Functional and Phylogenetic Properties of the Pore-forming β-Barrel Transporters of the Omp85 Family

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β-Barrel-shaped channels of the Omp85 family are involved in the translocation or assembly of proteins of bacterial, mito-
chondrial, and plastidic outer membranes. We have compared these proteins to understand the evolutionary development of the translocators. We have demonstrated that the proteins from proteobacteria and mitochondria have a pore diameter that is at least five times smaller than found for the Omp85 in cyanobac-
teria and plastids. This finding can explain why Omp85 from cyanobacteria (but not the homologous protein from proteobac-
teria) was remodeled to become the protein translocation pore after endosymbiosis. Further, the pore-forming region of the Omp85 proteins is restricted to the C terminus. Based on a phy-
logenetic analysis we have shown that the pore-forming domain displays a different evolutionary relationship than the N-termi-
nal domain. In line with this, the affinity of the N-terminal domain to the C-terminal region of the Omp85 from plastids and cyanobacteria differs, even though the N-terminal domain is involved in gating of the pore in both groups. We have further shown that the N-terminal domain of nOmp85 takes part in homooligomerization. Thereby, the differences in the phylogeny of the two domains are explained by differ-
ent functional constraints acting on the regions. The pore-
forming domain, however, is further divided into two func-
tional regions, where the distal C terminus itself forms a di-
meric pore. Based on functional and phylogenetic analysis, we suggest an evolutionary scenario that explains the origin of the contemporary translocon.

Polypeptide transport and assembly of proteins into or across the outer membrane of endosymbiotic organelles or Gram-negative bacteria depend on β-barrel-shaped chan-
nels (1–4). One class of these proteins is composed of polypeptide-transporting β-barrel (PTB)6 channels, whose topology was determined by modeling (5–7). PTBs of recent interest are, e.g. outer membrane proteins (which secrete adhesins such as hemagglutininin) (8, 9) and bacterial (1, 7, 10–12), mitochondrial (Tob55/Sam50) (5, 13, 14), and chloro-
plast outer membrane proteins (Toc75) (15) of the Omp85 fam-
ily. The PTBs are partitioned into two functional categories, namely in translocation of precursor proteins across the mem-
brane and in the assembly of outer membrane proteins (3). Fur-
thermore, comparison between chloroplastic, mitochondrial, and bacterial Omp85 protein sequences revealed a high simi-
laruty of these PTBs (14, 16, 17).

The PTB Toc75 forms a complex with Toc34, Toc64, and Toc159 (3). A precursor protein-binding site at Toc75 (15, 18), together with the action of Toc159 (19), facilitates the translo-
cation of precursor proteins across the membrane. In contrast, the Omp85 proteins from Neisseria meningitidis, Escherichia coli, and mitochondria are involved in the assembly of outer membrane proteins (1, 5, 7, 11–14). As found for Toc75, the mitochondrial PTB is a component of a larger complex with Mas37 (20, 13) and Tob38/Sam35 (21, 22).

Recently, it was demonstrated that the C-terminal portion of both a bacterial and plastidic member of the PTBs forms the pore, whereas the N-terminal portion takes part in the recog-
nition of substrates or complex assembly (Fig. 1A) (18). This observation raised two important questions: (i) what is the functional and phylogenetic relationship between prokaryotic and eukaryotic PTBs and (ii) which evolutionary scenario may have led to the divergency resulting in proteins with different functions? Here, we present a detailed analysis of the domain assembly of PTBs, the electrophysiological characterization of the pore-forming region, and a phylogenetic analysis of func-
tional domains and of the full sequences from eukaryotic and

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prokaryotic members of the Omp85 family. Based on our results, we propose an evolutionary scenario that explains the possible origin of the PTB Omp85 family.

**MATERIALS AND METHODS**

**General**—The generation of cDNA constructs encoding for regions of nOmp85 or psToc75 (Fig. 1A), their expression, purification, and subsequent antibody production has been described elsewhere (18). The native gel electrophoresis was adopted from Ref. 23 and blue native-PAGE from Ref. 24.

**Cloning of YaeT and Sam50**—The open reading frame coding for *Drosophila melanogaster* Sam50 was amplified from cDNA derived from Schneider cells (American Type Culture Collection, CRL-1963) and cloned (BamHI-SmaI) into a derivative of the pRSET6d vector (Invitrogen) using the BamHI cloning site, placing the coding sequence under the control of the promoter encoding the full-length YaeT was amplified by PCR from genomic DNA of *E. coli* (XL1-Blue). The C-terminal truncated version of YaeT was produced by amplification of the DNA fragment corresponding to YaeT starting with the amino acid 473. Both DNA fragments were subsequently cloned into a derivative of the pRSET6d vector (Invitrogen) using the BamHI cloning site, placing the coding sequence under the control of the T7 promoter. The proteins were overexpressed in *E. coli* BL21(DE3)RIL cells (Stratagene, La Jolla, CA). The cells were lysed by sonication, and inclusion bodies were pelleted by centrifugation for 20 min at 20,000 revolutions/min. Inclusion bodies were washed with detergent-containing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 0.5% lauryl-(dimethyl)-aminoa- xide) and subsequently with a buffer without detergent (20 mM Tris, pH 8.0, 100 mM NaCl). The purified inclusion bodies were solubilized in a buffer containing 8 M urea, 100 mM Tris, pH 8.0, 0.1 M L-arginine, 20 mM Tris, pH 8.0, with heating to 70 °C.

**Chemical Cross-linking**—Outer membranes of vegetative cells from *Nostoc* sp. PCC7120 or proteins reconstituted into liposomes (18) were incubated with dithiobis(succinimidyl propionate) (DSP, Perbio, Bonn, Germany) in 50 mM NaPi, pH 8.0, and 100 mM NaCl. The reaction was quenched after 30 min (4 °C) by the addition of 25 mM glycine or 20 mM dithiothreitol. Cross-linking products were immunodecoated with antibodies.

**Electrophysiological Measurements**—Measurements were performed as described previously (18). To analyze the subconductances, the current distribution, at an indicated holding potential, was analyzed for a single channel, considering a time period of at least 2 s. The resulting distribution was analyzed by least square fits to one, two, three, or four gaussians for a non-filtered (shown) or low pass-filtered data set (not shown). The mean variance plots were calculated as described in Ref. 25.

**In Vitro Binding Analysis**—Liposomes were prepared with the lipid composition of the outer envelope of chloroplasts without monogalactosyl-diacylglycerol and proteins reconstituted as described previously (18) in 20 mM Hepes, pH 7.6, and 200 mM sucrose. nOmp85-N and psToc75-N (in vitro translated) using TNT/reticulocyte lysate (Promega, Mannheim, Germany) in the presence of [35S]Met was centrifuged at 250,000 × g for 15 min at 4 °C, and 5 μl of each was incubated with proteoliposomes for 10 min at room temperature in the presence of I mM methionine, loaded onto 1 ml of sucrose cushion, and centrifuged for 35 min at 50,000 × g at 4 °C. The pellet was resuspended and subjected to SDS-PAGE analysis.

**Phylogenetic Analysis**—Sequences of 213 putative transmembrane β-barrel-shaped polypeptide transporters with homology to Omp85 (17) were identified by BLAST (26) searches using the sequences of the proteins from *N. meningitidis*, *E. coli*, *Nostoc* sp. PCC7120, *Saccharomyces cerevisiae*, *Homo sapiens*, *Arabidopsis thaliana*, and *Pisum sativum* as bait. The relevance of the BLAST result was confirmed by a reciprocal BLAST analysis. Sequences were aligned with the program MAFFT, version 5.664 (27). The resulting multiple sequence alignment of the Omp85 proteins contains 2399 sites. From this alignment, we extracted three subalignments, e.g. the 160-amino-acid C-terminal region (distal C terminus), the 350-amino-acid C-terminal region (C terminus), and the remaining N-terminal region (N terminus), with corresponding sequence lengths of 767, 1269, and 1130 aa, respectively. For each alignment, including the full alignment, the IQPNNI program (28) was used to reconstruct a maximum likelihood phylogeny, assuming the WAG model (29) of amino acid substitutions with constant rates across sites. Moreover, we analyzed the four alignments by MrBayes (30). That is, for each alignment, we sampled 1,000,000 trees according to a Markov chain. After a burn-in of 300,000 trees, we sampled every 100th iteration to ensure independence of successive trees. These 7,000 trees were used to build a consensus tree to obtain support values of the branches by means of posterior probabilities.

**RESULTS**

**Omp85 from *Nostoc* sp. PCC7120 Forms Oligomeric Complexes in Vitro and in Vivo**—In *Nostoc* sp. PCC7120, three open reading frames encode for Omp85 PTBs. The ubiquitous Omp85 protein with 90 kDa is encoded by *alr2269* (16–18). In line with the suggested name for the bacterial proteins (1), we named the protein nOmp85 (*n* stands for *Nostoc*). The remaining reading frames encode two proteins with a molecular weight below 85 kDa and a yet unknown function. Similar to the observations for the proteobacterial Omp85 protein (11, 23, 32), the nOmp85 assembles into higher oligomeric complexes *in vivo*, as determined by non-denaturing SDS-PAGE (Fig. 1B, *lanes* 2 and 4) and chemical cross-linking (not shown). In line with this, a homodimerization of the N-terminal region was reported (18). We subsequently investigated whether purified nOmp85 forms oligomeric complexes *in vitro* after reconstitution into artificial membranes. The addition of (chemical) cross-linker to the reconstituted protein revealed two cross-link products (Fig. 1C, *lane* 2, *white* and *gray triangles*), suggesting the presence of at least homotrimERIC complexes. Interestingly, analyzing the pore-forming C terminus (18, nOmp85-C) by the same approach, only one stabilized assembly was obtained under any conditions tested. The cross-link product had a molecular weight that we expected for the dimeric complex (Fig. 1C, *lane* 4). The distal C-terminal sequence of nOmp85 (Fig. 1A, *distal* (D)) contains conserved regions and contributes the last eight membrane-inserted β-strands (6, 17, 18). The C-terminal domain, however, facilitates the transport...
The Omp85 Family

FIGURE 1. Complex formation of nOmp85. A, the constructs are indicated as bars (18). B, cell walls of vegetative cells of Nostoc sp. PCC7120 were analyzed by denaturing (2% SDS, 95 °C; lanes 1 and 3) or semidissociative (0.1% SDS, 4 °C; lanes 2 and 4) SDS-PAGE, stained with Coomassie Blue (lanes 1 and 2), or probed with nOmp85 antibodies. A black triangle marks the monomeric and the gray triangle the complexed nOmp85 species. C, liposomes containing nOmp85 (lanes 1 and 2), nOmp85-C (lanes 3–6), or nOmp85-D (lanes 7 and 8) were incubated with 300 μM dithiobis(succinimidyl propionate) (lanes 2, 4, 6, and 8) after the addition of nOmp85-N (lanes 5 and 6). After blocking, probes were separated by SDS-PAGE and probed with nOmp85 antibodies. Triangles mark the cross-link product, where black indicates the highest and white the smallest product found here and using other cross-linkers (not shown). D, nOmp85-C liposomes (bottom) were incubated with nOmp85-N (top), solubilized by 1% dodecylmaltoside, and subjected to blue native (BN)-PAGE. The first dimension was transferred to SDS-PAGE followed by silver staining (bottom) or immunodetection using nOmp85 antibodies. E, nOmp85-N (top) or psToc75-N (bottom, for both (lane 2); lane 1 shows 5% translation product) were incubated with liposomes (lane 2) containing nOmp85-C (top) or psToc75-C (bottom), and binding was determined as described under "Materials and Methods." Liposomes (lane 7) after reconstitution of C-terminal construct (lane 8) were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies against nOmp85 (top) or psToc75 (bottom). MW, molecular weight standard; WB, Western blot; SG, silver-stained gel.

of sucrose or NaCl (18). Analyzing the potential for complex formation of the distal C-terminal domain by cross-linking revealed a 60-kDa cross-linking product (Fig. 1C, lane 8), suggesting a tetrameric ensemble of the 15-kDa domain. This observation, on the one hand, confirms that the cross-linking time and cross-linker concentration was sufficient to stabilize assemblies larger than dimeric structures, an observation important for the interpretation of the results for nOmp85-C. On the other hand, this result suggests an oligomeric structure of the distal C terminus, which is expected, because the nOmp85-D transports large molecules (18), which cannot be explained by a pore formed by 8-transmembrane β-strands (31).

These results suggest that nOmp85 indeed forms larger complexes in native membranes, and this complex formation depends on the N-terminal domain. As far as we know, other protein components are not required for complex formation. However, it cannot be excluded that other proteins are involved in vivo to enhance the kinetics of the assembly or to stabilize the final complex. To further explore the influence of the N-terminal domain on oligomerization, the heterologous expressed N terminus (nOmp85-N) was added to liposomes containing nOmp85-C. Subsequent cross-linking experiments revealed products (Fig. 1C, lane 2 versus 6) not obtained while cross-linking nOmp85-C (Fig. 1C, lane 4) or nOmp85-N only (not shown). To confirm the complex formation between N- and C-terminal domains, liposomes containing nOmp85-C were incubated with the N-terminal construct, solubilized, and subjected to blue native-PAGE. Again, nOmp85-C and nOmp85-N co-migrated in a complex with an apparent molecular mass of 270 kDa (Fig. 1D, top). In the absence of the N-terminal domain, a migration of nOmp85-C at ~100 kDa was observed (Fig. 1D, bottom), which agrees with a dimeric ensemble. To further support the interaction of the N- and C-terminal domains, an analysis by in vitro binding of nOmp85-N to proteoliposomes carrying nOmp85-C (Fig. 1E, top) followed. A strong interaction of the N terminus to these liposomes (Fig. 1E, lanes 4–6) but not to “empty” liposomes (lane 2) was observed. Therefore, the N-terminal portion of nOmp85 has an affinity for the C-terminal portion of the protein. Thus, the formation of the homotrimeric structure of nOmp85 requires the interaction of the pore (C terminus) and the N terminus.

Finally, the interaction of the N-terminal domain of PTB Toc75, located in chloroplasts, to its C-terminal portion was investigated. Strikingly, no interaction between these two domains was observed by blue native-PAGE, cross-linking (not shown) or liposome titration (Fig. 1E, bottom). Thus, the N-terminal domain of Toc75 has a drastically reduced affinity to its C terminus. This observation is surprising, because it was suggested that Omp85 from Nostoc sp. PCC7120 and Toc75 is homologous and traceable back to a common ancestor (17, 18).

Channel Properties of nOmp85—The results obtained so far suggest an influence of the N terminus on the complex formation of the PTB. To explore an influence of the interaction between the N- and the C-terminal domains on channel properties, the electrophysiological activity of the C-terminal pore-forming domain (nOmp85-C) was analyzed both in the absence and presence of nOmp85-N. The pore-forming region of the Nostoc PTB exhibits no subconductance states, and the current is very stable both in the open and closed state (Fig. 2A, left). At 100 mV, the open state has a current of ~57 pA. After the addition of nOmp85-N, the current trace of nOmp85-C becomes very active (Fig. 2B, left). This activation is specific, because the addition of similar amounts of nOmp85-N to membranes containing psToc75-C did not alter the current profile (not shown). Therefore, the observed activity reflects the influence of the N terminus on the pore properties. Similarly active current traces were obtained for the full-length protein (18). Thus, the electrophysiological results corroborate the tight interaction of the N- and C-terminal portions as shown (Fig. 1). In-depth analysis of the current distribution of nOmp85-C after the addition of nOmp85-N reveals three prominent opened states at 31, 42, and 58 pA (Fig. 2B, right), irrespective of whether non-filtered (Fig. 2B, right) or low pass-filtered data (not shown) were analyzed. The open state with a current of 58 pA is rather broad and does not contribute substantially to the observed distribution, even though this was the only current obtained for nOmp85-C (Fig. 2A, right). The detailed analysis of the full-length protein nOmp85 also revealed three open states and exhibits a similar distribution as in Fig. 2B. The voltage-current relationship (Fig. 3B) of the open states of nOmp85...
induced by conformational changes due to the interaction of the N- and C-terminal domains.

The ability of the distal C terminus of nOmp85 and psToc75 (Fig. 1, construct D) to transport sucrose or NaCl has been previously reported (18). To support this observation, the electrophysiological properties of the distal C terminus were examined. The distal C-terminal portion of nOmp85 containing eight \( \beta \)-strands showed a reverse potential of 39 mV, suggesting a cationic selectivity (nOmp85-D) (Fig. 3C) and a main conductance of \(~470\) pS (not shown). This conductance can be explained by a pore formed from two distal C-terminal monomers, because the conductance of the entire C-terminal portion or the full-length protein containing 16 \( \beta \)-strands is in a similar range (Table 1) (18).

Further, the previously observed reverse potential of 17 mV for nOmp85 and for the entire C-terminal region (18), respectively, reflects a slight cationic selectivity (\( P_K/P_C \)) of 2.2, according to the Goldman-Hodgkin-Katz voltage equation. The reverse potential of the distal C terminus (39 mV) reveals an increased cationic selectivity of 7.0. For psToc75-D, a reverse potential of 47 mV was obtained (Fig. 3D), which is similar to that observed for psToc75-C (40 mV) (18). Hence, the ion selectivity (\( P_K/P_C \)) decreases from 14.3 for psToc75 (15) to 12.9 or 7.7 for the pore-forming region psToc75-D (18) or -C, respectively. Therefore, both proteins (nOmp85 and psToc75) possess a selectivity filter within the conserved part forming the pore, whereas the ion selectivity is further influenced by the N-terminal domain.

The Pore Characteristics of Different PTBs—To explore differences and similarities of the PTBs from different species, we compared one PTB each from cyanobacteria, proteobacteria, chloroplasts, and mitochondria. Hence, we cloned, expressed, and purified YaeT from E. coli and Tob55/Sam50 from D. melanogaster to compare their electrophysiological properties with nOmp85 and psToc75. For YaeT, an N-terminal truncation starting at amino acid 473 was constructed as well. The purified proteins were subsequently analyzed by electrophysiological means. YaeT exhibits a conductance of 100 pS (Table 1). This resembles the recently reported value (33) and is \(~5\) times smaller than the conductance of the cyanobacterial homolog nOmp85. Considering similar channel features as for nOmp85 (18), a diameter of 0.54 nm was calculated. Such a diameter would be too small to transport polypeptide chains and suggests a different function for the two proteins found in

Table 1

| Protein | Species | Full-length | Pore region |
|---------|---------|-------------|-------------|
|         |         | Full-length | Pore region |
|         |         | \( U_{\text{rev}} \) | \( g \) | \( U_{\text{rev}} \) | \( g \) |
| Omp85   | Nostoc  | 17\( ^{a} \) | 520 | 17\( ^{a} \) | 640\( ^{a} \) |
| YaeT    | E. coli | 45 100      | 45 100      |
| Tob75   | P. sativum | 48 500 45 100 690 |
| Sam50   | D. melanogaster | 40 140 ND ND |

\( a \) Values taken from Ref. (18).
\( b \) Values for the C-terminal portion.
\( c \) Values for the distal C-terminal portion. ND, not determined.

is linear. The conductances are calculated as 640, 520, and 430 pS, where the main conductance (520 pS) corresponds to the value previously reported for nOmp85, where one open state was assumed (18). Interestingly, the largest conductance (640 pS) was the state observed for nOmp85-C, suggesting that the other conductances of nOmp85 (520 and 430 pS) are

FIGURE 2. The N-terminal domain of nOmp85 influences the pore activity. The current recording of a bilayer containing nOmp85-C (A) after the addition of 0.1 \( \mu \)M nOmp85-N (final) (B) at +100 mV holding potential is shown (left). The current distribution of the non-filtered data set (right) was calculated as described. The current distribution profile was analyzed by two (A) or four (B) gaussian distributions (gray for individual gaussians, dark gray for fitted curve gaussians), and peak values are given.

FIGURE 3. Properties of the pore formed by nOmp85 or the distal C terminus of psToc75 and nOmp85. A, the current distribution of the open state of nOmp85 (holding potential \( ~75 \) mV, black line) is shown. The distribution was analyzed by one gaussian (gray dotted) or three gaussians (gray dashed for individual curves, dark gray dashed for the entire curve). The residuals of the least square fit results are presented on the bottom to judge the quality of the fitted curves. B, the voltage-current relationship of the three different conductances observed for nOmp85 was analyzed by linear regression (lowest (circle), medium (square), and highest (triangle) current observed). Voltage ramps (\( \Delta V = 10 \) mV/s) were applied across bilayers containing nOmp85-D (C) or psToc75-D (D) in asymmetrical 250 mM/20 mM KCl (cis/trans), 10 mM Mops/Tris (pH 7.0) buffer. The line represents the linear regression.
proteobacteria and mitochondria. The removal of the N-terminal domain did not alter the conductance, which parallels the finding for nOmp85.

The reverse potential of YaeT and Sam50 is similar to that found for Toc75 (Table 1). The analysis of the Sam50 homolog from D. melanogaster revealed similar properties as found in YaeT (Table 1). The conductance is 140 pS and the reverse potential 40 mV. As reported (33), we also noticed that the single channel conductances of YaeT and dmSam50 displayed a higher variability than those of cyanobacterial and plastidic PTBs, further supporting the suggested different functional behavior.

One functional model considers the insertion of the folded outer membrane proteins into the Omp85 channels and a subsequent lateral diffusion into the bilayer (34). However, the size of the monomeric Omp85 pore contradicts an insertion, which in turn could only be realized by a fusion of the Omp85 molecules present in one complex. Hence, such fusion in vivo would require a cooperative action of the channels in vitro. Therefore, we analyzed whether we could detect cooperative effects in opening and closing of the channel, because multimeric structures were found after reconstitution into artificial liposomes (Fig. 1). To this end, we analyzed the current recordings of membranes containing multiple inserted channels for cooperative openings or closings. However, we did not detect any sign of cooperativity (Fig. 4). In contrast, comparing the expected distribution for independent gating behavior (line and dots) with the measured distribution of subsequent events suggested that single events are more frequent than multiple events. Therefore, all investigated PTBs have similar ion selectivity and do not reveal an obvious cooperative behavior. Sam50 and YaeT have a significantly smaller pore dimension compared with nOmp85 and Toc75. This alteration, however, is not a result of a different behavior of the N-terminal portion on the pore-forming region, because the removal of this domain does not alter the conductance. Furthermore, the obtained results support a “chaperoning” of the insertion of β-barrel proteins rather than an insertion of the Omps into the channel.

Phylogenetic Analysis of the Omp85 Family—To integrate the electrophysiological properties of the four PTBs (nOmp85, EcYaeT, dmSam50, and psToc75) into a phylogenetic view, we inferred a phylogenetic tree for the Omp85 family. Up to now, the phylogeny of Omp85 was based on a small number of sequences (14, 16, 17). Here, we present a phylogenetic analysis based on 213 sequences that comprise 37 mitochondrial, 10 chloroplastic, and 166 bacterial Omp85 PTBs. Fig. 5 displays the maximum likelihood tree based on IQPNNI (28) and the multiple sequence alignment of Omp85 proteins. This tree reflects the well known groups of plastids, mitochondria, and bacteria. All shown branches are supported by posterior probabilities of at least 0.5, as obtained by MrBayes (30). Thus, further evolutionary conclusions are justified. The grouping of cyanobacteria and plastids is highly supported, as is the grouping of mitochondria and α-proteobacteria. Although the branching pattern of the maximum likelihood tree agrees by and large with the NCBI taxonomy, some relationships differ. For example, the branching pattern of the proteobacteria deviates slightly from that of the NCBI taxonomy. Another observation is that plastids are not monophyletic. The clade containing the Cercozoa sequence (group A) splits off before the commonly assumed split of plastids and cyanobacteria. This may be due to the fact that group A sequences are responsible for protein import, whereas the remaining “blue/green” sequences (group B) are involved in protein export in analogy to the function of Sam50 proteins (3). Therefore, a functional separation of the sequences rather than a true phylogenetic classification is obtained.

To shed light on the functional influence of the branching of the gene tree, the maximum likelihood trees for various subalignments were studied. More precisely, the trees for the N terminus, the entire C terminus, and the distal C terminus of the 213 selected sequences (supplemental Table 1) were reconstructed. The resulting maximum likelihood trees for groups A and B are displayed in Fig. 6, where Fusobacteria serves as an outgroup. Remarkably, the tree topology from C-terminal domains is similar to multiple sequence alignment of the Omp85 proteins, whereas the tree of the N terminus contradicts the commonly assumed taxonomy. Here, the plastidic PTBs do not group with cyanobacterial PTBs but with mitochondrial Omp85 homologs, which in turn do not cluster with the sequences found in proteobacteria. However, the branching pattern receives less support (see supplemental material). One exception constitutes the N termini of Atha-Toc75-V and Osat-Toc75-I. This might suggest that the N terminus of Atha-Toc75-V has a function related to that of the cyanobacterial PTBs. Moreover, the total branch length (measured in numbers of substitutions per site) of the maximum likelihood trees differs considerably. That is, for the distal C terminus, the C terminus, the full tree, and the N-terminal tree, total branch lengths of 76, 86, 85, and 92, respectively, were measured. This
supports the observation that the distal C terminus reflects the most conserved region (14, 17, 35).

By analyzing the phylogenetic relationship of the distal C terminus of the PTBs, we observed that the groups containing Toc75-V/Toc75-III switch position with respect to the entire tree. However, plastidic and cyanobacterial PTBs together remain monophyletic in both trees (C and distal C termini). Hence, the pore-forming domain (C terminus) clearly reconstructs the phylogenetic relationship expected, whereas the N-terminal domain does not.

DISCUSSION

Relationship between Prokaryotic and Eukaryotic Members of the Omp85 Family—The phylogenetic relationship of the Omp85 family agrees with the commonly assumed groupings of plastids with cyanobacteria and mitochondria with proteobacteria (Figs. 5 and 6). Therefore, the mitochondrial Omp85 evolved from an ancestral Omp85-type molecule present in the symbiont leading to mitochondria. The same holds true for the Omp85 of plastids. This proposal is in accordance with the classical endosymbiont theory (36, 37). The common ancestry of plastidic and cyanobacterial Omp85s is also reflected by their physical properties. The Omp85s of this branch exhibit a conductivity of ~500 pS (Figs. 2 and 3; Table 1) (15, 18). In contrast, the E. coli YaeT, a member of the Omp85 family, and the D. melanogaster Sam50 have a conductivity of ~100 pS (Table 1), which again supports our phylogenetic analysis.

The conductances of YaeT and Sam50 reflects a pore diameter ~5 times smaller than the diameter of the cyanobacterial or plastid Omp85 proteins. Loosely speaking, the big diameter preadapted the cyanobacterial Omps to act as a protein translocator in eukaryotes. In contrast, the proteobacteria did not contain an Omp85 homolog with a sufficient pore size to facilitate protein translocation. These observations explain why the Omp85 from cyanobacteria but not from proteobacteria was remodeled to act as a eukaryotic protein translocator.

Besides some differences between cyanobacterial and proteobacterial Omp85s, some common features are identified. For instance, nOmp85 forms complexes with a putative trimeric structure (Fig. 1) similar to that of other known PTBs from proteobacteria (23, 38). We have shown that the N-terminal domain is required for the complex formation in vitro (Fig. 1), because the C-terminal pore-forming domain only forms dimeric complexes (Fig. 1). Furthermore, complex assembly does not induce a cooperative gating of the cyanobacterial and proteobacterial channel (Fig. 4). Especially the latter finding supports a chaperone-type function for the insertion of Omps into the outer membrane.

In contrast to the full alignment or the C-terminal alignment, the phylogenetic tree of the N-terminal alignment clusters mitochondrial and plastidic Omp85s to the exclusion of bacte-
rial homologs (Fig. 6, 1st panel from the left, and supplemental material). We would like to argue that this is a long branch attraction phenomenon (39) because of the strong selection pressure acting on this part of the molecule. This argument is supported by the different physiological properties measured for the N terminus of the plastidic and cyanobacterial proteins. More explicitly, we measured a high affinity of the N-terminal domain from nOmp85 to the C-terminal pore-forming region (Figs. 1 and 2), which could not be detected in the plastidic PTB (Fig. 1). We further demonstrated that the N-terminal region of nOmp85 induces subconductances not measured for the pore-forming region itself (Fig. 2). The data are interpreted as a reduction of the pore size due to the presence of a region in the N-terminal domain that is inserted in the pore. This notion is supported by the fact that the main conductance of the full-length protein does not show the largest conductance (Fig. 3). Hence, our data confirm the hypothesis (18) that the N terminus has a structural and functional influence on the pore-forming region. For the plastidic Omp85-N, it is suggestive to assume that it has the same effect, because psToc75-C does not reveal any subconductance (18). However, because of the missing affinity of psToc75-N for the C-terminal pore-forming region, the influence could not be studied by electrophysiological means.

Hence, the following functions for the N termini of the Omp85 proteins are proposed. First, the N terminus of all Omp85 proteins regulates the flux through the pore. Second, the N terminus is involved in complex formation at the outer membrane. However, the N terminus of the bacterial proteins is involved in homo-oligomerization (Fig. 1), whereas the N terminus of the eukaryotic Toc75 associates with the other Toc complex components. This association is essential for the formation of a functional Toc complex (19) but had to be evolutionarily developed, because these components did not exist in the prokaryotic membranes. The same holds true for the Sam complex, where proteins exposed to the cytosol regulate the function of Sam50. This necessitated changes in the N terminus leading to a higher similarity between eukaryotic proteins than to their ancestor, which is supported by the inferred tree (Fig. 6, 1st panel from the left). Third, the N terminus also acts as a docking site for the incoming precursor proteins (18). However, the importance of the interaction of Omp85 with the incoming protein displays a further difference between the Omp85 proteins of prokaryotes and higher eukaryotes. Whereas the Omp85 in prokaryotes acts as a primary docking site recognizing the phenylalanine of the incoming outer membrane protein (1, 2), precursor proteins are delivered to the eukaryotic Omp85 transporters (3, 5) by other components of the translocation machinery. Hence, the specificity selection does not occur any longer at the N-terminal domain of the eukaryotic Omp85, reducing the evolutionary pressure for the recognition of the sequence motif. This concept is also supported by the observation that phenylalanine is only essential for protein translocation into plastids of cyanelles, diatoms, and crytomonads but not of higher plants (2). Therefore, the phylogenetic distribution of the N-terminal domain (Fig. 6, 1st panel from the left) represents a changed functionality of this region.

The Hypothetical Evolutionary Development of Omp85 Proteins—The electrophysiological properties of the distal C-terminal region representing eight-transmembrane β-strands suggest a dimeric pore structure (Fig. 1) (18). This
The evolutionary scenario described in the discussion is visualized. The first step (1) shows the initial homo- or heterodimerization of two eight-transmembrane-sized β-strand proteins. The second step (2) shows the association of this pore with a periplasmic protein functioning as a plug or regulator of the pore. In parallel, the operon formation of the two components might have occurred. The third step (3) is envisioned as the formation of an operon of all three components, and the final step (4) marks the gene fusion leading to one gene encoding for the regulated pore-forming PTB. LPS, lipopolysaccharide layer; PL, phospholipid layer; OM, outer membrane.

![Diagram of the evolutionary concept of translocon development](image)

**Protein level**

**Gene level**

FIGURE 7. Schematic view of the evolutionary concept of translocon development. The evolutionary scenario described in the discussion is visualized. The first step (1) shows the initial homo- or heterodimerization of two eight-transmembrane-sized β-strand proteins. The second step (2) shows the association of this pore with a periplasmic protein functioning as a plug or regulator of the pore. In parallel, the operon formation of the two components might have occurred. The third step (3) is envisioned as the formation of an operon of all three components, and the final step (4) marks the gene fusion leading to one gene encoding for the regulated pore-forming PTB. LPS, lipopolysaccharide layer; PL, phospholipid layer; OM, outer membrane.

Such an interaction regulates the activity of the TonB-dependent outer membrane proteins (40). This complex of the pore proteins and the periplasmic protein allows the fine tuning of transport processes. Thereby, the periplasmic protein regulated the flux through the pore. One can envision that the modern Omp85 proteins evolved subsequently by gene fusion from the gene encoding for the periplasmic protein to form the N-terminal “plug” domain, and the genes encoding the pore that we now call C terminus (Fig. 7, steps 3 and 4). This assumption is in line with the proposal of Cavalier-Smith (41), who considers the development of the Omp85 a majorpromoter for the formation of complex outer membranes but still subsequent to the formation of the outer membrane leading to the development of a “cenancestral prokaryote.”

In the subsequent course of evolution, the bacterial proteins were adapted to respond to different environmental conditions but not to entirely new functions. In contrast, after endosymbiosis, the Omp85s of the symbiont had to adapt to new environmental conditions and a change in function. For example, the plastidic protein psToc75 translocates chloroplast-targeted precursor proteins across the membrane. Hence, the N terminus was remodeled to interact efficiently with complex components, a challenge not encountered for cyanobacterial PTBs. Additionally, the affinity of the N-terminal domain for the pore-forming region had to be reduced to accommodate the new function as a protein importer. However, the physical connection between N- and C-terminal domains still guarantees the interaction. The original high affinity between these two domains might be of similar magnitude as the one in cyanobacterial PTBs (Figs. 1 and 2). However, the final understanding of the evolutionary history of PTBs deserves further investigation.

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The Omp85 Family

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