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R. C. Hogg · M. Raggenbass · D. Bertrand

Nicotinic acetylcholine receptors: from structure to brain function

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Abstract Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels and can be divided into two groups: muscle receptors, which are found at the skeletal neuromuscular junction where they mediate neuromuscular transmission, and neuronal receptors, which are found throughout the peripheral and central nervous system where they are involved in fast synaptic transmission. nAChRs are pentameric structures that are made up of combinations of individual subunits. Twelve neuronal nAChR subunits have been described, $\alpha 2$–$\alpha 10$ and $\beta 2$–$\beta 4$; these are differentially expressed throughout the nervous system and combine to form nAChRs with a wide range of physiological and pharmacological profiles. The nAChR has been proposed as a model of an allosteric protein in which effects arising from the binding of a ligand to a site on the protein can lead to changes in another part of the molecule. A great deal is known about the structure of the pentameric receptor. The extracellular domain contains binding sites for numerous ligands, which alter receptor behavior through allosteric mechanisms. Functional studies have revealed that nAChRs contribute to the control of resting membrane potential, modulation of synaptic transmission and mediation of fast excitatory transmission. To date, ten genes have been identified in the human genome coding for the nAChRs. nAChRs have been demonstrated to be involved in cognitive processes such as learning and memory and control of movement in normal subjects. Recent data from knockout animals has extended the understanding of nAChR function. Dysfunction of nAChR has been linked to a number of human diseases such as schizophrenia, Alzheimer's and Parkinson's diseases. nAChRs also play a significant role in nicotine addiction, which is a major public health concern. A genetically transmissible epilepsy, ADNFLE, has been associated with specific mutations in the gene coding for the $\alpha 4$ or $\beta 2$ subunits, which leads to altered receptor properties.

R. C. Hogg (✉) · M. Raggenbass · D. Bertrand
Department of Physiology, CMU, 1 rue Michel Servet, 1211 Geneva 4, Switzerland
e-mail: hogg1@etu.unige.ch · Tel.: +41-22-7025355 · Fax: +41-22-7025402

Introduction

Ligand-gated ion channels mediate fast synaptic transmission in the central nervous system (CNS) and at ganglionic and neuromuscular synapses. The nicotinic acetylcholine receptor (nAChR) is a member of the ligand-gated ion channel superfamily, which includes the 5-HT_3, glycine and GABA type A and C receptors. These receptors are known as Cys-loop receptors, as all of them have a conserved sequence containing a pair of cysteines separated by 13 residues and linked by a disulfide bridge. nAChRs can be divided into two groups: muscle receptors, which are found at the skeletal neuromuscular junction where they mediate neuromuscular transmission, and neuronal receptors, which are found throughout the peripheral and central nervous system. Many of the early studies carried out on the subunit composition and structure of the nAChRs were performed on receptors isolated from the electric organ of *Torpedo californica*, as this tissue is very rich in nAChRs, and they were found to have a high degree of homology with the embryonic vertebrate muscle type receptor.

Muscle nAChRs are made up of five subunits arranged around a central pore (Fig. 1A, B). In *Torpedo* electric organ and vertebrate fetal muscle, the subunit composition is $(\alpha 1)_2\beta 1\gamma\delta$, and in adult muscle the γ subunit is replaced by the ε to give an $(\alpha 1)_2\beta 1\varepsilon\delta$ composition (Mishina et al. 1986). The order of assembly of the subunits that form the pentameric muscle receptor is highly constrained, with a clockwise sequence of $\alpha 1\varepsilon\alpha 1\beta 1\delta$ (Fig. 1A). To date 12 neuronal nAChR subunits have been described; $\alpha 2$–$\alpha 10$ and $\beta 2$–$\beta 4$; these are differentially expressed throughout the nervous system. The assembly of subunits in the neuronal nAChR is less tightly constrained than that of the muscle receptor. $\alpha 7$, $\alpha 8$ and $\alpha 9$ subunits have been shown to form functional homomeric pentamers when expressed in mammalian or amphibian cells (Fig. 1A). The other subunits combine in an $(\alpha)_2(\beta)_3$ stoichiometry to form functional channels (Anand et al. 1991), with the exception of $\beta 3$ and $\alpha 5$, which do not form functional receptors when expressed alone or with a single type of α or β subunit.

The majority of receptor complexes identified contained a single type of α and a single type of β subunit; however, heteromeric receptors involving three types of subunit can form. The $\beta 3$ and $\alpha 5$ subunits have been shown to form 'triplet' receptors when co-expressed with other α or β subunits in the *Xenopus* expression system (Boorman et al. 2000; Groot-Kormelink et al. 1998, 2001; Ramirez-Latorre et al. 1996). These triplet receptors had single-channel properties that were distinct from those of receptors containing a single type of α and β subunit. The $\alpha 5$ subunit has been shown to be incorporated into native nAChRs purified from chick optic lobe (Balestra et al. 2000). The $\beta 3$ subunit is expressed throughout the central nervous system (CNS) (Forsayeth and Kobrin 1997) and the $\alpha 5$ subunit is expressed in autonomic ganglia (Conroy and Berg 1995; Vernallis et al. 1993); however, the precise subunit composition of $\beta 3$- and $\alpha 5$-containing receptors in vivo has yet to be confirmed.

It is known that the ligand-binding site is made by the interface of the α subunit and its adjacent subunit (see Corringer et al. 2000 for recent review). It is thus predictable that the different subunit combinations of the neuronal nAChRs will have distinct pharmacological and biophysical properties (McGehee and Role 1995). This capacity of heteromeric receptor assembly contributes to the great diversity of receptor properties and represents an excellent example of the extraction of a maximal amount of functional diversity from a minimal amount of genetic information. Heteromeric nAChRs with a $(\alpha)_2(\beta)_3$ subunit stoichi-

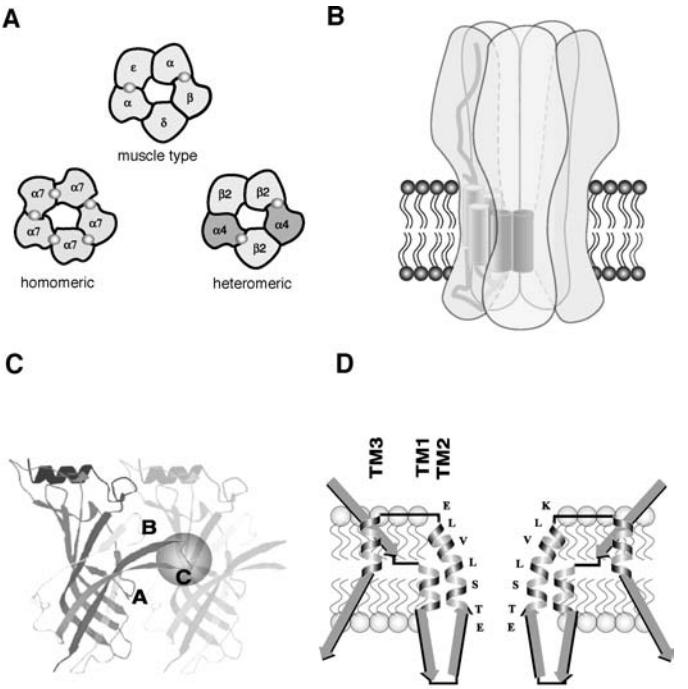


Fig. 1A–D Organization and structure of the nAChR. **A** Organization of subunits in muscle type receptor and neuronal homomeric and heteromeric receptors, showing the locations of the ACh binding sites. **B** Lateral view of subunit organization and membrane insertion. The membrane spanning portion is made up of the transmembrane segments TM1–4 and the N-terminal ligand-binding domain faces the extracellular space (adapted from Unwin 1993). **C** The structure of the ACh binding site on the homomeric AChBP, the binding site is located at the subunit interface and the 3 loops, *A*, *B* and *C*, which form the principal component of the binding site are marked (adapted from Brejc et al. 2001). **D** A schematic diagram of the nAChR pore, one side of the α–helical TM2 domain contributes the lining of the pore, amino acids facing the pore are indicated by their *single-letter codes* (adapted from Le Novere et al. 1999)

ometry are supposed to have two ACh-binding sites. In homomeric receptors, although a total of five ACh-binding sites must be present, the number of agonist molecules that should be bound to activate the receptor is unknown.

The elucidation of the secondary structure of the nAChR protein has been the focus of intense research over the last 30 years. Due to the hydrophobic nature of the membrane-spanning regions, it has so far been impossible to crystallize the pure protein, and structural data has come from a number of different approaches. Cryoelectron microscopy of membrane-bound receptors revealed a circular arrangement of five subunits forming a central pore (Toyoshima and Unwin 1988). Computer modeling suggests, however, that these subunits may not be equally arranged (Le Novere et al. 1999). Each nAChR subunit consists of an extracellular N-terminal that participates in the formation of the ligand-binding domain, transmembrane regions that are made up of four hydrophobic membrane spanning sections (M1–M4), and an intracellular loop between M3 and M4 that contains consensus sequences of phosphorylation sites (Fig. 1B).

The structure of the ACh-binding site has been investigated using a number of different approaches, including photoaffinity labeling, investigation with ligand probes and muta-

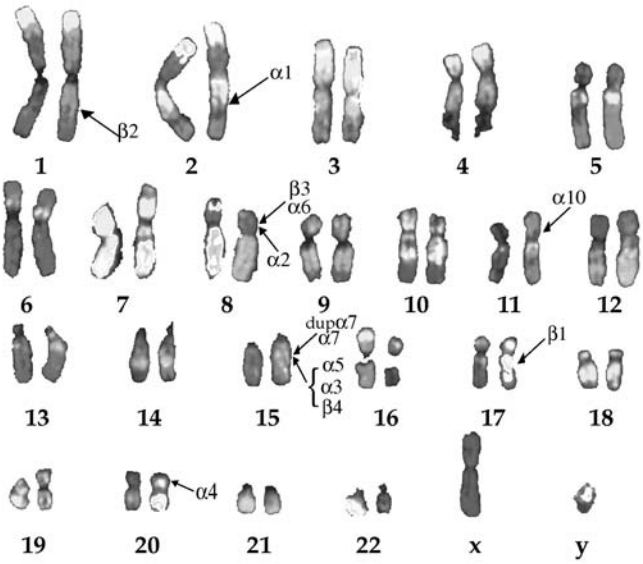
tion of residues believed to be involved in ACh binding. Photoaffinity labeling experiments demonstrated that agonists mainly bind to the α subunit (Middleton and Cohen 1991; Oswald and Changeux 1982; Reynolds and Karlin 1978), indicating that this subunit contributes the principle component of the binding site, with the adjacent subunit making up the complementary component (Corringer et al. 1995). The binding site has been investigated using ligands of different dimensions coupled with mutation of amino acid residues in the binding site region (Chiara et al. 1999; Galzi et al. 1990; Middleton and Cohen 1991; Tomaselli et al. 1991; D. Wang et al. 2000). These studies have identified residues involved in agonist and antagonist binding. A similar approach has also been applied to investigate the binding site of the α-neurotoxins, which are competitive inhibitors of nAChRs. Recently the crystal structure of an ACh-binding protein (AChBP) from the fresh water snail, *Lymnaea stagnalis*, has been published (Brejc et al. 2001). The AChBP is secreted into the synaptic cleft, where it regulates neurotransmission by quenching ACh. The protein is 210 residues long and is analogous to the extracellular ligand-binding domain of the nAChR (Brejc et al. 2001; Smit et al. 2001). The AChBP is soluble, forming stable homopentamers and is closely related to the α subunit of the nAChRs with almost all canonical residues conserved in the nAChR, including the Cys-loop, which contains 12 rather than 13 residues. Structural data from the crystallized AChBP has revealed that the topology of the ACh-binding site is remarkably similar to that predicted by mutation studies and computer modeling (Le Novere et al. 1999). The ACh-binding site is located at the interface between adjacent subunits. The principal component of the ACh-binding site comprises three loops A, B and C, which are involved in the binding of ACh. The complementary component is located on the adjacent subunit (α in homomeric or β in heteromeric receptors) and also contributes three loops (D, E and F) to the ACh-binding site (Fig. 1C). An improved 4.6-Å structure of the nAChR determined by electron microscopy of tubular crystals of *Torpedo* postsynaptic membranes embedded in amorphous ice identifies channels surrounded by twisted β-sheet strands in the channel extracellular domain. These form the extracellular mouth of a channel, connecting the water-filled vestibule at the mouth of the pore to the ACh-binding pockets (Miyazawa et al. 1999).

Information on the secondary structure of the channel from electron microscopy of crystalline arrays of membrane-bound receptors has shown that the receptor contains five rod-like segments arranged around a central axis; these rods were thought to be the pore lining M2 segments (Unwin 1993). The membrane spanning the M2 domain borders the ionic pore and there is strong evidence to suggest that it has a mainly α-helical structure (Fig. 1D). The four membrane-spanning segments were originally thought to be α-helices, as predicted from their amino acid sequence (Claudio et al. 1983; Devillers-Thiery et al. 1983; Noda et al. 1983). The functional effect of substitution of residues in the M4 domain of the α subunit of the *Torpedo* receptor indicated a periodicity consistent with an α-helical structure (Tamamizu et al. 2000). A computational approach suggested that although the M1, M3 and M4 domains contain α-helix, part of these membrane-spanning domains are also made up of β strand. The residues exposed to the lumen of the muscle type nAChR have been investigated with the radioactive channel blocker [^{3}H]-chlorpromazine. After digestion, peptide fragments with bound [^{3}H]-chlorpromazine were found to correspond to the M2 domain of the α, β and δ chains (Giraudat et al. 1986, 1987, 1989). The accessibility of residues in the M2 domain has also been investigated using the substituted cysteine accessibility method (SCAM), which identifies residues exposed on the surface of the protein. Residues along the entire length of the M2 domain (Akabas et al. 1994;

Zhang and Karlin 1998) and the N-terminal end of the M1 domain (Akabas and Karlin 1995; Zhang and Karlin 1997) were labeled supporting the view that these form the ion-conducting pore. Moreover, site-directed mutagenesis has identified residues in the transmembrane domain involved in ion selectivity, permeability and channel gating. In the chick $\alpha 7$ receptor, the ionic selectivity of the receptor can be converted from cationic to anionic by a minimum of three amino acid changes in the M2 domain and the M1–M2 loop (Cordero-Erausquin et al. 2000; Galzi et al. 1992). The electrostatic profile of the channel pore has been investigated and comprises a tunnel of negative electrostatic potential, which favors the entry of cations (Pascual and Karlin 1998). This negative charge is contributed by a ring of glutamic acid residues and if it is reduced, by substituting glutamic acid for amino acids with uncharged sidechains, the cation conductance of the receptor is decreased (Imotot et al. 1998). The region of the receptor that comprises the activation gate, which opens to allow ion permeation when the receptor is in the conducting state, has been mapped using SCAM, and this is located in the middle of the M2 domain (Wilson and Karlin 1998). The area of the pore that obstructs ion permeation in the desensitized state has also been identified using SCAM and is also found in the M2 domain closer to the extracellular mouth of the pore (Wilson and Karlin 2001). Site-directed mutagenesis has highlighted some of the elements of the receptor that are involved in structural reorganization upon channel activation. SCAM has revealed that changes in the M1 and M2 domains are involved in coupling the ACh-binding site and the channel activation gate (Karlin and Akabas 1995). The gating pathway has also been investigated by examining how single amino acid substitutions in the protein affect the rate equilibrium free energy relationships during channel gating. This approach suggests that the conformational change associated with channel activation proceeds in a wave-like manner from the region of the ACh-binding site towards the pore (Grosman et al. 2000). Recent results obtained with electron microscopy of nAChRs in the resting state and in the presence of bound ACh indicate that the α subunit undergoes a conformational change upon activation. This involves a 15°–16° rotation of the pore-lining region of the subunit, which is likely to be the trigger for opening the channel activation gate (Unwin et al. 2002).

nAChR genes and expression

The chromosomal location of the genes coding for the human neuronal nAChR subunits are shown in Fig. 2 with their respective GenBank accession numbers. The expression of *nAChR* genes in different tissues can be detected by the presence of functional receptors and by measurement of mRNA; however, caution must be exercised as the measurement of mRNA levels does not necessarily reflect the level of incorporation of the subunits in membrane receptors. It is clear that numerous receptor subtypes are expressed within individual neurons leading to a heterogeneous nAChR population. These different receptor subtypes vary in their desensitization characteristics and permeability to divalent ions, allowing subtle differences in response to ACh between neurons. The expression of functional recombinant nAChRs in cell lines is a multistep process; the assembly of muscle type nAChRs has been shown to involve folding, maturation, association and assembly (Blount et al. 1990; Paulson et al. 1991). The expression of certain neuronal nAChRs is highly dependent on the host cell and this is more evident with the homomeric $\alpha 7$ nAChRs than with other receptor subtypes. The necessary machinery for the insertion of functional



Subunit	Localisation	Access number
α2	8p21.2	NM_000742
α3	15q24.3	NM_000743
α4	20q13.33	NM_000744
α5	15q24.3	NM_000745
α6	8p11.21	NM_004198
α7	15q13.1	NM_000746
α9	Unknown	NM_017581
α10	11p15.4	NM_020402
β2	1q23.1	NM_000748
β3	8p11.21	NM_000749
β4	15q24.3	NM_000750

Fig. 2 Locations of the genes coding for nAChR subunits in humans. *Top*: The chromosomal locations of the genes coding for neuronal nAChR subunits. *Bottom*: The loci for each gene is shown with its respective GenBank access number

receptors in the cell membrane is not present in all cell types. It has been demonstrated that different strains of PC12 cells express dramatically different amounts of surface receptors without differences in the amounts of $\alpha 7$ mRNA expressed (Blumenthal et al. 1997). In five different host cell types that expressed mRNA for $\alpha 7$ and $\alpha 4\beta 2$ receptors, only two expressed surface $\alpha 7$ receptors, whereas all expressed $\alpha 4\beta 2$. $\alpha 7$ Protein was present intracellularly but was not formed into functional receptors (Sweileh et al. 2000). These data indicate that the machinery required for post-translational modification and assembly is not the same for different subunits.

nAChRs in the peripheral nervous system

Subunit composition

nAChRs mediate ganglionic neurotransmission in the autonomic peripheral nervous system (PNS) in mammals. A number of studies have investigated the presence of nAChR subunits in autonomic ganglia. The expression of mRNA coding for nAChR subunits was investigated using RT-PCR in rat olfactory bulb (OB) and trigeminal ganglion (TG). In the OB, all animals expressed $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$ subunit mRNAs, with $\alpha 6$, $\beta 3$, and $\alpha 9$ transcripts were expressed in 17%, 28%, and 33% of animals. In the TG, all animals expressed $\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 2$, and $\beta 4$ subunit mRNAs, whereas $\alpha 9$, $\beta 3$, $\alpha 4$, and $\alpha 5$ transcripts were expressed in 4%, 38%, 88%, and 92% of animals (Keiger and Walker 2000). Intracardiac ganglia neurons have been shown to express mRNA for $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ subunits with individual neurons, each expressing a different compliment of receptor subunits (Poth et al. 1997).

These studies, however, provide little information on the amount of functional receptors at the cell membrane or which subunits are involved in ganglionic neurotransmission. In vitro and in vivo studies using specific inhibitors of nAChR subtypes indicate that the principal receptors involved in neurotransmission in the intracardiac ganglia contain $\alpha 3\beta 2$ and $\alpha 3\beta 4$ subunits with a smaller contribution from $\alpha 7$ receptors (Bibevski et al. 2000; Hogg et al. 1999). In the bladder, parasympathetic neurons produce contraction of the detrusor muscle. Bladder strips from $\alpha 3$ and $\beta 4$ knockout mice did not contract to nicotine but responded to a muscarinic agonist or electric field stimulation. Bladders from mice lacking the $\beta 2$ subunit contract in response to nicotine, suggesting that nAChRs containing the $\alpha 3$ and the $\beta 4$ subunits mediate ganglionic transmission in the bladder (De Biasi et al. 2000). Although the amount of functional data available is relatively small, it appears that receptors involving $\alpha 3$ with $\beta 2$ or $\beta 4$ subunits are the principal receptors involved in neurotransmission in peripheral ganglia; however, data from measurements of mRNA levels suggest that there is a heterogeneous receptor population. The presence of these additional subunits and their different levels of expression between neurons likely contributes to modifying the characteristics of the nicotinic response in individual neurons.

nAChR distribution

nAChRs are necessary for synaptic transmission in the PNS. In the preceding section, we have seen that although peripheral ganglion neurons express a wide range of receptor subunits, the absence or blockade of $\alpha 3$-, $\beta 2$- and $\beta 4$-containing neurons can be sufficient to inhibit neurotransmission. Other subunits may not be located in the synaptic cleft but may contribute to extrasynaptic modulation. The localization of nAChR subunits at synapses has been investigated at the neuromuscular junction and in neurons. Skeletal muscle fibers express a single type of nAChR; targeting of these muscle type receptors to the endplate region has been shown to involve interaction with rapsyn (Apel et al. 1997; Maimone and Merlie 1993), which is an important molecule for synaptic organization. In contrast, neurons express a range of nAChR subtypes that are capable of forming receptors with different functional characteristics. The targeting of nAChRs to different areas of the neuron has been investigated in chick ciliary ganglion (CG) neurons, where they mediate excitatory ganglionic transmission. Individual CG neurons express two nAChR subtypes, $\alpha 3$-con-

taining nAChRs, which are predominantly localized at the postsynaptic membrane (Loring et al. 1985; Vernallis et al. 1993), and α7 nAChRs, which are excluded from the postsynaptic membrane and restricted to the perisynaptic dendritic membrane (Horch and Sargent 1995; Shoop et al. 1999). It is believed that the intracellular domain of the receptor is responsible for determining its location; however, the precise mechanism is still unclear (see Temburni et al. 2000).

nAChRs in the central nervous system

A growing body of evidence indicates that neuronal nAChRs are present in a variety of regions of the central nervous system. These receptors are located on cell bodies or dendrites, where they may mediate direct postsynaptic effects, or on axon terminals, where they could play a role in modulating synaptic transmission. The distribution, biophysical properties, pharmacological profiles and possible function(s) of neuronal nAChRs have been recently reviewed by several authors (Jones et al. 1999; MacDermott et al. 1999; McGehee and Role 1995; Role and Berg 1996; Wilson and Karlin 2001). Here, we shall summarize only very recent data.

The hippocampus, a cerebral structure involved in learning and memory, contains high amounts of nAChRs (Fabian-Fine et al. 2001). Most of them are located on GABAergic interneurons, but some are also present in pyramidal neurons (Ji et al. 2001). The majority of neurons express the α7-containing subtype. In a minority of interneurons, however, evoked nicotinic currents are slowly decaying and can be suppressed by mecamylamine, indicating that they are mediated by non-α7 nAChRs (Ji and Dani 2000; McQuiston and Madison 1999). Hippocampal nAChRs can modulate the induction of synaptic plasticity and may thus at least in part explain the effect of nicotinic agonists on learning and memory (Ji et al. 2001).

The ventral tegmental area (VTA) and one of its target regions, the nucleus accumbens (NAc), are thought to play a crucial role in reward (Koob et al. 1998). The VTA is rich in nAChRs and by increasing dopaminergic transmission in the NAc, these receptors probably play a role in nicotine addiction (Dani et al. 2001. By acting on presynaptic α7 nAChRs, nicotine can induce long-term enhancement of glutamatergic transmission in the VTA (Mansvelder and McGehee 2000), whereas activation of postsynaptic non-α7 receptors can facilitate GABAergic transmission (Mansvelder et al. 2002). The differential desensitization properties of these two nicotinic actions probably explain why nicotine tends to drive the activity of VTA neurons toward excitation rather then inhibition.

Magnocellular endocrine neurons located in the hypothalamic supraoptic and paraventricular nuclei possess functional nAChRs of the α7 type (Zaninetti et al. 2000a, 2002). These receptors probably play a role in ACh-induced enhancement of vasopressin release from the neurohypophisis. In addition, due to the high Ca^{2+} permeability of α7-containing nAChRs, these receptors could facilitate the Ca^{2+}-dependent release of vasopressin and oxytocin from the dendrites of magnocellular neurons (Pow and Morris 1989).

Brainstem motoneurons located in the VII, X and XII nuclei are sensitive to nicotinic agonists (Zaninetti et al. 2002). Whereas nAChRs present in VII and XII nuclei are of the heteromeric type, those present in the X nucleus contain the α7 subunit. Although the precise function of motoneuron nAChRs is still unknown, it is tempting to speculate that, via recurrent axon collaterals, motoneurons located in a specific brainstem nucleus could influence the activity of neighboring motoneurons via these receptors. In newborn mice, fa-

cial nerve axotomy causes nAChR down-regulation in the facial nucleus by interfering negatively with the expression of the α4 subunit (Zaninetti et al. 2000b). This raises the possibility that peripheral nerve injury may promote motoneuron degeneration by reducing motoneuron sensitivity to nicotinic agonists.

nAChRs and synaptic transmission

nAChRs present on cell bodies and/or dendrites of central neurons could play a role in mediating fast nicotinic synaptic transmission. The classic example is that of nAChRs present in Renshaw cells, a subpopulation of spinal glycinergic interneurons. Activation of these neurons by motoneuron axon collaterals results in recurrent inhibition of motoneurons (Curtis and Ryall 1966a, 1966b). More recently, the existence of nicotinic synaptic transmission has been examined in vitro, using brain slices and whole-cell recordings. In the CA1 region of the hippocampus, electrical stimulation of Shaffer's collaterals, performed in the presence of blockers of fast glutamatergic and GABAergic transmission, evoked small excitatory synaptic currents that could be suppressed by the selective α7 antagonists methyllycaconitine (MLA) and α-bungarotoxin (α-BgTX). This indicates that these synaptic currents were mediated by α7-containing nAChRs (Alkondon et al. 1998; Frazier et al. 1998; Hefft et al. 1999). Spontaneous and evoked nicotinic excitatory synaptic currents were also evidenced in the developing visual cortex (Roerig et al. 1997). These were suppressed by α-cobratoxin, but not by α-BgTX, suggesting that heteromeric nAChRs were involved. In hypothalamic slices, electrical stimulation of an area anterior to the supraoptic nucleus, which is rich in cholinergic neurons, evoked fast, atropine-insensitive excitatory synaptic currents in supraoptic neurons (Hatton and Yang 2002). These currents were probably mediated by α7-containing nAChRs, as they were suppressed by MLA and α-BgTX, but not by dihydro-β-erythroidine (DHβE).

Extrasynaptic nAChRs

In spite of these data, the evidence concerning the presence of functional nicotinic synapses in the brain remains relatively weak, suggesting that mediation of fast excitatory transmission may not be the main function of postsynaptic nAChRs. At least two alternative possibilities should be taken into consideration: (a) nAChRs present on neuronal cell bodies and/or dendrites may be activated by ACh diffusing from synaptic clefts (spillover) or by ACh released from axonal nonsynaptic varicosities (Descarries et al. 1997; Vizi and Lendvai 1999), (b) In hypothalamic neuronal cultures, chronic blockade of ionotropic glutamate receptors induced an increase in cholinergic transmission and up-regulation of AChRs, both of the nicotinic and of the muscarinic type (Belousov et al. 2001). This suggests that ACh may play a compensatory role and become a major excitatory transmitter in case of reduced or suppressed glutamatergic activity.

Modulation of synaptic transmission by presynaptic nAChRs

The poor evidence for the involvement of nAChRs in the generation of excitatory postsynaptic potentials (EPSPs) in the brain led many to proposing that the principal location of nAChRs may be presynaptic. The different locations of nAChRs on neurons are shown in

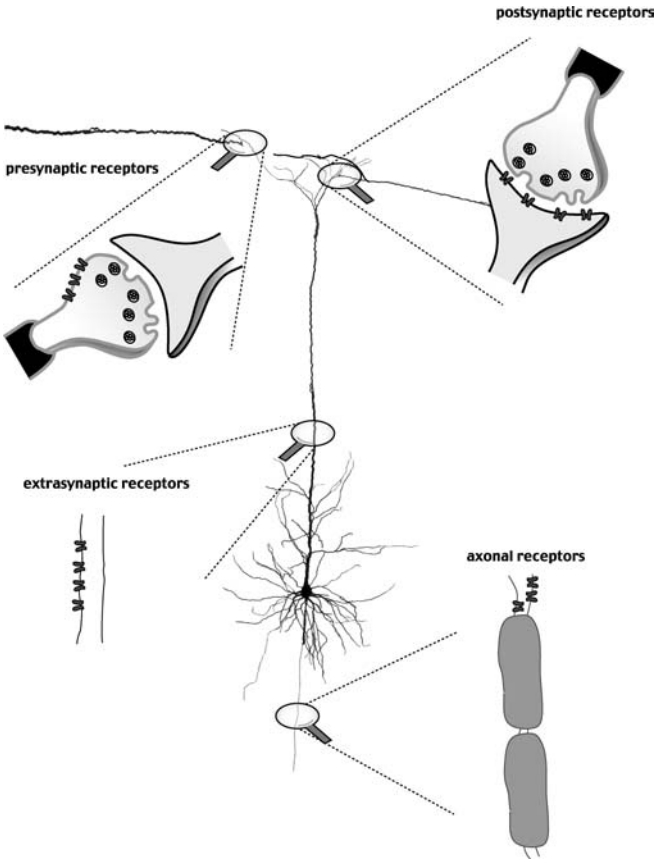


Fig. 3 Putative locations of nAChRs on a central neuron. Schematic diagram of a neuron showing postsynaptic receptors which would mediate classic synaptic transmission. Presynaptic receptors can mediate neurotransmitter release from the synaptic bouton and can regulate release of ACh from the same neuron (autosynaptic regulation) or the release of other neurotransmitters (heterosynaptic regulation). Extrasynaptic and axonal receptors can be activated by ACh released from varicosities on *en-passant* fibers. Local depolarization of the axon could lower the threshold for action potential initiation (excitatory); alternatively depolarization could cause inactivation of voltage-dependent channels and a decrease in the membrane resistance, leading to inhibition of action potential propagation

Fig. 3. The presence of presynaptic nAChRs in the CNS has been confirmed by autoradiography in nigrostriatal and mesolimbic dopaminergic neurons (Clarke and Pert 1985). In terminal neurons of the medial habenula, α-BgTX and nicotine-binding sites indicate that as much as 50% of α-BgTX binding may be presynaptic (Clarke et al. 1986). In guinea-pig prefrontal cortex presynaptic $\alpha 7$ receptors have been identified by immunogold labeling (Lubin et al. 1999). Immunostaining has shown that $\alpha 7$ nAChRs are present on somatic and dendritic regions in the hippocampus and immunogold labeling for $\alpha 7$ subunits was present both pre- and postsynaptically (Fabian-Fine et al. 2001). There is considerable evidence to suggest that in cultured rat hippocampal neurons, the two principal subunits, $\alpha 7$ and $\beta 2$, have different expression patterns on the cell surface. $\alpha 7$ subunits were found to co-localize with the presynaptic marker, synaptotagmin, whereas the $\beta 2$ subunits were

confined mainly to the cell soma and proximal processes and not specifically localized at presynaptic areas. These results indicate that $\alpha 7$ subunits are found at presynaptic terminals, where they may be involved in modulation of neurotransmitter release (Zarei et al. 1999). Immunolabeling with tyrosine hydroxylase (TH) indicated that the cell bodies and axon terminals of nigrostriatal neurons were also immunoreactive for the $\beta 2$ subunit. Analysis of double immunogold-labeled sections indicated that 86% of TH-positive axonal boutons are also labeled for the nAChR $\beta 2$ subunit (Jones et al. 2001). These results demonstrate that $\beta 2$ immunoreactivity is located presynaptically in nigrostriatal dopaminergic neurons, providing morphological evidence for presynaptic modulation of dopamine release by nAChRs.

Modulation of neurotransmitter release via nAChRs was first demonstrated in sympathetic ganglion preparation (Collier and Katz 1975) and subsequently in isolated motor nerves where nicotinic receptor activation increased the release of radiolabeled neurotransmitter (Wessler et al. 1992). In the mammalian CNS, evidence for the functional importance of presynaptic nAChRs has come both from measurements of labeled neurotransmitter release and from electrophysiological recordings from in vivo preparations, slices and acutely isolated and cultured neurons. Without exception, presynaptic nAChR activation was observed to facilitate neurotransmitter release (for review see (MacDermott et al. 1999). Facilitation of ACh release by nAChR activation has been termed autoregulation, whereas for other neurotransmitters, including noradrenaline, GABA, serotonin and glutamate, it has been termed heterosynaptic modulation.

In the striatum there is pharmacological evidence to support the presence of functional presynaptic $\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChRs, which modulate dopamine release from nigrostriatal terminals (Luo et al. 1998; Soliakov et al. 1995; Soliakov and Wonnacott 1996; Wonnacott et al. 2000). Nicotine- and (+/–)-anatoxin-a-evoked release of [^{3}H]-dopamine from rat striatal synaptosomes and slices was inhibited by the α-conotoxin MII. Although interpreted at first as a demonstration of the presence of $\alpha 3\beta 2$ nAChRs on dopaminergic terminals (Kaiser et al. 1998; Kulak et al. 1997), recent studies suggest that these processes may harbor $\alpha 6$-containing nAChRs (Champtiaux et al. 2002). Inhibition of [^{3}H]-dopamine release was incomplete, indicating the involvement of other nAChR subtypes (Kaiser et al. 1998; Kulak et al. 1997). The selective $\alpha 4\beta 2$ receptor agonist UB-165 also stimulated release of [^{3}H]-dopamine from striatal synaptosomes, implicating this receptor subtype in presynaptic modulation of dopamine release (Sharples et al. 2000). The α-conotoxin ImI, which is a selective inhibitor of $\alpha 7$ nAChRs, had no affect on [^{3}H]-dopamine release in the same preparation (Kulak et al. 1997). Luo et al. (1998) reported that the α-conotoxin AuIB, an inhibitor of $\alpha 3\beta 4$ nAChRs, did not effect nicotine-evoked [^{3}H]-dopamine release in striatal synaptosomes, but inhibited nicotine-induced noradrenaline release in hippocampal synaptosomes by 20%–35%, indicating the presence of $\alpha 3\beta 4$ receptors. The α-conotoxin MII also inhibited nicotine-induced release of noradrenaline from hippocampal synaptosomes, indicating the involvement of $\alpha 3\beta 2$ receptors in these neurons (Kulak et al. 1997).

At the excitatory synapse between co-cultured neurons of the medial habenula nucleus and the interpenduncular nucleus, a central limbic relay proposed to be involved in arousal and attention, nanomolar concentrations of nicotine enhanced glutamatergic and cholinergic synaptic transmission by activation of presynaptic nAChRs. This facilitation was blocked by α-BgTX application, and subunit deletion experiments confirmed that these presynaptic nAChRs contained the $\alpha 7$ subunit (McGehee et al. 1995). These findings support the fact that nAChRs enhance fast excitatory transmission in the CNS.

Nicotine has been demonstrated to activate presynaptic, postsynaptic, and somatic nAChRs in brain slices and cultured neurons. In cultured hippocampal neurons, rapid application of nicotine enhanced the amplitude of glutamate receptor-mediated excitatory postsynaptic currents. Glutamate release was shown to be enhanced via activation of presynaptic nAChRs (Radcliffe and Dani 1998). However, in the same preparation, activation of somatic or postsynaptic nAChRs has been shown to reduce the NMDA receptor component of the EPSCs and involved reduced responsive of NMDA receptors. This effect was blocked by the α7 antagonist, MLA, and was shown to be dependent on the entry of extracellular Ca^{2+} and involve a calmodulin-dependent process (Fisher and Dani 2000). In rat hippocampal slices, ACh-induced excitation of GABAergic interneurons caused either inhibition or disinhibition of pyramidal neurons. Both inhibition and disinhibition were sensitive to MLA or mecamylamine, indicating that α7 nAChRs on interneurons are involved in the regulation of hippocampal circuits (Alkondon et al. 2000; Ji and Dani 2000).

Presynaptic modulation of neurotransmitter release ultimately involves the regulation of intracellular Ca^{2+} in the presynaptic bouton. Ca^{2+} imaging experiments confirm that even in the absence of postsynaptic neurons, nicotinic activation increases intracellular calcium in presynaptic structures (McGehee et al. 1995). The cation permeability of the nAChRs causes depolarization of the presynaptic membrane and may trigger Ca^{2+} entry through voltage-activated Ca^{2+} channels. Anatoxin-a-stimulated release of dopamine from rat striatal synaptosomes is inhibited by Cd^{2+}, which blocks voltage-activated Ca^{2+} channels (Soliakov et al. 1995). Soliakov and Wonnacott (1996) investigated the subtypes of Ca^{2+} channels involved in facilitation of [^{3}H]-dopamine release in this preparation and the results indicate that the entry of Ca^{2+} is through N-type Ca^{2+} channels. Alternatively, it has been proposed that the high Ca^{2+} permeability of the α7 receptors could permit the direct entry of Ca^{2+} into the presynaptic bouton and directly facilitate neurotransmitter release (Bertrand et al. 1993; Seguela et al. 1993; Vernino et al. 1992). This increased entry of Ca^{2+} is consistent with the facilitation of neurotransmitter release that is observed following the presynaptic nAChR activation. The rapid desensitization of the α7 receptor makes it ideally suited to a positive feedback mechanism, especially autoregulation at cholinergic synapses, where fast desensitization would prevent uncontrolled escalation of the response.

While these numerous studies demonstrate without ambiguity that nAChRs modulate neuronal activity and synaptic transmission, the intimate mechanisms have not been fully identified. Firstly, it should be remembered that receptor subtypes involved in the different brain areas analyzed in these studies were based on their pharmacological characterization. However, given the lack of absolute specificity of the compounds employed and in some cases the use of high drug concentrations, care must be taken in the final analysis. Secondly, while clearer experiments can be carried out in cultured neurons, their results may reflect abnormal gene expression and synaptic organization. Nonetheless, the bulk of evidence reveals the broad mode of action of the nAChRs and their involvement in the regulation of neuronal network activity.

Up-regulation of nAChRs

Up-regulation of nAChR ligand binding

The chronic administration of nicotine to animals has been shown to result in an increase in the density of nicotine-binding sites in brain tissue. Postmortem autoradiographs of human brain have revealed that the density of [^{3}H]-epibatidine and [^{3}H]-cytisine binding is increased in brains from smokers compared to matched controls (Perry et al. 1999). This effect of nicotine is unusual in that it is contrary to the typical down-regulation of receptor-binding sites usually observed in neurotransmitter receptors following chronic agonist administration (Creese and Sibley 1981). Development of labeled ligands specific for nAChR subunits, [^{125}I]-α-BgTX for $\alpha7$ and [^{3}H]-cytisine and [^{3}H]-epibatidine for $\alpha4\beta2$ and other heteromeric nAChRs revealed that binding to both these receptor subtypes is up-regulated (Barrantes et al. 1995; Kawai and Berg 2001; Perry et al. 1999; Sparks and Pauly 1999). However, estimates of receptor density obtained by labeled ligand binding should be treated with caution: nicotine, cytisine and epibatidine are liposoluble and are known to penetrate inside the cell where they may label intracellular nAChRs or other proteins.

Nicotine-induced up-regulation of [^{3}H]-nicotine and [^{125}I]-α-BgTX binding in fetal rat brain was independent of de novo protein synthesis (Miao et al. 1998). The nicotine-evoked up-regulation of $\alpha4\beta2$ responses in K177 cells (Gopalakrishnan et al. 1997), permanently transfected clonal cell cultures of fibroblasts (Bencherif et al. 1995; Peng et al. 1994) and *Xenopus* oocytes (Peng et al. 1994) indicate that the necessary elements for up-regulation are present in cells of nonneural origin. Up-regulation of nicotinic binding sites has also been observed with other ligands that activate nAChRs, including DMPP, (–)-cytisine, ABT-418, A-85380 and (±)-epibatidine, the affinity of the ligand does not appear to be related to the efficacy in producing up-regulation (Gopalakrishnan et al. 1997). The competitive antagonists DHβE and *d*-tubocurarine (Gopalakrishnan et al. 1997) and the open channel blocker mecamylamine (Peng et al. 1994) have been shown to cause up-regulation of $\alpha4\beta2$ nAChRs in HEK cells and a fibroblast cell line, indicating that ionic current flow through the nAChR is not required for up-regulation. Mecamylamine acts synergistically with nicotine to cause up-regulation (Peng et al. 1994). GTS-21, (±)-epibatidine, DMPP and the antagonist MLA caused up-regulation of [^{125}I]-α-BgTX binding in HEK cells expressing $\alpha7$ receptors (Molinari et al. 1998). DMPP and d-tubocurarine are membrane impermeant, indicating that for these compounds binding to the extracellular domain of the nAChR is sufficient for up-regulation. Recently, in vivo up-regulation of neuronal nAChR binding was measured in baboons with dynamic SPECT studies using 5-[^{123}I]-iodo-A-85380. Following chronic nicotine treatment, binding was significantly increased in the thalamus and cerebellum (Kassiou et al. 2001).

The binding of labeled ligands to nAChRs shows both high- and low-affinity components, as does the activation of nAChRs by agonists in *Xenopus* oocytes and mammalian cells. Presently it is not known if these represent two distinct receptor populations or if a single receptor can present both high- and low-affinity sites as a result of an interaction with an allosteric modifier. The $\alpha4\beta2$ nAChR subtype represents more than 90% of the high-affinity [^{3}H]-nicotine-binding sites in mammalian brain (Flores et al. 1992; Whiting and Lindstrom 1986). It is unclear if the nicotine-induced increase in binding represents an increase in the number of receptors at the cell membrane or if it is due to an increase in the ratio of high-affinity to low-affinity ligand-binding sites.

Functional up-regulation of nAChR responses in vitro

In vitro recordings from neurons expressing native nAChRs, mammalian cell lines and nAChRs expressed in *Xenopus* oocytes demonstrate that the nicotinic currents induced by brief pulses of nicotine or ACh decrease during agonist application: this is known as desensitization (Fenster et al. 1997, 1999a; Olale et al. 1997; Peng et al. 1994; Pidoplichko et al. 1997). nAChRs in the desensitized state are less able to be activated by nicotinic agonists. Desensitization occurs in vitro at nicotine concentrations comparable to plasma nicotine levels in smokers (100 nM) (Henningfield et al. 1993). It has been proposed that desensitization may be necessary for up-regulation and that up-regulation following chronic nicotine exposure may compensate for this loss of receptor function (Fenster et al. 1999b). Paradoxically, studies on animal models of addiction report that chronic nicotine exposure causes an increased sensitivity to an acute nicotine pulse (Balfour et al. 2000; Benwell et al. 1995) and there is evidence from in vitro studies in mammalian cells to support this. Increased density of $\alpha 7$ binding to embryonic rat cortical neurons in culture was accompanied by an increased whole-cell response, and no evidence was found for long-lasting desensitization (Kawai and Berg 2001). This up-regulation was inhibited by inhibitors of protein synthesis, indicating that protein transcription is necessary. Human $\alpha 4\beta 2$ nAChRs stably expressed in HEK-293 cells (K-177 cells) show nicotine-induced up-regulation of nAChR function and remained functional even after prolonged exposure to nicotine (Gopalakrishnan et al. 1996, 1997). The $\alpha 4\beta 2$ receptors did not show desensitization following chronic nicotine exposure and could be activated in the presence of 100 nM nicotine (Buisson and Bertrand 2001). Moreover, following nicotine removal, the nAChRs had a higher apparent affinity for ACh; current amplitudes were increased and exhibited slower desensitization (Buisson and Bertrand 2001). These findings are contradictory to those from $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes (Fenster et al. 1996b; Olale et al. 1997) where nicotine exposure results in functional down-regulation of the nicotine response. In addition, chronic nicotine exposure has been reported to differentially inhibit the function of receptor subunits expressed in *Xenopus* oocytes: $\alpha 4\beta 2$ and $\alpha 7$ receptors are inhibited to a greater extent than $\alpha 3$-containing receptors (Olale et al. 1997). Using chimeras of the N-terminal region of the $\alpha 3$ subunit with the C-terminal of the $\alpha 4$ subunit, the same group demonstrated that the N-terminal extracellular domain determines the sensitivity to nicotine-induced activation (Kuryatov et al. 2000). This difference in the effect of nicotine in mammalian versus amphibian expression systems suggests that some of the chronic effects of nicotine exposure may be dependent on the host cell. Possible reasons for these differences include the different temperatures at which the proteins are expressed in amphibian versus mammalian cell lines, the presence or absence of serum in the culture medium, different protein kinase modulation of channels expressed in the two systems and differences in post-translational modification. Nicotine is lipophillic and may cross cell membranes and accumulate inside cells. The possibility that intracellular nicotine may inhibit nAChRs in *Xenopus* oocytes remains to be investigated.

Two hypotheses have been proposed to explain the possible mechanism of functional up-regulation. The first hypothesis puts forward that $\alpha 4\beta 2$ receptors might be recycled rapidly from the plasma membrane and that nicotine may cause a slowing of endocytosis from the membrane while receptors are added from an intracellular pool of presynthesized receptors, thereby increasing the total number of receptors in the cell membrane. The second hypothesis postulates that the total number of receptors in the membrane remains constant and that chronic nicotine exposure stabilizes receptors in a conformation, which has a higher affinity for the ligand. There is evidence from in vitro pharmacological experi-

ments in whole rat brain membrane preparations (Lippiello et al. 1987), *Xenopus* oocytes (Covernton and, Connolly 2000; Shafaee et al. 1999) and single-channel recordings in mammalian cell lines (Buisson and Bertrand 2001; Buisson et al. 2000) to indicate that both the rat and human $\alpha 4\beta 2$ receptors can exist in at least two different states: a high-conductance high-affinity state and a low-conductance low-affinity state. Although high- and low-affinity binding has been identified in different preparations, the state of the receptor to which they correspond is still unclear, and further studies are needed to clarify if a single receptor harbors both a high- and a low-affinity binding site or if they belong to different receptor states. While the mechanism underlying the up-regulation of nicotinic responses has not been elucidated, the appearance of an increased number of binding sites at the cell surface does not involve mRNA transcription, indicating that if receptors are inserted in the membrane these must come from some intracellular pool. This is confirmed by in vivo (Marks et al. 1992) and in vitro (Bencherif et al. 1995; Peng et al. 1994) studies, which show that steady state mRNA levels do not change during nAChR up-regulation following chronic nicotine exposure; however, the rate of protein synthesis or degradation may change.

Up-regulation of neurotransmitter release and functional up-regulation in vivo

In vitro studies in rat striatal synaptosomes confirm that these up-regulated nicotine-binding sites are functional receptors (Rowell and Wonnacott 1990). The up-regulation of the nicotinic binding sites has led many researchers to believe that up-regulation may be related to the addictive effects of nicotine, and there is evidence to suggest that this may be partly true. Nicotinic activation of nAChRs in the ventral tegmentum enhances glutamatergic inputs to dopaminergic neurons and has been demonstrated to produce long-term potentiation of excitatory inputs in the dopaminergic reward centers (Mansvelder and McGehee 2000).

The mechanisms underlying the up-regulation of ligand-binding sites and functional up-regulation are still unclear. The ability of molecules, such as mecamylamine, which bind to the extracellular domain of the nAChR without activating it, to cause up-regulation suggests that binding of certain ligands to receptor is sufficient to either slow down the removal of receptors from the membrane or to stabilize the receptor in a state which is consistent with the presence of a high-affinity ligand-binding site.

Nicotine-induced up-regulation was consistently observed both in vitro and in vivo, indicating that this is not a peculiarity caused by the experimental conditions. Since up-regulation of the amount of binding has been observed in postmortem human tissue, this increase may represent a determining step underlying nicotine addiction. Moreover, this observation is particularly illustrative of the long-term effects and possible brain reorganization, which can take place during prolonged drug intake. However, before understanding the physiological role of such receptor adaptation it remains to be demonstrated in vivo if receptors are also functionally up-regulated.

Modulation of nAChRs

The nAChR is an allosteric protein

The nAChRs have often been presented as a prototype of allosteric membrane protein (Changeux 1990). This type of model postulates that the protein can exist in different states and undergoes spontaneous conformational transitions. At rest, the equilibrium between these conformational states is in favor of the resting (closed) state. Exposure to an agonist preferentially stabilizes the receptor in the active (open) state. This behavior of the receptor can be described by the Monod-Wyman-Changeux model of allosteric interactions (Monod 1965) in which the structure of the molecule moves in concerted transitions between pre-existing conformational states, which may involve so-called rigid body movement of the subunits (Changeux and Edelstein 2001). The minimum model that adequately describes the nAChR function predicts that binding of the agonist molecule preferentially stabilizes the receptor in the active (A) or desensitized closed state (D) (see Edelstein et al. 1996). In his early work, Karlin (Karlin 1967) has described the binding of competitive ligands that stabilize the receptor in a closed conformation. It is interesting to note that in such model transition from one state to another depends upon both the presence of a ligand and/or the isomerization coefficient (L_0–L_3). Binding of a molecule at a site distinct from the agonist-binding site may therefore interact with the isomerization coefficient. Because they displace the equilibrium in favor of the active (open) state, molecules that reduce the isomerization coefficient L_0 are termed positive allosteric effectors. Similarly, compounds that increase the isomerization coefficient L_0, displacing the equilibrium in favor of the closed state, are termed negative allosteric effectors.

Numerous examples of positive and negative allosteric effectors acting at neuronal nAChRs have been reported and thereby illustrate the importance of this model. For example, it was shown that progesterone acts as negative allosteric effector at the $\alpha4\beta2$ receptor subtype (Valera et al. 1992), whereas the 17-β-estradiol acts as positive effector at this receptor subtype (Curtis et al. 2002; Paradiso et al. 2001). Predictions from the allosteric model indicate that receptors displaying a high L_0 coefficient, such as the $\alpha7$ receptor, must be more sensitive to positive effectors and that presence of such compounds must both increase the agonist sensitivity and the amplitude of the response. Experiments carried out with ivermectin, a powerful allosteric effector at the $\alpha7$ receptor (Krause et al. 1998) have confirmed these predictions. In addition, as expected from the model, ivermectin potentiation was accompanied by a change of the receptor pharmacological profile. Table 1 resumes our current knowledge of positive and negative allosteric effectors.

Steroid modulation of nAChRs

In addition to regulating gene expression, steroids are known to interact with ion channels. Some endogenous and synthetic steroids are potent allosteric modulators of the $GABA_A$ receptor, causing increased ionic current flow and enhancing inhibitory synaptic transmission. This enhancement of inhibitory $GABA_A$ neurotransmission is consistent with the sedative, anxiolytic and anticonvulsant affects of neurosteroid administration (see Lambert et al. 2001a, 2001b). Pregnane steroids are synthesized by neurons and glial cells in the CNS and may play an important role in modulating neuronal excitability (see Robel and Baulieu 1995). Steroids can interact directly with the ion channel protein or by perturba-

Table 1 Allosteric modulators of nAChRs

Compound	Effect	Reference
Ivermectin	Potentiation of ACh-evoked currents at chick and human $\alpha 7$ nAChRs in *Xenopus* oocytes	Krause et al. 1998
Ca^{2+}	Potentiation of ACh-evoked currents at $\alpha 7$ receptors in *Xenopus* oocytes	Eisele et al. 1993
	Potentiation of ACh-evoked currents at $\alpha 3\beta 4$, $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$ receptors in *Xenopus* oocytes	Vernino et al. 1992
	Potentiation of ACh-evoked currents at $\alpha 4\beta 2$ receptors in HEK 293 cells	Buisson et al. 1996
17β-Estradiol	Inhibition of ACh-evoked $^{86}Rb^+$ efflux in TE671/RD clonal or SH-SY5Y neuroblastoma cells expressing $\alpha 3\beta 4$ and muscle type receptors	Ke and Lukas 1996
	No effect at $\alpha 4\beta 2$ receptors in *Xenopus* oocytes	Nakazawa and Ohno 2001
	Potentiation of ACh-evoked currents at $\alpha 4\beta 2$ and $\alpha 4\beta 4$ receptors, but not at $\alpha 3\beta 2$ or $\alpha 3\beta 4$ receptors in *Xenopus* oocytes	Curtis et al. 2002
Corticosterone	Inhibition of ACh-evoked $^{86}Rb^+$ efflux in TE671/RD clonal or SH-SY5Y neuroblastoma cells expressing $\alpha 3\beta 4$ and muscle type receptors	Ke and Lukas 1996
Dexamethasone	Inhibition of ACh-evoked $^{86}Rb^+$ efflux in TE671/RD clonal or SH-SY5Y neuroblastoma cells expressing $\alpha 3\beta 4$ and muscle type receptors	Ke and Lukas 1996
Progesterone	Inhibition of ACh-evoked currents at chick $\alpha 4\beta 2$, $\alpha 3\beta 4$ and muscle type receptors	Bertrand et al. 1991; Garbus et al. 2001; Ke and Lukas 1996; Paradiso et al. 2001; Valera et al. 1992
Hydrocortisone	Shortens open times of adult and embryonic muscle type receptor expressed in HEK-293 cells	Bouzat and Barrantes 1996; Garbus et al. 2001
Tacrine	Increase in the number of $\alpha 4\beta 2$ receptors in SH-SY5Y and M10 cells	Svensson 2000; Svensson and Nordberg 1996, 1998
Galantamine	Potentiates ACh-evoked currents at human $\alpha 4\beta 2$ receptors in HEK–293 cells	Maelicke et al. 2001; Samochocki et al. 2000
Lynx-1	Increases sensitivity and rate of desensitization of $\alpha 4\beta 2$ receptors in *Xenopus* oocytes and BOSC cells	Ibanez-Tallon et al. 2002

tion of the lipid membrane environment. A number of natural steroids, which are synthesized in the brain, are known to interact with nAChRs and may represent an important physiological mechanism by which nAChR function is regulated in the brain. The lipophillic nature of steroids means they can readily cross the blood–brain barrier, thus presenting modulation of nAChRs by natural and synthetic steroids as a promising therapeutic strategy.

Promegestone has been shown to be a noncompetitive antagonist at the *Torpedo* nAChR (Blanton et al. 1999). The glucocorticoid hydrocortizone and mono-hydroxylated steroids such as progesterone shortened the open times of the muscle nAChR by up to 60% and displayed an inverse relationship between lipophilicity and their inhibitory potency, suggesting that their effects are not caused by a perturbation of the lipid membrane (Bouzat and Barrantes 1996; Garbus et al. 2001). Steroids did not affect binding of radio-

labeled cytisine to cell homogenates containing neuronal $\alpha4\beta2$ receptors (Sabey et al. 1999) and steroid effects at the nAChR were not affected by the presence of agonists, indicating a noncompetitive block mechanism. Analysis of the inhibitory potency of a range of different steroids has shown that potency is dependent on the structure of the steroid, with a hydroxyl group at position 11 being essential for inhibition of the muscle nAChR (Barrantes et al. 2000). A wide range of steroids has also been reported to inhibit ACh-induced currents at rat $\alpha4\beta2$ neuronal nAChRs expressed in HEK 293 cells (Paradiso et al. 2000; Sabey et al. 1999). The structure activity relationship of this effect has been investigated to determine the regions of the steroid molecule, which are important for activity at the nAChR. The inhibitory effect of $3\alpha,5\alpha,17\beta$-3-hydroxyandrostane-17-carbonitrile (ACN) was enantioslective, indicating an interaction with a stereoselective site on the nAChR. The potency was found to be strongly dependent on the orientation of the group at position 17. Progesterone also inhibits neuronal nAChRs from avian brain expressed in *Xenopus* oocytes in a noncompetitive manner (Bertrand et al. 1991; Valera et al. 1992).

ACh-activated currents were potentiated by 17β-estradiol through human $\alpha4\beta2$ and $\alpha4\beta4$ receptors expressed in *Xenopus* oocytes by increasing the apparent affinity of the receptor for ACh (Curtis et al. 2002; Paradiso et al. 2001). This effect was rapid in onset, concentration-dependent and readily reversible. In addition, 17β-estradiol increased the probability of opening of human $\alpha4\beta2$ nAChRs expressed in HEK 293 cells. The effect of 17β-estradiol showed enantioselectivity, indicating that its effects are not mediated by a disruption of the membrane lipid environment. The structural features of the steroid necessary for potentiation were an unsaturated A ring and a free hydroxyl group at positions 3 and 17 (Paradiso et al. 2001). No effect on the part of 17β-estradiol was observed at $\alpha3\beta2$ or $\alpha3\beta4$ receptors, suggesting that the $\alpha4$ subunit was necessary for these effects. It was reported that human but not rat $\alpha4\beta2$ receptors were potentiated by estradiol (Paradiso et al. 2001). Rat and human $\alpha4$ subunits differ in the C-terminal tail of the $\alpha4$ subunit, suggesting a likely location of the steroid-binding site. Chimeric subunits containing the N-terminal domain of the $\alpha4$ subunit and the C-terminal of the $\alpha3$ subunit were not potentiated by 17β-estradiol, whereas the $\alpha4$ C-terminal attached to a $\alpha3$ N-terminal was potentiated by 17β-estradiol. Truncation of the C-terminal tail of the $\alpha4$ subunit abolished the ability of 17β-estradiol to cause potentiation (Curtis et al. 2002). Mutation of residues in the C-terminus inhibited estradiol potentiation, the nature and position of the final four residues was critical, and extending the terminal sequence by inserting residues also abolished potentiation (Paradiso et al. 2001). The 17β-estradiol-binding site on the C-terminus was found to be distinct from the progesterone-binding site (Paradiso et al. 2001).

The sensitivity of nAChRs to modulation by steroids suggests that nAChRs may be implicated in conditions involving changes in hormone levels. Catamenial epilepsy is a condition that is associated with an increased incidence of epileptic seizures during particular phases of the menstrual cycle, and it has been estimated that 70% of women with focal epilepsies have catamenial tendencies (Herzog et al. 1997). Catamenial epilepsy has been linked to changes in plasma levels of sex hormones, including increased estrogen and decreased progesterone levels, a drop in serum progesterone levels, and to various metabolites of hormone breakdown (Herzog et al. 1997). Given the sensitivity of nAChRs to hormone modulation and the involvement of nAChRs in the generation of epileptiform activity, it is possible that modulation of nAChRs by neurosteroids could be involved in catamenial epilepsy.

Modulation of nAChRs by divalent cations

A number of divalent cations, including Ca^{2+}, Zn^{2+}, Mg^{2+}, Pb^{2+} and Cd^{2+} have been shown to modulate nAChR function (Eddins et al. 2002; Galzi et al. 1996; Hsiao et al. 2001; Vernino et al. 1992; Zwart et al. 1995). Physiologically, Ca^{2+} is likely to be the most important modulator of nAChRs. Ca^{2+} is released exocytotically from presynaptic neurons along with neurotransmitter (Brown et al. 1995). The extracellular Ca^{2+} concentration is maintained at approximately 1 mM; however, concentrations in the synaptic cleft can rise as high as 10 mM during periods of increased synaptic activity. ACh-induced currents through $\alpha 7$ receptors expressed in *Xenopus* oocytes are potentiated by elevated extracellular Ca^{2+} (Eisele et al. 1993). In tissue from mouse brain, Ca^{2+} modulated nAChR activation, increasing the maximal nicotine-induced response in a concentration-dependent manner (Booker et al. 1998). The increase in receptor activation could not be explained by changes in the ratio of activatable to desensitized receptors as assessed by the kinetics of ligand binding. Moreover, nAChR desensitization and recovery from desensitization was not modulated by Ca^{2+}. In cultured hippocampal neurons, extracellular Ca^{2+} was reported to modulate the activation and inactivation at $\alpha 7$ nAChRs. Increasing extracellular Ca^{2+} concentration from a starting level of 0.01 mM increased the efficacy of ACh at nAChRs with an EC_{50} of 0.1 mM; however, at higher concentrations (>1 mM), Ca^{2+} caused inactivation of nAChRs with an IC_{50} of 11 mM (Bonfante-Cabarcas et al. 1996). Neuronal $\alpha 3\beta 4$, $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$ nAChRs expressed in *Xenopus* oocytes (Vernino et al. 1992) and $\alpha 4\beta 2$ nAChRs in HEK 293 cells (Buisson et al. 1996) show potentiation of ACh-induced responses in raised extracellular Ca^{2+} and ACh-induced responses in neurons from the medial habenula are potentiated by increased concentrations of extracellular Ca^{2+} (Mulle et al. 1992).

Chimeric $\alpha 7$-V201-$5HT_3$ receptors containing the C-terminal end of the $5HT_3$-receptor are also potentiated by Ca^{2+}, whereas native $5HT_3$ receptors are not (Eisele et al. 1993), indicating that a binding site for Ca^{2+} is located on the N-terminal part of the $\alpha 7$ subunit. Single-point mutations in $\alpha 7$ receptors expressed in *Xenopus* oocytes have identified the Ca^{2+}-binding site in the 160–174 region of the receptor extracellular domain. This is located in close proximity to the ACh-binding site (Galzi et al. 1996). Ca^{2+} ions have been shown to increase the apparent affinity of nicotine binding to $\alpha 7$ receptors in both the active and desensitized states of the channel (Fenster et al. 1997). $\alpha 7$ receptors are highly permeable to Ca^{2+} and entry of Ca^{2+} ions through $\alpha 7$ nAChRs may activate other ion channels and intracellular Ca^{2+} cascades, possibly altering phosphorylation of the nAChRs. These effects would be most likely associated with long-term modulation of nAChR function.

Zinc is found in neurons throughout the brain, with the highest concentrations of zinc-containing neurons in the cerebral cortex and limbic system (Frederickson et al. 2000). Zinc is located in vesicles that are released from synaptic terminals by an increase in intracellular calcium. Zinc concentrations at the synapse have been estimated to reach 300 μM (Assaf and Chung 1984). Zinc has been shown to inhibit the current through homomeric $\alpha 7$ receptors expressed in *Xenopus* oocytes in a concentration-dependent manner (Palma et al. 1998). Hsiao and colleagues (Hsiao et al. 2001) reported that the effects of Zn^{2+} on nAChRs in *Xenopus* oocytes were dependent on the combination of subunits expressed. $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 4$ $\alpha 4\beta 2$ and $\alpha 4\beta 4$ receptors all showed biphasic modulation by Zn^{2+}. At 1–100 μM Zn^{2+}, the ACh-evoked response was potentiated with inhibition occurring at higher concentrations. Zinc is also known to modulate glutamate, GABA, glycine and

ATP channels (Bloomenthal et al. 1994; Cloues et al. 1993; Draguhn et al. 1990; Mayer et al. 1989; Paoletti 1997), suggesting it may play a role in modulating synaptic transmission.

Modulation by protein kinases

Phosphorylation is an important mechanism in the regulation of ligand-gated ion channels. This regulation includes modulation of receptor expression, subcellular localization and channel properties such as desensitization and recovery from inactivation. The functional role of nAChR phosphorylation was first investigated in receptors isolated from *Torpedo*. Both serine and tyrosine residues can be phosphorylated (Huganir 1991; Qu et al. 1990; Wagner et al. 1991), and this was shown to regulate receptor desensitization (Albuquerque et al. 1986; Middleton et al. 1986, 1988; Mulle et al. 1988). Subsequent investigation in chick ciliary ganglion demonstrated that $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ subunits are phosphorylated by PKA and PKC. Consensus sequences for PKA and PKC phosphorylation sites have been identified on the major intracellular loop between the M3 and M4 transmembrane segments of the rat, chick and human $\alpha 7$ and $\alpha 4$ subunits and two isoforms of the human $\alpha 1$ subunit (Wecker et al. 2001). At each of these putative phosphorylation sites the phosphorylated residue is a serine. Chronic nicotine exposure has been reported to cause phosphorylation of $\alpha 4$ subunits by PKA (Hsu et al. 1997). There is also direct evidence to indicate the involvement of PKA-mediated phosphorylation in the nicotine-induced up-regulation of nAChRs in PC12 cells. Chronic nicotine exposure caused an increase in the amount of [^{3}H]-nicotine binding in wild-type PC12 cells, which did not involve protein synthesis. This effect could be mimicked by an increase in intracellular cAMP; however, in mutant cells deficient in PKA, no increase in binding was seen (Madhok et al. 1994, 1995). Functional down-regulation of $\alpha 4\beta 2$ nAChRs in permanently transfected HEK 293 cells, in response to chronic nicotine exposure, was mediated by down-regulation of PKC activity. Recovery from down-regulation could be accelerated by inhibitors of protein phosphatases 2A and 2B, suggesting that nicotine-induced down-regulation of nAChRs involves dephosphorylation at PKC phosphorylation sites (Eilers et al. 1997). Activation of PKA and PKC in HEK 293 cells increased the surface expression of $\alpha 4\beta 2$ receptors and acted synergistically with nicotine to increase expression (Gopalakrishnan et al. 1997). $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes in which the serine at position 368 was replaced with an alanine residue did not recover from the nicotine-induced desensitized state (Fenster et al. 1999a). Viseshakul and colleagues (Viseshakul et al. 1998) provided direct evidence that the $\alpha 4$ subunit is phosphorylated in vivo and reported that in $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes, the $\alpha 4$ subunit is preferentially phosphorylated over $\beta 2$. Potentiation of rat $\alpha 7$, $\alpha 3\beta 2$ $\alpha 4\beta 2$, $\alpha 4\beta 4$ nAChRs expressed in *Xenopus* oocytes by arachidonic acid was via activation of PKC (Nishizaki et al. 1998). In rat and chick $\alpha 7$ nAChRs expressed in *Xenopus* oocytes, a conserved serine residue, Ser342, was phosphorylated by PKA; however, neither PKC, PKG or CAM KII caused significant phosphorylation (Moss et al. 1996). Phosphorylation of $\alpha 7$ nAChRs in vivo remains to be demonstrated; however, given that the intracellular domain of neuronal nAChRs is the most divergent region of the molecule, the conservation of the phosphorylation site suggests that phosphorylation may also play a role in modulating $\alpha 7$ receptor function.

Endogenous protein modulators

An endogenous peptide has been isolated from the mammalian CNS, which has a three-fingered structure and is analogous to the snake α-neurotoxins (Miwa et al. 1999). This protein, termed Lynx-1, is expressed in pyramidal neurons of the cortex and CA3 pyramidal neurons (Miwa et al. 1999). Lynx-1 has been shown to form stable complexes with $\alpha 7$ and $\alpha 4\beta 2$ nAChRs and to potentiate ACh-evoked currents and increase the rate of desensitization in vitro (Ibanez-Tallon et al. 2002; Miwa et al. 1999). The discovery of an endogenous peptide in humans with a high structural homology to snake α-neurotoxins raises the question of whether these molecules are involved in regulation of nAChR function in vivo. Much of the data obtained from brain slice recordings implicate nAChRs in a modulatory role. Modulation of these receptors by a variety of endogenous ligands may therefore represent a fine-tuning mechanism by which the activity of neuronal circuits can be subtly changed. The conservation of the three-fingered peptide structure over such a long period of divergent evolution hints that these molecules are likely to be physiologically important.

The wide expression of nAChRs in the brain, their contribution to the release of neurotransmitters and their sensitivity to a number of endogenous and exogenous substances indicate the complexity of the neuronal networks. Although no conclusion can yet be deduced from these observations, all point to the existence of complex regulatory processes. Thus, it can be proposed that nAChRs can be viewed as a system, which allows the fine-tuning of various brain areas either by circulating compounds or with a higher spatial resolution, by substances that are locally released.

nAChRs in brain function

Role of nAChRs in normal cognition

Although it is widely accepted that nicotine is the addictive compound contained in tobacco leaves and that it mediates its action through nAChRs in the CNS, little is known about the mechanisms involved in addiction. Nicotine elicits dopamine release in the VTA, a brain region known to play a role in addiction; however, the role of nAChRs in normal brain function is difficult to assess. The expression pattern of nAChR subtypes in the brain has been extensively investigated using labeled ligands specific for different subunits. The density of labeled ligand binding has provided an indication of which nAChR subunits may be involved in the function of a particular brain area.

Nicotine enhancement of cognition

Nicotine and a variety of nicotinic agonists, ABT-418, (Decker et al. 1994) SIB-1553A, (Bontempi et al. 2001) lobeline, (Decker et al. 1993) epibatidine, have been found to improve memory and learning in human and experimental animal models, whereas a block of nicotinic receptors impairs memory function (Newhouse et al. 1992; Rusted et al. 1994; Rusted and Warburton 1992). The effects of nicotine-induced improvements in cognition in humans can be most clearly seen in tests of attention (Levin and Rezvani 2000). Transdermal nicotine caused significant improvements in both nonsmokers and patients suffer-

ing from attention-deficit hyperactivity disorder, Alzheimer's disease and schizophrenia in computerized tests measuring response time and ability to distinguish between different visual stimuli (Levin and Rezvani 2000).

Animal models of cognitive function usually measure memory performance and learning and nicotine has been shown to increase memory performance in a variety of animal models, including rats and monkeys (Levin and Simon 1998). Both acute and chronic administration of nicotine was found to improve memory performance of rats in a radial-arm maze working memory test (Levin et al. 1996, 1997) and these effects were inhibited by the nAChR antagonist mecamylamine (Levin et al. 1993). The contributions of hippocampal $\alpha 7$ and $\alpha 4\beta 2$ receptor subtypes to increased memory performance have been assessed. Hippocampal infusion of the selective $\alpha 7$ antagonist MLA caused significant dose-related memory impairment in a radial-arm maze working memory test, which was not reversed by concurrent nicotine administration (Bettany and Levin 2001; Levin et al. 2002). Infusion of the antagonist DHβE (which is selective for $\alpha 4\beta 2$ receptors at low concentrations) also impaired working memory (Arthur and Levin 2002; Felix and Levin 1997; Levin et al. 2002) these effects were attenuated by acute systemic injection of nicotine. The specific roles played by the different nAChR subtypes in memory remain, however, to be determined.

Following neonatal exposure to nicotine, mice developed significant memory impairment; however, this did not appear until the age of 7 months (Ankarberg et al. 2001). The mechanism behind this is unclear. Nicotine binding in human hippocampus has been reported to be maximal in the late fetal stages, with a subsequent drop in binding density during the first 6 months of life followed by a plateau, (Court et al. 1997). This suggests that in neonatal stages of development there may be an excess of functional nAChRs and that memory impairment is revealed only when the density drops below a critical level.

What *nAChR* gene knockouts can reveal about CNS function

The use of genetic modifications targeted to nAChR subunits, in particular knockout (KO) mice lacking specific nAChR subunits, has provided new information on the possible receptor functions in the brain. The use of subunit-selective radioligand probes to label brain areas that are rich in particular receptor subtypes giving clues to which brain functions might be affected in the KO animal. KO mice have been created lacking the $\alpha 3$ (Xu et al. 1999a), $\beta 4$ (Xu et al. 1999b), $\beta 3$, $\alpha 6$ (Champtiaux et al. 2002) and $\alpha 9$ subunits (Vetter et al. 1999). KO mice lacking the principal subunits identified in the CNS, $\alpha 7$ (Franceschini et al. 2000; Orr-Urtreger et al. 1997; Paylor et al. 1998), $\alpha 4$ (Marubio et al. 1999; Ross et al. 2000) and $\beta 2$ (Xu et al. 1999b; Zoli et al. 1998) have also been created.

KO mice lacking the $\alpha 3$ subunit usually die in the 1st week of life due to multiorgan autonomic dysfunction (Xu et al. 1999a). $\alpha 3$-containing receptors are thought to be essential in mediating fast synaptic transmission in peripheral autonomic and sensory ganglia. Comparison of brains from $\alpha 3$ KO mice that survived to 8 days showed a significant loss of specific binding in the medial habenula and fasciculus retroflexus compared to wild-type mice (Whiteaker et al. 2002).

Mice deficient in the $\beta 4$ subunit survived to adulthood with no significant visible abnormalities; however, in these animals the nicotine-activated current in superior cervical ganglion neurons was significantly reduced (Xu et al. 1999b). Immunoprecipitation studies have shown that the principal nAChRs in sensory ganglia are made up of $\alpha 3$ and $\beta 4$ sub-

units (Flores et al. 1992). Studies of expression of mRNA in parasympathetic ganglia reported that all neurons expressed $\alpha 3$ mRNA, whereas just over half expressed $\beta 4$ mRNA (Poth et al. 1997), suggesting that in the $\beta 4$ KO, $\alpha 3$ may combine with other β subunits such as $\beta 2$ and be sufficient for adequate neuronal functioning. This is supported by the observation that mice with the double $\beta 2$ and $\beta 4$ KO die from multiorgan autonomic dysfunction similar to that observed in mice with the $\alpha 3$ KO (Xu et al. 1999b). $\beta 3$ KO mice showed loss of α-conotoxin MII and epibatidine-binding sites in the interpeduncular nucleus and loss of nicotine-induced dopamine release in the striatum. However, no anatomical or behavioral data is available for this subunit KO (see Cordero-Erausquin et al. 2000).

Mice lacking the $\alpha 6$ subunit did not display any obvious neuroanatomical or behavioral abnormalities. High-affinity [^{3}H]-nicotine, [^{3}H]-epibatidine and [^{3}H]-cytisine binding was decreased in retinal ganglion cells. High-affinity binding sites for [^{125}I]-α-conotoxin MII completely disappeared in the brain, indicating that these sites must correspond to $\alpha 6$-containing receptors (Champtiaux et al. 2002) or that remodeling of nAChR expression had taken place due to the $\alpha 6$ removal. [^{125}I]-α-conotoxin binding was lost from the mesostriatal dopamine system and the habenulo-interpeduncular system. The α-conotoxin MII was previously thought to be selective for $\alpha 3\beta 2$ receptors (Cartier et al. 1996); however, given the high sequence homology between the $\alpha 6$ and $\alpha 3$ subunits, it would not be surprising that the toxin could bind to both receptors. α-conotoxin MII has previously been demonstrated to inhibit nicotine-induced dopamine release in striatal synaptosomes (Kaiser et al. 1998; Kulak et al. 1997), and it was assumed that nicotine was acting through $\alpha 3\beta 2$ receptors. In light of these recent findings, it is likely that the $\alpha 6$ subunit forms functional receptors with $\beta 2$ subunits. Given the location of these receptors in the dopaminergic reward system, these results suggest that $\alpha 6$-containing receptors may play a role in nicotine addiction.

$\alpha 9$ Subunits are thought not to be expressed in the brain. $\alpha 9$ KO mice showed abnormal development of synaptic connections in cochlear outer hair cells as well as abnormal cochlear responses following efferent fiber activation (Vetter et al. 1999), suggesting $\alpha 9$ receptors play a role in this cholinergic system.

nAChRs containing the $\alpha 7$ or $\alpha 4\beta 2$ subunits are the most abundant and therefore presumably the most important nAChRs present in the CNS. A number of disease states and abnormal behavioral conditions have been linked to these receptors in humans. mRNA transcripts coding for the $\alpha 7$ subunit can be detected at embryonic day 13 in the rat cortex. $\alpha 7$ mRNA expression increased into the 1st postnatal week and was followed by a steady decline into adulthood (Broide et al. 1995), suggesting that receptors containing the $\alpha 7$ subunit may play a role in development. $\alpha 7$ KO mice showed no detectable α-BgTX-binding sites, confirming that the $\alpha 7$ nAChR contributes most of the α-BgTX-binding sites in the CNS. The $\alpha 7$ KO did not show any significant up-regulation of high-affinity nicotine-binding sites and appear to have normal growth, viability and brain neuroanatomy (Orr-Urtreger et al. 1997). Behavioral assessments in $\alpha 7$ KO mice demonstrated that the $\alpha 7$ subunit was not required for normal behavioral responses (Paylor et al. 1998). However, the $\alpha 7$ KO lacked rapidly desensitizing nicotinic currents in hippocampal neurons, suggesting that $\alpha 7$-containing receptors may be involved in learning and memory (Orr-Urtreger et al. 1997). A threonine for leucine substitution at position 247 in the M2 channel domain of the chick $\alpha 7$ nAChR renders one of the desensitized states of the receptor conductive, increasing the apparent affinity for ACh. This mutation also slows the rate of

desensitization, creating a gain-of-function model for the α7 receptor (Bertrand et al. 1992; Revah et al. 1991). Mice heterozygous for the analogous α7L250T mutation (+/T) survived to adulthood. Hippocampal neurons from these mice had α7 type currents with increased amplitude and slowed desensitization (Broide et al. 2002). +/T mice showed increased sensitivity to nicotine-induced seizures, which could be abolished by pretreatment with the α7 antagonist MLA (Broide et al. 2002; Gil et al. 2002). In contrast, the $\alpha7^{-/-}$ mice showed normal sensitivity to nicotine-induced seizures, suggesting that the properties of α7 receptors rather than their presence is important in susceptibility to nicotine-induced seizures. Mice homozygous for the L250T gene (T/T) die within 1 day of birth and their brains exhibit a marked reduction in α7 nAChR expression and show extensive apoptotic cell death throughout the somatosensory cortex, with abnormal layering of the cortex. The α7L250T nAChRs were functionally expressed on neurons within the brains of neonatal mice and have properties consistent with rat α7L250T and the homologous chick α7L247T nAChRs expressed in *Xenopus* oocytes (Orr-Urtreger et al. 2000). These results suggest that neurons expressing the α7L250T nAChRs may die from excessive calcium entry through the mutant nAChRs. Mice carrying a single copy of the L250T gene (–/T) have normal levels of apoptosis and cortical development, but die within 1 day of birth, similar to T/T homozygous mice, suggesting apoptotic cell death is not the cause of death in the T/T mice (Broide et al. 2001).

nAChRs containing the α4 and β2 subunits are the most common in the brain and contribute the high-affinity ACh-binding site. In mice lacking the β2 subunit, locomotion was significantly reduced, suggesting that this subunit may be involved in the physiology of motor control. Nicotine stimulates dopamine release in the ventral striatum of wild type mice, but not in β2 KO mice. Patch clamp recordings from mesencephalic dopaminergic neurons (Picciotto et al. 1998) and thalamic neurons (Picciotto et al. 1995) from β2 KO mice no longer responded to nicotine. Self-administration of nicotine is reduced in β2 KO mice trained to respond for cocaine in a substitution protocol, suggesting that nAChRs containing the β2 subunit participate in mediating the reinforcing properties of nicotine (Picciotto et al. 1998). Aged (22 –24 months old) β2 KO mice exhibited alterations in cortical regions, including neocortical atrophy, loss of hippocampal pyramidal neurons, astro- and micro-gliosis and elevation of serum corticosterone levels. These mice showed significantly impaired spatial learning, suggesting that β2 subunit-containing nAChRs contribute to neuron survival and maintenance of cognitive performance and learning during aging (Zoli et al. 1999).

KO mice lacking the α4 subunit survived to adulthood, but expressed less high-affinity [^{3}H]-nicotine and [^{3}H]-epibatidine-binding sites in the brain. These KO mice displayed a reduced antinociceptive effect of nicotine on the hot-plate test and a reduced sensitivity to nicotine in the tail-flick test. Patch-clamp recordings from raphe magnus and thalamic neurons showed that the high-affinity nicotine-induced response was no longer present (Marubio et al. 1999).

Perhaps the most surprising result from the study of mice with *nAChR* gene knockouts is the high level of viability, with only the α3 subunit being necessary for survival. Although interesting information on the physiological role of specific receptors may be obtained from studying the phenotypes of KO mice, the question remains of whether other receptor subtypes or signaling mechanisms can compensate for the loss of a specific subunit in these animals. KO mice lack the subunit of interest throughout their entire development and it is possible that other neuronal circuits adapt or the expression of other receptor

subunits is altered to compensate for the loss of a receptor subtype. The use of conditional and inducible KO may give additional information on the involvement of individual subunits in brain function.

Because high-affinity nAChRs, which are thought to correspond to the $\alpha4\beta2$ subtype, have been shown to be reduced in neurodegenerative diseases, it is interesting to compare our knowledge between KO mice and human clinical phenotypes. For example, the impairment of motility observed in the $\beta2$ KO could resemble that observed in neurodegenerative diseases such as Parkinson's disease. The specific loss of nAChR-expressing neurons could correspond to a reduction of cholinergic tone at this nucleus.

nAChRs in brain dysfunction

Diseases affecting nAChR function can lead to serious impairment of physiological processes. The importance of nAChRs in human disease of the nervous system has already been addressed in several studies (Lena and Changeux 1997; Lindstrom 1997; Paterson and Nordberg 2000; Weiland et al. 2000) and thereby illustrates the relevance of this fundamental topic. An example of this is myasthenia gravis, which is a disease characterized by muscular weakness caused by impaired neuromuscular transmission (see Vincent et al. 2001 for recent review). Myasthenia gravis can be divided into two types according to the presence or absence of genetic transmission. In the genetically transmissible form, it has been shown that pathologies are caused by the alteration of genes coding for the neuromuscular nAChR. In contrast, the large majority of myasthenia gravis cases are not genetically transmissible but arise from the production of autoantibodies that bind to the extracellular domain of the muscle nAChR leading to loss of nAChR function. This has been confirmed by immunoprecipitation of [^{125}I]-αBgTX-labeled receptors from human muscle (Lindstrom et al. 1976). Although no autoimmune diseases that affect neuronal nAChRs have been identified so far, loss of central nAChR function is believed to contribute to a number of disease states. Therefore similar mechanisms to myasthenia gravis may be involved in CNS disorders.

Diseases involving neuronal nAChRs can be divided into two broad categories: those in which nAChR function is altered, such as autosomal dominant frontal lobe epilepsy (ADNFLE), and those involving an apparent reduction in the number of nAChRs present, including schizophrenia and neurodegenerative conditions such as Alzheimer's and Parkinson's disease. Although the direct involvement of nAChRs in many of these diseases has not been demonstrated, the beneficial effect of drugs acting at nAChRs suggests that these receptors are directly or indirectly involved.

Epilepsy

Epilepsy affects around 1% of the general population. ADNFLE is genetically transmissible in an autosomal dominant mode and in which seizures originate from the frontal lobe. This form of epilepsy is characterized by clusters of seizures that occur mainly during sleep, either in the first hour of falling asleep or within the final hour before awakening, but rarely while the patient is awake. The first symptoms of the disease usually occur before the age of 20, with a mean age of onset of 12 years (Scheffer et al. 1994). In some

families ADNFLE has been directly associated to mutations in the gene coding for either the α4 or β2 subunit of the nAChR.

The autosomal dominant transmission of ADNFLE indicates that sufferers are heterozygous for this locus and carry one normal and one mutated allele. The penetrance of the disease is approximately 70%, with different members of the same family affected to different extents (Hayman et al. 1997). The gene coding for the α4 subunit (*CHRNA4*) is located on chromosome 20 (*20q 13.33*) and the gene coding for the β2 subunit (*CHRNB2*) is on chromosome *1q23.1*. To date, five different mutations that lead to ADNFLE have been identified in different families. These mutations are single nucleotide polymorphisms (SNPs), which cause either the exchange of a single amino acid or in one case the insertion of an extra codon that corresponds to an extra amino acid in the transcribed protein. The effects of these changes in the amino acid sequence on the functioning of the nAChR have been investigated in recombinant nAChRs expressed in *Xenopus* oocytes using the two-electrode voltage-clamp technique. The initial characterization of nAChR mutants was done using single allele expression of mutant subunits (Bertrand et al. 1998; Figl et al. 1998; Kuryatov et al. 1997; Picard et al. 1999; Weiland et al. 1996). These receptors displayed phenotypes with rapid desensitization (α4-S248F) (Kuryatov et al. 1997; Weiland et al. 1996) and use dependent functional up-regulation (α4-S248F, α4-L-776ins3) (Figl et al. 1998; Kuryatov et al. 1997). However, as individuals suffering from ADNFLE are heterozygous and carry only one copy of the mutated gene, these experiments are not consistent with an accurate representation of the nAChR subunit composition in vivo.

The electrophysiological properties of heterozygous nAChRs containing ADNFLE mutations have been characterized in *Xenopus* oocytes co-injected with both mutant and wild-type DNA. The maximal currents in the *α4-S248F*, *α4-L-776ins3*, *α4-S252L* and *β2-V287M*, *β2-V287L* mutants were not significantly different from control $\alpha4\beta2$ receptors; however, sensitivity of the mutant receptors to ACh was significantly increased for all mutations (Moulard et al. 2001; Phillips et al. 2001). The Australian α4-S248F and Norwegian α4-L-776ins3 mutant receptors have been reported to have a lower calcium permeability than control receptors (Kuryatov et al. 1997; Steinlein et al. 1997); however, this property was not common to all the ADNFLE mutations. A varying sensitivity to the antiepileptic drug carbamazepine (CBZ) has been reported among ADNFLE sufferers. In vitro experiments show that the *α4-S248F* and *α4-L-776ins3* mutations are approximately fourfold more sensitive to inhibition by CBZ than control receptors (Picard et al. 1999); however, the CBZ sensitivity of the *α4-S252L* mutation was unchanged (Moulard et al. 2001). To date, however, given the small number of cases, no correlation can be made between CBZ sensitivity and the patients' condition.

Armed with this knowledge of the ADNFLE mutation at the nAChRs level, it is tempting to consider how these altered receptor properties can lead to seizures. Data from EEG and SPECT studies indicate that seizures originate in the frontal cortex during stage 2 of sleep (Scheffer et al. 1995). The thalamocortical system is rich in cholinergic fibers and has been demonstrated to play an important role in sleep regulation (Steriade et al. 1993). nAChRs are highly expressed in the cortex and given the lack of evidence for the involvement of nAChRs in EPSP generation, the modulation of cortical neurons by extrasynaptic nAChRs should be considered. Acetylcholine (ACh) has been demonstrated to have facilitatory effects on pyramidal neurons, which are the principal excitatory cells of the cortex (McCormick and Prince 1986). Two models of cholinergic innervation have been proposed in the cerebral cortex. The first is that communication is not mediated via classic

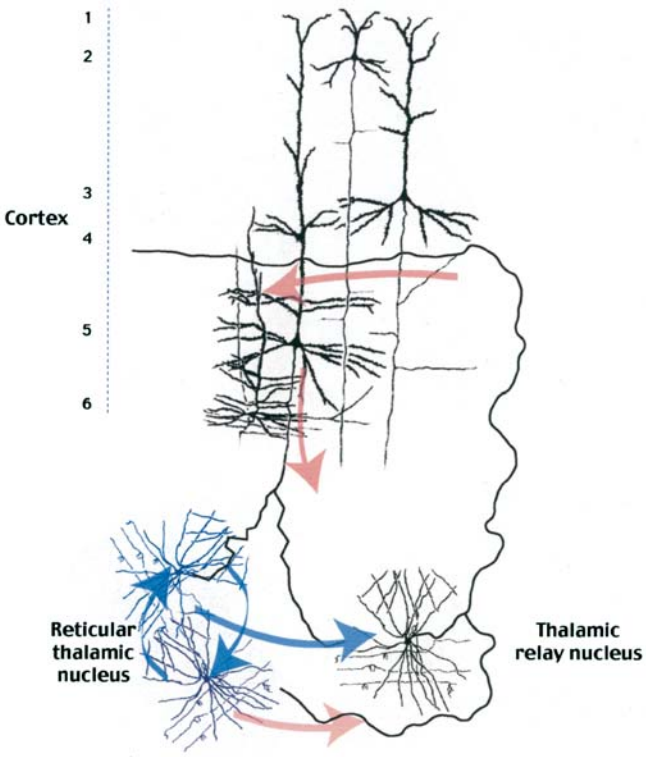


Fig. 4 Schematic diagram of cholinergic neurons involved in the thalamocortical loop (TC) and corticoreticular thalamic circuit (CRT). The positive feedback of the TC loop and the negative feedback of the CRT are balanced. Release of ACh from *en-passant* fibers causes synchronization of the CRT loop leading to increased stimulation of TC neurons, thus drawing the circuit into oscillation

synapses and involves extrasynaptic nAChRs. This has been termed volume transmission and the majority of studies support this model. Diffuse cholinergic transmission has been reported in many regions of the CNS and Descarries and co-workers (Descarries et al. 1997) have highlighted the possible role of ACh release from the varicosities of *en-passant* fibers in modulating the activity of cortical pyramidal cells. However, there is a second model based on evidence from ultrastructural studies that cholinergic innervation of the cortex can be mediated by synaptic contacts as opposed to volume transmission (Turrini et al. 2001). A schematic representation of the connections between the thalamus and cortex is shown in Fig. 4 and illustrates the positive feedback of the thalamocortical loop and the negative feedback of the corticoreticular thalamic circuit. In the normal brain, the positive and negative circuits are in balance. The firing of the thalamic neurons is under a strong cholinergic influence. If mutant nAChRs on the thalamic neurons have an increased sensitivity to ACh the firing of this positive feedback loop will be increased, resulting in stronger stimulation of the cortical pyramidal cells. This imbalance may provoke a difference in the phase of the circuit and promote its oscillation. Currently the relative distribution of nAChRs and their effects on synaptic or extrasynaptic cholinergic transmission are unknown. However, it can be proposed that the increased sensitivity of the mutant nAChRs

to ACh on thalamic neurons may play the key role in triggering the synchronization of cortical neurons. Mutations in gene coding for the $GABA_A$ receptor $\gamma 2$ subunit have also been found in families suffering from general epilepsy and febrile seizures (Baulac et al. 2001; Harkin et al. 2002; Wallace et al. 2001). The $GABA_A$ receptor is a ligand-gated ion channel and the $\gamma 2$ subunit is structurally analogous to the nAChR subunits having four transmembrane domains. To date, two mutations have been identified: *GABAG2(R43Q)*, which reduces the sensitivity of the receptor to benzodiazipines, and *GABAG2(Q351X)*, which introduces a premature stop codon and inhibits the surface expression of functional $GABA_A$ receptors. Since $GABA_A$ receptors act to reduce neuronal excitability, gene mutations that alter the functioning of these receptors fit the overall scheme of impaired inhibitory neurotransmission in individuals suffering from epilepsy.

Schizophrenia

Schizophrenia is a chronically deteriorating psychosis which begins in late adolescence or early adulthood and involves hallucinations, disturbances of thought, self-awareness, and perception and is characterized by bizarre behavior and abnormal social interaction. Around 1% of the population is affected, with a further 2%–3% of the population suffering schizotypal personality disorder, which is a milder form of the disease. Abnormalities in dopaminergic synaptic transmission are thought to be involved in schizophrenia, with the hypothesis that excessive release of dopamine causes overactivity at synapses in the mesolimbic system. Drugs that increase dopamine levels such as cocaine and amphetamines can induce psychotic episodes, which resemble those in paranoid schizophrenia.

Involvement of nAChRs in schizophrenia was first suggested by the high percentage of smokers among schizophrenics, 90% as compared to 33% in the general population, (Lohr and Flynn 1992). It was observed that the amount of [^{125}I]-α-BgTX and [^{3}H]-cytisine binding was reduced in the CA3 region of the hippocampus in the brains of schizophrenics (Freedman et al. 1995). Postmortem studies have shown that high-affinity nicotinic binding sites are increased in the brains of smokers (see "Up-regulation of nAChR ligand binding"). In contrast, the brains of schizophrenic smokers had reduced numbers of nicotinic receptors compared to control smokers (Breese et al. 2000). It has been suggested that the high percentage of smokers in the schizophrenic population may represent a form of self-medication to compensate for a deficit in nicotinic neurotransmission (Goff et al. 1992). Schizophrenia has a strong genetic component; however, the patterns of inheritance are complex and to date a number of loci have been linked to the disease. The disease is characterized by a number of symptoms, which most likely represent a group of related disorders, making it difficult to link any particular loci to a specific clinical trait. A characteristic of some schizophrenics and their nonschizophrenic relatives is decreased inhibition of P50 brain waves in response to paired auditory stimuli (Freedman et al. 1983, 1987; Siegel et al. 1984). This is manifested as an acute sensitivity to the second auditory stimulus, which is normally reduced in nonschizophrenics. In schizophrenics this abnormal response becomes normal following nicotine administration or smoking (Adler et al. 1992, 1993), and in rodents the $\alpha 7$ antagonists α-BgTX and MLA induce a loss of gating (Leonard et al. 2000). DBA/2 mice spontaneously exhibit a similar deficit in sensory inhibition and can be viewed as an animal model for schizophrenia. DMXB-A, a partial agonist at the $\alpha 7$ nAChR, improved sensory inhibition in DBA/2 mice. This improvement was blocked by

α-BgTX, but not by mecamylamine, suggesting that DMXB-A acts through the α7 nAChR (Simosky et al. 2001). The α7 subunit was found to be significantly decreased in the frontal cortex but not in the parietal cortex of schizophrenics, suggesting that a deficit of α7 subunits in the frontal cortex might contribute to the pathophysiology of schizophrenia (Guan et al. 1999). Abnormal smooth pursuit eye movement is one of the most common abnormalities associated with schizophrenia (Levy et al. 1993). Nicotine has been shown to improve the defects associated with this condition in schizophrenics (Adler et al. 1993).

Using genome-wide analysis, schizophrenia has been linked to a dinucleotide polymorphism at chromosome *15q13-14*, which is the site of the α7-subunit gene *CHRNA7* (*15q13.1*). This suggests that the *α7 nAChR* gene may be responsible for the inheritance of at least one aspect of the disease (see "nAChR genes and expression"). Other studies, however, have found no evidence for genetic linkage (Curtis et al. 1999; Neves-Pereira et al. 1998). Although the pharmacological evidence for the involvement of the α7 nAChR in schizophrenia is strong, the data concerning the involvement of the gene coding for the α7 nAChR subunit in schizophrenia is unclear and the positioning of the locus on chromosome 15 is unreliable. Several areas on chromosome 15 have obtained support from individual samples; however, a susceptibility gene for schizophrenia remains to be demonstrated (Gejman et al. 2001).

One of the difficulties in trying to correlate schizophrenia to a possible alteration of the α7 coding gene arises from a peculiarity at chromosome 15. Studies have shown that in the majority of the population chromosome 15 displays a duplication of the α7 exons 6–10. This partial duplication of the α7 gene prevents qualitative trait loci (QTL) analysis and has therefore slowed down the research in this field.

Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease characterized by motor dysfunction resulting in muscular rigidity, tremor and difficulty in initiating and sustaining movement. Patients suffering from PD show reduced dopamine levels in the striatum and this has been shown to be caused by degeneration of neurons in the substantia nigra pars compacta. Evidence from in vivo experiments in rodents demonstrates that prolonged nicotine administration can prevent degeneration of dopaminergic neurons (Janson et al. 1988, 1989, 1992). Nicotine has been shown to markedly improve the symptoms of PD patients (Kelton et al. 2000) and the beneficial effects appear to be due to increased synaptic dopamine levels in the substantia nigra (Lichtensteiger et al. 1982) and mesolimbic system (Kita et al. 1992) and possibly inhibition of monoamine oxidase B (Mihailescu et al. 1998). Smokers were observed to have a lower incidence of PD than the general population, with half the risk of developing the disease (Baron 1986; Morens et al. 1995). A recent study examining the relationship between smoking and PD in twins has shown that the risk of developing PD is inversely correlated with the number of cigarettes smoked. This effect was most pronounced in monozygotic twins (Tanner et al. 2002). Patients with PD showed significantly reduced high-affinity nicotine binding in the pars compacta, substantia nigra and dorsolateral tegmentum. The down-regulation of the nAChR was closely associated with primary histopathological changes in PD, Alzheimer's disease and Lewy body dementia, suggesting that down-regulation of the nAChR may precede neurodegeneration in these diseases (Perry et al. 1995).

Nicotine has been demonstrated to have a wide range of neuroprotective effects in the CNS, including delaying the aging process of nigrostriatal neurons (Prasad et al. 1994) and protecting against exocytotic cell death (Marin et al. 1994). The neuroprotective effects of nicotine have been attributed to a wide range of effects, including an increase in the expression of neurotrophic factors (Freedman et al. 1993), inhibition of nitric oxide production (Shimohama et al. 1996) and activation of protein kinase C (Li et al. 1999). Some studies have shown that neuroprotective effects are correlated with the ability to activate $\alpha 7$ nAChRs (Jonnala and Buccafusco 2001) and could be blocked by the selective $\alpha 7$ inhibitor MLA (Dajas-Bailador et al. 2000). $\alpha 7$ nAChRs are highly permeable to Ca^{2+} and nicotine has been shown to increase in intracellular Ca^{2+} in primary hippocampal cell cultures (Dajas-Bailador et al. 2000). It is possible that entry of extracellular Ca^{2+} through activation of $\alpha 7$ nAChRs has effects that promote neuron survival.

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative condition that affects almost 10% of individuals over the age of 65 (Evans et al. 1989) and is characterized by a progressive loss of short-term memory and higher cognitive functions. Postmortem brains from AD sufferers characteristically show intracellular neurofibrillary tangles and extracellular neuritic senile plaques as well as cell loss, cell atrophy, cortical shrinkage and changes in ACh, GABA, glutamate and 5HT neurotransmission. However, one of the most consistent and marked changes in the neurotransmitter system is the degeneration of the cholinergic innervation of the hippocampus and cerebral cortex (Delacourte and Defossez 1986). The activity of choline acetyl transferase (ChAT) is significantly reduced in the hippocampus and cerebral cortex of AD sufferers (Coyle et al. 1983) and a linear correlation is seen between the reduction in cortical ChAT activity (Perry et al. 1978) and the progress of dementia. The cholinergic hypothesis for AD attributes the deterioration of cognitive functions to degeneration of the cholinergic pathways from the basal forebrain to the cortex and hippocampus (Bartus et al. 1982), with specific loss of neurons expressing nAChRs. Muscarinic receptor-binding activity remained unchanged with increasing senile plaque formation, indicating these receptors are little affected (Perry et al. 1978). Significant reductions in [^{3}H]-nicotine, [^{3}H]-cytisine and [^{3}H]-epibatadine binding have been observed in the temporal cortex of AD sufferers (Sihver et al. 1999). Competitive binding studies with the agonists (–)nicotine, epibatidine, ABT-418 in competition with [^{3}H]-nicotine and [^{3}H]-cytisine suggested that the $\alpha 4\beta 2$ nAChRs were preferentially lost in AD (Warpman and Nordberg 1995).

Data presented so far suggest that reduced function of neuronal nAChRs in the brain is implicated in AD. The current treatment for AD uses cholinesterase inhibitors, which act by slowing the breakdown of ACh, making more ACh available at the synapse to interact with receptors. Other evidence also suggests that some cholinesterase inhibitors (tacrine and galantamine) interact directly with the nAChRs to cause potentiation via an allosteric mechanism (see Table 1) (Maelicke et al. 2001; Samochocki et al. 2000; Svensson 2000; Svensson and Nordberg 1996).

There is circumstantial evidence to suggest that nAChRs are involved in memory mechanisms (see "Nicotine enhancement of cognition"). Nicotine has been observed to improve the performance of humans in memory-related tasks (Rusted and Warburton 1992; Rusted et al. 1994) and the nicotinic antagonist mecamylamine has been reported to

impair short-term memory (Newhouse et al. 1992). It has also been suggested that activation of nAChRs can have neuroprotective effects (Janson et al. 1989; Nakamura et al. 2001) and improve memory and cognition in individuals suffering from AD (Potter et al. 1999; Rusted et al. 2000).

Neuritic plaques found in the brains of AD sufferers contain β-amyloid peptides, which have been reported to be neurotoxic (Allen et al. 1995; Forloni 1996; McKee et al. 1998). The majority of the β-amyloid peptide found in the brains of AD sufferers is 40 amino acids in length ($A\beta_{1–40}$) and a small fraction is made up of peptide that is 42 amino acids in length ($A\beta_{1–42}$) (Kuo et al. 1996). There is evidence to suggest that activation of $\alpha7$ and $\alpha4\beta2$ nAChRs can reduce β-amyloid toxicity (Kihara et al. 1997, 1998). Recently $A\beta_{1–40}$ and $A\beta_{1–42}$ were shown to bind to $\alpha7$ nAChRs with high affinity (HY Wang et al. 2000a, 2000b). Nanomolar concentrations of $A\beta_{1–40}$ and $A\beta_{1–42}$ have been demonstrated to inhibit hippocampal $\alpha7$ nAChRs (Liu et al. 2001; Pettit et al. 2001); conversely, $A\beta_{1–42}$ activated rat $\alpha7$ nAChRs expressed in *Xenopus* oocytes (Dineley et al. 2002). The effects of β-amyloid peptides on different nAChRs in the CNS remain to be described.

Tourette's syndrome

Tourette's syndrome is a neuropsychiatric disorder characterized by persistent motor and verbal tics and commonly associated with aggression, hyperactivity, obsessive–compulsive behavior, phobias, and general anxiety. It has been estimated that the prevalence of Tourette's syndrome in school-age children is 0.15%–1.1% (Kadesjo and Gillberg 2000). Tourette's syndrome usually appears before the age of 18 and leads to severe learning difficulties. The disease is believed to be transmitted in an autosomal dominant manner; however, the penetrance is variable, with a broad range of expression within families. The pathogenesis of Tourette's syndrome is as yet unknown, but is believed to be associated with abnormal neurotransmission in the basal ganglia. Several studies have reported that orally or transdermal administration of nicotine reduced the severity of the tics associated with the disease (Dursun and Reveley 1997; McConville et al. 1992; Sanberg et al. 1989), suggesting that nAChRs may play a role in the etiology of Tourette's syndrome. The effects of nicotine were seen immediately and persisted during chronic administration. Nicotine also potentiated the effects of haloperidol (Sanberg et al. 1989; Silver et al. 2001). The mechanisms by which nicotine exerts its beneficial effects in Tourette's syndrome are unclear. The nAChR antagonist mecamylamine also had a beneficial effect on tics (Sanberg et al. 1998; Shytle et al. 2000; Silver et al. 2000) suggesting nicotine may be causing a down-regulation of nAChR function, possibly due to desensitization or via interaction with another neurotransmitter system.

Depression and attention-deficit hyperactivity disorder

Depression and attention-deficit hyperactivity disorder (ADHD), although not related, are both believed to involve nAChR dysfunction. Smoking is more prevalent in patients suffering from ADHD (40%) and depression (46%) than in the general population (26%) (Pomerleau et al. 1995) and may represent self-medication. Transdermal nicotine has been demonstrated to cause a significant improvement in nonsmoking depressive patients (Salin-Pascual and Drucker-Colin 1998) and to improve symptoms in patients suffering

from ADHD (Pomerleau et al. 1995). A direct involvement of nAChRs in either disease remains to be demonstrated. A recent study has identified a polymorphic gene duplication on chromosome *15q24–26* (named DUP25), which has been linked with panic and phobic disorders (Gratacos et al. 2001). This is close to the loci identified for the human α7 gene (*15q13.1*) and α5, α3 and β4 (*15q24.3*) *nAChR* genes. Although there is no direct evidence of altered nAChR function in individuals suffering from these disorders, it is possible that the genes coding for these receptor subunits could be affected. Alternatively, the duplication of *nAChR* genes could lead to overexpression of certain nAChR subunits. This change in the expression ratio of subunits may be enough to alter the electrophysiological properties of the neurons expressing these nAChRs.

Conclusions

Work reviewed herein illustrates the importance of nAChRs in the central and peripheral nervous system. To date, eleven genes coding for the nAChRs have been identified in the human genome and the range of possible subunit combinations has been shown to provide a wide range of physiological and pharmacological profiles. Functional studies have revealed that nAChRs contribute to the control of resting membrane potential, modulation of synaptic transmission and mediation of fast excitatory transmission. These mechanisms depend upon the mode of ACh release as well as the receptor localization.

The widespread expression of nAChRs and their highly specific organization, both subcellularly and with respect to different neuronal populations, illustrates their relevance in normal brain function. The contribution of nAChRs to cognitive processes such as learning and memory, movement control, etc., has been demonstrated in normal subjects. Supported by evidence from self-administration of the natural agonist nicotine, these receptors are assumed to play a determinant role in tobacco addiction. Moreover, numerous human brain diseases have been linked to nAChR dysfunction. Diseases such as schizophrenia, Alzheimer's and Parkinson's disease affect a substantial proportion of the population and are major public health issues. A particular form of genetically transmissible epilepsy (ADNFLE) has been associated with a mutation in the gene coding either for the α4 or the β2 subunit and is an excellent example of how collaboration between clinical and molecular neuroscientists can lead to breakthroughs in our understanding of disease states. These studies highlight the need for a multidisciplinary approach to investigate nAChR function and dysfunction and the need for collaboration between neuroscientists at the basic and clinical level.

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O.-M. H. Richter · B. Ludwig

Cytochrome *c* oxidase – structure, function, and physiology of a redox-driven molecular machine

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Abstract Cytochome *c* oxidase is the terminal member of the electron transport chains of mitochondria and many bacteria. Providing an efficient mechanism for dioxygen reduction on the one hand, it also acts as a redox-linked proton pump, coupling the free energy of water formation to the generation of a transmembrane electrochemical gradient to eventually drive ATP synthesis. The overall complexity of the mitochondrial enzyme is also reflected by its subunit structure and assembly pathway, whereas the diversity of the bacterial enzymes has fostered the notion of a large family of heme-copper terminal oxidases. Moreover, the successful elucidation of 3-D structures for both the mitochondrial and several bacterial oxidases has greatly helped in designing mutagenesis approaches to study functional aspects in these enzymes.

Abbreviations
EPR electron paramagnetic resonance · *mt* mitochondrial

Perspective

Crystallization and X-ray diffraction analysis of the mitochondrial and of several bacterial cytochrome *c* oxidases have set the stage for a better understanding of the complex functions of this enzyme. Being responsible for catalyzing the reduction of more than 95% of the oxygen taken up by aerobically growing higher organisms, this terminal complex of the respiratory chain is at the same time an efficient energy-transducing device, the complexity of which we are only beginning to understand. This review makes frequent use of the obvious advantages of studying the structurally simpler bacterial enzyme complexes by site-directed mutagenesis approaches, but focuses in many aspects on the mitochondrial enzyme from higher eukaryotes. To this end, a general view on structure and function of

O.-M. H. Richter (✉) · B. Ludwig
Institute of Biochemistry, Biocenter, J.W. Goethe-Universität,
Marie-Curie-Str. 9, 60439 Frankfurt, Germany
e-mail: O.M.Richter@em.uni-frankfurt.de
Tel.: +49-69-79829240 · Fax: +49-69-79829244

cytochrome *c* oxidase will be given, and integrated with specific aspects of assembly, regulation, and pathology of the mammalian enzyme.

Introduction

Redox-linked proton pumps in mitochondria, as well as their prokaryotic counterparts, have evolved different mechanistic strategies to use the free energy of respiratory electron transfer for the generation of an electrochemical proton gradient across the coupling membrane (Trumpower and Gennis 1994; Schultz and Chan 2001). While complex-II (succinate: ubiquinone oxidoreductase) is not energy-transducing at all, the mechanism exerted by complex-I (NADH: ubiquinone oxidoreductase) is largely unknown at present. Complex-III (ubiquinol: cytochrome *c* oxidoreductase; cytochrome bc_1 complex) is fairly well explained in this respect by the Q-cycle mechanism, whereas for oxidase (cytochrome *c*: oxygen oxidoreductase; complex-IV) the presence of two different proton pathways or channels was disclosed even before X-ray data became available. Nevertheless, despite an excellent structural background, the molecular mechanism of proton translocation in oxidase, in particular the nature of the coupling device between the redox steps and the proton pump, is still unknown. This also holds true for other members of the superfamily of heme-copper oxidases, such as the quinol oxidases found in many bacteria; contrasting their obvious differences in heme composition and in electron entry into the enzyme complex (Calhoun et al. 1994; Pereira et al. 2001), basic structural and spectroscopic properties suggest a largely identical mechanism of oxygen reduction at the binuclear site, and of transmembrane proton translocation (see below).

We will first describe the fundamental structural and functional aspects of cytochrome *c* oxidase as such, based on the recent crystallization data, mutagenesis studies, and a wealth of spectroscopic evidence compiled over a period of several decades. This will be followed by a short treatment of the specific properties of the mitochondrial enzyme and its implications for the energy supply to the mammalian organism.

Further reviews focus on particular aspects of this enzyme, and are referred to for more detailed information (Capaldi 1990; Saraste 1990, 1991; Haltia 1997; Kitagawa and Ogura 1997; Michel et al. 1998; Wikström 1998; Michel 1998; Ludwig et al. 2001; Carrozzo and Santorelli 2002).

Structure and function – the basics

3-D structure-derived features

To date, structures of five different heme-copper terminal oxidases have been solved. The four cytochrome *c* oxidase structures (Iwata et al. 1995; Tsukihara et al. 1995, 1996; Ostermeier et al. 1997; Yoshikawa et al. 1998; Harrenga and Michel 1999; Soulimane et al. 2000; Svensson-Ek et al. 2002) reveal a striking degree of identity among each other, irrespective of the fact that the mitochondrial enzyme is the product of two separate genetic systems and displays a tremendous increase of subunit complexity. The enzymes from *Paracoccus denitrificans* and *Rhodobacter sphaeroides* may be viewed as simple bacterial model systems that perfectly match the mitochondrial enzyme, at least in all the basic enzymatic functions, while the *Thermus thermophilus* oxidase appears more distant in terms

of characteristic common structural and functional signatures (see below). Despite obvious differences in the electron entry branch of the *E. coli* bo_3-type quinol oxidase (Abramson et al. 2000), this enzyme shares all the main structural properties of its binuclear site and most likely its mechanism of proton translocation with the other members of the terminal oxidase family. We will first describe general properties of cytochrome *c* oxidases, on the basis of the *Paracoccus* enzyme X-ray-derived structure, and specify differences to other enzymes, whenever necessary.

Subunit composition and structure of the *Paracoccus* enzyme

Originally isolated as a two-subunit enzyme complex (Ludwig and Schatz 1980) from the cytoplasmic membrane of the soil bacterium *Paracoccus denitrificans* (Baker et al. 1998), this oxidase preparation has been characterized extensively and shown to be fully active in electron transport and coupled proton translocation (Pardhasaradhi et al. 1991; Hendler et al. 1991). Replacing Triton X-100 by dodecyl maltoside as the detergent for solubilization of the bacterial membranes, a four-subunit complex is obtained (Haltia et al. 1988; Hendler et al. 1991): its three largest protein components show a high degree of sequence identity to the three largest, mitochondrially coded subunits of the eukaryotic oxidase (Saraste 1990; and see "Mitochondria and oxidase–increasing the level of complexity"). This four-subunit oxidase has been crystallized in the presence of dodecyl maltoside as a complex with a monoclonal antibody fragment (Fv) directed against an epitope on the hydrophilic domain of subunit II, and its structure determined at 2.8 Å (Ostermeier et al. 1995; Iwata et al. 1995). Later, the two-subunit complex structure was solved at 2.7 Å, again using the Fv approach to increase the polar surfaces of the protein complex and undecyl maltoside as detergent (Ostermeier et al. 1997).

Subunit I is largely embedded in the membrane, with its 12 transmembrane helices shaped in a three-winged propeller arrangement (Iwata et al. 1995). Its N-terminus and the long, exposed C-terminus of the 558 amino acid polypeptide face the cytoplasmic side. Three redox centers, the two *a*-type hemes and copper B, are liganded by amino acid side chains of this subunit and are located about one third into the membrane depth from the periplasmic surface (the intermembrane space face, for the mitochondrial enzyme). Two histidines (see Table 1 for the positional numbering in three reference oxidases) provide the axial ligands to the low-spin heme *a*, whereas a histidine and a presumed hydroxyl or a water molecule provide ligands to the high-spin heme a_3 moiety. Both hemes are oriented perpendicular to the membrane plane, and their interplanar angle is 108°; from this, an edge-to-edge distance of 4.7 Å or 13.2 Å between both iron atoms ensues.

Heme a_3, together with a copper ion (Cu_B) in its immediate vicinity, forms the binuclear center where oxygen binding and reduction take place (see below). The copper ion is liganded by three histidines, both in the oxidized and the reduced forms of the enzyme (Harrenga and Michel 1999), and has been determined to be 5.2 Å away from the heme a_3 iron in the four-subunit enzyme, and 4.5 Å in the two-subunit complex (with a different pH used in the crystallization buffer for both preparations).

Subunit II has a bipartite structure: its N-terminal two transmembrane helices are followed by a hydrophilic, 10-stranded β-barrel domain extending into the periplasm, housing the Cu_A center. It is composed of two Cu ions in a mixed-valence state ($Cu^I \cdot Cu^{II}$) 2.6 Å apart, giving rise to a characteristic EPR (electron paramagnetic resonance) signal

Table 1 Amino acid numbering for selected residues of heme-copper oxidases from three different organisms (*SU* subunit, amino acids listed in one-letter code)

Function	SU	*P. denitrificans*		*R. sphaeroides*	Bovine heart
Electron entry	II	W	121	143	106
Cu_A ligands	II	C	216	252	196
		C	220	256	200
		E	218	254	198
		H	181	217	161
		H	224	260	204
		M	227	263	207
Heme *a* ligands	I	H	94	102	61
		H	413	421	378
Heme a_3 ligand	I	H	411	419	376
Cu_B ligands	I	H	276	284	240
		H	325	333	290
		H	326	334	291
Cross-linked tyrosine	I	Y	280	288	244
D-pathway	I	D	124	132	91
		N	131	139	98
		E	278	286	242
K-pathway	I	K	354	362	319
Side entry	II	E	78	101	62

in the oxidized state observed in other enzymes as well (Epel et al. 2002). The structure of an engineered Cu_A fragment has been determined independently (Wilmanns et al. 1995).

Subunit III is fully embedded in the membrane. Its seven helices are grouped into two bundles of two and five helices in a V-shape arrangement, with its N-terminus located on the inside. No redox cofactors are associated with this subunit, and both subunits III and IV may be removed from the *Paracoccus* complex (see above) without loss of its catalytic functions. For *Rhodobacter*, it has been concluded that this subunit stabilizes the integrity of the binuclear center in subunit I (Bratton et al. 1999). When, on the contrary, the gene coding for this subunit is deleted in *Paracoccus* (Haltia et al. 1989), a partially assembled complex results.

Subunit IV of the bacterial enzyme (both in *Paracoccus* and in *Rhodobacter*) consists of a single transmembrane helix in contact with subunits I and III; its sequence does not resemble any of the nuclear-coded subunits of the mitochondrial complex, but a high degree of sequence identity in the pairwise comparison between the two bacterial sequences has been noted (Svensson-Ek et al. 2002). On deletion of its gene (Witt and Ludwig 1997), the resulting three-subunit *Paracoccus* complex reveals no structural or functional defects.

Nonredox active metal ion binding sites have been found both in the bacterial and in the mitochondrial oxidases. The enzyme isolated from *P. denitrificans* grown under standard medium conditions contains a manganese ion, giving rise to a characteristic EPR signal (Seelig et al. 1981; Käß et al. 2000). Mn itself is not required for function of oxidase, and may be replaced by Mg. However, mutations in its binding site (Witt et al. 1997) located at the hydrophilic interface between subunits I and II in the vicinity of the Cu_A center show a moderately decreased electron transfer activity. A similar site is encountered in the *Rhodobacter* oxidase (Hosler et al. 1995; Florens et al. 2001) and the mammalian en-

zyme structure (see below) where a Mg ion occupies this site. A structural role for this divalent ion liganded by residues both from subunit I and II has been discussed to tether both subunits together, or to contribute to a proton or water exit channel.

From structures of both bacterial and the mitochondrial cytochrome *c* oxidases, another metal ion binding site was suggested at the periplasmic side in transmembrane helix I of subunit I (Pfitzner et al. 1999; Riistama et al. 1999; Lee et al. 2002). In the *Paracoccus* and *Rhodobacter* enzymes, this site was shown to be populated by a tightly bound Ca ion, whereas the mitochondrial oxidase revealed only a partial occupancy. Ca binding to the bovine heart enzyme, and to site-specific mutants of both bacterial enzymes, was shown to elicit a heme *a* spectral shift, with Na ions competing with Ca binding (Pfitzner et al. 1999; Lee et al. 2002). These data point to discrete differences in ligand environment in these three oxidases, while the *Thermus thermophilus* ba_3 oxidase completely lacks this site (Soulimane et al. 2000).

Structure of the mitochondrial oxidase

The structure of the mitochondrially coded subunits I–III of the bovine heart cytochrome *c* oxidase revealed an amazing similarity to their bacterial counterparts (Tsukihara et al. 1995, 1996; Yoshikawa et al. 1998), and may be considered the functional core of the eukaryotic oxidase. Initially crystallized in decyl maltoside and resolved to 2.8 Å resolution, the mitochondrial enzyme structure was further refined to 2.3 Å in different redox and ligand states. In the oxidized state, its redox site metal environments in subunits I and II were almost identical to the structure of the *Paracoccus* enzyme (see below), and the Mg site discussed above, and an additional Zn binding site were identified for the mammalian enzyme.

Ten nuclear-coded subunits surround the central core structure, and two identical oxidase monomers of 13 subunits each form the functional dimer of more than 400 kDa in mass (see Fig. 1). Subunits IV, VIa, VIc, VIIa, VIIb, VIIc, and VIII each traverse the membrane in a single helical arrangement, whereas Va and Vb (Zn binding) face the matrix side, and VIb is oriented towards the intermembrane space. Both the subunits VIa and VIb are mainly responsible for the contacts between monomers in the dimer. It is noteworthy that the bovine heart structure also shows a total of eight rather well-defined phospholipid molecules, and two cholate moieties, one of them possibly accounting for a nucleotide binding site with steric requirements similar to an ADP group (see "Physiology and regulation").

It is interesting to note that a supercomplex arrangement has been observed both for the mitochondrial and the bacterial cytochrome *c* oxidase (Schägger 2001).

The general mechanism: electron transfer and proton translocation

Based on a large body of spectroscopic and kinetic data (Kitagawa and Ogura 1997; Wikström 1998; Michel et al. 1998; Schultz and Chan 2001) it is generally accepted that internal electron transfer in oxidase proceeds, in four repetitive steps, from the Cu_A center located in subunit II to the three redox sites in subunit I, heme *a* and the binuclear center composed of heme a_3 and Cu_B where dioxygen reduction to water is catalyzed:

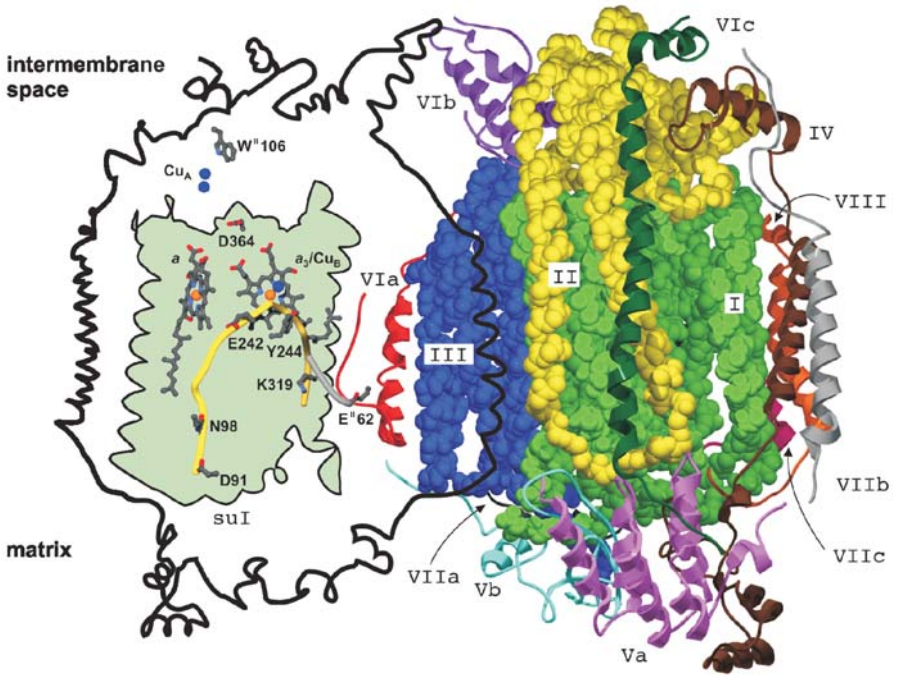


Fig. 1 Structure of the dimeric 13-subunit mitochondrial cytochrome *c* oxidase from bovine heart with its electron and proton pathways. Based on accession number 1OCC coordinates, the structure of the dimeric bovine heart cytochrome *c* oxidase is represented as a *space-filling* (subunits I–III) and *ribbon* model (nuclear coded subunits) for the one monomer. The other monomer is presented as a *contour line*, with its redox centers and essential residues shown to indicate spatial arrangement of electron and proton transfer pathways within subunit I (*green shading*). Residues E62 and W106 as well as Cu_A belong to subunit II. Both the D- and K-channel (starting at residue D91 and below K319) for proton uptake from the matrix side are approximated by *yellow tubes*, with an assumed alternative entry at $E^{II}62$ (for details, see text). *Blue* and *orange spheres* specify copper and heme iron atoms

$$4\ \text{cyt}\ c^{red} + 8\ H^{+}_{(in)} + O_2 \rightarrow 4\ \text{cyt}\ c^{ox} + 2\ H_2O + 4\ H^{+}_{(out)}$$

With the three redox centers of subunit I deeply buried into the depth of the hydrophobic membrane environment (to about 15 Å from the positively charged side of the membrane; see Fig. 1), two conclusions become immediately evident: (a) intramolecular electron transfer to the binuclear center is electrogenic as such, and (b) will require charge compensation for thermodynamic reasons (see below).

Electron transfer pathways

Electrons enter the oxidase complex exclusively via its Cu_A center, as proven also by mutation experiments involving residues liganding Cu_A (Malatesta et al. 1998). As deduced from early cross-linking and chemical modification studies using the mammalian enzyme (reviewed in Capaldi 1990; Trumpower and Gennis 1994), subunit II has been described as the main docking site for cytochrome *c*. Its interaction with oxidase is based on long-

range electrostatic preorientation between the highly basic mitochondrial cytochrome *c* (in particular, its cluster of positively charged residues located around the heme crevice) and an extended lobe of acidic residues on the surface of subunit II close to the Cu_A site. Recent mutagenesis studies with the bacterial oxidases have identified individual residues of this negatively charged patch relevant for electrostatic steering (Witt et al. 1998b; Zhen et al. 1999; Wang et al. 1999; Drosou et al. 2002b), as well as the corresponding docking sites on the bacterial cytochrome *c* donor molecule (Drosou et al. 2002b). The same mutational experiments have also revealed (a) a cluster of hydrophobic residues exposed on the surface of subunit II involved in fine-tuning the interaction with cytochrome *c* (Witt et al. 1998b; Drosou et al. 2002b), required for efficient electron transfer after the initial electrostatic interaction step, and (b) an additional contribution of residues from subunits I and III, to the docking site for cytochrome *c*. Further support for this ionic strength-dependent interaction is obtained by analyzing the relevant soluble fragments in their kinetic behavior, suggesting the involvement of 2–3 charges on either component (Maneg et al., in preparation). NMR studies indicate chemical shifts of residues involved in complex formation (Reincke et al. 2001; Wienk et al., in preparation), and theoretical calculations describe possible configurations of the 1:1 complex (Roberts and Pique 1999; Flöck and Helms 2002).

The first residue crucial for electrons to enter the oxidase complex from cytochrome *c* is a conserved tryptophan (see Table 1). A series of specific mutations in this residue, introduced both into the *Paracoccus* and the *Rhodobacter* enzymes (Witt et al. 1998a; Zhen et al. 1999; Wang et al. 1999; Drosou et al. 2002b), drastically diminished presteady state and turnover rates of the enzyme, at the same time leaving K_M for cytochrome *c* unaffected and not disturbing the integrity of the Cu_A center located at a distance of about 5 Å.

The further path for the electron from reduced Cu_A to the heme *a* iron in subunit I covers a distance of 19.5 Å, yet is characterized by a fast exchange rate of about 10^4 s^{-1} (Szundi et al. 2001b). Several suggestions have been made for discrete electron pathways (Iwata et al. 1995; Tsukihara et al. 1996), but a participation of the Mg/Mn site to electron transfer could be excluded (Käß et al. 2000). The alternative pathway, a direct transfer to heme a_3, is strongly disfavored due to a longer distance of 22.1 Å from Cu_A; yet, in a mutant (R54M in *Paracoccus*) where the redox potential of the heme *a* is markedly lower, this pathway has been suggested to operate, yielding a turnover activity in the mutant of 2% (Kannt et al. 1999).

The reduction of the binuclear site, heme a_3 and Cu_B, requires electron transfer from heme *a*. Both heme ring systems are only separated by a distance of 4.5 Å, and one of the ligands for either porphyrin is located on the same transmembrane helix X, providing a reasonable and short pathway.

It should be noted at this point that attempts to crystallize oxidase(s) in different redox states (Yoshikawa et al. 1998; Harrenga and Michel 1999) have not provided any evidence that major structural differences accompany the (complete) reduction of the enzyme cofactors. In the *Paracoccus* oxidase, the binuclear center conformation remains virtually unchanged, with a distance between metal ions of 5.2 Å and all three Cu_B-liganding histidines in place in both redox states. In the bovine heart enzyme structure, this distance is 5.2 Å for the reduced/reduced · CO/oxidized · azide forms, but slightly decreased to 4.9 Å for the fully oxidized state; however, under the latter condition, an electron density between both metal ions, interpreted as a peroxy ligand, is observed (Yoshikawa et al. 1998). Yoshikawa reported a single structural feature for the mammalian enzyme to change with reduction: D51 on the cytoplasmically oriented surface of subunit I gains ac-

cess to the aqueous phase, while being connected (through a transmembrane pathway spanning this subunit; see below) to the matrix in the oxidized state.

Oxygen reaction sequence

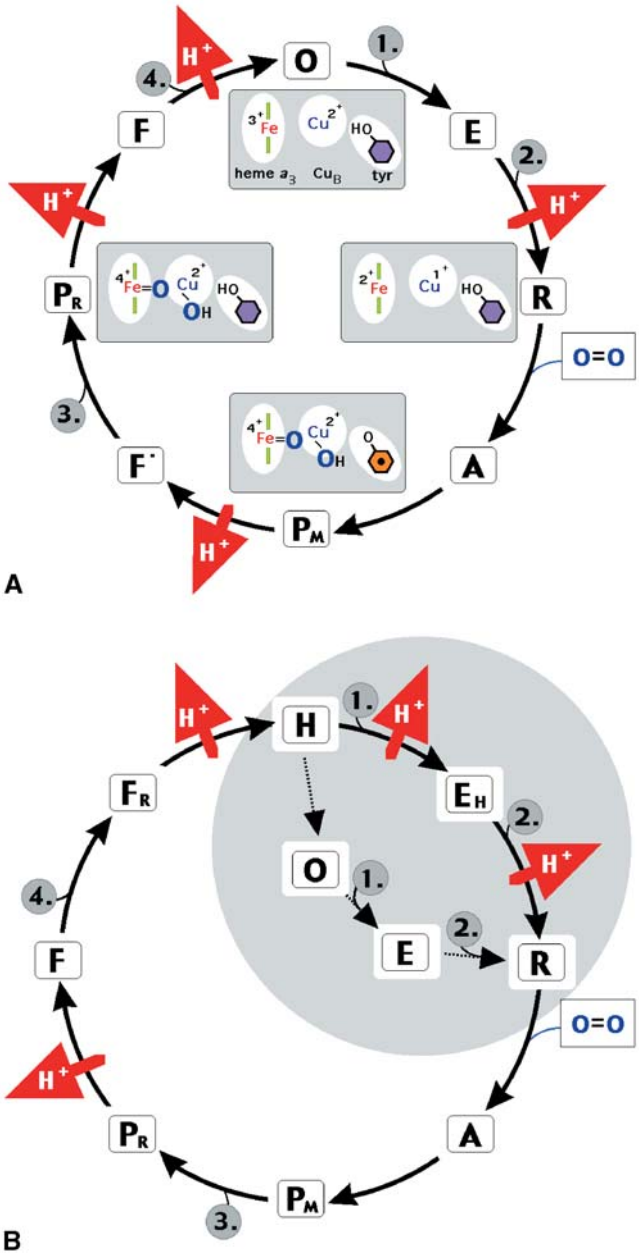
With the binuclear center reduced by two electrons (or all four redox sites carrying their reduction equivalents), oxygen is able to bind (see Fig. 2) at this site, and the reaction proceeds to the full reduction of dioxygen to yield two water molecules. For a more detailed description of kinetic and spectroscopic aspects of the reaction sequence, see, for example, Wikström and Babcock 1990; Kitagawa and Ogura 1997; Michel et al. 1998; Proshlyakov et al. 1998, 2000; Han et al. 2000; Szundi et al. 2001a, 2001b; Rogers and Dooley 2001. Here, the discussion will focus on only a few relevant aspects partly originating from the recent X-ray structures of the enzyme(s).

Oxygen access to the binuclear center is generally facilitated by its high solubility in the hydrophobic membrane phase, but seems to be further promoted by a distinct access channel from the membrane lipid into the protein transmembrane helix arrangement of subunits I and III (Riistama et al. 1996; Tsukihara et al. 1996; Hofacker and Schulten 1998; Svensson-Ek et al. 2002) and has also been supported by functional analysis for the *Paracoccus* oxidase (Riistama et al. 2000).

The nature of the P_M state (see Fig. 2), initially assigned to a putative peroxy intermediate, has been reinvestigated recently (Proshlyakov et al. 1998, 2000) and conclusively shown to carry a ferryl (Fe^{4+}) heme a_3, with the O–O bond already split in a fast time course (<200 μs) and both oxygen atoms present at the formal oxidation state of water. This immediately raised the question of the origin of the four redox equivalents required to reduce the dioxygen molecule, while previous interpretations could only account for three electrons available in the binuclear center for this reductive step ($Fe^{2+} \rightarrow Fe^{4+}$ / $Cu^{1+} \rightarrow Cu^{2+}$). Careful inspection of the 3-D structures both of the *Paracoccus* and the bovine heart enzymes has provided the additional information that a tyrosine and one of the Cu_B-liganding histidine (see Table 1) side chain densities were close enough for a bonding distance, thereby forming a side chain ring structure (Ostermeier et al. 1997; Yoshikawa et al. 1998). Protein chemical evidence confirmed this unusual cross-link in the two enzymes and in two further cytochrome *c* oxidases from *Thermus thermophilus* (Buse et al. 1999), and redox Fourier Transform Infrared spectroscopic analysis with the *Paracoccus* enzyme supported the presence of such a modified tyrosine as well (Hellwig et al. 2002). The status of this cross-link remains unresolved for the *Rhodobacter* enzyme where electron density evidence is not conclusive (Svensson-Ek et al. 2002).

This finding opened the view for a potential role of this tyrosine side chain, due to its presumably unusual properties in terms of its redox potential and dissociation behavior, to act as an electron (and/or proton) donor in the reaction cycle, transiently acquiring a radical state. Experimental confirmation came from two studies using either chemical derivatization (Proshlyakov et al. 2000) or EPR spectroscopy (MacMillan et al. 1999), the latter demonstrating that indeed a tyrosine residue elicits a radical signal. After studying the

Fig. 2A, B Oxygen reduction cycle catalyzed by cytochrome *c* oxidase. **A** *Boxed letters* along the *circular line* represent key reaction intermediates identified spectroscopically, referring to the redox state of the binuclear center: *O* oxidized, *E* single-electron reduced, *R* reduced, *A* adduct after dioxygen binding, P_M "peroxy" state (defined historically, but with O–O bond already split), $F \cdot$ *ferryl,* P_R *reduced "peroxy," F ferryl.*



Four intermediate states are further detailed in their electronic configuration at the binuclear center (*gray boxes*): heme a_3, Cu_B, and a tyrosine side chain of subunit I (Y244 in the bovine heart enzyme) presumably contributing to the redox cycle in its radical form (*orange* coloring in P_M state). Formal charges at oxygen atoms and charge-compensating proton uptake steps are omitted for clarity. The four electron entry steps (*gray circles*) are *numbered*, and transmembrane proton translocation steps highlighted by *red arrowheads*. **B** Modified redox cycle (Wikström 2000; Wikström and Verkhovsky 2002), differing mostly in the sequence of electron entry and proton translocation steps. *H* High-energy state with hydroxyl group bound to Fe(a_3). For further details, see text

Tyr→His mutant in this position in the *Paracoccus* enzyme by spectroscopic means (Pinakoulaki et al. 2002), it was concluded that this cross-link is primarily required to fix the distance between the two metals in the binuclear center. Whether or not the radical form is a kinetically competent and therefore compulsory intermediate in the oxygen reaction under turnover conditions (Han et al. 2000) remains to be established.

An immediate consequence of this finding is the fact that oxygen, only able to bind to the reduced binuclear center, can be reduced to water in a single and fast step, even if further supply of electrons from the Cu_A/heme *a* couple is delayed or stalled, thus avoiding the production and possible liberation of reactive oxygen species such as hydrogen peroxide.

The further steps in the oxidative part of the reaction cycle, from state P_M to state O (see Fig. 2), require two more electron input steps to reduce the tyrosine side chain radical (leading to F) and the ferryl state of the high-spin heme, completing the oxygen cycle. Three more proton translocation events are assumed to occur during these steps (Michel 1998), but an alternative scheme has been put forward by Wikström (Wikström 2000; Wikström and Verkhovsky 2002; see Fig. 2B).

Proton translocation pathways

A total of eight protons for every dioxygen molecule reduced are taken up from the inside (matrix side of mitochondria, or the cytoplasm of a bacterium), half of them to be delivered to the binuclear center for water formation, and another four for translocation across the membrane. Early concepts of functionally "uncoupling" electron transport from proton pumping by (a) chemical derivatization (reviewed in Capaldi 1990), (b) the requirement for physically separating "pumped" protons to avoid shortcuts to water formation, and (c) electrostatic calculations (Kannt et al. 1998a, 1998b) all support the notion that two separate pathways or channels should exist in oxidase. Early mutagenesis studies, mostly on a related member of the heme-copper oxidases, the *E. coli* heme bo_3 quinol oxidase, seemed to confirm the finding of two functionally distinct pathways: when a widely conserved aspartate residue (corresponding to D91 in the bovine sequence; see Table 1 and Fig. 1) located at the periplasmic face of subunit I was mutated, the enzymés electron transfer activity was diminished, and partly uncoupled from proton pumping. Another mutation in a conserved lysine (K319, located within the membrane section of subunit I) led to a complete loss of electron transfer. The original assignment that the "D-pathway" would be used exclusively for translocated protons, and protons destined for water formation would travel along the "K-pathway" no longer holds today (see below). However, the initial definition of internal pathways as chains of hydrophilic residues, of fixed water molecules and hydrogen bond networks acting as a proton wire within an otherwise hydrophobic protein environment, gained considerable support from X-ray structural data. Both in the bacterial structures (Iwata et al. 1995; Svensson-Ek et al. 2002) and that of the mitochondrial enzyme (Tsukihara et al. 1996), the matrix-oriented entrances for the K- and the D-pathways were observable, and their further course traced to some extent within subunit I. Moreover, the bovine heart structure was discussed to display another transmembrane channel (Tsukihara et al. 1996), later termed "E" or "H" (see below).

The D-pathway is now rather well defined from mutagenesis studies, extending from its matrix entrance, D91 (bovine heart numbering, see Fig. 1; all mutations actually introduced into the respective residues of the bacterial enzymes, see Table 1), to E242 close to

the binuclear center (e.g., Thomas et al. 1993; Hosler et al. 1993; Garcia-Horsman et al. 1995; Fetter et al. 1995; Pfitzner et al. 1998, 2000; Wikström 1998). In several positions along this pathway, the introduction of site-directed mutations has provided functional evidence for proton conduction. Mutant phenotypes are typically characterized by a loss of proton pumping capacity and a concomitant decrease in electron transfer rate. In one case (N98; see Fig. 1), an ideally uncoupled mutant was obtained for the *Paracoccus* oxidase with wild type redox behavior, but completely lacking proton pumping (Pfitzner et al. 2000). The importance of the highly conserved position E242 for proton conduction has been acknowledged early on, and a role in redox-dependent shuttling of protons into the binuclear center was suggested recently by demonstrating redox-dependent protonation or conformational changes of this residue (e.g., Hellwig et al. 1998; Jünemann et al. 1999; Lübben et al. 1999; Wikström 2000). For the *Rhodobacter* enzyme, the structure of the E→Q mutant in this position could be solved (Svenssson-Ek et al. 2002) showing a loss of a hydrogen bond, along with several other subtle conformational changes and relocation of water molecules.

With many more oxidase sequences becoming available also from genome sequencing projects (reviewed in Pereira et al. 2001), it was stressed recently that several representatives do not share the canonical D-pathway residues discussed above, most notably in the ba_3-type cytochrome *c* oxidase from *Thermus thermophilus*. Its structure is known to 2.4 Å resolution (Soulimane et al. 2000), and a careful determination of its proton pumping efficiency resulted in an unusually low H^+/e^- ratio of 0.5 (Kannt et al. 1998c). Subsequently, both in the *Paracoccus* and the *Rhodobacter* enzymes, mutations were introduced to "redesign" a functional pump by relocating crucial side chains to different helices (Aagaard et al. 2000; Backgren et al. 2000).

The exit pathway for protons to reach the opposite side of the enzyme is less well defined, and most likely comprises the area above both hemes characterized by a hydrophilic cluster of negative charges including the heme propionates (Puustinen and Wikström 1999; Behr et al. 2000). Mutants in an aspartate residue (corresponding to D364) at the periplasmic exit region of the *Paracoccus* enzyme (Pfitzner et al. 2000) revealed a lack of proton pumping, and the Mg site in the *Rhodobacter* enzyme has been linked to the proton/water exit path of oxidase (Florens et al. 2002).

The K-pathway (see Pecoraro et al. 2001, and references above) is named for a conserved lysine residue (see Fig. 1, Table 1) halfway between its cytoplasmic entrance and the binuclear center along helices VI and VIII of subunit I. It also comprises the tyrosine residue (Y244) cross-linked to a Cu_B histidine (see above). Recently, the question has been brought up whether a side entrance, or even the exclusive entrance, exists via a glutamate residue (E62) in subunit II (Ma et al. 1999; Bränden et al. 2002).

An additional proton relay system has been suggested from inspection of the bovine heart structure (Tsukihara et al. 1996; Yoshikawa et al. 1998), linking the cytoplasmic side of subunit I to D51 on the opposite surface (see "Oxygen reaction sequence"). It was hypothesized that this path consisted of individual, small-diameter cavities under redox control. While some of the relevant residues have no conserved counterparts in the bacterial enzymes, experimental evidence from introducing mutations into several conserved residues in the *Paracoccus* and *Rhodobacter* oxidases provided no hints for a functional contribution of those residues to proton pumping (Pfitzner et al. 1998; Lee et al. 2000).

Pathway utilization

A clear-cut "single-purpose" channel operation, separate for "chemical" and for "pumped" protons, as previously envisaged (see above), could not be maintained any longer when differential effects of mutants in either pathway were analyzed and related to specific partial reactions in the redox cycle of oxidase (e.g., Konstantinov et al. 1997). During the "eu-oxidase" reaction steps (reductive phase from O to R; see Fig. 2) the K-pathway was suggested to be operative, while during the "peroxidase" reaction part (P_M to O), the D-pathway should dominate for proton uptake (and pumping). This view has been modified upon focussing on the individual steps of electron entry into oxidase. In line with the above assumption, electrometric measurements on reconstituted oxidase mutants showed a clear delay in the proton uptake reaction during the first electron reduction step from O→E (see Fig. 2) for the K-pathway mutant, indicating a charge compensation via the K-channel (Ruitenberg et al. 2000). In going from E→R, both the K- and the D-channel mutants showed delayed kinetics, arguing for a compensatory proton movement via the K-path and a transmembrane pumping step across the D-channel (Ruitenberg et al. 2002). Thus, for a full oxygen reaction cycle, up to 7 (out of 8) protons may travel through the D-channel.

Present concepts of coupling electron transfer to proton translocation

Despite a vast amount of overall structural and functional information on oxidase, little definitive insight has been attained for the actual step of coupling the free energy of the redox event(s) to proton translocation, most likely due to the fact that the redox cycle apparently is not accompanied by major conformational changes detectable by standard procedures. We do not understand the molecular mechanisms of gating, i.e., (a) which precise step of electron transfer really powers proton translocation, (b) how the concerted action of proton delivery, and its vectoriality, is achieved, and (c) how proton pathways are safeguarded against back-leak. A few hypothetical concepts have been put forward to date, which are briefly described here.

A notion central for understanding processes proceeding at the membrane-embedded heme/copper site is the electroneutrality principle (reviewed in Rich 1995). Stabilizing a charge introduced into a well-insulated membrane environment is only feasible in a thermodynamically compatible way by concomitant charge compensation, i.e., an electron transferred to a heme site should immediately pull in a proton, via one of its respective pathways (see above).

Along this line, Michel presented an intricate set of individual steps of alternating electron entry reactions followed by charge-compensating movements of protons (Michel 1998). Two separate acceptor sites (A and B), viewed as protonic networks located above, and including the two hemes, were assumed to store one proton each. With every electron entry step leading to heme *a* reduction, a compensatory proton would move in and be stored in A/B. Driven by electrostatic repulsion following each step in oxygen chemistry proceeding at the binuclear center, a proton was assumed to be expelled to the outside. As depicted in Fig. 2A, one of the four proton translocation steps would already have taken place in the reductive part of the cycle, i.e., during the E→R transition. Experimental evidence for this translocation step, starting with the "E state" enzyme, has been presented (Ruitenberg et al. 2002).

Modifying his earlier "power stroke" model (Morgan et al. 1994), Wikström subsequently presented another histidine-based scheme (Wikström 2000; Wikström and Verkhovsky 2002), where the imidazole side chain of one of the Cu_B ligands was assumed to be flexible, existing in an input and an output conformation; by this, it could relay to the E242 residue at the binuclear end of the D-channel in a concerted way to recruit a proton and deliver it to an output location above the hemes. In terms of the timing of the proton pumping steps, it was suggested that the free energy provided in the oxidative part of the cycle is stored (state "H" in Fig. 2B; highlighted in gray) and only released (in two pumping steps) if immediately followed by another round of reduction. If the supply of electrons is stalled, the stored energy would dissipate at a timescale of seconds, and rereduction by the "first" two electrons (dashed thin arrows in Fig. 2B) proceed without any proton translocation during the reductive phase.

Mitochondria and oxidase – increasing the level of complexity

Mitochondria are the key players in supplying energy to almost every eukaryotic cell. Following their description in the late nineteenth century (Altmann 1890), mitochondrial DNA was discovered only in 1963 (Nass and Nass 1963) and the sequence of human mitochondrial DNA (mtDNA) was published in 1981 (Anderson et al. 1981).

The size and coding capacity of mitochondria from different organisms vary considerably. For example, the 366,924-base pair (bp) mitochondrial genome of *Arabidopsis thaliana* codes for 57 gene products (Unseld et al. 1997), while the human mitochondrial genome has a size of 16,569 bp. Analysis revealed genes coding for 13 proteins (7 subunits of complex-I, cytochrome *b* of complex-III, 3 subunits of complex-IV, and 2 subunits of complex-V) and 24 RNAs (2 rRNAs and 22 tRNAs).

Each mitochondrion of a human cell may contain between 2 and 10 copies of the mtDNA, and up to 10^5 mitochondria have been estimated for the human oocyte (Smeitink et al. 2001). The distribution of mitochondria in dividing cells is not a random process but involves a cellular machinery of unknown complexity for its coordination (Yaffe 1999). Segregational processes within the germline are responsible for the observation that even siblings may show different levels of mutations in the mitochondrial genome. Similar events later in development may lead to differences among mitochondria of the same organ or even the same cell (Larsson and Clayton 1995), an observation known as heteroplasmy (for example, see DiMauro and Andreu 2001). This phenomenon is important for the onset and severity of certain pathological disorders discussed elsewhere (see "Pathology and disease"). Usually, mitochondria are inherited exclusively maternally, although sperm mitochondria are detectable in the zygote (Smeitink et al. 2001). Therefore, an unknown selection process eliminating paternal mitochondria seems operative as is obvious from the study of abnormal embryos where paternal mtDNA is still detectable (St. John et al. 2000).

Several unique features of the mitochondrial genetic system of higher eukaryotes are known today (Garesse and Vallejo 2001; Larsson and Oldfors 2001; Smeitink et al. 2001) and deserve a brief mentioning. The coding density of genetic information is considerably higher than in nuclear DNA. There are no introns and hardly any intergenic gaps in addition to partially overlapping genes. Two major polycistronic transcripts encompass all genes of either strand of mtDNA and are processed to mostly monocistronic mRNAs in

addition to tRNAs and the two rRNAs. mRNAs lack 5' untranslated regions and cap structures. The mitochondrial genetic system uses a simplified decoding mechanism with only 22 of the usual 31 tRNAs, allowing translation of all mitochondrial codons. Last but not least, there are deviations from the standard genetic code in mitochondrial translation as, for example, the general stop codon TGA codes for the amino acid tryptophan. In addition, the overall mutation frequency of mtDNA is about ten times higher than that observed in the nucleus (Brown et al. 1979). Proteins necessary for replicative, transcriptional, and translational processes, as well as all proteins required for the biogenesis and functional maintenance of mitochondria are nuclear encoded. Around 1000 proteins of nonmitochondrial origin might be involved in this complex and intricate regime (Foury and Kucej 2002).

In addition to the classical cytochrome *c* oxidase, many organisms express a so-called alternative oxidase (AOX), transferring electrons directly from ubiquinol to oxygen without being proton-motive (Berthold et al. 2000; Affourtit et al. 2001; Affourtit 2002). An even broader spectrum of terminal oxidases is seen with the parasitic yeast *Candida parapsilosis*, where a complete parallel respiratory chain seems to be operative under the appropriate conditions (Milani et al. 2001).

A cell cycle-regulated correlation of mitochondrial transcription with synthesis of nuclear DNA and retrograde communication might signal changes in the activity of mitochondria to the nucleus (Garesse and Vallejo 2001). Growing evidence indicates that mitochondria, in addition to their role in energy transduction, might play a role in regulating the expression of several “hypoxic” genes in the nucleus (Dagsgaard et al. 2001).

Biogenesis: subunit and cofactor assembly

Assembly of a functional cytochrome *c* oxidase apparently is an ordered multistep process in humans and probably other eukaryotes as well, while the sequence of assembly events is less strict in prokaryotic organisms. This obviously relates to the fact that with prokaryotes there is only one genome coding for all oxidase subunits and subunit composition of prokaryotic oxidases is comparatively simple. Import of nuclear-encoded oxidase subunits into mitochondria clearly adds to the complexity of eukaryotic oxidase assembly. For recent reviews on protein import into mitochondria, see Truscott et al. (2001) and Paschen and Neupert (2002).

The assembly process of the human cytochrome *c* oxidase complex seems to follow a defined path with an ordered sequence of discrete intermediates leading to a homodimeric complex with 13 subunits each. Association of subunits I, IV, and Va appears to constitute the crucial initial assembly event (Nijtmans et al. 1998; Taanman 2001; Taanman and Williams 2001) due to mutual stabilization of these subunits, as observed by immunoblotting. This initial complex already involves products of both the nuclear and mitochondrial genome. Although further assembly intermediates are likely, the next intermediate detectable with some certainty is a complex lacking only subunits VIa and VIIa or VIIb (Nijtmans et al. 1998). Association with these remaining subunits yields the fully assembled oxidase found to be a dimer at least in mammals.

Some details are known as to the role of certain oxidase subunits and their involvement in the assembly process. At least in yeast, subunit VIa apparently is not required for oxidase assembly but instead is supposed to be involved in oxidase dimerization (Nijtmans et al. 1998) and together with VIIc is essential for maximal activity of the oxidase complex

(Taanman and Williams 2001). The importance of VIa and VIb for dimerization of bovine heart oxidase is substantiated by experiments converting the monomeric oxidase to the dimeric complex (Musatov and Robinson 2002). Individual null-mutants for subunits IV, Va, Vb, VIc, or VIIa, on the other hand, do not show any oxidase activity. Therefore, these subunits are considered essential for an ordered sequence of assembly events (Taanman and Williams 2001).

Assembly of the eukaryotic oxidase is by no means a process of self-association, but instead a set of assembly factors supports this process. In *Saccharomyces cerevisiae* about 30 different gene products seem to participate in processing mitochondrial mRNAs, translation of mitochondrially encoded COX subunits, processing of oxidase subunit precursors, their membrane insertion and cofactor incorporation (reviewed in Barrientos et al. 2002a).

Incorporation of subunit I into the membrane is promoted by Oxa1 (Bonnefoy et al. 1994), which may also function in inserting subunits II (together with Cox18; Souza et al. 2000) and III (Hell et al. 2001). In addition, incorporation of subunit II was reported to depend on the membrane potential (Hell et al. 2001) and on processing its precursor by Cox20 (Hell et al. 2000) and the Imp2 peptidase (Nunnari et al. 1993). Furthermore, absence of the assembly factor Surf1 disfavors addition of subunit II and III to the growing complex (Robinson 2000). Residual oxidase activity in Surf1 mutants indicates that this protein is not absolutely required for oxidase assembly (Shoubridge 2001). Presence of Shy1 (the yeast homologue of Surf1), on the other hand, is necessary for full expression of CoxI possibly due to protection against proteolysis (Barrientos et al. 2002b). Incorporation of heme *a* into eukaryotic oxidase may accompany the folding of subunit I within the membrane, even promoting binding of subunit II, which in turn could stabilize the binuclear heme a_3/Cu_B center of subunit I (Taanman and Williams 2001). In yeast heme *a* is synthesized by a three-component monooxygenase composed of Cox15, ferredoxin, and the corresponding reductase (Barros et al. 2002) from heme *o*, which in turn is produced by a Cox10-catalyzed addition of a farnesyl moiety to heme *b* (Tzagaloff et al. 1993). It seems likely that farnesylation of the heme by the membrane-integral Cox10 and conversion to heme *a* occurs before its incorporation into the oxidase, although this particular aspect is not yet settled (Tzagaloff et al. 1993).

Copper is taken up by eukaryotic cells via specific transport proteins and is translocated as Cu(I) to mitochondria by Cox17 (reviewed in Harrison et al. 2000), which is also detectable in the intermembrane space of mitochondria. In humans and yeast, two homologous proteins, Sco1 and Sco2, are involved in transfer of copper to cytochrome *c* oxidase. Sco1 is a membrane-integral protein with one copper binding site per monomer and is supposed to assist in copper incorporation into the Cu_A center of cytochrome *c* oxidase subunit II accepting copper from Cox17 (Robinson 2000). A complex of Sco1 and CoxII corroborates the function of Sco1 (Lode et al. 2000). In yeast, Sco2 is able to substitute Sco1 to a certain extent, although its precise function still remains obscure (Barrientos et al. 2002a). In humans, Sco2 may be of higher importance for delivery of copper to the oxidase in heart and other organs, while a mutation in Sco1 predominantly affects liver. Sco2 was also shown to physically interact with CoxII and to bind copper in a 1:1 stoichiometry. Addition of copper to the growth medium of Sco2-deficient myoblasts surprisingly restored cytochrome *c* oxidase activity (Jaksch et al. 2001).

Another membrane-integral protein, Cox11, seems to be important for the delivery of copper to the Cu_B site of the binuclear center in oxidase subunit I. In yeast, this protein

probably acts as a heterodimer, first with the copper donor Cox17, then directly with oxidase subunit I as the final acceptor. A mitochondrial ribosome protein, Rsm22, might assist in this particular transfer (Carr et al. 2002). The exact timing of copper incorporation into the two oxidase subunits remains to be established, but it seems reasonable to assume that these cofactors (like the hemes in case of CoxI) are incorporated in the process of folding the corresponding subunit and insertion into the membrane and/or assembly with additional subunits (Taanman and Williams 2001). Mrs2 is another membrane-integral protein which obviously constitutes a Mg^{2+} transporter (Grivell et al. 1999) as its deletion leads to a significantly lowered mitochondrial concentration of this ion, compromising mitochondrial energy transduction (Zsurka et al. 2001). Mutations of human Cox10, Sco1/Sco2, and Surf1 are all associated with separate clinical features (reviewed in Barrientos et al. 2002a) indicating their importance in the assembly and/or maturation of the cytochrome *c* oxidase.

A minimum of two (core) subunits may be sufficient to constitute a functional energy conserving oxidase (see "Structure and function–the basics"). Assembly of the four subunit cytochrome *c* oxidase of *Rhodobacter sphaeroides*, a typical member of bacterial oxidases, does not seem to follow such a strict regime as described above for the eukaryotic counterparts. Copurification of subunit I with either subunit II or III (from corresponding deletion mutants) reflects several stable intermediates that assemble even in the absence of heme *a*, indicating a less ordered and sequential process (Hiser and Hosler 2001). Furthermore, a *Rhodobacter* Cox11 null-mutant still assembled oxidase to completion and contained Cu_A and normal amounts of heme *a* and a_3 but no Cu_B. In addition, the content of Mg^{2+}/Mn^{2+} was severely decreased (Hiser et al. 2000).

Little is known about further assembly factors in prokaryotes. Identification of genes homologous to *surf*1 (Poyau et al. 1999) and of several Sco-homologue proteins in a number of bacteria (SenC: Buggy and Bauer 1995; YmpQ: Mattatall et al. 2000) suggests on the other hand that the basic events observed with eukaryotes will have equivalent counterparts in prokaryotes. Possible differences in the assembly of pro- and eukaryotic oxidases definitely need to be established in more detail and on the basis of a broader selection of species.

Physiology and regulation

Although mitochondrial cytochrome *c* oxidase may by itself be part of a regulatory circuit (see end of this section) more is known about mechanisms that directly or indirectly modulate either its level of expression, its assembly as a functional complex, or its activity.

Eukaryotic cytochrome *c* oxidase is the product of two different genetic systems. All ten peripheral subunits are encoded by the nuclear genome, while the three central subunits of the mature complex are coded for and expressed in the mitochondrion. No specific regulatory mechanism seems to coordinate gene expression from both genomes. In fact, the steady-state level of cytochrome *c* oxidase seems to be influenced mainly by mitochondrial turnover of unassembled subunits (D'Aurelio et al. 2001). Excess unassembled cytochrome *c* oxidase subunits are prone to turnover by mitochondrially located proteases (Nijtmans et al. 1998), depending on which mitochondrially encoded subunit is affected (Taanman and Williams 2001). On the other hand, expression of nuclear-encoded genes for respiratory complexes are regulated by transcription factors (nuclear respiratory factors) like Nrf1/2 (reviewed in Smeitink et al. 2001; Garesse and Vallejo 2001). Nrf1 was

also shown to regulate the expression of mitochondrial transcription factors and could communicate a nucleo-mitochondrial interaction (Garesse and Vallejo 2001).

Isoforms of several genes exist in higher vertebrates. In humans, tissue-specific isoforms (designated H and L for the isoforms dominating in heart and liver) are known for subunit VIa and VIIa genes (Capaldi 1990; Kadenbach and Reimann 1992). In the fetus, predominantly the L isoforms are expressed, while in later stages a switch to the H isoforms occurs, almost completely in skeletal muscle and to a lesser extent in heart (Bonne et al. 1993). In addition, two isoforms for human oxidase subunit IV, termed IV-1 and IV-2, are found. The latter is detectable in fetal muscle, and in fetal as well as adult lung, while IV-1 is ubiquitously and constitutively expressed (Hüttemann et al. 2001).

A diverse set of mechanisms is reported to regulate the activity of cytochrome *c* oxidase. Amongst them are nucleotides, hormones, and several other small molecules. A total of ten nucleotide-binding sites have been identified on cytochrome *c* oxidase by equilibrium dialysis (Napiwotzki et al. 1997). A decrease in H^+/e^- stoichiometry from 1 to 0.5 was observed with reconstituted bovine heart cytochrome *c* oxidase at high ATP/ADP ratio inside the vesicles and was explained by exchange of bound ADP by ATP at the matrix domain of subunit VIaH (Frank and Kadenbach 1996). This nucleotide-binding site corresponds to one cholate molecule identified in the crystal structure of the bovine oxidase (Tsukihara et al. 1995, see "3-D structure-derived features"). With oxidase from bovine liver which contains the liver-specific isoforms VIaL, VIIaL, and VIIIL (in contrast to humans isoforms exist also for subunit VIII) the H^+/e^- ratio was 0.5 when measured under identical conditions and found to be independent of the ATP/ADP ratio (see Ludwig et al. 2001). With bovine oxidase, an allosteric inhibition was reported at high intramitochondrial ATP/ADP ratios and attributed to binding of ATP to the matrix domain of subunit IV (Napiwotzki and Kadenbach 1998; Kadenbach and Arnold 1999), enhanced by cAMP-dependent phosphorylation of subunits II and/or III and Vb and reversed by Ca^{2+}-activated dephosphorylation (Bender and Kadenbach 2000). Not only dephosphorylation, but binding of 3,5-diiodothyronine to subunit Va abolishes the allosteric inhibition by ATP as well (Arnold et al. 1998). Overall, a hormonally controlled equilibrium between two states of energy metabolism is proposed with varying efficiency of oxidative phosphorylation (reviewed in Ludwig et al. 2001). Selective removal of bovine VIb increased the enzymatic activity of the remaining complex, indicating an inhibitory function of this subunit (Weishaupt and Kadenbach 1992).

Several hormones modulate the rate of transcription of respiratory genes. For example, thyroid hormones are known to increase the amount of mitochondrial mRNA and the expression of several nuclear-encoded genes (reviewed in Garesse and Vallejo 2001). At least in rat this also holds true for expression of COX subunits from both genomes (Wiesner et al. 1992). Estrogen is reported to regulate the expression of the gene for CoxVIIa (Watanabe et al. 1998), while the level of CoxVb was found to be proportional to expression of the gastrin gene as gastrin-antisense RNA reduced the level by a factor of 5 (Wu et al. 2000).

A variety of other low-molecular-weight components influence oxidase activity as well. D-2-Hydroxyglutaric acid is a potential regulator of oxidase as a specific inhibition of oxidase activity was observed already at concentrations within the physiological range, at least for rat cerebral cortex and human skeletal muscle (da Silva et al. 2002). Another example is the dependence of cytochrome *c* oxidase activity on cardiolipin (Fry and Green 1980). Increased production of reactive oxygen species, like superoxide anion radicals, by

the mitochondrion itself results in a decreased oxidase activity due to peroxidation of cardiolipin (Milatovic et al. 2001). At least for bovine liver-type cytochrome *c* oxidase (with subunit VIaL), cardiolipin increases the H^+/e^- ratio from 0.5 to 1.0, while palmitate antagonizes this stimulatory effect (Lee and Kadenbach 2001). NO, like other small molecules, binds readily to heme iron and is known to inhibit cytochrome *c* oxidase activity. There is growing evidence that NO exerts an inhibitory effect in vivo as well (reviewed in Cooper 2002). A somewhat peculiar example of possible regulation of cytochrome *c* oxidase is the observation that at least in yeast CoxII is a substrate for the protease Yme1, which has its active site in the intermembrane space and causes a decrease in oxidase activity (Manon et al. 2001).

With those events resulting in a diminished cytochrome *c* oxidase activity, it has to be kept in mind that already a small decrease in oxidase activity may cause alterations of the mitochondrial respiratory rate as the excess capacity of oxidase under physiological conditions may be as low as 1.5-fold (Kunz et al. 2000). Therefore, the respiration rate of mitochondria seems to be tightly regulated by the amount and/or activity of cytochrome *c* oxidase.

Finally, there is growing evidence that mitochondria, in addition to their role in energy transduction, might play a role in regulating the expression of several genes in the nucleus (Dagsgaard et al. 2001). This invokes the existence of a signaling pathway from the mitochondrion to the nucleus, involving the cytochrome *c* oxidase (and maybe others) as a sensory molecule not only for oxygen. For example, it has been shown that cytochrome *c* oxidase is required for the induction of carbon monoxide-sensitive genes (Dagsgaard et al. 2001).

Pathology and disease

Carrying its own genetic information in conjunction with an elevated mutation frequency, the mitochondrial DNA is an ideal target for mutations to occur. On the other hand, the heteroplasmic situation usually requires a reasonably high proportion of mitochondria (sometimes as high as 80–90%) which carry the same mutation before a threshold level is reached to exhibit phenotypical, and very often pathological, changes. In addition, other age-related changes in the mitochondrial DNA occur, like rearrangements (deletions and/or duplications) of varying extent and position. Again, the heteroplasmic nature of the cellular pool of mitochondria is protective to a certain threshold. Another aspect that has to be considered for the onset of mutational effects is mitotic segregation. The level of mutant mitochondrial DNA in daughter cells may shift as may the threshold for a pathogenic phenotype, giving rise to age-related variability of clinically relevant features.

Given the fact that the vast majority of proteins required for biogenesis and maintenance of mitochondria are coded for in the nucleus, it is obvious that mutations in those genes are detrimental, usually affecting all mitochondria in the same way. Mitochondrially related disorders either caused by accessory proteins or by mutations of nonoxidase and tRNA genes, like LHON, MELAS, Friedreich's ataxia, Huntington's disease, Parkinson's disease, Alzheimer's disease, and apoptosis, have been reviewed elsewhere (Beal 2000; DiMauro and Andreu 2000; DiMauro and Schon 2001; Munnich and Rustin 2001; Penta et al. 2001; Smeitink et al. 2001). Most subunits for respiratory complexes are nuclear-encoded. To date, one defect in such a subunit of complex-II is known, while there are several known mutations for corresponding subunits of complex-I (Munnich and Rustin 2001).

Table 2 Mitochondrial mutations affecting cytochrome *c* oxidase subunits (*s* introduction of a stop codon, *p* point mutation, *MELAS m*itochondrial *e*ncephalomyopathy, *l*actic acidosis *a*nd *s*trokelike episodes)

Gene	Type	Phenotype	References
*cox*I	s	Motor neuron disease	Comi et al. 1998
*cox*I	s	Exercise intolerance, myoglobinuria	Karadimas et al. 2000
*cox*I	p	Epilepsia partialis	Varlamov et al. 2002
*cox*I	p	Acquired sideroblastic anemia	Gattermann et al. 1997
*cox*I	p	Acquired sideroblastic anemia	Gattermann et al. 1997
*cox*I	s	Deafness, ataxia, blindness	Bruno et al. 1999
*cox*I	(s)	McArdle's disease	Aguilera et al. 2001
*cox*II	p	Myopathy, ataxia, dementia	Clark et al. 1999
*cox*II	p	Proximal limb weakness	Rahman et al. 1999
*cox*II	s	Early onset multisystem disease	Campos et al. 2001
*cox*II	s	Lactic acidosis	Wong et al. 2001
*cox*III	15-bp deletion	Exercise intolerance, myoglobinuria	Keightley et al. 1996
*cox*III	s	Leigh-like syndrome	Tiranti et al. 2000
*cox*III	s	Exercise intolerance	Hanna et al. 1998
*cox*III	p	MELAS	Manfredi et al. 1995

On the other hand, not a single mutation is known in any of at least ten (see "Physiology and regulation") human nuclear cytochrome *c* oxidase genes (D'Aurelio et al. 2001).

Mutations occurring in vertebrate mitochondria can affect RNA genes or those 13 structural genes coding for subunits of mitochondrial protein complexes. Mutations in RNA genes usually will affect mitochondrial protein synthesis in general and will lead to so-called multisystem disorders with observable consequences in a wide range of different tissues and are beyond the scope of this article. Within this review only mutations in the three mitochondrial genes coding for subunits of cytochrome *c* oxidase will be considered.

A general observation with all the mutations listed in Table 2 is that most of them become manifest only late in childhood or even at the onset of adolescence [with the exception of 8042delAT (Wong et al. 2001) which was identified in a child that died shortly after birth]. Another general feature is that even when the same mutation is present in different individuals, the clinical consequences may vary considerably (Shoubridge 2001). Furthermore, only few of the studied mitochondrial mutations affecting COX genes seem to be inherited maternally. All other reported cases are thought to reflect spontaneous mutations having occurred in a small number of individuals (Karadimas et al. 2000). Lastly, even in affected individuals the tissue distribution of these mutations varies considerably, with some tissues showing no deviations from healthy controls.

All listed mutations lead either to point mutations or premature termination of translation, the only exception being the 15-bp deletion within subunit III, which leads to loss of five amino acids (Keightly et al. 1996) and a point mutation in the stop codon of subunit I. With the latter mutation, activity of the cytochrome *c* oxidase was found to be normal and the significance of this mutation for the reported disease is still controversial (Aguilera et al. 2001). Mutations that lead to premature termination are in most cases accompanied by loss of redox centers of subunit I or II. In one extreme case subunit I is lost almost completely (mutation G5920A, Karadimas et al. 2000) or amino acid side chains considered essential ligands to both hemes of subunit I are absent (G6930A, Bruno et al. 1999). Irrespective of the consequences of these truncations for assembly of the remaining complex, loss of activity is inevitable. The same holds true for truncated forms of subunit II which

have lost the Cu_A center (mutation G7896A, Campos et al. 2001; deletion of AT at position 8042, Wong et al. 2001). The truncations usually lead to severe reduction of immunological detectability of the affected subunits.

An interpretation is less obvious with truncations occurring in subunit III of the cytochrome *c* oxidase. Even the loss of roughly 150 amino acids of this subunit did not cause complete loss of oxidase activity in cybrid cells considered to carry 100% of the respective mutation (insertion of C at position 9537, Tiranti et al. 2000). This is somewhat surprising as the completely assembled complex was found to be absent. A considerably smaller loss of only 13 residues at the C-terminal end of subunit III (G9952A, Hanna et al. 1998) nevertheless seems to impair assembly of the holoenzyme as levels of subunit I, II, and VIc were reduced in comparison to controls, while those of subunit IV and Va did not differ (Hanna et al. 1998).

Several point mutations in the three mitochondrially coded cytochrome *c* oxidase subunits are known that were attributed to pathological situations. Mutation L196I in oxidase subunit I leads to lowered maximal rates of respiration at more than 90% of mutated mtDNA and seems to cause a decrease in the stability of the entire COX enzyme complex (Varlamov et al. 2002). For a limited number of mutations that were found in human mitochondrial oxidase, equivalent amino acid substitutions were also introduced into bacterial or yeast complexes to study the consequences of the corresponding exchange. Two amino acid substitutions (M273T and I280T) were reported in human oxidase subunit I that could be the cause of respiratory chain dysfunction, assuming that a functional oxidase is required to maintain the necessary level of reduced iron for heme synthesis (Gattermann et al. 1997). These two residues are highly conserved and located in the vicinity of the K-channel of subunit I (see "Proton translocation pathways"). Introduction of the same mutations into yeast cytochrome *c* oxidase showed a mildly deleterious effect on respiration with some aspects of their properties being similar to the human counterparts. Such mutant yeast strains might prove useful as an alternative model to study consequences of human oxidase mutations (Meunier 2001). Alteration of the start codon for subunit II (mutation T7587C) is expected to compromise translation of the corresponding protein. Consequently, a decrease in COX activity was observed at a mutant load of more than 55%, and in cells with more than 95% mutated mtDNA no mature CoxII polypeptide could be detected (Clark et al. 1999). Exchange of methionine by threonine in the first transmembrane helix of cytochrome *c* oxidase subunit II causes the oxidase assembly to be partially arrested at the level of the first assembly intermediate (Nijtmans et al. 1998). Furthermore, a marked decrease in heme a_3 was observed indicating that association of subunits I and II is necessary to stabilize binding of heme a_3 (Rahman et al. 1999). The 15-bp in-frame deletion (9480del15, loss of the amino acid sequence FAGFF) in the third transmembrane section of subunit III causes a severe respiratory chain defect by interfering with the proper assembly of the core subunits into a multisubunit complex, especially in a homoplasmic situation (Keigthley et al. 1996). This observation could be confirmed by introducing the same deletion in yeast cytochrome *c* oxidase. Cells with this mutation were respiratory growth deficient and enzyme assembly was abolished (Meunier 2001). Strikingly, no limitation of energy production was observed even though oxidase activity was reduced by 85% in the patients' skeletal muscle (Hoffbuhr et al. 2000).

Typically, the MELAS (see Table 2) clinical phenotype is due to mutations in mtDNA-encoded tRNA genes. There is one report that MELAS can also be caused by a missense mutation (F251L) in the gene for cytochrome *c* oxidase subunit III (Manfredi et al. 1995).

A corresponding mutation introduced into the *Paracoccus denitrificans* aa_3 cytochrome *c* oxidase could give a hint for interpretation of the clinical observation as the H^+/e^- ratio was reduced in the mutant oxidase from 0.8 to 0.5 when compared to wild type (Mather and Rottenberg 1998).

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J. H. Eckert · R. Erdmann

Peroxisome biogenesis

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Abstract Peroxisome biogenesis conceptually consists of the (a) formation of the peroxisomal membrane, (b) import of proteins into the peroxisomal matrix and (c) proliferation of the organelles. Combined genetic and biochemical approaches led to the identification of 25 *PEX* genes-encoding proteins required for the biogenesis of peroxisomes, so-called peroxins. Peroxisomal matrix and membrane proteins are synthesized on free ribosomes in the cytosol and posttranslationally imported into the organelle in an unknown fashion. The protein import into the peroxisomal matrix and the targeting and insertion of peroxisomal membrane proteins is performed by distinct machineries. At least three peroxins have been shown to be involved in the topogenesis of peroxisomal membrane proteins. Elaborate peroxin complexes form the machinery which in a concerted action of the components transports folded, even oligomeric matrix proteins across the peroxisomal membrane. The past decade has significantly improved our knowledge of the involvement of certain peroxins in the distinct steps of the import process, like cargo recognition, docking of cargo-receptor complexes to the peroxisomal membrane, translocation, and receptor recycling. This review summarizes our knowledge of the functional role the known peroxins play in the biogenesis and maintenance of peroxisomes. Ideas on the involvement of preperoxisomal structures in the biogenesis of the peroxisomal membrane are highlighted and special attention is paid to the concept of cargo protein aggregation as a presupposition for peroxisomal matrix protein import.

Introduction

Appreciation of the peroxisome as a highly versatile and important multirole player in the cellular landscape has come a long way since Rhodin (1954) first described the "spherical and oval microbodies" in the 1950s. Later on these organelles were renamed by de Duve

J. H. Eckert · R. Erdmann (✉)
Institut für Physiologische Chemie, Medizinische Fakultät, Ruhr-Universität Bochum,
44780 Bochum, Germany
e-mail: Ralf.Erdmann@ruhr-uni-bochum.de · Tel.: +49-234-3224943 · Fax: +49-234-3214937

and Baudhuin (1966) after the hydrogen peroxide-based respiration reaction, which is one of its conserved functions. Despite the fact that already in 1973 it was discovered that cells derived from patients suffering from Zellweger's syndrome lacked morphologically distinguishable peroxisomes (Goldfischer et al. 1973), the perception of the peroxisome as the site of many vital biochemical reactions did not evolve before the mid 1980s (reviewed in de Duve 1996). Today, the importance of peroxisomes can be estimated much more clearly. Peroxisomes are structurally and functionally related organelles that are found in virtually all eukaryotic cells and also comprise the glyoxysomes of plants and fungi and the glycosomes of kinetoplastida. Substantial differences in the metabolic functions between man and trypanosomes may lead the way towards novel strategies against malaria, and a wide variety of genetically inherited diseases in humans underline the impact which this organelle has on our lipid metabolism. The range of defects attributed to defects and deficiencies of the peroxisome nowadays extends from the various complementation groups of Zellweger's syndrome and different types of adrenoleukodystrophy to Refsum disease or rhizomelic chondrodysplasia punctata (Fujiki 2000; Gould and Valle 2000; Wanders et al. 1995). Despite the progress which has been made over the past two decades in understanding the peroxisome, a central question remains unanswered: How do peroxisomes arise?

In this article, we review the current state of knowledge on the origins of the peroxisomal membrane, its membrane proteins, and import of matrix proteins. A new model for the import of matrix proteins based on recent observations on the aggregation of import substrates is presented and discussed.

Morphology and metabolism

The morphology of peroxisomes differs significantly among various tissues and species. They are surrounded by a single lipid bilayer, are mostly spherical bodies with a range of 0.2 μm–1 μm in diameter in typical human cells, or around 0.5 μm in oleate-grown yeast. In glucose-grown yeast, the peroxisomes tend to be significantly smaller—between 0.1 μm and 0.2 μm. The occurrence of tubular structures has been reported in several organisms, some seeming to interconnect the spherical compartments (Angermüller et al. 1986; Makita 1995; Yamamoto and Fahimi 1987). Peroxisomes have been reported to have a very electron-dense matrix, and in several studies a paracrystalline structure of this matrix has been described (Fig. 1). The Woronin body of the filamentous fungi *Neurospora crassa* is a new type of specialized peroxisome which contains a hexagonal crystalloid core and appears to plug septal pores of the syncytium upon cellular damage (Jedd and Chua 2000).

Metabolic tasks of peroxisomes are as widespread as are their morphologies throughout the eukaryotic kingdom. One function well-conserved throughout all of these is the hydrogen peroxide respiration. Naturally, much effort has been invested into determining the functional properties of peroxisomes in man (Wanders and Tager 1998). They comprise the β-oxidative degradation of a specific set of lipids which cannot be processed by mitochondria. These lipids include (but are not limited to) very long chain fatty acids (VLCFA), long chain dicarboxylic acids, various unsaturated fatty acids, di- and trihydroxycholestanoic acids, and pristanoic acids. Plasmalogens (ether-phospholipids) are synthesized cooperatively in the endoplasmic reticulum and the peroxisome, with the peroxisome being the site of introduction of the ether linkage into the plasmalogens. While the precise

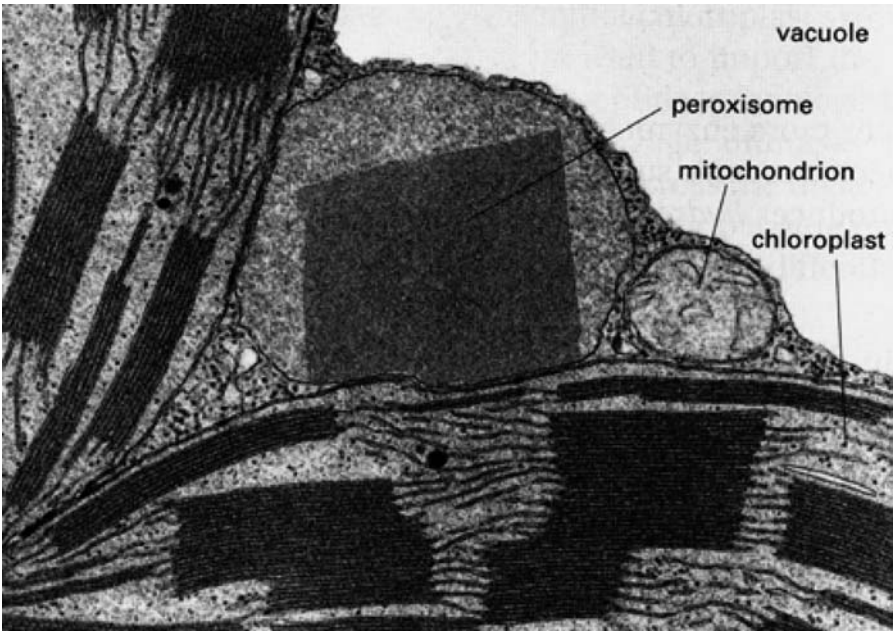

Fig. 1 Ultrastructural appearance of peroxisomes. A peroxisome with a paracrystalline core of a tobacco leaf mesophyll cell. Its proximity to chloroplasts and mitochondria is thought to facilitate the exchange of metabolites between these organelles during photorespiration (from Frederick and Newcomb, 1969)

role of the plasmalogens remains mysterious, the severe pathological consequences of a deficiency in plasmalogen synthesis hint at a central role in the human organism.

Human peroxisomes take part in the de novo synthesis of cholesterol from different precursor molecules and play a major role in isoprenoid metabolism (Biardi et al. 1994; Krisans 1992; Krisans et al. 1994). Pipecolic acid is catabolized in peroxisomes of man, and phytanic acid is α-oxidized here to pristanic acid, which can be β-oxidized in peroxisomes as described above. Furthermore, glyoxylate aminotransferase, which transforms toxic glyoxylate into alanine, is localized in the peroxisome in man (Noguchi and Fujiwara 1988).

In plants, the glyoxylate cycle takes place in the peroxisomes of seedlings, which thus are referred to as "glyoxysomes." In leaf tissues, photorespiration takes place in peroxisomes, and in root tissues of uninfected leguminoses part of the nitrogen metabolism is carried out by peroxisomes, which play a key role in ureide production (Johnson and Olsen 2001; Reumann 2000).

In yeasts, several steps of the glyoxylate cycle also occur in the peroxisome. Other pathways which are at least partially described to occur in peroxisomes include the biosynthesis of lysine and the degradation of amino acids, methanol, and hydrogen peroxide (Mannaerts and Van Veldhoven 1993). The β-oxidation of fatty acids exclusively occurs in peroxisomes in yeasts, a discovery first made by Kunau and coworkers (Veenhuis et al. 1987) and later exploited for genetic screening for genes essential for peroxisome biogenesis (Erdmann et al. 1989).

Peroxisome assembly: approaches and model organisms

The proteins which are essential for the biogenesis of the peroxisome demonstrate a broad level of similarity from yeast to man and have been named "peroxins." Their nomenclature was unified in 1996 (Distel et al. 1996; Table 1). Due to early observations of peroxisomal matrix and membrane proteins being synthesized on free ribosomes in the cytosol and posttranslationally imported into the matrix or membrane of the organelle, the biogenesis of peroxisomes was thought to occur similar to that of the endosymbiotic mitochondria and plastides (reviewed in Lazarow and Fujiki 1985). However, the biogenesis of peroxisomes differs from mitochondria and plastides in several particular aspects. First, all peroxisomal proteins are encoded by the nuclear DNA; peroxisomes do not contain their own DNA. Second, there is a widespread understanding that peroxisomes can arise newly in cells which apparently lack peroxisomes or peroxisomal remnants. Third, the mechanism of matrix protein import into the peroxisome differs considerably from the mitochondrial one as the peroxisomal import machinery accommodates folded, even oligomeric proteins from the cytosol. Thus, protein unfolding is not a prerequisite for import of peroxisomal matrix proteins, suggesting novel mechanisms for the translocation of polypeptides across the peroxisomal membrane.

Genetic screens in various model organisms, yeast peroxisomal proteomics, and in vitro import assays have been applied in order to study the biogenesis of peroxisomes, especially the import of proteins into the organelle and its membrane. After it had been found that *S. cerevisiae* relied on peroxisomes to utilize oleate as the sole carbon source and that growth on oleate induced the proliferation of peroxisomes in this yeast (Veenhuis et al. 1987), screens for mutants affected in the assembly of peroxisomes and therefore unable to survive on these media were identified by Erdmann et al. (1989). Fujiki and coworkers screened for Chinese Hamster Ovary (CHO) cell mutants affected in plasmalogen synthesis which also turned out to be affected in peroxisome biogenesis (Tsukamoto et al. 1990). Complementation of yeast and CHO-mutants with genomic libraries led to the isolation of numerous genes related to peroxisomal biogenesis (Erdmann et al. 1991; Tsukamoto et al. 1991; reviewed by Titorenko and Rachubinski 2001b). The genetic approaches were successfully extended to other yeasts such as *Hansenula polymorpha, Pichia pastoris*, and especially to *Yarrowia lipolytica* (Distel et al. 1996; Purdue and Lazarow 2001a). Twenty-three of the 25 genes known to be involved in the biogenesis of peroxisomes have been identified with these genetic methods (Table 1). Novel peroxins were also discovered by a proteomic approach to identify components of the yeast peroxisomal protein translocation apparatus (Erdmann and Blobel 1995; Erdmann and Blobel 1996) and more recently by microarray screenings for oleic acid-induced yeast genes (Smith et al. 2002).

In vitro import assays have apparently been established for plant peroxisomes (Baker 1996; Brickner et al. 1997; Pool et al. 1998; reviewed by Baker et al. 2000), which will allow further studies on the tasks of single components of the import machinery. A limiting factor was the low number of plant peroxins which had not been identified until recently (Baker et al. 2000; Charlton and Lopez-Huertas 2002; Table 1). On the other hand, in vitro import systems have so far failed to work in yeast cell systems, for which more information on peroxins and their interactions are available (Tables 1 and 2). In vitro import systems using streptolysin-O (SLO)-permeabilized mammalian semi-intact cells demonstrated the ATP-dependence and the requirement for cytosolic factors of the protein import into the peroxisomal matrix (Terlecky et al. 2001; Wendland and Subramani 1993). Surprisingly, the available plant and mammalian in vitro systems have not yet provided a

Table 1 Peroxins (*IMP* integral membrane protein, *MAP* membrane attached protein, *SP* soluble protein)

Peroxin	Other Names	Species							Proposed functions	Type of protein/localization
		S.c.	*N.c.*	*Y.l.*	Other yeast	Mammals	Plants	Other		
Pex1p	*PpPAS1, ScPAS1*	X	X	X	X	X	X	*D.m.*	Matrix protein import, vesicle fusion	SP, MAP, (dep. on species)
Pex2p	*HsPAF1 RnPAF1 PaCAR1 PpPER6 ScPAS5 YlPAY5*	X	X	X	X	X	X	*C.l. L.d. T.b.*	Receptor recycling/ translocation	IMP
Pex3p	*HpPER9 PpPAS2 ScPAS3*	X	X		X	X	X		PMP targeting/import	IMP
Pex4p	*PpPAS4, ScPAS2*	X	X		X	X	X		Ubiquitin conjugase	SP/MAP
Pex5p	*CaPAS10 HpPAH2 HpPER3 HsPTS1R HsPXR1 MmPTS1R MmPXR1 PpPAS8 ScPAS10 YlPAY32*	X	X	X	X	X	X	*C.l. D.m. T.b.*	PTS1 receptor, PTS3 receptor (in *S.c.*)	SP/MAP
Pex6p	*HsPAF2 HsPXAAA1 PpPAS5 RnPAF2 ScPAS8 YlPAY4*	X	X	X	X	X	X	*C.l.*	Matrix protein import, vesicle fusion	SP, MAP, (dep. on species)

Table 1 (continued)

Peroxin	Other Names	Species							Proposed functions	Type of protein/localization
		S.c.	*N.c.*	*Y.l.*	Other yeast	Mammals	Plants	Other		
Pex7p	*ScPAS7* *ScPEB1* *SpPAS7*	X	X		X	X	X		PTS2 receptor	SP/MAP
Pex8p	*HpPER1* *PpPER3* *ScPAS6* *YlPEX17*	X	X	X	X				Matrix protein import	MAP (matrix face)
Pex9p	*YlPAY2*			X					Matrix protein import/Perox. enlargement	IMP
Pex10p	*AtPER8* *HpPER8* *PpPAS7* *ScPAS4*	X	X	X	X	X	X		Receptor recycling/ translocation	IMP
Pex11p	*CbPMP30/31/32,* *RnPMP26* *ScPMP27*	X	X		X	X	X	*C.l. T.b.*	Proliferation, lipid metabolism	IMP
Pex12p	*HsPAF3* *PpPAS10* *RnPAF3* *ScPAS11*	X	X		X	X	X		Receptor recycling/ translocation	IMP
Pex13p	*PpPAS6* *ScPAS20*	X	X		X	X	X	*C.l.*	Matrix protein import/ docking/recycling	IMP
Pex14p	*HpPER10*	X	X	X	X	X	X	*T.b.*	Matrix protein import/docking	IMP
Pex15p	*ScPAS21*	X								IMP
Pex16p			X	X	X	X	X		PMP targeting/import	IMP/MAP (matrix face)
Pex17p	*ScPAS9*	X			X		X		Matrix protein import/docking	IMP

Table 1 (continued)

Peroxin	Other Names	Species							Proposed functions	Type of protein/localization
		S.c.	*N.c.*	*Y.l.*	Other yeast	Mammals	Plants	Other		
Pex18p		X							*S.c.* PTS2–dep. protein import/Cargo aggregation	SP/MAP
Pex19p	*ScPAS12*	X	X	X	X	X	X	*C.l.*	PMP targeting/import	SP/MAP
Pex20p			X	X					*Y.l.* PTS2–dep. protein import	MAP
Pex21p		X							*S.c.* PTS2–dep. protein import/Cargo aggregation	SP/MAP
Pex22p		X			X				Recruiting Pex4p to membrane	IMP
Pex23p			X	X					Matrix protein import	IMP
Pex24p			X	X					Matrix protein import/PMP targeting/import	IMP
Pex25p		X							Proliferation and division of peroxisomes	MAP
Djp1p		X							Folding and/or aggregation of import substrates	SP/MAP

Given are the names in peroxin nomenclature, their previous names, the organisms from which these peroxins have been identified, the proposed function(s) as well as the localization and type of protein. Of the 14 mammalian genes listed, 12 have been shown to cause peroxisome biogenesis disorders (PDB) (Fujiki 2000). This list covers the organisms studies most thoroughly and is not complete (the data on *Neurospora crassa* peroxins are from Sichting et al. 2003)

Abbreviations of species and groups of species: *C.l,. Caenorhabditis elegans*; *D.m.*, *Drosophila melanogaster*; *L.d.*, *Leishmania donovani*; *M,* mammals; *N.c.*, *Neurospora crassa*; *Pl*, plants; *S.c.*, *Saccharomyces cerevisiae*; *T.b.*, *Trypanosoma brucei*; *Y*, yeasts; *Y.l.*, *Yarrowia lipolytica*

Table 2 Overview of interactions between peroxins. Denoted are interactions which have been clearly demonstrated in any organism by two-hybrid analysis, coprecipitation (unless the interaction has been shown to be indirect) and in vitro binding studies. An interaction denoted in this table does not necessarily imply that these two peroxins have been found to interact in all species in which both partners have been described

	Pex 1p	Pex 2p	Pex 3p	Pex 4p	Pex 5p	Pex 6p	Pex 7p	Pex 8p	Pex 9p	Pex 10p	Pex 11p	Pex 12p
Pex1p						X						
Pex2p												
Pex3p												
Pex4p												
Pex5p					X		X	X				X
Pex6p	X											
Pex7p					X							
Pex8p					X							
Pex9p												
Pex10p										X		X
Pex11p											X	
Pex12p					X					X		
Pex13p					X		X					
Pex14p					X		X					
Pex15p												
Pex16p												
Pex17p												
Pex18p							X					
Pex19p		X	X							X	X	X
Pex20p							X	X				
Pex21p							X					
Pex22p				X								
Pex23p												
Pex24p												
Pex25p												

clue about the principle of translocation of folded proteins across the peroxisomal membrane and about the order of events during the peroxisomal protein import process (see below).

Yeast two-hybrid studies and coprecipitation analysis revealed direct and indirect interactions among known peroxins (Table 2). Several of the interactions observed in vivo have been confirmed by in vitro binding studies as well as by the analysis of the composition of isolated cross-linked or native preparations of membrane-bound peroxisomal protein complexes (Albertini et al. 2001; Albertini et al. 1997; Girzalsky et al. 1999; Gouveia et al. 2000; Hazra et al. 2002; Reguenga et al. 2001; Urquhart et al. 2000). These and other studies allowed postulation of the existence of stable and transient protein complexes of the peroxisomal protein import machinery (Subramani et al. 2000; Fig. 6).

The biogenesis of the peroxisomal membrane: distinct import machineries for peroxisomal matrix and membrane proteins and morphological appearance of peroxisomal membrane ghosts

The first hint that the targeting and insertion of peroxisomal membrane proteins might require different proteins than the import into the peroxisomal matrix came from the dis-

Table 2 (continued)

Pex 13p	Pex 14p	Pex 15p	Pex 16p	Pex 17p	Pex 18p	Pex 19p	Pex 20p	Pex 21p	Pex 22p	Pex 23p	Pex 24p	Pex 25p
						X						
						X						
									X			
X	X											
X	X				X		X	X				
							X					
						X						
						X						
						X						
	X					X						
X	X			X		X						
						X						
	X					X						
X	X		X	X					X			
						X						

covery of "empty peroxisomal membranes" ("ghosts") in cells from Zellweger patients which still contained peroxisomal membrane proteins but lacked most if not all matrix proteins (Santos et al. 1988a; Santos et al. 1988b; Yamasaki et al. 1999). This was finally verified by studies in yeast, demonstrating that the deficiency in central components of the import machinery for peroxisomal matrix proteins did not affect the topogenesis of peroxisomal membrane proteins (Albertini et al. 1997; Erdmann and Blobel 1996; Gould et al. 1996; Salomons et al. 2001). The peroxisomal ghosts are not easily recognized morphologically because they often are difficult to distinguish from other cell membranes. However, when labeled immuncytochemically, they can be easily detected and usually appear somewhat larger and less abundant than normal peroxisomes and frequently have an onion-like structure consisting of multiple layers of peroxisomal membranes (Fig. 2). A systematic electron microscopic examination of ghosts in *pex6* mutant CHO cells revealed that they are complex membrane structures with usually one central spherical body and several layers of double membrane loops, with endoplasmic reticulum (ER) present alongside the outer loop (Fig. 3). Most interestingly, in the early stages of complementation, peroxisomal matrix proteins accumulated in the lumen of the double membrane loops (Hashiguchi et al., 2002). Characteristic matrix-deficient peroxisomal membrane loops which could be immunolabeled with peroxisomal membrane proteins previously have been reported to reside in close proximity to mature peroxisomes

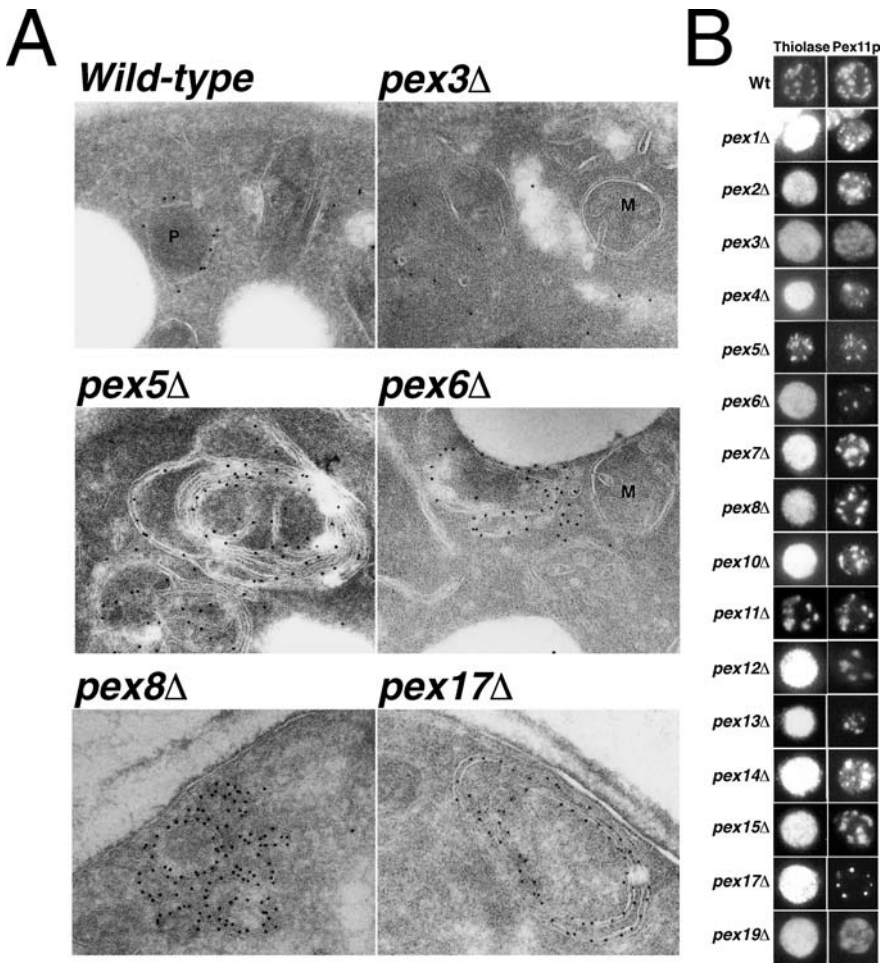

Fig. 2A, B Peroxisomal membrane ghosts. Ultrastructural appearance of peroxisomal membrane ghosts and subcellular localization of peroxisomal membrane and matrix proteins in wild-type and selected *pex*-mutants of *S. cerevisiae*. Oleic-acid induced wild-type and mutant cells expressing hemagglutinin-tagged Pex11p as a marker for peroxisomal membranes were processed for **A** immunogold electron microscopy and **B** double immunofluorescence microscopy, both with mouse monoclonal antibodies specific for the HA-epitope. For the double-immunofluorescence microscopy, thiolase was labeled with rabbit polyclonal antibodies against the protein. Cells lacking Pex3p or Pex19p were characterized by mislocalization of Pex11p-HA to the cytosol which led to their function in the biogenesis of the peroxisomal membrane. In most other *pex*-mutants, Pex11p-HA was present in onion-shell like multilayered membrane structures. Secondary antibodies were CY3-conjugated antimouse IgG and FITC-conjugated antirabbit IgGs. *P* peroxisome, *M* mitochondrion. *Bar*, 0.25 μm (adapted from Hettema et al. 2000)

and have to be considered to be true intermediate structures for the generation of peroxisomes (Baumgart et al. 1989; Erdmann and Blobel 1995). We also have to consider them to be directly involved in the import process of folded and oligomeric proteins of the peroxisomal matrix (see below).

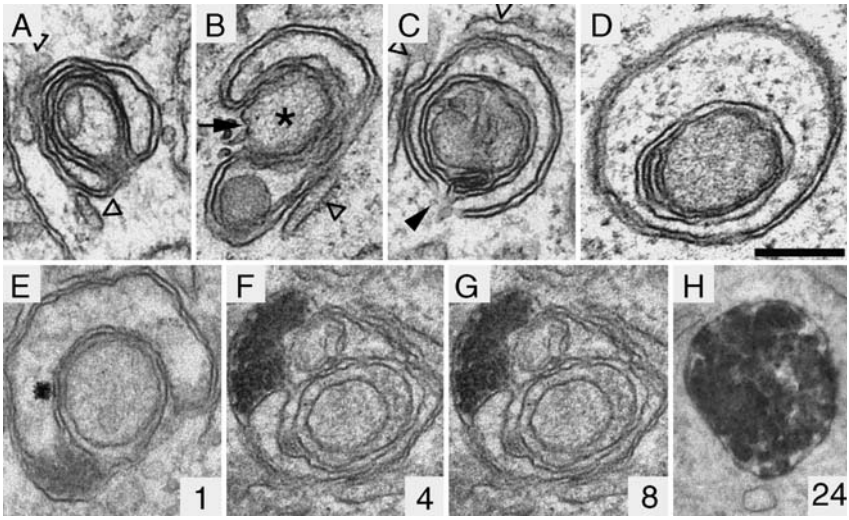

Fig. 3A–F Complex membrane structures in Pex6p-deficient cells. **A–D** Ultrastructural appearance of peroxisomal membrane ghosts in Chinese Hamster Ovary *pex6* mutant cells. Typically, sets of one central spherical body and two layers of double-membrane loops were observed, with endoplasmic reticulum present alongside the outer loop. In **C** and **D**, a spherical body (*asterisk*) is invading the double-membrane loop. **E**, **F** Morphological appearance of the peroxisomal membrane ghosts upon complementation. Cells were cotransfected with catalase- and *PEX6*-cDNA under the control of an inducible promoter. The numbers indicate the time (h) after induction. Catalase-positive signals were not observed in the spherical body but appeared in the lumen of the double-membrane loops. *Bars*, 200 nm (adapted from Hashiguchi et al. 2002)

Peroxins contributing to the formation of the peroxisomal membrane

Of the currently known 25 peroxins, only three have been identified to play a particular role in the biogenesis of the peroxisomal membrane: several studies indicated that human cells lacking either a functional copy of *PEX3*, *PEX16*, or *PEX19* contain neither peroxisomes nor peroxisomal remnants (Ghaedi et al. 2000a; Honsho et al. 1998; Matsuzono et al. 1999; Sacksteder et al. 2000; Shimozawa et al. 1998b; South and Gould 1999). In all other peroxin deletion strains assayed, spherical membranous structures containing other peroxisomal membrane proteins ("ghosts") and in some cases also a fraction of imported matrix proteins (e.g., in *pex5*Δ or *pex7*Δ cells) were found (Hettema et al. 2000; Fig. 2). The same study indicates that in *S. cerevisiae* only Pex3p and Pex19p are required for the biogenesis of the peroxisomal membrane, based on the observation that among all *pex*-mutants known, only *pex3*Δ and *pex19*Δ cells seem to completely lack detectable peroxisomal membrane structures and mislocalize their peroxisomal membrane proteins (PMPs) to the cytosol, where they are rapidly degraded. To our present knowledge, there is no homologue to the *PEX16* gene in *S. cerevisiae*.

Upon expression of the respective peroxin gene from plasmids introduced into *pex3*, *pex16*, or *pex19* cells, fully functional peroxisomes are formed again. These experiments were accepted as proof for the de novo synthesis of peroxisomes in the absence of peroxisomes (Matsuzono et al. 1999; Sacksteder et al. 2000; South and Gould 1999; South et al. 2000).

More recent studies, however, seem to challenge this view. Snyder et al. (1999a), for example, found tiny vesicles and tubules containing Pex3p in *pex19*Δ cells of *P. pastoris* by deconvolution microscopy. While these structures are morphologically distinct from fully developed peroxisomes, they might resemble an "early peroxisome." Also, Lambkin and Rachubinski (2001) found structures resembling peroxisomes and containing a number of peroxisomal proteins in cells deficient for *PEX19* in *Y. lipolytica*, from which they assigned a role for Pex19p in stabilizing membrane protein complexes, which would be different from what had been deduced for other organisms. Recently, several PMPs (Pex8p, Pex12p, Pex13p, Pex14p, Pex17p) have been described to associate with membranes that are different from mitochondria in *pex3*Δ cells of *Pichia pastoris* (Hazra et al. 2002). Whether these membranes represent peroxisomal remnants or other cellular compartments still has to be shown. So far, we cannot exclude the possibility that PMPs are artificially targeted to other cellular structures in the absence of the normal target membrane in *pex3*Δ or *pex19*Δ cells. However, based on the evidence mentioned above, we also have to consider that there are protoperoxisomes in the cells of any species lacking *PEX3*, *PEX16*, or *PEX19*. Thus, it still remains uncertain today whether there actually is a de novo formation of peroxisomes. Irrespective of this question, Pex3p, Pex16p, and Pex19p seem to play an early role in the biogenesis of peroxisomes.

Pex3p has been identified as an integral membrane protein in yeast and mammals, exposing both termini to the cytosol (Ghaedi et al. 2000b), or with the N-terminus residing in the peroxisomal matrix and an externally exposed C-terminus (Höhfeld et al. 1991; Soukupova et al. 1999). It has been shown to interact with Pex19p, a farnesylated protein which is localized in the cytosol and at least partially at the peroxisomal membrane (Götte et al. 1998; Sacksteder et al. 2000; Snyder et al. 1999a; Soukupova et al. 1999). To date, it is widely believed that the interaction between the two is required for the targeting of other PMPs. Pex19p has been proposed to function as a PMP recognition factor which might bind PMPs in the cytosol and subsequently targets them to Pex3p, which might assist in the membrane insertion of other PMPs (Sacksteder et al. 2000). In line with this assumption, Snyder et al. (1999a) suggest that in *P. pastoris*, Pex3p functions upstream of Pex19p. Because Pex3p is found in early remnants in *pex19*Δ cells of this yeast, its incorporation into membranes could not be dependent on Pex19p. In addition, in mammals Pex3p does not seem to require Pex19p for its own targeting (Ghaedi et al. 2000a). Thus, while the question is still not fully answered, the hints are accumulating of a concurrent function of Pex19p and Pex3p in the earliest stages of peroxisome biogenesis, namely the insertion of other PMPs into the membrane.

Pex16p has been identified in several fungi, plants, and human cells. In humans, Pex16p is an integral membrane protein which appears to expose both termini to the cytosol and plays a role in membrane assembly (Honsho et al. 2002; Honsho et al. 1998; South and Gould 1999). In *Y. lipolytica*, however, Pex16p is a peripheral membrane protein which does not seem to participate in this process (Eitzen et al. 1997)**.** The expression of human *PEX16* in fibroblasts of complementation group D lacking a functional *HsPEX16* gene restored the biogenesis of the peroxisomal membrane as well as peroxisomal matrix protein import, the first prior to the latter (Honsho et al. 1998; South and Gould 1999). While it seems obvious that *PEX16* is involved in the biogenesis of the peroxisome membrane, it is not clear whether its role lies within peroxisome membrane protein import or within the formation of the lipid bilayer. Recently, it was reported that expression of the C-terminal cytosolic part of Pex16p in human cell lines severely affected the complemen-

tation of peroxisome assembly in *pex3*Δ mutant cells by *PEX3* expression. This data might imply that the Pex16p truncation might interfere with the targeting and/or integration of newly synthesized Pex3p into membranes of preperoxisomes. Thus, Pex16p is likely to be involved in a very early stage of peroxisomal membrane assembly, maybe even upstream of Pex3p (Honsho et al. 2002). Interestingly, *Y. lipolytica* cells deficient in Pex16p can be complemented by the expression of Sse1p, a protein which is required for protein and lipid body biogenesis in *Arabidopsis* (Lin et al. 1999). Lipid and protein bodies generate by budding from the ER (Lin et al. 1999; Murphy and Vance 1999). However, whether the observed interchangeability of Pex16p and Sse1p refers to a related biogenesis of lipid or protein bodies and peroxisomes needs to be further analyzed. While the task which Pex16p fulfills in peroxisome biogenesis remains to be determined, it is clear that the protein does not target other PMPs via the endoplasmic reticulum (South and Gould 1999), a theory that will be discussed later in this article.

Taken together, these three peroxins (or these two in the case of *S. cerevisiae* and others) were seen as the factors essential for the assembly of the peroxisomal membrane including all other PMPs. They are suggested to recognize newly synthesized membrane proteins in the cytosol (Pex19p) and integrate them into the membrane (Pex3p) (Hettema et al. 2000; Sacksteder et al. 2000).

On the origin of peroxisomes: the combinatorial model: de novo synthesis combined with division?

In 1985, Lazarow and Fujiki (1985) summarized evidence for the posttranslational import of matrix and membrane proteins into pre-existing peroxisomes and postulated that peroxisomes proliferate and multiply by growth and division. Since then, the posttranslational targeting and insertion of peroxisomal membrane proteins was convincingly confirmed by several in vivo and in vitro studies (Imanaka et al. 1987; Pause et al. 1997; Suzuki et al. 1987a; Suzuki et al. 1987b). In line with the growth and division model, Heinemann and Just (1992) demonstrated that newly synthesized proteins in the cytosol are being imported mainly into peroxisomes of small to medium density and that these peroxisomes thereby maturate to larger peroxisomes of higher density. In yeast, Pex3p and thiolase containing peroxisomes of 1.15 g/ml density were shown to significantly increase in density up to 1.23 g/ml upon oleic acid induction (Erdmann and Blobel 1995). The ability of peroxisomes to multiply by division was recently demonstrated convincingly in vivo by time-lapse microscopy (Hoepfner et al. 2001). Pex11p was assigned a role as a regulator of peroxisome division, while the protein itself was found to contribute neither to the biogenesis of the membrane nor to the import of matrix proteins (Erdmann and Blobel 1995; Marshall et al. 1995; Passreiter et al. 1998; Schrader et al. 1998).

In the second half of the 1990s, evidence accumulated for a de novo synthesis of peroxisomal membranes, (e.g., Elgersma et al. 1997; reviewed in Kunau and Erdmann 1998).

To account for these different findings, a two-way model for the biogenesis of peroxisomes was developed (Erdmann et al. 1997; Subramani 1996). The principle of the two-way model, including some new ideas, are outlined in Fig. 4. In the first pathway, which refers to the original growth and division model of Lazarow and Fujiki (1985), existing peroxisomes grow by continuous import of PMPs and matrix proteins and finally divide, possibly controlled directly or indirectly by Pex11p (Fig. 4, lower part). In the second route of peroxisome biogenesis, preperoxisomes are supposed to originate from an en-

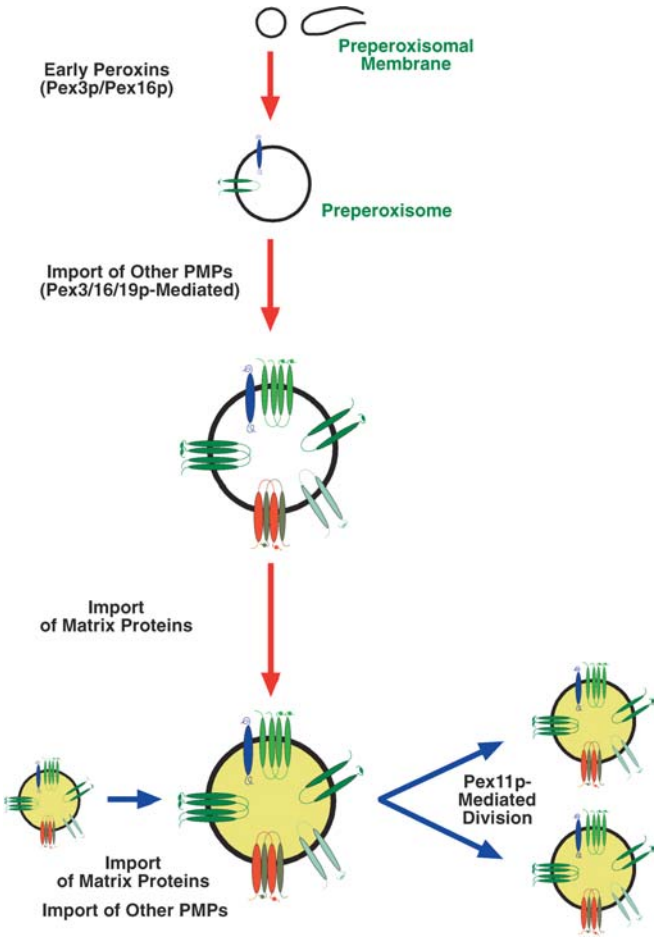

Fig. 4 Two-way model of peroxisome biogenesis. The model describes two ways in which new peroxisomes might be generated. In the first way (*red arrows*), a preperoxisomal membrane vesicle originates from an endomembrane, which could be the ER or a peroxisomal prestructure. The preperoxisomal vesicle represents the template for the growing and maturing organelle. It might already carry early peroxins such as Pex3p or Pex15p, and further PMPs might be incorporated with the help of Pex3p and Pex19p. The thus matured peroxisomal membrane imports peroxisomal matrix proteins, which complete the maturation process. The second way of peroxisome formation (*blue arrows*) is based on the growth and division model of Lazarow and Fujiki (1985) and describes the generation of new peroxisomes by fission of mature peroxisomes and maybe also preperoxisomes. Matrix protein-filled organelles are indicated in *yellow*

domembrane which could be the ER or a peroxisomal structure that by itself could be part of a peroxisomal reticulum (Fig. 4, upper part). The newly formed preperoxisomes might already contain or first import the early peroxins Pex3p, Pex16p, and possibly Pex15p into their membranes. Then, mediated by Pex19p and the early peroxins, other PMPs would be integrated into the membrane of the preperoxisome, which then would start to import peroxisomal matrix proteins and thereby maturate. This model implies that at least one early peroxin might be directed to the peroxisomal membrane by a distinct, most likely Pex19p-independent pathway, while the majority of PMPs, including most peroxins, would be di-

rected to the peroxisomal membrane by a more general, Pex3p- and Pex19p-dependent pathway. This is in line with the observations that targeting signals and Pex19p binding region are distinct in Pex3p (Fransen et al. 2001; Snyder et al. 1999a), which is the most likely candidate for being responsible for the first step in the generation of new peroxisomes (Shimozawa et al. 2000; South et al. 2000). At any point of time after the beginning of late PMP import, the Pex11p-controlled division of vesicles might take place. With exception of the data on vesicle fusion by Titorenko et al. (2000a) in the yeast *Y. lipolytica*, the peroxisomes of which seemed to escape conserved mechanisms in various ways, this model accounts for all recent observations at that stage (Erdmann and Blobel 1995; Götte et al. 1998; Honsho et al. 1998; Kunau 1998; Marshall et al. 1995; Matsuzono et al. 1999; Shimozawa et al. 1998a; Shimozawa et al. 1998b; Shimozawa et al. 1998c; South and Gould 1999)

Is the ER involved in peroxisome formation?

If peroxisomes could arise de novo, the source for membranes and preperoxisomal compartments would have to be identified. The membrane of the ER was seen as a likely candidate (Erdmann et al. 1997; Holroyd and Erdmann 2001; Tabak et al. 1999; Titorenko and Rachubinski 1998a; Titorenko and Rachubinski 2001b); especially inspired by (a) early electron micrographs showing a close physical proximity and apparently continuous membranes between the ER and peroxisomes (Novikoff and Shin 1964; Zaar et al. 1987) and by (b) data from Elgersma et al. (1997), which described Pex15p as residing in both the peroxisomal membranes and the membranous extensions of the ER, referred to as karmellae. In the same publication, it was mentioned that the deletion of the C-terminal 30 amino acid residues of Pex15p targeted the remaining protein to the ER. Later evidence by the same group proved that the ER localization was an artifact due to the overexpression of the protein. They also showed the occurrence of Pex15p in the peroxisomal membrane to depend on Pex3p and Pex19p (Hettema et al. 2000), which made a passage of Pex15p through the ER membrane rather unlikely. On the other hand, there are studies showing that Pex15p from *Saccharomyces cerevisiae* and pAPX from cottonseed in vitro posttranslationally insert into plant ER membranes only, though not into peroxisomal or other organelle membranes. In a direct comparison, the peroxisomal adenine nucleotide transporter Ant1p of *Candida boidinii* (PMP47) was imported into the peroxisomal membrane (Mullen et al. 1999), while Pex15p and pAPX were transported into the ER, supporting the assumption of distinct import pathways for early peroxins and other PMPs. This insertion of Pex15p and pAPX into the ER membrane was dependent on the provision of the cytosolic Hsp70p machine and ATP supply, but independent of the signal recognition particle, indicating that the process might occur posttranslationally (Mullen et al. 1999). In the same and a later study (Mullen and Trelease 2000), it was shown that pAPX from cottonseed localizes to the peroxisomal and the ER membrane when expressed at native levels. It still remains to be shown whether the ER-localized pAPX is in transit on the way to the peroxisome or whether it is also an ER-resident protein in cottonseed.

Other studies with partially truncated peroxisomal proteins or peroxisomal fusion proteins yielded mistargeting to the ER and other organelles, which was considered as evidence for the ER-involvement in peroxisome biogenesis. The N-terminal 16 amino acids of *Hansenula polymorpha* Pex3p, while insufficient for targeting to the peroxisomal membrane, direct a reporter protein to the ER membrane (Baerends et al. 1996). On the other

hand, a GFP fusion protein containing the first 16 amino acids of the human Pex3p, as well as several other truncated PEX3 fusion proteins, were localized to mitochondria in human fibroblasts (Soukupova et al. 1999).

Several experiments on peroxisome synthesis in the absence of pre-existing peroxisomes (namely carried out in human *pex16* cells) did not yield indications for an involvement of the ER (South and Gould 1999). Further investigations of the COP I- and COP II-dependence of Pex3p-mediated peroxisome synthesis upon expression of the protein in *pex3* cells found no effect of brefeldin A on the process. Brefeldin A blocks anterograde movement in the secretory pathway, which would have to be seen if preperoxisomal vesicles should bud from the ER, yet this treatment did not affect the targeting of Pex16p to peroxisomes (South et al. 2000). On the contrary, Salomons et al. (1997) discovered that brefeldin A does indeed interfere with the peroxisomal protein sorting in the yeast *Hansenula polymorpha*. PMPs Pex3p, Pex8p, and Pex14p and some matrix proteins accumulated in the ER in cells treated with brefeldin A and could be chased from the ER to peroxisomes after the removal of brefeldin A. Similar results were obtained in cottonseed with regard to the PMP pAPX (Mullen and Trelease 2000).

However, when cells were incubated at 15°C, Pex16p was still targeted to peroxisomes, even though at this temperature the exit of proteins from the ER-Golgi intermediate compartment is blocked. Thus, it was concluded that Pex16p cannot be targeted to peroxisomes via the ER (South and Gould 1999; South et al. 2000). Voorn-Brouwer et al. (2001) extended these studies on blocking COPI- and COPII-mediated vesicular transport and found that under these circumstances Pex2p, Pex3p, and Pex16p were still targeted properly to the peroxisome and that peroxisome morphology or integrity were not affected by COPI or COPII inhibitors.

A central prediction of an ER-involvement would be that the formation of nascent peroxisomes would require protein translocation into the ER. This assumption was tested by South et al. (2001) and they found that inactivation of the ER protein translocation factors Sec61p or Ssh1p does not affect peroxisome biogenesis. This result indicates that peroxisome biogenesis might not require protein import into the ER. The *SEC61*/*SSH1* double mutant, however, has not yet been analyzed. Even so, some proteins may enter the ER independently of Sec61p and Ssh1p. In this respect, it is interesting to note that tail-anchored protein insertion into the ER-membrane is not dependent on the classical *SEC* translocation machinery but rather occurs via an ATP-dependent pathway involving at least one novel membrane protein factor (Steel et al. 2002). This result indicates that there might still be ER targeting pathways to discover; whether the newly identified one is involved in the biogenesis of peroxisomes needs to be investigated.

Significant support for the involvement of the ER comes from the yeast *Yarrowia lipolytica*. The pulse-labeled PMPs Pex2p and Pex16p from this organism are targeted from the cytosol to the ER when produced at normal levels. There, they are N-glycosylated in the lumen and could be chased to peroxisomes. Furthermore, mutations in *PEX1* and *PEX6* of *Y. lipolytica* affect peroxisome biogenesis, prevent the exit of Pex2p and Pex16p from the ER or lead to a mistargeting of these proteins to the ER, and cause a proliferation of the ER membrane (Titorenko et al. 1997; Titorenko and Rachubinski 1998b).

Taken together, there is evidence that the ER might be involved in the biogenesis of peroxisomes, but a requirement for the known ER-protein import and export pathways has not yet been established. If the ER is involved in the formation of peroxisomal structures, alternative pathways for ER-targeting and insertion of the responsible proteins, as well as

COPI- and COPII-independent vesicle-mediated transport pathways from the ER to peroxisomes, have to be considered.

Lipid transport to peroxisomes

The peroxisome is not capable of synthesizing the constituents of its own membrane. An analysis of the lipid molecular species composition of yeast subcellular membranes by Schneiter et al. (1999) found phosphatidyl choline and phosphatidyl ethanolamine to be the most abundant phospholipids of the peroxisomal membrane, both undoubtedly synthesized in the ER. As phospholipid exchange proteins are too inefficient to cover for the turnover required in proliferating peroxisomes (Wirtz 1982; Wirtz 1991), other means of transport have to account for the flow of phospholipids from the ER to the peroxisomal membrane. There is evidence that membrane constituents slip from the ER membrane into the mitochondrial membrane at sites of close apposition (Achleitner et al. 1999; Ardail et al. 1993; Shiao et al. 1995; Voelker 1993), which also has to be taken into consideration for the peroxisomal membrane. Purdue and Lazarow (2001a) suggest that specialized vesicles might carry phospholipids from the ER to peroxisomes and that such vesicles might contain a few proteins—possibly made in the ER—that assist in the targeting of the vesicle. There is, however, no evidence supporting this idea to date.

What triggers the proliferation of peroxisomes?

Exciting new insights into the role of the actin cytosceleton by Hoepfner et al. (2001) revealed that the dynamin-like protein Vps1p regulates the number of peroxisomes in the cell. Apart from actually visualizing peroxisomal fission events by in vivo time-lapse microscopy, the group found that few giant peroxisomes evolved in *vps1* mutant cells, a picture known from the effects of deleting the *PEX11* gene. Pex11p has been postulated to play a regulatory role in peroxisome proliferation after finding that *pex11*Δ cells also contain few giant peroxisomes and appear to be unable to segregate the giant peroxisomes to daughter cells (Erdmann and Blobel 1995). Furthermore, Marshall et al. (1995) and Schrader et al. (1998) noted that peroxisomes are hyperproliferated upon overexpression of Pex11p. While the extent to which Pex11p promotes peroxisome proliferation is disputed (Erdmann and Blobel 1995; Marshall et al. 1995), the function in proliferation control is clearly established and has been shown to also work in the absence of extracellular stimuli (Schrader et al. 1998). Marshall et al. (1996) proposed that the redox-sensitive homodimerization of Pex11p might be a mechanism to regulate peroxisome proliferation. Recently, Pex25p was identified as a novel peroxin involved in peroxisome proliferation (Smith et al. 2002). Cells lacking Pex25p had fewer and larger peroxisomes. Thus, as Pex11p, Pex25p seems to be required for the regulation of peroxisome size and maintenance. Whether the two proteins act in tandem in this cellular process has not yet been analyzed.

Recently, a metabolic role for Pex11p in MCFA oxidation was also described (van Roermund et al. 2000), which hints at a bifunctional character of the protein. It has been speculated that the metabolic role might be a direct function of Pex11p and the role in peroxisome division a secondary effect due to ongoing ß-oxidation (van Roermund et al. 2000). Recent studies on mice and murine cells have demonstrated that the overexpression

of Pex11βp can promote peroxisome division even in the absence of metabolic activity of peroxisomes and that cells lacking the PEX11β gene display reduced peroxisome abundance under the same conditions (Li and Gould 2002). Therefore, the authors propose that Pex11p acts directly in peroxisome division.

Van Roermund et al. (2001) observed less peroxisomal structures in cells lacking the adenine nucleotide transporter Ant1p (YPR128cp) and, therefore, postulated that the protein plays a role in a process that affects peroxisomal number or proliferation. However, Li and Gould (2002) could not find any evidence for overexpressed Ant1p to induce peroxisome proliferation in the absence of oleate, whereas overexpression of Pex11p clearly did. In line with this observation, Rottensteiner et al. (2002) found that the signal for induction of Ant1p expression did not depend on the biogenesis of the peroxisomal compartment.

Thus, Pex11p, Pex25p, and Vps1p are clear candidates for playing a role in proliferation and inheritance of peroxisomes. Whether these proteins function independently or cooperatively remains to be elucidated.

Peroxisome maturation?

As described above, there are both spherical and tubular structures in peroxisome morphology in various species, including yeasts, depending on the growth conditions. Often these tubes seem to interconnect the spherical bodies. There is significant evidence from electron microscopy to support the concept of a peroxisomal reticulum, a compartment in which there is a connection between the single peroxisomal bodies, be it transient or permanent (Erdmann and Blobel 1995; Lüers et al. 1993; Purdue and Lazarow 2001a; Yamamoto and Fahimi 1987). Recently, evidence from the yeast *Yarrowia lipolytica* supports the idea of the existence of a dynamic population of organelles that differ in various aspects such as import competence for matrix proteins (Titorenko et al. 2000a; Titorenko et al. 1996; Titorenko and Rachubinski 2000; Titorenko et al. 2000b). From six structurally and functionally distinct subforms of peroxisomes which this group could isolate and characterize, they derived the picture of a compartment in which the various subforms were conversed to mature peroxisomes in a temporally ordered manner. This maturation process involved the import of different proteins into distinct intermediates (reviewed in Titorenko and Rachubinski 2001b). While the data obtained in the fractionation experiments could still be challenged by the possible explanation that the peroxisomes or peroxisomal reticulum might have been torn into vesicles of various size and density during the isolation and fractionation process, the data presented from pulse-chase experiments and on the dependency of membrane fusion between peroxisomal membranes on Pex1p and Pex6p appear to be convincing (Titorenko et al. 2000a; Titorenko and Rachubinski 2000; Titorenko et al. 2000b). In addition, the heterogeneous nature of peroxisomes in mammalian and yeast systems was demonstrated in several previous studies. Heinemann and Just (1992) did provide evidence for two fractions of peroxisomes in vivo, differing in density. The fraction of higher density represented mature peroxisomes, the one of lower density consisted of translocation-competent peroxisomes. Similarly, Lüers et al. (1993) identified distinct populations of peroxisomes from rat liver, also differing in shape, diameter, density, protein content, and import competency. Thus, while too little is known to date about a peroxisomal compartment structured temporally towards mature peroxisomes or about fusion

events among peroxisomes, the idea cannot be dismissed easily. Further research into the matter will be required in the future to solve the question.

Topogenesis of peroxisomal membrane proteins

As outlined above, the pathway for the import of PMPs is distinct from the matrix protein import pathway. This is also supported by in vitro studies showing that the import of peroxisomal matrix and membrane proteins have different biochemical characteristics such as the requirement for ATP, which is necessary for the import of all matrix proteins but seems to be dispensable for most membrane proteins (Diestelkötter and Just 1993; Imanaka et al. 1996). PMPs such as PMP22, PMP70, and Pex2p are synthesized on free polysomes (Fujiki et al. 1984; Suzuki et al. 1987a; Tsukamoto et al. 1994b). The targeting of most PMPs is believed to occur posttranslationally and directly from the cytosol to the peroxisome (Diestelkötter and Just 1993; Imanaka et al. 1996; Pause et al. 2000), although, as mentioned above, a targeting of some early peroxins via the ER cannot be fully excluded.

Membrane peroxisomal targeting sequences (mPTS) have been described for several proteins including *Cb*Pmp47p, *Rn*Pmp22p, *Hs*Pmp34p, and Pex3p from various species, yet these sequences do not exhibit a clear consensus (Honsho and Fujiki 2001; Pause et al. 2000; Wang et al. 2001). The first mPTS which was identified is a hydrophylic loop of 12 amino acids from CbPmp47 (Dyer et al. 1996), the peroxisomal adenine nucleotide transporter of *Candida boidinii* (Nakagawa et al. 2000). The hydrophilic loop comprised a basic cluster of amino acids of the sequence KIKKR, which was described to be the most important attribute for the peroxisomal targeting of Pmp47 (Dyer et al. 1996). However, a more detailed study from the same group revealed that efficient targeting to peroxisomes also required a membrane-anchoring transmembrane domain adjacent to the cytoplasmic-oriented basic cluster (Wang et al. 2001). For PMP34, the putative human orthologue of CbPMP47, an intervening basic loop plus three transmembrane domains were shown to be sufficient for targeting and membrane insertion of the protein (Honsho and Fujiki 2001). Jones et al. (2001) described two independent peroxisomal targeting signals in the same PMP34 as well as in the peroxin Pex13p. A basic cluster of the ER- and peroxisome-localized cottonseed ascorbate peroxidase (pAPX) fused to a synthetic membrane span and reporter protein, was shown to target to punctate and reticular structures; a peroxisomal localization, however, was not demonstrated (Mullen and Trelease 2000).

A 22 amino acid region localized within the N-terminal region of the rat Pmp22 was reported to function as mPTS. Comparison with orthologous proteins revealed the conserved motif $YX_3LX_3PX_3$(KQN), which has been suggested to represent the core of the signal sequence (Pause et al. 2000). Brosius et al. (2002) found a second mPTS at the C-terminal part of rat as well as human Pmp22. Both mPTS consist of two transmembrane domains and adjacent protein loops with almost identical basic clusters.

In a search for the Pex16p topogenic signal, a basic amino acid cluster (RKELRKKLPVSLSQQK) and a transmembrane segment located 40 amino acids downstream were defined as being essential for peroxisomal targeting and insertion (Honsho et al. 2002).

The mPTS of Pex3p from several species was shown to reside at the N-terminal region of the protein, comprising a conserved block of positively charged amino acids (Ghaedi et al. 2000b; Huang and Lazarow 1996; Kammerer et al. 1998; Wiemer et al. 1996; reviewed

in Purdue and Lazarow 2001a). Baerends et al. (2000) demonstrated that this amino acid cluster is required for peroxisomal targeting.

According to the sequences which were recognized in the PMPs, a mPTS consists of a hydrophilic peptide containing a group of positively charged amino acids adjacent to at least one hydrophobic patch or transmembrane domain. The length of the PMP targeting signals differs drastically from the small size of the PTS1 and PTS2 of peroxisomal matrix proteins. However, retention of matrix proteins is provided by the barrier of the peroxisomal membrane, while PMPs need to be anchored into the membrane for retention. Thus, it is not surprising that the presence of a transmembrane span is a common property of all mPTS.

The integral peroxisomal membrane protein Pex3p and Pex19p, a farnesylated, predominantly cytosolic factor which is also found attached to the peroxisomal membrane, were the first components of the peroxisomal transport machinery for peroxisomal membrane proteins to be identified (Götte et al. 1998; Hettema et al. 2000). The assumption of a functional role of these peroxins in peroxisomal membrane biogenesis was based on the following observations: First, the consequences of deleting the *PEX3* and the *PEX19* gene for the targeting of PMPs were quite similar. For example, Pex11p or Pex14p are mislocalized at similar protein levels to the cytosol or mitochondria, respectively, in both cases (Hettema et al. 2000; Muntau et al. 2000; Sacksteder et al. 2000). Thus, in contrast to all other *pex* mutants analyzed, cells lacking Pex3p or Pex19p were not only affected in the import of matrix proteins, but also mislocalized most if not all peroxisomal membrane proteins (Hettema et al. 2000). Second, both proteins were found to functionally interact (Götte et al. 1998), and third, Sacksteder et al. (2000) found most PMPs to be recognized by Pex19p (see also Table 2 for interaction partners of Pex19p). For the interaction of Pex19p with Pex14p, the binding affinity was estimated to be around 500 nM, a figure which is considered to lie well within the physiological range (Sacksteder et al. 2000).

The cellular distribution of the two interacting peroxins, Pex3p and Pex19p, the similar phenotypes of the corresponding deletion strains as well as the observation of Pex19p binding to a number of PMPs, triggered the idea that Pex19p might function as a chaperone and/or import receptor for newly synthesized PMPs (Hettema et al. 2000; Sacksteder et al. 2000). According to this idea, Pex19p was supposed to recognize and bind PMPs in the cytosol and subsequently to contribute to the targeting of the PMPs to the peroxisomal membrane, possibly realized by the Pex19p/Pex3p interaction.

Interestingly enough, Pex3p interacts with Pex19p via its C-terminal domain, not its mPTS, which is found on the very N-terminus (Ghaedi et al. 2000b; Muntau et al. 2000; Soukupova et al. 1999). Both regions are, however, essential for the function of Pex3p (Baerends et al. 1996; Ghaedi et al. 2000b; Wiemer et al. 1996). This observation was in line with the assumption that the Pex19p/Pex3p interaction does not simply reflect receptor/cargo recognition, but would be consistent with the proposed receptor/docking factor binding.

Whether farnesylation is required for the function of Pex19p or not is heavily disputed. Götte et al. (1998) demonstrated that in *S. cerevisiae*, Pex19p was farnesylated at the C-terminal CAAX-box signal and that this farnesylation was required for proper function. In man, Pex19p is also farnesylated (Kammerer et al. 1997), but while Matsuzono et al. (1999) found it to be essential for function, Sacksteder et al. (2000) found that it was not. In *P. pastoris*, Snyder et al. (1999a) found no signs of farnesylation of Pex19p under the conditions tested. A recent study by Fransen et al. (2001), however, demonstrated that the

CAAX prenylation motif of human Pex19p is an important determinant in the affinity of Pex19p for Pex10p, Pex11pβ, Pex12p, and Pex13p.

As mentioned above, it has been suggested that Pex19p is a peroxisomal membrane protein receptor which has the ability to solubilize newly synthesized PMPs in the cytosol for their transport to the peroxisomal membrane (Sacksteder et al. 2000). In line with this assumption, it has been established that Pex19p binds both minimal mPTS of PMP34 and this interaction has been shown to take place in the cytosol (Jones et al. 2001). The observations that (a) many PMPs are undetectable or exist at reduced levels in *pex19* cells, and (b) that cells overexpressing a PMP together with Pex19p retain this PMP stable in the cytosol, while previously synthesized PMPs do not accumulate in the cytoplasm under such conditions, contribute to this assumption (Gould and Valle 2000; Sacksteder et al. 2000).

It should, however, be noted that Pex19p does not seem to bind to the mPTS of all PMPs. Snyder et al. (1999a), for example, described *P. pastoris* PMPs (Pex10p, Pex13p, and Pex22p) truncations which still properly targeted to the peroxisomal membrane but did not interact any more with Pex19p, indicating that the mPTS and the Pex19p binding sites are distinct. Similar results were also obtained by Fransen et al. (2001), who showed by deletion studies and random mutagenesis analysis that the peroxisomal sorting determinants and the Pex19p-binding domains of a number of PMPs (Pex3p, Pex12p, and Pex13p) in human cell lines are distinct entities. However, it remains unclear whether the observed interactions between Pex19p and other peroxins take place in the cytosol or at the peroxisomal membrane. Therefore, it still remains to be investigated whether Pex19p is also binding newly synthesized peroxins as has been shown for newly synthesized PMP34 (Jones et al. 2001).

In summary, after it has been suggested frequently that Pex19p functions as a soluble receptor for the targeting of peroxisomal membrane proteins (PMPs) to the peroxisome (e.g., Gould and Valle 2000), Fransen et al. (2001) propose an alternative view in that Pex19p might function as a chaperone at the peroxisomal membrane. It is of course conceivable that Pex19p and its PMP substrate form a complex with other proteins, possibly chaperones or targeting factors. Hydrophobic membrane-spanning domains cannot be exposed to the aqueous environment of the cytoplasm. Molecular chaperones are clear candidates for shielding hydrophobic domains and preventing aggregation. In addition to Hsp70 and Hsp40 chaperons which have been implicated in protein import into the peroxisomal matrix (Corpas and Trelease 1997; Walton et al. 1994; Wendland and Subramani 1993), the multimeric cytosolic ring complex TriC has been shown to interact with newly formed PMPs (Pause et al. 1997). However, TriC is a more general chaperone and therefore it is unlikely to contribute to the peroxisomal targeting specifity. Thus, additional peroxisomal targeting factors, such as Pex19p, are expected to associate with newly formed PMPs to guarantee proper targeting to the peroxisome. At this time, it is unknown at which stage of the targeting process Pex19p binds to PMPs. Pause et al. (1997) observed a very early association of newly formed PMPs with an unknown 40 kDa protein. It would be not surprising if this protein turned out to be Pex19p.

According to the results summarized above, an alternative view on the function of Pex19p, which combines the different observations, is proposed and outlined in Fig. 5. There might be at least two different types of peroxisomal membrane proteins. Pex19p might function as an import receptor for PMPs of type I, like PMP34 (PMP47), and Pex11p, which usually do not associate with components of the peroxisomal import machinery. Pex19p recognizes the newly formed type I PMPs and directs them to a docking

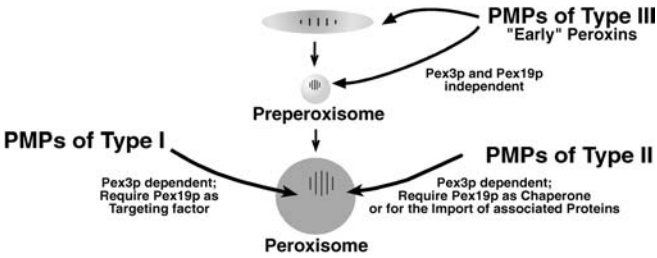

Fig. 5 Model of branched pathways for the topogenesis of peroxisomal membrane proteins. There might be three different types of peroxisomal membrane proteins. For membrane proteins of type I, Pex19p functions as a signal recognition and targeting factor. Pex19p recognizes the mPTS of newly formed PMPs of type I, such as PMP34 (PMP47) and Pex11p, and directs them to a docking site at the peroxisomal membrane, which presumably contains Pex3p and which contributes to membrane insertion of the membrane cargo proteins. For PMPs of type II, Pex19p could function like a chaperone and might be required for targeting efficiency. In the case of PMPs of type II, usually components of the peroxisomal import machineries, thus mostly peroxins, the Pex19p interaction might contribute to shielding of the hydrophobic transmembrane segments. Thus targeting might mainly be realized by complexation of these proteins with other PMPs, presumably also peroxins at the peroxisomal membrane. The targeting signals of type II PMPs are expected to represent contact regions to peroxins with which these PMPs form a complex at the peroxisomal membrane. The third type of PMPs comprises early peroxins which are proposed to efficiently target to peroxisomes or preperoxisomes completely independent of Pex19p

site at the peroxisomal membrane, which presumably contains Pex3p and which contributes to membrane insertion of the PMPs. In the case of PMPs of type II, usually components of the peroxisomal import machineries, thus mostly peroxins, the Pex19p interaction might contribute to shielding of the hydrophobic transmembrane segments but peroxisomal retention and thus targeting might mainly be realized by complexation of these proteins with other PMPs, presumably also peroxins at the peroxisomal membrane. For PMPs of type I, Pex19p functions as a real targeting factor, thus, the Pex19p binding sites represent the mPTS of these proteins. For PMPs of type II, Pex19p might function like a chaperone. The targeting signals of type II PMPs are expected to represent contact regions to peroxins with which these PMPs form a complex at the peroxisomal membrane. As an alternative to the function of Pex19p as a chaperone, peroxin interactions might already take place prior to their peroxisomal targeting, opening the possibility for a piggy-backing of PMPs. In this scenario, Pex19p would also function as a signal receptor for PMPs of type II, and a complex of PMPs would be targeted to the peroxisome if at least one of the complexed components contains a binding site for Pex19p.

A third type of PMPs might comprise early peroxins which could efficiently target to peroxisomes or preperoxisomes completely independent of Pex19p.

The role of Pex16p in PMP targeting or membrane insertion remains fully enigmatic to date. In human cells, South and Gould (1999) studied the targeting of Pex16p itself, which did not seem to differ significantly from that of any other PMP. It seemed to insert directly into existing peroxisomal membranes without a detour via the ER. Its role in membrane assembly, however, remains uncertain; it might lie in the insertion of the PMPs into the membrane or in the formation of the lipid bilayer itself. Pex16p from *Y. lipolytica* seems not to be involved in the biogenesis of the membrane and has different properties (Eitzen et al. 1997).

Finally, Pex17p has been suggested to interact with Pex19p and to play a role in the import of both peroxisomal membrane and matrix proteins in *P. pastoris* (Snyder et al.

1999b). These findings contradict previous results by Huhse et al. (1998) in *S. cerevisiae*, who found the biogenesis of membrane proteins unaffected upon deletion of the *PEX17* gene. The systematic analysis of deletion mutants by Hettema et al. (2000) in *S. cerevisiae* also failed to detect such properties of Pex17p. Finally, Harper et al. (2002) re-examined the *pex17*Δ mutant of *P. pastoris* and found a mild reduction in PMP stability and slightly aberrant behavior in subcellular fractionation experiments, though indistinguishable from a *pex5*Δ mutant and distinctly different from a *pex3*Δ mutant. The group, therefore and based on other results, proposed that Pex17p acts primarily in the matrix protein import pathway and does not play an important role in PMP import.

As outlined above, although in recent years our knowledge of the principle mechanisms of PMP recognition, targeting, and insertion into the peroxisomal membrane has dramatically increased, it is still fragmentary and far from being complete. Ideas about (a) the nature of different mPTS and the functioning of the corresponding targeting factors, (b) protection of hydrophobic transmembrane segments of newly formed PMPs from aggregation, (c) how PMPs assemble into complexes in the membrane, and (d) whether or not all PMPs are transported posttranslationally and directly from the cytosol to the peroxisomal membrane have been proposed, but need to be proven.

Import of matrix proteins

The majority of peroxins are thought to be involved in the posttranslational import mechanism for peroxisomal matrix proteins. Some 50 different proteins have to be selectively shuttled from their place of synthesis by free ribosomes in the cytosol across the membrane of the organelle, and they seem to be imported in a manner distinctly different from other import mechanisms described so far, such as those across the membranes of the ER or of the mitochondria. The peroxisomal import machinery accepts folded proteins, oligomerized proteins, and items of large diameter such as gold particles fused to import signals as substrates (McNew and Goodman 1994; Walton et al. 1995). In recent years, considerable progress has been made in the elucidation of the function of peroxins and cargo proteins in the different stages of the protein import process.

Distinct peroxisomal targeting signals (PTS1 and PTS2), which direct a protein from the cytosol to the peroxisomal matrix, have been identified. Proteins harboring one of the two PTS are recognized in the cytosol by either one of the two PTS-receptors, Pex5p or Pex7p. According to the original "hypothesis of shuttling receptors," the PTS receptors cycle between the cytosol and the peroxisomal membrane. They recognize and bind their cargo proteins in the cytosol and deliver them to a common docking and translocation complex at the peroxisomal membrane. After the release of the cargo proteins to the translocation machinery, the receptors are supposed to shuttle back to the cytoplasm. The import receptors Pex5p and Pex7p are predominantly localized in the cytosol. However, fractions of the receptors are frequently found in the peroxisomal lumen, which gave rise to the so-called extended shuttle hypothesis, which suggests that the receptors do not release the cargo after the docking step but instead reach the peroxisomal lumen together with the cargo, where cargo/receptor dissociation takes place. After the cargo release, the receptors are shuttled back to the cytoplasm (Fig. 6).

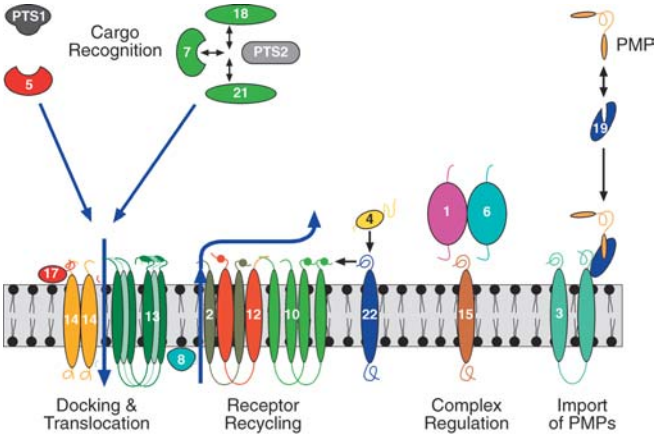

Fig. 6 Model of the peroxisomal protein import cascade. Overview of transient or stable protein complexes of the peroxisomal protein import machinery. It can be imagined that the different complexes involved in the same aspect of peroxisome biogenesis do not form separate entities but are expected to interact at least transiently during their performance. The steps of the import of proteins into the peroxisomal matrix can be subdivided into consecutive steps: First, the early events in the import cascade comprise the cytosolic recognition of import substrates by the import receptors Pex5p and Pex7p, formation of an import-competent complex and transport to the peroxisome. This step is followed by the membrane docking process, in which substrate and receptor bind to a complex of peroxisomal membrane proteins. The late events consist of dissociation of the receptor-cargo complexes from the docking complex, translocation and release of the cargo into the lumen and, finally, recycling of the receptors to the cytosol. The machinery for the targeting and insertion of peroxisomal membrane proteins (PMPs) is distinct from the peroxisomal import machinery for matrix proteins. Most newly formed PMPs are supposed to bind to cytosolic Pex19p, which might function as a chaperone or targeting factor and contributes to the directed transport of PMPs to the peroxisomal membrane. The membrane-standing Pex3p interacts with Pex19p and is expected to contribute to the membrane targeting of PMPs. Pex15p has been found to interact with the AAA-ATPases Pex1p and Pex6p (W.H. Kunau, personal communication; Birschmann et al. 2003). AAA-ATPases function in the formation, organization and/or dissociation of protein complexes (Neuwald et al. 1999)

Early events: recognition of substrates and cargo transport to the membrane

Most peroxisomal proteins contain a peroxisomal targeting signal (PTS) that is necessary and sufficient to target the protein to the peroxisomal matrix.

PTS1

The majority of peroxisomal matrix proteins carries a (S/A/C)-(K/R/H)-L tripeptide consensus sequence at its extreme C-terminus which is referred to as PTS1 (Gould et al. 1989). Later studies found extended sequence lengths as well as species-dependent ranges of possible conservative exchanges of the residues (Elgersma and Tabak 1996; Gould et al. 1989; Lametschwandtner et al. 1998; Miura et al. 1992; Subramani et al. 2000). Proteins carrying a PTS1 are recognized and bound by the receptor protein Pex5p in the cytosol. Pex5p has been described in a wide range of species (Table 1). From yeast to man, from invertebrates to plants, the tetratricoide peptide repeats (TPRs) have been conserved. TPR-proteins are characterized by six direct repeats of the degenerate, typically 34-amino

acid TPR motif. Proteins of this family tend to be associated with multisubunit complexes, and the TPR motif is thought to mediate protein–protein interactions (Goebl and Yanagida 1991). Indeed, it has been shown that this region interacts with the PTS1 of the cargo proteins (Brocard et al. 1994; Dodt et al. 1995; Fransen et al. 1995; Gatto et al. 2000a; Otera et al. 2002; Terlecky et al. 1995), while highly conserved N-terminal pentapeptide repeats were shown to be essential for the interaction with the members of the docking complex (Otera et al. 2002; Saidowsky et al. 2001). The importance of the TPR domain region of Pex5p for PTS1 recognition was confirmed by extensive random mutagenesis studies (Klein et al. 2001). A single amino acid change in the sixth TPR domain abolishes the Pex5p/PTS1 interaction and represents one molecular cause for Zellweger's syndrome, one of the most severe peroxisome biogenesis disorders (Dodt et al. 1995). The crystal structure of human Pex5p in complex with a PTS1 revealed that two clusters of three TPRs almost completely surrounded the peptide (Gatto et al. 2000b). The binding affinity of Pex5p to PTS1 has been determined to be approximately 500 nM in *Pichia pastoris* (Terlecky et al. 1995).

There is some dispute as to where Pex5p is localized in the cell. Pex5p from *P. pastoris*, *H. polymorpha*, and man have been determined to be localized primarily in the cytosol with small fractions associated with the peroxisome (de Walque et al. 1999; Dodt et al. 1995; Jardim et al. 2000; McCollum et al. 1993; van der Klei et al. 1995; Wiemer et al. 1995). In contrast, Terlecky et al. (1995) found Pex5p from *P. pastoris* to be tightly associated with the matrix face of the peroxisomal membrane. Szilard et al. (1995) found Pex5p from *Y. lipolytica* to be localized entirely inside the peroxisome. However, significant species-dependent differences in the distribution and additional tasks of Pex5p are thinkable. For example, studies on mammalian cells (both CHO and human cell lines) identified two isoforms of Pex5p. The long isoform Pex5pL contains additional 37 amino acids compared to the short isoform Pex5pS (Braverman et al. 1998; Otera et al. 2000). Otera et al. (2000) and Matsumura et al. (2000) were able to show that in mammalian cells the interaction of the complex between a PTS2 import substrate and its receptor with Pex5pL was essential for import of the PTS2-protein. Nito et al. (2002) recently demonstrated similar results in *Arabidopsis thaliana*. This is remarkable as the routes of PTS1- and PTS2-dependent import in yeasts meet at the docking complex, in mammals and plants, however, this convergence of pathways might start ahead of docking to the peroxisomal membrane.

PTS2

The PTS2 is an amino-terminal nonapeptide with the consensus motif (R/K)-(L/I/V)-X_5-(H/Q)-(L/A/F) and is found in only a few matrix proteins (Swinkels et al. 1991). While PTS1 requires to be localized at the extreme C-terminus for function, PTS2 can function as a topogenic signal in any position within the protein; however, it appears to occur mostly at or near the N-terminus (Rehling et al. 1996). While studies in some organisms indicate that the consensus may be more complex than indicated, the length of the five-amino acid linker between the conserved residues seems to be crucial for recognition by the PTS2 receptor (Flynn et al. 1998; Glover et al. 1994b; Tsukamoto et al. 1994a).

The PTS2 is recognized by the receptor Pex7p, a member of the family. It contains six WD repeats, which are each approximately 40 amino acids long and contain a central tryp-

tophan (W)-aspartate (D) motif. As for Pex7p, these repeats comprise the entire protein except for approximately 60 amino acids at its N-terminus.

Again, the localization of Pex7p is disputed. Reports by Marzioch et al. (1994) and Elgersma et al. (1998) found that Pex7p from *S. cerevisiae* and *P. pastoris* are both localized in the cytoplasm and at the peroxisome. Another report suggests that Pex7p from *S. cerevisiae* is entirely localized in the peroxisomal lumen (Zhang and Lazarow 1996). Recently, the distribution of Pex7p was studied in human and CHO cells and the protein found to be localized both in the lumen of the peroxisome and in the cytosol (Ghys et al. 2002; Mukai et al. 2002). Marzioch et al. (1994) also found that in the presence of the PTS2-protein thiolase, Pex7p is associated with peroxisomes in *S. cerevisiae*. However, in the absence of thiolase, the receptor was entirely localized in the cytosol.

There is a substantial variance between the PTS2 import pathways of different species. In the nematode *Caenorhabditis elegans*, the PTS2 import pathway is probably completely lost. In this nematode, all matrix proteins of the peroxisome – including those that are PTS2 proteins in other organisms—carry the PTS1 and are recognized by Pex5p (Motley et al. 2000). In mammals, plants, and the yeast *Y. lipolytica*, cleavage of some PTS2 proteins by a peroxisomal peptidase has been described (Subramani 1993; Titorenko and Rachubinski 2000); however, this PTS2 processing does not take place in *S. cerevisiae.* Most importantly, PTS2 protein import requires additional factors to Pex7p. Most recently, Pex7p of *Arabidopsis thaliana* was shown to functionally interact with Pex5p. *At*Pex5p was demonstrated to bind to members of the docking complex, while Pex7p did not. Thus, the PTS2 pathway in plants seems also to depend on the PTS1 pathway (Nito et al. 2002). In *S. cerevisiae*, the redundant proteins Pex18p and Pex21p are required (Purdue et al. 1998); as mentioned above, mammalian cells need Pex5pL for PTS2-dependent import (Braverman et al. 1998; Otera et al. 1998), and in *Y. lipolytica* Pex20p is essential for this branch of import (Titorenko et al. 1998). These proteins from the latter three groups are thought to fulfill a common function. Pex5pL, Pex18p, and Pex21p can interact with Pex7p, while Pex7p binds to the PTS2-containing cargo proteins (Dodt et al. 2001; Matsumura et al. 2000; Otera et al. 2000; Stein et al. 2002). Einwächter et al. (2001) recently found that a *pex18*Δ/*pex21*Δ double mutant could be partially complemented by the expression of Pex20p from *Y. lipolytica*, which supports the idea of a conserved function of Pex18p, Pex21p, and Pex20p. In the same work, it was found that Pex5pL, Pex18p, Pex20p, and Pex21p all contain a conserved sequence region which most likely represents a common PTS2-receptor binding site. Since Pex7p has not yet been identified in *Y. lipolytica*, Pex20p might fulfill the tasks of Pex7p as well as of Pex18p and Pex21p. This idea is supported by the observation of Smith and Rachubinski (2001), who reported that Pex20p is required for the cytosolic oligomerization of thiolase, which seems to be a prerequisite for import, and also interacts with the intraperoxisomal protein Pex8p. However, *Neurospora crassa* recently has been shown to contain both Pex20p and Pex7p (Sichting et al. 2003). These proteins were shown to act in tandem in the import of PTS2 dependent proteins. Complementation studies provided compelling evidence that Pex18p/Pex21p and Pex20p perform similar functions in the PTS2-dependent import process. Based on these data, it is anticipated that also *Y. lipolytica* will need Pex7p for the targeting of PTS2 proteins to peroxisomes. Recent studies by Stein et al. (2002) on the interplay of Pex7p and Pex18p in cargo recognition and targeting as well as on the binding of Pex7p, Pex18p, and Pex21p to members of the docking complex indicated that Pex18p/Pex21p are required

prior to the docking event for the formation of a thiolase-containing import-competent complex (see below).

PTS3 and others

There are also a few peroxisomal matrix proteins which contain neither a PTS1 nor a PTS2 (for review see Subramani 1998). For some of these, new PTS have been identified. Peroxisomal acyl-CoA oxidase (Pox1p) from *P. pastoris*, for example, was found to be targeted into the peroxisomal matrix via Pex5p, interacting with the C-terminal nonconsensus APKI-region (Koller et al. 1999b). Pox1p from *S. cerevisiae* is also targeted via Pex5p, but, with its internal PTS3, uses a completely different region of Pex5p for its interactions (Klein et al. 2002; Skoneczny and Lazarow 1998; Small et al. 1988). The interaction region in Pex5p is located in a defined area of the amino-terminal part of the protein, clearly distinct from the TPR domain that is involved in the PTS1 recognition. These results demonstrate that Pex5p is a multifunctional PTS receptor and it seems plausible that other peroxisomal proteins which apparently lack a PTS are also directed to peroxisomes by binding to Pex5p in an unconventional manner.

For other proteins lacking both PTS1 and PTS2, import into the peroxisome may be mediated by virtue of an association with bona fide PTS-containing molecules. This is expected to function analogous to the coimport of PTS-truncated and full-length proteins demonstrated by McNew and Goodman (1994) and Glover et al. (1994a) which provided the first evidence for the now general understanding that peroxisomes can import folded and oligomeric proteins. Such a “piggyback” targeting has also been demonstrated for the enoyl CoA- isomerases Dci1p and Eci1p in *S. cerevisiae* (Yang et al. 2001) and has been identified as the native import pathway for isocitrate lyases in oilseed (Lee et al. 1997).

Apart from the assumed targeting factors such as Pex18p, Pex20p, and Pex21p, further factors have been identified to assist in the peroxisomal import receptors in substrate recognition and/or peroxisomal targeting: the requirement for both the DnaJ-like protein Djp1p as well as the Hsp70/Hsp40 chaperone system has been established (Crookes and Olsen 1999; Hettema et al. 1998; Preisig-Müller et al. 1994). Hsp73 has been reported to play a role in peroxisomal matrix protein import in human fibroblasts and hepatocytes from rat (Walton et al. 1994). Whether these chaperones are required to keep the import substrates in an import-competent state, to fold them into their native state prior to import, or to assist in the recognition of substrates by the receptor remains enigmatic to date. So far, no chaperones have been identified inside the peroxisome, with the exception of one Hsp70 protein which is targeted to a minor extent to peroxisomes via a weak PTS2 sequence (Wimmer et al. 1997). While it is established that chaperones do play a role in peroxisomal matrix protein import, the nature and extent of this role remain unknown.

Docking: attaching the cargo-receptor complex to the membrane

In addition to their ligands, Pex5p and Pex7p also bind to components of the docking machinery in the peroxisomal membrane. The transmembrane proteins Pex13p and Pex14p, as well as the peripheral protein Pex17p, have been established as members of the docking complex (Albertini et al. 1997; Brocard et al. 1997; Elgersma et al. 1996; Erdmann and Blobel 1996; Girzalsky et al. 1999; Gould et al. 1996; Huhse et al. 1998; Schliebs et al.

1999; reviewed in Hettema et al. 1999and Holroyd and Erdmann 2001). Pex13p and Pex14p are nonredundant peroxins, both of which each provide binding sites for Pex5p and Pex7p (Albertini et al. 1997; Girzalsky et al. 1999; reviewed in Purdue and Lazarow 2001a). Pex13p is a transmembrane protein which exposes both termini to the cytosol. The N-terminal domain has been shown to provide the binding site for Pex7p (Stein et al. 2002). Pex5p and Pex14p bind to the C-terminal Src homology (SH3) domain of Pex13p (Albertini et al. 1997; Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996). A typical proline-rich SH3-ligand motif in Pex14p is responsible for the binding to the SH3 domain of Pex13p (Girzalsky et al. 1999; Pires et al. 2003). The yeast Pex5p interacts with the SH3 domain of Pex13p in an unconventional, non-PXXP-related manner (Barnett et al. 2000; Bottger et al. 2000). Human Pex5p, however, does not bind to the SH3 domain of Pex13p, but instead interacts via two WXXXF/Y motifs (see below) with the N-terminal domain of human Pex13p, which also binds Pex7p (Otera et al. 2002; Stein et al. 2002). The interactions of Pex5p with Pex13p and Pex14p have been shown to be direct (Barnett et al. 2000; Otera et al. 2002; Saidowsky et al. 2001; Urquhart et al. 2000), and so are the ones of Pex7p (Albertini et al. 1997; Brocard et al. 1997; Girzalsky et al. 1999; Otera et al. 2002; Stein et al. 2002; Will et al. 1999).

Schliebs et al. (1999) further characterized the interaction between Pex5p and Pex14p and found the N-terminus (residues 1–78) of Pex14p to be responsible for the interaction and the binding affinity in the nanomolar range. They also proposed that human Pex5p possesses multiple binding sites for human Pex14p within its N-terminal half and demonstrated by in vitro and in vivo studies that the conserved seven WXXXF/Y motifs within this region form individual high affinity sites for Pex14p (Saidowsky et al. 2001). The interaction between Pex5p and Pex13p has been studied intensively (Barnett et al. 2000; Urquhart et al. 2000). In their characterization of the binding of Pex5p to Pex13p, Urquhart et al. (2000) demonstrated that the interaction was stronger with Pex5p alone than with cargo-loaded Pex5p. In contrast, Pex5p bound stronger to Pex14p if loaded with cargo than alone. These data indicate that that cargo-loaded Pex5p might make first contact with Pex14p rather than with Pex13p on its way to the translocation machinery. Pex13p and Pex14p have also been reported to form a complex with cargo-loaded Pex5p, but dissociate in the presence of unloaded Pex5p, implying that PTS cargoes are released from Pex5p at a step downstream of Pex14p, but upstream of Pex13p (Otera et al. 2002). Moreover, these data might indicate that Pex13p and Pex14p only transiently interact during the import process, but very likely form mutually and temporally distinct subcomplexes. This is also supported by native gel electrophoresis and gradient sedimentation analysis indicating that Pex14p together with Pex5p, and the RING zinc finger proteins Pex2p and Pex12p on the one side and the majority of Pex13p on the other, are subunits of distinct stable complexes which might interact with each other transiently (Reguenga et al. 2001). The group found small, though no stoichiometric amounts of Pex13p in the complex containing Pex14p, which might account for the interaction of the two proteins previously observed by two-hybrid analysis or coprecipitation. However, an interaction of Pex13p and Pex14p seems to be required for the import process. Cells harboring a mutation within the SH3 domain of Pex13p which abolishes the Pex14p binding are impaired in the import of peroxisomal matrix proteins (Elgersma et al. 1996). Also, both proteins seem to be required in stoichiometric amounts as overexpression of either Pex13p or Pex14p leads to a blockage of import, while overexpression of both together does not (Bottger et al. 2000; Komori et al. 1997). In mammalian cells, overexpression of Pex14p, but not of Pex13p, Pex10p, or Pex12p caused the accumulation of Pex5p in the peroxisomes of mammalian cells, and

Pex5p accumulated in the lumen of peroxisomal remnants lacking Pex13p or the RING finger peroxins (Otera et al. 2000). Therefore, Pex14p most likely provides the initial docking site for cargo-loaded Pex5p, which subsequently is expected to be transported to other components of the import machinery. Evidence that Pex14p may in fact represent the initial docking site also for PTS2 proteins comes from studies by Stein et al. (2002), who demonstrated that thiolase, a PTS2 protein, interacts via Pex7p with Pex14p but not with Pex13p. Girzalsky et al. (1999) reported that Pex13p in *S. cerevisiae* was required for the targeting of Pex14p to the peroxisomal membrane.

Interestingly enough, the two import pathways may also converge further downstream than previously assumed (see above) as findings by Stein et al. (2002) indicated that the binding site for Pex7p is located in the N-terminal 100 amino acids, while binding to Pex5p and Pex14p occurs at the SH3 domain in the C-terminal part of the protein and that these sites can be functionally separated. Remarkably, expression in *pex13*Δ cells of a truncated Pex13p lacking the Pex7p- but not the Pex5p-binding site does complement the defect in the import of PTS1- but not PTS2 proteins. These data indicate that the PTS1 and PTS2 import branches can still be separated on the level of Pex13p.

Late events: translocation, dissociation, and receptor recycling

After docking of the receptor-cargo complexes to the peroxisomal membrane, a translocation of the cargo has to be achieved. The receptor has to dissociate from the cargo either prior to the transport process or posttranslocationally. In the latter case, the receptor then has to be recycled through another translocation step.

The mechanism underlying the translocation process of peroxisomal matrix proteins has not yet been determined. It is not impossible that the docking proteins Pex13p, Pex14p, and Pex17p are also a part of the translocon. Gouveia et al. (2000) reported that the membrane-associated Pex5p behaves like an integral membrane protein and is associated with Pex14p in a ratio of 1:5. Pex14p, therefore, has been suggested to shield the PTS1 receptor from the hydrophobic environment of the membrane. In *pex14*Δ mutants of *H. polymorpha*, however, the import defect for PTS1 proteins could be partially complemented by overexpression of Pex5p (Salomons et al. 2000). Thus, at least in *H. polymorpha*, Pex14p might be dispensable under certain conditions which would be rather unlikely for a central component of the translocation machinery. Nevertheless, the idea that Pex14p and Pex13p might be directly involved in the translocation process certainly deserves a deeper investigation.

Pex5p has been shown to interact—apart from Pex7p, Pex13p, and Pex14p—with other membrane-bound peroxins as well; namely with Pex8p (Rehling et al. 2000) and with Pex12p (Albertini et al. 2001; Chang et al. 1999). This might hint at the existence of an import cascade in which the cargo-loaded PTS receptors are translocated from one component of the import machinery to the next (Erdmann et al. 1997; Hettema et al. 1999; Holroyd and Erdmann 2001).

Pex12p is one of the three “RING finger” peroxins which have been suggested to play a role in the translocation of peroxins. Like Pex2p and Pex10p, it contains a C3CHC4 zinc-binding motif which is thought to mediate protein–protein interactions in all three RING finger peroxins (Chang et al. 1997; Huang et al. 2000; Kalish et al. 1995; Okumoto et al. 2000; Okumoto et al. 1997; Okumoto et al. 1998a; Okumoto et al. 1998b; Patarca

and Fletcher 1992; Tan et al. 1995; Tsukamoto et al. 1991). The interaction of Pex12p with Pex10p and Pex5p has been established by Chang et al. (1999). Reguenga et al. (2001) demonstrated the existence of a complex comprising Pex2p, Pex5p, Pex12p, and Pex14p in rat liver peroxisomes. Interestingly, as mentioned previously, Pex13p was not detected in stoichiometric amounts in the complex. The interaction of Pex10p with Pex12p was also observed by Eckert and Johnsson (J. Eckert and N. Johnsson, submitted), who could also coprecipitate Pex10p with itself, indicating that the protein is also homo-oligomerizing. Data from split-ubiquitin-, two-hybrid- and coimmunoprecipitation experiments in this work also hint at a (possibly transient) interconnection of Pex4p with the complex of RING finger peroxins.

The effects of deleting or overexpressing Pex10p and Pex12p have been studied extensively (Chang and Gould 1998; Dodt and Gould 1996; Okumoto et al. 2000; Otera et al. 2000). Surprisingly, the level of Pex5p associated with the peroxisomal membrane appears to be largely unaffected by either, demonstrating that these peroxins are not required for receptor docking (Chang et al. 1999). However, Dodt and Gould (1996) isolated cells from a patient suffering from a peroxisomal biogenesis disorder caused by a mutation in Pex12p (S320F) and found Pex5p accumulating inside the peroxisome. From this observation and later studies, it was concluded that Pex12p actually appears to have a role in recycling Pex5p across the membrane (Chang et al. 1999; Dodt and Gould 1996). Pex2p has been described to act downstream of Pex10p/Pex12p by Okumoto et al. (2000). Its deletion does not impair the docking of Pex5p to the peroxisomal membrane, yet abolishes PTS1- and PTS2-mediated import (Huang et al. 2000; Otera et al. 2000). Pex5p also accumulated in the lumen of peroxisomal remnants lacking Pex13p or the RING finger peroxins Pex2p and Pex12p, which would implicate that also Pex13p could be involved in the recycling process (Otera et al. 2000).

Pex4p is a member of the E2 family of ubiquitin-conjugating enzymes and is also referred to as Ubc6p. It has been shown to bind ubiquitin in vitro and to be essential for both PTS1 and PTS2-import in all organisms studied, except for *H. polymorpha*, where only PTS1-mediated import seems affected by the deletion of the *PEX4* gene (Crane et al. 1994; Koller et al. 1999a; van der Klei et al. 1998; Wiebel and Kunau 1992). Koller et al. (1999a) also found that Pex22p was required to recruit the soluble Pex4p to the peroxisomal membrane. Eckert and Johnsson (J. Eckert and N. Johnsson, submitted) found the interaction of Pex4p with Pex10p to depend on the presence of Pex22p in the membrane. As described above, Pex10p and Pex12p contain RING finger domains, which are characteristic elements of E3 proteins, the so-called recognins, which are the substrate recognition proteins in the ubiquitination process (Joazeiro and Weissman 2000; Xie and Varshavsky 1999). It could be speculated that Pex22p recruits the E2 Pex4p to the E3-recognins Pex2p, Pex10p, and/or Pex12p. Okumoto et al. (2000) and Albertini et al. (2001) have demonstrated that the function of Pex12p depends on its RING finger domain. Recent findings by Purdue and Lazarow (2001b) indicate that Pex18p is constitutively degraded with a half-time of less than 10 min in *S. cerevisiae*. Several experiments strongly support the idea that this degradation probably occurs in proteasomes and that it is dependent on the presence of Pex4p, and also Pex1p, Pex13p, and Pex14p. This fosters the assumption that Pex18p turnover is associated with its normal function. Mono- and diubiquitinated forms of Pex18p have been detected in wild-type cells and dependence on ubiquitin homeostasis has been established. These data suggest that Pex18p might present a likely target for Pex4p and its cofactors, possibly Pex2p, Pex10p, and/or Pex12p. Also, there is the

observation that the effects of a *PEX4* deletion on PTS1 protein import of *H. polymorpha* cells could be suppressed by overproduction of Pex5p in a dose-response related manner, and that in such cells the peroxisome-bound Pex5p, specifically accumulated at the inner surface of the peroxisomal membrane (van der Klei et al. 1998). The authors therefore hypothesized that in *H. polymorpha*, Pex4p plays an essential role for normal functioning of Pex5p, possibly in mediating recycling of Pex5p from the peroxisome to the cytosol. How Pex4p-related ubiquitination might facilitate Pex5p recycling remains fully enigmatic and highly speculative to date, however. In this respect, it is interesting to note that Pex5p frequently is detected in two forms, one of which migrates slightly slower on SDS-Page (K. Schulz and R. Erdmann, unpublished). It is tempting to speculate that like Pex18p, Pex5p also might be ubiquitinated during the protein import process. However, attempts to substantiate this assumption have not been successful so far.

Pex8p has been cloned in several yeasts and has been found to be a peripheral membrane protein on the matrix face of the peroxisome in three species (Liu et al. 1995; Rehling et al. 2000; Smith and Rachubinski 2001), while it has been found to be a peroxisomal matrix protein in *H. polymorpha* (Waterham et al. 1994). Thus, all reports find the protein on the luminal side of the peroxisomal membrane. Rehling et al. (2000) found Pex8p to be required for both PTS1- and PTS2-mediated import pathways, but dispensable for the insertion of peroxisomal membrane proteins. The group provided evidence for a direct involvement of Pex8p in peroxisomal matrix protein import by showing that Pex8p interacts directly with Pex5p, independently of the C-terminal SKL-motif of Pex8p. In the same study, it was also shown that Pex8p was not required for the docking of Pex5p, suggesting that the interaction between Pex5p and Pex8p follows at a later point of time than the interaction of Pex5p to Pex13p and Pex14p. The *pex8-1* mutant of *P. pastoris* exhibits an import defect for PTS1, but not for PTS2 proteins (Liu et al. 1995). This discriminating import defect suggests that the PTS1 and PTS2 import branches still can be separated at the stage of Pex8p. The nature of this Pex8p mutation, however, has not yet been investigated. The fact that Pex8p contains both a PTS1 and a PTS2 fuels the idea that it might be the point of separation of receptor and cargo. Recently, interaction of the RING complex and the docking complex was shown to depend on Pex8p (Agne et al. 2003).

Smith and Rachubinski (2001) characterized the interactions of Pex8p in *Y. lipolytica* and demonstrated that Pex8p and Pex20p form a complex by a direct interaction. As the phenotype of cells lacking Pex8p is more severe than that of cells lacking Pex20p, the protein is not necessary for the targeting of Pex8p to peroxisomes. In the absence of Pex8p, thiolase is mostly cytosolic while the usually predominantly cytosolic Pex20p and a small amount of thiolase associate with peroxisomes, suggesting that Pex8p is involved in the import of thiolase after docking of the Pex20p-thiolase complex to the membrane. As peroxisomal thiolase and Pex20p are protected from the action of externally added protease in *pex8*Δ cells and Pex8p was found to be intraperoxisomal, these results indicated that Pex20p might accompany thiolase into peroxisomes during the import process and that Pex8p is likely to play a role in separation of the two or recycling of Pex20p to the cytosol.

Both the results from *S. cerevisiae* and *Y. lipolytica* suggest that Pex8p might interact with Pex5p or Pex20p on the luminal side of the peroxisomal membrane, therefore supporting the extended hypothesis of shuttling receptors. Originally, Marzioch et al. (1994) and Dodt and Gould (1996) proposed a shuttle model for the cycling of the PTS-receptors between the cytosol and the outer face of the peroxisomal membrane. The free receptor in

the cytosol binds the cargo, the receptor-cargo complex then associates with the docking complex at the membrane, upon which separation of cargo and receptor occurs. The cargo then is imported, while the receptors return to the cytosol. As discussed above for Pex5p, Pex7p, and Pex20p, these receptors were found at different localizations in the cell; ranging from solely cytosolic, over membrane attached, to solely inside the peroxisome, the latter localization being hard to reconcile with the original shuttle model. Furthermore, Dammai and Subramani (2001) found human Pex5p to be translocated into the peroxisomal matrix and recycled back to the cytosol. Altogether, these results support an extended shuttle of the receptors, meaning that the receptors enter the peroxisomal matrix together with their cargo. In the matrix, uncoupling of cargo and receptor takes place and the receptors are recycled to the cytosol (Fig. 6). While some of the methods applied to obtain the data are under criticism, the model still engulfs and explains all the different observations both on the localization of the receptor proteins as well as on the interactions of Pex8p.

Finally, Pex1p and Pex6p are thought to participate in the late events in peroxisomal matrix protein import, as well. These membrane-bound peroxins belong to the AAA ATPases and have been shown to interact with each other (Erdmann et al. 1991; Faber et al. 1998; Titorenko and Rachubinski 1998b; Voorn-Brouwer et al. 1993). Members of this AAA-family are involved in various cellular activities, including protein degradation and vesicle-mediated protein transport. Their characteristic highly conserved AAA domain of 230 amino acids contains Walker ATP binding sequences and imparts ATPase activity. They are also known to be able to specifically bind to and disrupt oligomeric membrane protein complexes (Ogura and Wilkinson 2001; Patel and Latterich 1998). Localization studies on Pex1p and Pex6p show remarkable differences between various organisms (Faber et al. 1998; Kiel et al. 1999; Tamura et al. 1998a; Tamura et al. 1998b; Titorenko and Rachubinski 2000; Tsukamoto et al. 1995; Yahraus et al. 1996), and there is dispute over the proposed function of these proteins, as well: two roles have been proposed so far for Pex1p and Pex6p. In *Y. lipolytica*, Pex1p and Pex6p are part of the peroxisome assembly pathway as they are required for the fusion of small peroxisomal vesicles (Titorenko and Rachubinski 2000; Titorenko and Rachubinski 2001a). In sharp contrast to this, Collins et al. (2000) found some evidence that Pex1p and Pex6p function in the translocation machinery of *P. pastoris*; downstream of receptor docking and translocation, yet upstream of Pex4p and Pex22p. To gain insights into the sequence of events in the late events of peroxisomal matrix protein translocation, Collins et al. (2000) carried out a series of experiments with *pex* mutants in the yeast *P. pastoris*. They had noted that the steady-state protein level of Pex5p remained unaffected in most *pex* mutants, but was altered in four: in *pex4*Δ and *pex22*Δ mutants it was severely decreased and moderately reduced in *pex1*Δ and *pex6*Δ mutants. This was exploited to determine the epistatic relationships among several groups of *pex* mutants. Pex4p thus appeared to act after the peroxisome membrane synthesis factor Pex3p, the Pex5p docking factors Pex13p and Pex14p, and after the matrix protein import factors Pex2p, Pex8p, Pex10p, Pex12p, and Pex17p. Pex1p and Pex6p were also found to act after Pex10p, but upstream of Pex4p and Pex22p. These results suggest that Pex1p, Pex4p, Pex6p, and Pex22p act late in peroxisomal matrix protein import, after matrix protein translocation, and likely in the recycling of the PTS1-receptor, as their function follows Pex8p (on the inside). All of these data combined led to the model for a hypothetical peroxisomal protein import cascade which is depicted in Fig. 6.

Cargo aggregation hypothesis

Numerous studies and reviews have been concerned with the import of peroxisomal matrix proteins. From the early observations that the peroxisomal import machinery accepts folded proteins, oligomerized proteins, and items of large diameter such as gold particles fused to import signals as substrates (Glover et al. 1994a; Häusler et al. 1996; McNew and Goodman 1994; Walton et al. 1995), the picture has steadily grown. Elgersma et al. (1995) found that carnitine acetyltransferase lacking its PTS1 was still able to interact with Pex5p in the two-hybrid assay. Pex5p and Pex7p were shown to interact, and in some species this interaction was essential for PTS2-mediated import, hinting at the formation of a complex of receptors and substrates of both pathways (Nito et al. 2002). Schliebs et al. (1999) found Pex5p to form homo-tetramers. Stewart et al. (2001) found that dihydroxyacetone synthase (DHAS) quickly dimerizes in the cytosol prior to import, and "piggyback" targeting has been described for various proteins, (e.g., Koller et al. 1999b; Lee et al. 1997). While alcohol oxidase has been shown to assemble inside the peroxisomal matrix (Faber et al. 2002; Stewart et al. 2001), indicating that import of oligomeric proteins seems not to be a universal pathway for all peroxisomal matrix proteins, it actually appears that more and more proteins reach the matrix fully folded and aggregated.

The whole chaperone machinery which has been described so far to be required for peroxisomal matrix protein import appears to be on the cytosolic face of the membrane, reviewed in Hettema et al. (1999). To date, there is no specific intraperoxisomal chaperone system known. This might hint at the fact that the folding and oligomerization of the matrix proteins into their final conformation occurs already in the cytosol and/or on the outer face of the membrane. Also, the possible role of Pex8p as the point of uncoupling of receptor and cargo lies on the matrix face of the peroxisomal membrane (Rehling et al. 2000), suggesting that in fact a multimer cargo-receptor complex enters the peroxisomal matrix.

The nature of the peroxisomal matrix has been described numerous times as "paracrystalline," and latency tests have undermined the notion that at least a number of the proteins of this compartment appear to be multimerized in vivo as well (Purdue and Lazarow 2001a; Reumann 2000; Subramani 1992; Thompson and Krisans 1990; Zaar et al. 1986). Also, studies on the rate of chemical reactions in the matrix of leaf peroxisomes resulted in the conclusion that a direct channelling of products from one enzyme to the next in a highly ordered multienzyme complex was the only possible explanation of the kinetics observed (Heupel and Heldt 1994; Reumann 2000). It is difficult to imagine that such a highly ordered and folded multienzyme complex can be arranged and even aggregated into a paracrystalline matrix without the help of chaperones, unless the newly imported matrix proteins reached the matrix side already in accordingly folded and assembled structures. The size of the aggregate complex would also determine requirements for the molecular mechanism of import.

Bellion and Goodman (1987) observed that several peroxisomal enzymes enter an extremely large membrane-associated protein complex prior to the import process, but were no longer associated after the import process. Based on these data, Gould and Collins (2002) recently postulated the formation prior to the import of larger Pex5p/cargo aggregates which were named preimport complexes (preimplexes). Stein et al. (2002) described that Pex18p and Pex21p promote the formation of a higher ordered import-competent PTS2 substrate complex. In the absence of Pex18p/21p, the PTS2 receptor Pex7p is only associated with a small amount of thiolase, while in the presence of Pex18p/21p a signifi-

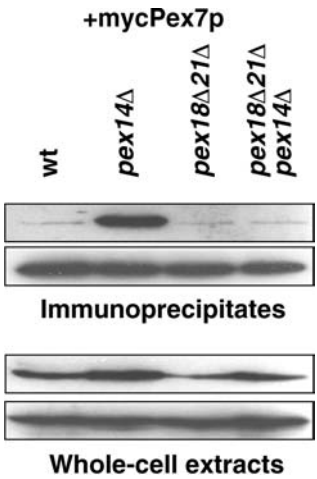

Fig. 7 Pex18p and Pex21p promote the formation of an import-competent PTS2 substrate complex. Accumulation of a Fox3p-Pex7p complex in *pex* mutants. The indicated myc-Pex7p-expressing strains were analyzed for the presence of Fox3p in immunoprecipitates of myc-Pex7p by immunoblotting (*upper panel*). In a wild-type strain, the PTS2 receptor Pex7p is only associated with a small amount of thiolase, while in the absence of Pex14p a significantly greater amount of thiolase can be precipitated. An accumulation of Fox3p was not observed in the *pex18*Δ, *pex21*Δ double-mutant and in the triple deletion strain (*pex18*Δ, *pex21*Δ, *pex14*Δ). This result indicates that the accumulation of the Pex7p-Fox3p complex that contains a surplus of thioase in a *pex14*Δ mutant is dependent on Pex18p/Pex21p. Furthermore, the data indicate that the function of Pex18p and Pex21p is already required in the import process prior to Pex14p, ergo before the Fox3p-Pex7p-Pex18p/Pex21p complex docks at the peroxisomal membrane. The *lower panel* shows an immunoblot of the cell lysates that were used for precipitation (adapted from Stein et al. 2002)

cantly greater amount of thiolase can be precipitated (Fig. 7). This observation triggers the idea described here that Pex18p/Pex21p as Pex5p are required for a cross-linking of receptor-cargo complexes in PTS2- and PTS1-dependent protein import respectively, therefore causing an aggregation of the cargo-receptor complexes which is suggested to be a presupposition for an efficient import process (Fig. 8).

The mechanism by which the import machinery could accommodate the translocation of such folded and oligomerized substrates still needs to be determined. One suggestion is, as previously mentioned, the existence of a pore. Its structural requirements have been excellently discussed in Hettema et al. (1999), who also hint at the fact that, so far, freeze-fracture electron microscopic studies have failed to identify such a complex (Kryvi et al. 1990). Size limitation of such a pore could represent the reason why the aggregated receptor/cargo complexes might be disassembled prior to the import process as observed by Bellion and Goodman (1987) and outlined by Gould and Collins (2002). This, however, raises the question why such aggregates would need to form in the first place.

Alternatively, an invagination process of the membrane might result in a sort of endocytic incorporation of these aggregates (McNew and Goodman 1994). This scenario, however, would be hard to reconcile with a requirement to disassemble the aggregates prior to the import process. Therefore, testing for the requirement of disassembly might provide evidence for either the pore or invagination hypothesis. According to the invagination model, the docking complex might represent the site of cargo aggregation and subsequently an invagination might release a vesicle-like structure into the matrix which then could

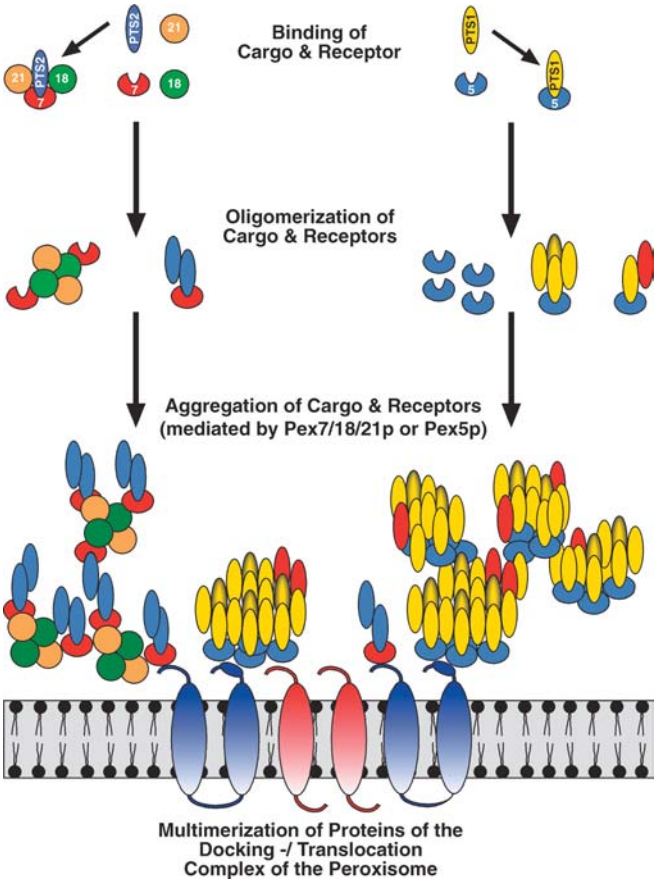

Fig. 8 Cargo-receptor aggregation in the peroxisomal protein import cascade. This model describes the idea that Pex18p/Pex21p as Pex5p are required for a cross-linking of receptor-cargo complexes in PTS2- and PTS1-dependent protein import, respectively, therefore causing an aggregation of the cargo/receptor complexes, which is proposed to be a presupposition for an efficient import process

be dissolved and the protein complex integrated into the paracrystalline structure of the peroxisomal matrix. However, there is no evidence yet for such a pathway. Alternatively, peroxisomal protein import could proceed in a way comparable to the cytosol to vacuole pathway of folded aminopeptidase 1 (Ape1p). Cytosolic aggregates of the enzyme have been shown to be incorporated into double-membrane vesicles which subsequently fuse with the vacuolar membrane in an autophagocytotic-like process (Kim and Klionsky 2000). The inner membranes of these vesicles are degraded within the organelle, therefore releasing the vesicle content into the lumen. However, there is little evidence for such a pathway in peroxisome biogenesis either. Nevertheless, the characteristic structural appearance of peroxisomal membrane ghosts as described by Hashiguchi et al. (2002) and Hettema et al. (2000) and depicted in Figs. 2 and 3, the heterogenous populations of peroxisomal prestructures identified by Titorenko et al. (2000a), as well as the aggregation hypothesis described here and by Gould and Collins (2002) cannot be easily dismissed and provide grounds for such speculations.

Concluding remarks

After the discovery of peroxisomes, the mechanisms underlying their biogenesis were thought to be simple variations of those of other organelles. The picture which evolved during the last years, however, made clear that this was an oversimplification. Genetic and proteomic approaches have led to the identification of most of the components required for the biogenesis of peroxisomes. Despite the fact that our knowledge of the function of single peroxins and peroxin complexes has clearly grown within the last decade, the "big picture" into which to fit these observations is still missing. The molecular mechanism of matrix protein import has still not been solved. Many interactions among peroxins have been identified, but how their concerted action results in the transport of folded and oligomeric proteins across the peroxisomal membrane remains a mystery. To date, we still don't know whether the transmembrane transport is performed by a translocation pore, nor is there striking evidence for or against the model of an invagination of the membrane. The elucidation of the principle mechanisms underlying peroxisomal matrix protein import and the topogenesis of peroxisomal membrane proteins still represents the ultimate challenge in peroxisomal biogenesis research.

The roles of the individual peroxins and peroxin complexes need to be established more clearly. Further epistatic studies such as the one by Collins et al. (2000) should help to understand the temporal sequence of events and thereby elucidate mechanistic questions. Similarly, the new players on the team, Pex23p, Pex24p, and Pex25p, need to be studied carefully (Brown et al. 2000; Smith et al. 2002; Tam and Rachubinski 2002). The effects of the *pex23* phenotype, which impairs both the matrix protein import and the biogenesis of the peroxisomal membrane, is particularly interesting. It might also reveal insights into the mechanism of biogenesis of the membrane, which is still heavily disputed as indicated in this article.

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C. Andersen

Channel-tunnels: outer membrane components of type I secretion systems and multidrug efflux pumps of Gram-negative bacteria

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Abstract For translocation across the cell envelope of Gram-negative bacteria, substances have to overcome two permeability barriers, the inner and outer membrane. Channel-tunnels are outer membrane proteins, which are central to two distinct export systems: the type I secretion system exporting proteins such as toxins or proteases, and efflux pumps discharging antibiotics, dyes, or heavy metals and thus mediating drug resistance. Protein secretion is driven by an inner membrane ATP-binding cassette (ABC) transporter while drug efflux occurs via an inner membrane proton antiporter. Both inner membrane transporters are associated with a periplasmic accessory protein that recruits an outer membrane channel-tunnel to form a functional export complex. Prototypes of these export systems are the hemolysin secretion system and the AcrAB/TolC drug efflux pump of *Escherichia coli*, which both employ TolC as an outer membrane component. Its remarkable conduit-like structure, protruding 100 Å into the periplasmic space, reveals how both systems are capable of transporting substrates across both membranes directly from the cytosol into the external environment. Proteins of the channel-tunnel family are widespread within Gram-negative bacteria. Their involvement in drug resistance and in secretion of pathogenic factors makes them an interesting system for further studies. Understanding the mechanism of the different export apparatus could help to develop new drugs, which block the efflux pumps or the secretion system.

Abbreviations *ABC* ATP-binding cassette · *CD* Circular dichroism · *Gsc* Single channel conductance · *GSP* General secretory pathway · *IM* Inner membrane · *MF* Major facilitator · *MIC* Minimum inhibitory concentration · *NMR* Nuclear magnetic resonance · *OM* Outer membrane · *PMF* Proton motif force · *RND* Resistance nodulation cell division · *RTX* Repeats in toxins · *SMR* Small multidrug resistance · *TMS* Transmembrane segment

C. Andersen (✉)
Department of Biotechnology, University of Würzburg, 97074 Würzburg, Germany
e-mail: andersen@biozentrum.uni-wuerzburg.de

Introduction

The cell envelope of Gram-negative bacteria consists of the cytoplasmic membrane (or inner membrane, IM) and the outer membrane (OM), which enclose the periplasmic space (Duong et al. 1997). Membranes form permeability barriers for hydrophilic substances and therefore transport of these substances out or into the cell needs the presence of selective transport proteins (Nikaido and Vaara 1985). Active transport processes are possible across the IM and the energy is provided by cellular ATP used by an ATP-binding cassette (ABC) transporter or by ion gradients across the membrane, which are used by sym-, anti-, or uniporter to accumulate substances at one side of the membrane (Saier 2000). In contrast to the energized IM is the OM a barrier, across which transport occurs by diffusion following a concentration gradient between the periplasmic space and the external environment. Active transport across the OM is only possible by interaction with energized proteins of the IM.

In 2000, Koronakis and coworkers (2000) crystallized an outer membrane protein belonging to a protein family, which is part of two distinct export systems: the type I protein secretion system and the multidrug efflux pumps (Koronakis et al. 2000). Its extraordinary crystal structure reveals how both systems are capable of transporting substrates actively across both membranes directly from the cytosol into the external environment. This review deals with these two export systems with special consideration of the role of the OM protein.

Export systems

Protein secretion systems

Protein secretion is required for several aspects in the bacterial life such as nutrient acquisition or virulence factor expression. Several pathways in the cell envelope of Gram-negative bacteria are known to transport proteins out of the cell (Thanassi and Hultgren 2000). They can be divided into six overarching groups. Four of them, the type II and type IV secretion system, the chaperone/usher and the autotransporter secretion pathway, use the general secretory pathway (GSP) for the transport across the IM. Here, proteins with a cleavable amino-terminal secretion signal are directed to the sec-system in the IM, which transports it into the periplasmic space (Fekkes and Driessen 1999; Manting and Driessen 2000). After or during the translocation, the secretion signal is cleaved. The transport of the periplasmic intermediate across the outer membrane is different for the four groups. Autotransporters do not need accessory factors to cross the OM. The mechanism lies in the exported protein itself. The carboxy-terminal β-domain inserts in the OM, forms a porin-like β-barrel through which the rest of the protein passes the membrane. The IgA1 protease of *Neisseria gonorrhoe* is a prototypical member of this secretion mechanism (Pohlner et al. 1987). The chaperone/usher pathway is used for the assembly and secretion of a broad range of adhesive virulence structures such as P and type 1 pili of uropathogenic *Escherichia coli* (Roberts et al. 1994; Langermann et al. 1997). Chaperones interact with the pili subunits in the periplasmic space, preventing the pilus assembly. They direct the subunits to the OM, in which the usher proteins form ring-shaped, oligomeric structures containing a central pore (Thanassi et al. 1998). Released from the chaperones, the subunits pass the pore and assemble at the bacterial surface.

The type II and type IV protein secretion systems are more complex. Recent studies suggest that both systems are closely related (Sauvonnet et al. 2000). Between 12 and 16 accessory proteins are used by the type II secretion system to export extracellular enzymes such as the *Klebsiella oxytoca* pullulanase or toxins such as the heat labile enterotoxin of enterotoxigenic *E. coli* (Pugsley et al. 1997; Russell 1998; Tauschek et al. 2002). Most of the components are associated with the IM and have extensive periplasmic domains playing a role in energy transduction to the OM. Only one protein is an integral OM protein belonging to the secretin superfamily, which assemble into highly stable ring-shaped oligomers analogous to the usher protein of the chaperone/usher pathway. The central channel has a diameter between 5 nm and 10 nm, large enough to accommodate folded substrates (Bitter et al. 1998; Nouwen et al. 1999; Nouwen 2000). The type IV secretion system is homologous to the bacterial conjugation system and the VirB system of *Agrobacterium tumefaciens* that facilitates the translocation of oncogenic T-DNA into plant cells (Burns 1999). It is also used for pertussis toxin secretion by *Bordetella pertussis*. The secretion apparatus consists of nine proteins. As in the type II secretion system, the transport across the OM is mediated by proteins of the secretin superfamily (Schmidt et al. 2001).

The two remaining protein secretion systems, the type I and type III secretion systems, translocate substrates in a sec-independent manner across the IM. The type III system is essential for the pathogenicity of several bacteria such as *Yersinia* or *Salmonella* by translocating antihost factors into the cytosol of target eukaryotic cells (Hueck 1998; Cheng and Schneewind 2000). Approximately 20 components assemble into a complex structure, which spans both membranes as visualized by electron microscopy (Kubori et al. 1998; Blocker et al. 1999). The needle-shape structure is similar to that of the flagellar basal body, and a relation between the two systems is confirmed by sequence homology and the finding that the flagellar export apparatus can function as a protein secretion system (Young et al. 1999). The only component, which is homologous to proteins of other systems, is a protein of the secretin superfamily forming a ring structure in the OM (Crago and Koronakis 1998). The type I secretion is the other sec-independent protein secretion system, which has in common with the type III secretion system the fact that no periplasmic intermediates are found. The composition of this secretion system is less complicated. The type I secretion system can be divided into two subtypes dependent on the location of the secretion signal in the allocrit. They are described in detail in the next sections.

Type I secretion with carboxy-terminal secretion signal

The type I secretion system is composed of only three proteins: an ABC-transporter in the IM, which forms a complex with an accessory protein in the periplasmic space, and an OM protein of the TolC family. The allocrits of the type I systems are RTX (Repeats in Toxin) toxins such as the *E. coli* hemolysin or *B. pertussis* adenylate cyclase, extracellular enzymes such as proteases or lipases, as well as proteins remaining attached to the cell surface such as S-layer proteins or certain glycanases (Wandersman and Delepelaire 1990; Glaser et al. 1988; Duong et al. 1994; Thompson et al. 1998; Awram and Smit 1998; Finnie et al. 1997). Recent findings add other protein classes to the allocrits of the type I secretion system: the cell-associated exopolysaccaride processing enzymes of *Rhizobium*, *Sphingomonas*, and *Azorhizobium* species (York and Walker 1997; Finnie et al. 1998) and bifunctional proteins secreted by *Caulobacter* species, containing an amino-terminal S-

layer protein fused to a carboxy-terminal domain with an RTX motif (Thompson et al. 1998; Kawai et al. 1998).

The allocrits have a secretion signal, which is in contrast to the amino-terminal secretion signal of proteins exported by the GSP located within the last 60 residues of the carboxy-terminus, and is not cleaved after secretion (Stanley et al. 1991). An exception has to be mentioned in this context. HasA, a comparatively small allocrit with no RTX repeats, possesses a carboxy-terminal signal sequence which is cleaved after secretion (Izadi-Pruneyre 1999). The size of the allocrits varies between a few hundred and more than 4,000 residues, and in some cases they are posttranslational acylated as it has been shown for hemolysin and adenylate cyclase toxin (Letoffe et al. 1994; Lin et al. 1999; Glaser et al. 1988; Stanley et al. 1994). It can be assumed that the proteins are partly folded when exported out of the cell. A role of cytoplasmic chaperons is also reported in some cases (Delepelaire and Wandersman 1998; Young and Holland 1999). The prototype of the type I secretion system is the hemolysin export apparatus of *E. coli*. The hly-operon codes for the 110-kDa hemolysin HlyA, the ABC transporter HlyB, the accessory protein HlyD (previously termed membrane fusion protein) and the acyl transferase HlyC, which activates the HlyA protoxin by fatty acylation at two lysine residues (Stanley et al. 1994). The OM protein TolC, which is essential for the secretion process, is not part of the hly-operon. It is part of the stress-induced mar-sox operon of *E. coli* (Aono et al. 1998; Alekshun and Levy 1999). TolC is described in more detail in the section "TolC of *E. coli*". The ABC transporter HlyB has two domains: a cytoplasmic domain, containing the nucleotide-binding site and a membrane domain predicted to consist of six transmembrane helices. The structure of its cytoplasmic domain was recently solved (Kranitz et al. 2002), while the structure of the membrane domain can only be assumed from the crystallized distantly related homologue MsbA (Chang and Roth 2001). HlyB is most likely assembled as homodimers in the IM. The accessory protein HlyD has a different symmetry; it has been shown to form trimers. Each HlyD monomer has three domains. Fifty-nine residues of the amino-terminus form a cytoplasmic domain, which is linked by a 21-residue-long transmembrane domain to the large periplasmic domain (residues 81–478). HlyB and HlyD form a stable complex in the IM. It has been shown by in vivo cross-linking that both proteins independently bind the allocrit HlyA and that HlyA binding induces bridging of the HlyB/D complex to TolC via HlyD (Thanabalu et al. 1998; Balakrishnan et al. 2001). Protease accessibility indicated that translocation induced conformational changes in each of the three exporter proteins. After substrate passage, TolC and the HlyB/D complex disengage. This means that the allocrit-dependent bridging is dynamic. It could be shown that the secretion process could be divided into two distinct stages: an early stage for allocrit binding, which requires the electrochemical potential, possibly reflecting the allocrit binding, and a late stage, which is independent of the electrochemical potential (Koronakis et al. 1991).

Type I secretion system with amino-terminal secretion signal

In addition to this classic type I secretion system, there is another group of proteins that has to be mentioned here. Colicins and microcins are described to be exported by systems, which are homologous to the type I secretion system (Hwang et al. 1997; Garrido et al. 1988; Azpiroz et al. 2001). However, the length of the allocrits is not sufficient to carry a 45-residue-long carboxy-terminal secretion signal and it is also known that the allocrits are

processed and an amino-terminal secretion signal is cleaved (Havarstein et al. 1994; Lagos et al. 1999). These facts clearly separate the secretion mechanism from the classic type I secretion system. Both IM proteins of the secretion apparatus are found to be homologous to proteins from Gram-positive lactic acid bacteria and *Streptococcus pneumoniae*, which are transporters for antibacterial peptides such as lantibiotics or bacteriocins (also termed nonlantibiotics) and competence-stimulating peptides (competence pheromone). These antibacterial and signaling peptides are produced by the processing of larger precursor peptides (Kleerebezem et al. 1997). A characteristic amino-terminal leader sequence, termed double-glycine-type leader sequence, is cleaved concomitant with export (Klaenhammer 1993; van Belkum et al. 1997). Responsible for this process is most likely an amino-terminal proteolytic domain of around 150 amino acids in their ABC transporter that is absent in other ABC transporters (Havarstein et al. 1995). This amino-terminal extension is also found in the ABC transporters of Gram-negative bacteria, which are involved in colicin and microcin secretion (Gilson et al. 1990; Solbiati et al. 1999) and these parallels allow the assumption that Gram-positive and Gram-negative systems have a common ancestor.

There are other microcin-producing plasmids of *E. coli* which carry the information for microcin synthesis and export. The mcjABCD operon codes for the microcin J25 precursor. The amino-terminal 37-amino-acid-long leader peptide, a variant of the double-glycine leader sequence, is cleaved and the remaining 21 residues become head-tail linked resulting in the cyclic microcin J25. mcjD codes for an ABC transporter. The remaining two genes, mcjB and mcjD, do not code for an accessory protein, but are somehow involved in microcin maturation. The lack of an accessory protein is surprising because it has been shown that TolC is necessary for the production of this peptide antibiotic (Delgado et al. 1999).

Multidrug transporter

Bacteria have established several different mechanisms to become resistant to antibacterial drugs: inactivating drugs by hydrolysis or modification, altering the target of the drug, preventing drug access to the cell, or preventing accumulation of drugs in the cell. The latter resistance mechanism, which belongs to one of the most frequently employed resistance strategies, is mediated by the multidrug transporter. By extrusion of the drugs out of the cell, they reduce the intracellular drug concentration to subtoxic levels. In general, the multidrug transporter can be associated to four superfamilies: the ABC (ATP-binding cassette), the MF (major facilitator), the SMR (small multidrug resistance), and the RND (resistance nodulation cell-division) (Table 1). Drug transporters of the ABC, RND, and MF superfamilies are found in prokaryotes, archaea, and eukaryotes, while drug transporters of the SMR superfamily are exclusively found in prokaryotes (Saier et al. 1998; Higgins 1992; Tseng et al. 1999). It should be mentioned that RND transporters are most likely not involved in nodulation of legumes as their name implies. It has been shown that the exopolysaccharides, which act as nodulation factors of *Rhizobium leguminosarum* are secreted by proteins, which are similar to capsular polysaccharide secretion proteins of *E. coli* (Vazquez et al. 1993) and not by the nolG and nolF gene products (the RND transporter NolG was previously thought to consist of three separate proteins NolG, NolH, and NolI; Baev et al. 1991).

The energy source for the transport process is ATP hydrolysis in the case of the ABC transporter; the transporters of the other superfamilies use ion gradients across the membrane. The transporters of the MF superfamily act as sym-, anti- or uniporters, and trans-

Table 1 Properties of superfamilies that include drug transporters (Saier et al. 1998)

Superfamily	Occurrence	Number of described subfamilies	Drug transporting subfamilies	Driving force	TMS	Residues	Well-characterized example
ABC	Bacteria, archaea, eukaryotes	>36	3–4	ATP	12 (6+6)	>1,000	HlyB, MDR
MF	Bacteria, archaea, eukaryotes	17	3	PMF	12–14	~400	TetB
RND	Bacteria archaea, eukaryotes	3	1	PMF	12	~1,000	AcrB
SMR	Bacteria	2	1	PMF	4[a]	~100	EmrE

[a] The oligomeric state of proteins of the SMR superfamily is dimeric or trimeric (Tate et al. 2001; Muth and Schuldiner 2000)

porters of the SMR and RND superfamily are proton antiporter (Saier et al. 1994, 2000; Griffith et al. 1992; Marger and Saier 1993).

The distribution of the superfamilies among the drug transporter within the prokaryotes is not uniform. For example, the 29 drug pumps of *E. coli* identified by sequence comparison with the established drug transporter distribute over two of the ABC superfamily, 18 of the MF, five of the SMR, and four of the RND. Similar relationships are found in *Haemophilus influenza* (0, 4, 1, 1). In contrast, *Mycobacterium genitalium* has exclusively transporters of the ABC family, which can be explained by the lack of an electron transport chain, which is necessary for generating a proton electrochemical gradient as a primary source for members of the other drug transporter superfamilies (Saier et al. 1998; Fraser et al. 1995).

The molecular structure of the transporter is known for the RND and the ABC superfamily. The structure of the RND transporter AcrB of *E. coli* was solved recently (Murakami et al. 2002). Three AcrB protomers assemble to a homotrimer, which can be divided into two domains. The 50-Å-thick transmembrane region is composed of twelve helices per monomer. Three transmembrane domains are arranged in a ring-like manner with a central 30-Å-wide hole. Large loops between helices 1 and 2, and 7 and 8 form the 70-Å-thick headpiece protruding into the periplasmic space. The headpiece can be divided into two parts. The upper part, named the TolC docking domain, forms a funnel-like structure open at the top. The funnel is connected by a central pore with a cavity formed by the lower part of the headpiece, the pore domain. This cavity is open to the periplasm by three vestibules near the membrane plane. These openings might be the entrance for allocrits located on the membrane plane or the outer leaflet of the membrane into the transporter. The structure of the ABC transporters MsbA and BtuBC reveals that the membrane-spanning part of the functional unit consists of twelve transmembrane helices (six per monomer; Chang and Roth 2001; Locher et al. 2002). Two ATP-binding domains (one per monomer) are linked to the cytoplasmic side of the membrane domain, where they provide energy for the translocation process. The relatively small proteins of the SMR superfamily arrange into four membrane-spanning helices. Its oligomerization state is dimeric or trimeric (Tate et al. 2001; Muth and Schuldiner 2000; Yerushalmi and Schuldiner 2000). Structure predictions of transporters of the MF superfamily are not yet confirmed by crystal structures. The MF superfamily is classified into two subfamilies according to the number of membrane-spanning helices: the 12-helix transporters such as the *E. coli* class B tetracycline transporter TetA(B) and the 14-helix transporters, such as the *Staphyloccoccus aureus* class K tetracy-

cline transporter TetA(K). The MF transporters are most likely evolved by gene duplication. They consist of two halves with usually related sequences containing 6 and 7 transmembrane helices respectively, connected by a cytoplasmatic loop.

The progress in genome sequencing has made it possible to study the origin of multidrug transporters. Because drug transporters are found in the genome of pathogenic as well as in nonpathogenic bacteria in comparable numbers, it is unlikely that these export systems have evolved recently as a result of extensive exposure to medically relevant drugs (Saier et al. 1998). Instead, they may play an important physiological role in the extrusion of naturally occurring toxic substances.

Multidrug efflux pumps

Gram-positive bacteria are shown to be more sensitive to a large number of antibiotics and chemotherapeutic agents than Gram-negative bacteria. The higher resistance of Gram-negative bacteria measured as a higher minimum inhibitory concentration (MIC) can be explained by the additional permeability barrier in the cell envelope of the bacteria. The OM limits the penetration of hydrophilic solutes by the narrow porin channels, and the low fluidity of the lipopolysaccharide leaflet slows down the inward diffusion of lipophilic solutes (Nikaido and Vaara 1985; Nikaido 1989; Plesiat and Nikaido 1992). Multidrug transporters described in the previous section transfer drugs from the cytosol or the IM into the periplasmic space. Thus, the bacteria do not benefit from the advantage of the additional permeability barrier. Analogous to the type I protein secretion system drug transporter of the ABC, MF, and RND superfamilies employ two proteins, a periplasmic accessory protein and an outer membrane protein of the TolC family, to form an export system termed multidrug efflux pump. This tripartite export apparatus excretes drugs directly into the external medium. Because the reentry of the drugs is slowed down by the outer membrane barrier, the multidrug efflux pumps can produce significant resistance levels in Gram-negative bacteria. For example, the MIC of carbenicillin is 32 μg/ml for *Pseudomonas aeruginosa* wild type. If the MexAB/OprM multidrug efflux pump is inactivated, the MIC is $\leq$0.25 μg/ml, whereas if the pump becomes overexpressed, it raises to 1,024 μg/ml (Li et al. 1995). Indeed, it has also been shown for other Gram-negative bacteria that inactivating the main multidrug efflux pump such as AcrAB/TolC of *E. coli* (Fralick 1996; Sulavik et al. 2001), MtrCDE of *Neisseria gonorrhoe* (Lucas et al. 1995), AmrAB/OprA of *Burkholderia pseudomallei* (Moore et al. 1999), or SmeDEF of *Stenotrophomonas maltophilia* (Zhang et al. 2001) increases the susceptibility to various drugs, showing that efflux pumps work with exceptional efficiency through their synergistic interaction with the outer membrane barrier (Nikaido 1996; Thanassi et al. 1995).

Cation efflux pumps

A variant of the multidrug efflux pumps are cation efflux pumps. The IM transporter belongs to a subfamily of RND transporters that is specific for cations. They assemble with an accessory protein and an OM protein into a cation efflux pump. In *Ralstonia* species and *P. aeruginosa* tripartite cation efflux pumps have been described, which enable the bacteria to grow in the presence of high concentrations of diverse toxic cations such as Cd, Zn, Ni, or Co (Nies et al. 1989; Hassan et al. 1999).

Table 2 Transporter and accessory proteins, which form a functional export apparatus with *E. coli* TolC

Transporter	Family	Accessory protein	Allocrite	Organism	Reference
HlyB	ABC	HlyD	Hemolysin (HlyA)	*E.coli*	Wandersman and Delepelaire 1990
			Colicin V (CvaC)	*E. coli*	Fath et al. 1991
			Hemolysin 1 (Hly1A)	*A. pleuropneumoniae*	Gygi et al. 1990
			Leukotoxin (LktA)	*P. hemolytica*	Highlander et al. 1990
			Metalloprotease (PrtB)	*E. chrysanthemi*	Delepelaire and Wandersman 1990
			Adenylat cyclase toxin (CyaA)	*B. pertussis*	Sebo and Ladant 1993
			Alcaline protease (AprA)	*P. aeruginosa*	Guzzo et al. 1991
			Nodulation factor (NodO)	*R. leguminosarum*	Scheu et al. 1992
			Lipase (AprA)	*P. fluorescens*	Duong et al. 1994
HasD	ABC	HasE	Hemophore (HasA)	*S. marcescens*	Binet and Wandersman 1996
			Metalloprotease (PrtB)	*S. marcescens*	Binet and Wandersman 1996
			Metalloprotease (PrtB)	*E. chrysanthemi*	Binet and Wandersman 1996
PrtD	ABC	HasE[a]	Metalloprotease (PrtC)	*E. crysanthemi*	Binet and Wandersman 1995
CvaB	ABC	CvaA	Colicin V (CvaC)	*E. coli*	Hwang et al. 1997
McjD	ABC	–	Microcin J25	*E. coli*	Delgado et al. 1999
MceG	ABC	MceH	Microcin E492	*K. pneumoniae*	Lagos et al. 2001
MchF	ABC	MchE	Microcin H47	*E. coli*	Azpiroz et al. 2001
AcrB	RND	AcrA	Drugs	*E. coli*	Sulavik et al. 2001
EmrB	MF	EmrA	Drugs	*E. coli*	Lomovskaya and Lewis 1992
MacA	ABC	MacB	Macrolides	*E. coli*	Kobayashi et al. 2001

[a] The accessory protein HasE is from *S. marcescens*

TolC of *E. coli*

TolC is the OM component of various type I secretion systems and multidrug efflux pumps (see Table 2). In addition to its contribution in these export systems, there are other phenotypes of TolC mutants described, showing the role of TolC as a multifunctional protein in the envelope of *E. coli*. In 2000, Koronakis and coworkers (2000) solved its structure. It was the first structure of a member of the family of OM proteins involved in the type I secretion and multidrug efflux. TolC is therefore the prototype of this family and is described in more detail in this section.

TolC functions

TolC as a component of different type I secretion systems

Most operons coding for genes of type I secretion systems comprise the three genes for the ABC transporter, for the accessory protein, and for the OM component. As mentioned above, the *E. coli* genome is different. TolC is not linked to any export operon, but is part of the mar-sox regulon (Aono et al. 1998). Its involvement in the export process of *E. coli*

hemolysin was first described in 1990 (Wandersman and Delepelaire 1990). The hemolysin secretion system still serves as the prototype of the type I secretion system and was described in detail in "Protein secretion systems." A dimer of the ABC transporter HlyB forms a complex with the trimeric accessory protein HlyD, which monomers are anchored in the IM by a transmembrane helix. The other two genes of the hly-operon code for the allocrit HlyA, a member of the RTX toxins, as well as for the acyl transferase HlyC, which posttranslationally acylates the toxin.

The HlyBD/TolC secretion system is also able to secrete a number of other proteins, which are normally secreted by other type I export systems. The transport of colicin V of *E. coli*, adenylate cyclase toxin of *B. pertussis*, metalloprotease of *Erwinia chrysanthemi*, lipase of *P. fluorescens*, or alkaline protease from *P. aeruginosa* is partially not as effective as the hemolysin secretion, but it shows that the secretion through the HlyBD/TolC export apparatus is not only specific for hemolysin.

The *E. coli* colicin V secretion complex in the IM formed by CvaB (ABC-transporter) and CvaA (accessory protein) also employs TolC as an OM component (Hwang et al. 1997). This secretion apparatus belongs to the type I secretions systems, which depend on an amino-terminal secretion signal (Fath et al. 1994). The secretion signal belongs to the double-glycine-type leader peptides, which are cleaved concomitant with secretion. The proteolytic domain resides in the amino-terminal part of the ABC-transporter (Havarstein et al. 1995). As mentioned above, the secretion of another microcin, microcin J25, is also TolC-dependent. It is still not clear if the secretion apparatus consists only of the ABC transporter and the OM protein, because a gene coding for an accessory protein could not be found in the corresponding operon (Delgado et al. 1999). In the case of genes involved in production and secretion of microcin E492, originally found in a *Klebsiella pneumoniae* strain, the ABC transporter (MceG) was genetically linked to an accessory protein (MceH). Expressed in *E. coli*, it has been shown that TolC is necessary for successful secretion into the external environment (Lagos et al. 2001).

There are reports which show that TolC is also involved in the secretion of the heat-stable enterotoxins I, STB and Ip (STIp) in *E. coli* (Yamanaka et al. 1998; Foreman et al. 1995; Okamoto et al. 2001; Yamanaka et al. 2001). Their secretion mechanism does not fit any of the described type I secretion systems. It is shown that the enterotoxins are transported secA-dependent into the periplasm concomitant with cleavage of the 18–23-residue-long amino-terminal signal peptide. In the periplasm, the proteins are processed by DsbA to form intramolecular disulfide bonds (STII and STB) or are further cleaved (enterotoxin I). The mature protein is then transported across the OM. This step involves TolC. In the absence of TolC, the proteins accumulate in the periplasmic space, which is atypical for the type I secretion system (Yamanaka et al. 1998). It remains unclear how the proteins cross the outer membrane considering that TolC might only be functional in conjunction with an ABC transporter and a corresponding accessory protein.

The compatibility of TolC with IM complexes of secretion systems derived from other bacteria was tested. It has been shown that the *Serratia* and *Erwinia* metalloprotease PrtB, as well as hemophore HasA, was secreted by the IM HasDE complex in the presence of TolC. In contrast, when the ABC transporter (PrtD) and the accessory protein PrtE of *E. chrysanthemii* were introduced in *E. coli* to export the *Erwinia* metalloprotease PrtB, secretion was not observed, showing that this hybrid secretion apparatus is not functional (Binet and Wandersman 1996; Letoffe et al. 1993). Interestingly, a hybrid system assembled by the ABC transporter of *E. chrysanthemi* (PrtD), the accessory protein of *Serratia*

marcescens (HasE), and TolC of *E. coli* was able to secrete the *Erwinia* metalloprotease (Binet and Wandersman 1995).

TolC is part of different multidrug efflux pumps

Altogether, 29 drug transporters (two of the ABC superfamily, 18 of the MF, five of the SMR, and four of the RND) were identified in the *E. coli* genome by sequence comparison with established drug transporters (Saier et al. 1998). For assembly into a multidrug efflux pump, it needs a periplasmic accessory protein and an OM protein of the TolC family. In addition to TolC, there are genes of three other homologues (yjcP, yohG, and ylcB) found in the genome of *E. coli*. None of them are genetically linked to genes of IM transporters and the corresponding accessory proteins, which apart from one (acrD) are always combined in an operon (Blattner et al. 1997). The contribution of each of those OM proteins for the multidrug efflux was investigated by determination of the MIC of *E. coli* in which the genes were deleted. It was found that only the lack of TolC had a big impact on the susceptibility of the bacteria to most toxic compounds tested (Sulavik et al. 2001). The dominant role of TolC can be explained by the fact that the other homologues are not expressed under the conditions tested or that they fail to interact with the complex in the IM. The need for TolC is shown for different drug transporters. The most effective multidrug efflux pump is formed by AcrAB/TolC (Fralick 1996). Its broad spectrum of extruded allocrits ranges from diverse antibiotics and cationic dyes to organic solvents (Sulavik et al. 2001; Tsukagoshi and Aono 2000). AcrB is a transporter of the RND superfamily. It forms a complex with the accessory protein AcrA as shown by chemical cross-linking (Zgurskaya and Nikaido 2000). The accessory protein is homologous to the accessory proteins involved in type I secretion systems, but in contrast to them, it has no cytoplasmic and transmembrane domain but is anchored by an amino-terminal bound fatty acid in the IM (Johnson and Church 1999).

The second multidrug efflux pump which depends on TolC in the OM is EmrAB/TolC. Studies of the specificity of this exporter are performed in an acrAB mutant strain because of the dominant role and the broad specificity of the AcrAB/TolC efflux pump. Known allocrits of the EmrAB/TolC pump are carbonylcyanide m-chlorophenylhydrazone or nalidixic acid (Lomovskaya and Lewis 1992). The transporter EmrB belongs to the MF superfamily, which is generally more specific than transporters of the RND superfamily. The accessory protein EmrA shares similarities with the accessory protein of the RND transporter-based efflux pumps, but possesses an amino-terminal transmembrane helix which anchors it in the IM. The homologous proteins EmrK and EmrJ also code for an MF transporter and an accessory protein. When overexpressed it could be shown that they also mediate drug resistance. Although it is not experimentally proven, it is most likely they also depend on TolC in the OM (Nishino and Yamaguchi 2001). Recently, another drug transporter was described which employs TolC (Kobayashi et al. 2001). Proteins coded by the macAB operon (former ybjYZ) were identified to significantly increase the resistance to macrolides such as erythromycin (Kobayashi et al. 2001). MacA is homologous to ABC transporters and MacB is the corresponding accessory protein anchored in the IM by a transmembrane helix. This is the first report of an ABC-type drug efflux transporter, which assembles with an accessory protein and an OM protein to form a tripartite efflux pump.

Other roles of TolC

In addition to its role as outer membrane component of different type I protein secretion systems and multidrug efflux pumps, other phenotypes of TolC-deficient mutants show that this outer membrane protein is involved in other cellular processes.

Originally, TolC got its name from the finding that TolC-deficient mutants are tolerant of certain colicins (de Zwaig and Luria 1967). Various colicins, which act as weapons against other bacteria and thus help to compete and survive the battle for resources, are isolated to date. Their length varies between 300 and 700 amino acids. One member, colicin Ia, is fully crystallized and forms a largely α-helical, highly elongated shape of approximately 200 Å (Wiener et al. 1997). Because of sequence similarities, one can assume that other colicins have a similar overall shape. The molecules are divided in three functional domains. The receptor domain is responsible for binding at a receptor on the bacterial surface, the translocator domain is necessary for the third domain, the activity domain, to cross the outer membrane. The activity domain kills the cells either by forming pores in the cytoplasmic membrane or by nuclease activity or inhibition of protein synthesis in the cytoplasm (Lazdunski et al. 1998). How this translocation across the outer membrane works is not understood. In the case of colicin E1 and colicin10, it is known that the translocator domain interacts specifically with TolC and that this is a prerequisite for the translocation (de Zwaig and Luria 1967; Pilsl and Braun 1995). Other colicins interact with their translocator domain with other outer membrane proteins such as the outer membrane porin OmpF (Bourdineaud et al. 1990), which would suggest that the uptake process is not related to the special shape and function of TolC. Knowing the structure of colicin N and its translocator OmpF suggests a passage of the activity domain along the side of the β-barrel of the porin and not through the central pore (Vetter et al. 1998).

TolC also acts as cell-surface receptor for the TLS bacteriophage (German and Misra 2001). As proven for other phage receptor proteins such as LamB, it could be shown that extracellular domains are responsible for this interaction (Etz et al. 2001). Another TolC-deficient phenotype is the occurrence of anucleate cells (Hiraga et al. 1989). A possible role of TolC in cell division is also supported by the fact that its expression is downregulated sixfold if SeqA, a protein shown to be involved in sequestration during chromosome segregation, is missing (Bahloul et al. 1996). One might speculate that TolC is one of the contact sites for the replicative origins, which appear to be tightly bound to regions in the outer membrane and move apart when the cells grow and divide (Hendrickson et al. 1982). Other phenotypes of TolC-deficient mutants are changes in gene expression. The absence of TolC increases the expression of ProU (proline uptake) and decreases the expression of the general diffusion pore OmpF (Dorman at el. 1989; Morona and Reeves 1982). A direct influence of TolC in gene regulation must be excluded because of the local separation from the genes. TolC has an indirect effect and it seems that DNA supercoiling might be the clue for altered gene expression. The proU promoter is supercoiling-sensitive and for OmpF expression it is also reported to be regulated by changes in DNA supercoiling (Dorman et al. 1989). How TolC could influence the supercoiling state of DNA is unclear, but a possible direct contact site between TolC and the origin of replication may be a hint. Another explanation could be that TolC mutants respond differently to environmental conditions because of their altered membrane integrity and this may somehow affect the supercoiling state of the DNA.

The TolC structure

The TolC crystal

The first structural information on TolC was derived from 2D crystals in 1997 (Koronakis et al. 1997). With a resolution of 13 Å it could be shown that TolC has a trimeric structure and that it most likely forms a single pore rather then three. The electron microscopy also gave a hint for the existence of a periplasmic domain. The breakthrough came in 2000, when Koronakis and coworkers (2000) solved the structure by x-ray crystallography with a resolution of 2.1 Å (Koronakis et al. 2000). TolC had to be treated with V8 protease, which cleaved specifically the 43 carboxy-terminal residues, before the protein crystallized. The structure was solved by multiple wavelength anomalous dispersion using oxidized selenomethionine derivatives (Sharff et al. 2000). In the crystal, the TolC trimers are loosely packed, resulting in a relatively high solvent content of 70%. In the unit cell of the crystal, a pattern of three trimers coming together in a triangular arrangement was visible. The intermolecular contact points are located at the top and near the bottom of the cannon-shaped structure. It should be mentioned here that the residues involved are at sites that are highly variable throughout the TolC family. It is assumed that the crystallization of TolC homologues might not necessarily follow the same protocol used for TolC (Koronakis et al. 2001).

The overall structure

The TolC trimer is cannon-shaped with a long axis measuring 140 Å (Fig. 1A). One end of the cylinder is open and has an inner diameter of about 20 Å (Fig. 1B, top). The diameter is uniform for a length of 100 Å, then becomes smaller so that the other end of the body is almost closed (Fig. 1B, bottom). The interior is mostly solvent, filled with a volume of roughly 43,000 $Å^3$. The structure can be partitioned into three domains. The 40-Å-long β or channel domain, the 100-Å-long α-helical or tunnel domain and the mixed α/β domain or equatorial domain, which forms a “strap” around the midsection of the tunnel domain. The two domains forming the continuous tube stand behind the name of this new structure: the channel-tunnel. The folding of the peptide chain of a monomer is shown in Fig. 1C. Each monomer adds four β-strands (S1, S2, S4, and S5) to the channel domain; the tunnel domain is formed by two long (H3 and H7) and four shorter helices (H2, H4, H6, and H8) and the equatorial domain comprising the amino- and carboxy-terminus consists of small β-strand and α-helical structures (S3 and S6, and H1, H5, and H9, respectively).

The channel domain

The channel domain is that part of the structure which anchors the protein in the OM. It consists of 12 β-strands, four per monomer. The β-strands are arranged in an antiparallel way to form the barrel, which is right twisted, that is, if seen from the top; the top portion of the barrel is rotated anticlockwise in respect to the bottom portion. Apolar residues are located at the outside of the barrel facing the lipids, whereas polar residues are found at the inside. This leads to typical amphipathic β-strands, which were already correctly predicted before the structure was solved (Johnson and Church 1999). The β-strand must both

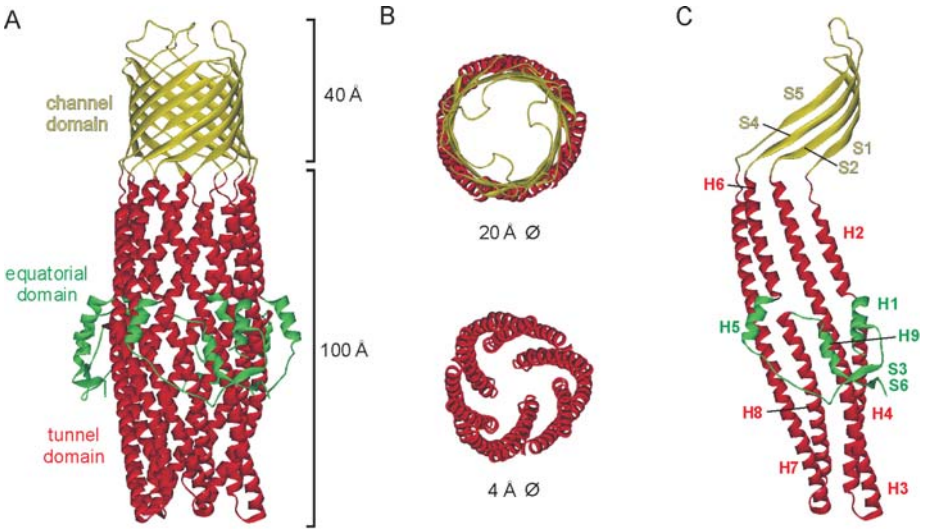

Fig. 1A–C The crystal structure of the TolC protein from *E. coli*. The protein consists of three domains colored in *yellow* (channel domain), *red* (tunnel domain), and *green* (equatorial domain). **A** Three TolC protomers are assembled into a canon-shaped structure termed the channel-tunnel. **B** Top view of the part above (*top*) and below (*bottom*) the equatorial domain. The inner diameter of the β- and α-barrel is uniform and measures approximately 20 Å. The coiled-coils below the equatorial domain taper and decrease the diameter to 4 Å at the tunnel entrance. **C** Structure of a TolC protomer. The secondary structural elements are labeled accordingly Koronakis et al. (2000). The figure was generated by using WEBLABVIEWER (Accelrys, Cambridge UK)

curve and twist to form the barrel. Small or unbranched side chains facing the interior allow a dense packing to accommodate the curvature. At the base of the channel domain are aromatic side chains, particularly tyrosine and phenylalanine, forming a ring facing outwards. Aromatic side chains are known to localize at the membrane-water interface (Killian and von Heijne 2000). Thus, the aromatic ring anchors the barrel domain in the OM, delimiting the inner edge of the lipid bilayer. A common parameter to characterize β-barrels is, in addition to the number of β-strands, the shear number. It is defined as the number of the residues encountered when traveling along a strand before re-encountering the same vector oriented perpendicular to the strand axis (Liu 1998). Because the 11–14 residue-long β-strands of TolC are not sufficient to form one turn around the barrel axis, the shear number is an extrapolation. The shear number for the β-barrel of TolC is 22.

The strands S1 and S2, and S4 and S5 respectively enclose 8- and 21-residue-long loops, which are located at the cell surface. The high crystallographic thermal disorder factors and the weaker electron density in this region correspond with a high flexibility of the loops. It is unlikely that they form a stable barrier, restricting the channel entrance from the extracellular side. It has been shown that residues within the loops are responsible for the interaction of TolC with TLS bacteriophage and colicin E1, which uses TolC as OM receptor and translocator into the periplasmic space, respectively (German and Misra 2001).

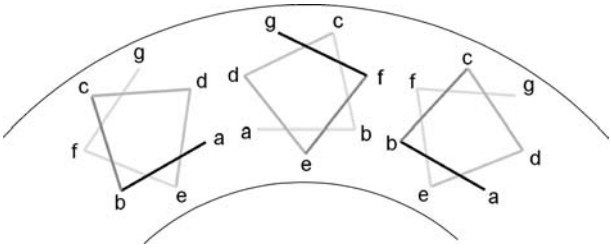

Fig. 2 Schematic presentation of three adjacent antiparallel helices of the α-helical barrel. Residues of the heptadic repeat have been labeled in sequence as *a*–*g*. For explanation, see text

The tunnel domain

The tunnel domain is the 100 Å-long extension of the β-barrel into the periplasmic space. It consists entirely of α-helices. Structurally, it is divided into two parts. Above the equatorial domain the helices assemble into an α-barrel; below the equatorial domain the helices form conventional coiled-coils. The α-barrel of TolC is a structure which is not found in any other crystallized protein. Twelve helices (H2, H3, H6, and H7 of each monomer) arrange antiparallel to form an almost uniform cylinder with an inner diameter of approximately 20 Å (Calladine et al. 2001). The axes of the helices are inclined at approximately 20° relative to the molecule axis. The helices are not straight, but are bent so that they do not move off tangentially from the curved surface of the cylinder. To maintain this structure the helices must adjust structurally. Firstly, they must bend in a curve, and secondly they must untwist. The local packing of the helices is similar to a conventional coiled-coil, that is, knobs-into-holes-interaction (Crick 1953). However, in the α-barrel the helices do not wrap around each other, but form a cylinder. In this assembly, one helix has two interfaces, resulting in two sequence patterns that are phased to match the two contact sites. The sequences of the parts of the molecule, which form the α-barrel, have a specific type of heptadic repeat. In the heptadic repeat a–g residues at positions a and d form one interface, residues at positions b and f the other (Fig. 2). Residues located at position e line the inside, residues at positions c and g the outside of the tunnel wall. A statistical analysis of the corresponding amino acids in heptadic repeats of TolC and TolC homologues show that at positions c, g, and e polar residues with long side chains (Glu, Asp, Gln, Asn, His, Arg, and Lys) are the most frequent amino acids. At positions a, b, and d are residues with small, not β-branched side chains (Ala, Ser, Gly, and Cys), and nonpolar, bulky residues (Leu, Met, Trp, Phe, Tyr, Thr, Ile, and Val) are found at position f. The distribution of the different amino acids influences the twisting and bending of the helix. The α-barrel structure requires a compression of the interior interface, which is achieved by small side chains at positions a and b, and an expansion of the exterior interface achieved by more bulky residues, especially at position f. Residues at positions b and d fit directly into the corresponding hole, while the residues at positions a and f are inclined with respect to the corresponding holes. Thus, the contacts of residues at positions a and f tend to compensate for the inclination by favoring residues with side chains that can rotate into the hole presented by the partner helix (Calladine et al. 2001).

The α-barrel structure finishes with the equatorial domain, where the small helices H2 and H6 end. Below the equatorial domain, the long helices H3 and H7 pair with helices H4 and H8. The long helix H3 is straight, below the equatorial domain, and helix H4 coils around it. This pair forms the outer coiled-coil. The other pair H7/H8 forms a conventional coiled-coil bending inwards. This inner coiled-coil is responsible for the tapering of the

periplasmic end of TolC. Seen from the top, the coiled-coils arrange like an iris to almost seal the periplasmic entrance (Fig. 1B, bottom).

TolC homologues

TolC homologues are found in almost all Gram-negative bacteria (for a list, see Sharff et al. 2001). This confirms the importance of this outer membrane protein for the cells. A phylogenetic tree based on a sequence alignment of 36 TolC homologues shows that the channel-tunnel family splits into three subfamilies (Andersen et al. 2000). Sequence similarity correlates with the export processes in which they are involved. Thus, there are the protein secretion, the drug efflux, and the cation efflux subfamilies. One known exception is TolC from *E. coli*. It groups into the protein secretion subfamily, but as mentioned above, it is also functional as OM component of multidrug efflux pumps. The reason that TolC is a multifunctional protein may be that its gene does not belong to any export operon. As a member of the mar-sox regulon, its basal expression is probably constitutive and so channel-tunnel-dependent export systems would not need an OM component for functionality.

The overall length of members of the channel-tunnel family varies between 414 and 541 residues. This is due to variable extensions at the amino- and carboxy-terminus. Gaps or insertions exist only in the extracellular loops or the equatorial domain. High variation within loop regions is also found in families of other outer membrane proteins, for example, the porin family (Koebnik et al. 2000). This is understandable because this part of the structure is the most flexible and its folding is not important for maintenance of its functionality. Variation in the loop structure is also helpful for escaping the immune system of a host organism. Changes in functionally or structurally important regions within a structure are poorly tolerated. Within the channel-tunnel family, it is striking that the length of the tunnel forming helices is constant. The long helices (H3 and H7) consist of 67 residues, the four shorter helices of 23 (H2 and H6) and 34 residues, respectively (H4 and H8). Experiments performed with OprM of *P. aeruginosa* showed that insertions or deletions within these regions lead to unstable proteins (Li and Poole 2001; Wong et al. 2001). The length of the helices is clearly determined because residues, which are important at the transitions between the different parts of the structure, are highly conserved. These are glycine residues between the coiled-coils of the lower tunnel domain, which are necessary for the turn between the helices H3 and H4, and H7 and H8, respectively. The transitions between the α-helices of the tunnel domain and the β-strands of the channel domain are characterized by highly conserved proline and glycine residues. Nearby are conserved aromatic residues, forming the aromatic ring around the bottom of the channel domain, delimiting the inner edge of the outer membrane. That all members of the channel-tunnel-family have a tapered periplasmic ending can be concluded from the conservation of small residues such as alanines and serines at the coiled-coil regions at the bottom part of the tunnel. They allow a dense packing of the coiled-coils, which determines the tapering and entrance closure. Other conserved residues are aspartates, which line the narrowest part of the periplasmic entrance and have strong influence on the electrophysiological behavior of channel-tunnels (see “Biophysical characterization of channel-tunnel proteins”). Their role in the functionality of the channel-tunnels is discussed below.

A characteristic of channel-tunnels belonging to the drug efflux subfamily is a conserved cysteine residue at the amino-terminus of the mature protein. It becomes acylated

and serves as a membrane anchor. Studies with mutants of OprM of *P. aeruginosa* show that it is not needed for function (Li and Poole 2001; Yoneyama et al. 2000).

Channel-tunnels are evolved by gene duplication

The TolC structure comprises an approximate structural repeat, which is evident in the figure of TolC monomer (Fig. 1C). The two halves are linked by parts of the equatorial domain. The structural repeat corresponds to a repeat in the primary structure. Without knowing the structure of TolC, this was first recognized for CyaE, the TolC homologue of *B. pertussis* (Gross 1995) and then further investigated for the whole channel-tunnel family (Johnson and Church 1999). These observations suggest that TolC and its relatives evolved by gene duplication from a common ancestor. It is remarkable that when comparing the primary sequence of the amino- and carboxy-terminal halves of the family members the strongest identity (29.1%) is found for CyaE. CyaE is also nearest to the root of the phylogenetic tree, suggesting that it is closest to the family progenitor (Andersen et al. 2001).

Folding and assembly of TolC

The TolC protomers are transported by the sec-system into the periplasmic space, where folding and assembly takes place. Three protomers are necessary to assemble into a functional unit. Little is known about the folding and trimerization process for TolC or another channel-tunnel. Because of the totally different structure of TolC compared to other OM proteins (see below), one can assume that the process is different. One hint is, for example, that all OM porins have at their carboxy-terminal end an aromatic amino acid, which has a big impact on folding and assembly (Struyve et al. 1991). In TolC or homologues such a conserved residue at the carboxy-terminus is missing. The theory as to how the assembly of the structure occurs is only speculative. It is known for OprM, a TolC-homologue from *P. aeruginosa*, that it refolds and assembles into trimers in vitro (Charbonnier et al. 2001). Therefore, self-assembly of channel-tunnels in the absence of a lipid bilayer and without any helper proteins seems possible. Only with a correctly folded monomer can the following trimerization be successful. The correct folding of the monomers is achieved by various intramolecular interactions. The intrinsic tendency of β-strands forming right-handed twist is already mentioned above (Branden and Tooze 1999). In the case of the strands of the TolC β-barrel, the twist is further supported by the small hydrophilic side chains facing inwards and more bulky side chains facing outwards. The size of the tunnel forming helices delimited by glycines or helix breaking prolines is constant within the channel-tunnel family. Gaps or insertions are not found, suggesting that changes within the length of the helices would inhibit the correct assembly of the structure leading to degradation of the protomers in the periplasmic space. The helices interact by knobs into holes connections. These interactions are further supported by a number of salt bridges formed between residues at position e at the inside or at position c or g at the outside of the tunnel. During folding of the monomer, these electrochemical interactions may play a major role for correct pairing of the helices. The same could be true for the assembly of monomers into trimers. Most of the interface between monomers is provided by the contact of H7 with H2 and H4 of the adjacent monomer. Charged residues found beside the hydrophobic interface of the contact site could direct the monomers in a way that the knobs find the corre-

sponding hole to assemble in the right configuration. The long periplasmic extension of the TolC trimer evokes another question. How does TolC protrude through the peptidoglycan layer? It is possible that it needs activity of muramidases for hydrolyzing the peptidoglycan structure as it is shown for flagella assembly in *S. typhimurium* (Nambu et al. 1999). However, so far, nothing is known about any muramidases dependence for channel-tunnel assembly. A hint for potential interaction sites with the peptidoglycan layer may be a ring of arginine residues above the equatorial domain, which might form salt bridges with the peptidoglycan layer.

Comparison to other outer membrane proteins

The TolC channel-tunnel adds a remarkable structure to the solved structures of bacterial OM proteins known so far. They can be divided into five functionally and structurally different families. Each family is represented by a prototype from *E. coli*. OmpA and OmpX belong to the family of small β-barrel membrane anchors. OmpA is structurally important by providing a physical linkage between the OM and the peptidoglycan layer (Koebnik 1995); OmpX is important for virulence by neutralizing host defense mechanisms (Heffernan et al. 1994). They form an eight strand β-barrel and exist as monomers (Pautsch and Schulz 1998; Vogt and Schulz 1999). The outer membrane phospholipase A (OMPLA) belongs to the family of membrane integral enzymes. It normally exists as a monomer, but has the potential to dimerize, with its dimeric form being catalytically active (Dekker et al. 1997). It forms a 12-strand β-barrel with a half moon-shaped cross section (Snijder et al. 1999). The barrel interior of OmpA, OmpX, and OMPLA is too small to allow the passage of ions or substrates. Thus, they do not act as pore-forming proteins. Another monomeric protein is FhuA, a member of the family of TonB-dependent receptors. It is involved in the uptake of iron-siderophore complexes and is, with 22 β-strands, the biggest β-barrel known so far. The inside of the barrel is filled by an amino-terminal domain forming a plug. The remaining two families are porins, subdivided into general (e.g., OmpF) and substrate-specific porins (LamB), and channel-tunnels (TolC) (Cowan et al. 1992; Schirmer et al. 1995; Koronakis et al. 2000). They are pore-forming proteins, that is, they form water-filled structures, which allow the diffusion of hydrophilic substrates across the OM. Specific porins have a binding site for substrates such as carbohydrates or phosphate inside the channel, which allows a facilitated diffusion of these substrates into the periplasmic space (Benz et al. 1987). Both porins and channel-tunnels are trimeric, but their architecture is totally different. Porins form one barrel of 16 (general porins) or 18 (specific porins) β-strands per monomer so that the trimeric assembly contains three separate channels. In the case of channel-tunnels, each monomer contributes four β-strands to form a single barrel. The interior of the TolC β-barrel is also different from those of porin barrels. The cross sectional area of the TolC channel domain is 960 $Å^2$, making it 15-fold larger than that of the general porin OmpF. That is because TolC lacks the characteristic structural element of porins, an inward-folded loop that constricts the internal diameter of the porin β-barrel. Residues located at this loop have a strong influence on channel properties, like diameter, ion-selectivity, or substrate affinity (Saint et al. 1996; Bauer et al. 1989; Jordy et al. 1996). In porins, the inward-folded loop is also important to stabilizing the β-barrel. This stabilization is missing in channel-tunnels. Here, a stable channel domain is achieved by the elongation of the cylinder into the periplasm. The right-twisted β-barrel and the left twisted α-barrel might mutually stabilize each other so that the structure does not collapse. The periplasmic extension of channel-tunnels is the most striking difference compared to

all other OM proteins. More than 80% of the TolC peptide chain is located in the periplasmic space. OmpA, as well as the sucrose specific porin ScrY, also have periplasmic domains (Forst et al. 1998; Pautsch and Schulz 1998). Their structure could not been solved, but they are too small to form such an impressive assembly.

The common feature of all families, including the channel-tunnel TolC, is the β-barrel (Koebnik et al. 2000; Buchanan 1999). To date there is no OM protein known containing a transmembrane helix. A possible explanation for this phenomenon is that a hydrophobic helix would remain in the IM when secreted (MacIntyre et al. 1988; Pugsley 1993).

Model for the channel-tunnel-dependent export

The dimension of the periplasmic space

Allocrits of the channel-tunnel-dependent export systems are secreted without periplasmic intermediates. Therefore, previous models already demanded a proteinaceous pathway through the periplasmic space, enabling the secretion in one step directly from the cytosol into the external environment (Johnson and Church 1999; Koronakis et al. 1997; Nikaido 1998). The periplasmic tunnel-domain of TolC now enlightens how 100 Å of the intermembrane space is bridged. However, is the periplasmic end of the tunnel already in close contact to the IM complex, or is there still some distance between the exit from the IM transporter and the entrance of the tunnel? If so, how big is the periplasmic space? Various studies with different invasive techniques have been performed and dimensions between 70 Å and 500 Å were proposed (Van Wielink and Duine 1990; Graham et al. 1991; Dubochet et al. 1983; Hobot et al. 1984). The most accurate size was determined by freeze-substitution, a cryotechnique which preserves the ultrastructure of the object. A periplasmic size ranging from 106 Å to 143 Å in width were observed in *E. coli*, *Aeromonas salmonicida*, *Campylobacter fetus*, *Proteus mirabilis*, *P. putida*, *H. pleuropneumoniae*, and *P. multocida* (Graham et al. 1991). The recently solved structure of AcrB, the IM transporter of the RND type, makes an indirect determination of the dimension of the periplasmic space possible. The periplasmic domain measures 70 Å (Murakami et al. 2002). Assuming a direct contact between the transporter and the tunnel domain of TolC, the minimal size of the periplasmic space is 170 Å. One should consider that the dimension of the periplasmic space is not necessarily uniform and constant. There can be local regions smaller or wider in size. It is also possible that size varies in response to external conditions. It might be that changes of the osmolarity cause expansion or contraction, which could make it impossible for the export systems to assemble properly. Therefore, this dynamic could have a regulatory function for secretion and efflux.

Allocrit binding

Allocrits of the channel-tunnel-dependent export systems are either proteins, all sorts of drugs, or cations. The transporter proteins are responsible for allocrit specificity. The initial step of every export process is the binding of the allocrit to the transporter.

Allocrit binding of protein transporters

There are two distinct type I protein secretion systems differing in the type of secretion signal of the allocrits. In one case, the secretions signal is located at the carboxy-terminal end and is uncleaved, in the other case it is analogous to the GSP located at the amino-terminus and is cleaved during secretion. It has been shown that the carboxy-terminal secretion signal is sufficient to direct proteins to the secretion apparatus. Hybrid constructs with the secretion signal fused to the carboxy-terminus of various proteins were successfully secreted by the corresponding export systems (Bingle et al. 1997; Gentschev et al. 1996; Hahn et al. 1998; Palacios et al. 2001). Various studies have been performed investigating the carboxy-terminal secretion signal and how it interacts with the inner membrane transporter complex (Stanley et al. 1991; Zhang et al. 1993; Ghigo and Wandersman 1994; Duong et al. 1996; Omori et al. 2001; Hui and Ling 2002). The primary sequence similarity between carboxy-terminal secretion signals of different allocrits is low. However, three conserved features of secondary structure can be found in RTX toxins and proteases: an extreme carboxy-terminal eight-residue hydrophobic region, proximal to this a 13-amino acid uncharged sequence, and proximal to both these features a 22-residue amphipathic α-helical domain (Stanley et al. 1991). Mutations in any of these regions affected the secretion efficiency.

Signal peptides have been subjected to CD and NMR spectra and shown to be highly flexible and unstructured in aqueous solution and in mimetic environments (Izadi-Pruneyre 1999; Delepelaire and Wandersmann 1998; Zhang et al. 1995; Wolff et al. 1994). Therefore, it is still unclear how interaction with the IM complex occurs. Cross-linking experiments with the most studied hemolysin secretion system of *E. coli* revealed that hemolysin interacts before secretion with the transporter HlyB and the accessory protein HlyD (Thanabalu et al. 1998). The binding site at the accessory protein is located at the 60-residue-long cytoplasmic domain, which is essential for secretion (Pimenta et al. 1999; Balakrishnan et al. 2001). Allocrit binding triggers a conformational change in the periplasmic domain of the accessory protein (Thanabalu et al. 1998; Balakrishnan et al. 2001). This means that there is a signal transduction from the cytosol into the periplasmic space, which is possibly mediated by the transmembrane helix. Detailed studies have shown that this amino-terminal domain consists of a potential amphipathic helix linked by a cluster of charged amino acids to the transmembrane helix of the accessory protein. Both the amphipathic helix and the charged cluster interact with hemolysin. While deletion of the amphipathic helix leads to a reduction of hemolysin secretion, it becomes completely abolished when the charged cluster is missing (Balakrishnan et al. 2001). The necessity of binding to the cytoplasmic part of the accessory protein could only be shown for the hemolysin secretion system and can not be generalized for all type I secretion systems. Recent results show that the transporter HlyB interacts with the secretion signal at the nucleotide-binding domain (L. Schmitt, personal communication). A potential interaction of the secretion signal with other parts of the transporter exhibits a study screening for mutants of the transporter HlyB, which complement the repressed secretion by a lack of 29 residues in the hemolysin secretion signal. It revealed fifteen HlyB point mutations, all located in the membrane domain of the transporter (Zhang et al. 1993). The fact that point mutations are able to compensate for drastic changes in the HlyA secretion signal suggests that substrate specificity of transporters may shift dramatically during evolution and may account for the diversity of substrates observed for the ABC transporter superfamily.

Experiments with hybrid secretion systems might suppose that there exist transporters which recognize a broader range of secretion signals and transporters which are more specific. It was shown that the hemolysin secretion system (HlyBD/TolC) is also able to secrete, for example, colicin V (CvaC), protease (PrtB) from *E. chrysanthemi*, or a nodulation factor (NodO) of *Rhizobium leguminosarum* (Fath et al. 1991; Delepelaire and Wandersman 1990; Scheu et al. 1992; see Table 2). In contrast, the natural export systems for colicin V (CvaAB/TolC) and for the *E. chrysanthemi* protease (PrtDEF) were not able to secrete hemolysin (Fath et al. 1991). The LipBCD exporter from *S. marcescens* and the *Caulobacter crescetus* S-layer protein secretion system RsaDEF seem also to be more general secretion systems. The Lip-system secretes the *Serratia* lipase and metalloprotease (LipA and PrtB), an S-layer protein SlaA, as well as the heme acquisition proteins from *P. aeruginosa* and *P. fluorescens* (Kawai et al. 1998; Omori et al. 2001). The Rsa-system enables the export of its natural substrate, an S-layer protein RsaA, as well as the *P. aeruginosa* alkaline protease AprA and the *E. chrysanthemi* metalloprotease (Awram and Smit 1998).

In the second type of type I secretion systems, the secretion signal is located at the amino-terminus of the allocrit. It is different from the leader peptides recognized by sec-system of the GSP and is characterized by a double-glycine motive, termed the double-glycine leader peptide. It is cleaved directly upstream of the glycine residues during secretion. The proteolytic function resides in the 150 amino-terminal residues of the transporter (Havarstein et al. 1995). Thus, the transporter has a dual function: in addition to the translocation of the allocrit across the IM, it is also involved in maturation of the allocrit by removing the leader peptide. This extra domain has not been found in most of the ABC transporters of the carboxy-terminal signal-dependent type I systems. One exception is HlyB and this could be a hint for HlyB also being proteolytically active. Another hint is that the hemolysin secretion system is capable of secreting colicin V, an allocrit with a double-glycine leader peptide (Fath et al. 1991). Indeed, it has been shown that an export signal in colicin V recognized by HlyBD is localized at the amino-terminal 57 residues (Fath et al. 1991).

The folding state of type I allocrits in the cytosol is unclear. They have to be at least partly unfolded to fit through the export machinery. There is strong evidence that the secretion of the heme-binding protein HasA from *S. marcescens* is dependent on the SecB chaperon, which prevents folding, allowing the export in a partly or completely unfolded state (Delepelaire and Wandersman 1998). However, this is possibly a specific case, because hemolysin and protease secretion are SecB-independent (Blight and Holland 1994). There might be other unidentified chaperons which are involved in secretion. This has to be clarified in the future.

Allocrit binding of drug transporters

Some of the efflux pumps exhibit a very wide allocrit specificity covering practically all antibiotics, chemotherapeutic agents, dyes, and detergents. It is still unclear how the drug transporter recognizes such a wide variety of substances. Generally, one can say that transporters of the RND superfamily are less specific than those of the MF superfamily. Allocrits of the AcrAB/TolC efflux pump of *E. coli* have structures that cover a wide range. Many of them carry a net negative charge, but there are also components with positive charges, such as the cationic dyes and erythromycin (Sulavik et al. 2001). What should be

noted is that very hydrophilic compounds such as aminoglycosides are not pumped out by this RND-type transporter. All allocrits seem to contain hydrophobic domains of significant size, which could result in a partial membrane insertion. It is supposed that this is the general characteristic for allocrits of the transporter. It binds any molecule that is at least partially inserted into the membrane bilayer but do not appear like the usual phospholipid (Nikaido 1996). It remains open, if the RND transporter can bind allocrits inserted in both leaflets of the IM. It could be shown for the AcrAB/TolC efflux pump of *S. thyphimurium* that it excretes even those drugs that fail to traverse the cytoplasmic membrane. Accordingly, the transporter must be able to bind molecules, which are inserted in the outer leaflet of the IM. It is speculated that the RND transporter has a flippinase-like activity, originally shown in the mouse multidrug-resistant protein MDR2, that flips over the phosphatidylcholine from the inner leaflet to the outer leaflet (Ruetz and Gros 1994). Drugs, which insert in the cytoplasmic side of the membrane, would be flipped over to the outer leaflet to bind at the binding site of the transporter (Nikaido et al. 1998). The topological models of RND transporters revealed charged amino acids in the membrane-spanning helices. In MexB, the multidrug transporter of the MexAB/OprM efflux pump of *P. aeruginosa*, there are five charged residues found in the twelve transmembrane helices. Substitution of the two charges in transmembrane segment (TMS) 2 had no effect on function, but changes of the two residues in TMS 4 and one charge in TMS10 had substantial effect on the drug resistance of the bacteria. It is supposed that residues in TMS4 and TMS10 form a charged network that is important for proton translocation and/or energy coupling (Guan and Nakae 2001). Similar results were obtained for the homologous multidrug transporter MexF from *P. aeruginosa* (Aires et al. 2002). The recently solved structure of AcrB, the RND transporter of *E. coli*, does not clarify the mechanism by which drugs are bound and secreted into the channel-tunnel (Murakami et al. 2002). Experiments with chimeric constructs of the two homologous drug transporters AcrD and AcrB could show that the large periplasmic loops are responsible for the specificity of the transporter, suggesting that the binding site is located in this part of the molecule (Elkins and Nikaido 2002).

Transporters of the cation efflux pumps also belong to the RND superfamily. For CzcA from *Ralstonia* sp. CH34 mediating heavy metal resistance, it could be shown that three negatively charged residues in TMS4 were absolutely required for a functional transporter. In a working model, the authors propose that binding of Zn^{2+} to the cytoplasmic metal-binding site triggers proton transport into the cytoplasm. This leads to an electronegative potential at the periplasmic end of the transporter driving the zinc cations through the cation channel. Exchange against protons at the periplasmic side releases the zinc cations from the transporter (Goldberg et al. 1999).

The *E. coli* multidrug transporter MdfA belongs to the MF superfamily and exports lipophilic cationic drugs. It possesses a single acidic residue in its putative TMS1, which provides drug selectivity (Edgar and Bibi 1999). The substitution for a basic residue abolishes transport of positively charged ethidium bromide but not uncharged chloramphenicol. Furthermore, many multidrug transporters belonging to the MF superfamily possess acidic residues at a similar position in TMS1, suggesting that they utilize a similar mechanism of drug selectivity (Zheleznova et al. 2000). In addition, in a member of the SMR superfamily, a single negatively charged residue in the first membrane-spanning helix was found to influence the drug efflux of these transporters (Muth and Schuldiner 2000).

Nothing is known about any drug transporter of the ABC superfamily involved in efflux pumps. However, studies with the ABC drug transporter from the Gram-positive *Lactococcus lactis* LmrA have revealed that the homodimeric LmrA possesses two drug-bind-

ing sites: a transport-competent site on the cytosolic surface and a drug-release site on the extracellular surface of the membrane. Drug transport is mediated by an alternating two-site transport (two-cylinder engine) mechanism driven by the hydrolysis of ATP (van Veen et al. 2000). One can assume that this mechanism may also be relevant for the ABC transporters, which are part of multidrug efflux pumps.

Some understanding as to how the binding site can accommodate structurally unrelated ligands comes from studies of BmrR, a regulator of *B. subtilis*, which upon drug binding activates the expression of the multidrug transporter Bmr. Crystal structures of BmrR alone and complexed with a drug revealed a drug-induced unfolding and relocation of an α-helix, which exposes an internal drug-binding pocket. Drug binding is mediated via interactions with many hydrophobic residues and an electrostatic interaction between the positively charged drug and a negatively charged residue inside the pocket. Modeling studies suggest that the binding pocket can accommodate a range of structurally dissimilar cationic drugs (Zheleznova et al. 1999). One can assume that the allocrit binding might operate similarly in drug transporters in the IM.

The role of the accessory protein

The family of the accessory proteins were previously named membrane fusion proteins. Without knowing the structure of the OM channel-tunnel, previous models of the type I secretion system and multidrug efflux pumps allocated the role of the accessory protein to form the bridge between the inner and outer membrane or to reduce the distance between the two membranes by pulling them together (Pimenta et al. 1996; Johnson and Church 1999). Even after the structure of TolC was solved, it was proposed that the accessory protein acts by pulling the IM and OM together so that the transporter can inject the allocrit directly into the channel-tunnel (Borges-Walmsley and Walmsley 2001). No study has ever shown that parts of the accessory protein inserts in the OM. The name membrane fusion protein was based on weak similarities to a protein of paramyxovirus involved virus penetration, hemolysis, and cell fusion (Dinh et al. 1994). Using this term for the accessory proteins implies a wrong function and should not be used anymore for this protein family. The role of the accessory protein is to link the IM transporter to the OM channel-tunnel and to stabilize this assembly. Furthermore, it has to induce a conformational change in the tunnel-domain of the channel-tunnel, which opens the entrance, allowing allocrits to enter the tunnel. Therefore, the accessory protein can be regarded as a dynamic adapter between the channel-tunnel and the energy-providing, allocrit-binding IM transporter (Fig. 3).

Accessory protein models

The channel-tunnel-dependent export systems are composed of three proteins. The structure of the OM component, the channel-tunnel, is solved. Recently, also the structure of the RND transporter AcrB was determined (Murakami et al. 2002). Structures of the other diverse IM transporters are not available except for the cytoplasmic nucleotide-binding domain of HlyB and two homologous ABC transporters (Kranitz et al. 2002; Chang and Roth 2001; Locher et al. 2002). However, there exist reasonably good topological models. The structure of the third component, the accessory protein, is still a mystery. Cross-linking

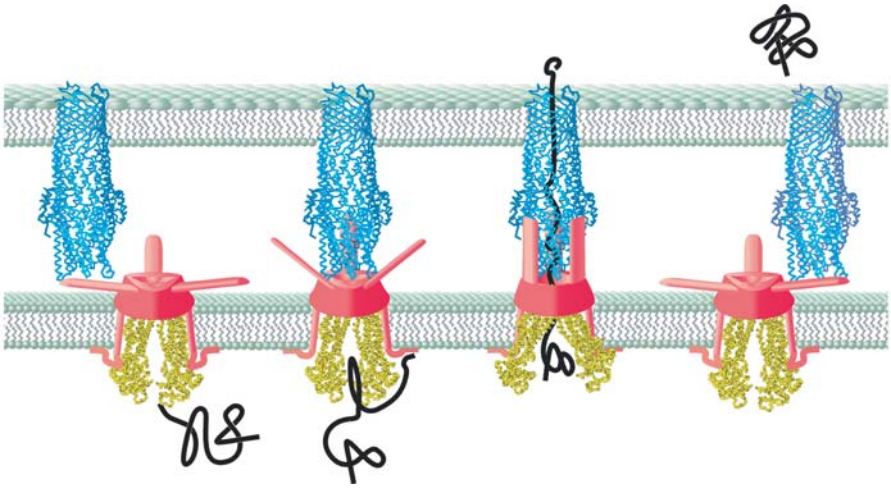

Fig. 3 Model of the type I protein secretion system. The channel-tunnel (*blue*) is anchored by its channel domain in the outer membrane. The accessory protein (*red*) and the ABC transporter (*yellow*) forming a complex in the inner membrane. The allocrit (*black*) binds to the transporter and the cytoplasmic part of the accessory protein and induces a conformational change in the periplasmic domain of the accessory protein, allowing the recruitment of the channel-tunnel. A tight tripartite complex is formed, the tunnel entrance of the channel-tunnel is opened and the allocrit is secreted. After the process is finished the proteins return into their resting states. For an animated model of the type I secretion system please go to: http://archive.bmn.-com/supp/ceb/ani1.html

experiments could show that accessory proteins assemble into trimers (Thanabalu et al. 1998; Zgurskaya and Nikaido 2000). They are located with their main part in the periplasmic space, forming a stable complex with the IM transport protein. With the amino-terminal end, they are anchored in the IM either by a transmembrane helix or by a lipid anchor. Accessory proteins complexed with transporters of the ABC and MF superfamilies have a transmembrane helix, which connects the periplasmic domain with a small cytoplasmic domain. Accessory proteins interacting with RND transporters possess a lipid anchor. Mutant experiments could show that the covalently bound fatty acid is not required for a functional efflux pump (Zgurskaya and Nikaido 1999a; Yoneyama et al. 2000). Major structural studies were performed with AcrA, the accessory protein of the *E. coli* multidrug efflux pump AcrAB/TolC. Analytical ultracentrifugation and dynamic light scattering have shown that AcrA exists in solution as a highly asymmetric molecule with an axial ratio of eight and a predicted length of 170 Å. The elongated shape of the protein is taken as a hint that it is able to span the periplasmic space and fulfill the role of membrane fusion protein (Zgurskaya and Nikaido 1999b). The AcrA protein used in this study was not in its natural configuration as trimers but was monomeric. Therefore, it is possible that the elongated structure is the result of a misfolding of the protein. Another study of the same protein using lipid-layer crystallization revealed a preliminary three-dimensional structure (Avila-Sakar et al. 2001). The protein was organized as a ring-like structure with a central opening of 30 Å. Two different models are presented modeling the protein architecture. The length of the protein within each model is consistent with the elongated structure of the monomer. The oligomeric state of AcrA in this study is dimeric, which leaves again doubts if the structure corresponds with the native structure.

Structure predictions have appointed different domains in the accessory proteins (Johnson and Church 1999). Every member of the accessory family has a central domain predicted to form coiled-coils. It consists of two regions with high coiled-coil probability separated by a gap of 5–10 residues. It is predicted that the coiled-coil domain forms an α-helical hairpin. This is confirmed by the fact that the two regions are of approximately equal length (4–5 heptad repeats) and that among different accessory proteins the length of the regions frequently differ by integers of seven. The helical content of accessory proteins leads to models predicting an α-barrel structure similar to that of the OM component. Surrounding the bottom part of the tunnel, it would seal the pathway from the transporter into the channel-tunnel (Sharff et al. 2001; Lewis 2000).

The coiled-coil domain is flanked by motifs found in domains of enzymes that transfer covalently attached lipoyl or biotinyl moieties between enzymatic components (Neuwald et al. 1997). In these proteins, the two ~30-amino-acid-long motifs consist of four β-strands, which assemble into a so-called lipoyl domain, as shown in several structures solved by X-ray crystallography or NMR (Dardel et al. 1993; Green et al. 1995; Athappilly and Hendrickson 1995). In these proteins, the two motifs are connected by a 3-amino-acid-long linker. In accessory proteins, the two motifs are separated by 60–300 amino acids. The motif at the amino-terminal end of the coiled-coils is close to the membrane anchor. If the hairpin model is right and the two motifs assemble to a lipoyl domain, this would mean that the carboxy-terminal end of the accessory protein is close to the IM. This is supported by an observation showing that mutations in the hemolysin transporter HlyB can be suppressed by a carboxy-terminal mutation of the accessory protein HlyD, suggesting a close contact between HlyB and the carboxy-terminus of HlyD (Schlör et al. 1997). Predictions of the secondary structure revealed a relatively high percentage of β-structure in the carboxy-terminal part of the accessory proteins. The importance of this part of the protein is shown by mutations that lead very often to instable or nonfunctional proteins (Hwang and Tai 1999; Schülein et al. 1994).

It is still unclear how the accessory protein assembles into trimers and what trimers must look like. Assuming that the IM transporter has a kind of channel and releases the allocrit at the periplasmic surface, one has to demand that the accessory protein also forms a kind of channel to bridge the gap between the transporter and the channel-tunnel for guiding the allocrit into the tunnel entrance. Therefore, the accessory proteins have to assemble into a ring-like structure. Assuming that the coiled-coil domains form a hairpin structure which is not involved in trimerization, it is the carboxy-terminal part of the accessory protein which trimerizes and forms a ring-like structure (Fig. 3). This would also explain why mutations in the extreme carboxy-terminal part have such a dramatic effect on the secretion. Future research will reveal if this model is correct.

Accessory protein – channel-tunnel interaction

The interaction between the IM complex formed by the transporter and the accessory protein with the OM channel-tunnel is necessary to assemble a functional export complex. For the hemolysin system, it is shown that without the OM component there is no secretion in the periplasmic space (Gray et al. 1989; Koronakis et al. 1989). In addition, other protein secretion systems provide evidence that translocation across the IM is disabled if the OM is not present. For multidrug efflux it is not shown in vivo that the drug transporter in the IM is only active when the drug efflux pump is correctly assembled. The absence of

the OM component makes the cells more susceptible to drugs, but this effect could be explained by a less efficient drug efflux if exported just into the periplasmic space compared to an export across two membranes (Nikaido 1996; Thanassi et al. 1995). Using proteoliposomes, it could be shown that the AcrB transporter was active in vitro and increased its transport rate in the presence of the accessory protein AcrA (Zgurskaya and Nikaido 1999a). Cross-linking experiments have proven a close interaction between the accessory protein and the channel-tunnel for the hemolysin and the colicin V secretion system of *E. coli* (Thanabalu et al. 1998; Hwang et al. 1997). In contrast, attempts to cross-link TolC with the IM complex AcrAB of the multidrug efflux pump failed (Zgurskaya and Nikaido 2000). This could be a hint that the assembly is not so stable, possibly due to small contact sites. It is known for the hemolysin secretion that the assembly between the IM complex HlyBD and TolC is only temporal (Thanabalu et al. 1998). After the secretion is finished, the secretion apparatus disassemble and TolC is released from the IM complex (Fig. 3).

Studies of the stability of the components in the presence or absence of the interacting partners provide another clue about the interaction between the three components of the export systems. Systematic studies were performed with the hemolysin secretion system (Thanabalu et al. 1998; Pimenta et al. 1999). They show that the IM transporter HlyB becomes unstable in the absence of the accessory protein HlyD independently of the OM component TolC. The accessory protein HlyD is most stable if both partners are present. Surprisingly, it is more stable if both partners are absent than if HlyB is present. This means that HlyB is a determinant of HlyD and provides evidence for at least two topological or organizational states of the accessory protein, depending on the presence or absence of the transporter. The stability of the complete secretion apparatus was tested in the presence and absence of the allocrit. It shows that the complex becomes more susceptible to proteases if hemolysin is present, meaning that there are conformational changes when the export machinery becomes active.

The interaction between the components of the colicin export system CvaAB/TolC was shown by similar experiments. The ABC transporter CvaB was less stable in CvaA-deficient strains than in TolC-deficient strains and the accessory protein CvaA was more unstable when TolC was absent then in the absence of CvaB (Hwang et al. 1997).

Another possibility to get information about the interaction between the accessory protein and the channel-tunnel is to study hybrid exporter systems. TolC, the channel-tunnel of *E. coli*, is capable of successfully interacting with various IM complexes for protein secretion and drug efflux (see Table 2). The *S. marcescens* channel-tunnel HasF complements some of the TolC phenotypes, including drug and detergent sensitivities and hemolysin secretion. This shows that it is also able to interact with the AcrAB and HlyBD complexes. One TolC phenotype missing in HasF-containing cells is the sensitivity to colicin E1, which means that HasF can not serve as translocator in colicin E1 uptake. Conversely, TolC can complement HasF in the HasDEF secretion apparatus (Binet and Wandersman 1996). Other secretion systems such as the lipase secretion systems of *P. fluorescens* (AprDEF) or *S. marcescens* (LipBCD) and the metalloprotease secretion system of *E. chrysanthemi* (PrtDEF) were not functional if TolC replaced the OM channel-tunnels (Akatsuka et al. 1995; Duong et al. 1994). Also, the OM components of these systems, AprF and PrtF, can not replace TolC in hemolysin and colicin V secretion systems (Wandersman and Delepelaire 1990; Binet and Wandersman 1996). In contrast, channel-tunnels of *S. typhimurium*, *S. typhi*, *Shigella flexneri*, and *V. cholera* were able to complement TolC in the hemolysin secretion system (Spreng et al. 1999). Pair-wise swapping of heterologous type I exporter subunits showed that the various components are not universally interchange-

able. Only components of protein secretion systems were combined in these hybrid systems. As already mentioned, the channel-tunnel family has three subfamilies corresponding to the export system in which they are involved. Hybrid experiments pairing protein secretion complexes and channel-tunnels of the drug efflux family have not yet been reported. Like TolC, which interacts with diverse IM complexes in *E. coli*, there is another example, which interacts naturally with two types of IM complexes for drug efflux. The channel-tunnel MtrE from *N. gonorrhoe* is not only functional in combination with the IM MtrCD complex comprising a transporter of the RND superfamily, but also in combination with the IM complex FarAB with FarB belonging to the MF superfamily (Lee and Shafer 1999). The knowledge of compatibilities of channel-tunnels with different accessory proteins can be a clue to find sequence patterns, which are common in channel-tunnels, that can replace each other. Another possibility is to look for common features in the sequence of the different accessory proteins, which specifies them for interaction with certain channel-tunnels. To date, no such sequence patterns have been found either in channel-tunnels or in accessory proteins. Thus, it is possible that the interaction probably relies on conformation rather than a specific amino acid sequence.

One can speculate about the potential interaction sites between the channel-tunnel and the accessory protein. One function of the accessory protein is to contact the channel-tunnel. A second function is to induce a conformational change in the tunnel domain to open it, allowing export of allocrits. There are suggestions that the equatorial domain of the channel-tunnel acts as a lever that moves the coiled-coils of the bottom portion of the channel-tunnel and dilates the closed end (Koronakis et al. 2000). It may be contacted by the potential hairpin structures of the accessory proteins. Recently, we discovered that important residues, which keep the tunnel in a closed conformation, are located close to the tunnel entrance (see “Biophysical characterization of channel-tunnel proteins”). They are accessible and may be contacted by parts of the accessory protein leading to a tunnel opening. Electrophysiological experiments have shown that the open state of the channel-tunnel is unstable and it is suggested that the potential coiled-coils of the accessory protein assemble with the coiled-coils of the tunnel domain to stabilize the open configuration (Andersen et al. 2002a).

In this context it has to be mentioned that the secretion of enterotoxins is also TolC-dependent (Yamanaka et al. 1998; Okamoto et al. 2001). Interestingly, enterotoxins are translocated across the IM by the GSP. Thus, it must be possible that periplasmic intermediates enter the channel-tunnel in an accessory protein-independent manner. A recent study shows that the 60 carboxy-terminal residues of TolC, which are part of the equatorial domain, are necessary for this process (Yamanaka et al. 2001).

Accessory proteins in Gram-positive bacteria

A surprising finding was the existence of accessory proteins in Gram-positive bacteria. They are genetically linked to an ABC transporter and are involved in the secretion of nonlantibiotics. Examples include the *Lactobacillus sake* SapTE complex for secretion of the sakacin, the *L. lactis* LncCD complex for secretion of lactococcin and the *Enterococcus faecium* EntTD complex for secretion of enterocin (Axelsson and Holck 1995; Varcamonti et al. 2001; O’Keeffe et al. 1999). The bacteriocins secreted by these transporters possess an amino-terminal signal peptide of the double-glycine leader sequence type, which is cleaved during secretion. Therefore, they are closely related to the bacteriocin-se-

creting systems of Gram-negative bacteria. The role of the accessory proteins is unclear. There is no OM protein, which has to be recruited. Therefore, accessory proteins must have an additional function. The closely related lantibiotics, which are also recognized by a double-glycine leader sequence, are exported by a single ABC transporter without an accessory protein. One explanation for the existence of accessory proteins in Gram-positive bacteria may be that the cytoplasmic part of the accessory protein plays an important role in binding of the nonlantibiotics before secretion analogous to the hemolysin secretion system. Another explanation is based on the different mode of action and the accompanying immunity systems associated with the two classes of bacteriocins (Baba and Schneewind 1998; Saris et al. 1996). Lantibiotics are proteolytically activated after secretion and acts by forming pores in cytoplasmic membranes of target cells without any need for a membrane-associated receptor. In contrast, nonlantibiotics are processed during their transport and rely on a membrane-bound receptor in the target cell for acting as a pore forming protein. The mode by which the bacteriocin-secreting cells protect themselves against the exported bacteriocin is different. The producer of lantibiotics have another ABC transporter that recognizes and expels the lantibiotics inserted in the membrane so that they can not accumulate in lethal concentrations, a mechanism resembling that of multidrug transporters. The immunity of nonlantibiotic secreting cells is not very well understood. They secrete an immunity protein, which inhibits the pore-forming action either by binding to the receptor or by blocking the pore. It is speculated that the accessory protein also plays a protective role by directing the activated nonlantibiotic away from the cytoplasmic membrane to prevent it from binding to the receptor and damaging its own cell (Young and Holland 1999).

Driving forces for the transport

The main energy source is of course the electrochemical potential at the IM. It is directly used by sym-, anti, or uniporters for translocation of allocrits by transporters of the RND, MF, or SMR superfamily or indirectly by synthesis of ATP, the substrate for ABC transporters. Not much is known about the direct impact of the electric potential of the IM on the translocation of allocrits. What argues against a major role is that drug transporters are able to expel positively as well as negatively charged allocrits (Nikaido 1998; Sulavik et al. 2001). In the hemolysin secretion system, two energetically distinct stages were found (Koronakis et al. 1991). An early stage depends on the electrochemical potential. Secretion was severely inhibited by the addition of an uncoupler. It is speculated that this reflects the binding of hemolysin to the transporter complex. A late stage of the secretion was identified which does not require an electrochemical potential. This process might be driven by ATP hydrolysis, but it is also possible that it is an energetically favorable transfer requiring no additional energy. The role of ATP hydrolysis was evaluated further by a HlyB mutant that binds ATP but can not hydrolyze it. It had the effect that hemolysin was not exported, but instead accumulated in the assembled IM-OM export complex. This suggests that release of the allocrit into the recruited TolC channel-tunnel is dependent upon ATP hydrolysis (Thanabalu et al. 1998).

Proteins exported by the type I secretion system can be over 1,700 residues long. It is questionable if the IM transporter works steadily to push the long peptide chain into the extracellular space. A clue for this problem may be the repeats that bind calcium to fold into a compact structure. This calcium binding at the surface concomitant with immediate folding of the protein may contribute to the translocation by pulling the remaining peptide

chain out of the export apparatus. It should be noted that similar refolding steps involving calcium appear to be implicated in secretion of subtilisin from *B. subtilis* and also in the secretion of several proteins into the endoplasmatic reticulum (Petit-Glatron et al. 1993; Nigam et al. 1994). RTX-repeats are found in various big allocrits of the type I secretion system, for example, the S-layer proteins RsaA and SlaA of *C. crescetus* and *S. marcescens*, the *B. pertussis* adenylate cyclase toxin CyaA, or the *E. coli* hemolysin HlyA (Coote 1992; Kawai et al. 1998; Sebo and Ladant 1993). This assumption is confirmed by experiments showing that in some cases the RTX repeats appear to be required for efficient secretion through the transport apparatus, especially for large, heterologous proteins (Baumann et al. 1993; Letoffe and Wandersman 1992; Duong et al. 1996). However, it has to be mentioned that these nonapeptide RTX repeats are not found in all proteins secreted by the type I secretion system. However, a proposed novel heptapeptide repeat is found in exopolysaccharid glycanases (PlyA and PlyB) secreted by *Rhizobium leguminosarum* (Finnie et al. 1998). Other cell-associated exopolysaccaride-processing enzymes of *Rhizobium*, *Sphingomonas*, and *Azorhizobium* species also contain these novel heptapeptide repeats, possibly defining a new set of type I allocrits (York and Walker 1997; Finnie et al. 1998).

Formation of disulfide bonds

Disulfide bonds formation is catalyzed by thiol:disulphide oxidoreductase DsbA (Bardwell 1994). The enzyme is located in the periplasmic space and this poses the question as to how proteins secreted by the type I secretion machinery form disulphide bonds without contacting the periplasmic space. Previously, the existence of disulfide bonds in secreted colicin V was taken as a hint that the protein has to pass the periplasmic space. In a recent study, the disulphide bond formation of allocrits of the type I system was investigated in detail using the hemolysin secretion system and, as a reporter protein, a hybrid containing the hemolysin secretion signal and an antibody single chain fragment with two disulphide bonds enclosed (Fernandez and De Lorenzo 2001). It shows that the periplasmic DsbA protein was not involved in disulfide formation. Deletion of cytoplasmic thioredoxins (TrxA and TrxC), which keep cysteine-containing proteins in their reduced state, also had no influence on the secretion. However, a reduced secretion was observed if the thioredoxin reductase (TrxB) was mutated, resulting in an accumulation of oxidized thioredoxins. This suggests that oxidized thioredoxins can promote a premature oxidation of the allocrit. Similarly, the formation of colicin V disulphide bonds may occur already in the cytoplasm. Furthermore, it shows that the type I system tolerates secretion of disulfide-containing proteins. Nevertheless, there must be a size restriction for the exported allocrits. Secretion of hybrids containing the carboxy-terminal secretion signal of the PrtB protease fused to diverse eukaryotic proteins by the *E. chrysanthemi* protease secretion system was dependent on the presence of disulfide bonds in the allocrits. Allocrits with disulfide bonds such as human erythropoietin or trout growth hormone were not secreted, while disulphide bond free proteins such as greenfluorescence protein from *Aequorea victoria* or endochitinase from *Trichoderma harzianum* were successfully exported (Palacios et al. 2001). This is another hint that disulfide bond formation can occur prior to secretion. An explanation of the inhibited secretion of proteins containing disulfide bonds may be that the type I channel is too narrow to permit the export of proteins with secondary structures stabilized by disulfide bonds.

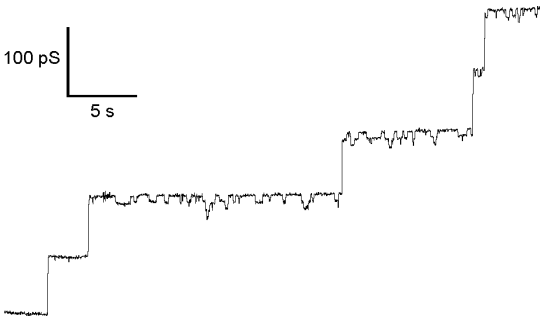

Fig. 4 Trace of a single-channel conductance measurement of TolC performed in black lipid bilayer. The membrane was formed by diphytanoyl-phosphatidylcholine/n-decane. The electrolyte is 1 M KCl, pH 7.5, the membrane potential −80 mV

Biophysical characterization of channel-tunnel proteins

The pore-forming ability of TolC was first described by Benz and coworkers in 1993 (Benz et al. 1993). TolC formed stable pores in planar lipid bilayer and single pores adopted up to three substates, which leads to the assumption that this trimeric protein has a porin-like structure (Fig. 4). This was disproved by solving the highly different structure of TolC (see "Comparison to other outer membrane proteins"). The most obvious difference is the 100-Å-long periplasmic extension. At the end of this tunnel domain is the narrowest part of the conduit. Thus, the restriction zone is not in the membrane-embedded channel domain like in porins, but 100 Å apart at the periplasmic opening. The asymmetric structure of TolC leads to a totally directed insertion into artificial membranes. It always inserts with the channel domain first, which makes it possible to interpret channel characteristics in respect to its orientation in the membrane. The new architecture of this pore-forming protein is reflected in the electrophysiological behavior of TolC.

TolC forms small cation selective pores

The single channel conductance (*Gsc*) of TolC under standard conditions (20 mV, 1 M KCl) is approximately 85 pS. It is more than 20-fold smaller than that of the general diffusion pore OmpF (Bauer et al. 1989). The cross-sectional area of the membrane-embedded β-barrel of TolC is 15-fold bigger than that of OmpF, but the 20-Å-wide inner diameter decreases towards the periplasmic entrance, which has an inner circular diameter of almost 4 Å. The narrow entrance explains the relatively low *Gsc*. Additionally, one has to consider that due to the distance to the membrane, the electric field across the tunnel entrance might be weaker than that across a porin channel, which has no extramembraneous extension. There are no experimental or theoretical data available which can quantify the electric field at this position, because it clearly depends on the unknown electrical properties of the tunnel wall. Measurements of the ion-selectivity show that the TolC channel-tunnel is cation-selective with a 16.5-fold preference of potassium ions over chloride ions (Andersen et al. 2002b). This is in agreement with most of the pore-forming proteins of the outer membrane of *E. coli*, which are also cation-selective (Benz 1994). It might be a general feature to protect the cells from uptake of negatively charged, harmful substances such as bile salts found in the environment of enteric bacteria. It is known that charged residues lining the channel interior are responsible for ion selectivity. The properties of the channel-tunnel interior show that the lower tunnel domain is highly electronegative

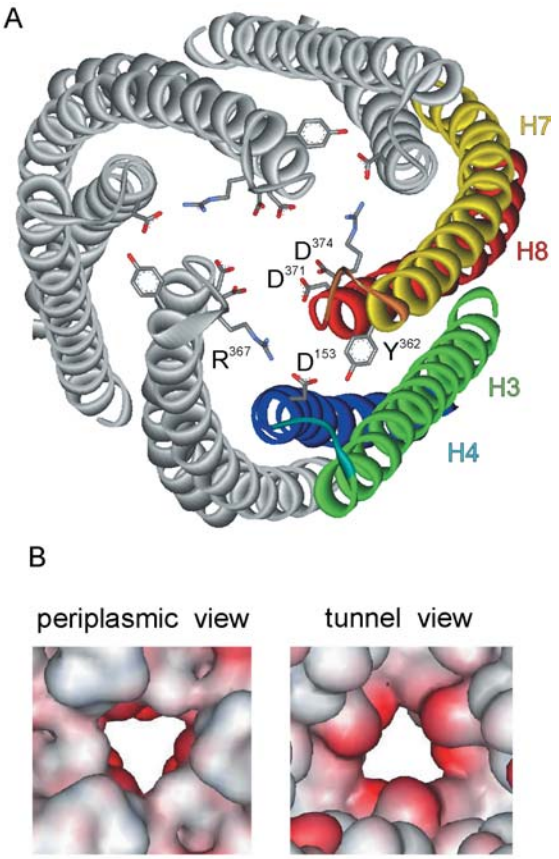

Fig. 5A The periplasmic entrance seen from the periplasmic side. One monomer is *colored*. Helices forming the outer coiled-coil are shown in *green* (H3) and *blue* (H4), helices forming the inner coiled coil are *yellow* (H7) and *red* (H8). Residues establishing the circular network (D153, Y362, and R367), which keeps the tunnel in a closed confirmation are shown in detail. Aspartates (D371 and D374) lining the tunnel entrance are also presented. **B** Surface representation of the periplasmic entrance seen form the periplasmic space and from the tunnel inside. The electronegative surface (*red*) of the aspartate residues is clearly visible. The figure was generated by using WEBLABVIEWER (Accelrys, Cambridge UK)

(Koronakis et al. 2000). The molecular basis is several aspartate residues located at this part of the tunnel. Particularly, the periplasmic entrance is lined by a ring of six aspartate residues (two per monomer, Fig. 5). The substitution of these residues for alanines clearly proved their role in ion selectivity. The resulting mutant TolC^{DADA} is anion-selective with a tenfold preference of chloride ions over potassium ions (Andersen et al. 2002c). Investigating the electrolyte concentration dependence of *Gsc* establishes another effect of the negative charges at the tunnel entrance (Fig. 6). The *Gsc* of TolC^{DADA} shows, in the range up to 1M KCl, a linear dependence on the electrolyte concentration. Such a linear correlation signifies that there is no ion-binding site inside the channel. Despite a smaller tunnel entrance, *Gsc* of TolC^{WT} is higher than that of TolC^{DADA} at small electrolyte concentrations. This behavior shows that the aspartates at the periplasmic entrance form an ion-binding site inside the molecule leading to ion accumulation and higher ion concentration inside the tunnel compared to the bulk phase, resulting in a higher *Gsc* of TolC^{WT}. At high electrolyte concentration, the influence of the charges becomes smaller and *Gsc* reflects the maximal ion flux through the channel-tunnel. The *Gsc* of TolC^{DADA} saturates at a higher level than TolC^{WT} because of the wider tunnel entrance.

Another characteristic of the TolC channel-tunnel is the pH-induced closure. A similar behavior is known from other porins of *E. coli*, leading to the assumption that it is an im-

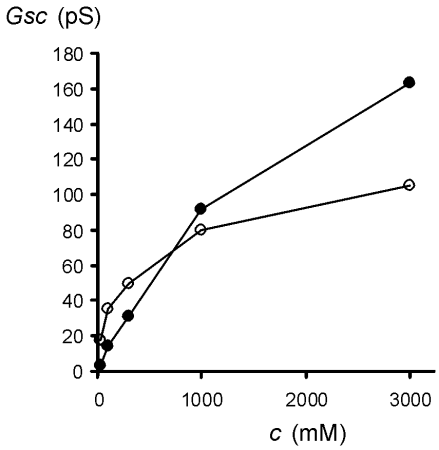

Fig. 6 Concentration dependence of the single-channel conductance of $TolC^{WT}$ (*open circles*) and $TolC^{DADA}$ (*closed circles*). The membrane was formed by diphytanoyl-phosphatidylcholine/n-decane. The electrolyte is KCl varying from 0.03 M to 3 M. The membrane potential is −60 mV

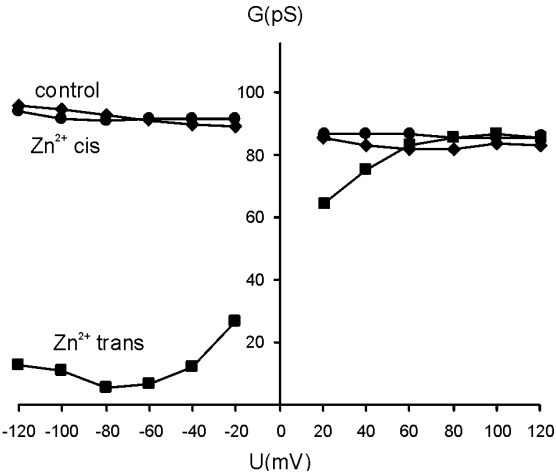

Fig. 7 Voltage dependence of the single-channel conductance of TolC in the absence (*diamonds*) and presence of 800µM $ZnCl_2$. Addition to the cis or periplasmic side had no effect (*circles*). Addition to the trans- or extracellular side of the membrane leads to a drastic decrease of the conductance when the membrane potential is negative (*squares*). For further explanation, see text. The electrolyte is 1 M KCl, pH 7.5

mediate response of the bacteria to protect the cell against sudden acidification of the environment (Andersen et al. 2002d). $TolC^{DADA}$ did not exhibit any pH dependence, which shows that the aspartate residues are responsible for the closing process.

Blocking of TolC by di- and trivalent cations

The ion-binding site at the tunnel entrance was further characterized by its ability to bind di-and trivalent cations. The potassium ion flux through TolC could be blocked when di- and trivalent cations were added to the side of the membrane, which corresponds to the "extracellular" side. Addition to the "periplasmic" side had no effect (Fig. 7). This means that the binding site for these cations is not accessible from the periplasmic side. The hydrated radii of the tested cations were all above 2 Å, too big to enter the tunnel through the periplasmic entrance with its cross-section below 4 Å. It could be shown that the binding of the cations is reversible and the binding constant was in the range between 460 M^{-1}

(Ca^{2+}) and 130,000 M^{-1} (Tb^{3+}) measured in 1 M KCl. The binding constant was dependent on two parameters. Firstly, it was dependent on the applied potential. Potential had to be negative on the periplasmic side to see the most effective binding. In this setup, the cations are pulled into the channel-tunnel. However, an inhibition of the potassium flux was observed also at small positive potential, which pulls the cations apart from the binding site, showing that di- and trivalent cations don't simply block the tunnel opening but bind to the binding site (Fig. 7). Secondly, the binding was dependent on the potassium chloride concentration. The higher the electrolyte concentration the smaller the binding constant. This shows that there is competition between the potassium ions and the di- and trivalent cations for the binding site. For one tested cation, the binding characteristics were different. Chromium ions block the potassium flux through the channel-tunnel not only when added to the extracellular side but also when added to the periplasmic side. This can be explained by the small hydrated radius of chromium ions. It is approximately 1.98 Å and thus they can enter the tunnel entrance and reach the binding site from the periplasmic side. Another difference compared to the other tested cations is that it binds irreversibly. Neither chelating substances nor high potentials pulling the ions apart from the binding site were able to abolish the binding. The $TolC^{DADA}$ mutant could not be blocked by large cations or anions, which is evidence that the six aspartates at the tunnel entrance form the binding site for cations in $TolC^{WT}$.

Opening of the TolC tunnel entrance

Exported proteins or drugs have to pass through the channel-tunnel. The narrow periplasmic entrance is too small to allow passage of the allocrits and therefore it has to be opened. Electrophysiological measurements have shown that the closed configuration is very stable. Experiments to open the tunnel entrance by high salt concentrations, high potentials, or urea failed. Bonds at the tunnel entrance were identified, which connects the coiled-coils of the tunnel domain. An aspartate residue at the outer coiled-coil (D153) forms an intramolecular hydrogen bond with a tyrosine residue (Y362) located at the inner coiled-coil of the same monomer. Additionally, the aspartate residue also forms an intermolecular salt bridge with an arginine residue (R367) of the adjacent monomer. All together, these links form a circular network, which keeps the tunnel in the closed confirmation (Fig. 5A). Disruption of the connections by substituting tyrosine for phenylalanine (YF) and arginine for serine (RS) leads to the opening of the tunnel entrance. The single mutations $TolC^{YF}$ and $TolC^{RS}$ lead to an increase of *Gsc* by a factor of 1.3 and 2.5, respectively, while the double mutation $TolC^{YFRS}$ had a synergistic effect and increased the conductance tenfold (Fig. 8). The voltage dependence of the single mutations is remarkable. It reflects the funnel-shaped architecture and its flexibility. Increasing negative potential results in an increase in *Gsc*. This behavior might illustrate the progressive opening of the entrance under the pressure of the high cation flux towards the periplasmic side. The reversed cation flux at positive potential is constant, representing the resting state of the conformation. The single channel recordings of the double-mutant $TolC^{YFRS}$ show that the open configuration of the channel-tunnel is very unstable. Various substates could be observed, which might reflect the collapse of coiled-coils into the tunnel interior (Fig. 9).

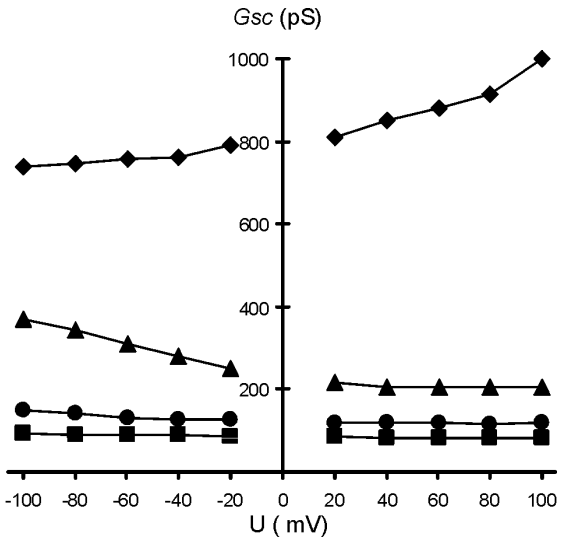

Fig. 8 Voltage dependence of single-channel conductance TolC^{WT}(*squares*), TolC^{YF} (*circles*), TolC^{R5} (*triangles*), and TolC^{YFRS} (*diamonds*) measured in 1 M KCl, pH 7.5. For further explanations, see text

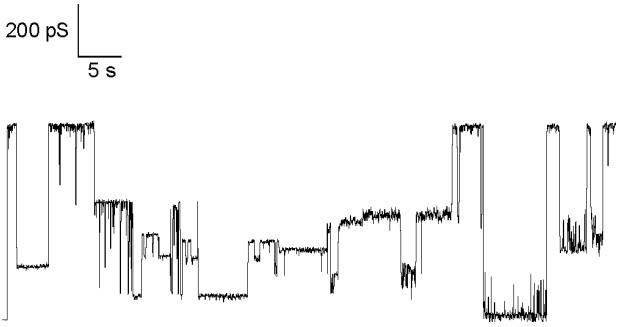

Fig. 9 Trace of the single channel conductance measurement of the TolC mutant TolC^{YFRS}. The open configuration of the pore is not stable and reversible switching into diverse substates could be observed. The electrolyte is 1 M KCl, pH 7.5, the membrane potential is −80 mV

Biotechnological and pharmaceutical relevance of channel-tunnel dependent export systems

Applications of the hemolysin secretion system

It has been shown that hybrid proteins are successfully exported by the type I secretion system. Diverse proteins could be fused to the amino-terminus of the secretion signal and were exported to the extracellular medium. The secretion of heterologous proteins via the hemolysin secretion system has mainly been applied to immunological and vaccine research. Because the hemolysin signal sequence represents a very weak antigen for B-cells and T-cells, this system is excellently suited for the presentation of secreted antigens in attenuated Gram-negative bacterial live vaccines (Gentschev et al. 1996). Examples of exported antigens from bacteria are listeriolysin and superoxid dismutase of *Listeria mono-*

cytogenes, Shiga toxin of *Shigella* species, toxin A of *Clostridium difficile*, diphtheria toxin of *Corynebacterium diphteriae*, 30-kDa antigen from *Mycobacterium bovis* BCG, and ESAT-6 of *M. tuberculosis* (Hess et al. 1996; 1997; Tzschaschel et al. 1996; Ryan et al. 1997; Orr et al. 1999; Hess et al. 2000; Mollenkopf et al. 2001). Hybrid proteins were also constructed with proteins from parasites such as the surface protein 2 from *Plasmodium falciparum* and with nucleocapsid protein from measles virus (Gomez-Duarte et al. 2001; Spreng et al. 2000a). The export via the hemolysin secretion system has advantages over other approaches. The epitopes do not remain in the cytoplasm like in most bacterial systems for the presentation of recombinant antigens, which need disintegration of the bacteria to become accessible for the host immune system. Furthermore, it is possible to secret even large heterologous antigens. Other systems which display epitopes inserted in surface proteins are restricted in size and only small peptides of the antigen can be used (Hormaeche and Khan 1996).

The effective secretion of fusion protein is also a useful tool for production of monoclonal and polyclonal antibodies (Mollenkopf et al. 1996) and can be applied for detection of protective antigens of pathogenic bacteria (Spreng et al. 2000b).

The multidrug efflux pumps as target for drugs

The emergence of resistance to the major classes of antibacterial agents is recognized as a serious public health concern. One cause for these resistances are the multidrug efflux pumps. Mutants lacking one of the three components of the multidrug efflux pumps have a higher antibiotic susceptibility compared to the wild type. The strategy to design drugs which put the pumps out of action is promising in the battle against resistant strains. In 1999, a broad spectrum inhibitor for the multidrug efflux pumps of *P. aeruginosa* was found that enhances the activity of fluoroquinolones (Renau et al. 1999). Inhibition of the efflux pump also reversed acquired resistance and decreased the frequency of emergence of *P. aeruginosa* strains that are highly resistant to fluoroquinolones (Lomovskaya et al. 2001). It is not known how the inhibitor inactivates the drug efflux pump, but it is supposed to inhibit the drug transporter in the IM. Knowing the structural basis of multidrug efflux pumps would make it possible to design drugs which deactivate the drug efflux apparatus. A possible target is the binding site of the drug transporter. In addition, the interaction between the accessory protein and the channel-tunnel could become disrupted by pharmaceuticals which could occupy the interaction sites and disable the assembly of a functional apparatus. Finally, the channel-tunnel itself could be blocked by substances which bind irreversibly and make an efflux of harmful substances impossible. The cation-binding site near the tunnel entrance could be a potential target for those substances.

Conclusion

Very rarely it happens that a protein structure illuminates and informs function and mechanism in a manner that is direct. The channel-tunnel structure of the multifunctional channel-tunnel TolC has big impact in understanding the export processes in which it is involved. Its conduit shape illustrates how proteins and drugs are exported in one step across the cell envelope of Gram-negative bacteria. The channel-tunnel-dependent export systems are widespread across the Gram-negative bacteria. The involvement in drug resis-

tance, in secretion of pathogenic factors, and also as a tool in vaccine development makes it an interesting system for further studies. The knowledge of the channel-tunnel structure helps to understand the mechanism of the export apparatus. By interaction with the IM transport complex, the tunnel entrance has to be opened, allowing export of the allocrits. In the near future, studies will focus on defining the sites of interaction with the accessory proteins and how this contact mediates opening. One can expect that the structure of members of the accessory protein family, as well as of other channel-tunnels and IM transporters, will be solved soon. This will help to understand the mechanism of the channel-tunnel-dependent export processes.

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