Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 15831–15836, December 1998
Plant Biology

Origin of a chloroplast protein importer

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Edited by Diter von Wettstein, Washington State University, Pullman, WA, and approved October 27, 1998 (received for review August 24, 1998)

ABSTRACT **During evolution, chloroplasts have relinquished the majority of their genes to the nucleus. The products of transferred genes are imported into the organelle with the help of an import machinery that is distributed across the inner and outer plastid membranes. The evolutionary origin of this machinery is puzzling because, in the putative predecessors, the cyanobacteria, the outer two membranes, the plasma membrane, and the lipopolysaccharide layer lack a functionally similar protein import system. A 75-kDa protein-conducting channel in the outer envelope of pea chloroplasts, Toc75, shares ≈22% amino acid identity to a similarly sized protein, designated SynToc75, encoded in the *Synechocystis* PCC6803 genome. Here we show that SynToc75 is located in the outer membrane (lipopolysaccharide layer) of *Synechocystis* PCC6803 and that SynToc75 forms a voltage-gated, high conductance channel with a high affinity for polyamines and peptides in reconstituted liposomes. These findings suggest that a component of the chloroplast protein import system, Toc75, was recruited from a preexisting channel-forming protein of the cyanobacterial outer membrane. Furthermore, the presence of a protein in the chloroplastic outer envelope homologous to a cyanobacterial protein provides support for the prokaryotic nature of this chloroplastic membrane.**

Mitochondria and plastids, chloroplasts in green plant organs, have relinquished most of their genes to the cell nucleus during evolution (1–4). The necessity to reimport the cytosolically synthesized gene products resulted in the establishment of distinct protein import machineries in both the outer and the inner chloroplastic envelope. The translocon at the outer envelope of chloroplasts (Toc complex) is a multisubunit oligomeric membrane structure (5–7) that consists of Toc86, a precursor receptor (8–10) potentially regulated by phosphorylation and nucleotide binding; Toc75, a preprotein translocation channel (11–13); and Toc34, a G-protein with GTPase activity (8, 14), which might regulate the transfer of precursor proteins from the recognition to the translocation site or the gating of the Toc75 channel. An Hsp70 homologue (15) and a member of the Cim/Com44 (16) family are further subunits of the Toc complex. The translocon at the inner envelope of chloroplasts (Tic complex) contains the functionally less well defined components Tic110, Tic55, Tic22, Tic21 and a member of the Cim/Com44 family (for review, see ref. 5).

Plastids and mitochondria originated most likely from independent endocytobiotic events (2, 3). Today's relatives of the photosynthetic prokaryotic endosymbiont are the Gram-negative-like cyanobacteria (1–3). Cyanobacteria are surrounded by two independent membrane systems, the plasma membrane and the outer membrane (lipopolysaccharide layer) separated by a peptidoglycan layer. The delimination of plastids by the inner and outer envelope membranes is reminiscent of this structure. The plastidic inner envelope is homologous to the prokaryotic plasma membrane whereas the origin of the outer envelope seems less clear (3, 17). The presence of galactolipids and carotenoids in both the prokaryotic outer membrane and the plastidic outer envelope supports it's prokaryotic origin (18–20). The "eukaryotic" type lipid phosphatidylcholine is found only in the chloroplastic outer envelope, where it represents the major polar lipid (21). This finding was taken as support for the eukaryotic nature of the plastid outer envelope (22). However, no information is available if protein homologues can be detected in the different membrane systems.

We recently have found an ORF (slr1227) in the *Synechocystis* PCC6803 genome that is homologous to Toc75 and might represent a phylogenetic intermediate of a protein import complex component (5). In this presentation, we show that SynToc75 is localized in the cyanobacterial outer membrane and that the reconstituted protein forms a channel with similar properties to the pea preprotein translocation channel Toc75.

MATERIALS AND METHODS

Membrane Isolation. *Synechocystis* cells were grown at 4% CO_2 (vol/vol), 25°C, and a 12-h/12-h light–dark regime (450 $\mu M/cm^2 \cdot s^1$). Cells were harvested by centrifugation (30 min at 5,000 × *g*) and were washed once with 5 mM Tris·HCl (pH 8.0) and 1 mM phenylmethylsulfonylfluorid (PMSF). Cells were resuspended in 10 mM Tris·HCl (pH 8.0), 1 mM PMSF, 600 mM sucrose, 5 mM EDTA, and 0.2% (wt/vol) lysozyme and were incubated for 2 h at 30°C under continuous shaking (18–20). Cells were recovered by centrifugation and were washed once in 20 mM Tris·HCl (pH 8.0), 600 mM sucrose, and 1 mM PMSF. Cells were resuspended in 25 ml of 20 mM Tris·HCl (pH 8.0), 600 mM sucrose, and 1 mM PMSF and were ruptured by two passages through a French Press at 1,200 psi (1 psi = 6.89 kPa). The lysate was brought to 10 mM NaAc, 1 mM $MgCl_2$, 1 mM PMSF, and 0.1% (wt/vol) DNase and was left on ice for 15 min. Intact cells were removed by centrifugation. The supernatant was brought to 50% sucrose. The dense cell lysate suspension was overlayed with 8 ml of a 39%, 7 ml of a 30%, and 3 ml of a 10% sucrose solution in 10 mM Tris·HCl (pH 8.0), 10 mM NaCl, and 1 mM PMSF and was centrifuged for 16 h at 130,000 × *g* in a swinging bucket rotor. The plasma membrane and the thylakoid fraction were recovered from 30 and 39% sucrose interphase, respectively, and were concentrated after dilution with 5 volumes of 10 mM Tris·HCl (pH 7.0) by centrifugation at 130,000 × *g* for 30 min. The pellet from the sucrose density gradient containing the cell wall with the adhering outer membrane was washed twice in 20 mM Tris·HCl (pH 7.0) and 1 mM PMSF and was recovered at 12,000 × *g* for 15 min. The pellet was resuspended in 10 ml 10 mM Tris·HCl (pH 7.0), 5 mM $MgCl_2$, and 2% Triton X-100 (vol/vol) and was stirred for 2 h at 29°C.

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Abbreviation: PMSF, phenylmethylsulfonylfluorid.
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Membranes were concentrated by centrifugation (30 min at 47,000 × *g*) and were washed 5 times in 20 mM Tris·HCl (pH 7.0). Membranes were resuspended in 0.5 ml of 50 mM NaP_i (pH 6.5), and 4 mg/ml lysozyme were added. The suspension was incubated for 24 h at 37°C under continuous agitation. Outer membranes were recovered by centrifugation (90 min at 47,000 × *g*) and were washed at least thrice with 20 mM Tris·HCl (pH 7.0).

Reconstitution of SynToc75. SynToc75 was expressed in *Escherichia coli* BL21(DE3) cells by using the pET21b vector (Novagen). To the purified SynToc75 [in 6 M urea and 10 mM KP_i (pH 6.0)], the detergent Mega-9 was added to a final concentration of 80 mM. Preformed stigmasterol saturated liposomes (23) were dissolved in 80 mM Mega-9 and 10 mM Mops/Tris (pH 7.0). Both samples were mixed to yield 0.8 mg of protein/10 mg of lipid and were dialyzed for 4 h in 5 liters of Mops/Tris (pH 7.0) buffer at room temperature and subsequently after changing the buffer overnight at 4°C.

Electrophysiological Measurements. Planar lipid bilayers were produced by using the painting technique (13). The resulting bilayers had a typical capacity of ≈0.5 $\mu F/cm^2$ and a resistance of >100 gigaohms (rms ≈ 1 pA at 5-kHz bandwidth). After a stable bilayer was formed in symmetrical solutions of 20 mM KCl and 10 mM Mops/Tris (pH 7.0), the solution of the cis chamber was changed to asymmetrical concentrations [cis chamber: 250 mM KCl, 10 mM $CaCl_2$, and 10 mM Mops/Tris (pH 7.0)] by adding concentrated solutions of KCl and $CaCl_2$. The liposomes were added to the cis compartment directly below the bilayer through the tip of a micropipette to allow the flow of the liposomes across the bilayer. If necessary, the solution in the cis chamber was stirred to promote fusion. After fusion, the electrolytes in both compartments were changed to the final composition by perfusion. The Ag/AgCl electrodes were connected to the chambers through 2 M KCl-agar bridges. The electrode of the trans compartment was connected directly to the headstage of a current amplifier (geneClamp500, Axon Instruments, Foster City, CA). Reported membrane potentials are referred to as the trans compartment. The amplified currents were recorded to hard disk by using the PCLAMP7 software (Axon Instruments). From the determined lipid/protein ratio (100:1, wt/wt) after reconstitution, it can be calculated (13) that each proteoliposome vesicle contains in average maximal 12 SynToc75 molecules (assuming 100% reconstitution yield and homogeneous distribution, d_{ves} = 50 nm). Because we did not observe any protein contamination in a silver stained SDS/PAGE gel, we may assume a maximal protein contamination of 1%. Thus, we expect considerably <0.1 molecules of any possible contaminant ($5 < M_r < 50$) per lipid vesicle. In the course of the experiments ($n \geq 40$), we observed that fusion of single liposomes always led to multiple incorporation of active channels ($3 < n < 10$). Coincident fusion of at least 30 vesicles containing in average 0.1 contaminant channel molecules within a time window of ≈1 ms would be required to produce the observed simultaneous appearance of channel activity. This is very unlikely to occur, and we never observed this simultaneous fusions with any reconstituted channel protein.

RESULTS

Reconstitution of SynToc75. The amino acid sequence of pea Toc75 shows detectable similarity (22% identity/55% similarity) to an ORF (slr 1227, D90906) of unknown function of the *Synechocystis* PCC6803 genome (24). The predicted ORF of SynToc75 begins with an ATG-codon at position 1031202 and ends at position 1033787 of the genome. Sequence conservation is observed primarily between the mature region of pea Toc75, which is synthesized as a precursor protein with an NH_2-terminal cleavable presequence (11, 12) and the deduced cyanobacterial protein starting at amino acid position 220. Secondary structure algorithms predict that SynToc75 can form 16 β-sheets, 10 of which are colinear with transmembrane β-sheets from pea Toc75 (13) (Fig. 1*A*). This motif would allow folding into a barrel-like structure characteristic of membrane pore proteins (25). To test whether SynToc75 could function as a channel protein, the gene was amplified by PCR and was subcloned into an expression vector. The recombinant protein was purified by anion exchange chromatography to apparent homogeneity (Fig. 1*B*) and was reconstituted into liposomes. When SynToc75 proteoliposomes were fused with planar bilayers, single channel activities were observed (Fig. 1*C*). The SynToc75 channels, which have the highest open

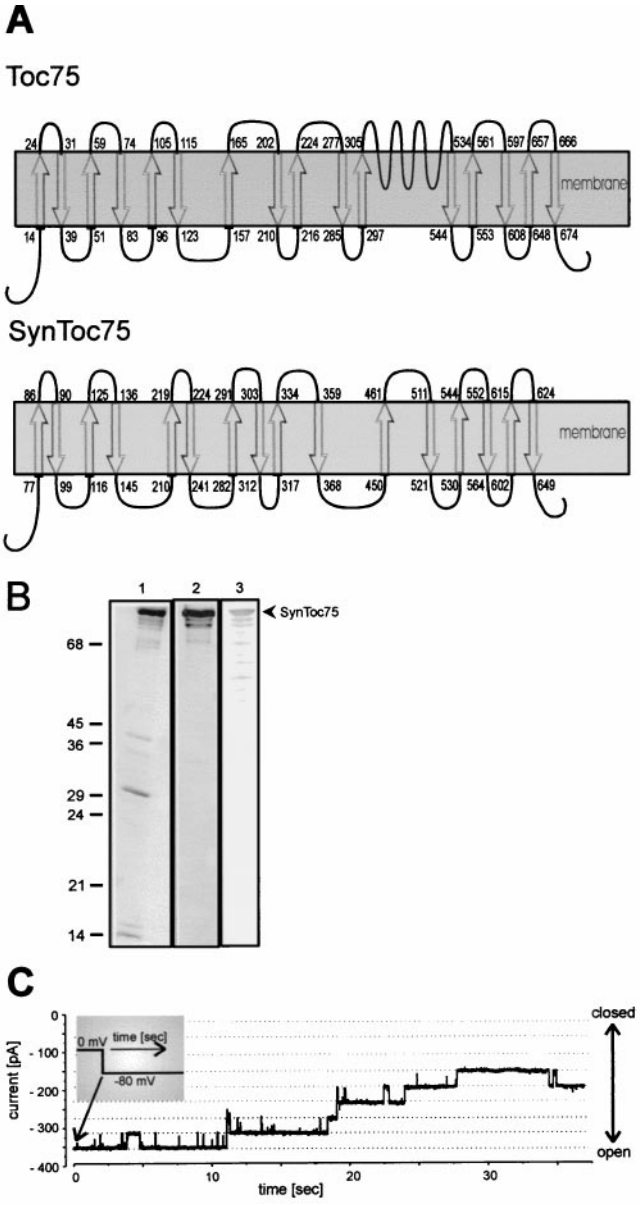

FIG. 1. Secondary structure prediction and function of SynToc75. (*A*) Toc75 from pea and SynToc75 are predicted to form multiple transmembrane β-sheets (13). (*B*) Heterologously expressed SynToc75 was recovered from inclusion bodies (lane 1) and was purified further by anion exchange chromatography (lane 2). A Coomassie brilliant blue-stained SDS/PAGE is shown. In lanes 3 is an immunoblot of purified SynToc75. Lanes: 1 and 2, 10 μg of protein; 3, 1 μg of protein. (*C*) Current trace from a bilayer containing eight active SynToc75 channels after a voltage step from 0 to −80 mV with 250 mM KCl, 10 mM $CaCl_2$, and 10 mM Hepes·Tris (pH 7.2) on both sides of the membrane.

probability ≈0 mV (Fig. 2*B*), close after a voltage jump to either negative or positive holding potentials (Fig. 1*C*).

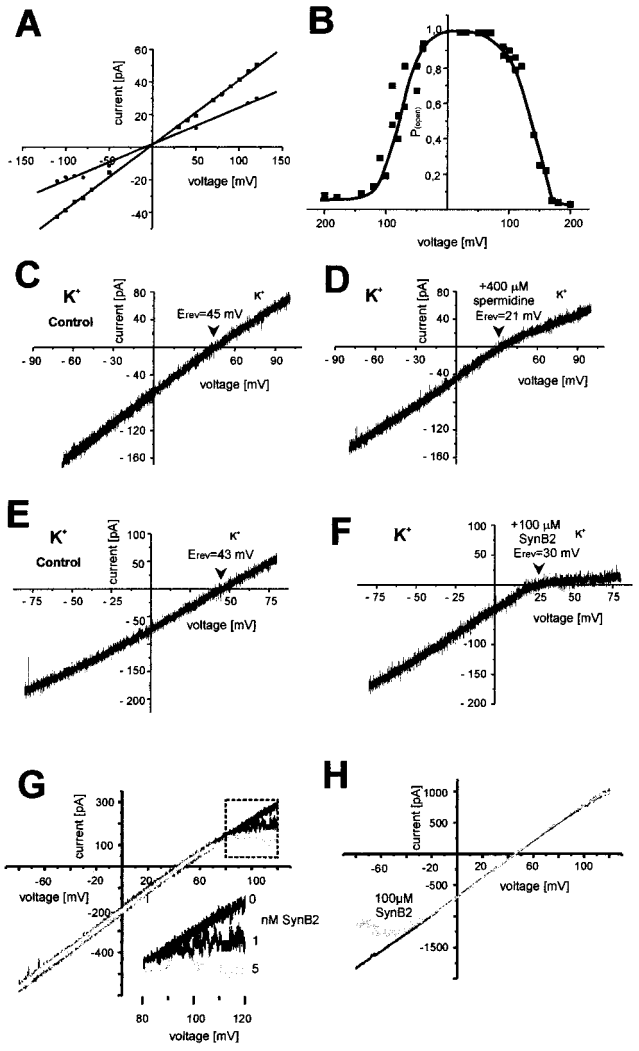

FIG. 2. SynToc75 forms a high conductance cation-selective channel. (*A*) Current–voltage relationship of the fully open single channel (squares) and the most frequent subconductance level (circles) with 250 mM KCl on both sides of the membrane (data points were averaged from five independent bilayers, with SEMs <3.5%). (*B*) Voltage dependence of the probability for the SynToc75 channel being in any of its open states. To approach equilibrium of channel gating with respect to the applied membrane, voltages were applied for 5 min, but only the current recordings of the last minute were used to calculate open probability from the amplitude histograms (averages of at least three independent bilayers, with SEMs <6% of the values). (*C* and *D*) Influence of SynB2 (MLSRQQSQRQSQQSQRQSRYLL, M_r = 2, 9) and spermidine on the SynToc75 conductivity. Voltage ramps (ΔV = 10 mV/s) were applied across bilayers containing multiple copies of the active SynToc75 channel. (*C*) Recording from a bilayer containing four active copies of the SynToc75 channel in asymmetrical 250 mM/20 mM KCl, 10 mM Mops/Tris (pH 7.0) buffer (cis/trans), control. (*D*) Same bilayer as in *C*, but after addition of 400 μM spermidine to the trans compartment. (*E*) Recording from a bilayer containing four active copies of the SynToc75 channel in asymmetrical 250 mM/20 mM KCl, 10 mM Mops/Tris (pH 7.0) buffer (cis/trans), control. (*F*) Same bilayer as in *E*, but after addition of 100 μM SynB2 to the trans compartment. (*G*) Recording from a bilayer containing 12 active copies of the SynToc75 channel in asymmetrical 250 mM/20 mM KCl, 10 mM Mops/Tris (pH 7.0) buffer (cis/trans). Black, control; gray/light gray, after addition of 1/5 nM SynB2 respectively to the trans compartment. (*H*) Recording from a bilayer containing 44 active copies of the SynToc75 channel in asymmetrical 250 mM/20 mM KCl, 10 mM Mops/Tris (pH 7.0) buffer (cis/trans). Black, control; gray, after addition of 100 μM SynB2 to the cis compartment.

Electrophysiological Properties of SynToc75. The current voltage relationship of reconstituted SynToc75 revealed a main conductance of Λ = 410 ± 40 pS (n > 15; Fig. 2*A*); additional subconductances [Λ = 230 ± 25 pS, n > 10 (Fig. 2*A*); and Λ = 330 ± 28 pS, n = 8 (data not shown); were observed frequently. The subconductance states show the same cation selectivity; it seems, therefore, unlikely that they respond differently. From the conductance of the fully open channel (Λ = 410 ± 40 pS), we may estimate the diameter by the cylindrical model (26) as $d_{channel}$ = 1.29 nm. Considering the more realistic case that the actual conductivity in the channel pore could be ≈5× lower than in the bulk medium (27), we obtain a value of $d_{channel}$ = 2.6 nm as an upper limit. The SynToc75 channel is cation-selective with P_{K^+}/P_{Cl^-} = 1: 0.09 as calculated from the reversal potential under asymmetric conditions (n > 40; Fig. 2 *C* and *E*).

Additional experimental information on the range of the pore diameter of the SynToc75 channel was gathered by measuring the permeability of large cations and positively charged peptides, tetraethylamine, tetrabutylamine, cadaverine, spermidine, spermine, $CoxIV_{1–23}$ (28), and SynB2 (29). In line with the estimated pore size the large cations, tetraethylamine (cross section, $d_{max} \times d_{min}$, 9 × 8.1 Å) and tetrabutylamine ($d_{max} \times d_{min}$, 13 × 13 Å) are able to permeate the SynToc75 channel, P_{K^+}: $P_{tetraethylamine^+}$ = 1: 0.3 (n = 2), P_{K^+}: $P_{tetrabutylamine^+}$ = 1: 0.1 (n = 3) as calculated by the Goldman-Hodgkin-Katz equation from the reversal potentials in biionic measurements (26). The polyamines cadaverine, spermidine, and spermine also were measured under biionic conditions, and, according to the respective reversal potentials, they also were able to permeate the SynToc75 channel. In line with this is the observation that addition of polyamines to the low salt trans compartment under asymmetric conditions (cis/trans: 250 mM/20 mM KCl) results in a shift of the reversal potential to a less positive value (n = 3 cadaverine, n = 8 spermidine, n = 5 spermine). Furthermore, the current is reduced at a positive membrane potential (Fig. 2*D*), and this effect becomes more pronounced with increasing positive charge of the polyamines: $spermine^{+4}$ > $spermidine^{+3}$ > $cadaverine^{+2}$. This current reduction is most likely caused by a slow permeation (as compared with KCl) of the polyamines, which indicates a high affinity to presumably negatively charged sites inside the pore. Furthermore, the addition of polyamines to the low salt trans compartment at asymmetric KCl concentrations (cis/trans: 250 mM/20 mM KCl) also resulted in a less positive reversal potential (e.g., 10 mM spermidine added to the trans compartment caused a decrease from reversal potential = 38 mV to reversal potential = 25 mV, n = 3). The only plausible explanation of this result is a change in the apparent selectivity of the SynToc75 channel (increasing anion permeability).

When the peptides $COX\ IV_{1–23}$ and SynB2 were added under asymmetric conditions (cis/trans: 250 mM/20 mM KCl) to the low salt trans compartment they also caused a shift of the E_{rev} to a less positive value and a marked reduction of the currents at positive potentials (Fig. 2*F*; n = 4 SynB2, n = 3 COX IV). The current reduction was more evident than with polyamines and was even noticeable at nanomolar concentrations of the peptide (Fig. 2*G*; n = 4 SynB2). But, in contrast to the results with polyamines, the addition of micromolar concentrations of SynB2 to the high salt cis compartment (n = 2) caused only a current reduction but not a change of the reversal potential (Fig. 2*H*). Therefore, the selectivity of the SynToc75 channel remains unchanged, and the only possible explanation for the above mentioned shift of the E_{rev} to less positive values on addition of SynB2 to the low salt trans compartment is that the peptide acts as a permeant polycation (SynB2 carries 5 positive charges at pH 7). Biionic measurements for further confirmation of these results were not feasible (the quantities of peptide that would have been necessary were not available).

Fractionation of Synechocystis Cells. To define the subcellular location of SynToc75, thylakoids, plasma membrane, and outer membrane from *Synechocystis* were separated and purified. Each fraction showed a distinct polypeptide composition (Fig. 3*A*). Membrane fractions were extracted with 80% (vol/vol) diethyl ketone, and the spectra were recorded from 350 to 700 nm. The characteristic but distinct absorption with three maxima between 350 and 550 nm indicated a unique carotenoid composition for each membrane fraction (18–20) (Fig. 3*B*). From the typical chlorophyll absorption at 660 nm, we estimated that the plasma membrane and the outer membrane were contaminated by thylakoids between 4–7% and 1–3%, respectively. Antisera against marker polypeptides of the thylakoids, ATPase α- and β-subunit, or of the stroma, Rubisco, reacted only with proteins in their native subcompartment (Fig. 3*C*), further indicating the purity of the isolated *Synechocystis* membranes.

Immunolocalization of SynToc75. An antiserum raised against heterologously expressed SynToc75 recognized selectively one protein in the outer membrane fraction from *Synechocystis* (Fig. 3*C*, lane 1). No cross-reaction with polypeptides could be detected in either the plasma membrane, the thylakoids, or the soluble protein fraction. The protein detected by the SynToc75 antiserum in the outer membrane has an apparent molecular mass of 75 kDa, which is ≈20 kDa smaller than the 95 kDa predicted from the DNA sequence and observed for the expressed recombinant protein (Fig. 3*C*, lane 5). Amino acids 1–32 are characteristic of a bacterial export signal; amino acids 32 to ≈180 might represent a propeptide necessary for productive translocation across the periplasmic space and insertion or assembly into the outer membrane (30). Several low abundance proteins are visualized in the range between 70 and 80 kDa in the outer membrane, which might represent SynToc75 (Fig. 3*A*, lane OM). However, insufficient protein was obtained to determine the N-terminal sequence of mature SynToc75 directly.

SynToc75 is resistant to extraction at pH 11.5 or 1 M NaCl, indicating that it is an integral membrane protein (31) (Fig. 3*D*). Both the outer membrane localization and the resistance to extraction at pH 11.5 of SynToc75 clearly resemble the properties of pea Toc75, which is localized exclusively in the chloroplastic outer envelope (Fig. 3*E*, lane OE) and is resistant to extraction at pH 11.5 or 1 M NaCl (Fig. 3*E*, lanes 5–8) (11, 12, 21).

To verify further the outer membrane localization of SynToc75 *in situ*, thin sections of *Synechocystis* cells were used for immunogold decoration with affinity-purified antibodies against SynToc75. In agreement with its low abundance (see Fig. 3*A*), the observed labeling density was low. Despite this, gold grains were confined almost exclusively to the outer membrane, as seen in the electron microscopic overview (see Fig. 4*A*) and higher magnifications (Fig. 4*B-D*). Control experiments from 10 independent preparations using either preimmune serum or omitting the SynToc75 antiserum showed no labeling (data not shown).

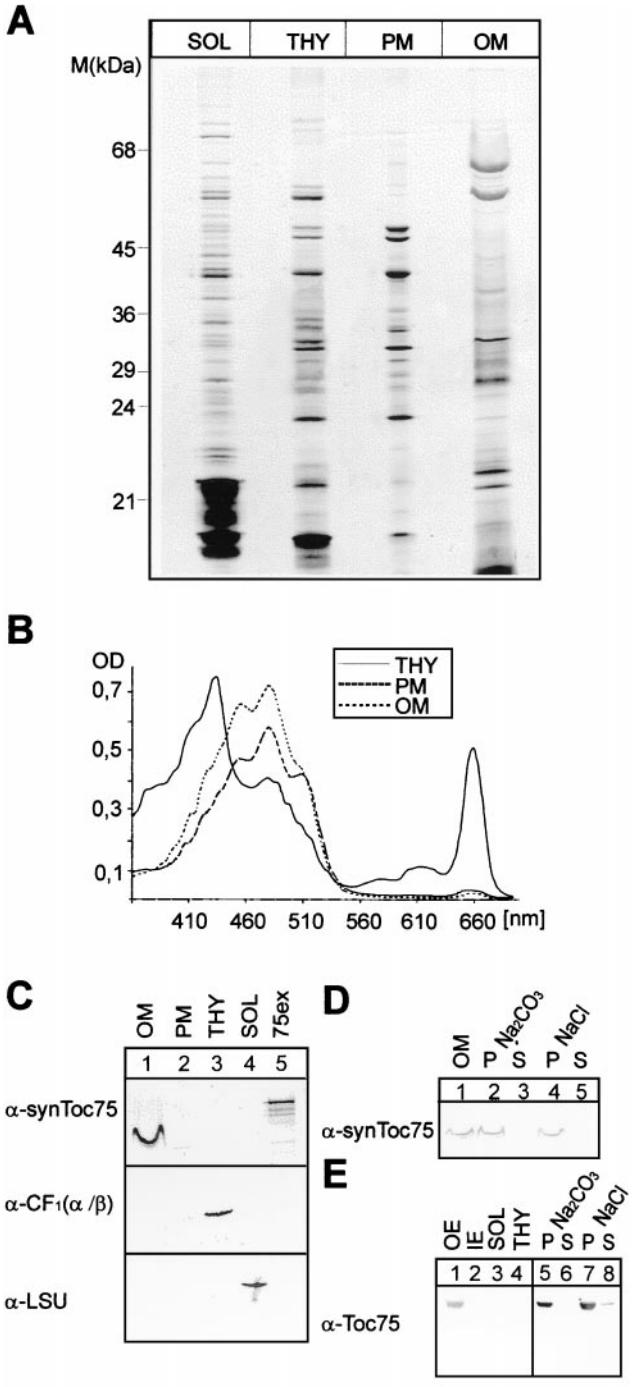

FIG. 3. Localization of SynToc75 in cellular subfractions of *Synechocystis* PCC6803. (*A*) The polypeptide composition of soluble proteins (SOL), thylakoids (THY), plasma membrane (PM), and outer membrane (OM) was analyzed by PAGE and silver-staining. (*B*) Adsorption spectra were recorded from cyanobacterial membranes extracted by 80% (vol/vol) diethyl ketone. (*C*) Immunoblot analysis using antisera (α) against SynToc75, ATPase α- and β- subunit from spinach (CF_1 α/β), Rubisco (LSU), and heterologously expressed SynToc75 (75ex). (*D*) *Synechocystis* outer membranes (lane 1) were extracted with 0.5 M Na_2CO_3 (pH 11.5) (lanes 2, 3) or 1 M NaCl (lanes 4, 5) and were separated into an insoluble (P) and a soluble (S) protein fraction. An immunoblot is shown. (*E*) Localization of Toc75 in pea chloroplast outer envelope (OE, lane 1), inner envelope (IE, lane 2), stromal proteins (SOL, lane 3), and thylakoids (THY, lane 4). Outer envelope membranes were treated with Na_2CO_3 or NaCl and fractionated as in *D*. An immunoblot is shown using an antiserum raised against peaToc75.

DISCUSSION

The data presented here address two important problems concerning the origin of plastids: First, the hypothesis that the chloroplastic outer envelope originated from the outer membrane of the endocytobiont has drawn support from the presence of galactolipids and carotenoids in both the cyanobacterial and the chloroplastic outer membranes (1, 3, 17, 18, 20). However, phosphatidylcholine, a "eukaryotic" type lipid, is the single most prominent lipid in the chloroplastic outer envelope, supporting the view of a host origin for the outer envelope (22). The demonstration that a polypeptide from a cyanobacterium is strongly related to the primary and the secondary structure level, as well as in its functional ability to form a high-conductance solute channel to a chloroplastic outer envelope protein, provides support for the prokaryotic nature of this chloroplastic membrane (Fig. 5).

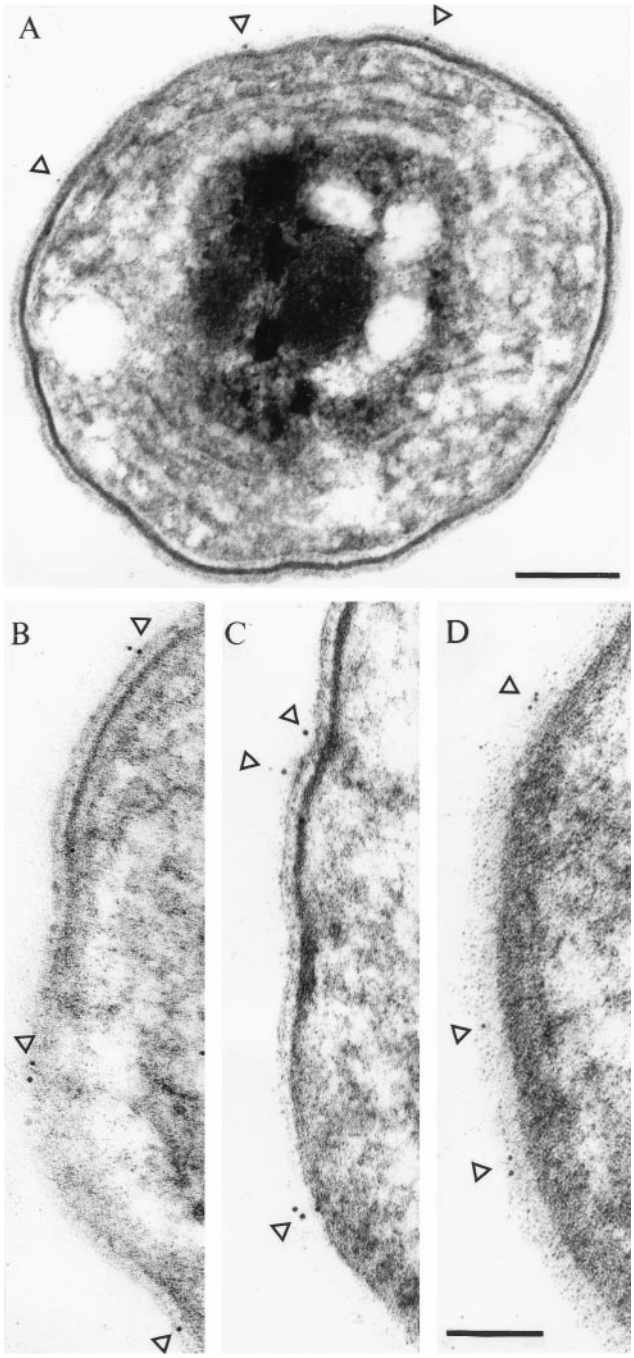

FIG. 4. Immunogold labeling of ultra-thin sections of *Synechocystis* PCC6803 by using αSynToc75. Cells were fixed with 0.5% paraformaldehyd and were embedded in LRWhite by using standard methods. Colloidal gold (5 nm) coupled to the secondary antibody was used for visualization. The position of gold grains is indicated by arrowheads. [Bars = 200 nm (*A*), and 100 nm (*B–D*).] (*A*, ×70,000; *B–D*, ×125,000.)

Second, the protein import machinery (translocon) of chloroplasts has no known functional equivalent in cyanobacteria. Yet, the evolution of such an import system was a prerequisite for the transfer of genes from plastids to the nucleus that occurred early in plant evolution (4). Toc75 and Tic55—a Rieske-type iron–sulfur protein (32)—share sequence similarity with proteins encoded in cyanobacterial genomes and thus apparently were recruited to perform novel protein import functions in the chloroplast membranes. Other components of the chloroplast translocon (Toc86, Toc36, and Tic110) share no similarity with known proteins, suggesting that they arose *de novo* in eukaryotes (5, 6). The ancestral protein import

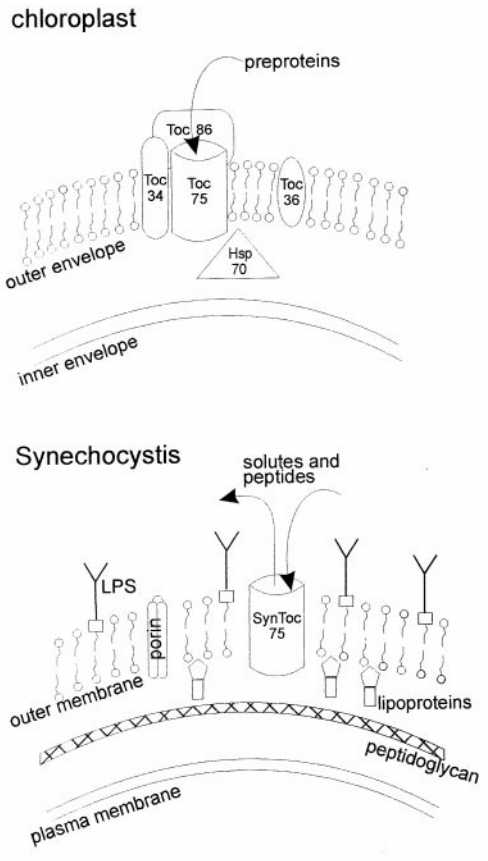

FIG. 5. Working model of the localization and origin of a protein importer in cyanobacteria and pea chloroplasts.

system of plastids was surely more primitive than its contemporary homologue. In principle, early protein import across the inner symbiont membrane may have entailed a simple reversal of the Sec-machinery-mediated protein secretion. In support of such a model is the finding that examples of such reversal can be observed among modern protein transport systems (33, 34). Yet ancestral protein transport across the outer symbiont membrane had no functional homologue, although certain bacteria and some cyanobacteria are known to export toxic peptides (35). In line with this notion is the fact that SynToc75 shows significant homology to other bacterial outer membrane proteins that are involved in polypeptide export: e.g., the hemolysine transporter ShlB from *Serratia marcescens* (ref. 36; accession no. M22618), HecB from *Erwinia chrysanthemi* (ref. 37; accession no. L39897), HpmB from *Proteus mirabilis* (ref. 38; accession no. M30186), or FhaC from *Bordella pertussis* (ref. 39; accession no. X64876) or the activation/secretion protein from *Edwardsiella tarda* (ref. 40; accession no. D89876). These proteins allow the permeation of polypeptides from the intermembrane space into the environment whereas Toc75 functions in the reverse direction. In general, solute channels or facilitators do not show a pronounced directionality in permeation. Unidirectionality of protein translocation could have been achieved by the addition of high affinity binding components to the translocation complex and a precursor protein recognition site to the cytosolic face of the Toc75 channel (9, 10, 41). No evidence exists regarding whether Toc75 operates only in one direction—i.e., anterograde transport—or whether it can function *in vivo* also in retrograde polypeptide transport.

SynToc75 also shares significant homologies to a class of outer membrane proteins from Gram-negative bacteria that represent major surface antigens but whose physiological function remains to be established: e.g., OMP1 from *Brucella*

abortus (S. W. Bearden and T. A. Ficht, direct submission to the National Center for Biotechnology Information databank;accession no. U51683), Omp85 from *Neisseria gonorrhoeae* (ref. 42; accession no. AF021245), D15 from *Haemophilus influenza* (S. M. Loosmore, Y. Yang, D. C. Coleman, J. M. Shortreed, D. M. England, and M. H. Klein, direct submission to the National Center for Biotechnology Information databank; accession no. U60834) or Oma87 from *Pasteurella multocida* (ref. 43, accession no. U60439).

Polyamines were transported by the SynToc75 channel, as proven by means of biionic measurements. The observed change of the E_{rev} on addition of positively charged peptides also leads to the conclusion that these peptides are translocated. The slow permeation of these peptides (as compared with KCl) then leads to the observed current reductions. Furthermore, the SynToc75 shows a remarkable affinity for these peptides, which already are inducing effectively a current reduction at nanomolar concentrations. This high selectivity and permeation of polycations and peptides by the SynToc75 channel is most likely related to its *in vivo* function (see above).

The similarities between Toc75 and SynToc75 displayed on an electrophysiological level are striking. Toc75 also constitutes a cation selective channel (13), and, when reconstituted as described here for SynToc75, it also has a main conductance of 400 ± 32 pS in symmetrical 250 mM KCl ($n > 20$) (S.H. and R.W., unpublished work). Toc75 shows an open probability that is highest at $V_m = 0$ mV. The electrophysiological properties of the mitochondrial outer membrane preprotein translocation channel Tom40 have been characterized in some detail (13, 29, 44). All three proteins have a slight selectivity for cations, interact specifically with positively charged peptides and polyamines, and share a pore diameter $\approx$20 Å. The underlying mechanism of how a polypeptide permeates through the protein translocation channel seems conserved in these cases.

The SynToc75 protein seems to be an essential gene product because it cannot be knocked out completely from the multicopy genome without loss of viability (K. Keegstra, personal communication). We conclude, therefore, that the functional properties, sequence conservation, and localization of SynToc75 in the outer cyanobacterial membrane suggest that it is an excellent candidate for a protein translocation pore and that it may have played an integral role in the origin of modern chloroplast protein import (Fig. 5).

We thank R. J. Berzborn for a gift of ATPase antiserum and L. Heins and A. Hansel for continuous advice and support. We thank W. Martin for critical reading of the manuscript. The research was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Human Frontier Science Program.

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