by competing with phosphatidylserine (Kiss et al., 1987, Shoji et al., 1988, Hofmann et al., 1989, Shoji et al., 1991, Überall et al., 1991). They are equally as potent against PKC in intact cells as in cell free extracts (Shoji et al., 1988; Hofmann et al., 1989; Überall et al., 1991). The assumption that inhibition of PKC is responsible for the inhibition of cell growth is further supported by the observation that the antiproliferative effect of miltefosine can be antagonized by TPA (Geilen et al., 1991). Quaternary ammonium analogues of edelfosine, which inhibited PKC activity in vitro, also inhibited cell proliferation. A quaternary ammonium analogue which did not inhibit PKC did not inhibit cell proliferation (Civoli and Daniel, 1998). However, the conclusion that PKC is the target of phospholipid analogues has been questioned recently. Treatment of HL-60 and K562 cell lines with miltefosine and edelfosine for 2 hours did not lead to PKC translocation. In the same experimental setting, dioctanoylglycerol-stimulated PKC translocation was not affected by miltefosine or edelfosine. These findings indicated that miltefosine and edelfosine do not interfere with PKC translocation but rather mediate a general decrease of the enzyme activity in the membrane and cytosol of the cells. Since the extent of PKC inhibition was similar in the sensitive HL-60 and in the more resistant K562 cell line, it was concluded that inhibition of PKC may not be a prerequisite for the antiproliferative action of miltefosine and edelfosine (Berkovic et al., 1994). Cells depleted of PKC showed similar

sensitivity to edelfosine on PKC activity in different cell types as cells expressing PKC. These results suggested that a role of PKC in the cytotoxic action of edelfosine is very unlikely (Heesbeen et al., 1994). These data may be explained by the fact that phospholipid analogues interfere with other signal transduction pathways as inositol phosphate formation (Überall et al., 1991; Powis et al., 1992) or phosphatidylcholine synthesis (Vogler et al., 1985).

Inhibition of cell proliferation of Chinese hamster ovary cells treated with the PKC inhibitor sphinganine correlated with PKC inhibition. Sphinganine blocked changes in protein phosphorylation patterns that occurred in response to TPA (and vice versa). Mutant cells that exhibited increased resistance to sphinganine lacked phorbol ester and sphinganine-induced phosphorylation changes and differed somewhat in the behavior of PKC in vitro. From these results it was concluded that the cytotoxicity of sphinganine may be a consequence of PKC inhibition (Stevens et al., 1990). *N,N*-dimethylsphingosine and *N,N,N*-trimethyl-D-*erythro*-sphinganine inhibited the proliferation of several human tumor cell lines in vitro and inhibited the growth of human gastric MKN74 carcinoma xenografts in athymic mice. Both compounds showed similar growth inhibitory effects, despite the fact that *N,N,N*-trimethyl-D-*erythro*-sphinganine showed a much stronger inhibitory effect than *N,N*-dimethylsphingosine on PKC activity (Endo et al., 1991).

2.3.3 Staurosporine-Derivatives

Staurosporine, isolated from Streptomyces (Omura et al., 1977) is a very potent inhibitor of PKC (Tamaoki et al., 1986). The compound is an unspecific kinase inhibitor and inhibits protein tyrosine kinases, cAMP-dependent kinases and myosin light-chain kinases with only moderately higher IC_{50} values than those for PKC (Meyer et al., 1989). Recently staurosporine-derivatives with higher specificity for PKC such as benzoylstaurosporine (CGP 41251, Meyer et al., 1989; Caravatti et al., 1994; Marte et al., 1994), RO 31-8220 and RO 31-7549 (Dieter and Fitzke, 1991), Gö 6976 and Gö 6850 (Martiny-Baron et al., 1993; Hu, 1996), GF 109203X (Toullec et al., 1991), 7-hydroxystaurosporine (UCN-01, Seynaeve et al., 1994), CGP 53506 and CGP 54345 (Zimmermann et al., 1996), rottlerin (Gschwendt et al., 1994) and LY333531 (Ishii et al., 1996) have been synthesized. With the exception of rottlerin all of them exhibit the lowest IC_{50} values for cPKCs (Mizuno et al., 1995; Hofmann, 1997). Bryostatins and phospholipid analogues modulate PKC activity by interaction with the regulatory domain. Staurosporine and its derivatives are believed to inhibit PKC by competition with ATP.

CGP 41251 inhibits preferentially cPKCs (IC_{50} = 0.022-0.031 µM; Marte et al., 1994; Meggio et al., 1995; Fabbro et al., 1999). It also inhibits vascular endothelial growth factor(VEGF)-inducible KDR-receptor tyrosine kinase (IC_{50} = 0.086 µM). PDGF receptor tyrosine kinase is inhibited with an IC_{50} of 0.08 µM (Fabbro et al., 1999). CGP 41251 inhibited the proliferation of human bladder carcinoma, leukemia, small cell lung cancer, non-small cell lung cancer, glioblastoma, gliosarcoma and breast carcinoma cell lines (Meyer et al., 1989; Courage et al., 1995; Ikegami et al., 1995; Ikegami et al., 1996a; Begemann et al., 1996; Fabbro et al., 1999). The compound exhibited antitumor activity in human tumor xenografts derived from bladder, melanoma, gastric, colorectal, breast or lung cancers in nude mice (Meyer et al., 1989; Ikegami et al., 1995). Consistent with the finding that CGP 41251 blocks the VEGF-dependent phosphorylation of KDR (one of the two major receptors for VEGF) in vitro was the result obtained in mice that received a subcutaneous VEGF-impregnated implant. The treatment with CGP 41251 completely inhibited the angiogenic response to VEGF, but not to bFGF. Thus, CGP 41251 may suppress tumor growth by inhibiting tumor angiogenesis in addition to directly inhibiting tumor cell proliferation via its effects on PKCs. This anti-angiogenic action may contribute to the broad antitumor activity displayed by the compound (Fabbro et al., 1999). In clinical phase I (Czendlik and Graf, 1996; McDonald et al., 1997) and phase II studies (Mehta et al., 1997) CGP 41251 has been well tolerated.

UCN-01 has been shown to inhibit the proliferation of MCF-7, MDA-MB453, SK-BR-3 (Seynaeve et al., 1993), A549 and MCF-7 (Courage et al., 1995) and glioma cell lines (Pollack et al., 1996) in vitro. The compound inhibited the growth of myeloid leukemia, fibrosarcoma and epidermoid carcinoma xenografts in mice. UCN-01 inhibited the down-modulation of epidermal growth factor receptor caused by TPA in A431 cells at a near 50% inhibitory concentration for cell growth. (Akinaga et al., 1991). Clinical trials using the compound as an antitumor agent are ongoing (Lush et al., 1997). A major problem in the treatment of patients with both CGP 41251 and UCN-01, may be the binding to plasma proteins such as α-acidic protein (Lush et al., 1997; Sausville et al., 1998; Utz et al., 1998).

Several data indicate that PKC is the major target of these compounds, others do not support this notion. Inhibition of cell proliferation by CGP 41251 correlated with the inhibition of PKC. CGP 42700, a derivative of CGP 41251 did not inhibit PKC and did not inhibit cell proliferation (Meyer et al., 1989; Utz et al., 1998). Human A549 lung carcinoma cells were exposed to CGP 41251, UCN-01 or Ro 31-8220 at gradually increasing concentrations. Cells acquired, 4.3-fold resistance against CGP 41251, 4.0-fold against UCN-01 and 14-fold against Ro 31-8220. Cells were neither collaterally cross-

resistant towards the PKC inhibitors nor resistant against the growth-inhibitory properties of TPA. PKC activity in these cells was decreased by between 57% and 96% compared to wild-type A549 cells. The levels of PKCα and PKCθ in all 3 resistant cell types and of PKCε in UCN-01-resistant cells were concomitantly reduced (Courage et al., 1997). Compounds with varying potencies for PKC inhibition were investigated for their antiproliferative activity in A549 and MCF-7 carcinoma cells. When the IC_{50} values for cell proliferation were plotted against the IC_{50} values for inhibition of cytosolic PKC activity, two groups of compounds could be distinguished. The group which comprised the more potent inhibitors of enzyme activity (calphostin C, staurosporine and its analogues UCN-01, RO 31-8220, CGP 41251) were the stronger growth inhibitors, whereas the weaker enzyme inhibitors (trimethylsphingosine, miltefosine, NPC-15437, H-7, H-7I) affected proliferation less potently. GF 109203X was exceptional in that it exhibited high potency for PKC inhibition but was only weakly cytostatic. The PKCα selective inhibitor CGP 53506 has been shown to inhibit growth of T24 bladder carcinoma cells (Zimmermann et al, 1996). Novel diaminoanthraquinones also have therapeutical potentials against human tumors (Jiang et al., 1992).

In cells depleted of PKC by incubation with bryostatin, growth arrest induced by staurosporine, RO 31-8220, UCN-01 or H-7 was slightly, but not significantly lower than that observed in control cells not treated with bryostatin 1. These results suggested that PKC is unlikely to play a direct role in the arrest of the growth of A549 and MCF-7 cells mediated by these agents (Courage et al., 1995). The antiproliferative activity of these compounds may be caused at least in part by inhibition of kinases involved in cell cycle regulation as CDKs or retinoblastoma protein. It has been reported that CGP 41251 and UCN-01 interact with cell cycle progression (Begemann et al., 1996; Ikegami et al., 1996a; Ikegami et al., 1996b; Courage et al., 1996; Kakawami et al., 1996; Wang et al., 1996; Shimizu et al., 1996; Akiyama et al., 1997; Yu et al., 1998). CGP 41251 and UCN-01 are also involved in the induction of apoptosis (Wang et al., 1995; Han et al., 1996; Hunakova et al., 1996; Shao et al., 1997) which may be PKC-dependent or PKC-independent.

2.3.4 Antisense Oligonucleotides

Down-regulation of PKCα by antisense phosphorothioate oligonucleotides significantly reduced the rate of proliferation of three human glioma cell lines. This reduction in growth rate was attributed to apoptosis (Dooley et al., 1998). Expression of antisense oligonucleotides directed towards PKCα in U-87 glioblastoma cells resulted in no detectable PKCα content, a 95% reduction in total PKC activity, an increase in doubling time in vitro, less

serum-dependent growth and reduced sensitivity to the selective PKC inhibitor Ro 31-8220 (Ahmad et al., 1994). Treatment of mice bearing U-87 xenografts with oligonucleotides against PKCα resulted in suppression of tumor growth (Yazaki et al., 1996). In T-24 human bladder carcinoma cells, expression of PKCα antisense led to a reduction of PKCα and was without effect on the expression of other PKC isoenzymes. The antisense oligonucleotide inhibited the growth of T-24 bladder carcinoma, A549 lung carcinoma and Colo 205 colon carcinoma cells in a dose-dependent manner in nude mice (Dean et al., 1996). The selective depletion of PKCα in smooth muscle cells by antisense towards PKCα inhibited proliferation, but did not induce apoptosis (Leszczynski et al., 1996). A 20-mer phosphorothioate oligodeoxynucleotide directed against human PKCα alone or in combination with established antitumor drugs were studied in nude mice that had been transplanted s.c. with a variety of human tumors (breast, prostate, large cell lung and small cell lung carcinomas, and melanomas). Additive antitumor effects with the antisense oligonucleotide and the cytotoxins were found for half of the combinations studied. The combination of the antisense oligonucleotides with vinblastine or cis-platin showed superadditive antitumor activities against MCF-7 human breast carcinomas and PC3 prostate carcinomas with complete responses, that with adriamycin resulted in superadditive antitumor effects against BT20 human breast carcinomas with complete tumor responses, and that with mitomycin C showed superadditive antitumor effects with cures observed against NCI-H460 human large cell carcinomas. The antisense oligonucleotide was completely inactive as single agent against A549 and NCI-H69 human lung carcinomas (Geiger et al., 1998). C8161 human melanoma cells were treated in vitro with a phosphorothioate antisense oligodeoxynucleotide that specifically inhibited PKCα. Northern blots demonstrated 70% inhibition of PKCα mRNA in treated cells compared to controls. Metastasis was suppressed by 75% when oligonucleotide-treated cells were injected intravenously into athymic mice (Dennis et al., 1998). The expression of antisense PKCα markedly inhibited the cell proliferation rate, colony forming efficiency in soft agar, and tumorigenicity of human LTEPa-2 lung carcinoma cells in nude mice (Wang et al., 1999a). Animals implanted with the pancreatic cancer cells overexpressing PKCα had a mortality rate almost twice that of those implanted with the parental non overexpressing cell line. Animals treated with antisense oligonucleotides directed against PKCα after orthotopic implantation of pancreatic cancer cells survived statistically longer than those treated with vehicle alone. Treatment with a scrambled oligonucleotide also conferred a survival benefit compared with vehicle alone (Denham et al., 1998). In a clinical phase I study with an antisense phosphorothioated oligonucleotide

to PKCα, dose limiting was complement activation. From 36 patients with advanced cancer, two patients with non-Hodgkins's lympoma achieved complete remissions (Nemunaitis et al., 1999).

Antisense oligonucleotides directed against PKCβII led to loss of proliferative capacity in K562 cells (Murray et al., 1993). Downmodulation of PKCζ by antisense had no effect on the proliferation and saturation density in K562 cells (Murray et al., 1997). However, Spitaler et al. (1999) found that down-modulation of PKCζ with antisense oligonucleotides in HeLa cells led to the induction of apoptosis.

2.3.5 Remarks and Conclusions

PKC isoenzymes seem to play important roles in cell proliferation and tumor growth. There are several explanations for the contradicting results. Some experiments show that a certain PKC isoenzyme seems to be involved in proliferation, but other results indicate that this isoenzyme may be involved in differentiation and inhibition of proliferation. These highly variable effects may be due to the different cells used in these experiments. Many conclusions were made by overexpression of a certain PKC isoenzyme. In many experiments overexpression of PKC isozymes was induced by DNA-transfection. This may lead to overexpression at the "wrong time" or in the "wrong cell line". The localization of the PKC isoenzymes by receptors for active C-kinase (RACKs) or receptors for inactive C-kinase (RICKs) is important for the PKC activity and the phosphorylation of specific targets (Mochley-Rosen and Gordon, 1998). The levels of these proteins may depend on the tissue. The lack of such proteins may lead to inappropriate localization of PKC. It has been shown that mislocation of PKC isoenzymes led to altered behaviour of the cells to TPA (Whelan et al., 1999). Overexpression of a PKC isoenzyme does not necessarily lead to elevated PKC activity because the overexpressed protein may be inactive in the cytosol (Mischak et al., 1993). Following activation and downmodulation in physiological conditions, the catalytic domains of PKC isoenzymes may have some functions in the nuclear envelope, nuclear matrix, nucleolus or chromatin (Buchner, 1995; Martelli et al., 1999). In tumor cells increased expression of PKC isoenzymes may be important for tumor growth. However, the expression of PKC may also be accidental because of the unstability of the genome in tumor cells. It is not known exactly whether tumor promotion is the result of short term activation, prolonged activation or down-modulation. PKC modulators such as bryostatins, phospholipid analogues, UCN-01, CGP41251 or PKCα antisense show antitumor activity. However, it is not clear whether PKC is the only or the major target or whether other kinases

are the main targets of these compounds. It was indeed presumed that growth inhibition by bryostatin does not result from interaction with PKC and that the interaction of bryostatin with PKC is responsible for the toxicity of the compound (Szallasi et al, 1996). Further investigations into the exact role of the different PKC isoenzymes in proliferation, differentiation and tumor growth are essential.

3 PKC in Apoptosis

3.1 Introduction

The control of cell numbers is regulated by cell proliferation, differentiation and apoptosis. Increased proliferation and/or decreased apoptosis result in neoplasia. In addition to inhibition of proliferation or induction of differentiation, the modulation of apoptosis can be employed for treatment for cancer. Several anticancer agents in use are potent inducers of apoptosis (Dive and Hickman, 1991; Fisher, 1994). Tumor promotion may result in decreased apoptosis. Because PKC activation by TPA induces carcinogenesis, it seems that PKC may be involved in apoptosis. There are many reports on the effects of PKC on apoptosis. However, the results are very controversial. Here an overview of these data is presented.

3.2 PKC Activation Promotes Apoptosis

Fragmentation of DNA induced by tyrosine-kinase inhibitors was enhanced by activation of PKC with TPA in mouse thymocytes (Azuma et al., 1993). Exposure of human myeloid leukemia HL-60 and KG-1 cells to mitoxantrone induced programmed cell death. Pretreatment with TPA enhanced mitoxantrone-induced apoptosis, whereas staurosporine and H-7 had no effect (Bhalla et al., 1993). Exposing HL-60 cells to TPA for 48 hours induced morphological changes characteristic of apoptosis. In contrast, TPA for five days did not induce apoptosis in a HL-60 mutant defective in its response to TPA. It was concluded that the promyelocytes have the capacity to undergo apoptosis in response to agents which activate PKC (MacFarlane and O'Donnell, 1993). In mouse but not in rat thymocytes, apoptosis was potentiated by TPA and prevented by H-7 (Shaposhnikova et al., 1994). In murine B lymphoma WEHI-231 cells signaling through anti-receptor antibodies led to growth arrest and apoptosis. Direct activation of PKC with phorbol esters also could mediate this response (Haggerty and Monroe, 1994). The deoxycytidine analogues ara-C and gemcitabine induced apoptosis in human ovarian BG-1 cancer cells and at the same time activated PKC. Short-term

exposure to TPA and gemcitabine did not alter the response to gemcitabine. However, a 24-hour exposure to TPA followed by gemcitabine resulted in synergistic cytotoxicity. Coincubation of TPA with a PKC inhibitor abrogated the synergistic response (Cartee and Kucera, 1998, Cartee et al., 1998).

Overexpression of PKCζ in U937 histiocytic lymphoma cells increased expression of PKCα and β isoforms. In response to TPA, parental U937 cells displayed growth arrest and differentiated into a monocyte/macrophage-like cell line, while PKCζ overexpressing cells underwent death. The ability of GF109203X to inhibit TPA-induced cell death suggested that activation of a conventional isoform was necessary to induce apoptosis (de Vente et al., 1995). Activation of PKC enhanced and down-modulation of PKC activity reduced apoptosis of neuronal cells (Mailhos et al., 1994). Ceramide, an activator of PKCζ is an inducer of apoptosis (Muller et al, 1995; Hannun, 1998, Obeid et al., 1993).

3.3 PKC Activation Prevents Apoptosis

Activation of PKC by TPA suppressed IL-6-starvation-induced apoptosis in T1165 and T1198 plasmacytoma cells (Romanova et al., 1996). Stimulation of PKC by TPA blocked DNA fragmentation and cell death induced by the Calcium-ionophore A23187 or glucocorticoids in thymocytes (McConkey et al., 1989). A23187 was found to induce apoptosis in immature mouse thymocytes. The addition of TPA at low concentration inhibited the DNA fragmentation induced by A23187 and was accompanied by an increase in DNA synthesis. The result suggested that PKC activation switched a suicide process induced by A23187 to an opposite process (Kizaki et al., 1989). A combination of A23187 and TPA inhibited corticosterone-induced apoptosis in lymphocytes (Iseki et al., 1991). Usually chronic lymphocytic leukemia cells undergo apoptosis during culture for 1 to 2 days. DNA fragmentation was greatly enhanced when cells were cultured in the presence of colchicine, etoposide, or methylprednisolone. Phorbol esters inhibited cell death. Phorbol ester action was prevented by H-7 (Forbes et al., 1992). Activation of PKC by short term treatment of HL-60 cells with TPA inhibited apoptosis triggered by topoisomerase I and II inhibitors (Solary et al., 1993) and by singlet oxygen (Zhuang et al., 1998). Induction of apoptosis by C_2-ceramide or TNFα was prevented by TPA activation of PKC in U937 cells (Obeid et al., 1993). Activation of PKC by TPA also inhibited DNA damage-induced apoptosis in U937 cells (Kaneko et al., 1999). The activation of PKC promoted cell survival of mature lymphocytes prone to apoptosis and protected them from radiation-induced apoptosis (Lucas et al., 1994). Apoptosis occurring at a high rate among B cells in germinal centers can be arrested by

TPA (Knox et al., 1993). Activation of PKC by TPA prevented Fas-induced apoptosis in human leukemic T cell lines (Ruiz-Ruiz et al., 1997). Apoptosis of B cells occurring in the ileal Peyer's patch follicles in sheep could be abrogated during the first 12 hours of culture by the addition of TPA or PdBu (Motyka et al., 1993). Translocation of PKC from the cytosol rescued center B cells in germinal centers from apoptosis (Knox and Gordon, 1994).

Serum deprivation of C3H 10T½ fibroblasts resulted in DNA fragmentation which was prevented by TPA. Palmityl carnitine, an inhibitor of PKC, reversed the effects of TPA (Kanter et al., 1984). bFGF and phorbol esters protected endothelial cells against radiation-induced apoptosis. bFGF mediated the translocation of PKCα form the cytosol into the membrane (Haimovitz-Friedman, 1994a). PKC activation blocked radiation-induced apoptosis, and apoptosis was restored by ceramide analogues added exogenously (Haimovitz-Friedman et al., 1994b). Treatment of U937 and HL-60 cells with 0.5–1 μM daunorubicin induced a greater than 30% activation of neutral sphingomyelinase activity within 4–10 min with concomitant sphingomyelin hydrolysis and ceramide generation. Activation of PKC by TPA and phosphatidylserine inhibited daunorubicin-induced neutral sphingomyelinase activation, sphingomyelin hydrolysis, ceramide generation, and apoptosis. The apoptotic response could be restored by the addition of 25 μM C_6-ceramide. Therefore, it was concluded that PKC activity negatively regulates the anthracycline-activated sphingomyelin-ceramide apoptotic pathway (Mansat et al., 1997).

3.4 PKC Inhibition Promotes Apoptosis

Granulocyte macrophage colony-stimulating factor or interleukin-3 suppressed apoptosis in hemopoietic cells. H-7, staurosporine, and sphingosine reverted this suppression of apoptosis. Conversely, TPA allowed a bypass of receptor activation in suppression of apoptosis (Rajotte et al., 1992). Induction of apoptosis by freezing and rewarming of confluent human synovial McCoy's cells was inhibited by activation of PKC and promoted by H-7 or sphingosine (Perotti et al., 1990). H-7 enhanced dexamethasone induced apoptosis in mouse natural killer cells and cytotoxic T lymphocytes (Migliorati et al., 1994). Expression of v-abl prevented apoptosis in a haemopoietic cell line. Calphostin C restored apoptosis. Chronic exposure to TPA did not alter survival of the cells (Evans et al., 1995). Addition of chelerythrine or calphostin C to murine B lymphoma WEHI-231 cells triggered apoptosis (Chmura et al., 1996a). GF109203X reverted the suppression of apoptosis mediated by IL-2 or IL-2 plus dexamethasone in murine T-cells. The use of TPA allowed a bypass of the IL-2/IL-2R interaction in the sup-

pression of apoptosis mediated by dexamethasone or IL-2 withdrawal in TS1 cells with medium but not in TS1 cells with high affinity IL-2 receptors. (Gomez et al., 1994). Bisindolylmaleimide VIII potentiated Fas-mediated apoptosis in human astrocytoma 1321N1 and in Molt-4 T cells, both of which were devoid of apoptosis induced by anti-Fas antibody in the absence of the PKC inhibitor (Zhou et al., 1999). Calphostin C and TNF-α were found to induce apoptosis in U937 histiocytic lymphoma cells. Treatment with TPA prevented apoptosis induced by these compounds. A peptide derived from the V1 region of PKCε specifically blocked translocation of PKCε and blocked also the inhibitory effect on apoptosis by TPA (Mayne and Murray, 1998). Staurosporine inhibition of PKC activity in lymphocytes correlated to some extent with the inhibition of [^{3}H]thymidine incorporation and the breakdown of DNA into oligonucleosome-sized fragments (Lucas et al., 1994). Calphostin C and chelerythrine induced apoptosis in HL-60 and U937 cells (Freemerman et al., 1996). In L1210 murine leukemia cell lines sensitive and resistant to apoptosis induced by cis-platin or 5-azacytidine, staurosporine induced apoptosis in both cell lines (Segal-Bendirdjian and Jacquemin-Sablon, 1996). Staurosporine and H-7 potentiated apoptosis triggered by singlet oxygen in HL-60 cells (Zhuang et al., 1998). Incubation of HL-60 cells with H-7, chelerythrine or calphostin C produced concentration-dependent increases in DNA fragmentation (Jarvis et al., 1994a). HL-60 cell transfected with bcl-2 were significantly less susceptible to apoptosis to ara-C-induced apoptosis than untransfected control cells. When bcl-2 overexpressing HL-60 cells were incubated with bryostatin, staurosporine or UCN-01, ara-C induced apoptosis was restored to levels greater than those observed in empty-vector cells (Wang et al., 1997). Staurosporine, H-7, calphostin C and chelerythrine were found to enhance ara-C-induced apoptosis in HL-60 and U937 cells (Grant et al, 1994). Staurosporine potentiated ara-C-related degradation of DNA to oligonucleosomal fragments in HL-60 and U937 cells, but was ineffective when given alone at these concentrations. In contrast, co-administration of H-7, calphostin C and chelerythrine, also increased the extent of DNA fragmentation observed in ara-C-treated cells, but these effects were evident only at inhibitor concentrations that were by themselves sufficient to induce DNA damage (Grant et al., 1994). Exposure of HL-60 cells to ara-C induced time- and concentration-related apoptosis. Treatment with bryostatin 1 alone failed to induce DNA damage, but promoted substantial time- and concentration-related increases in the extent of apoptosis induced by a subsequent exposure to ara-C. Concentrations of bryostatin 1 that maximally potentiated ara-C-related DNA fragmentation were associated with virtually complete down-regulation of total cellular PKC activity, whereas diglyceride and phospholipase C, which suppressed

the response to ara-C, moderately increased total PKC activity (Jarvis et al., 1994b; Jarvis et al., 1998). Apoptosis of freshly isolated rat hepatocytes was induced by either the omission of fetal bovine serum in the culture medium or addition of polymyxin B or staurosporine. This effect was partially prevented by short-term treatment with TPA. After eight hours of incubation, TPA failed to counteract this action and itself produced the apoptosis of rat hepatocytes (Sanchez et al., 1992). UCN-01 enhanced cis-platin cytotoxicity and apoptosis in ovary cancer cells. This occurs regardless of p53 status, but in wild-type p53 cells the degree of sensitization seemed to be increased (Husain et al., 1997). Staurosporine and H-7 augmented TNF-mediated DNA fragmentation in HEL human embryonic lung fibroblast cells which are normally TNF resistant (Kobayashi et al., 1997). Staurosporine increased the TNF-mediated cytotoxicity in two renal cell lines (Woo et al., 1996). Staurosporine and tamoxifen induced apoptosis in glioma cell lines (Couldwell et al., 1994). Tamoxifen significantly enhanced adriamycin-induced cytotoxicity and apoptosis of hepatocellular Hep-3B MDR1 expressing cells. (Cheng et al., 1998). Safingol increased apoptosis in SK-GT-5 and MKN-74 gastric cancer cells after exposure to the alkylating agent mitomycin C. Simultaneous exposure of SK-GT-5 cells to safingol and TPA abrogated the safingol-mediated ehancement of mitomycin C-induced apoptosis. (Schwartz et al, 1995). Down-regulation of PKCα by antisense phosphorothioate oligonucleotides led to apoptosis in glioma cells (Dooley et al., 1998). The PKC inhibitor Ro 31-8220, which preferentially inhibits the PKCα, β and γ isoenzymes, induced apoptosis mainly in glioblastoma cells expressing high levels of PKCα. PKCα suppressed apoptosis in theses cells by restricting the accumulation of p53 and the expression of insulin-like growth factor-1-binding protein as well as by maintaining the retinoblastoma protein in an inactive hyperphosphorylated state (Shen and Glazer, 1998). Exposure of cells to a genotoxic stimulus that induced apoptosis, led to an inhibition of PKCζ (Berra et al, 1997). The product of the par-4 gene interacted with PKCζ and inhibited its enzymatic activity. The expression of par-4 correlated with growth inhibition and apoptosis (Diaz-Meco et al, 1996).

3.5 PKC Inhibition Prevents Apoptosis

In contrast to the reports by Wang et al. (1997) and Grant et al. (1994), (see page 28) it was also reported that H-7 and staurosporine blocked apoptosis following induction by ara-C in HL-60 cells (Kharbanda, et al, 1991). The calcium ionophore A23187 induced apoptosis in immature mouse thymocytes. H-7 inhibited the DNA fragmentation and cell death (Kizaki et al.,

1989). In murine thymocytes radiation-induced DNA fragmentation could be prevented by treatment with H-7 or staurosporine during incubation time. Incubation of irradiated cells with HA-1004, an inhibitor of cAMP-dependent protein kinase, with a minor effect on PKC did not affect the DNA fragmentation induced by irradiation. Incubation of cells with PdBu gave a dose-dependent induction of DNA fragmentation. This effect could be inhibited by staurosporine (Ojeda et al., 1992). During metanephric development the metanephric mesenchyme is programmed for apoptosis. Incubation of mesenchyme with a heterologous inducer, embryonic spinal cord prevented this DNA degradation. Phorbol esters mimicked the effects of the inducer and staurosporine prevented the effect of the inducer (Koseki et al., 1992). Downmodulation of PKC by TPA prevented apoptosis in DU-145 human prostatic carcinoma cells (Rusnak and Lazo, 1996).

3.6 PKC Isoenzymes and Apoptosis

Different PKC isoenzymes seem to be involved in the induction of apoptosis. However, the exact role of each isoenzyme is not clear at present (McConkey et al., 1994; Lucas and Sanchez-Margalet, 1995; Lavin et al., 1996; Grant et al., 1996; Deacon et al., 1997). Here the known effects of different PKC isoenzymes on apoptosis are summarized.

There are reports indicating that inhibition of PKCα activity seems to be associated with apoptosis as shown by a series of antisense oligonucleotides directed towards PKCα (see section 2.3.4). Other examples are: The PKC inhibitor Ro 31-8220, which preferentially inhibits the PKCα, β and γ isoenzymes, induced apoptosis mainly in glioblastoma cells expressing high levels of PKCα (Shen and Glazer, 1998). Tamoxifen significantly enhanced adriamycin-induced cytotoxicity and apoptosis of hepatocellular Hep-3B cells. Tamoxifen inhibited the activation of PKCα. TPA restored the membrane translocation of PKCα and abrogated the synergistic cytotoxicity of tamoxifen and adriamycin (Cheng et al., 1998). In Jurkat cells induction of apoptosis by Fas-activation was found to inhibit the ability of PKCα to phosporylate histone H1, but did not inhibit PKCε (Chen and Faller, 1999). In human erythroleukemia TF-1 cells PKCα was inactivated within 5 minutes of treatment with apoptosis-inducing levels of ionizing radiation. This postirradiation inactivation did not occur when cells were rescued from apoptosis by GM-CSF. The survival signal seemed to be mediated by PKCα but not by PKCβII or PKCε (Kelly et al., 1998). A dominant negative mutant of PKCα induced apoptosis in COS-1 cells. Expression of wild-type PKCα was able to rescue the cells from apoptosis (Whelan and Parker, 1998). bFGF and phorbol esters protected endothelial cells against radiation-induced

apoptosis. bFGF mediated the translocation of PKCα form the cytosol into the membrane (Haimovitz-Friedman et al., 1994a). IL-6-starvation of T1165 and T1198 plasmacytoma cell lines led to apoptosis which was suppressed by TPA-induced PKC-activation that involved PKCα and/or PKCδ (Romanova et al., 1996). PKCα activity was found to be essential for the prevention of apoptosis in Ramos-BL Burkitt's lymphoma cells (Keenan et al. ,1999).

On the other hand there are also data showing that PKCα activity seems to be essential for apoptosis. In human prostate cancer cells, the presence of PKCα in the membrane correlated with apoptosis, the absence correlated with resistance to apoptosis (Powell et al., 1996). Safingol potentiated mitomycin C-induced apoptosis in MKN-74 gastric cancer cells. Mitomycin treatment alone resulted in a complete loss of PKCα from the membrane and the cytosol and of PKCε from the membrane. Treatment with safingol and mitomycin C together resulted in complete restoration of PKCα and PKCε to the levels of untreated controls. TPA blocked the enhancement of mitomycin C-induced apoptosis by safingol and was accompagnied with loss of PKCα but not of PKCε. From these experiments it was concluded that restoration of PKCα but not PKCε is essential for enhancement of apoptosis (Danso et al., 1997). The steady-state population of stratified squamous epithelium is maintained by balanced cell proliferation and apoptosis. The surface epithelium of the human tonsil expressed cytoplasmic PKCα, β, δ, ε, and ζ. PKCδ and ε were most abundant in viable epithelial cells while PKCα and β were most intense in cells undergoing apoptosis (Knox et al., 1993). Treatment of parental U937 cells with TPA displayed growth arrest and differentiation into a monocyte/macrophage-like cell line, while PKCζ-overexpressing cells underwent cell death following treatment with TPA. The ability of GF109203X to inhibit TPA-induced death of PKCζ overexpressing cells suggested that activation of a conventional isoform was necessary to induce apoptosis (Ways et al., 1994).

One possible explanation for the contrasting results obtained with PKCα may be that in addition to PKCα also other PKC isoenzymes may be involved in apoptosis. The selective depletion of PKCα in smooth muscle cells by antisense oligonucleotides against PKCα inhibited proliferation without concomitant induction of apoptosis (Leszczynski et al, 1996). However, induction of apoptosis by ceramide was associated with inactivation of PKCα and activation of PKCζ (Lee et al, 1996a). PKCα and PKCζ seem to be involved in the phosphorylation of I-κB kinase β which leads to the induction of NF-κB-inducable genes (Lallena et al., 1999). Another possible explanation may be that PKCα is activated and down-modulated during different stages of the apoptic process. For example, it was reported that caspases regulate PKCα activity in HL-60 cells. PKCα activity was initially inhibited at

one hour and subsequently activated during apoptosis. PKCβI and PKCδ were proteolytically cleaved and activated during apoptosis in these cells (Shao et al., 1997), indicating also that several PKC isoenzymes may concomitantly be involved in apoptosis. The effects of PKCα inhibition or activation may also depend on the presence and status of other factors such as raf-1 or bcl-2. PKCα phosphorylates raf-1 (Kolch et al, 1993) as well as bcl-2 (Ruvolo et al, 1998) and raf-1 also interacts with the apoptosis-preventing bcl-2 protein (Blagosklonny et al, 1997).

Expression of v-abl prevented apoptosis in a haemopoietic cell line. Calphostin C restored apoptosis. Chronic exposure to TPA did not alter survival of the cells. The PKC isoenzyme responsible for these effects was identified as PKCβII (Evans et al., 1995). Induction of apoptosis by growth to high density or by etoposide in U937 cells was associated with dephosphorylation and downmodulation of PKCβI (Whelan and Parker, 1998). However, spontaneously apoptotic U937 cells from exponentially growing cell cultures exhibited increased PKCβ and reduced PKCζ expression (Pongracz et al., 1995b). 12-deoxyphorbol-13-O-phenylacetate-20-acetate (DOPPA), an activator of PKCβI, induced apoptosis in U937 cells (Pongracz et al., 1996). In contrast to the parental HL-60 cells, PKCβ-deficient HL-525 cells were resistant to TNFα-induced apoptosis but sensitive to anti-Fas monoclonal antibody-induced apoptosis. Both cell types expressed similar levels of the TNF-receptor I, whereas the Fas receptor was detected only in HL-525 cells. Transfecting the HL-525 cells with an expression vector containing PKCβ reestablished their susceptibility to TNF-alpha-induced apoptosis (Laouar et al., 1999). Ara-C induced apoptosis and concomitantly increased membrane-bound (activated) PKCβII, but not PKCα or PKCδ. Ara-C or TPA-induced translocation of PKCβII was inhibited by edelfosine, and ara-C-induced apoptosis was stimulated by pretreatment of the cells with edelfosine. Edelfosine or antisense oligonucleotides directed toward PKCβII, but not the sense contol, enhanced ara-C-induced apoptosis. Edelfosine also inhibited stimulation of bcl-2 by TPA and enhanced the decrease in bcl-2 observed in ara-C-treated cells (Whitman et al., 1997).

PKCδ is cleaved by caspase 3 during apoptosis. This leads to the generation of an active fragment of PKCδ and growth inhibitory effects (Emoto et al., 1995; Ghayur et al., 1996). PKCδ was also activated by a caspase-dependent proteolysis during UV-induced apoptosis (Denning et al., 1998). Upon treatment with TPA, the growth of CHO cells overexpressing the PKCδ subspecies was markedly inhibited, whereas cell lines overexpressing PKCα, PKCβII, and PKCζ subspecies were not significantly affected (Watanabe et al., 1992). Bistratene A, an activator of PKCδ, induced translocation of PKCδ to the nucleus and induced growth arrest in G2/M in HL-60 cells, suggesting

that activation of PKCδ can induce growth arrest and apoptosis in HL-60 cells (Griffiths et al., 1996). In rat vascular smooth muscle cells apoptosis was found to be regulated by PKCδ and ζ but not by PKCα and ε (Leszczynski et al., 1995). PKCδ was shown to be a growth and tumor repressor in rat colonic epithelial cells (Perletti et al., 1999). The tyrosine kinase and oncogene src promoted cell proliferation and DNA synthesis. Src promote tyrosine phosphorylation of PKCδ and its subsequent degradation (Blake et al., 1999).

In glucocorticoid-induced apoptosis in murine thymocytes, PKCε is selectively activated (Iwata et al., 1994). Recently also proteolytic activation of PKCε during chemotherapeutic agent-induced apoptosis by caspase 3 was reported in U937 cells (Koriyama et al., 1999). Activation of PKCε is also critical for cardiac myocyte protection from hypoxia-induced apoptosis (Gray et al., 1997). Calphostin C and TNF-α were found to induce apoptosis in U937 histiocytic lymphoma cells. Treatment with TPA prevented apoptosis induced by these compounds. A peptide derived from the V1 region of PKCε specifically blocked translocation of PKCε and blocked also the inhibitory effect on apoptosis by TPA (Mayne and Murray, 1998). These results indicate that PKCε is activated during apoptosis. However, R6 cells stably transfected with PKCε were shown to prevent cis-platin-induced apoptosis (Basu and Cline, 1995).

PKCθ is cleaved by caspase 3 during apoptosis induced by diverse agents (Datta et al, 1997). In Jurkat cells, induction of apoptosis by activation of Fas inhibited the TPA-induced translocation of PKCθ from the cytosol to the membrane (Chen and Faller, 1999). In murine thymocytes treatment with the diterpene diester, ingenol-3,-20-dibenzoate induced selective translocation of nPKCδ, ε, θ and μ from the cytosolic fraction to the particulate fraction and induced apoptosis. This induction of apoptosis was inhibited by non-isoform-selective PKC inhibitors, but not by their structural analogs with weak PKC-inhibitory activity or the selective inhibitor of cPKCs and PKCμ, Gö 6976 (Asada et al., 1998).

K562 chronic myelogenous leukemia cells are highly resistant to chemotherapeutic drugs, such as taxol, that induce cell death by apoptosis. This resistance is mediated by the chimeric tyrosine kinase oncogene bcr-abl. PKCι overexpression protected K562 cells against ocadaic acid- and taxol-induced apoptosis, whereas overexpression of PKCζ did not exhibit this resistance (Murray and Fields, 1997). Treatment of K562 cells with taxol led to sustained activation of PKCι. In contrast, bcr-abl-negative HL-60 myeloid leukemia cells, which are sensitive to taxol-induced apoptosis, did not exhibit sustained PKCι activation in response to taxol. Treatment of K562 cells with tyrphostin AG957, a selective bcr-abl inhibitor, blocked taxol-induced

PKCι activation and sensitized these cells to taxol-induced apoptosis, indicating that PKCι is a relevant downstream target of bcr-abl-mediated resistance. Furthermore, expression of constitutively active PKCι by adenovirus-mediated gene transfer rescued AG957-treated K562 cells from taxol-induced apoptosis (Jamieson et al., 1999). If focal adhesion kinase or the correct extracellular matrix was absent, cells entered apoptosis through a p53-dependent pathway. PKCλ/ι was required for this apoptosis. It was concluced that PKCλ/ι phosphorylates p53 and increases its stability enabling it to induce apoptosis (Ilic et al., 1998).

Exposure of cells to a genotoxic stimulus that induced apoptosis led to an inhibition of PKCζ (Berra et al, 1997). The product of the par-4 gene interacts with PKCζ and inhibits its enzymatic activity. The expression of par-4 correlated with growth inhibition and apoptosis (Diaz-Meco et al, 1996). Loss of the ζ isoenzyme triggered apoptosis (Leszczynski et al, 1995). Overexpression of PKCζ did not protect cells from taxol-induced apoptosis (Murray and Fields, 1997), indicating also that elevated levels of this isoenzyme are not necessarily correlated with resistance to apoptosis. PKCζ is cleaved by caspase 3 following induction of apoptosis by UV. The PKCζ fragment generated is enzymatically inactive. PKCι is not cleaved following UV treatment (Frutos et al., 1999). DNA fragmentation was associated with inhibition of PKC by H-7, chelerythrine, calphostin C in HL-60 cells promyelocytic cells. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway led to increased ceramide levels (Jarvis et al., 1994a). TPA induced apoptosis and rapid ceramide generation in LNCaP prostate cancer cells. Treatment with fumonisin B1, a specific inhibitor of ceramide synthase, abrogated both ceramide production and TPA-induced apoptosis. Ceramide analogues bypassed fumonisin B1 inhibition to initiate apoptosis directly. Thus, ceramide appeared to be a neccesary signal for TPA-induced apoptosis in LNCaP prostate cancer cells (Garzotto et al., 1998). Exposure of murine B lymphoma WEHI-231 cells to chelerythrine or calphostine C induced apoptosis and increased ceramide production. This suggested an antagonistic relationship between PKC activity and ceramide in the signaling events preceding apoptosis (Chmura et al., 1996a; Chmura et al., 1996b). Ceramide a potent inducer of apoptosis (Hannun, 1998), is an activator of PKCζ (Lozano et al., 1994). An explanation for this discrepancy may be that ceramide concentrations as low as 0.5 nM activated PKCζ, whereas concentrations above 60 nM led to a downmodulation. Based on these data it was suggested that PKCζ may act as a molecular switch between mitogenic and growth inhibitory signals (Muller et al., 1995). In addition to PKC, ceramide also interacts with other targets as ceramide-activated protein kinase or ceramide-activated protein phosphatase (Mathias et al.,

1998). The exact role of ceramide in apoptosis seems not to be clear because of problems detecting ceramide levels (Kolesnick and Hannun, 1999; Hofmann and Dixit, 1999; Watts et al, 1999). Ganglioside GD3 is an important mediator of Fas-induced apoptosis in hematopoietic cells. Ceramide triggers the synthesis of ganglioside GD3 (DeMaria et al., 1997). So it is difficult to explain whether the inducer of apoptosis is ceramide or ganglioside.

3.7 Remarks and Conclusions

These contrasting results point to a great variability depending on cell type, cell environment, agent, phase of the cell cycle, and intracellular signaling pathways causing apoptosis. One reason for the conflicting results may be the use of different cell lines or even different clones of the same cell line for these investigations, and the expression of different PKC isoenzymes in these cells. For example, it has been reported that PC12 cells express PKCα, β, δ, ε, and ζ (Wooten et al., 1994). Others reported that the PC12 cells they used also expressed PKCγ (O'Driscoll et al., 1995) or PKCη (Borgatti et al., 1996). This may lead to contradicting results obtained in similar experiments employing the same cell line. The reason for controversial results may also be due to cells used from different species. For example, apoptosis was potentiated by TPA and prevented by H-7 in mouse thymocytes but not in rat thymocytes (Shaposhnikova et al., 1994). Death suppression induced by IL-6-starvation in T1165 and T1198 plasmacytoma cells by transient TPA-induced PKC activation occured when a significant number of cells were in "competent" G1 state, allowing them to pass the restriction point safely without initiating the cell death program (Romanova et al., 1996).

The microenvironment may also influence apoptosis. Exposure of isolated thymocytes to TPA plus ionomycin for 24 hours enhanced apoptosis. On the other hand, when thymocytes were cultured in intact lobes, a 24 hour TPA plus ionomycin exposure only marginally induced apoptosis. Therefore, it appears that removing thymocytes from their thymic microenvironment makes the cells more susceptible to certain stimuli, possibly by altering their physiological status. (Moore et al., 1992). Viral infection may also alter apoptosis. Epstein-Barr virus infected human Burkitt's lymphoma cells were particularly sensitive to treatment with PdBu (42% apoptosis at 72 hours), whereas its virus free counterpart displayed only 12% apoptosis (Ishii and Gobe, 1993).

Apoptosis can be induced or prevented through different pathways (TNFα-induced, Fas-induced, TRAIL-induced). In lymphokine-activated killer cells Fas-mediated cytotoxicity could be dissociated from perforin-mediated cytotoxicity by their different requirement of TPA-sensitive PKC

isoforms (Ohmi et al., 1997). Apoptotic death in T cell hybridomas can be induced by glucocorticoids or the stimulation via the TCR/CD3 complex. The two apoptotic processes are mutually antagonistic (Iseki et al., 1991). Interleukin-3 and TPA rescued differentiating myeloid leukemic cells by different pathways. (Lotem et al., 1991). An ATP-dependent and an ATP-independent apoptotic pathway was found by Eguchi et al. (1999). It was reported that both, an increase in the intracellular Ca^{2+} level and an activation of PKC are essential for the TCR/CD3-mediated apoptosis. Either reduction of extracellular Ca^{2+} or addition of H-7 inhibited anti-CD3-induced but not dexamethasone-induced DNA fragmentation. The combination of ionomycin and TPA, but neither one alone nor the combination of ionomycin and cyclic nucleotide analogs, induced DNA fragmentation. On the contrary, only an increase in the intracellular Ca^{2+} level was essential for the inhibition of glucocorticoid-induced apoptosis, because ionomycin alone as well as the combination of ionomycin and TPA inhibited dexamethasone- but not anti-CD3-induced DNA fragmentation (Iseki et al., 1991). The mechanism of induction of apoptosis by staurosporine, UCN-01, and UCN-02 was clearly different from the mechanism that mediated induction of apoptosis by etoposide and dexamethasone (Harkin et al., 1998).

Staurosporine, UCN-01, and UCN-02 (a weak PKC inhibitor) induced a concentration- and time-dependent increase in apoptosis, whereas neither CGP 41251, RO 31-8220, nor GF 109203X induced apoptosis in immature rat thymocytes. In the human cell line BM13674 the specific inhibition of PKA gave rise to significantly increased levels of apoptosis at postirradiation compared to values after radiation exposure only. Calphostin C which caused 68% inhibition of PKC activity in irradiated cells, did not alter the level of radiation-induced apoptosis (Findik et al., 1995).

It was also shown that different concentrations of a drug or a protein may elicit different effects. The culture of insulin-secreting RIN m5F cells with 0.1–1 nM of staurosporine inhibited DNA synthesis but were unable to trigger apoptosis. 0.1–1 μM of staurosporine which abolished DNA synthesis almost completely, were needed to trigger apoptosis. TPA failed to inhibit this effect. (Sanchez-Margalet et al., 1993). Low concentrations of ceramide activated PKCζ, high concentrations led to a downmodulation (Muller et al., 1995). Bryostatins 1 and 2 inhibited cell proliferation at low concentrations, but showed no effect at high concentrations (Dale et al., 1989). Low levels of raf-1 activity induced proliferation whereas high levels caused growth arrest (Sewing et al., 1997). Low levels of bcl-2 were found to be antiapoptotic, high levels not (Shinoura et al., 1999).

The induction or prevention of apoptosis may depend, in addition to or independent of PKC, on calcium, pH, p53, cyclin-dependent kinases, cyclin-

dependent kinase inhibitors, caspases, raf, bax, bcl-2, bcl-x, mdm2, NF-κB, pRB, STAT-factors, and many others (Fig. 2). The expression levels of all these factors are usually not determined in the cells used for experiments but they may lead to different results in similar experiments. Contrasting results may also arise from the presence or absence of wild-type or mutated p53. One hypothesis is that PKC may phosphorylate p53 and keep it in a latent inactive state (Magnelli and Chiarugi, 1997), although this has been questioned (Blattner et al., 1999). It is not clear at present whether p53 is a target for PKC phosphorylation (Livneh and Fishman, 1999).

Oncogenes or growth factors, such as c-myc, c-fos, E2F, cyclin D or E1A can induce proliferation and cellular survival but can also cause apoptosis and growth arrest. Quiescent cells may respond to these signals by proliferation whereas proliferating cells may respond by growth arrest or apoptosis. (Lavin et al., 1996; Blagosklonny, 1999). TPA is mitogenic for mature T cells and normal melanocytes. However, it causes cell cycle arrest in tumorigenic T cells and malignant melanoma cells (Burger et al., 1994; Coppock et al., 1995; Desrivieres et al., 1997).

Another reason for conflicting results may be the that the PKC mudulators available so far are not specific. Even the new generation of more specific PKC inhibitors may affect yet uncharacterized kinases (Dieter and Fitzke, 1991; Yu et al., 1998). Different cells or different clones of the same cell line express different PKC isoenzymes (Wooten et al., 1994; O'Driscoll et al., 1995; Borgatti et al., 1996) and, therefore, unspecific inhibitors display different effects. For example, dermal papilla cells underwent apoptosis in a dose-dependent manner when treated with staurosporine but not when treated with H-7 (Ferraris et al., 1997). Chelerythrine inhibited taurine uptake in the retina in a PKC-independent way (Militante and Lomardini, 1999). Induction of apoptosis by PKC modulators may be independent of PKC. For example, in human DU-145 prostatic carcinoma cells, sphingosine induced apoptosis through down-regulation of bcl-2 or bcl-X_L, independently of PKC inhibition (Shirahama et al., 1997, Sakakura et al., 1996). Harkin et al. (1998) found that inhibition of PKC alone is insufficient for induction of apoptosis in thymocytes. Data obtained with TPA are difficult to interpret, because short term treatment with the compound leads to an activation and long term treatment to a downmodulation of PKC (Rodriguez-Pena and Rozengurt, 1984). TPA also interacts with other proteins (Ahmed et al., 1993), PKD (Valverde et al., 1994) and β2-chimaerin (Caloca et al., 1997) and induces the expression of different genes (Rahmsdorf and Herrlich, 1990; Schlatterer et al., 1999). The activation or down-modulation of PKC by TPA may be vary in different cells due to the expression of different PKC isoenzymes. Usually PKC is activated and subsequently downmodu-

lated. It was reported that during apoptosis PKC is inhibited first and subsequently activated (Shao et al., 1997).

Overexpression of one PKC isoenzyme may lead to altered expression and activity of one or more of the other PKC isoenzymes. For certain effects several PKC isoenzymes may be involved. For example, the combined effects of PKCλ, ε and ζ are essential for the transcriptional activation of c-fos by oncogenic H-ras (Kampfer et al., 1998). PKCλ and ζ participate in the ras-mediated reorganization of the F-actin cytoskeleton (Überall et al., 1999). PKCζ can control the phosphorylation and activation of PKCδ (Ziegler et al., 1999). It may be that not the levels of PKCs, but the levels of the dephosphorylated forms of PKCs are important for apoptosis (Whelan and Parker, 1998).

4 PKC and MDR

4.1 Introduction

Cells selected for resistance against a single cytostatic drug may simultaneously acquire cross-resistance to a range of other drugs (Gottesman and Pastan, 1993). Cross-resistance is related to decreased intracellular drug accumulation that is correlated with the presence of a plasma membrane 170-kilodalton glycoprotein (PGP) (Juliano and Ling, 1976). This protein is a broad specificity efflux pump encoded by multidrug resistance genes. Multidrug resistance results in resistance to major classes of anticancer drugs in clinical use, e.g., Vinca alkaloids, anthracyclins, podophyllotoxins and actinomycin D (Gottesman and Pastan, 1993).

There are many reports showing an influence of PKC on MDR. On the other hand, many publications indicated that PKC seems not play a role. Possible interferences between PKC and MDR1 or PGP are indicated in Fig. 3. Here the results showing an involvement of PKC and that arguing against it are summarized.

4.2 PKC-Activity in MDR1-Mediated Drug Resistance

Fine et al. described that the activity of PKC was 7-fold higher in multidrug resistant cells compared with the sensitive parental breast cancer cells (Fine et al., 1988). An over 6-fold increase in PKC activity in the MDR human breast cancer subline MCF-7/DOXR was confirmed when compared with the sensitive parent cell line, MCF-7/WT (Schwartz et al., 1991). Aquino et al. found that multidrug resistant HL-60/ADR cells contained 2-fold more PKC than the parental cell line (Aquino et al., 1988). In four murine UV-2237M

fibrosarcoma cell lines a positive correlation between the level of PKC activity and resistance to ADR was found (O'Brian et al., 1989b). Posada et al. found that the multidrug resistant sarcoma 180A10 subline had the same quantity of PKC as the parent Sarcoma 180 cells, but the resistant cells had significantly higher intrinsic PKC activity and an altered ability to translocate the enzyme (Posada et al., 1989a). It was also shown that multidrug resistant sarcoma 180 and KB cell lines exhibited 80-90% increases in basal PKC activity (Posada et al., 1989b). Enzyme assays showed that multidrug resistant KB-V1 cells exhibited 4-fold higher PKC activity compared with the drug sensitive KB-3 subline (Chambers et al., 1990a, Chambers et al., 1990b). An elevated level of nuclear PKC was found in multidrug resistant MCF-7 human breast carcinoma cells (Lee et al., 1992). The PKC level in the MDR1-expressing cell line K562/D1-9 was higher than that in parental 562 cells (Urasaki et al., 1996).

4.3 Arguments Against a Role of PKC Activity in MDR

In HL-60 cells containing increased levels of PGP it was found that not PKC, but a novel membrane-associated protein kinase phosphorylates and regulates PGP activity (Staats et al., 1990). Staurosporine, at both subtoxic and toxic concentrations as well as at concentrations shown to be inhibitory to PKC, failed to increase drug resistance of parent and resistant MOLT-3 cells and to decrease drug resistance of MCF-7/WT and MCF-7/DOXR cells (Schwartz et al., 1991). Short-term exposure to TPA, which activated PKC 7.0-fold and 4.7-fold, respectively, in the membrane of MOLT-3 and resistant cells, resulted in small increases (rather than decreases) in resistance to adriamycin, whereas for vincristine no consistent trend was observed. Identical results were also obtained with PdBu. These results indicated that PKC activity can be decreased or increased in multidrug resistant cells. Both staurosporine inhibition and phorbol ester activation failed to produce changes in drug resistance that would be considered consistent with the resulting degree of PKC activity. PKC activity in these cells may then be unrelated to MDR (Schwartz et al., 1991). In MDR KB-A1 and KB-A10 cells 100- and 1000-fold resistant to adriamycin, respectively, PKC acitivity was similar in both resistant lines (Dolci et al., 1993). Total cytosolic PKC activity was 400% and 350%, PKCα protein expression was increased by 600% and 375% in KB-A1 and KB-C1, respectively, over the parent KB-3-1 line. A correlation between PKC and multidrug resistance was found only for cells selected in colchicine and not with those selected in other drugs (Drew et al., 1994). A combination of adriamycin and CGP 41251 reduced the number of lung metastases produced by i.v. injection of murine CT-26P or drug-

resistant CT26R500 cells into nude mice. PKC activity was reduced in tumors derived from mice treated with either adriamycin or CGP 41251, but not from those derived from mice treated with the combination (Killion et al., 1995). In drug-sensitive CCRF-CEM, KB-3-1, Hela-WT, multidrug resistant CCRF–ACTD400, CCRF–VCR1000, CCRF–ADR5000, KB-8–5, KB–C1 and HeLa cells transfected with the MDR1 gene, the expression of PGP was the determinant of resistance and PKC did not contribute to the resistance (Utz et al., 1996). An increase in PKC activity in the MDR cell lines KB-A1 and KB-8–5, but not in the MDR lines C6–0.5 and C6–1V compared with their parental cell lines was observed. Cyclosporin A and S-9788 were the most active compounds on MDR reversal and were also able to inhibit PKC activity in the resistant KB as well as in all C6 cell lines. PKCα, γ and δ were increased in the resistant KB sublines. In contrast PKCα and γ were decreased in C6–1V cells, δ in the C5–0.5 line. It was concluded that an increase in PKC activity is not an absolute requirement for expression of the MDR phenotype provided that the basal level be high enough. However, it was also concluded that some modulators may act on PGP, not only through direct PGP interaction, but also through PGP phosphorylation or expression. (Hu and Robert, 1997). A human leukemia K562 mutant 100-fold resistant to the induction of apoptosis by the phosphatase inhibitor okadaic acid showed similar levels of phosphatases but lacked PKCε and overexpressed PGP indicating that MDR is not necessarily associated with increased cPKC activity (Zheng et al., 1994).

4.4 PKC Inhibition or Downmodulation in MDR

Inhibition of PKC activity by staurosporine resulted in decreased resistance to adriamycin. (Posada et al., 1989b; Sato et al., 1990; Sampson et al., 1993). H-7 completely reversed the protection against ADR cytotoxicity conferred on UV-2237M-ADRR cells by deoxycholate, providing evidence that deoxycholate exerts its protective effects by a mechanism that involves stimulation of protein phosphorylation and not merely by detergent effects on membrane permeability (O'Brian et al., 1991a). The PKC inhibitory peptide N-myristoyl-RKRTLRRL reversed adriamycin-resistance in UV-2237M-ADRR cells (O'Brian et al., 1991b). Drug accumulation assays demonstrated that in multidrug resistant KB-V1 cells TPA caused a decrease, wheras staurosporine and calphostin C caused an increase, in accumulation of $[^3H]$vinblastine. These compounds did not alter $[^3H]$vinblastine levels in drug-sensitive KB-3 cells (Chambers et al., 1992). An intrinsic resistant LoVo colon adenocarcinoma cell line showed a significant increase of PKC activity compared with the parental sensitive cell line. Preincubation with H-7 induced PKC inhibi-

tion and reversal of drug resistance (Dolfini et al., 1993). Inhibition of PKC with calphostin C, staurosporine or prolonged treatment with TPA decreased phosphorylation of PGP and impaired transport of vinblastine. Calphostin C also inhibited transport of actinomycin D, vincristine, rhodamine and azidopine in SW620 Ad300 multidrug-resistant human colon carcinoma cells. Photoaffinity labeling of PGP with azidopine was decreased by calphostin C. From these results it was concluded that dephosphorylation alters the affinity of PGP for its substrates (Bates et al., 1993). Daunorubicin resistance in differentiated blast cells was not correlated with the level of PGP expression but rather with the ability to extrude rhodamine 123. Staurosporine used at subtoxic concentrations induced a twofold to threefold enhancement of daunorubicin cytotoxicity, increased rhodamine accumulation and decreased rhodamine efflux kinetics in resistant AML cells. These effects were observed for staurosporine concentrations much lower than those required to displace the PGP-binding probe azidoprazosin, suggesting that staurosporine might act through its PKC inhibitory effect and not through PGP binding (Laredo et al., 1994). Safingol, a lysosphingolipid PKC inhibitor competitively interacts at the regulatory phorbol binding domain of PKC. Safingol treatment of sensitive MCF-7 and resistant MCF-7 DOXR cells inhibited phosphorylation of the myristoylated alanine-rich PKC substrate in both cell lines, suggesting inhibition of PKC. However, only in MCF-7 DOXR cells safingol treatment increased accumulation of [^{3}H]vinblastine and enhanced toxicity of Vinca alkaloids and anthracyclines. Drug accumulation changes in MCF-7 DOXR cells treated with safingol were accompanied by inhibtion of basal and PdBu stimulated phosphorylation of PGP. Treatment of MCF-7 DOXR cell membranes with safingol did not inhibit [^{3}H]vinblastine binding. Therefore, it was concluded that enhanced drug accumulation and sensitivity in MCF-7 DOXR cells treated with safingol are correlated with inhibition of PKC rather than competitive interference with PGP drug binding through direct interaction with PGP (Sachs et al., 1995). Sphingosine stereoisomers increased vinblastine accumulation up to 6-fold in MCF-7/ADR cells, but did not alter it in sensitive MCF-7 wild type cells. PdBu treatment of MCF7/ADR cells increased phosphorylation of PGP, and this increase was inhibited by prior treatment with sphingosine stereoisomers. Sphingosine stereoisomers did not inhibit specific binding of [^{3}H]vinblastine or [^{3}H]azidopine photoaffinity labeling, suggesting inhibition of PKC-mediated phosphorylation (Sachs et al., 1996). Three N-myristoylated peptides corresponding to the pseudosubstrate region of PKCα restored intracellular accumulation of chemotherapeutic drugs in MCF-7/MDR cells in association with inhibition of the phosphorylation of PKCα substrates. A fourth peptide did not affect drug accumulation and

failed to inhibit the phosphorylation of the PKCα substrates. An effective peptide did not bind to PGP as shown by its inability to inhibit [^{3}H]azidopine photoaffinity labeling (Gupta et al., 1996). Nontoxic concentrations of CGP 41251 significantly enhanced the cytotoxic properties of adriamycin, actinomycin D, vinblastine, vincristine but not those of 5-FU (5-fluorouracil). CGP 41251 increased intracellular adriamycin but did not cause significant differences in PGP expression. Pretreatment of MCF-7/ADR cells with TPA reduced CGP 41251-mediated intracellular accumulation of adriamycin and decreased the level of PGP phosphorylation but did not compete with azidopine. The conclusion of this investigations was that CGP 41251 reverses the MDR phenotype by modulating the phosphorylation of PGP or other PKC substrates critical to the maintenance of the MDR phenotype (Beltran et al., 1997).

4.5 Arguments Against a Role of PKC Inhibition in MDR

The isoquinoline PKC-inhibitors H-7, H-8 and H-9 did not reverse resistance to vinblastine in multidrug resistant K562–ADR and P388/ADR cells. It was found that reversal of MDR by isoquinoline derivatives did not correlate with the reversal of resistance and that they reverse resistance due to the suppression of drug binding to PGP (Wakusawa et al., 1992). In another study, H-8, H-9 and H-86 reversed the resistance of P388/ADR cells. These compounds dose-dependently inhibited photaffinity labeling, indicating a direct interaction with PGP (Nakamura et al., 1993). Several staurosporine derivatives enhanced accumulation of vinblastine in P388/ADR cells in a dose-dependent manner. The potency of these compounds correlated with inhibition of [^{3}H]azidopine photolabeling, but was not correlated with their inhibitory activity on protein kinases (Miyamoto et al., 1993; Wakusawa et al., 1993). The PKC inhibitory staurosporine-derivative NA-382 completely reversed the vinblastine resistance of P388/ADR cells without effect on the parental P388 cells. Photolabeling experiments with [^{3}H]azidopine suggested that NA-382 reverses MDR by direct inhibition of the drug efflux system of PGP (Miyamoto et al., 1992). Calphostin C inhibits PKC only if it is activated by light. Calphostin C (without prior exposure to light) increased the accumulation of daunorubicin in P388/ADR cells in a concentration-dependent manner. This effect was not observed in HL-60/AR cells expressing MRP. Calphostin C increased the uptake and decreased the efflux of rhodamine 123 in P388/ADR cells but had no such effect in drug-sensitive P388 cells. These data suggested that calphostin C may reverse drug resistance independently of its effect on PKC activity (Gupta et al., 1994). In mouse lymphoma HU-1 cells transfected with MDR1 cDNA, among the tested indole

carbazole (K-252a) family of protein kinase inhibitors, only KT-5720 could overcome MDR. Since other protein kinase A, C and G modulators did not reverse MDR, it was concluded that the chemosensitising activity of KT-5720 on these cells is apparently independent of its kinase inhibitory effects (Galski et al., 1995). The PKC inhibitors staurosporine, CGP 41251, UCN-01, Ro-31-8820 and GF 109203X were compared in terms of their MDR-reversing properties, their susceptibility towards PGP-mediated efflux from MCF-7/ADR cells, their binding to PGP and their ability to inhibit PKC. The results suggested that these compounds affect PGP directly and not via inhibition of PKC (Budworth et al., 1996). The same conclusions were drawn after comparisons of MDR reversal, azidopine-competition and PKC inhibition of GF 109203X, dexniguldipine and dexverapamil (Gekeler et al., 1996). In HL-60 wild-type cells, HL-60 cells expressing MDR1 and HL-60 cells expressing MRP, araC-induced apoptosis can be stimulated by PKC- and PTK-inhibitors, suggesting that this process is mediated at least partially, also by PKC and PTK-independent mechanisms (Hunakova et al., 1996). The protein kinase C inhibitor NPC 15437 led to nuclear accumulation of daunorubicin and decreased LD_{50} for vincristine in multidrug resistant CH(9)C5 and MCF-7/ADR cells. Treatment with TPA partially reversed the effect of NPC 15437, suggesting that NPC 15437 was exerting an effect through protein kinase C. However, NPC 15437 inhibited the binding of [^{3}H]azidopine to PGP (Sha et al., 1996). Ilmofosine, inhibiting PKC by interaction with the regulatory region did not compete with azidopine and did not reverse multidrug resistance (Hofmann et al., 1997). Dexniguldipine-HCl reversed resistance by direct interaction with PGP (Hofmann et al., 1995). The PKC-inhibitory N-myristoylated PKCα psuedosubstrate peptide potently and selectively induced the uptake of cytotoxic drug in colon cancer cells devoid of PGP expression (Bergman et al., 1997). The staurosporine-derivatives CGP 41251 and CGP 42700 reversed multidrug resistance to a similar extent, although CGP 42700 did not inhibit PKC (Utz et al., 1998). In LoVo cells long term inhibition of PKCα and βI by Gö6976 (Martiny-Baron et al., 1993) led to increased survival following treatment with adriamycin (La Porta et al., 1998). There are no reports to date that UCN-01 also reverses MDR. The bisindolylmaleimide protein kinase inhibitor Ro 32–2241 was found to reverse MDR1-mediated MDR by acting directly on PGP rather than, or in addition to, an effect on PKC (Merrit et al., 1999).

4.6 Phorbol Ester and MDR Modulation

As mentioned in chapter 2.3.1, in many cases it is difficult to distinguish whether the effects of phorbol esters are due to activation or inactivation of

PKC. Despite this disadvantage many experiments in MDR1-mediated resistance have been performed with TPA or PdBu. Experiments with TPA suggested that the PKC effect is linked to drug sensitivity, since activation of the enzyme by short TPA exposure enhanced adriamycin's cytotoxicity as well as its ability to provoke DNA damage. Likewise, down-regulation of PKC by extended TPA exposure partially protected the cells from adriamycin-induced cytotoxicity as well as from DNA damage. Thus, the ability of cells to be injured by adriamycin appeared to be correlated with the activity of PKC (Posada et al., 1989b). TPA-induced downregulation of PKC activity was less in MDR UV-2237M-ADRR cells (due to reduced rates of PKC degradation) compared to the parental UV-2237M cells (Ward and O'Brian, 1991). The MDR1-expressing cell line KM12L4a exhibited significantly reduced sensitivity to adriamycin, vincristine and vinblastine, but not to 5-FU, following 96-hour incubation with 15 nM PdBu. Because 15 nM PdBu did not downmodulate PKC, it was concluded that this effect was due to activation of PKC. Treatment of the cells with diacylglycerol reduced [^{14}C]adriamycin and [^{3}H]vincristine accumulation significantly, which were completely reversed by H-7 (Dong et al., 1991). A 2.5 hours exposure to PdBu activated PKC and induced a 4-fold transient MDR (Morgan et al., 1991). The PKC activators TPA and DAG increased the activity as well as the levels of PGP in several cell lines derived from leukemias and solid tumors. The increase was observed at the mRNA and protein level and was suppressed by staurosporine (Chaudhary and Roninson, 1992). In K562 cells TPA was found to increase the transcription of the MDR1 gene through activation of the MDR1 promoter (McCoy et al., 1995; McCoy et al., 1999). Cells exposed to 100 nM of TPA for one hour were approximately 3-fold more resistant to adriamycin than cells exposed to adriamycine alone. The PKC inhibitor H-7 completely blocked the TPA-induced effect, but did not reverse the MDR phenotype. TPA-treated cells showed significantly higher levels of expression of PGP when compared to those from control cells (Ahn et al., 1996). Drug accumulation assays revealed that TPA treatment of KB-V1 cells significantly reduced [^{3}H]vinblastine accumulation induced by verapamil. From these results it was concluded that PKC-mediated phosphorylation stimulates the drug transport activity of PGP (Chambers et al., 1990a, Chambers et al., 1990b). Pretreatment of MCF-7/ADR cells with TPA reduced the CGP 41251 mediated intracellular accumulation of [^{14}C]adriamycin. At concentrations that induced drug uptake, CGP 41251 significantly decreased the level of PGP phosphorylation in the cells but did not compete with [^{3}H]azidopine for photoaffinity labeling of PGP. From these data it was concluded that CGP 41251 reverses the MDR phenotype by modulating the phosphorylation of PGP and/or other PKC substrates critical

to the maintenance of the MDR phenotype (Beltran et al., 1997). The results presented in this chapter indicate that phorbol esters or modulation of PKC by phorbol esters alter MDR1-mediated resistance. However, there are also results indicating that phorbol esters seem not to be involved in the modulation of resistance.

4.7 Results Indicating no Influence of Phorbol Esters in MDR Modulation

The human HL-60 R1B6 subclone resistant to PdBu exhibited PKC activity following twenty four to thirty six hours after removal of PDBu from the medium. Despite differences in PKC activity there was no difference in the cellular accumulation of daunomycin or in the sensitivity to the toxic effects of adriamycin between the R1B6 cells and the parental HL-60 cell line (Hait and DeRosa, 1991). Rat1 cells treated with TPA showed neither increased MDR1 mRNA expression nor stimulation of PGP function (Kopnin et al., 1995). Phorbol esters sensitized MCF-7/ADR cells to PGP substrate drugs, however, there was no correlation with activation of PKC (Smith and Zilfou, 1995). In Rat1 fibroblasts, rat IAR2 epithelial and rat McA RH 7777, MDCK, K562 and LIM1215 (human colon carcinoma) cells, TPA showed effects in opposite directions (Stromskaya et al., 1995). TPA reduced daunomycin accumulation in both drug-sensitive KB-3-1 and multidrug resistant KB-C1 cells. TPA had no effect on daunomycin efflux and did not induce PGP expression. The results suggested that PKC may regulate drug resistance by reducing drug influx in a PGP-independent manner. This may represent a mechanism of drug resistance independent of, or in addition to, PGP-mediated drug efflux (Drew et al., 1996). TPA caused a decrease in the cellular accumulation of daunorubicin and etoposide, both in PGP-overexpressing and wild-type cells. Since treatment of cells with staurosporine reversed this effect and the non-PKC-stimulating 4α-phorbol-12,13-didecanoate did not result in a decreased daunorubicin accumulation it was concluded that this effect is the result of kinase activity. Accumulation of the PGP substrate calcein-acetoxymethyl-ester was not influenced by TPA in wild-type cells. Activation of PKC with TPA or inhibition of protein phosphatases 1 and 2A by okadaic acid did not affect the accumulation of calcein-acetoxymethyl-ester in the MDR or wild-type cells. Staurosporine increased the calcein acetoxymethyl ester accumulation only in the MDR cells. Neither stimulating PKC with TPA nor inhibiting phosphatases with okadaic acid led to a decreased inhibition of PGP by staurosporine, indicating that staurosporine inhibits PGP directly. From these experiments it was concluded that PKC and phosphatase activity do not regulate the drug transport activity of PGP

and that TPA-induced PKC activity decreases cellular drug accumulation in a PGP-independent manner (Wielinga et al., 1997). TPA significantly reduced the uptake of adriamycin and vincristine in human colon cancer cells devoid of PGP activity (Bergman et al., 1997). Bryostatin, similar to TPA, modulates PKC activity (Blumberg, 1991). Bryostatin 1 affected PGP phosphorylation, but did not reverse multidrug resistance multidrug resistance (Scala et al., 1995). It was shown that bryostatin is a potent modulator of multidrug resistance in two cell lines overexpressing a mutant MDR1-encoded PGP (valine instead of glycine in position 185), namely KB-C1 cells and HeLa cells transfected with a MDR1-V185 construct. This reversal was independent of PKC. Bryostatin 1 was not able to reverse the resistance of cells overexpressing the wild type form (glycine in position 185) of PGP, namely CCRF-ADR5000 cells and HeLa cells transfected with a MDR1-G185 construct. Treatment of mutant PGP expressing HeLa-MDR1-V185 cells with bryostatin 1 was accompanied by an increase of the intracellular accumulation of rhodamine 123, whereas no such effect could be observed in HeLa-MDR1-G185 cells (expressing wild-type PGP). HeLa-MDR1-V185 cells expressed the PKC isoforms α, δ and ζ. Downmodulation of PKCα and δ by TPA did not affect the drug accumulation by bryostatin. In HeLa-MDR1-V185 cells, short-term exposure to bryostatin 1 which led to a PKC activation was as efficient in modulating the pumping activity of PGP as long-term exposure leading to PKC depletion. These results suggested that PGP is another target of bryostatin in addition to and independent of PKC (Spitaler et al., 1998). Neither TPA, dioctylglycerol, nor staurosporine of H-7 altered intracellular drug accumulation in renal proximal tubule cells (Miller et al., 1998).

4.8 Influence of PGP Phosphorylation on MDR

The multidrug resistant sublines from both mouse sarcoma 180 and human KB lines exhibited 80-90% increases in basal PKC activity due to higher levels of PKCα protein. Inhibition of endogenous PKC activity by staurosporine resulted in decreased resistance to adriamycin. Phosphorylation of MDR cell membrane vesicles by purified PKC resulted in a level of phosphorylation of P-glycoprotein that was greater than the endogenous phosphorylation level. (Posada et al., 1989b). In HL-60 cells isolated for resistance to vincristine, staurosporine induced a major inhibition in the phosphorylation of PGP. Further studies showed that under the same conditions in which staurosporine inhibited PGP phosphorylation there has been a concomitant increase in cellular drug accumulation and a major inhibition in drug efflux (Ma et al., 1991). Staurosporine inhibited the effects

of TPA on the phosphorylation of PGP and on the accumulation of vinblastine (Aftab et al., 1994). In isolated membranes, phosphorylation of PGP by purified PKC was rapid, and time-dependent dephosphorylation was inhibited by okadaic acid, an inhibitor of type 1 and type 2A protein phosphatases. In [^{32}P]-labeled intact KB-V1 cells, PGP phosphorylation was stimulated by both TPA and okadaic acid. Two-dimensional thin layer tryptic phosphopeptide maps indicated that the sites of phosphorylation were similar in control, TPA-treated, and okadaic acid-treated cells and that they corresponded to those phosphorylated by PKC in vitro. Staurosporine, calphostin C and edelfosine, inhibited PGP phosphorylation in vitro and in intact cells. Drug accumulation assays demonstrated that in KB-V1 cells TPA caused a decrease, whereas staurosporine and calphostin C caused an increase, in accumulation of [^{3}H]vinblastine. These compounds did not significantly alter [^{3}H]vinblastine levels in drug-sensitive KB-3 cells. These results suggested that PKC is chiefly responsible for PGP phosphorylation in KB-V1 cells, that membrane-associated protein phosphatases 1 and 2A are active in dephosphorylation of PGP, and that phosphorylation of PGP may be an important mechanism for modulation of drug-pumping activity (Chambers et al., 1992). Membrane vesicles from multidrug resistant KB-V1 cells were incubated with purified PKC and [γ-^{32}P]ATP. PGP was purified and serines 661, 667, 671, 675 and 683 were found to be phosphorylated (Chambers et al., 1993). Okadaic acid and calyculin A, inhibitors of protein phosphatases 1 and 2A caused mitotic arrest of HL-60, HL-60/ADR and K562 cells by chromatid scattering/overcondensation and abnormal mitotic spindles. Protein phosphorylation experiments in intact cells revealed that in multidrug resistant HL-60/ADR cells overall phosphorylation of nuclear proteins was higher than that in drug -sensitive HL-60 cells (Sakurada et al., 1992). TPA increased the phosphorylation of PGP 6-fold and selectively decreased the accumulation of vinblastine in MCF-7/ADR cells. The actions of TPA did not require new synthesis of PGP, and had similar effects in MCF-7/BC-19 cells transfected with a cDNA for PGP. Transfection of MDR1 expressing MCF-7 cells with an expression vector containing PKCα antisense reduced PKCα levels and decreased total PKC activity by 75%. This was accompanied by reduced phosphorylation of PGP, a 2-fold increase in drug retention, and a 3-fold increase in adriamycin cytotoxicity (Ahmad and Glazer, 1993). Increased resistance in PKCα-transfected BC-19 cells was associated with enhanced PKC activity, phosphorylation of PGP and decreased drug accumulation (Yu et al., 1991). PGP and PKC were coimmunoprecipitated from the multidrug resistant cell lines MCF-7/ADR and KB-V-1, using antibodies to either protein. PKCα, β, γ, ε and θ, but not δ, μ, ζ and λ were found to coimmunoprecipitate with PGP. These studies indicated that

PGP closely interacts with PKC and serves as a substrate. The association between the two proteins was enhanced by TPA (Yang et al., 1996).

4.9 Arguments Against an Involvement of PGP Phosphorylation

Transfection of BC-19 cells with PKCγ led to a 19-fold increase in PKC activity, but did not confer increased resistance to adriamycin (Ahmad et al., 1992). Ser-667, Ser-671 and Ser-683 of PGP were not only phosphorylated by PKC (Chambers et al., 1993), but also by PKA in a cAMP-dependent manner (Chambers et al., 1994). Several phorbol esters sensitized MCF-7/ADR cells to PGP substrate drugs. However, there was no correlation with activation of PKC and the sensitization was not antagonized by staurosporine. Mezerein, K-252a and H-89 sensitized MCF-7/ADR cells, increased intracellular accumulation of [^{3}H]vinblastine and antagonized photolabeling of PGP by [^{3}H]azidopine. Therefore, phosphorylation did not appear to play a significant role in regulation PGP activity in these cells (Smith and Zilfou, 1995). In P388 cells treated with increasing concentrations of bryostatin 1 the phosphorylation of PKC-specific substrate was decreased up to 94%, compared with the parental cell line. Similar decreases were observed for PKC isoenzyme expression. There was no significant degree of cross-resistance to daunorubicin in the bryosatin 1-resistant cell lines (Prendiville et al., 1994). Bryostatin 1 was also found to affect PGP phosphorylation, but did not reverse MDR (Scala et al., 1995). A matched pair of mammalian cell lines was generated expressing wild-type PGP or a non-phosphorylatable mutant protein. Mutation of the phosphorylation sites did not alter PGP expression or its subcellular localization. The transport properties of the mutant non-phosphorylatable and wild-type proteins were indistinguishable (Goodfellow et al., 1996; Germann et al., 1996). A MDR variant of the human Saos-2 osteosarcoma cell line exhibited increased levels of PGP at the plasma membrane and in the nucleus. Cellular and nuclear PKC were not modified with respect to sensitive cells indicating that resistance is not dependent on PKC (Zini et al., 1997). PGP seems to be associated with volume-activated chloride currents. It seems to be a PKC-mediated channel regulator. Therefore, phosphorylation of PGP might be important only for regulation of chloride currents (Hardy et al., 1995) and not for regulation of drug efflux.

4.10 Effects of PKC on the Levels of MDR1-mRNA and PGP

Drug-mediated MDR1 induction was blocked by nonspecific PKC inhibitors that are active against PKC, but not by a protein kinase inhibitor ineffective against PKC (Chaudhary and Roninson, 1993). In KB cells transfected with

the MDR1 gene promoter and a chloramphenicol acetyltransferase reporter gene, H-7 inhibited the activation of the promoter by ethylmethane sulfonate, 5-FU or UV irrradiation (Uchiumi et al., 1993). In 18 primary renal cell carcinoma cell lines a high PKC expression significantly correlated with both resistance to adriamycin and high PGP expression (Efferth and Volm, 1992). Between the expression of PKC in 83 untreated solid human non-small cell lung carcinomas and the resistance to adriamycin a statistically significant correlation was found (Volm and Pommerenke, 1995). Davies et al. concluded from their experiments that some regulation of PGP expression at the post-translational level and a coregulation of PGP expression by PKC occurred (Davies et al., 1996). The PKC level in the MDR1-expressing cell line K562/D1-9 was higher than that in parental 562 cells (Urasaki et al., 1996). Staurosporine prevented araC-induced MDR1 overexpression (Walter et al., 1997). MCF-7/ADR cells cultivated in absence of drugs lost their resistance gradually with time, so that by week 24 they had almost completely regained the drug sensitivity seen in wild-typ MCF-7 cells. PGP levels measured by Western blot mirrored the change in adriamycin sensitivity. PGP was not detectable anymore at week 24. MCF-7/ADR cells expressed more PKCα and PKCθ than wild-type cells and possessed a different cellular localiziation of PKCε. The expression and distribution pattern of these PKCs reverted back to that seen in wild-type cells by week 24. The results suggested that MCF-7/ADR cells lost MDR1 gene expression and PKC activity in a co-ordinate fashion, consistent with the existence of a mechanistic link between MDR1 and certain PKC isoenzymes (Budworth et al., 1997).

4.11 Arguments against a Role of PKC on MDR1-mRNA and PGP Levels

The staurosporine-derivative CGP 41251 has been shown to exert a high degree of selectivity for inhibition of PKC and PKC-mediated cellular events. The IC_{50}-values of enzyme inhibtion in vitro for PKC, protein kinase A, phosphorylase kinase, S6 kinase and tyrosine-specific protein kinase of the epidermal growth factor receptor are 50 nM, 2.4 μM, 48 nM, 5 μM and 3 μM, respectively (Meyer et al., 1989). CGP 41251 reversed multidrug resistance in CCRF-VCR1000 and KB-8511 cells. Preincubation with the compound for 12 or 24 hours did not alter MDR1-mRNA levels (Utz et al., 1994). The PKC inhibitors CGP 41251 (Utz et al., 1994), dexniguldipine-HCl (Hofmann et al., 1995) and ilmofosine (Hofmann et al., 1997) did not alter the MDR1 expression. CGP 41251 has been found to increase intracellular adriamycin but did not cause significant differences in PGP expression (Beltran et al., 1997). Inhibition of PKCα and βI by Gö6976 even increased the expression of PGP and the resistance to adriamycin (La Porta et al., 1998).

4.12 The Role of PKC Isoenzymes on MDR1-Mediated MDR

PKCα was modestly increased (approximately 65%) in the multidrug resistant KB-V1 cell line compared with the sensitive KB-3 cells (Cloud-Heflin et al., 1996). In MCF-7-MDR cells the MDR phenotype was associated with a 10-fold increase in PKCα activity and a 10-fold decrease in calcium-independent PKC activity due to decreased expression of PKCδ and PKCε. Phosphorylation of PGP was increased more than 20-fold in the MCF-7-MDR cell line and its phosphorylation corresponded to the increases in PGP pump function underscoring the role of PKCα (Blobe et al., 1993). MCF-7/ADR cells expressed more PKCα and PKCθ than wild-type cells and possessed a different cellular localiziation of PKCε (Budworth et al., 1997). Dolfini et al. (1993) concluded from experiments with human LoVo colon adenocarcinoma cells a contribution of PKCα to the resistance. Transfection of the MDR1 gene in sensitive breast carcinoma cells did not contribute to a high degree of resistance, whereas cotransfection of the MDR1 gene with the PKCα gene induced a high degree of resistance to anticancer drugs (Yu et al., 1991). Expression of antisense cDNA for PKCα reduced drug resistance (Ahmad and Glazer, 1993). Activation of PKC by PdBu or thymeleatoxin induced resistance to multiple anticancer drugs in the metastatic human colon cancer cell line KM12L4a cells. The induction of resistance by thymeleatoxin was associated with a reduction in cytotoxic drug accumulation in KM12L4a cells. These cells contain only PKCα from the thymeleatoxin activating isoforms. Thus, it was concluded that that activation of PKCα is sufficient for the induction of resistance observed in KM12L4a cells (Gravitt et al., 1994). Basal PKC activities and immunoreactivities of PKCα and PKCζ were higher in a multidrug resistant compared to three multidrug sensitive human glioma cell lines. PKCβ, γ and ε were not detected in these cell lines. Treatment of multidrug resistant glioma cells with 100 nM TPA for 2 hours resulted in activation of PKCα, but not of PKCζ, with concomitant decrease in vincristine accumulation and increase in PGP phosphorylation. The treatment of multidrug resistant cells with 100 nM calphostin C for 2 hours decreased immunoreactive PKCζ, but not PKCα, inducing an increase in vincristine accumulation with a concomitant decrease in PGP phosphorylation. There was no significant change in vincristine accumulation in sensitive cells treated with TPA or calphostin C. It was concluced that PKCα and ζ are involved in PGP phosphorylation and vincristine efflux (Matsumoto et al., 1995). On the other hand, the expression of PKCα did not correspond to the expression of MDR1 or to the drug-sensitivity of sensitive and multidrug resistant CCRF and KB cell linies (Gekeler et al., 1996).

Rat embryo fibroblasts transfected with PKCβI displayed elevated PKC activity. These cells exhibited significant resistance to adriamycin, actinomycin D, vinblastine and vincristine but not to 5-FU. Intracellular accumulation of the MDR-drugs was decreased, but this was not associated with an altered level of PGP expression (Fan et al., 1992). P388/ADR and drug sensitive P388 cells were permeabilized and incubated with rabbit anti-PKCα or anti-PKCβ antibodies. An anti-PKCβ antibody partially corrected the drug accumulation defect and completely reversed resistance to daunorubicin. An anti-PKCα antibody had no effect (Gollapudi et al., 1995). MDR OAW-tax cells exhibited higher levels of PKCβII compared with parental OAW-42 and OAW-dox cells (Masanek et al., 1997). In relapsed state acute myelogenous leukemias highly significant positive correlations between the expression of MDR1/PKCη were found (Beck et al., 1996; Beck et al., 1998; Spitaler et al., 1999).

4.13 Remarks and Conclusions

One of the possible explanations of the contradicting results is the fact that most of the results were obtained with tumor cells. These cells have very unstable genoms and alterations in gene expressions are common. Overexpression of MDR1 and one of the eleven PKC isoenzymes may be accidential. For example, it was found that a correlation between PKC and multidrug resistance exists only in cells selected in colchicine and not with those selected in other drugs (Drew et al., 1994). In LoVo cell clones exhibiting MDR1-mediated resistance PKCα was increased and PKCε was decreased. However, a similar pattern of PKC expression was also observed in a LoVo clone exhibiting MRP-mediated resistance (Dolfini et al., 1997). Antisense oligonucleotides directed to PKC isoenzymes reduce MDR. However, used in non-MDR1 expressing cells they also induce cell death (Ahmad et al., 1994; Yazaki et al., 1996; Dean et al., 1996; Leszczynski et al., 1996; Dooley et al., 1998).

Several PKC effects were obtained from conclusions which are not substantiated by experiments. If the photoaffinity labeling with azidopine is decreased by a PKC inhibitor which reverses MDR, it may be concluded that the inhibitor competes with azidopine by binding to PGP. However, it can also be concluded that the PKC inhibitor reduces the phosphorylation of PGP and that, due to altered affinity of unphosphorylated PGP to drugs, less azidopine binds to PGP. If a PKC inhibitor reverses MDR, it may be concluded that the reversal is due to inhibition of PKC and reduced PGP phosphorylation. However, a PKC inhibitor might also reduce the drug efflux by direct interaction with PGP. There are several possibilities how a PKC inhibi-

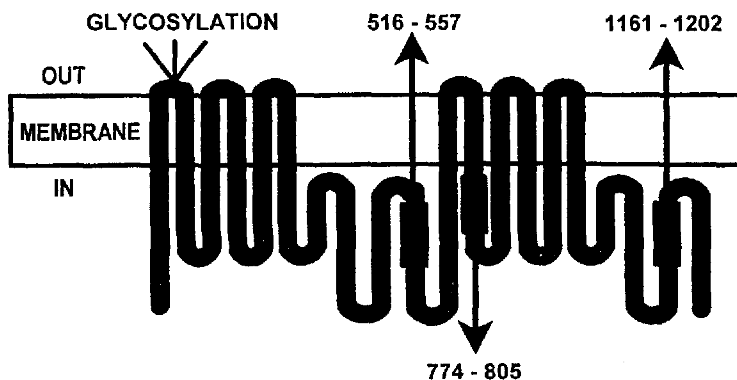

Fig. 4. Schematic representation of potential ATP binding sites in human PGP

tor might reduce drug efflux by direct interaction with PGP. Such an inhibitor might block the drug efflux by interaction with the drug binding site. In theory it might block a potential phosphorylation site which is responsible for activation of the efflux. Another possibility is that the PKC inhibitor interacts with the ATP binding site of PGP. Many PKC inhibitors such as staurosporine, CGP 41251 or UCN-01, are believed to compete with ATP at the ATP binding site of PKC. The drug efflux mediated by PGP is also dependent on ATP. PGP contains two ATP binding sites (amino acids 516–557 and 1161–1202; Gottesman and Pastan, 1993) to which binding of ATP is essential for drug transport (Fig. 4). As shown in the sequence comparison in Table 1 (below), several amino acids in the ATP binding sites of PKC and PGP are identical. So it is conceivable that a competition of a PKC inhibitor for the ATP binding site of PGP reduces drug efflux. A sequence analysis shows that in addition to these two ATP binding sites in PGP, there is a third sequence which is related to the ATP binding sites of PKCs (amino acid sequence 775–805, Table 1). Fig. 4 shows the location of these sequences. In theory, a PKC inhibitor could also bind to this site (775–805) and affect the drug efflux. So PKC inhibitors might interfere with MDR in different ways which are not known at present. The lack of this knowledge may lead to different conclusions as described above.

The effects of PKC on PGP may be time-dependent. For example, it has been reported that long term inhibition of PKC enhanced PGP expression and MDR (La Porta et al., 1998). If PKC activity is elevated in MDR1 overexpressing cells and this leads to increased resistance compared with sensitive cells, it could be speculated that PKC activity might increase the resistance by mechanisms independent of MDR1, for example, by reduction of apoptosis (due to expression of bcl-2). Castro et al. (1999) reported that PKC

Table 1. Sequence comparisons of potential ATP binding sites in PKC isoenzymes and PGP (Amino acids occurring in both, PKC and PGP, in a similar position are printed in bold)

PKC α	LGKG--S-FGKVM--LADR-KG-TEELY--AIKIL-KK------D	
PKC β	LGKG--S-FGKVM--LSER-KG-TDELY--AVKIL-KK------D	
PKC γ	LGKG--S-FGKVM--LAER-RG-SDELY--AIKIL-KK------D	
PKC δ	LGKG--S-FGKVL--LGEL-KG-RGE-YS-AIKAL-KK------D	
PKC ε	LGKG--S-FGKVM--LAEL-KG-KDEVY--AVKVL-KK------D	
PKC θ	LGKG--S-FGKVF--LAEF-KK-TNQFF--AIKAL-KK------D	
PKC L	LGKG--S-FGKVM--LARV-KE-TGDLY--AVKVL-KK------D	
PKC ι	IGRG--S-YAKVL--LVRL-KK-TDRIY--AMKV-VKK------E	
PKC ζ	IGRG--S-YAKVL--LVRL-KK-NDQIY--AMKV-VKK------E	
PGP 775	L-QGF-T-FGKAGEILT---KRLR---YMVFRSML-RQ------D	805
PGP 516	LPHKFDTLVGERGAQLSGGQKQ-R--IA-IAR-ALVRNPKILLLD	557
PGP 1161	LPNKYSTKVGDKGTQLSGGQKQ-R--IA-IAR-ALVRQPHILLLD	1202

inhibitors, in addition to direct interaction with PGP, may reverse MDR by a pathway that involves inhibition of PKC, but is independent of PGP phosphorylation. This might be due to PKC-mediated phosphorylation of one or more proteins that modulate PGP (Castro et al., 1999). Another possible reason for contradictory results may be that increased intracellular drug concentrations may not represent the drug concentrations in the nucleus where the DNA binding occurs.

Overexpression of MDR1 may influence experimental results through additional effects of PGP, independent of drug efflux. In addition to the development of MDR, exposure of tumor cells to antimitotic agents produces further cellular changes, as activation of the MAPK pathway and acceleration of the cell cycle machinery, that may contribute to the failure of chemotherapy (Emanuel et al., 1999). It has been reported that overexpression of PGP delays the apoptotic cascade in CHO fibroblasts (Robinson et al, 1997), leads to altered expression of genes regulating apoptosis in human myeloid leukemia cells (Kim et al., 1997) and protects CCRF-CEM and K562 cells from caspase-dependent apoptosis (Smyth et al., 1998; Johnstone et al., 1999). On the other hand, it has been reported that the somatostatin analogue TT-232 induced apoptosis in multidrug resistant and sensitive hepatocellular carcinoma cell lines to a similar level, indicating that the machinery involved in apoptosis is functional in all these cells to a similar extent (Diaconu et al., 1999). 2-deoxy-D-glucose preferentially induced apoptosis in MDR1-expressing cells (Bell et al., 1998). MDR cells typically have elevated

intracellular pH and decreased plasma membrane potential and this is apparently due to PGP overexpression and not exposure to chemotherapeutic drugs. One model for PGP's function (partitioning model) suggests that alterations in pH and/or membrane potential that accompany the overexpression of PGP, indirectly affect partitioning of chemotherapeutic drugs, but PGP does not directly pump them. It has also been shown that PGP expression leads to significant resistance to complement-mediated cytotoxicity induced by the elevation of the intracellular pH and that PGP expression alone cannot account for MDR1-mediated resistance (Weisburg et al., 1999). Multidrug resistant MCF-7 cells exhibited marked accumulation of glucosylceramide compared with parental cells. Reversal of resistance by tamoxifen, verapamil and cyclosporin A, decreased the levels of glucosylceramide. The glucosylceramide synthesis inhibitors 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol or tamoxifen sensitized resistant MCF-7 cells, indicating that the presence or absence of other targets in addition to PGP may influence reversal of resistance (Lavie et al., 1997).

In K562 cells TPA activated the MDR1 promoter and led to increased MDR1 mRNA levels. This activation was mediated by the zinc finger transcription factor EGR1. This activation by TPA was inhibited by the Wilms' Tumor suppressor WT1 (McCoy et al., 1995; McCoy et al., 1999). Controversial response to TPA may depend on the presence or absence of EGR1 or WT1 in the cell lines investigated.

5 PKC Modulation in Drug Resistance not Mediated by MDR1

5.1 PKC Inhibition Enhances the Sensitivity to Antitumor Drugs

Down-modulation of PKC by long-term treatment with TPA, PKC inhibitors, such as quercetin, tamoxifen, staurosporine and the ether lipid ilmofosine enhanced the antiproliferative activity of antitumor drugs in vitro and in human tumor xenografts in nude mice (Hofmann et al, 1988; Hofmann et al., 1989; Hofmann et al., 1990). Noseda et al., (1988) found additivity in a combination of ilmofosine with cis-platin. Quercetin synergistically inhibited the growth of HL-60 cells if combined with ara-C (Teofili et al., 1992). However, Freund et al. (1998) questioned these results using the same cell line. Enhancement of cytotoxic drugs by quercetin may be due to the induction of transforming growth factor β1 or inhibition of tyrosine kinases (Larocca et al., 1995; Ferry et al., 1996). A combination of quercetin with carboplatin has been investigated in a clinical phase I trial (Ferry et al., 1996; Fyfe et al., 1996). The PKC inhibitor SPC-100270 enhanced the antiprolifera-

tive activity of adriamycin or cis-platin on murine isografts and human melanoma, lung adenocarcinoma or protstate tumor xenografts (Adams et al., 1993). The compound did not potentiate the cytotoxicity of either adriamycin or cis-platin on human myeloid, erythroid, or megakaryocyte lineages (Susick et al., 1993). Threo-dihydrosphingosine potentiated the in vivo antitumor efficacy of cis-platin and adriamycin in 16C mammary tumors and SCCVII carcinomas in mice (Siemann et al., 1993). PKC is activated rapidly and transiently following ionizing radiation. In two human squamous cell carcinoma cell lines exposed to graded doses of X-rays the presence of staurosporine or sangivamycin enhanced cell killing, while H-7 did not (Hallahan et al., 1992). In human ovarian cancer cell lines with intrinsic resistance to cis-platin, PKC activities in the cytosol and membrane were approximately 4- to 5-fold higher than that of sensitive cells. Proliferation of sensitive and resistant cells was inhibited in a dose-dependent manner by TPA. The membrane PKC activities in the cis-platin-sensitive cells were rapidly activated and down-regulated 24 hours after exposure to TPA, while those in the resistant cells were not down-regulated even after exposure to TPA for 24 hours, suggesting that the membrane form of PKC may be involved in the intrinsic resistance. In these cell lines cis-platin sensitivity was reduced by TPA when cellular PKC rose, the sensitivity was increased when cellular PKC decreased (Hirata et al, 1993). In human osteosarcoma U2-OS cells and a cis-platin resistant U2-OS/Pt variant, a 24 hours exposure to TPA caused a potentiation of cis-platin toxicity in sensitive and resistant cells. A short term exposure to TPA did not affect cis-platin cytotoxicity in both cell lines (Perego et al., 1993). Activation of PKC by TPA increased the resistance of PAM 212 keratinocytes transformed by the v-H-ras oncogene to vincristine and adriamycin, while it significantly decreased the resistance in E1a transformed PAM 212 cells to cis-platin. Staurosporine increased the cytotoxicity of vincristine, doxorubicin and cis-platin in the E1a transformed keratinocytes (Sanchez-Prieto et al., 1995). Short term treatment of Chinese hamster ovary cells with TPA increased the resistance to methotrexate in a dose dependent manner. H-7, staurosporine and calphostin C decreased the TPA-induced resistance (Noe and Ciudad, 1995). In C6 glioma cells staurosporine and H-7 increased the sensitivity to irradiation, whereas the PKA inhibitor HA1004 failed to affect the radiosensitivity of these cells (Zhang et al., 1993).

Combination studies with PKCα antisense and standard chemotherapeutic agents (cis-platin, mitomycin-C, vinblastine, estracyt and adriamycin) in nude mice that had been transplanted with a variety of human tumors (breast, prostate, large cell lung and small cell lung carcinomas, and melanomas) were found to be additive or superadditive (Geiger et al, 1998).

Exposure of U251 and LN-Z308 glioma to nitroprusside (a NO-generating agent) resulted in significant cytotoxicity. U343 cells were resistant to the compound and exhibited higher basal levels of PKCα and bcl-2 than U251 and LN-Z308 cells. Introduction of PKCα antisense oligonucleotides into U343 cells decreased the levels of bcl-2 and and also decreased the resistance to nitroprusside. PKCα transfected U251 clones displayed increased PKC acitivty, bcl-2 expression and resistance to nitroprusside (Blackburn et al. 1998).

As described above (section 2.3.3), UCN-01 inhibited the proliferation of human leukemia, breast cancer, malignant glioma, and small cell lung cancer cell lines (Akinaga et al., 1991; Seynaeve et al., 1993; Courage et al., 1995; Pollack et al., 1996). The compound exhibited antitumor activity against human tumor xenografts in nude mice (Akinaga et al., 1991). Clinical trials with the compound are ongoing (Lush et al., 1997). UCN-01 enhanced the antitumor activity of mitomycin C in human A431 epidermoid carcinoma, human xenografted colon carcinoma Co-3 and murine sarcoma 180 cells in vitro and in vivo (Akinaga et al, 1993). UCN-01 also blocked the proliferation of glioma cells in vitro and in vivo and potentiated the effects of BCNU and cis-platin (Pollak et al., 1996). In an investigation by Husain et al., (1997) in all cell lines studied UCN-01 was effective as a cytotoxic agent alone and in combination with cis-platin. The combination of UCN-01 plus cis-platin was effective in increasing the cytostatic and cytotoxic effects of irradiation at 4 Gy (Sommers and Alfieri, 1998). In Chinese hamster ovary cells UCN-01 abrogated the G2 arrest induced by the DNA-damaging agent cis-platin. UCN-01 concentrations that resulted in abrogation of the cis-platin-induced G2 arrest also enhanced cis-platin-induced cytotoxicity. UCN-01 enhanced cis-platin cytotoxicity up to 60-fold and reduced by 3-fold the concentration of cis-platin required to kill 90% of the cells. The concentrations of UCN-01 required for this enhancement have been shown to be well tolerated in animal models, suggesting that this combination may represent an effective strategy for enhancing cis-platin-based chemotherapeutic regimens (Bunch and Eastman, 1996).

Pretreatment of human T24 bladder or human colorectal adenocarcinoma cells with 5-FU followed by CGP 41251 showed a synergistic drug interaction in both cell lines (Fabbro et al., 1999). CGP 41251 or 5-FU were ineffective as a single agent against COLO 205 tumors up to 200 mg/kg/day p.o. and 75 mg/kg/week i. v., respectively. Combination of both compounds at these concentrations showed significant antitumor activity in this tumor model (Fabbro et al., 1999).

5.2 PKC Activation Enhances Sensitivity to Antitumor Drugs

Short term treatment with TPA sensitized human 2008 ovarian carcinoma cells to cis-platin. This sensitization disappeared completely by seven hours after treatment, indicating that not inhibition, but activation of PKC sensitizes 2008 cells to the antiproliferative activity of cis-platin (Isonishi et al., 1990). Pretreatment of HeLa cells with TPA or PdBu caused a 9-fold increase in cellular sensitivity to cis-platin and 2.5-fold to melphalan, but had now effect on the antiproliferative activity of bleomycin, adriamycin, vincristine, or mitomycin C. The sensitization of HeLa cells by TPA was associated with a 6-fold stimulation of PKC activation and a concentration- and time-dependent increase in cellular platinum content. (Basu et al. 1990). PKC activity was found to be decreased significantly in cis-platin-resistant human small cell lung H69/CP cancer cells compared to the drug-sensitive variant. A similar reduction in PKC activity was noted in ovarian carcinoma 2008 cells that were resistant to cis-platin. A modest decrease in PKC activity was also observed in etoposide-resistant H69 cells but not in taxol-resistant H69 cells or bleomycin-resistant human head and neck carcinoma A-253 cells (Basu et al., 1996), indicating that reduced PKC activity leads to decreased sensitivity in this system.

5.3 PKC Isoenzymes Involved in the Modulation of Antiproliferative Activity

TPA induced pleiotropic resistance against antitumor drugs in human colon cancer cells. The resistance was triggered by the activation of PKCα. PKCα is expressed abundantly in surgical specimens of human colon cancer, indicating that PKCα-mediated drug resistance may contribute to the intrinsic resistance of clinical colon cancer (O'Brian et al., 1995). Downmodulation of PKCα, but not of PKCβ, with antisense oligonucleotides sensitized human colon carcinoma cells to mitomycin C, 5-FU and vincristine (Chakrabarty and Huang, 1996). H69 human small cell lung cancer cells expressed PKCα, β, δ, ζ, ι and μ. A decrease in PKCα and β and an increase in PKCδ were observed in a cis-platin-resistant subline. The abundance of PKCζ or ι was unaffected. H69 cells resistant to etoposide also displayed a reduction in PKCβ and an increase in PKCδ. Taxol-resistant H69 cells showed no alteration in the expression of any of the PKC isozymes (Basu et al., 1996). R6 cells stably transfected with PKCε were shown to prevent cis-platin-induced apoptosis and protected cells against cis-platin cytotoxicity (Basu and Cline, 1995). PKCε was overexpressed in human lung A549 adenocarcinoma cells following irradiation (Kim et al., 1992). The parental ovarian carcinoma 2008

cells expressed the PKCα, ε, and ζ isoforms. In resistant 2008 cells PKCα decreased significantly, whereas the amount of PKCε increased moderately, with no alteration in the PKCζ content. Therefore, the authors concluded that a reduction in PKCα and/or an increase in PKCε expression may be associated with the drug-resistant phenotype (Basu and Weixel, 1995). Antisense oligodeoxynucleotides against PKCε down-regulated the PKCε level, blocked drug-induced translocation, and reduced cis-platin-mediated cytotoxicity 3-fold compared to that of sense-treated cells. Antisense PKCε also decreased SKBR-3 cell sensitivity to carboplatin but not to adriamycin and taxol (Ohmori and Arteaga, 1998). Therefore, the effects of PKCε may be cell type specific and also drug specific.

5.4 Remarks and Conclusions

Contradicting results on the effects of PKC on drug resistance in different publications may have several reasons. One reason may be the use clonogenic assays or short term assays. For example, treatment of mouse embryo fibroblasts from wild-type ($p53^{+}/^{+}$) and from p53 knockout mice ($p53^{-}/^{-}$) with etoposide led to increased sensitivity of wild-type expressing cells tested in a short-term XTT-assay. However, no difference in sensitivity was observed following etoposide treatment if tested in a clonogenic assay (Brown and Wouters, 1999). In a comparison between matched $p53^{+}/^{+}$ and $p53^{-}/^{-}$ mouse cells, five investigations found $p53^{-}/^{-}$ cells more radioresistant, nine found no difference, and three found $p53^{-}/^{-}$ more sensitive than $p53^{+}/^{+}$ cells (Brown and Wouters, 1999).

UV-2237MM cells exhibited high sensitivity to adriamycin in vitro. If these cells were transplanted into different organs of mice, tumors growing in the subcutis and the spleen were sensitive, tumors growing in the lung were resistant to adriamycin. If tumor cells from the lung were isolated and grown in vitro they were sensitive again. Nearly identical levels of PKC activity in tumors growing in the subcutis, spleen and lung were observed, indicating that PKC activity levels did not account for the different responses to adriamycin (Staroselsky et al., 1990).

The influence of PKC on cellular sensitivity or resistance to cis-platin, adriamycin or irradiation may arise from the status of phosphorylation of raf-1, bcl-2, IκB; IκB kinase β, and many others (Grant and Jarvis, 1996; Schönwasser et al., 1998; Lallena et al., 1999). It has been shown that PKCα phosphorylates raf-1 (Kolch et al, 1993) and bcl-2, leading to resistance (Ruvolo et al, 1998). Raf-1 also interacts with the apoptosis-preventing bcl-2 (Blagosklonny et al, 1997). Phorbol ester treatment of quiescent Swiss 3T3 cells led to cell proliferation, a response thought to be mediated by PKC. In

addition to activation of raf-1 by PKCα, it was described that this isoenzyme induced also raf desensitization to prevent further raf stimulation by growth factors (Schönwasser et al., 1998). In 15 human cell lines high cyclin D1 expression was related to cis-platin resistance but had no relationship with radiation responsiveness, whereas high c-raf-1 expression, although related to radiosensitivity has no relationship with cis-platin responsiveness (Warenius et al., 1996). When transferred from monolayer to three-dimensional culture, a consistent upregulation (up to 15-fold) of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ protein was observed in a panel of mouse and human carcinoma cell lines which was accompagnied by resistance to antitumor drugs and radiation, implicating that $p27^{KIP1}$ is a regulator of drug resistance in solid tumors (StCroix et al., 1996; StCroix and Kerbel, 1997). These are indications that, in addition to PKC, many factors influence drug resistance and cell death.

Treatment of cultured mammalian cells with serum growth factors and activators of PKC, led to induction of metallothionein mRNA. One of the required steps in the signal transduction pathways triggered by these agents ending in metallothionein gene induction, appears to be the activation of protein kinase C (Imbra and Karin, 1987). Resistance to cis-platin may be due to induction of metallothionein genes by PKC and inhibition of PKC may sensititize cells by inhibition of metallothionein gene expression (Yu et al., 1997).

6 Summary

PKC isoenzymes were found to be involved in proliferation, antitumor drug resistance and apoptosis. Therefore, it has been tried to exploit PKC as a target for antitumor treatment. PKCα activity was found to be elevated, for example, in breast cancers and malignant gliomas, whereas it seems to be underexpressed in many colon cancers. So it can be expected that inhibition of PKC activity will not show similar antitumor activity in all tumors. In some tumors it seems to be essential to inhibit PKC to reduce growth. However, for inhibition of tumor proliferation it may be an advantage to induce apoptosis. In this case an activation of PKCδ should be achieved. The situation is complicated by the facts that bryostatin leads to the activation of PKC and later to a downmodulation and that the PKC inhibitors available to date are not specific for one PKC isoenzyme. For these reasons, PKC modulation led to many contradicting results. Despite these problems, PKC modulators such as miltefosine, bryostatin, safingol, CGP41251 and UCN-01 are used in the clinic or are in clinical evaluation. The question is whether PKC is the

major or the only target of these compounds, because they also interfere with other targets.

PKC may also be involved in apoptosis. Oncogenes and growth factors can induce cell proliferation and cell survival, however, they can also induce apoptosis, depending on the cell type or conditions in which the cells or grown. PKC participates in these signalling pathways and cross-talks. Induction of apoptosis is also dependent on many additional factors, such as p53, bcl-2, mdm2, etc. Therefore, there are also many contradicting results on PKC modulation of apoptosis. Similar controversial data have been reported about MDR1-mediated multidrug resistance. At present it seems that PKC inhibition alone without direct interaction with PGP will not lead to successful reversal of PGP-mediated drug efflux. One possibility to improve chemotherapy would be to combine established antitumor drugs with modulators of PKC. However, here also very contrasting results were obtained. Many indicate that inhibition, others, that activation of PKC enhances the antiproliferative activity of anticancer drugs. The problem is that the exact functions of the different PKC isoenzymes are not clear at present. So further investigations into the role of PKC isoenzymes in the complex and interacting signalling pathways are essential. It is a major challenge in the future to reveal whether modulation of PKC can be used for the improvement of cancer therapy.

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Compartment-Specific Functions of the Ubiquitin-Proteasome Pathway

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1 Introduction

Intracellular protein degradation, which was thought to be an unspecific and uncontrolled event, is now known to constitute a tightly regulated process involved in numerous basic cellular functions. The vast majority of specific proteolysis in the cell is executed by the ubiquitin-proteasome pathway. Among the numerous short-lived substrates that have been described, are cell cycle regulators, modulators of transcription, components of signal transduction cascades, enzymes of metabolic pathways, foreign antigens cleaved for antigen presentation but also damaged or mutated proteins. To guarantee specificity, the ubiquitin system is structured in form of a complex cascade of enzymes and recognition factors (Hershko and Ciechanover, 1998; Hochstrasser, 1996). Given the multitude of substrates, it is not surprising that ubiquitin-dependent proteolysis is a key control element in many central cell biological processes. Moreover, aberrations of this system seem to be implicated in the pathogenesis of several important human diseases (Ciechanover, 1998).

Eukaryotic cells represent highly ordered three-dimensional structures in which specific functions are confined to certain areas. In contrast, it was assumed for a long time that the ubiquitin system would be evenly dispersed throughout cytoplasm and nucleus. A number of recent results have changed this view. It is now widely accepted that the ubiquitin-proteasome pathway mediates also the degradation of secretory proteins, which occurs mainly at the endoplasmic reticulum (ER). In this process, malfolded and short-lived secretory proteins are first transported back into the cytosol where they are subjected to ubiquitin-conjugation and proteolysis by the 26S proteasome. It is generally assumed that this proteolytic pathway is restricted to the cytoplasmic surface of the ER (Sommer and Wolf, 1997; Kopito, 1997; Bonifacino and Weissman, 1998). Another example of compartment specific ubiquitin-conjugation takes place at the plasma membrane: ubiquitin-conjugation is an essential step during endocytosis of proteins of the plasma membrane (Hicke, 1999; Strous and Govers, 1999). Moreover, some recent reports have pointed to the fact that degradation of nuclear substrates may depend on the transport of the substrate into or out of the nucleus. In this review we will summarize the current knowledge on the spatial organization of the ubiquitin-proteasome pathway.

2 The Ubiquitin System

Hershko, Rose and colleagues identified the ubiquitin system more than 20 years ago in a cell-free system from rabbit reticulocytes. They demonstrated

in vitro that the previously known energy dependent degradation of proteins required the covalent modification of the substrate with the polypeptide ubiquitin. In addition, the enzymological framework leading to ubiquitin-protein conjugates was biochemically dissected, basic enzymes were purified and their activity defined in vitro (Hershko and Ciechanover, 1998). Later, the in vivo relevance of the system was demonstrated first in mammalian cells and then in the yeast *Saccharomyces cerevisiae*. Especially the analysis of yeast mutants in the components of the ubiquitin-conjugation cascade revealed the large variety of processes that are controlled by proteolysis (Hochstrasser, 1996). Thus, a lot of examples described in this review are derived from studies in the yeast system.

Ubiquitin is a highly conserved protein of 76 amino acids, which differs in only three amino acids between yeast and mammals. It is a tightly folded, globular protein with a protruding C-terminus. Determination of the three-dimensional structure revealed that the globular domain exposes patches of different physical properties that probably allow interactions with surfaces of other proteins (Vijay-Kumar, 1985). In all eukaryotic cells, ubiquitin is encoded by three classes of genes that are rapidly processed after translation to yield free ubiquitin. The two classes of genes that provide ubiquitin under normal growth conditions code for fusions to proteins of the large and the small subunit of the ribosome, respectively. The third class encodes head-to-tail fusions of several ubiquitin molecules in which the last unit carries an additional asparagine residue at the C-terminus. These poly-ubiquitin genes are induced under stress conditions. The processing of all these precursors to individual ubiquitin molecules is performed by a group of enzymes called de-ubiquitinating enzymes (see below). Hereby, a Gly-Gly di-peptide is exposed at the C terminus of ubiquitin that is essential for the conjugation to substrate proteins (Jentsch, 1992).

The usual ubiquitin-substrate linkage is an isopeptide bond between internal lysine residues of the substrates and the protruding C-terminus of ubiquitin. However, also the N-terminus of substrate molecules appears to be a possible acceptor for modification with ubiquitin since the ubiquitin-dependent degradation of the transcription factor MyoD proceeded after removal of all the protein's lysine residues (Breitschopf et al. 1998). Ubiquitin itself has no enzymatic activity but when it is covalently linked to other proteins it functions as a tag, marking proteins for destruction by the 26S proteasome. This large protease consists of two types of subparticles: a cylindrical 20S core that harbors the catalytic activities and a 19S regulatory complex attached at either end of the core cylinder (Baumeister et al. 1998). Ubiquitin-conjugated proteins are recognized by the 26S proteasome efficiently, only when multiple ubiquitin molecules are attached to them. Such a

poly-ubiquitin chain is formed in successive rounds of conjugation, in which the C-terminus of ubiquitin is linked to a lysine residue of a ubiquitin molecule that has been attached to the substrate in a previous conjugation reaction. The proteolytic relevant ubiquitin-ubiquitin linkage involves Lys48 (Hochstrasser, 1996). Chains linked in such manners create unique hydrophobic patches which are probably recognized by the 19S cap of the proteasome (Pickart, 1997). This entrance control prevents the unspecific degradation of cytosolic proteins. Poly-ubiquitin chains are not static elements, but highly dynamic structures with rapid addition and removal of ubiquitin moieties. Although poly-ubiquitination seems to be the predominant form of conjugation, mono-ubiquitinated species have also been observed in vivo (Hochstrasser, 1996).

In a brief and simplified view, the formation of ubiquitin-conjugates requires the successive action of three classes of enzymes: The E1 or ubiquitin-activating enzyme, E2s or ubiquitin-conjugating enzymes (Ubc) and occasionally E3s or ubiquitin-protein ligases. E1 hydrolyses ATP to first adenylate the C-terminal glycine of ubiquitin and then link it to the side chain of its central cysteine residue, yielding a high energy E1-ubiquitin thioester, free AMP and pyrophosphate. From the E1, the thioester is transferred onto the Ubc's. In vitro, the Ubc's are able to directly link ubiquitin to substrate proteins. However in most cases described in vivo, the conjugation of substrates requires the function of an E3 (Fig. 1; Hershko and Ciechanover, 1998).

The three classes of enzymes build a cascade initiated by the E1 enzyme that is required for all subsequent steps. The second enzymatic activity (E2) comprises a family of enzymes related in sequence. Each of them participates in the turnover of only a limited number of substrates, indicating that these enzymes are among those components that mediate specificity. Given the numerous proteolytic substrates that have to be degraded with different kinetics at any given timepoint in a cell, it is not surprising that the third class of enzymes (E3) constitute a highly diverse group. At least two types can be distinguished by their enzymatic mechanism. One type of E3 enzymes is unable to form transfer intermediates with ubiquitin. However, they possess substrate-binding properties and thus direct the Ubc's to their targets. Representative for this class is the first E3 described (E3α) and its yeast homologue Ubr1 but also the multisubunit E3 complexes (RING-H2-finger E3) which are involved for instance in the proteolysis of cyclins (Deshaies, 1999). Another class of E3s, in addition to substrate recognition, is able to form a DTT-sensitive adduct with ubiquitin (HECT E3s, see below; Scheffner et al. 1995). They are able to directly interact with the substrate or they need ancillary factors that mediate this association (Ciechanover, 1998).

The characteristics of the ubiquitination cascade suggest that the substrate specificity of the ubiquitin system may be extended through association of a limited number of E2 and E3 enzymes into multiple oligomeric complexes (Hochstrasser, 1996).

In addition to this well characterized cascade, a modulator of polyubiquitination has been described. This factor, termed E4, promotes the formation of high molecular weight ubiquitin-substrate conjugates in conjunction with E1, E2 and E3. However, E4 is not directly involved in the transfer of ubiquitin to the substrate but binds the ubiquitin-conjugate. In the yeast system, this factor turned out to be identical to Ufd2 (Ubiquitin fusion degradation), which was found previously in a genetic screen. Though it belongs to a family of conserved proteins found in different organisms, it does not perform essential functions in yeast. However, a function under stress conditions can be assumed, possibly by altering the length of the ubiquitin chain (Koegl et al. 1999).

2.1 Ubiquitin-Activating and Ubiquitin-Conjugating Enzymes

The first step in the ubiquitin conjugation cascade is the activation by the E1 enzyme. E1 enzymes and specifically their active site cysteine residue are essential for cell viability (Finley et al. 1984; Ciechanover et al. 1984).

The E2 activity comprises a highly conserved family of enzymes found in all eukaryotic organisms. A domain of about 150 amino acids termed Ubc-domain characterizes them. At a central cysteine residue of this region, the thioester bond with ubiquitin is formed (Jentsch et al. 1990). In addition to the core, Ubc's may carry extensions at the C- or N-terminus or both. These extensions apparently contribute to the substrate specificity of the enzymes. In yeast, where only C-terminal extensions exist, it could be shown that the swapping of the extensions between Ubc3/Cdc34 and Ubc2/Rad6 also switches their substrate specificity (Silver et al. 1992). Furthermore, some of the extensions contain stretches of hydrophobic amino acids integrating the enzymes into a specific cellular membrane (Sommer and Jentsch, 1993).

The analysis of cells lacking a specific E2 demonstrated that they are involved in a large variety of cellular processes, like cell cycle control, DNA repair, peroxisome biogenesis, stress response and resistance to heavy metals. However, the analysis of specific proteolytic substrates revealed that Ubc's seemed to be redundant in function. Furthermore, ubiquitin-conjugating enzymes may function in dimeric complexes with other Ubc's, which may alter the substrate specificity of the involved enzymes (Chen et al. 1993). The current knowledge on the yeast Ubc's is summarized in Table 1.

Table 1. The *Saccharomyces cerevisiae* ubiquitin-conjugating enzymes

Protein	kDa	Functions
Ubc1	24	essential in absence of Ubc4 and Ubc5, important for outgrowth of spores, endocytosis of membrane proteins
Ubc2/Rad6	20	DNA repair, induced mutagenesis, sporulation, repression of retrotransposition, N-end rule pathway
Ubc3/Cdc34	34	mainly nuclear localization, essential for viability, G1-S cell cycle progession, DNA replication, spindle pole body separation, degradation of p40 (Sic1), Gcn4, and Clns
Ubc4 Ubc5	16 16	92% identity between Ubc4 and Ubc5, degradation of abnormal proteins, sporulation, resistance to stress conditions, degradation of MATα2 and ubiquitin-fusion proteins, endocytosis of membrane proteins
Ubc6/Doa2	28	localized at the ER-membrane/nuclear envelope, involved in ERAD, degradation of MATα2
Ubc7	18	localized at the ER-membrane via interaction with Cue1p, central ERAD component, degradation of MATα2, resistance to cadmium
Ubc8	25	catabolite degradation of fructose-1,6-bisphosphatase
Ubc9	18	Smt3 conjugating enzyme, mainly nuclear localization, essential for viability, mutants arrest at G2-M transition of the cell cycle, deficient in degradation of Clb2 and Clb5 cyclins
Ubc10/Pas2	21	peroxisome biogenesis
Ubc11	17	not essential also in combination with Ubc4
Ubc12	21	Rub1 conjugating enzyme
Ubc13	18	DNA repair, modified by Mms2 to form K63 linked polyubiquitin chains

2.2 Ubiquitin-Ligases

In vivo participation of E3 enzymes can be assumed generally and it seems likely that proteolysis is controlled primarily by regulating the activity of the E3's. Although they are thought to be most directly involved in substrate recognition this class of enzymes is least well understood. E3s might be divided into distinct families with regard to sequence class and enzymatic

mechanisms. The first ubiquitin ligase characterized was E3α of rabbit reticulocytes. It is the likely counterpart of yeast Ubrl, a protein of 225 kD. Ubrl is the recognition component of the so-called N-end rule pathway (Bartel et al. 1990). Both enzymes stimulate substrate degradation, and bind both the substrate and the respective Ubc. In Ubrl a RING-H2 finger domain is found which is critical for the formation of a poly-ubiquitin chain on N-end rule substrates (Xie and Varshavsky, 1999).

An important group of E3s is characterized by a conserved element, the HECT-domain. HECT-domain proteins carry a C-terminal region of about 100 amino acids exhibiting strong similarities to human E6 associated protein (E6AP; Huibregtse et al. 1995). Cellular E6AP together with an ancillary factor, the papilloma virus E6 oncoprotein, is needed for the turnover of the tumor suppressor p53 (Scheffner et al. 1993). E6AP is able to form a thioester bond with ubiquitin at a cysteine residue within the C-terminal conserved region (Scheffner et al. 1995). It receives the ubiquitin moiety from a specific Ubc. Thus, E6AP is integrated into the transfer cascade for ubiquitin. Because of this, a stable interaction of E6AP with a certain Ubc might not be necessary.

A major input into the architecture and function of E3's were derived from studies of the yeast cell cycle. In an in vitro reconstituted system the multimeric ubiquitin ligase SCF was identified. The minimal unit consists of four different subunits: Cdc53, Hrtl, Skpl and Cdc34. Cdc53 and Hrtl recruit the ubiquitin-conjugating enzyme Cdc34 into the complex. Skpl helps to link the minimal SCF via interaction with Cdc53 to a member of the family of F-box proteins. The F-box proteins are characterized by the F-box that binds to Skpl. In addition, they comprise other protein-protein interaction domains that specifically recognize phosphorylated substrates. Thus, the different F-box proteins integrate SCF into substrate specific degradation pathways. Hrtl also belongs to a family of proteins that are characterized by a zinc-binding RING-H2 finger domain. Hrtl dramatically increases the activity of SCF and enables the complex to synthesize unanchored poly-ubiquitin chains and promotes auto-ubiquitination of Cdc34. Cdc53 contains a Cullin domain, which is also found in many other proteins. Some of these protein motives are also found in another ubiquitin ligase complex that functions at the end of mitosis, the APC complex. Apc2 belongs to the family of Cullins and Apcll contains a RING-H2 finger domain (Deshaies, 1999).

2.3 Ubiquitin Specific Proteases

Most of the work of the past years has focused on the enzymes attaching ubiquitin to substrate proteins. Recent results suggest that regulatory events also occur at the level of de-ubiquitination. De-ubiquitinating enzymes are thiol proteases which can be divided into two classes: The so-called ubiquitin-specific processing proteases (Ubp) and the ubiquitin carboxyl-terminal processing hydrolases (Uch) (Wilkinson, 1997). The first class is extremely divergent but all members contain short conserved sequence motives, termed the Cys and His boxes, respectively. These motives are likely to form parts of the active site of the enzymes (Hochstrasser, 1996). Two representants of this enzyme family have been investigated more closely. One of them is the yeast Ubp4/Doa4, a de-ubiquitinating enzyme cleaving linear ubiquitin-protein fusions and isopeptide linkages. In *doa4* mutants ubiquitin-dependent protein degradation of certain N-end-rule substrates is affected (Papa and Hochstrasser, 1993). In addition, ubiquitin species accumulate that are slightly larger than free ubiquitin, suggesting that they represent ubiquitinated peptides that are proteolytic remnants of the 26S proteasome activity. Furthermore, the proteolysis of ubiquitin is increased in *doa4* mutants, suggesting that Doa4 is required to recycle ubiquitin from proteasome-bound substrates (Swaminathan et al. 1999). In agreement with this hypothesis, Ubp4/Doa4 could be co-purified with the 26S proteasome (Papa et al. 1999). Interestingly, Ubp4/Doa4 exhibits sequence similarities to the mammalian tre-2 oncogene and it could be shown that tre-2 is indeed an Ubp (Papa and Hochstrasser, 1993).

The second investigated enzyme of this class is yeast Ubp14, a functional homologue of human isopeptidase T. Cells lacking this activity exhibit no obvious growth defect but are hypersensitive to canavanine, an amino acid analog which leads to aberrant translation products. Furthermore, the turnover of some typical substrates of the ubiquitin system is reduced. Cells lacking Ubp14 accumulate unanchored poly-ubiquitin chains. These ubiquitin chains do not only derive from the proteolysis of ubiquitinated substrates. Instead cells seem to synthesize unanchored ubiquitin chains de novo. Accumulation of these chains in *ubp14* cells inhibits degradation probably by competing for the substrate-binding sites of the proteasome. Overexpression of Ubp14 and thus reducing free ubiquitin chains also reduces protein breakdown indicating that the pre-assembled ubiquitin chains contribute significantly to the efficiency of ubiquitination (Amerik et al. 1997).

2.4 Ubiquitin-Like Proteins

One of the major developments of the last years was the identification of proteins that display similarities to ubiquitin (Ubl, ubiquitin-like proteins). Like ubiquitin, some of them are covalently linked to a number of cellular proteins and the enzymatic activities required for this modification parallel those linking ubiquitin to substrates. Because the Ubl's have been described only recently, their function is less clear. From the current knowledge it can be speculated that Ubl-conjugation is not directly linked to proteolysis. Instead, at least one of them has been implicated in protein localization. Ubl proteins are less conserved than ubiquitin among species. One of them is the 11.5 kD mammalian protein SUMO-1 which shares 18% sequence identity with ubiquitin. In yeast a homologous protein exists, Smt3, sharing 48% identical amino acids with SUMO-1. Many amino acids involved in interaction of ubiquitin with the proteasome are not present in SUMO-1 and Smt3, respectively. Nevertheless, both can be found either free or conjugated to cellular proteins. Cells lacking Smt3 are inviable, and thus it can be assumed that this Ubl fulfills essential cellular functions.

Another Ubl is NEDD8 and its yeast equivalent Rub1 (59% sequence identity). Unlike Smt3, Rub1 is not essential for viability. NEDD8 displays 58% sequence identity with ubiquitin and 20% with SUMO-1. Two observations suggest that Rub1 is conjugated analogously to ubiquitin: First, identical residues between ubiquitin and Rub1 are clustered on one surface of the three-dimensional structure of ubiquitin and particularly at the C terminus through which ubiquitin is conjugated. Second, Lys48 and Lys29 of ubiquitin are conserved in Rub1. Both residues are required for the formation of a poly-ubiquitin chain. Indeed it turned out that Rub1 is also conjugated to a small number of cellular substrates. Interestingly, one of the substrates is Cdc53, a protein functioning in ubiquitin-protein ligation (Hochstrasser, 1998).

Ubl's are activated by distinct E1 activities but this activity is not provided by a single polypeptide chain. Instead, a heterodimeric enzyme performs the Smt3 or Rub1 activation. The two subunits of these dimers exhibit homologies to either the N- or C-terminal part of the ubiquitin-activating enzyme. In the case of yeast Smt3, Uba2 and Aos1 provide the activating activity (Johnson et al. 1997). Another activating enzyme is formed by Uba3 and Ula1/Enr2 that activates Rub1 (Liakopoulos et al. 1998). A similar activity has been purified from Arabidopsis indicating a high degree of conservation. It comprises Axr1 and Ecr1 that are similar to Ula1/Enr2 and Uba3 respectively. When combined they are able to activate NEDD8 in vitro through thioester formation at Ecr1. Both Ubl's are also conjugated by dis-

tinct ubiquitin-conjugating enzymes. Ubc9 conjugates Smt3 (Schwarz et al. 1998) while Ubc12 links Rub1 to substrates (Liakopoulos et al. 1998). In vitro, both Ubc9 and Ubc12 are unable to form thioester bonds with ubiquitin. Ubiquitin ligases specific for Smt3 and Rub1 have not been described yet.

Furthermore, Ubl-specific proteases have been identified in yeast (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000) and also from higher eukaryotic cells (Gong et al. 2000). Yeast Ulp1 and Ulp2 (Ubl-specific protease) cleave Smt3, but not ubiquitin, from substrates. Both enzymes shows no

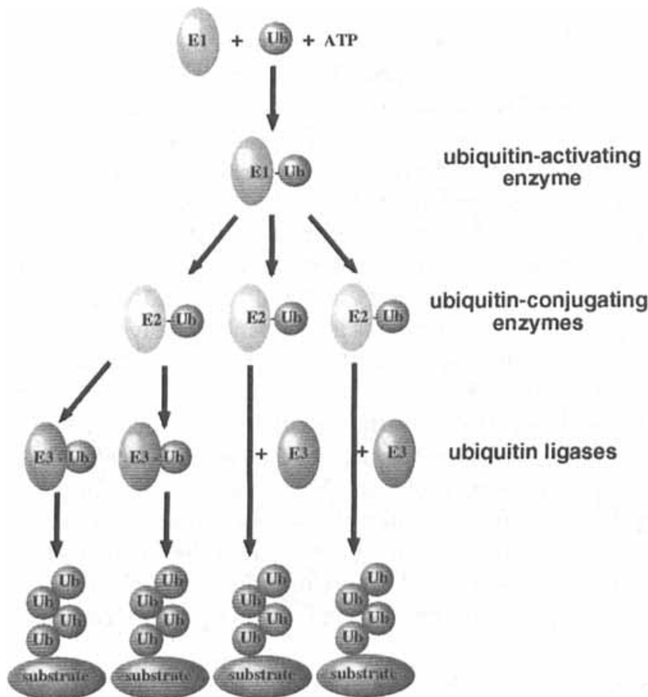

Fig. 1. A schematic diagram outlining the hierarchic structure of the ubiquitin system. In an ATP-dependent manner a thioester bond is formed between the C-terminus of ubiquitin and an internal cystein residue of the ubiquitin-activating enzyme. Subsequently, ubiquitin is transferred to a member of the family of ubiquitin-conjugating enzymes, which are also able to form a thioester bond with ubiquitin. The third class of enzymes, the ubiquitin ligases, direct ubiquitin to the proteolytic substrates. Different families of this class of enzymes are known, some of which are also able to form a thioester intermediate with ubiquitin (HECT-domain ligases). The final ubiquitin-substrate linkage is an isopeptide bond between the C-terminus of ubiquitin and internal lysine residues in the substrate proteins

homology to any known de-ubiquitinating enzymes. Intriguingly, Ulp1 plays an essential role at the G2/M transition of the cell cycle (Li and Hochstrasser, 1999).

3 Retrograde Transport From the ER

3.1 ER Proteins Are Degraded by the Ubiquitin-Proteasome System

The endoplasmic reticulum (ER) constitutes the site of synthesis and maturation of proteins destined for the secretory pathway. Proteins that enter this pathway are inserted into the ER as nascent polypeptide chains. Subsequently, these proteins adopt their proper folding within the ER and are in some cases assembled into multimeric protein complexes, which are then transported to other organelles. Misfolded or aberrantly assembled polypeptide chains are recognized by a specific quality control system that retains such proteins within the ER and eventually channels them to degradation. The importance of ER protein degradation for cellular processes is emphasized by severe diseases, which are accompanied with the breakdown of misfolded proteins, such as cystic fibrosis. Proteolysis of ER proteins has been shown to be highly selective (Klausner and Sitia, 1990). Additionally, it was observed that degradation of ER proteins is independent of lysosomal or vaccuolar proteases and does not require ER to Golgi transport. Thus it was long believed that breakdown of ER proteins occurs within the compartment itself (Klausner and Sitia, 1990). However, the specificity and efficiency of ER degradation was difficult to explain with a model that would place the vast amount of loosely and partly folded proteins, which should be susceptible to proteolysis, and highly active proteases into the same compartment. At the same time, ER localized proteases, which would catalyze the breakdown of substrate proteins, could not be isolated. Therefore, the mechanisms of ER protein degradation and the proteases involved in this process remained mysterious.

In 1993 the identification of a yeast ubiquitin conjugating enzyme, Ubc6p, as an integral protein of the ER membrane with the catalytic part facing the cytosol was reported (Sommer and Jentsch, 1993). Based on the observation that a mutation within the Sec61 protein, which causes a translocation defect, was suppressed by loss of function mutants of Ubc6p the authors proposed for the first time a link between ER protein degradation and cytosolic ubiquitin-proteasome mediated proteolysis (Sommer and Jentsch, 1993). Another line of evidence for an involvement of the ubiquitin-proteasome machinery in the breakdown of ER proteins came from the investigation of the turnover of the cystic fibrosis transmembrane conduc-

tance regulator (CFTR). CFTR constitutes a chloride channel of the ATP-binding-cassette class protein family in epithelial cells. Wild type CFTR gets transported to the plasma membrane whereas a mutant form CFTR ΔF508 is retained within the ER and rapidly degraded (Kopito, 1999). Ward et al. and Jensen et al. noticed that treatment of cells expressing CFTR ΔF508 with specific proteasome inhibitors resulted in a remarkable stabilization of the protein (Jensen et al. 1995; Ward et al. 1995). The overexpression of a mutant form of ubiquitin UbK48R, which fails to form poly-ubiquitin chains and thus does not efficiently label substrate proteins for recognition by the 26S proteasome, also resulted in a reduced turnover of CFTR ΔF508. Finally, in the presence of the proteasome inhibitor lactacysteine CFTR accumulated with covalently attached poly-ubiquitin chains (Ward et al. 1995). Recent findings indicate, that the ubiquitin-proteasome machinery also contributes to the breakdown of soluble substrate proteins in the ER lumen (Hiller et al. 1996; Qu et al. 1996; Bonifacino and Weissman, 1998). It is worthy to note that not only misfolded and aberrant proteins are degraded by this mechanism. Proteasome mediated ER protein degradation also has a regulatory function, as has been demonstrated for the catabolite induced degradation of HMG-CoA reductase (Hampton et al. 1996), and accounts for the turnover of otherwise stable host-proteins after viral infections (Wiertz et al. 1996a,b).

Although studies exist which report an association of the 26S proteasome with the ER membrane, proteasomes have never been found inside the ER. It was therefore hard to envisage, how a cytosolic multienzyme protease like the 26S proteasome should catalyze the proteolysis of ER proteins, which are integrated into a membrane by the virtue of several transmembrane segments or are located within the ER lumen and thus separated from the proteasome by a lipid bilayer. Consequently, one can propose certain steps, which an ER degradation substrate should pass in order to become accessible by a cytosolic protease (for a schematic view see Fig. 2). Proteolysis of aberrant ER proteins should be initiated by the recognition of the substrate by the quality control system within the ER. Genetic and biochemical evidence implicates chaperones and folding enzymes as well as components of the glycosylation machinery with this process (Ellgaard et al. 1999). Subsequently, a mechanism should exist that shuttles luminal substrate proteins through the ER membrane into the cytosol. Integral ER membrane proteins must get dislocated from this membrane to become fully accessible by the 26S proteasome. Although we lack a detailed knowledge on these processes, recent studies indicate that the machineries mediating protein import into the ER and protein dislocation from the ER share at least some components, such as the Sec61 translocation channel (Wiertz et al. 1996b; Plemper et al.

1997). Protein translocation through biological membranes depends on targeting signals, which are recognized by specific receptors and initiate the insertion of the substrate into a transport channel. In the case of protein translocation into the ER these signals are often proteolytically removed from the substrates during import (Rapoport et al. 1996). To date, we have no knowledge on the nature of such signals in protein export from the ER, however, it is obvious that import into the ER and dislocation from the ER must depend on different signals. Active transport of proteins through membranes also relies on a driving force, which determines the direction of protein movement. No such driving force has been described in retrograde transport, so far, although ER protein degradation depends on several components, which hydrolyze nucleotide-tri-phosphates during their enzymatic activity. Once in the cytosol the substrates are labeled by the covalent attachment of poly-ubiquitin and are finally degraded by the 26S proteasome. In the following chapters we will try to give an overview on the current knowledge on proteasome mediated degradation of ER proteins and summarize the implications resulting from the models proposed for the mode of action of the ubiquitin-proteasome machinery on ER substrate proteins.

3.2 Systems to Study ER Protein Degradation

3.2.1 Yeast

Genetic and biochemical studies on the breakdown of a variety of substrate proteins in the yeast *Saccharomyces cerevisiae* have resulted in major contributions for the understanding ER protein degradation (Brodsky and McCracken, 1997; Sommer and Wolf, 1997; Bonifacino and Weissman, 1998; Plemper and Wolf, 1999). Sec61, a multispanning ER membrane protein, constitutes an essential component of the Sec61 complex that mediates translocation of proteins into the ER. A mutation in Sec61, termed sec61-2, results in an unstable protein which causes the dissociation of the Sec61 complex and a translocation defect (Sommer and Jentsch, 1993; Biederer et al. 1996). Degradation of Sec61-2 was shown to depend on poly-ubiquitination by the E2 enzymes Ubc6 and Ubc7 and on proteasomal activity (Biederer et al. 1996). Similarly, a mutant of the multidrug-resistance-mediating protein Pdr5, an ATP binding cassette type plasma membrane channel that is integrated into the membrane by the virtue of 12 transmembrane segments, undergoes rapid turnover in the ER. The degradation of Pdr5* parallels the breakdown of Sec61-2 in it‘s need for ubiquitination and proteasome activity (Plemper et al. 1998). The turnover of a mutant form of

cells secrete a small glycosylated protein, termed α pheromone or α-factor, into the medium which binds to the pheromone receptors of a cells and causes morphological changes required for mating. By removal of the glycosylation sites pre-pro-α-factor becomes an unstable protein that undergoes ER protein degradation (Caplan et al. 1991). Yeast pre-pro-α-factor has served as a substrate protein in a reconstituted system to study ER protein degradation in vitro (see later).

Physiological regulation of cellular processes by controlled protein degradation by the 26S proteasome has been observed in several cases (Hochstrasser, 1995; King et al. 1996; Hershko, 1997). 3-hydroxy3-methylglutaryl-CoA reductase (HMG-CoA reductase), an integral protein of the ER membrane involved in the mevalonate pathway, is subjected to feedback regulation and undergoes regulated degradation via the ubiquitin-proteasome pathway in mammals and yeast (Hampton and Rine, 1994; Hampton et al. 1996; McGee et al. 1996). Interestingly, degradation of the yeast HMG-CoA reductase was shown to depend on the same factors which are required for the proteolysis of malfolded or aberrant ER proteins (Hampton et al. 1996; Bordallo et al. 1998). This finding indicates that regulated ER protein degradation and the breakdown of proteins sorted out by the quality control system are at least partly mediated by a common mechanism.

3.2.2 MammalianCells

The turnover of several unstable proteins in the secretory pathway has been intensively examined in mammalian cells by biochemical techniques (Kopito, 1997; Bonifacino and Weissman, 1998). Proteins, that have been reported to be short-lived, were expressed in cell-cultures to allow the detailed study of their maturation and proteolysis. Cystic fibrosis, a human autosomal recessive genetic disorder, is caused by the dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-binding cassette-type chloride channel in the plasma membrane (Kopito, 1999). Mutant ΔF508 of CFTR is of special interest: The majority of patients suffering from cystic fibrosis carry the CFTR ΔF508 allele. CFTR ΔF508 does not get transported to the cell surface but is retarded in the ER and rapidly degraded in a ubiquitin-proteasome dependent manner (Jensen et al. 1995; Ward et al. 1995). Overexpression of CFTR ΔF508 in transfected cells results in the appearance of functional channel molecules in the plasma membrane indicating that the pathogenesis of cystic fibrosis is most likely caused primarily from mislocalization and rapid degradation of the CFTR ΔF508 mutant protein (Dalemans et al. 1991). Indeed, CFTR ΔF508 is transported to the cell surface and forms active Cl-channels, when certain arginine residues

present in the luminal part of protein were changed to lysines thereby masking a potential ER retardation signal and allowing the protein to escape ER protein degradation (Chang et al. 1999). Surprisingly, maturation and transport of the wild-type CFTR is also ineffective in transfected cells indicating the requirement for additional factors for folding and membrane delivery that may be limiting in these cells (Lukacs et al. 1994; Ward and Kopito, 1994). A mutant in α1-antitrypsin (α1-ATZ), that causes retardation of this soluble secretory protein within the ER lumen, has been shown to account for chronic liver injuries and hepatocellular carcinoma. Biochemical investigation of α1-ATZ revealed, that it is degraded in an ubiquitin-proteasome dependent manner (Qu et al. 1996).

In the ER proteins are not only folded into their mature structure but are in some cases integrated into multimeric enzyme complexes. Components of such complexes, which are present in excess and will therefore not be correctly assembled, are removed from the ER and degraded. A model substrate for this process is the T-cell antigen receptor, which represents a hetero-oligomeric plasma membrane complex consisting of at least seven transmembrane subunits (Klausner and Sitia, 1990). In the absence of other subunits, the TCRα, an integral membrane protein, is retarded within the ER and rapidly degraded. A detailed analysis of TCRα proteolysis revealed that it paralleled the breakdown of CFTR ΔF508 in the requirement for polyubiquitination and proteasomal function, thus representing another example of ubiquitin-proteasome mediated ER protein degradation (Huppa and Ploegh, 1997; Yang et al. 1998; Yu and Kopito, 1999).

3.2.3 Viral Induction of Proteolysis

MHC class I restricted antigen presentation provides a potential of the cellular immune system to selectively label virus infected cells at the surface and kill them by CD8+ T-lymphocytes. This mechanism requires that MHC class I molecules are loaded with virus derived peptides in the ER and transported by the secretory pathway to the plasma membrane. The human cytomegalo virus (HCMV) employs a strategy to interfere with this process and escape detection by the immune system. HCMV encodes two proteins, US2 and US11, which interact with MHC class I molecules in the ER membrane and target them to proteasome mediated degradation (Wiertz et al. 1996a). This process depends on the ubiquitination of the substrate protein as well as proteasomal functions. Breakdown of ER proteins by the expression of viral gene-products features several mechanistic differences when compared to the extraction and degradation of ER substrates sorted out by the quality control. In US2 expressing cells de-glycosylated MHC class I molecules have

been shown to associate transiently with Sec61β, whereas the proteolysis of MHC class I molecules due to misfolding of the substrate, e.g. after treatment with DTT, involves a Sec61β associated and glycosylated degradation intermediate (Wiertz et al. 1996b). Furthermore, membrane-extraction of MHC class I molecules in US11 expressing cells has been reported to be initiated independently from poly-ubiquitination (Shamu et al. 1999). On the contrary, attachment of ubiquitin constitutes a pre-requisite for the dislocation of other degradation substrates in mammalian and yeast cells, such as TCRα and CPY* (Biederer et al. 1997; Yu and Kopito, 1999).

3.2.4 In Vitro Systems

McCracken and Brodsky have established a reconstituted system to examine the turnover of an ER protein in vitro (McCracken and Brodsky, 1996). Radio-labeled mutant pre-pro-α-factor, which has been depleted of all glycosylation sites and thus becomes an unstable protein of the ER lumen (see above), was in vitro translocated into microsomes isolated from yeast cells. After import and removal of substrate molecules that did not get translocated into the vesicles addition of cytosol and an ATP-regenerating system resulted in the export and degradation of pre-pro-α-factor. Proteolysis of pre-pro-α-factor in this system depended on proteasomal functions, as cytosol derived from yeast proteasomal mutants failed to promote degradation. However, breakdown of mutant pre-pro-α-factor in vitro most likely represents a special case of ER protein degradation. The removal of glycosylation sites results in de-stabilization of the pro-α-factor, whereas such mutations in other substrates cause accumulation within the ER (Knop et al. 1996a). Moreover, turnover of pre-pro-α-factor has been shown to occur independently from poly-ubiquitination and the involvement of other known components of the ER protein degradation machinery, like the Hrd and Der proteins described below, has not been demonstrated, yet. Similar in vitro systems to study the degradation α1-ATZ and CFTR ΔF508, respectively, have been recently reported (Qu et al. 1996; Sato et al. 1998; Xiong et al. 1999).

3.3 The ER Quality Control System

Not much is known about the mechanisms that ensure efficient discrimination between ER proteins, which are in the process of folding and will finally form stable enzymes, and those, which fail to acquire their mature structure and have to be degraded. For example, small peptide stretches, which are buried in the mature but exposed in a native conformation of a protein, have

been proposed to serve as an indicator for the correct folding and maturation state of a substrate. Yet, such stretches are likely to be exposed also in intermediates of the folding process of functional enzymes that have not yet acquired their final structure. Therefore, breakdown of a substrate protein must be delayed for the time needed to become at least partly folded. Newly translocated proteins associate with chaperones and folding enzymes within the ER lumen such as BiP, calnexin, calreticulin, the protein disulfide isomerase, GRP94, ERp57 and ERp72 (Ellgaard et al. 1999). Prolonged association with these folding enzymes due to an inability to fold the substrates appropriately may constitute one mechanism to sort out proteins for degradation (Knittler et al. 1995; Ellgaard et al. 1999). Indeed, the involvement of ER localized chaperones for the efficient turnover of ER degradation substrates has recently been demonstrated. Microsomes harboring mutant versions of calnexin (McCracken and Brodsky, 1996) or Kar2 (Brodsky et al. 1999), the yeast homologue of BiP, were unable to induce efficient export and proteolysis of pre-pro-α-factor in vitro. Degradation of α1-ATZ in vivo involves the association of the substrate with ubiquitinated calnexin, suggesting role of this chaperone in recognition and targeting of ER degradation substrates to proteolysis (Qu et al. 1996). A function of BiP in ER protein degradation was genetically demonstrated in the yeast *Saccharomyces cerevisiae*. Mutants of BiP failed to degrade the substrate protein CPY* under conditions that did not affect import of this protein into the ER (Plemper et al. 1997). The activity of ER chaperones and folding enzymes depends on the Ca^{2+} concentration within the ER lumen. In yeast, disruption of the ion transporter Pmr1, which supplies the ER with the divalent cations Ca^{2+} and Mn^{2+}, has been shown to cause stabilization of the ER degradation substrate CPY* (Dürr et al. 1998). However, the effects of a *pmr1* deletion on ER protein degradation are most likely pleiotropic because changes in the ionic milieu within the ER lumen probably affect chaperone activity as well as other enzymatic reactions such as glycosylation. Recently, degradation of mutated pre-pro-α-factor in vitro was shown to require the function of protein disulfide isomerase (PDI) (Gillece et al. 1999). The retrograde transport of pre-pro-α-factor depended on the interaction with PDI, because microsomes harboring different deletion mutants of PDI were unable to export this substrate. Interestingly, export and proteolysis of pre-pro-α-factor was not affected by a point mutation within PDI, which disrupts only the catalytic activity, but still allowes binding of PDI to substrate proteins. Controversingly, degradation of CPY* was hardly affected in the PDI deletion mutants, however, CPY* breakdown still depended on disulfide bond formation (Gillece et al. 1999). Protein folding and disulfide bond formation may thus represent distinct enzymatic functions of PDI. The exact role of chaperones

in ER protein degradation has to be determined further. It was speculated that BiP or other ER folding enzymes contribute to the dislocation of ER degradation substrates by constituting a motor to push substrate proteins out of the ER and into the cytosol (see later). There exists no biochemical evidence for such a mode of action, so far. More likely, these enzymes may primarily fulfill other functions such as preventing the aggregation of aberrant proteins and keeping them in a dislocation competent conformation, or the targeting of substrates to an export channel. BiP may also act as a gating factor in protein import and protein dislocation that seals the translocation pore to prevent the uncontrolled diffusion of small molecules trough the Sec61 channel (Hamman et al. 1997). The capability of BiP to bind to nascent chains in yeast depends on the interaction with Sec63, an essential protein required for post-translational import into the ER (Scidmore et al. 1993; Rapoport et al. 1996). Interestingly, mutants in Sec63 have also been shown to stabilize ER degradation substrates in yeast, suggesting a function of this enzyme in the turnover of ER proteins (Plemper et al. 1997). It is not clear, however, whether Sec63 and BiP interact during ER protein degradation.

The correct folding of the cytoplasmic domains of integral ER membrane proteins requires the action of cytosolic chaperones. There exist controversial reports about an involvement of these enzymes in ER protein degradation. For example, degradation of an unstable mutant form of the insulin receptor was found to depend on cytosolic Hsp90 (Imamura et al. 1998). Injection of specific anti-Hsp90 antibodies into cells resulted in a remarkable stabilization of the receptor. On the contrary, disruption of the binding of cytosolic Hsp90 to CFTR by the drug Geldanamycin resulted in an accelerated breakdown of CFTR as well as the unstable mutant CFTR ΔF508 (Loo et al. 1998). Cytosolic Hsp70 and Hsp90 have also been found in association with cytoplasmic aggregates of CFTR ΔF508 in cells treated with proteasome inhibitors. Still, these Hsps probably did not promote dislocation of CFTR but rather associated with the substrate afterwards. A function of these chaperones in the degradation of luminal ER proteins has not been reported, so far, questioning a general role for these proteins in the dislocation of ER substrates.

3.3.1 Glycosylation

Certain proteins destined for the plasma membrane or other organelles are modified by the covalent attachment of sugar residues within the ER. This glycosylation represents a dynamic structure, which is repeatedly trimmed and re-synthesized by a multitude of enzymes. Surprisingly, the inhibition of

mannose trimming in the ER results in the stabilization of otherwise unstable glycoproteins which indicates that only glycoproteins containing completely maturated mannose residues will be accepted as substrates of the degradation machinery. (Su et al. 1993; Helenius, 1994; Knop et al. 1996a). The degradation of unassembled subunits of the asialoglycoprotein receptor is severely affected by inhibiting the trimming of mannose chains with chemicals (Ayalon Soffer et al. 1999). Binding of the substrate to calnexin was not affected under these conditions. This suggests that the mannosidases localized in the ER may serve as sensors for the folding state of a glycoprotein. In yeast, mutations, that prevent the mannose trimming catalyzed by mannosidase I impair the proteolysis of CPY* (Knop et al. 1996a; Jakob et al. 1998). Strikingly, the processing of glycan-residues present in CPY* in the ER takes about 20 min., which is in the range of the observed half live (Jakob et al. 1998). This finding suggests that mannose trimming may cause a delay in the action of the quality control system which is required for the correct folding of proteins by the folding machinery and allows functional enzymes to acquire their mature structure before degradation is initiated. Similar results have been obtained in mammalian cells by the investigation of mannose trimming during the breakdown of mutant ribophorin I (de Virgilio et al. 1999). Inhibition of glucose chain trimming in mammalian cells has the opposite effect: Applying the drug Castanospermine to cells increases the degradation of substrate proteins as has been demonstrated for unassembled subunits of the asialoglycoprotein receptor (Ayalon Soffer et al. 1999) and the α-subunit of the nicotinic acetylcholine receptor (Keller et al. 1998).

3.4 Specific Factors Involved in ER Protein Degradation

The search for yeast mutants, which were defective in the turnover of HMG-CoA reductase and CPY*, respectively, resulted in the identification of several genes, whose function had not been described at that time. (Hampton et al. 1996; Hiller et al. 1996; Knop et al. 1996b). Surprisingly, both genetic screens resulted in the isolation of an overlapping spectrum of genes which indicated the existence of a single machinery required for the breakdown of misfolded proteins sorted out by the quality control system as well as substrates that are degraded in a regulated manner. Disruption of these newly isolated genes did not cause a growth defect or other detectable phenotype, suggesting that ER protein degradation is not essential for the viability of yeast cells. *HRD1/DER3* encodes an integral protein of the ER membrane with a large luminal domain (Bordallo et al. 1998). Interestingly, this domain harbors a region with high similarity to the so-called RING-H2 finger motif, which is also found in a subunit of the E3-SCF complex. The RING-H2 motif

of Hrd1/Der3 is essential for its function because deletion of this region or a point mutation changing a single cysteine residue to serine results in a biologically inactive protein (Bordallo et al. 1998; Bordallo and Wolf, 1999). This led to the speculation that Hrd1 may be a component of an E3 enzyme, which catalyzes the poly-ubiquitination of ER degradation substrates. All features found for Hrd1/Der3 point to such a function. The RING-H2 domain in the SCF complex has been demonstrated to mediate the association with E2 enzymes. Whether the Hrd1/Der3 RING-H2 motif is able to interact with cytosolic E2 enzymes has still to be shown. Meanwhile, the Hrd1/Der3 protein has shown to be necessary for the proteolysis of all yeast ER degradation substrates investigated so far, which points to a central function of Hrd1/Der3 in ER protein turnover. Hrd3 constitutes an integral protein of the ER membrane with one transmembrane segment. Surprisingly, deletion of *HRD3* results in stabilization of HMG-CoA reductase and CPY* but rapidly increases the turnover of Hrd1/Der3. Accelerated degradation of Hrd1 in *HRD3* disrupted cells depends on proteasomal activity as well as Ubc7 and requires Sec61 function (Plemper et al. 1999b). It was therefore speculated that Hrd1 and Hrd3 form a protein complex in the ER membrane, which dissociates in the absence of Hrd3 and leads to Hrd1 degradation. Another integral membrane protein, which is required for the degradation of CPY*, is Der1 (Knop et al. 1996b). Surprisingly, Der1 function is dispensable for the breakdown of other ER degradation substrates tested so far (Bordallo et al. 1998) but constitutes a necessity for the rapid turnover of Hrd1/Der3 in *HRD3* deletion strains (Plemper et al. 1999b). The function of Hrd3 and Der1 in ER protein degradation has not been established yet. These two proteins together with Hrd1/Der3 have been suggested to associate in a complex, which promotes the dislocation and proteolysis of ER degradation substrates. The luminal domain of Hrd1/Der3 may also be involved in the targeting of substrate proteins to the translocation pore. Further biochemical studies will be needed to determine the function of Hrd1, Hrd3 and Der1 in ER protein turnover.

Two ubiquitin conjugating enzymes (E2s) have been shown to contribute to the degradation of ER protein substrates in yeast (Sommer and Jentsch, 1993; Biederer et al. 1996; Hiller et al. 1996). One of these, Ubc6, is integrated into the ER membrane by a single transmembrane segment, the catalytic domain facing the cytosol (Sommer and Jentsch, 1993). The other one, Ubc7, constitutes a soluble protein, which is recruited to the ER membrane by the interaction with the integral membrane protein Cue1 (Biederer et al. 1997). Both enzymes have been shown to be also required for the breakdown of soluble non-ER proteins (Chen et al. 1993). The interaction with Cue1 is essential for Ubc7 function: In a Cue1 deletion strain Ubc7 is mislocalized to

the cytosol and degraded (Biederer et al. 1997). As a consequence, substrate proteins, which are degraded in an Ubc7 dependent manner, are stabilized. Interestingly, deletion of Cue1 does not only affect the breakdown of ER degradation substrates, but also results in the stabilization of non-ER proteins. Overexpression of Ubc7 in the absence of Cue1 to obtain wild type protein levels does not overcome the loss of Cue1. This finding indicates that localization of Ubc7 at the ER membrane is an essential prerequisite for its function, emphasizing the importance of correct cellular placement for enzymatic activities. Disruption of either *UBC6* or *UBC7* affects the breakdown of all yeast ER degradation substrates tested so far, stressing the important role of these E2s in ER protein turnover.

To date, no mammalian factors specifically involved in ER protein degradation have been isolated. There exist entries in the sequence databases, which share similarity to the yeast Hrd1/Der3, Hrd3 and Der1 proteins, respectively, however, the function of these proteins has not been determined so far. Therefore, it remains an open question whether ER protein degradation in mammalian cells requires the activity of similar cellular components that have been shown to mediate turnover of ER proteins in yeast.

3.5 Retrograde Transport and De-Glycosylation

In order to become accessible to cytosolic proteasomes, integral proteins have to be extracted from the ER membrane prior to degradation. Additionally, there should exist a machinery, which shuttles luminal substrate proteins into the cytosol. This transport apparatus must be capable to confer selective and vectorial movement of proteins through a lipid bilayer, which is opposite to protein import into the ER. The first hint on the nature of this export machinery came from the investigation of a membrane bound ER substrate. Wiertz et al. noticed that during breakdown of MHC-class I molecules in cells expressing the HCMV US2 gene-product a de-glycosylated form of the substrate could be co-precipitated with subunits of the Sec61 translocon (Wiertz et al. 1996b). This association was only observed in the presence of proteasome inhibitors indicating that it indeed represented a MHC class I degradation intermediate. The engagement of Sec61 in ER protein degradation was demonstrated in the yeast *Saccharomyces cerevisiae*. Breakdown of CPY* was shown to be diminished in mutants of Sec61 and Sec63, respectively, under conditions where the import into the ER is not affected (Plemper et al. 1997). Mutations in other components of the translocon impaired the import, but had no effect on CPY* degradation. Similar results were obtained using the yeast reconstituted system. After import,

pre-pro-α-factor could be chemically crosslinked to mutant versions of Sec61, which were shown to selectively inhibit retrograde transport but not to affect protein import into the ER (Pilon et al. 1997). These findings suggested a transient interaction of ER degradation substrates and the Sec61 translocation pore during retrograde transport. An association of degradation intermediates with Sec61 may indicate that these substrates were still in the process of import into the ER and had in fact never been released from the translocon. A CPY* mutant harboring an additional glycosylation site at the very carboxy-terminus was completely glycosylated before degradation, demonstrating that this molecule had indeed been fully translocated into the ER lumen and dissociated from the Sec61 import machinery before degradation (Plemper et al. 1999a). Recently, an interaction of CFTR ΔF508 with Sec61ß, which was dependent on the presence of proteasome inhibitors, was reported (Bebök et al. 1998). The Sec61-containing export machinery seems therefore to mediate the extraction of membrane bound substrates was well as the dislocation of soluble luminal proteins. Studies on the Sec61 translocon revealed that in vitro the pore is equally traversible for proteins in both ways (Johnson and van Waes, 1999). Therefore, the Sec61 channel is likely to require additional factors, which determine the design as an import channel or as a dislocation machinery, respectively. It was speculated that ER membrane proteins like the previously characterized Hrd1/Der3, Hrd3 or Der1 (see above) interact with Sec61 and thereby modulate the channel's activity for retrograde transport. Recent work has led to the isolation of conditional Sec61 mutants in yeast that specifically affect protein dislocation from the ER into the cytoplasm but do not impair translocation into the ER (Wilkinson et al. 2000; Zhou and Schekman, 1999). These mutations may provide a valuable tool to elucidate the role of Sec61 in retrograde transport and to isolate interacting factors, which contribute to the formation of the protein export channel.

Proteasomal degradation of glycoproteins is preceded by the removal of the glycan-residues. The characterization of a cytosolic N-glycanase activity (Suzuki et al. 1994) and the finding that the Sec61 pore is large enough to mediate transport of glycosylated polypeptide chains (Johnson and van Waes, 1999) has led to the common view that the removal of sugar residues before proteolytic breakdown takes place in the cytoplasm. Indeed, glycosylated and poly-ubiquitinated forms of CPY* have been found at the cytoplasmic side of the ER membrane in yeast (Hiller et al. 1996). Proteolysis of MHC class I molecules after DTT treatment and in the presence of proteasome inhibitors resulted in a glycosylated intermediate associated with Sec61β (Wiertz et al. 1996b). However, there seems to exists no strict order of de-glycosylating and ubiquitinating reactions for a given substrate, be-

cause MHC class I molecules in HCMV US2 expressing cells were found to be already de-glycosylated before ubiquitination (Wiertz et al. 1996b, Shamu et al. 1999).

3.6 Ubiquitination

The attachment of poly-ubiquitin to substrate proteins is a prerequisite for efficient recognition and subsequent proteolysis by the 26S proteasome. In a cell line defective in E1 function, CFTR ΔF508 was not efficiently degraded (Ward et al. 1995). Overexpression of the dominant negative ubiquitin mutant UbK48R, which is impaired in the formation of polyubiquitin chains, diminishes the turnover of ER proteins in mammalian (e.g. Jensen et al. 1995; Ward et al. 1995; deVirgilio et al. 1998) and yeast cells (e.g. Biederer et al. 1996; Hiller et al. 1996). Finally, proteolysis of unstable yeast ER proteins is significantly impaired in the absence of the E2 enzymes Ubc6 and Ubc7, respectively (Biederer et al. 1996; Hiller et al. 1996). Surprisingly, deletion of *UBC6*, *UBC7* or the disruption of both genes does not completely abolish the degradation of ER substrates (Biederer et al. 1997). This raises the question, whether other, so far unknown, E2s contribute to poly-ubiquitination of ER proteins. Recently, yeast Vph1p has been shown to be degraded independently from Ubc6p and Ubc7p function (Hill and Cooper, 2000). There may also exist an additional ER degradation mechanism, which does not involve E2 functions. For example, yeast pre-pro-α-factor was shown to be degraded in vitro in an ubiquitination-independent manner, although proteolysis still depended on an active proteasome (McCracken and Brodsky, 1996; Werner et al. 1996).

3.7 Vectorial Transport – A Function for Poly-Ubiquitination in Protein Dislocation?

The translocation of proteins through membranes has been shown to rely on the hydrolysis of nucleotide-tri-phosphates, which provides the driving force for protein transport and ascertains the vectorial nature of this process (Schatz and Dobberstein, 1996). In bacteria the SecA protein pushes substrates through a channel in the cell membrane, thereby hydrolyzing ATP. Co-translational protein import into the ER is accompanied by GTP hydrolysis of the translating ribosome, which pushes the emerging nascent chain through the ER translocon. In the case of post-translational ER import and the translocation into mitochondria the substrate proteins are supposed to traverse the translocation channel with the help of a pulling mechanism excerted by ATP hydrolyzing chaperones on the other side of the membrane.

So far, the nature of the driving force mediating retrograde transport of ER degradation substrates is unknown. An involvement of ER chaperones in a transport machinery analogous to bacterial SecA is unlikely. SecA shares no homology to the ER chaperones described so far, and an enzymatic activity, which parallels the reversible membrane insertion of SecA, has not been characterized from the ER. Furthermore, ER chaperones have an essential function in protein import, which makes an active role in the transport of proteins in the opposite direction unlikely. Cytosolic Hsp70 and Hsp90 have been shown to contribute to the folding of the cytosolic domains of integral ER membrane proteins, but so far there exists no evidence for an involvement of these enzymes in the dislocation of ER proteins. For example, degradation of luminal pre-pro-α-factor occurred independently from cytosolic Ssa1p in vitro, indicating that this chaperone was not required for protein export (Brodsky et al. 1999).

There exists growing evidence that the attachment of polyubiquitin on the cytosolic side of the ER membrane plays a crucial role in the retrograde transport of ER proteins. Deleting the genes for Ubc6, Ubc7 and Cue1, which should affect the ubiquitin conjugating activity at the yeast ER membrane, resulted in the accumulation of CPY* within the ER (Biederer et al. 1997; Bordallo et al. 1998). This result indicates that proper export of ER proteins destined for degradation depends on the conjugation with polyubiquitin. Indeed, similar results have been obtained for the dislocation of membrane bound degradation substrates. The retrograde transport and subsequent proteolysis of a mutant version of ribophorin A and of unassembled TCRα was impeded after overexpression of UbK48R (deVirgilio et al. 1998; Yu and Kopito, 1999). Yet, the role of polyubiquitination in protein dislocation is not clear. Covalent attachment of ubiquitin to a degradation substrate emerging from the translocation pore may assist to anchor this protein at the cytosolic side of the ER membrane and prevent it from slipping back into the ER. Subsequently, such arrested intermediates may be pulled out of the ER by a so far unknown mechanism and degraded. Polyubiquitination of substrate proteins may thus work like a molecular ratchet and assist to define the direction of retrograde transport. Additionally, attachment of ubiquitin molecules to the cytoplasmic domains of integral ER proteins was proposed to initiating dislocation of these substrates from the membrane. In the case of virally induced degradation of MHC-class I molecules this has been shown not to be the case: Mutations replacing lysine residues present in the cytoplasmic domain of MHC-class I molecules did not affect extraction and degradation of this protein (Shamu et al. 1999). However, as mentioned above HCMV induced turnover of MHC-class I molecules exhibits mechanistic differences when compared to the breakdown of ER proteins

sorted out by the quality control system. In addition to the described mechanism, there may exist pathways for protein export from the ER, which do not depend on polyubiquitination. Yeast pre-pro-α-factor was shown to be degraded in a proteasome dependent manner in vitro, however export and proteolysis occurred independent from polyubiquitination (McCracken and Brodsky, 1996; Werner et al. 1996).

Jentsch and co-workers proposed a direct involvement of the proteasome in the retrograde transport of ER proteins (Mayer et al. 1998). Breakdown of an artificial substrate protein, which was composed of a non-ER degradation signal fused to a membrane anchor, occurred in two steps: In proteasomal mutants the cytosolic part of the fusion protein was degraded more rapidly than the transmembrane segment which resulted in the accumulation of a proteolytic fragment (Mayer et al. 1998). This experiment indicated that the proteasome is able to extract a protein from the ER membrane and raised the speculation that the ATPases located in the 19S cap particle of the proteasome may be a part of an ER protein dislocation machinery. Such a function would also explain the need for poly-ubiquitination in the dislocation of ER proteins, because the 26S proteasome displays high affinity only for polyubiquitinated substrates. However, as Mayer et al. used a non-ER degradation signal to initiate proteolysis of their substrate, the impact of their results on the breakdown of other ER substrates remains to be determined. Indeed, the degradation of other ER membrane proteins (e.g. CFTR, Pdr5*, Sec61-2) does not involve a proteolytic intermediate. Very recently, the retrograde translocation from the ER preceding proteasomal degradation of unassembled immunoglobulin light chains has been shown to depend on the proteolytic activity of the proteasome (Chillaron and Haas, 2000). This again suggests an involvement of this protease in the export process of ER proteins.

3.8 Degradation by the Proteasome

The involvement of the 26S proteasome in the degradation of ER proteins was demonstrated by the use of specific inhibitors in mammalian cells (Jensen et al. 1995; Ward et al. 1995) and the investigation of conditional proteasome mutants in yeast (Biederer et al. 1997; Hiller et al. 1996). It is still an open question, how substrate proteins, that have been dislocated from the ER, are targeted to the degrading 26S proteasome. Several observations suggest a localization of 26S proteasomes at the cytosolic side of the ER membrane. Impairing the catalytic activity of the proteasome by specific inhibitors results in the accumulation of ubiquitinated forms of CFTR ΔF508 in insoluble structures surrounded by ER membranes (Johnston et al. 1998).

A closer investigation of these aggregates revealed, that they also contained cytosolic Hsp70, Hsp90 and at least some proteasomal subunits suggesting that degradation of CFTR ΔF508 is catalyzed by proteasomes localized at or near to the ER (Wigley et al. 1999). Indeed, an association of the 26S proteasome with ER membranes in yeast and permeabilized rat liver cells, respectively, was recently observed (see later; Enenkel et al. 1998; Sakata et al. 1999). It is not clear, whether this association is mediated by the interaction with specific receptors or if proteasomes are recruited by other mechanisms to the ER. For example, the appearance of poly-ubiquitinated substrate proteins associated with the ER may target 26S proteasomes, which bind to polyubiquitin with high affinity, to this membrane. However, the proteolysis of ER substrate proteins need not necessarily involve membrane bound 26S proteasomes. MHC class I molecules, for example, accumulate in HCMV US11 expressing cells and the presence of proteasome inhibitors as soluble ubiquitinated intermediates in the cytosol indicating that this substrate may be degraded by proteasomes, that are not associated with the ER (Wiertz et al. 1996b; Shamu et al. 1999). However, protein breakdown mediated by viral gene-products is likely to follow different pathways than the turnover of misfolded ER degradation substrates and the usage of proteasomal inhibitors may affect the cellular localization of substrate proteins and the 26S proteasome.

It has not been conclusively ruled out that in addition to the 26S proteasome other proteases contribute to the degradation of ER proteins. A function of the signal peptidase, which removes the amino-terminal signal sequences during ER protein import, in the proteolysis of ER membrane proteins has been proposed. Lodish and co-workers observed that the breakdown of an unassembled subunit of the asialoglycoprotein receptor involves a proteolytic intermediate that was most likely generated by the enzymatic action of the signal peptidase complex (SPC) (Yuk and Lodish, 1993). The cleavage site, which led to the formation of the breakdown fragment, shared similarity with the processing sites for SPC in signal sequences. Moreover, proteolytic breakdown of an artificial fusion protein was significantly delayed in mutants of the Sec11 and Spc3 subunits of the signal peptidase, respectively (Mullins et al. 1995; Fang et al. 1997). SPC does not seem to constitute an essential factor for the breakdown of other degradation substrates and may thus contribute only to the turnover of a small subset of ER proteins. A putative protease, termed ER-60, from the ER of mouse liver cells was recently identified, although an involvement of this enzyme in protein degradation has not been convincingly demonstrated (Otsu et al. 1995).

3.9 Cellular Role of ER Protein Degradation

Although ER protein degradation seems not be essential for yeast cells, the breakdown of mutated and thus malfolded ER proteins is often associated with severe diseases in human (Ciechanover, 1998; Plemper and Wolf, 1999). The importance of this process in the understanding of genetic disorders like cystic fibrosis and α1-antitrypsin deficiency has been outlined above. In the following we will give further examples on how viruses or toxins may misuse the machinery for the ER protein degradation to interfere with cellular processes.

3.9.1 Vpu Mediated Degradation of CD4

HIV has developed a method to reduce the amount of its receptor protein CD4 at the plasma membrane of infected cells. The virus encodes a protein Vpu that inserts into the ER membrane and targets CD4 for destruction (Bour et al. 1995; Schubert et al. 1998). Interestingly, the Vpu protein contains a recognition signal for the human-ß-transducin-repeats-containing protein (h-βTrCP), which is also found in cellular proteins like β-catenin and IκBα (Laney and Hochstrasser, 1999). H-βTrCP, a F-box and WD domain containing protein, binds to this signal in a phosphorylation-dependent manner and induces ubiquitination followed by proteolysis of the substrate. In this case, however, the h-βTrCP signal does not initiate breakdown of Vpu, but rather induces the proteolysis of interacting CD4. Expression of Vpu causes the formation of a protein complex that contains CD4, Vpu and the h-βTrCP (Margottin et al. 1998). This complex in turn is thought to associate with SKP1, a component of a cytosolic E3 complex, which mediates ubiquitination and subsequent proteolysis of CD4. Thus, Vpu seems to induce the degradation of CD4 by a mechanism, which normally mediates the breakdown of soluble non-ER proteins. It remains to be determined, whether Vpu dependent CD4 turnover matches the path observed in the proteolysis of misfolded proteins of the ER or represents a new system of ER protein degradation. Breakdown of the CD4 receptor in HIV infected cells is thought to prevent viral super-infection and also to promote the maturation and release of newly assembled virus particles.

3.9.2 Toxins

Several cellular toxins of the AB type have been shown to enter the cell by endocytosis, followed by intracellular transport to the Golgi compartment and the ER (Hazes and Read, 1997). Among this subgroup are the cholera

toxin, heat-labile enterotoxin, pertussis toxin, shiga toxin and ricin. Within the ER, these toxins get activated by the formation of disulfide bonds. However, the mode of translocation through the ER membrane into the cytosol, where they exert their toxic function, is unclear so far. Several observations led to the speculation, that toxins may utilize the Sec61 retrograde transport machinery to enter the cytoplasm (Hazes and Read, 1997; Lencer et al. 1999). Export of a heterologously expressed version of the plant toxin ricin subunit A, which was translocated into the ER of yeast cells by fusion to a signal sequence, was significantly reduced in cells expressing proteasomal mutants as well as Sec61 alleles, that were shown to specifically inhibit retrograde translocation of ER degradation substrates (Simpson et al. 1999). Even though, transport was not affected by a deletion of *UBC6* and *UBC7* a large portion of the toxin was degraded by the 26S proteasome after membrane traversal. It was speculated that the remarkable low content of lysine residues in the A subunits may enable a sufficient number of molecules to escape ubiquitination and proteolysis. Recent work revealed an association of ricin subunit A with Sec61α in mammalian cells (Wesche et al. 1999) and a dependence of cholera toxin A1 transport on Sec61 complex function in vitro (Schmitz et al. 2000), which further supports the idea, that AB-type toxins enter the cytosol by a mechanism related to the dislocation of ER protein degradation substrates.

4 A function of Ubiquitin-Conjugation at the Cell Surface

4.1 Signal Transducing Receptors Are Down-Regulated by Internalization

The activity of receptors, transporters or ion channels of the plasma membrane is tightly regulated. Very often, such a regulation comprises variations in the abundance of these proteins at the cell surface. Therefore, controlling the rates of protein internalization is an important tool for regulation of activities in the plasma membrane. In the case of signal transducing receptors, particularly, cells return to an unstimulated stage through accelerated endocytosis of the involved receptors, a mechanism known as down-regulation. The importance of these regulatory issues is underlined by the fact that a misregulation will cause severe diseases. For example, an inherited form of hypertension is caused by the lack of internalization of an epithelial Na+ channel in human kidney cells (Snyder et al. 1995). Reduced internalization of the epidermal growth factor receptor (EGF receptor) results in phenotypes characteristic of transformed cells (Vieira et al. 1996).

Once internalized, receptors can either be transported to the lysosome for degradation or be recycled back to the cell surface.

Endocytosis is initiated by modification of the involved receptor in response to certain stimuli. One of these modifications is the attachment of ubiquitin. The first membrane proteins that were shown to be conjugated with ubiquitin were the platelet-derived growth factor β-receptor (PDGFRβ) and the growth hormone receptor (GHR), respectively (Yarden et al. 1986; Leung et al. 1987). When these receptors were sequenced, a second N-terminal sequence was observed that correlated with that of ubiquitin. The T-cell receptor was among the first examples for which it was shown that ligand binding triggered ubiquitin-conjugation on the cytosolic tail (Cenciarelli et al. 1992). Similar results have been observed for other receptors (see Table 2). The conjugation of PDGFRβ with ubiquitin was dependent on the kinase activity of the receptor (Mori et al. 1993). In the case of the high-affinity receptor for IgE (FcεRI receptor) and the T-cell receptor (TCR)

Table 2. Ubiquitinated plasma membrane proteins

Protein	Reference
In yeast	
ABC peptide transporter Ste6	Kölling and Hollenberg, 1994
Multidrug transporter Pdr5	Egner and Kuchler, 1996
α-factor receptor Ste2	Hicke and Riezman, 1996
a-factor receptor Ste3	Roth and Davis, 1996
Uracil permease Fur4	Galan et al. 1996; Marchal et al. 1998
Galactose permease Gal2	Horak and Wolf, 1997
Maltose transporter	Lucero et al. 2000
General amino acid permease (Gap1)	Springael and André, 1998
In mammalian cells	
PDGF receptor	Yarden et al. 1986
Prolactin receptor	Cahoreau et al. 1994
T-cell receptor	Cenciarelli et al. 1992
Fcε receptor 1	Paolini and Kinet, 1993
SLF receptor (c-kit)	Miyazawa et al. 1994
EGF receptor	Galcheva-Gargova et al. 1995
FGF receptor	Mori et al. 1995
CSF-1 receptor	Mori et al. 1995
Growth hormone receptor	Strous et al. 1996
Rhodopsin	Obin et al. 1996
p185 (c-erbB-2)	Mimnaugh et al. 1996
Met tyrosine kinase receptor	Jeffers et al. 1997
Epithelial Na-channel (ENaC)	Staub et al. 1997
Complement receptor 2	Hein et al. 1998

the modification with ubiquitin was reversible upon disengagement of the ligand (Paolini and Kinet, 1993).

The function of ubiquitin-conjugation during endocytosis remained obscure for many years, although it became clear that, in contrast to ER-degradation, it did not result in degradation by the 26S proteasome (Hicke, 1999; Strous and Govers, 1999). In 1996 experiments performed in yeast pointed to a very unexpected function of ubiquitin-conjugation at the plasma membrane: It constitutes a signal for the endocytosis of cell surface receptors (Hicke and Riezman, 1996).

4.2 Ubiquitin-Conjugation Serves Essential Functions for Internalization of Receptors of the Yeast Plasma Membrane

The first yeast membrane protein, which was shown to be a target of ubiquitin conjugation, was the ABC-transporter Ste6. Kölling and Hollenberg demonstrated that high molecular weight forms of this protein accumulated in mutants affected in endocytosis (Kölling and Hollenberg, 1994). Surprisingly, the short-lived Ste6 was stabilized in cells lacking the ubiquitin-conjugating enzymes Ubc4/Ubc5 but also by mutations in proteases of the vacuole, the lysosome-like compartment of yeast. Later, other proteins of the yeast plasma membrane where found to be ubiquitin-conjugated, too (Table 2). Among them are the G-protein-coupled signal-transducing receptors for the yeast mating factors α (Ste2) and a (Ste3) (Hicke and Riezman, 1996; Roth and Davis, 1996). Interaction of these receptors with their ligands initiates a signaling cascade required for the mating reaction and stimulates the internalization of the receptors. Both receptors are ubiquitin-conjugated constitutively. However, Hicke and Riezman could show that ligand binding stimulates phosphorylation and further ubiquitination of the α-factor receptor. Modified Ste2 is transported to the vacuole where the receptor is permanently inactivated by degradation. The proteasome is not required for this proteolysis. It was concluded, that ubiquitin-conjugation may either promote endocytosis or the vacuolar degradation of Ste2. Since ubiquitination was increased in an end4 mutant, which blocks endocytosis, it was assumed that the modification occurred at the plasma membrane. Evidence pointing to a function of ubiquitination in internalization came from experiments in which ubiquitination was abrogated: Mutants in *UBC4/UBC5* are specifically unable to internalize Ste2. Further proof for such a function was provided by the analysis of Ste2 mutants that do not contain cytoplasmic lysine residues. These modified receptors cannot be conjugated with ubiquitin and are not internalized and degraded. Roth and Davis have made similar observations for the a-factor receptor (Roth and Davis, 1996; Roth et

al. 1998). Besides these two receptors, a number of transporters and permeases have been shown to undergo ubiquitin-dependent internalization (Hicke, 1999; Strous and Govers, 1999). Moreover, experiments with the general amino acid permease (Gapl) and with the uracil permease (Fur4) have implicated a ubiquitin ligase of the HECT family, Rsp5, in this process (Hein et al. 1995; Galan et al. 1996). These data demonstrated for the first time that ubiquitin-conjugation had a function other than tagging a protein for degradation by the proteasome.

Obviously, these results raise the question why ubiquitin-conjugated receptors are not degraded by the proteasome. Proteasomal degradation of a membrane protein has two requirements: i. It would require the attachment of a Lys48 linked poly-ubiquitin chain (Pickart, 1997) and ii. Most likely it would be dependent on an export channel, as it was demonstrated for ER-bound membrane proteins (see above and Sommer and Wolf, 1997). However, most receptors are modified with only mono- or di-ubiquitin, which was demonstrated with cells lacking the de-ubiquitinating enzyme Doa4. In this mutant, the formation of poly-ubiquitin chains is largely reduced due to a reduction in the pool of free ubiquitin. Ste2 internalization is abrogated in *doa4* cells, but can be restored by expression of additional ubiquitin. When lysine-free versions of ubiquitin, which do not allow the formation of poly-ubiquitin chains, are expressed in the doa4 mutant, endocytosis of Ste2 is restored. In similar experiments, versions of Ste2 have been used that contain only one lysine residue in the cytoplasmic tail. Also in this case, internalization could be restored with the lysine-free ubiquitin (Terrell et al. 1998). These results confirm that mono-ubiquitination on a single lysine residue is sufficient for rapid internalization of Ste2. In support of this, it was shown that also the a-factor receptor is mono- or di-ubiquitinated (Roth and Davis, 1996). Slightly different results have been found for the uracil permease (Fur4), a protein that undergoes regulated endocytosis. This protein was modified with oligo- or poly-ubiquitin chains linked through Lys63. Mono-ubiquitination seems to be sufficient for internalization of Fur4 but formation of the Lys63 linked chain increases the efficiency of endocytosis (Galan and Haguenauer-Tsapis, 1997). Taken together, it can be assumed that the ubiquitin modification of these receptors at the cell surface is different from a modification that leads to proteolysis by the 26S proteasome.

In many cases of regulated protein degradation it was shown that ubiquitination is positively regulated by phosphorylation. This holds true also for endocytosis. Internalization of the α-factor receptor, for example, was shown to depend on specific serine residues in the cytoplasmic tail. They are found in a 9 amino acid motive, SINNDAKSS, which is essential for endocytosis. Mutant versions of Ste2 in which the three serine residues have been

eliminated are not phosphorylated, ubiquitin-conjugated and internalized. In agreement with that, mutants in casein kinase I, the kinase which phosphorylates Ste2, are deficient in phosphorylation, ubiquitination and internalization of the receptor (Hicke et al. 1998). Consequently, it can be assumed that phosphorylation precedes ubiquitination. Similarly, internalization of the uracil permease is also triggered by phosphorylation. In this case, a PEST like sequence is required for phosphorylation, ubiquitin-conjugation and internalization (Marchal et al. 1998). Also Ste3 carries such a PEST like sequence which is required for ubiquitin-conjugation and endocytosis. However, it is not required for phosphorylation of the receptor (Roth and Davis, 1996; Roth et al. 1998).

The experiments described above clearly demonstrate a function of ubiquitin-conjugation in endocytosis. However, it remained unclear how ubiquitin triggers internalization. It could either induce a conformational change in the target protein, which exposes a previously masked endocytosis signal, or ubiquitin itself may contain the signal for internalization. Recently, experiments have been published that strongly support the second hypothesis. Truncated versions of the Ste2 receptor lacking cytoplasmic sites for ubiquitin-conjugation are internalized when ubiquitin is fused to them. The SINNDAKSS element is not necessary for endocytosis of Ste2 chimera that contain ubiquitin moieties. If ubiquitin would indeed serve as a signal for endocytosis it should be transferable signal. This was tested with the plasma membrane proton ATPase, encoded by *PMA1*. This protein undergoes rapid internalization when fused to sequences of Ste6 or Ste3 that are needed for ubiquitination. In an alternative approach, Hicke and coworkers fused ubiquitin to Pma1 and observed degradation in the vacuole, which was dependent on endocytosis (Shih et al. 2000). Taken together, these observations indicate that ubiquitin itself carries signals for endocytosis. However, the signal could not be confined to a linear stretch of amino acids. Instead, the three-dimensional structure of ubiquitin seems to be important and especially the residues Ile44 and Phe4 are essential for endocytosis. Furthermore, it can be speculated that phosphorylation of the receptors is only required to trigger ubiquitin-conjugation (Shih et al. 2000).

4.3 Ubiquitin-Dependent Down-Regulation of Receptors in Mammalian Cells

Although ubiquitin-conjugation of receptors in mammalian cells has been observed, the function of this modification seems less clear. In the case of the growth hormone receptor (GH-receptor) and the epithelial Na+ channel (ENaC), it seems likely that ubiquitin-conjugation triggers their endocytosis.

For the GH-receptor, the most thoroughly studied example, Strous and co-workers have investigated ligand stimulated endocytosis of this receptor in a cell line expressing a temperature sensitive ubiquitin-activating enzyme. In these cells, internalization is impaired and the receptor accumulates at the cell surface in a non-ubiquitinated form, suggesting that endocytosis and ubiquitin-conjugation are related (Strous et al. 1996). Surprisingly, it was found that the receptor undergoes ligand stimulated ubiquitin-conjugation, but the lysine residues present in the cytoplasmic tail were not essential for ubiquitin-dependent internalization. Instead, a 10 amino acid motive lacking lysine residues was found to be necessary both for ubiquitin-conjugation and endocytosis (Govers et al. 1999). Moreover, inhibitors of the proteasome blocked both degradation and endocytosis of the wild type as well as the lysine-less variant (van Kerkhof et al. 2000). Thus, it was speculated that ubiquitin-conjugation and/or proteasomal removal of an unknown factor is required prior to endocytosis of the GH-receptor. Its own ubiquitin-conjugation might be a byproduct of the interaction with the conjugation machinery mediated by the 10 amino acid motive.

Another ubiquitin-conjugated protein of the plasma membrane is ENaC. Increased activity of this channel at the cell surface causes an inherited form of hypertension known as Liddle's syndrome. All identified mutations causing this syndrome are concentrated in a proline-rich motive of the cytoplasmic tails of the channel β and γ subunits. In the wild-type channel these motives mediate the interaction with Nedd4, the mammalian homologue of the ubiquitin ligase Rsp5 (Staub et al. 1996). Furthermore, it was demonstrated that ENaC is poly-ubiquitinated and that mutations in the cytoplasmic lysine residues reduce the degradation rates (Shimkets et al. 1997; Staub et al. 1997). Thus, the generation of this human disease seems to be directly linked to a disturbed interaction of the channel protein with the ubiquitin-conjugation machinery.

Protein degradation of ENaC, the GH-receptor, and a number of other proteins including the PDGF-receptor, the Met-receptor and p185c-erbB2 proto-oncogene is delayed upon treatment with inhibitors specific for the proteasome as well as for the lysosomal proteases (Hicke, 1999; Strous and Govers, 1999). This observation can be explained in two ways. First, it is possible that different fractions of these proteins are degraded through different proteolytic systems. Alternatively, the proteasome might attack parts of the proteins that are orientated towards the cytosol while those parts that are located to the lysosomal lumen are directly digested by the resident proteases. This is in contrast to observations with the yeast G-protein-coupled receptors, which seem to be degraded exclusively by vacuolar proteases (Hicke and Riezman, 1996; Roth and Davis, 1996). However, studies

with yeast Ste6 protein also pointed to an influence of the proteasome (Loayza and Michaelis, 1998).

Recent experiments have addressed the nature or the mammalian machinery involved in modifying proteins of the plasma membrane with ubiquitin. Yarden and co-workers have established an in vitro reconstituted system to ubiquitinate and degrade the EGF receptor (Levkowitz et al. 1999). It consists of purified ubiquitin-activating enzyme, ubiquitin-conjugating enzyme H5B or H5C and of a known factor, the c-Cbl protein. Previously, it has been shown that overexpression of c-Cbl increased the down-regulation of EGFR and PDGFR. C-Cbl is a protein of 120 kDa that contains an N-terminal SH2 domain and a C-terminal proline-rich region and several tyrosine phosphorylation sites. Between these region a C3HC4 type RING finger is found. Cbl-b and Cbl-3, the two other members of this protein family, also contain the RING finger but in the two oncogenic forms, v-Cbl and 70Z-Cbl it is corrupted. While all three members of the Cbl family are able to mediate ubiquitination of EGFR the oncogenic forms are not. Based on the in vitro observations a model was postulated how ubiquitin-conjugation of EGFR is regulated. Binding of EGF to the extracellular part of the receptor stimulates its kinase activity and results in phosphorylation of the receptor at Tyr-1045. This creates a binding site to which c-Cbl binds via its SH2 domain. Bound c-Cbl becomes phosphorylated at a site near the RING finger domain. Modified c-Cbl is now able to recruit the respective E2 enzyme to the complex, which in turn mediates the conjugation of the receptor with ubiquitin. Since RING finger containing proteins have been found as subunits of E3 complexes, it is feasible to speculate that also c-Cbl might function as part of an E3 complex. Thus, the c-Cbl mediated degradation of EGFR might represent the first example of a regulated ubiquitin-conjugating event in which both the substrate as well as the E3 complex are activated by tyrosine phosphorylation. The c-Cbl docking site in EGFR is found in a similar manner in Erb-B1 and Erb-B2. Both proteins undergo ubiquitination and lysosomal degradation. However, it is absent from Erb-B3, a receptor that is not modified with ubiquitin and constitutively shuttles between endosome and plasma membrane. Most interestingly, EGFR mutants that are unable to interact with c-Cbl and thus exhibit no ubiquitination or degradation, constantly shuttle between cell surface and an early endosomal compartment. In consequence, Yarden and co-workers speculated that ubiquitination plays a critical role in sorting of EGFR from the early endosomal compartment to the lysosomes rather than during endocytosis at the plasma membrane (Yarden et al. 1999).

5 Subcellular Distribution of Components of the Ubiquitin-Proteasome System

There has been rapid progress in understanding the involvement of the ubiquitin-proteasome system in the metabolism of eukaryotic cells, but some important questions on the cellular localization of the degradation machinery still remain. The general view for many years was that the activity of the ubiquitin-proteasome pathway is limited to the cytosol and to the nucleus. Despite lacking direct evidence for nuclear degradation pathways, this idea was supported by the occurrence of poly-ubiquitinated proteins in both compartments. However, some recent reports have demonstrated specific nuclear pathways and pointed to a linkage of ubiquitin-mediated protein degradation to nuclear transport functions. In this context, two complexes of questions are of special interest: First, in which cellular compartment do poly-ubiquitination and final breakdown of the target proteins actually occur? Is for a given nuclear protein the site of its function identical with the site of its ubiquitination and/or degradation? And, second: What are the relevant nucleo-cytoplasmic transport pathways and how are they linked to the ubiquitin-proteasome pathway to gain spatial control on the degradation machinery?

5.1 Ubiquitin-Activating Enzyme, Ubiquitin-Conjugating Enzymes and Ubiquitin-Ligases

In mammalian cells the E1 ubiquitin-activating enzyme exists in two isoforms, E1a (110 kDa) and E1b (117 kDa), which are derived from a single gene and mRNA (Cook and Chock 1992, Handley Gearhart et al. 1994). The isoform E1a is predominantly found in the nucleus and has been shown to harbor a functional nuclear localization sequence (NLS) required for nuclear targeting and phosphorylation. In contrast, E1b lacks the NLS, is not phosphorylated and localized in the cytoplasm (Handley-Gearhart et al. 1994; Stephen et al. 1997). Phosphorylation of E1a was demonstrated to occur in a cell cycle-dependent manner, being maximal in G2 phase (Stephen et al. 1996). Since the enzymatic activity of E1a was independent from increased phosphorylation the function of E1 phosphorylation remains unclear. From the three E1 homologs in the yeast *Saccharomyces cerevisiae* only Uba2p harbors a putative NLS. Uba2p is largely localized to the nucleus (Dohmen et al. 1995) and was shown to be essential for Smt3p-activation (Johnson et al. 1997).

Although E2s and E3s appear to be generally present in the nucleus and in the cytoplasm (Schwartz and Ciechanover, 1999), this finding does not

exclude that the activity of individual E2 and E3 enzymes may be restricted to a certain site. With the exception of the membrane-bound E2s (see below), there is presently no experimental evidence for a functional targeting sequence within a single E2 or E3 that could direct the protein to a distinct subcellular compartment. However, in yeast, immunochemical localization of the gene product and biochemical data have shown that the E2 Ubc3/Cdc34 is a predominantly nuclear protein (Goebl et al. 1994). This finding matches the observation that relevant substrates of Ubc3/Cdc34 reside within the nucleus. Several cell cycle events have been shown to be under control of Ubc3/Cdc34 (Deshaies et al. 1995; Plon et al. 1993; Goebl et al. 1988). Furthermore this E2 regulates the function of the transcripition factors Gcn4p and Rgt1p (Kornitzer et al 1994; Li and Johnston, 1997). In mammalian cells, transfected human Cdc34 has been proposed to serve a function analogous to its yeast counterpart (Pagano et al. 1995; Plon et al. 1993). Human Cdc34 represents a nuclear protein and is suggested to mediate the ubiquitination of the transcripton factor MyoD (Lisztwan et al. 1998; Song et al. 1998). Yeast Ubc4 and Ubc5 are required for the degradation of many abnormal and short-lived proteins including nuclear mitotic cyclins and the Matα2 repressor protein (Hochstrasser et al. 1999). Thus, at least in the latter cases both enzymes should have enzymatic activity on target proteins in the nuclear compartment.

In yeast cells, the E2 Ubc6 is membrane-bound at the ER (Sommer and Jentsch, 1993) and is assumed to be involved in ubiquitination processes at the cytosolic face of the ER. The soluble ubiquitin-conjugating enzyme Ubc7 has to be recruited to the ER surface by its receptor, Cue1 (Biederer et al. 1997), to catalyze ubiquitination and finally proteolysis of both ER proteins and soluble non-ER proteins (see chapter ER degradation). In addition, Ubc7p was also proposed to interact physically with Ubc6p forming a membrane-associated heteromeric complex (Chen et al. 1993). Ubc8 is located in the cytoplasm and one of its function has been shown to reside in catabolite degradation of the gluconeogenic enzyme fructose-1.6-bixphosphatase (Schüle et al. 2000). Ubc9 conjugates the ubiquitin-like proteins SUMO-1/Smt3 (Johnson and Blobel, 1997; Schwarz et al. 1998). From current data it seems to be predominantly nuclear.

Some ubiquitin ligase activities are coupled with large-multisubunit protein complexes. The substrates of these E3 complexes represent a broad spectrum of proteins that participate in a variety of cellular functions, e.g. regulation of CDK activity, activation of transcription, signal transduction, assembly of kinetochores, and DNA replication. Concerning those substrates relevant for the cell cycle the critical issue is how and when these proteins are ubiquitinated. Studies of cell cycle regulation have demon-

strated that two E3 complexes play a crucial role for the timing of cell cycle regulator proteolysis: the cyclosome/anaphase-promoting complex (APC) and the Skp1-cullin-F-box protein ligase complex (SCF) (reviewed in Deshaies, 1999; Koepp et al. 1999, Winston et al. 1999). Regulatory proteins of the cell cycle fulfill their function mainly in the nucleus, but at present experimental evidence for specific nuclear pathways of ubiquitin-mediated degradation is lacking. However, in *Saccharomyces cerevisiae*, the feedback-regulated degradation of the transcriptional activator Met4 has been shown to be triggered by the SCF^{Met30} ubiquitin-protein ligase. The F-box protein Met30p itself is short-lived and localizes to the nucleus (Rouillon et al. 2000), also indicating a nuclear localization of the whole complex. From this example we can assume a defined cellular distribution of the E3 complexes and, consequently, a spatial regulation of their activity.

5.2 Proteasomes

As discussed in the previous chapter there is some evidence both for a defined spatial arrangement of the ubiquitin-conjugating machinery and for the compartment-specific activity of several components. For understanding of ubiquitin-proteasome-mediated proteolysis a further fundamental issue is the spatial relationship within the cell between the protease itself and its substrates. Thus the questions arise whether active proteasomes are distributed homogeneously throughout the cell or if there exist defined cellular sites of proteasome-dependent degradation? In general, studies of proteasomal distribution revealed differences in the localization of proteasome subpopulations, demonstrated the functional importance of endoplasmic reticulum-associated proteasomes, and investigated the role of the putative nuclear localization signals on proteasome transport into the nucleus (reviewed in Rivett, 1998). Some still existing disagreement on the details could be explained by a variation with cell types, cell cycle stage and development. For example, a cell-cycle-dependent change in the distribution of nuclear proteasomes was observed in higher eukaryotes, with a co-localization of the proteasome with chromosomes and the spindle during metaphase (Kawahara and Yokosawa, 1992).

In mammalian cells, previous reports proved the localization of proteasomes both in the cytoplasm and in the nucleus (Palmer et al. 1996; Peters et al. 1994). In a more recent study GFP-tagged 20 S proteasomes in fibrosarcoma cells were shown to be dispersed over the cytoplasm and nucleoplasm wherein they seem to diffuse rapidly (Reits et al. 1997). Reits et al. used a fusion protein of GFP with the proteasome subunit LMP2, which replaces a β subunit of the proteasome upon induction with interferon γ, a stimulator of

MHC class I presentation (Belich et al. 1994; Nandi et al. 1996). The authors also demonstrated that proteasomes were transported slowly and unidirectionally from the cytoplasm to the nucleus, but can enter the nucleus rapidly upon its re-assembly during cell division. Several other studies also provided evidence for a preferential localization to nuclear substructures (Grossi de Sa et al. 1988, Pal et al. 1988). Putative nuclear localization signals (NLS) that have been found in some α subunits of the 20S proteasomal core complex (Tanaka et al. 1990), might function in targeting proteasomes into the nucleus, which was shown for single proteasome subunits in vitro (Knuehl et al. 1996; Wang et al. 1997). A distinct fraction of proteasomes was also reported to be associated with the cytoplasmic face of the ER membrane (Rivett et al. 1997; Rivett et al. 1992; Yang et al. 1995). There it is thought to degrade mutant proteins extracted from the ER (see chapter ER degradation) or generate antigenic peptides for presentation on MHC class I molecules (Coux et al. 1996; Lehner and Cresswell, 1996; Schild and Rammensee, 2000; Schubert et al. 2000; Reits et al. 2000).

Recently, nuclear and perinuclear substructures were suggested as sites of concentration of proteasomes. Johnston et al. (1998) reported the formation of a novel pericentriolar structure, termed "aggresome". In transfected human embryonic kidney (HEK293) or Chinese hamster ovary cells overexpression of the integral membrane protein CFTR, which is inefficiently folded and exported from the ER, or inhibition of proteasome activity resulted in the accumulation of stable, ubiquitinated aggregates of CFTR. Once formed, these aggregates are delivered to an ubiquitin-rich structure at the centrosome/microtubule-organizing center (MTOC). The microtubule depolymerizing drug nocodazole prevents the perinuclear formation of aggresomes and causes the formation of smaller protein inclusions dispersed in the periphery of the cells, indicating that protein aggregates move on microtubles to the MTOC to form the aggresome (Garcia Mata et al. 1999; Johnston et al. 1998). Further investigations on the generality and dynamics of aggresome formation suggested that aggresomes can be formed by soluble, non-ubiquitinated proteins as well as by integral transmembrane ubiquitinated ones. In conclusion, aggresomes are proposed as a general response of the cell to the presence of misfolded and aggregated proteins (Garcia Mata et al. 1999). Regarding the formation of protein aggregates within the cell it is noteworthy that neuronal intranuclear inclusions (NIs) were described as a ultrastructural feature of several neurodegenerative polyglutamine repeat expansion disorders, including Huntington's disease and the spinocerebellar ataxias (SCAs). NIs show high ubiquitin content and colocalization with proteasomes. Recent work showed that these inclusions are not required for expanded polyglutamine pathology (reviewed in Floyd and Hamilton 1999).

Cummings et al. (1999) observed that mutant ataxin-1, the expanded protein causing SCA type 1, is selectively resistant to degradation though it is equally well poly-ubiquitinated as the normal protein. Inhibiting proteasome activity promotes mutant ataxin-1 aggregation in transfected cells indicating that ataxin-1 is degraded by the ubiquitin-proteasome pathway.

To study proteasomal degradation of nuclear proteins and viral antigens in human osteosarcoma cells Anton et al. (1999) used a mutated form of influenza virus nucleoprotein (dNP) as a model protein with a nuclear localization sequence that is ubiquitinated and degraded by proteasomes. Immunoflourescence and biochemical analysis revealed that in the presence of proteasome inhibitors dNP accumulates in highly insoluble ubiquitinated and non-ubiquitinated species in nuclear substructures known as promyelocytic leukemia oncogenic domains (POD) and at the centrosome/MTOC. The authors could show by immunoflourescence that dNP recruits proteasomes and the chaperone HSC70 to both sites. Moreover, after restoring proteasome activity while blocking protein synthesis dNP disappeared from PODs and the MTOC resulting in the generation of MHC class I-bound peptide derived from dNP. These findings suggest PODs and MTOC as sites of proteasomal degradation of misfolded NP and probably other cellular proteins and indicate that antigenic peptides are generated at these sites.

By immunoflourescence and cell fractionation Wigley and co-workers (Wigley et al. 1999; Fabunmi et al. 2000) have shown that active proteasomal complexes degrading ubiquitinated protein and proteasome-specific peptide substrates associate with centrosomes in HEK 293 and HeLa cells. The structure is perinuclear, surrounded by endoplasmic reticulum, adjacent to the Golgi and co-localizes with the centrosomal marker γ-tubulin. Besides misfolded CFTR (Johnston et al. 1998; Wigley et al. 1999), by immunocytochemistry a number of other proteasome substrates have been localized to the centrosome, including the tumor suppressor p53 (Brown et al. 1994), cyclins (Koepp et al. 1999), presenilin 1 (Johnston et al. 1998), and IκB (Crepieux et al. 1997). Moreover, the substrates cyclins A, B1, and E are associated with the centrosome in a cell cycle dependent manner (Bailly et al. 1992, Lacey et al. 1999, Hinchcliffe et al. 1999). Thus, the centrosomal co-localization of active proteasomes with multiple physiological substrates not only suggested the centrosome/MTOC as a novel site for proteasome function, but also provided evidence that proteasome-catalyzed proteolysis may be regulated by its selective partitioning to this structure.

The cellular distribution of 26S proteasome subunits in yeast appears to be similar as in higher eukaryotes but there also exist some differences in the details. Indirect immunoflourescence studies localized the GFP-tagged yeast 26S proteasome primarily to the yeast nucleus and the nuclear periph-

ery throughout the cell cycle (Enenkel et al. 1999; Enenkel et al. 1998; McDonald and Byers, 1997; Russell et al. 1999; Wilkinson et al. 1998). Co-immunolocalization studies revealed overlapping localizations of proteasomes and marker proteins of either ER translocon or nuclear pore components (Enenkel et al. 1998; Wilkinson et al. 1998). In living cells of *Schizosaccharomyces pombe*, GFP-tagged 26S proteasomes were found predominantly at the nuclear periphery both in interphase and throughout mitosis. However, a dramatic change in the localization was observed during meiosis (Wilkinson et al. 1998). Surprisingly, in fission yeast by electronmicroscopy the proteasome was shown to be concentrated on the inner side of the nuclear membrane (Wilkinson et al. 1998). Together with biochemical fractionation experiments the localization data gave evidence that the majority of yeast proteasomes (about 80%) is structurally bound to the contiguous network of nuclear envelope (NE) and ER membranes (Enenkel et al. 1999). These results were also supported by immunoflourescence data provided by (Russell et al. 1999) who estimated that the fraction of cytoplasmic proteasomes represents at most 20% of the total proteasome population. These findings raise not only questions relating to the function of proteasomes at the NE and their targeting to this compartment but also about the cytoplasmic activity of proteasomes. It is possible that the small cytoplasmic fraction of proteasomes is sufficient to degrade cytoplasmic substrates. This is consistent with the observation that yeast cells continue to grow despite inhibiting of 70–80% of the proteasome activity (Lee and Goldberg, 1997), suggesting that proteasomes should not be rate limiting for degradation. Otherwise multi-ubiquitinated proteins in the cytoplasm are likely to be transported to the NE/rough ER or the nucleus for degradation by the proteasome. Additionally, assuming that most short-lived substrates of the proteasome in yeast are cellular regulators functioning primarily in the nucleus, these proteins have to be directed to the NE/rough ER before degradation. This not only implies that the ubiquitin-conjugation machinery has to be active in the nucleus but also that nucleo-cytoplasmic trafficking is needed for transport of the nuclear substrates to the site of degradation.

In summary, proteasomes are generally found at subcellular sites together with the vast majority of substrates. The intracellular distribution of proteasomes has been characterized as flexible but the sites of proteasomal concentration are strictly associated with proteolytic activity. This might be due to a guiding process for all degradation components resulting in the formation of large units which could be necessary to facilitate the controlling of the whole proteolytic machinery. Indeed, there is growing evidence for a defined spatial regulation of proteasome-catalyzed proteolysis in yeast and in higher eukaryotic cells. Thus, subcellular distribution, a common mecha-

nism for the regulation of enzymatic systems, appears to be an important feature of regulation of proteasomal proteolysis. However, up to now it is as yet poorly understood what determines the recruitment of proteasomes to different cellular compartments like the NE/rough ER, and if the translocation process represents an active mechanism or simply occurs due to diffusion. The latter possibility is probably unlikely because molecules of the size of the 26S proteasome are thought to be too large to simply diffuse through the cytosol (Janson et al. 1996).

5.3 A Link Between Nucleo-Cytoplasmic Transport and Degradation

A fundamental problem in understanding ubiquitin/proteasome-mediated degradation of regulatory proteins is how individual substrates are selectively targeted at a given time. In principle, protein degradation can be timed by regulating the interaction between the target and components of the ubiquitin system that recognize specific degradation signals or other modifications. The phosphorylation status of the target is suggested to play an important role in selective substrate recognition. Indeed, proteins whose activity is required only at certain stages of the cell cycle are often phosphorylated, modified by covalent attachment of ubiquitin molecules and finally digested by the proteasome (Koepp et al. 1999; Hershko and Ciechanover, 1998). However, some recent reports indicated that phosphorylation followed by polyubiquitination is not sufficient to degrade cell cycle regulatory proteins. Additionally, regulated nuclear import or export has been shown to be involved in the timing of substrate turnover. Considering the preferential localization of proteasomes to the nuclear periphery (see chapter above) the connection between nuclear transport functions and proteasomal degradation is not surprising. With respect to a probable nucleus-specific ubiquitin-proteasome pathway the role of nuclear transport processes in the targeting of substrates and in the selective partitioning of proteasomes is of special interest.

Regulated transport of proteins between the nucleus and the cytosol is determined by their ability to interact with specific nuclear import and export factors. Nuclear import and export proceed through nuclear pore complexes (NPC) (Pante and Aebi, 1996; Doye and Hurt, 1997; Fabre and Hurt, 1997), and can occur via a great number of distinct pathways (reviewed in Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). The selective transport of proteins requires energy and depends on targeting signals within the amino acid sequence. Signal-mediated nuclear import is a three-step process: docking at the NPC, translocation, and nuclear deposition of the cargo. In the docking step the cargo in the cytoplasm binds to a

soluble import receptor (importin). The cargo-importin complex is targeted to the NPC and translocated to the other side. Upon entering the nucleus the cargo is released and the importin is returned to the cytoplasm for another round of transport. Protein export shares mechanistic similarities with protein import. In the nucleus export receptors (exportins) are able to recognize and bind cargos bearing a nuclear export signal (NES). The complex is then rapidly translocated to the cytoplasm, where it dissociates.

By regulating the transport of proteins into and out of the nucleus eukaryotic cells manage numerous biological processes. Several modes of such regulated nuclear transport had been shown to control cell cycle progression and signalling pathways (reviewed in Kaffman and O'Shea, 1999; Hood and Silver, 1999; Görner et al. 1999; Hopper, 1999). The phosphorylation of the cargo represents a common mechanism regulating the ability of the cargo to interact with specific import or export receptors. Examples for this model of regulation are the yeast transcription factor Pho4 (Kaffman et al. 1998), and the mitotic cyclin B (Hagting et al. 1998, Hagting et al. 1999; Toyoshima et al. 1998; Yang et al. 1998). Cargo-receptor complex formation could also be regulated by intermolecular association of the cargo with itself, with RNA or with other proteins. Other mechanisms of regulated localization are cytoplasmic anchoring, the regulation of the soluble transport machinery and the regulation of the NPC (reviewed in Kaffman and O'Shea, 1999).

Recent studies on cell cycle regulatory proteins have indicated a spatial control of short-lived regulators. For example, Yap1p (Yan et al. 1998), Far1p (Blondel et al. 1999), Cdc6p (Petersen et al. 1999) and Cdc25p (Lopez-Girona et al. 1999) were shown to be down-regulated by specific export from the nucleus. Other reports not only underline the importance of regulated nuclear import and export for controlling the compartment-specific level of regulatory proteins, but also point to the fact that the degradation kinetics of a regulator might be different in the cytosol and the nucleus. First clue for a connection between regulated nucleo-cytoplasmic transport and ubiqitin-mediated degradation came from Loeb et al. (1995) who reported on nuclear transport mutants that could not degrade mitotic cyclins. They showed that the function of Srp1, a yeast importin-α ortholog, is required for the execution of mitosis and suggested that the import of cell cycle regulators into the nucleus is critical for cell cycle progression. In mammalian cells, the down-regulation of two members of the family of basic helix-loop-helix/Per-ARNT-Sim proteins was demonstrated to be ubiquitin-proteasome mediated and to depend on nuclear import and export functions (Davarinos and Pollenz, 1999; Roberts and Whitelaw, 1999). Following exposure to ligands both the aryl hydrocarbon receptor (AHR) and the dioxin receptor (DR) were imported into the nucleus triggering them for phosphorylation and subse-

quent rapid degradation via the ubiquitin-proteasome pathway. Inhibition of proteasomal function led to nuclear accumulation of both proteins. Stabilization of a constitutively nuclear short-lived form of the DR (DRNLS) was observed when it was co-expressed with the ubiquitin-mutant UbK48R (Roberts and Whitelaw, 1999). In addition, the mutation of a putative CRM1-specific NES present in the AHR or inhibition of CRM1-mediated export by Leptomycin B (Davarinos and Pollenz, 1999) resulted in reduced degradation level and nuclear accumulation. Thus, Davarinos and Pollenz (1999) concluded that ligand bound AHR is degraded in the cytoplasm via the proteasome after being exported from the nuclear compartment. Several other reports have also linked nuclear export with regulated proteolysis. Diehl et al. (1998) reported that ubiquitin-dependent degradation of the cell-regulatory protein cyclin D correlates with its nuclear export. Cyclin D1 accumulates in the nucleus during G1 phase, but it is redistributed into the cytoplasm as cells move through S phase. The authors demonstrated that the specific phosphorylation of cyclin D1 by the glycogen synthase kinase-3β (GSK-3β) not only triggers rapid cyclin D1 turnover by the ubiquitin-proteasome pathway, but also enforces the cytoplasmic localization of cyclin D1. Cyclin D1 does not contain nuclear import or export signals. Thus, Diehl et al. (1998) speculate that GSK-3β-mediated phosphorylation of nuclear cyclin D1 may result in its export by facilitating the interaction with an exportin. Furthermore, the results suggest that GSK-3β actively shuttles between nucleus and cytoplasm and that its observed cell cycle dependent partitioning to the nuclear compartment may be necessary for the relocalization of cyclin D1.

The cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the p53 tumor suppressor were also shown to interact with nuclear export mediating factors, which appears to be a requirement for these proteins to be degraded (reviewed in Lain 1999b). Phosphorylated $p27^{Kip1}$ is recognized by an SCF-type E3 ligase, which promotes ubiquitination and degradation (Amati and Vlach 1999; Carrano et al. 1999; Sutterlüty et al. 1999; Tsvetkov et al. 1999). Moreover, Tomoda and co-workers (Tomoda et al. 1999) found that phosphorylated $p27^{Kip1}$ binds to a novel protein, Jab1, which directs movement of $p27^{Kip1}$ from the nucleus to the cytoplasm. This Jab1-dependent relocalization is necessary for proteolysis because a mutant $p27^{Kip1}$, which is unable to bind to Jab1, is neither exported nor degraded. This was also supported by the observation that nucleo-cytoplasmic transportation per se is not sufficient for degradation of $p27^{Kip1}$, as the fusion of $p27^{Kip1}$with a heterologous NES led to efficient export to the cytosol but not to accelerated degradation. Impairing proteasomal function by specific inhibitors prevented not only the breakdown of $p27^{Kip1}$ but also its movement from the

nucleus to the cytoplasm. An explanation could be that the proteasome might degrade either a factor interfering specifically with the export of $p27^{Kip1}$ or a more general inhibitor of export. The exact function of Jab1 in $p27^{Kip1}$ degradation remains to be elucidated. It may simply serve as a shuttle for $p27^{Kip1}$, or probably Jab1 itself physically interacts with components of the ubiquitin/proteasome system (reviewed in Scheffner, 1999). The latter possibility seems quite attractive, especially when keeping in mind that Jab1 shows sequence similarity to the yeast protein Rpn11p/Mpr1p, which represents a subunit of the 19S regulatory particle of the proteasome (Lenk et al. unpublished)

The proto-oncoprotein Mdm2 (Freedman et al. 1999) was recently shown to harbor an E3 ubiquitin ligase activity, that directly transfers ubiquitin onto the p53 tumor suppressor protein (Honda et al. 1997). Export of Mdm2 from the nucleus or at least the interaction of Mdm2 with the export machinery is required for the ubiquitin/proteasome dependent degradation of the p53 (Lain et al. 1999a; Freedman and Levine, 1998; Roth et al. 1998; Stommel et al. 1999). Moreover, not only nuclear export but also import of Mdm2 is needed to promote p53 export and degradation, because a mutant Mdm2 protein deficient for nuclear import was unable to reduce p53 levels in transfected cells (Tao and Levine, 1999). Blockage of nuclear export results in accumulation of p53-Mdm2 complexes in subnuclear domains adjacent to the PODs (Lain et al. 1999a), which have been shown to function as sites of proteasomal activity (see above).

Going into some details of these studies there are arising some questions concerning the spatial organization of the ubiquitin-proteasome machinery. The connection of nuclear export and ubiquitin-dependent degradation indicates that proteolysis of the reported substrates itself is a strictly cytoplasmic event. This might be due to the cytoplasmic localization of active proteasomes, of components of the ubiquitin-conjugation system or of factors sensitizing the substrate protein for ubiquitination and/or degradation. However, in response to treatment with specific proteasome inhibitors both p53 and $p27^{Kip1}$ accumulate primarily in the nucleus instead of in the cytoplasm (Freedman and Levine, 1998; Lain et al. 1999a; Smart et al. 1999; Tomoda et al. 1999). These observations can be explained by the assumption, that the exported protein, which is not degraded due to inhibition of cytoplasmic proteasomes, may be rapidly re-imported and therefore accumulate preferentially in the nucleus. However, since proteasomes seem to be present both in the cytoplasm and in nuclear compartments, why degradation of substrates should only occur in the cytoplasm? Scheffner (1999) proposed for $p27^{Kip1}$-breakdown that the export of the substrate is controlled in a proteasome-dependent manner and that ubiquitination and degradation

take place in the cytoplasm. However, up to now there exists no experimental evidence for a regulation of nuclear export by the proteasome. Alternatively, breakdown of substrate proteins may not proceed equally distributed over the cytoplasm but rather within the subnuclear or nucleus-associated structures where active proteasomes have been shown to accumulate (see above). In this case, the nuclear substrates must be targeted by nuclear export factors, which facilitate substrate translocation to these proteolytic centers.

Regarding the localization of the majority of proteasomes to the nuclear rim and nuclear envelope, it seems likely, that proteasomal degradation requires nuclear export or translocation processes, which directs nuclear proteins at least to the nuclear pore or to the inner surface of the nucleus. This implies that all components of the ubiquitination machinery have to be active inside the nucleus, which seems to be a prerequisite for the specific export process. It is also possible that nucleus-specific kinases or E2/E3 enzymes promote the triggering of nuclear proteins for rapid degradation.

In summary, the principle, that nuclear proteins might have to be exported for ubiquitin-dependent degradation, seems to act as a common mechanism for controlling the level of short-lived proteins (reviewed in Pines, 1999; Scheffner, 1999; Lain et al. 1999b). With respect to our knowledge so far about the cellular localization of proteasomes and parts of the ubiquitin-conjugation system there remain some still open questions concerning the mechanistic link of nuclear export and degradation via the ubiquitin-proteasome pathway.

5.4 Ubiquitin-Like Proteins

In contrast to ubiquitin, the covalent attachment of ubiquitin-like molecules (Ubls) to other proteins seems to be more important for post-translational protein modification than for protein degradation. There is not much known yet about the subcellular distribution of these proteins but since they are frequently involved in targeting functions it can be assumed that they are also subjected to a spatial organization.

It is known from the interferon-inducible ubiquitin cross-reactive protein (UCRP) that it is conjugated to a small subset of intracellular proteins probably targeting them to the cytoskeleton (Loeb and Haas, 1994).

The function of SUMO conjugation is only beginning to be understood. So far, available data suggest that SUMO-1 is likely to play a role in either targeting modified proteins and/or inhibiting their degradation. This is also supported by the observation that yeast cells deficient in SUMO/Smt3 conjugation are inviable and arrest in the cell cycle before anaphase (Seufert et

al. 1995; Li and Hochstrasser, 1999). SUMO-1 conjugation covalently modifies a number of target proteins, including the inflammatory response regulatory protein IkB_ (Desterro et al. 1998), the acute promyelocytic leukemia protein PML (Sternsdorf et al. 1997; Kamitani et al. 1998; Muller et al. 1998), the p53 tumor suppressor (Gostissa et al. 1999), and the GTPase activating protein RanGAP1 (Mahajan et al. 1997; Matunis et al. 1996). SUMO-modified RanGAP1 is recruited to the cytoplasmic face of the nuclear pore where it tightly associates with the nucleoporin RanBP2 (Majahan et al. 1997; Saitoh et al. 1997; Mahajan et al. 1998; Matunis et al. 1998). Thus, the attachment of SUMO-1 might have a targeting function for RanGAP1, which is important for the function of RanGAP1 in nuclear transport. Ran GAP1, together with the Ran GDP/GTP exchange factor, RanGEF, regulates the GTPase Ran, which switches between a GDP- and a GTP-bound form and is required for both nuclear import and export. RanGAP1 and RanGEF are localized to the opposite sides of the nuclear envelope. The asymmetric distribution of both enzymes results in a RanGTP gradient across the nuclear envelope with a high RanGTP concentration in the nucleus and low levels in the cytoplasm (Görlich et al. 1996; Izaurralde et al. 1997). The Ran system likely defines the direction of transport, while the specificity of nuclear import and export is achieved by interaction of signal sequences with importins and exportins. Modification of RanGAP1 by SUMO-1 requires the Ubc9 homologue in Xenopus eggs (Saitoh et al. 1997) and in human cells (Lee et al. 1998). Accordingly, the human homologue of Ubc9 has been shown to co-localize with RanGAP at the nuclear envelope (Lee et al. 1998). Besides its perinuclear localization SUMO-1 also has been shown to be nuclear-diffuse as well as nuclear-punctate, with SUMO-1 concentrated foci corresponding to PML nuclear bodies (Boddy et al. 1996; Duprez et al. 1999; Matunis et al. 1996). Interestingly, in mammalian cells, accumulation of proteasomal substrates together with active proteasomes was observed adjacent to PML oncogenic domains (POD) (Anton et al. 1999; Lain et al. 1999a). Since both the SUMO/Smt3 activating E1 enzyme, Uba2, and the SUMO/Smt3 conjugating E2 enzyme, Ubc9, were localized to the nucleus (see above), covalent modification by SUMO/Smt3 seems to represent a specific modification for nuclear targets. However, in the yeast S.cerevisiae, during distinct phases of mitosis the SUMO homologue Smt3p also has been found specifically attached to septins, which are components of filaments encircling the yeast bud neck (Johnson and Blobel, 1999).

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Transgenic Models of α_2-Adrenergic Receptor Subtype Function

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Abbreviations

α_{2A}-KO, α_{2A}-adrenergic receptor knockout; α_{2A}-D79N, aspartic acid 79 to asparagine mutation of the α_{2A}-adrenergic receptor; ERK, extracellular signal-regulated kinase; G_i, inhibitory G protein; GRK, G protein-coupled receptor kinase; G_s, stimulatory G protein; MAPK, mitogen-activated protein kinase; N_2O, nitrous oxide; PC12 cells, rat pheochromocytoma cell line; WT, wild-type

1 Introduction

Adrenergic receptors are located throughout the body on neuronal and non-neuronal cells where they mediate a diverse range of responses to the endogenous catecholamines adrenaline and noradrenaline. To date, nine

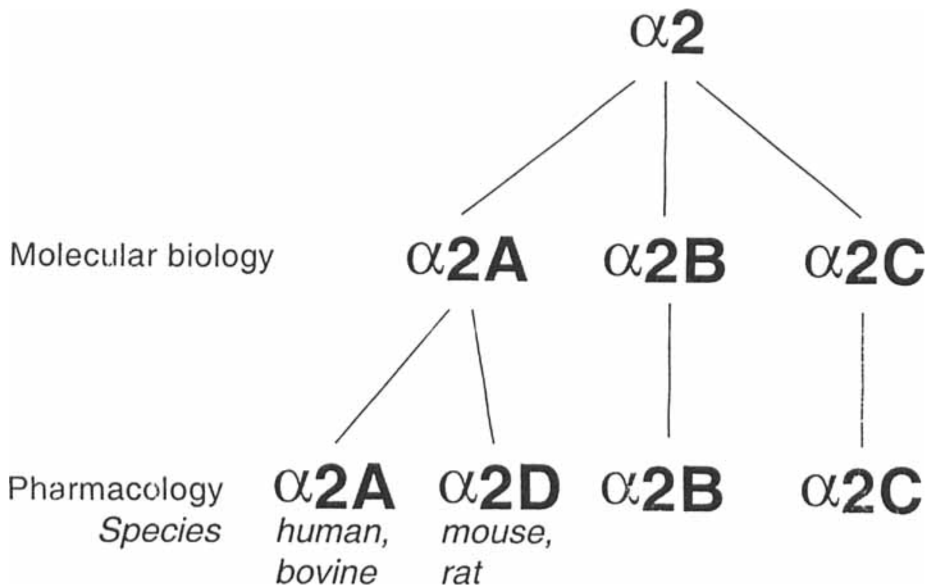

Fig. 1. Overview of α_2-adrenergic receptor subtype nomenclature. In many species, three genes encoding for distinct α_2-adrenergic receptor subtypes have been identified. These receptors are termed α_{2A}, α_{2B}, and α_{2C}. Pharmacological experiments with subtype-preferring antagonists have led to a further subdivision into α_{2A}- and α_{2D}-receptors (Bylund et al. 1994). These two receptors are species variants of the same gene, with the pharmacological α_{2A}-subtype being expressed in human and bovine tissues and the α_{2D}-subtype in mouse and rat. Point mutation of a serine residue in the fifth transmembrane domain of the α_{2A}-receptor to alanin confers decreased affinity to rauwolscine and yohimbine and thus constitutes α_{2D}-pharmacology (Link et al. 1992)

adrenergic receptors have been cloned. Based on pharmacological and molecular biology nomenclature, the adrenergic receptor family can be subdivided into three α_1-receptors (α_{1A}, α_{1B}, α_{1D}), three α_2-receptors (α_{2A}, α_{2B}, α_{2C}) and three β-adrenergic receptors (β_1, β_2, β_3). Physiological functions of α_1- and β- receptor subtypes have been reviewed extensively elsewhere (Docherty 1998; Lowell 1998; Rohrer 1998; Rohrer and Kobilka 1998; Dzimiri 1999; Liggett 1999; Rohrer, 2000). This review focusses on the significance of α_2-receptor subtype diversity.

1.1 α_2-Receptor Subtype Nomenclature

Three genes encoding for α_2-adrenergic receptor subtypes have been identified from several species, termed α_{2A}, α_{2B}, and α_{2C}, respectively (Fig. 1, 2).

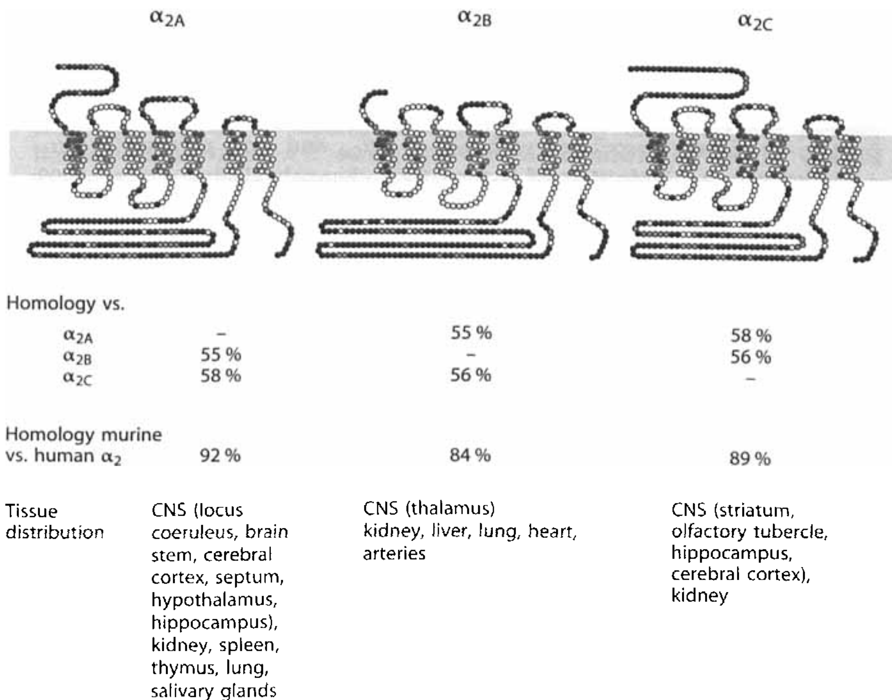

Fig. 2. α_2-Adrenergic receptor subtypes. The putative membrane topology of the three mouse α_2-receptor subtypes is schematically depicted. Open circles represent amino acids which are identical between all subtypes, gray circles represent amino acids which are conserved among two subtypes, dark circles are non-identical amino acids. Tissue distribution of α_2-receptor subtypes is based on mRNA and protein expression; references are given in the text

The pharmacological profile of the α_{2A}-subtype differs significantly between species, thus giving rise to the pharmacological subtypes α_{2A} in humans, rabbits and pigs and α_{2D} in rats, mice and guinea pigs (Bylund et al. 1994). Part of the pharmacological difference between α_{2A}- and α_{2D}-receptors can be explained by a Ser-Ala mutation in the fifth transmembrane helix of the α_{2A}-receptor rendering this receptor less sensitive to rauwolscine and yohimbine binding (Link et al. 1992). For the purpose of this review, the genetic nomenclature of α_{2A}, α_{2B}, and α_{2C} receptor subtypes will be used.

1.2 Intracellular Signal Transduction

α_2-Adrenergic receptors can regulate a wide range of signalling pathways via interaction with multiple heterotrimeric G_i proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$) including inhibition of adenylyl cyclase (Cotecchia et al. 1990; Wise et al. 1997), stimulation of phospholipase D (MacNulty et al. 1992), stimulation of ERK/mitogen-activated protein kinases (Alblas et al. 1993; Anderson and Milligan 1994; Koch et al. 1994), stimulation of K^+ currents (Surprenant et al. 1992) and inhibition of Ca^{2+} currents (Surprenant et al. 1992). In neurons, G protein $\beta\gamma$-dimers associated with $G\alpha_{OA}$, $G\alpha_{OB}$ and $G\alpha_{i2}$ mediate the α_2-receptor-induced inhibition of N-type Ca^{2+} channels (Delmas et al. 1999; Jeong and Ikeda 2000). In addition to N-type Ca^{2+} channels, neuronal α_2-receptors inhibit voltage-gated P- and Q-type Ca^{2+} channels (Waterman 1997). Furthermore, at least with high receptor expression levels and high receptor occupancy, effector regulation via activation of other G proteins can be uncovered (Eason et al. 1992; Pepperl and Regan 1993; Eason et al. 1994; Eason and Liggett 1995; Pierce et al. 2000).

In the α_{2A}-receptor, mutation of an aspartic acid residue (Asp^{79}) which is highly conserved among G protein-coupled receptors to asparagine (D79N) led to selective uncoupling of the α_{2A}-D79N receptor from K^+ channel activation in AtT20 cells without an alteration in adenylyl cyclase inhibition or Ca^{2+} current inhibition (Fig. 3) (Surprenant et al. 1992). This aspartate is conserved among all G protein-coupled receptors and mutation of this aspartate to asparagine reduces agonist affinity (Strader et al. 1987; Chung et al. 1988; Fraser et al. 1989; Wang et al. 1991) and prevents the modulation of agonist binding by cations (Guyer et al. 1990) and nonhydrolyzable guanosine triphosphate analogs (Chung et al. 1988). Recombinant fusions of the α_{2A}-D79N receptor with pertussis toxin-resistant forms of the G proteins $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ have demonstrated that point mutation of the aspartic acid residue non-selectively disrupted the capacity of the α_{2A}-receptor to activate these closely related G proteins (Ward and Milligan 1999). Recent studies have also demonstrated that the α_{2A}-D79N mutation is conforma-

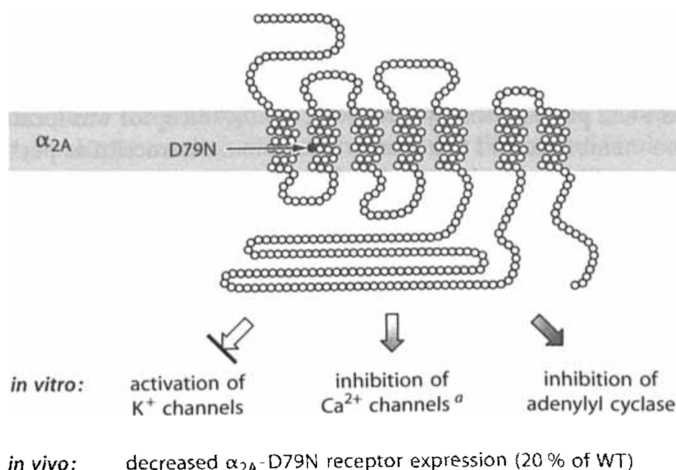

Fig. 3. Signal transduction of the α_{2A}-D79N adrenergic receptor. Mutation of the aspartic acid residue 79 in the second transmembrane domain of the mouse α_{2A}-adrenergic receptor to asparagine (D79N) selectively uncoupled this receptor mutant from activation of K^+ currents in vitro without interfering with Ca^{2+} channel or adenylyl cyclase inhibition (Surprenant et al. 1992). In vivo, the α_{2A}-D79N receptor was expressed at 20% of the level of the wild-type α_{2A}-receptor (MacMillan et al. 1996), and Ca^{2+} channel inhibition was also blunted (*a*) (Lakhlani et al. 1997)

tionally unstable and readily turns over on the cell surface (Wilson and Limbird, 2000). The observed phenotype of the α_{2A}-D79N mutation may thus be due to differential levels of signal amplification required to produce K^+ channel activation, Ca^{2+} channel and adenylyl cyclase inhibition in different cell types or due to differences in G protein expression levels or localization and/or to intrinsic differences in receptor stability and turnover.

Upon stimulation with agonist, α_{2A}-adrenergic receptors undergo subtype-specific, short-term desensitization (Eason and Liggett 1992; Kurose and Lefkowitz 1994). Desensitization of α_{2A}- and α_{2B}-receptors is mediated by phosphorylation of intracellular receptor domains by specific G protein-coupled receptor kinases type 2 and 3 (GRK2 and GRK3; Jewell-Motz and Liggett 1996; Wu et al. 1997). No phosphorylation of the α_{2C}-subtype by GRKs could be detected (Eason and Liggett 1992; Jewell-Motz and Liggett 1996). In neurons, inhibition of voltage-dependent Ca^{2+} channels mediated by α_2-adrenergic receptors desensitizes slowly with prolonged exposure of transmitter and desensitization is mediated by GRK3 kinase (Diverse-Pierluissi et al. 1996).

α_2-Adrenergic receptors are differentially targeted to membrane domains in cells (for review see Saunders and Limbird 1999). While α_{2A}- and α_{2B}-receptors were targeted to the plasma membrane in most cell lines, including differentiated PC12 pheochromocytoma cells, the α_{2C}-receptor was localized at the plasma membrane and to a greater extent in an intracellular perinuclear compartment (Daunt et al. 1997; Olli-Lähdesmäki et al. 1999). Upon agonist stimulation, α_{2B}- and α_{2C}-receptors underwent reversible internalization into endosomes, while the α_{2A}-subtype remained in the plasma membrane (Daunt et al. 1997). Internalization of α_{2B}-receptors was dramatically enhanced by coexpression of arrestin-2 and arrestin-3, and redistribution of receptors to clathrin-coated vesicles and endosomes was observed (DeGraff et al. 1999). Internalization of α_{2B}- and α_{2C}-receptors was inhibited by coexpression of dominant negative dynamin-K44A (DeGraff et al. 1999); for the α_{2B}-receptor, coexpression of dynamin-K44A gave variable results (DeGraff et al. 1999; Schramm and Limbird 1999). Endocytosis did not appear to be required for α_2-adrenergic receptor-mediated p42/p44 MAP kinase activation (DeGraff et al. 1999; Schramm and Limbird 1999).

1.3 α_2-Receptor Tissue Distribution

The three α_2-receptor subtypes have unique patterns of tissue distribution in the central nervous system and in peripheral tissues (Fig. 2) (Nicholas et al. 1993; Aoki et al. 1994; Scheinin et al. 1994; MacDonald and Scheinin 1995; Nicholas et al. 1996; Rosin et al. 1996; Talley et al. 1996; Uhlen et al. 1997). These studies argue against complete functional redundancy for these receptor subtypes. The α_{2C}-receptor appears to be expressed primarily in the central nervous system (striatum, olfactory tubercle, hippocampus and cerebral cortex), although very low levels of its mRNA are present in the kidney. In contrast, the α_{2B}-receptor shows primarily peripheral expression (kidney, liver, lung, heart) and only low level expression in thalamic nuclei. The α_{2A}-receptor is expressed more widely throughout the central nervous system, including the locus coeruleus, brain stem nuclei, cerebral cortex, septum, hypothalamus, hippocampus) and in the periphery (kidney, spleen, thymus, lung, salivary gland).

In mice, expression of α_2-receptors can be detected during early embryonic development, starting at day 9.5 postcoitus for the α_{2A}-receptor, day 11.5 for the α_{2B}-, and 14.5 for the α_{2C}-subtype (Wang and Limbird 1997). α_{2A}-receptor mRNA showed a widespread distribution in the developing embryo, including the developing stomach and cecum, craniofacial regions and central nervous system. Also, α_{2A}-mRNA was expressed in the interdigital mesenchyme in parallel with digital separation (Wang and Limbird

1997). The α_{2B}-receptor could be detected in the developing liver when the liver is the principal site for hematopoiesis. α_{2C}-receptor mRNA was found in the nasal cavity and cerebellar primordium. The functional role of α_2-receptors for embryonic development is unclear at present. However, the α_{2A}-subtype can induce apoptosis in vitro (Wang and Limbird 1997).

2 Transgenic Mouse Models

Using molecular genetics and transgenic techniques, several mouse lines overexpressing or lacking α_2-adrenergic receptor subtypes have been generated (for review, see MacDonald et al. 1997; Kable et al. 2000). The genes encoding for the three murine α_2-receptors were disrupted in embryonic stem cells and knockout mouse lines lacking α_{2A}-, α_{2B}-, and α_{2C}-receptors were established (Link et al. 1995; Link et al. 1996; Altman et al. 1999). Mice with homozygous deletions in single α_2-receptor genes were viable and appeared grossly normal. From heterozygous crosses, homozygous α_{2A}-KOs and α_{2C}-KOs were born at the expected Mendelian ratios, however, α_{2B}-KOs were less abundant in these crosses than expected (Link et al. 1996). Thus, α_2-receptors may play an important role during embryogenesis and development. As the three α_2-receptor genes are localized on different chromosomes, knockout mouse lines could be crossed to generate strains lacking two α_2-receptor subtypes. So far, only mice lacking α_{2A}- and α_{2C}-receptors (α_{2AC}-KO) could be recovered from these crosses (Hein et al. 1999). All other combinations, i.e. α_{2AB}-KO and α_{2BC}-KO, seemed to be lethal during intrauterine development, further emphazising the significance of α_2-adrenergic receptors for embryonic development.

In order to investigate the in vivo significance of individual signalling pathways of the α_{2A}-subtype, Limbird and colleagues used gene-targeting to mutate the α_{2A}-receptor gene to express an Asp79->Asn (D79N) α_{2A}-receptor in mice (Fig. 3) (MacMillan et al. 1996). The D79N mutation substitutes asparagine for an aspartate residue at position 79, which is predicted to lie within the second transmembrane region of the α_{2A}-receptor and is highly conserved among G protein-coupled receptors (Fig. 2). In vitro, the α_{2A}-D79N receptor was found to be selectively uncoupled from activation of K^+ currents, but remained coupled to inhibition of voltage-gated Ca^{2+} channels and of cAMP production characteristic for the wild-type α_{2A}-receptor (Surprenant et al. 1992). Similar to the α_{2A}-KO mice, mice carrying the α_{2A}-D79N mutation were viable and developed normally (MacMillan et al. 1996). Surprisingly, the density of α_{2A}-D79N receptors was greatly reduced by 80% in brain membranes of homozygous transgenic mice, although there was no change in the abundance of mRNA encoding for α_{2A}-D79N compared with

the wild-type mRNA (MacMillan et al. 1996). These results indicate that, in vivo, the α_{2A}-D79N receptor may be improperly processed or stabilized in its target cells (MacMillan et al. 1998). In fact, subsequent studies suggest that the diminished steady state densitity of the α_{2A}-D79N receptor is due to accelerated turnover of this conformationally unstable mutant (Wilson and Limbird 2000). As expected from in vitro experiments, the α_{2A}-D79N receptor was uncoupled from K^+ channel activation in locus coeruleus neurons (Lakhlani et al. 1997). However, the inhibitory effect of α_2-agonists on Ca^{2+} channels in superior cervical ganglion cells was also blunted while the agonist-dependent inhibition of adenylyl cyclase appeared to be normal in these mice (Lakhlani et al. 1997). Thus for most, but not all, physiological function of α_2-receptors tested, the α_{2A}-D79N mutation turned out be behave like a "functional knockout" (MacMillan et al. 1996).

In addition to these gene-targeted mouse models, mouse lines overexpressing the α_{2C}-adrenergic receptor under control of its own promoter were generated by Kobilka and colleagues (Sallinen et al. 1997). The promotor elements present in the α_{2C}-transgene construct were capable of directing the increased receptor expression to those brain regions, and probably also to those cells, which normally express endogenous α_{2C}-receptors. In these transgenic mice, 3-fold overexpression of the α_{2C}-receptor was detected in caudate-putamen and in the stratum radiatum of the CA1 region of the hippocampus (Sallinen et al. 1997).

3 Presynaptic α_2-Adrenergic Receptors

Prejunctional α_2-receptors on adrenergic nerves mediate a negative feedback whereby released noradrenaline inhibits its own further release (for reviews, see Langer 1974; Starke et al. 1975; Westfall 1977; Starke et al. 1989; Langer 1997). Using a series of antagonists, measurements of ^{3}H-noradrenaline release in isolated tissues had suggested that presynaptic α_2-receptors belong – at least predominantly – to the pharmacological α_{2A}- or α_{2D}-subtypes (for review, see (Trendelenburg et al. 1993). This finding was confirmed in mice lacking α_{2A}-receptors by two experimental approaches: first, inhibition of the electrically evoked twitch response of isolated vasa deferentia by exogenous α_2-agonists was tested (Altman et al. 1999), second, the stimulated release of ^{3}H-noradrenaline from isolated tissues was measured (Fig. 4) (Hein et al. 1999; Trendelenburg et al. 1999). With both methods, the maximal inhibition of presynaptic transmitter release by a non-subtype selective α_2-agonist was decreased in mice lacking α_{2A}-receptors but not in lines lacking α_{2B}- or α_{2C}-receptors. These data indicate,

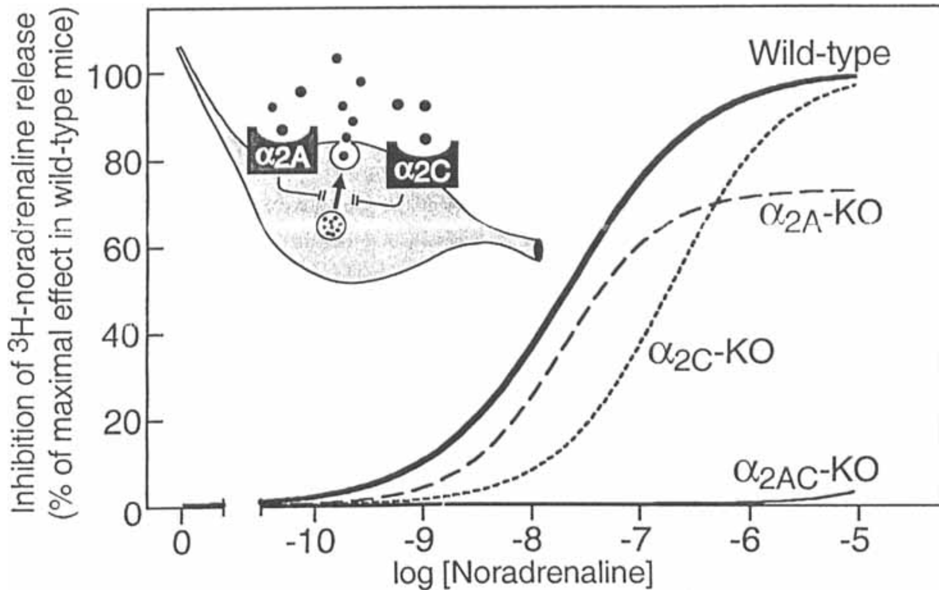

Fig. 4. Inhibition of noradrenaline release by α_{2A}- and α_{2C}-receptors. In atria from wild-type mice, exogenous noradrenaline completely inhibited release of ^{3}H-noradrenaline from sympathetic nerves. After deletion of the α_{2A}-receptor gene, the maximal inhibitory effect of noradrenaline was decreased, and knockout of the α_{2C}-receptor gene led to a rightward shift of the noradrenaline concentration response curve. Only in atria from mice lacking α_{2A}- and α_{2C}-receptors (α_{2AC}-KO) was the inhibitory effect of noradrenaline completely abolished, indicating that α_{2A}- and α_{2C}-adrenergic receptors are both required for presynaptic feedback inhibition of transmitter release (adapted from Hein et al. 1999)

that indeed the α_{2A}-subtype is the main inhibitory presynaptic autoreceptor on adrenergic neurons. However, in all tissues investigated from α_{2A}-KO mice α_2-agonists could still inhibit the release of noradrenaline (Altman et al. 1999; Trendelenburg et al. 1999). This finding suggested the presence of an additional presynaptic receptor. In α_{2A}-KO tissues, any correlation of antagonist pK_d values with those of α_{2D}-pharmacology was lost and the remaining receptors displayed α_{2B}- or α_{2C}-pharmacology (Trendelenburg et al. 1999). By crossing mouse lines deficient in single α_2-receptors, double transgenic mice could be generated which lacked both α_{2A}- and α_{2C}-receptors (α_{2AC}-KO) (Hein et al. 1999). Using these α_{2AC}-KO mice, the second presynaptic receptor could be identified as the α_{2C}- receptor, as no agonist effect remained in tissues from mice lacking α_{2A}- and α_{2C}-receptors (Fig. 4) (Hein et al. 1999). Both, α_{2A}- and α_{2C}-receptors operated as presyn-

aptic inhibitory receptors in a wide range of tissues investigated so far, including central adrenergic neurons, e.g. brain cortex and hippocampus, and peripheral sympathetic neurons in heart atria and vas deferens (Hein et al. 1999; Trendelenburg et al. 1999). Pharmacological studies in human atria suggested that indeed α_{2C}-receptors are involved in presynaptic autoregulation of noradrenaline from sympathetic nerves (Rump et al. 1995). In the brain, subtle changes have been observed in dopamine and serotonin metabolism in transgenic mice with altered expression of the α_{2C}-receptor (Sallinen et al. 1997). Lack of α_{2C}-receptor expression (α_{2C}-KO) slightly decreased the rate of monoamine turnover in the brain and overexpression of α_{2C}-receptors increased dopamine stores and metabolism (Sallinen et al. 1997).

Several lines of evidence suggest that α_{2A}- and α_{2C}-receptors operate also in wild-type mice as integral parts of the presynaptic feedback loop. Experiments on peripheral tissues, e.g. heart atria, demonstrated that the α_{2C}-subtype mediates autoinhibition by low concentrations of noradrenaline in wild-type mice, whereas the potency of noradrenaline at the α_{2A}-subtype was lower (Fig. 4) (Hein et al. 1999). This potency difference of noradrenaline for the α_2-receptors correlated with the affinity difference of noradrenaline for the α_{2A}- and α_{2C}-subtypes, respectively. Furthermore, there is no evidence so far that the expression of the remaining α_2-receptor subtypes was altered in mice carrying deletions in α_{2A}- or α_{2C}-receptor genes (Link et al. 1995; Altman et al. 1999).

Presynaptic α_{2A}- and α_{2C}-receptors can be distinguished functionally and may thus serve independent presynaptic functions. In mouse atria, the α_{2A}-subtype inhibited noradrenaline release at high stimulation frequencies whereas the α_{2C}-receptors operated at lower levels of sympathetic nerve stimulation (Hein et al. 1999). Moreover, inhibition of noradrenaline release mediated by the α_{2A}-subtype occurred much faster than inhibition by the α_{2C}-receptor. These findings indicate that two presynaptic receptors in the inhibitory feedback loop of transmitter release may differentially regulate synaptic transmission.

In mice carrying the mutated α_{2A}-D79N receptor gene, presynaptic function in the vas deferens did not differ from wild-type control mice (Altman et al. 1999). This finding indicates (1) that K^+ channel coupling is not required for presynaptic function of α_{2A}-receptors in sympathetic nerves and (2) that there is a high number of spare receptors at the presynaptic site, as expression of the α_{2A}-D79N receptor was found to be reduced to 20% of the wild-type level (MacMillan et al. 1996). Thus, Ca^{2+} channels in presynaptic nerve terminals are essential for neurotransmitter release, and current

research has provided evidence for the involvement of a multitude of Ca^{2+} channel subtypes (Reuter 1996).

4 Cardiovascular Function of α_2-Receptors

4.1 Hemodynamic Effects of α_2-Agonists

α_2-Adrenergic receptors in the rostral ventrolateral medulla of the brain respond to noradrenaline and adrenaline to decrease sympathetic outflow and reduce arterial pressure and heart rate (Ruffolo et al. 1993). This hypotensive effect of α_2-agonists has been the rationale for the therapeutic use of clonidine in the treatment of hypertension (Ruffolo et al. 1993). However, especially after rapid intravenous injections, clonidine may initially cause a transient hypertensive response mediated by α_2-receptor-elicited contraction of the peripheral vasculature. Using α_2-subtype-specific knockout mice, these two opposing hemodynamic effects of an α_2-agonist could be identified as being mediated by the α_{2A}- (hypotension) and the α_{2B}-receptor (hypertension). In conscious, unrestrained wild-type mice, infusions of the α_2-agonist brimonidine (UK14,304) or dexmedetomidine into the carotid artery resulted in a transient pressor response followed by an extended hypotensive response. The hypotensive response was essentially absent in α_{2A}-KO and in α_{2A}-D79N mice (MacMillan et al. 1996; Altman et al. 1999). However, the rapid initial hypertensive response to α_2-agonist infusion was abolished in α_{2B}-KO mice and the hypotensive response occurred immediately and was significantly greater in α_{2B}-KOs than in wild-type mice (Link et al. 1996). These results demonstrate that stimulation of α_{2B}-receptors counteracts the therapeutic antihypertensive effect of drugs acting at central α_{2A}-receptors in the central nervous system (Link et al. 1996). In addition, the α_{2A}-receptor may contribute to the vasoconstriction in some vascular beds, as the hypertensive response to α_2-agonists in α_{2A}-D79N mice was absent after femoral administration but unchanged after carotid administration after carotid injection of α_2-agonists (MacMillan et al. 1996).

4.2 Baseline Hemodynamics of α_2-Receptor-Deficient Mice

At baseline, α_{2B}-KO, α_{2C}-KO, and α_{2A}-D79N mice had similar heart rate and blood pressure as compared with wild-type mice (Link et al. 1996; MacMillan et al. 1996). However, α_{2A}-KO mice were tachycardic at rest and showed a significant increase in arterial pressure (Makaritsis et al. 1999). The increased heart rate in α_{2A}-KO was due to an increase in sympathetic activity, as infusion of the β-receptor antagonist propranolol could completely abol-

ish the chronotropic effect of the gene deletion (Altman et al. 1999). As expected from the presynaptic location of the α_{2A}- and α_{2C}-receptor subtypes, plasma noradrenaline levels were slightly elevated in α_{2A}-KO mice and were 3-fold above the control value in α_{2AC}-KO mice lacking both presynaptic receptors (Hein et al. 1999). Increased noradrenaline turnover was also observed in mice expressing the α_{2A}-D79N receptor (Lakhlani et al. 1997).

4.3 Long-Term Effects of α_2-Receptor Deletion on the Cardiovascular System

The long-term physiological consequences of altered sympathetic transmitter release were studied in mice lacking α_{2A}- and α_{2C}-receptors. The heart is very sensitive to chronic elevations of catecholamines, and abnormal activity of the sympathetic nervous system has been implicated in the pathogenesis of heart failure (Cohn et al. 1984; Francis et al. 1990; Packer 1992; Lechat et al. 1998). In α_{2AC}-KO mice, left ventricular maximal contractility decreased to 70% of the wild-type value, while mice lacking single α_2-receptor subtypes did not show an impairment of cardiac function (Hein et al. 1999). In addition, cardiac hypertrophy developed in α_{2AC}-KO mice. These findings indicate that the heart is exposed to significantly higher levels of catecholamines in α_{2AC}-KO mice than in either α_{2A}-KO or α_{2C}-KO mice.

The role of the α_{2B}-receptor in cardiovascular regulation was further emphazised by results from experiments using a hypertension model (Makaritsis et al. 1999). The increase in blood pressure after subtotal nephrectomy and replacement of the drinking water with 1% saline was significantly smaller in α_{2B}-KO than in wild-type or α_{2C}-KO mice (Makaritsis et al. 1999). Thus, α_{2B}-receptors are necessary to raise blood pressure in response to salt-loading. It is unclear whether this process is of central origin (inability to increase sympathetic outflow), vascular origin (inability to vasoconstrict) or renal origin (inability to retain excess salt and fluid) (Makaritsis et al. 1999).

4.4 Imidazoline Receptors

In 1984, Bousquet et al. suggested that a characteristic cerebral effect of clonidine-like imidazoline derivatives, hypotension, might be mediated not by α_2-adrenergic receptors but by separate "imidazoline-preferring" receptors. Imidazoline receptors are now being discussed as potential sites of drug action in many tissues and are thought to comprise several distinct types, of which two have been termed I_1 and I_2 (for review, see Molderings 1997). There is controversy concerning whether agents such as clonidine, which contain an imidazole moiety, elicit their hypotensive effects by inter-

acting with α_2-receptors or with a separate imidazoline receptor population (Bousquet et al. 1992). To address this question, the hypotensive effect of several imidazoline ligands was tested in α_{2A}-D79N mice (Zhu et al. 1999). In wild-type mice, rilmenidine, moxonidine and clonidine decreased blood pressure and heart rate. In α_{2A}-D79N mice, hypotensive responses to rilmenidine and moxonidine were completely absent. After clonidine infusion, hypotension was absent in α_{2A}-D79N animals. In contrast, dose-dependent hypertension and bradycardia were observed. Thus, there was no evidence for imidazoline receptor-mediated hemodynamic effects in mice carrying a mutated α_{2A}-receptor gene (Zhu et al. 1999).

5 Central Nervous System Function of α_2-Receptor Subtypes

5.1 Sedation

α_2-adrenergic agonists are used in the anaesthetic management of the surgical patient for their sedative and hypnotic properties. The sedative effects of α_2-agonists was investigated in α_{2A}-D79N mice and in mice with altered expression of the α_{2C}-receptor (knockout and overexpression) (Hunter et al. 1997; Lakhlani et al. 1997). α_{2A}-D79N mice showed no sedative response to the α_2-agonist, dexmedetomidine, indicating that the α_{2A}-subtype is responsible for the clinically used hypnotic effect of α_2-agonists (Lakhlani et al. 1997). In mice lacking α_{2B}- or α_{2C}-receptors, the sedative response to dexmedetomidine was unaltered when compared with wild-type mice (Hunter et al. 1997; Sallinen et al. 1997). Transgenic overexpression of the α_{2C}-receptor did not affect cortical EEG delta amplitudes at baseline and after α_2-receptor activation (Björklund et al. 1998).

A clinically useful action of α_2-agonists is their ability to reduce the requirements for other anaesthetic agents during anaesthesia. In control mice, non-sedative doses of dexmedetomidine reduced the concentrations of the volatile anaesthetic, halothane, to induce anaesthesia by 30% (Lakhlani et al. 1997). This anaesthetic-sparing effect of α_2-agonists was completely abolished in α_{2A}-D79N mice.

Several lines of evidence suggest that the locus coeruleus is the site of the α_{2A}-agonist-mediated sedative response. The α_{2A}-subtype is abundantly expressed in locus coeruleus neurons (Wang et al. 1996). Administration of antisense oligonucleotides for the α_{2A}-receptor subtype into the locus coeruleus of rats attenuated the hypnotic effect of dexmedetomidine reversibly (Mizobe et al. 1996). In the locus coeruleus of wild-type mice, α_2-agonists suppressed the spontaneous firing rate of neurons but did not alter

spontaneous activity or membrane potential in neurons from α_{2A}-D79N mice (Lakhlani et al. 1997). In addition, α_2-receptor activation reduces Ca^{2+} channel currents in numerous neuronal preparations. The inhibitory effect of the α_2-agonists, clonidine and dexmedetomidine on voltage-gated Ca^{2+} channels in locus coeruleus or superior cervical ganglion cells was significantly blunted, but not abolished in α_{2A}-D79N mice (Lakhlani et al. 1997). In α_{2A}-D79N neurons, the inhibitory effect of clonidine on Ca^{2+} currents was reduced to 30% of the response recorded in cells from wild-type mice and this effect was sensitive to the α-receptor antagonist prazosin, indicating that α_{2B}- or α_{2C}-receptors are involved in this response.

5.2 Analgesia

Analgesia is a clinically important use of α_2-receptor agonists. α_2-Agonists mediate analgesia (Yaksh 1985) and they interact synergistically with opioids (Sullivan et al. 1987; Wilcox et al. 1987; Drasner and Fields 1988; Ossipov et al. 1989; Monasky et al. 1990). Pharmacological studies have suggested that activation of α_{2A}-receptors mediates the α_2-agonist-induced analgesia (Millan 1992; Millan et al. 1994), others have suggested that the site of action may be α_{2A}- or non-α_{2A}-receptors dependent on the agonist used (Takano and Yaksh 1992). Subtype-selective antisera have localized the α_{2A}-subtype in the rat spinal cord to terminals of capsaicin-sensitive, substance P-containing primary afferent fibers (Stone et al. 1998). The α_{2C}-subtype was found to be expressed in a subset of spinal interneurons. In situ hybridization studies have localized mRNA for α_{2A}- and α_{2C}-subtypes in dorsal root ganglion neurons (Nicholas et al. 1993), thus one or both subtypes may mediate spinal analgesia at a presynaptic site on primary afferent fibers.

In comparison to control mice, dexmedetomidine was completely ineffective as an antinociceptive agent in the tail immersion test in the α_{2A}-D79N transgenic mice (Hunter et al. 1997a; Hunter et al. 1997b). In α_{2A}-D79N mice, intrathecal administration of brimonidine (UK14,304) had no analgesic effect in the tail-flick test, whereas the analgesic potency of morphine was not altered in these mice (Stone et al. 1997). The α_{2A}-D79N mutation also decreased α_2-agonist-mediated spinal analgesia and blocked the synergy seen in wild-type mice with δ-opioid or μ-opioid agonists in the substance P behavioral test (Stone et al. 1997). However, some α_2-agonist effect remained in the substance P test in α_{2A}-D79N mice which could be attributed to residual adenylyl cyclase coupling of the α_{2A}-D79N receptor or to another α_2-subtype. Combinations of α_{2A}-agonists and μ-opioid agonists may prove useful in maximizing the analgesic efficacy of opioids while decreasing total dose requirements.

Supraspinal opioid receptors and spinal α_2-receptors are involved in the analgesic mechanism for nitrous oxide (N_2O). It has been suggested that activation of opioid receptors in the periaqueductal gray activates descending noradrenergic pathways which release noradrenaline onto α_2-receptors in the dorsal horn of the spinal cord (Zhang et al. 1999). After exposure of rats to N_2O a fourfold increase in noradrenaline release could be detected in the dorsal horn of the spinal cord (Zhang et al. 1999). N_2O produced antinociception in the tail flick test in wild-type and in α_{2A}-D79N mice, although the response was less pronounced in α_{2A}-D79N mice (Guo et al. 1999). The antinociceptive response to N_2O in α_{2A}-D79N mice could be antagonized by opioid receptor antagonists and by prazosin, which blocks α_{2B}- and α_{2C}-receptors. Adrenergic agonists have been shown to inhibit neurotransmitter release from spinal cord preparations by a prazosin-sensitive receptor, suggesting a role for the α_{2B} or α_{2C} subtypes (Ono et al. 1991). Thus, the analgesic effect of α_2-agonists seems to be mediated by the α_{2A}-receptor subtype. In addition, α_{2B}- and/or α_{2C}-receptors may be involved in the antinociceptive effect of nitrous oxide.

5.3 Inhibition of Epileptic Seizures

Noradrenaline is unique among the monoamine transmitters in that it exerts powerful antiepileptogenic actions (McNamara et al. 1987) which are mediated by the α_2-receptor (Gellman et al. 1987). Mice carrying a mutated α_{2A}-receptor (α_{2A}-D79N) showed marked enhancement of epileptogenesis in a mouse kindling model, and the proepileptogenic actions of the α_2-antagonist idazoxan were abolished (Janumpalli et al. 1998). These data suggest that the α_{2A}-subtype is the only α_2-receptor involved in modulating seizure threshhold.

5.4 Hypothermia

α_2-Agonists lower body temperature dose-dependently (Hunter et al. 1997b; Sallinen et al. 1997). Dexmedetomidine showed a hypothermic effect in mice lacking α_{2B}- or α_{2C}-receptors but failed to decrease body temperature in α_{2A}-D79N mice (Hunter et al. 1997). Contrary to these results, the hypothermic effect of dexmedetomidine was slightly blunted in α_{2C}-KO mice (Sallinen et al. 1997) and it had no effect on body temperature in α_{2C}-KO mice at a low dose that produced significant hypothermia in wild-type mice (Hunter et al. 1997b). Thus, two α_2-receptor subtypes, α_{2A} and α_{2C}, may be involved in the regulation of body temperature.

5.5 Behavioural Functions

α_2-Adrenergic receptors mediate many physiological functions and pharmacological effects in the central nervous system, mainly by inhibiting neuronal firing and release of noradrenaline and other neurotransmitters. Locus coeruleus noradrenaline neurons send noradrenergic fibers into different forebrain structures and modulate different cognitive functions, such as attention, arousal, and planning (Crow 1968; Arnsten and Goldman-Rakic 1985; Arnsten and Leslie 1991; Riekkinen et al. 1992; Arnsten et al. 1996; Coull et al. 1996). A variety of behavioural paradigms were tested in mice lacking or overexpressing α_{2C}-adrenergic receptor, but no data have been obtained for α_{2A}- or α_{2B}-receptor-deficient mice.

Activation of α_2-receptors resulted in locomotor inhibition. The α_2-agonist dexmedetomidine did not alter spontaneous motor activity or diurnal rhythm of motor activity of α_{2C}-KO or α_{2C}-overexpressing mice (Sallinen et al. 1997). Thus, the α_{2C}-subtype does not seem to be involved in the effect α_2-agonists on locomotor behaviour. However, D-amphetamine stimulated locomotor activity to a greater extent in α_{2C}-KO mice than in wild-type mice (Sallinen et al. 1998). The behavioural serotonin syndrome and head twitches to injection of the serotonin precursor 5-hydroxytryptophan were inhibited by α_2-agonists with similar magnitude in wild-type and α_{2C}-KO mice, suggesting that the α_{2A}-subtype rather than the α_{2C}-receptor may be involved in α_2-mediated inhibition of the serotonin syndrome (Sallinen et al. 1998).

Experimental data indicate that antagonists selective for the α_{2C}-subtype and agonists devoid of any α_{2C}-receptor affinity can modulate cognition more favourably than subtype-nonselective drugs. Mice overexpressing α_{2C}-receptors were impaired in spatial or nonspatial water maze tests, and an α_2-antagonist fully reversed the water maze escape defect in α_{2C}-receptor overexpressing mice (Björklund et al. 1998; Björklund et al. 1999; Björklund et al. 2000). The α_2-agonist dexmedetomidine increased swimming distance more effectively in wild-type mice than in α_{2C}-KO mice (Björklund et al. 1998). These results suggest that α_{2C}-receptors can modulate navigation to a hidden or visible escape platform. Activation of the α_{2C}-subtype disrupts execution of spatial and non-spatial search patterns (Björklund et al. 1999).

Altered startle reactivity and attenuation of the inhibition of the startle reflex by an acoustic prepulse has been observed in psychiatric patients, e.g. in schizophrenia (Braff et al. 1978). Disrupted prepulse inhibition in rats can be normalized by antipsychotics and this paradigm is being used as an animal model for drug development. Interestingly, α_{2C}-KO mice had enhanced startle responses, diminished prepulse inhibition, and shortened attack

latency in the isolation-aggression test (Sallinen et al. 1998). The opposite effect was observed in mice overexpressing the α_{2C}-receptor. Thus drugs acting via the α_{2C}-receptor might have therapeutic value in disorders associated with enhanced startle responses and sensorimotor gating deficits, such as schizophrenia, attention deficit disorder, post-traumatic stress disorder, and drug withdrawal. Activation of α_{2C}-receptors reduces hyperreactivity and impulsivity of mice, indicating that the α_{2C}-subtype has an inhibitory role on reactivity of the central nervous system. It is tempting to speculate that the therapeutic benefit and clinical acceptance of clonidine in neuropsychiatric disorders might have been restricted by the adverse effects of hypotension and sedation, which seem to be mediated solely by α_{2A}-receptors. However, recent studies in mouse behavioural models suggest that the α_{2A}-receptor has a protective role in some forms of depression and anxiety, and that this subtype mediates the antidepressant effects of imipramine (Schramm, McDonald, Limbird, personal communication). Thus, α_{2A}- and α_{2C}-receptors may complement each other to integrate central nervous system function.

6 Conclusion

Gene targeting in mice represents a unique approach to delineate the physiological functions of individual α_2-adrenergic receptor subtypes in vivo (Fig. 5). In the cardiovascular system, stimulation of α_{2B}-receptors in vascular smooth muscle cells produces hypertension and counteracts the clinically beneficial hypotensive effect of stimulating α_{2A}-receptors in the central nervous system. In addition to the hypotensive action of α_2-agonists, their hypnotic, antiepileptogenic, and analgesic effects are mediated via the α_{2A}-adrenergic subtype. However, some evidence accumulates that the α_{2C}-receptor may be an important target for fine tuning of neurotransmitter release in the central and peripheral nervous system. In addition, several aspects of cognitive function which are modulated by α_2-agonists are mediated via the α_{2C}-subtype. However, further studies are required, as not all of the clinically relevant functions of α_2-receptors have been systematically investigated in all three lines of α_2-receptor-deficient mice simultaneously. Rather, many assignments of α_{2A}-receptor functions have been made by using mice carrying a point mutation (D79N) in the α_{2A}-receptor gene, which partially uncouples this receptor mutant from intracellular signalling pathways and dramatically reduces receptor expression in vivo (MacMillan et al. 1996; Lakhlani et al. 1997). The α_{2A}-D79N transgenic mouse provides important in vivo insight into the physiological significance of individual signalling pathways of an individual receptor. However, caution should be

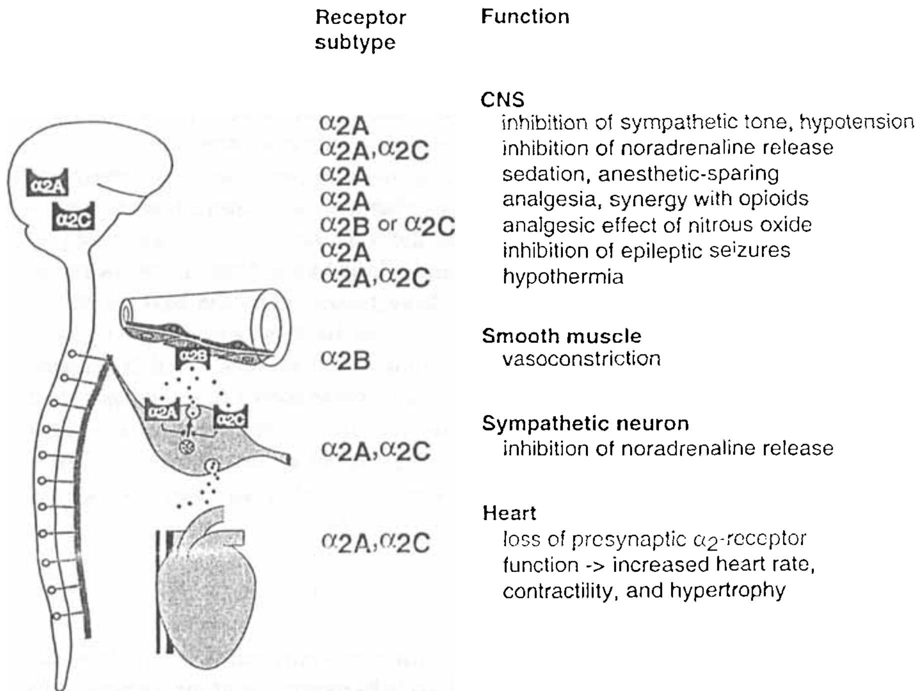

Fig. 5. In vivo functions of α_2-adrenergic receptor subtypes. This overview assigns physiological effects of α_2-receptor stimulation to individual α_2-receptor subtypes based on experiments performed in transgenic mice carrying deletions in α_2-receptor gene (see text for references)

used when interpreting data obtained with this mouse model as a "functional knockout". In addition, double knockout mice lacking two of the three α_2-receptor subtypes will be important tools to assign α_2-receptor functions more precisely to individual subtypes. The combined deletion of α_{2A}- and α_{2C}-receptor (α_{2AC}-KO) highlights the importance of combining transgenic lines to define the function of the presynaptic α_2-receptor feedback loop for neurotransmitter release. With these tools, in vivo gene targeting will be further exploited to identify the pharmacological significance of specific receptor subtypes in vivo and to guide pharmaceutical development of novel subtype-selective drugs.

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