Chloroplast SecY Is Complexed to SecE and Involved in the Translocation of the 33-kDa but Not the 23-kDa Subunit of the Oxygen-evolving Complex*

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SecY is a component of the protein-conducting channel for protein transport across the cytoplasmic membrane of prokaryotes. It is intimately associated with a second integral membrane protein, SecE, and together with SecA forms the minimal core of the preprotein translocase. A chloroplast homologue of SecY (cpSecY) has previously been identified and determined to be localized to the thylakoid membrane. In the present work, we demonstrate that a SecE homologue is localized to the thylakoid membrane, where it forms a complex with cpSecY. Digitonin solubilization of thylakoid membranes releases the SecYE complex in a 180-kDa form, indicating that other components are present and/or the complex is a higher order oligomer of the cpSecYE dimer. To test whether cpSecY forms the protein-conducting channel of the thylakoid membrane, translocation assays were conducted with the SecA-dependent substrate OE33 and the SecA-independent substrate OE23, in the presence and absence of antibodies raised against cpSecY. The antibodies inhibited translocation of OE33 but not OE23, indicating that cpSecY comprises the protein-conducting channel used in the SecA-dependent pathway, whereas a distinct protein conducting channel is used to translocate OE23.

Thylakoid membranes consist of proteins synthesized by both nuclear and chloroplast genomes. Nuclear encoded thylakoid proteins are first targeted to the chloroplast by means of the transit peptide, which initiates the translocation of the protein across the envelope membranes into the stroma (1). Translation initiation of chloroplast encoded thylakoid proteins appears to occur in the stroma (2, 3), and then synthesis appears to continue on thylakoid bound ribosomes through a co-translational targeting mechanism (4). Considerable progress has been made in defining mechanisms by which nuclear encoded thylakoid proteins insert or translocate posttranslationally across the membrane. One class of proteins insert into the membrane in the absence of an energy supply, soluble factors, or membrane components (5–8). A second class of proteins does not require any soluble factors but requires a trans-thylakoid pH gradient and the membrane protein encoded by the gene hcf106 (9–13). A third class of proteins requires ATP and a chloroplast homologue of the bacterial protein, SecA (14, 15). Finally, a fourth class of proteins requires GTP (16), chloroplast homologues of the bacterial proteins SRP54† (17, 18) and FtsY,‡ and a novel protein, cp SRP43 (19, 20). Little is known about the targeting of chloroplast encoded proteins; however, it is likely that they share many of the same translocation components described above (21–24). In two of the cases mentioned above, no soluble factors are required (6, 10); in two other cases, reconstitution has been achieved in the presence of purified soluble components instead of stroma (14, 22) thereby defining the minimum soluble-factor requirements. However, membrane components remain to be elucidated for the ΔpH, cpSec, and cpSRP pathways.

Protein export across the bacterial inner membrane is catalyzed by a membrane-embedded translocation apparatus consisting of SecY, SecE, SecG, SecF, SecD, and YajC in conjunction with the peripheral protein, SecA (25, 26). The essential core of the translocase is SecYSecE and SecA (27, 28). SecE/Y forms a transmembrane channel through which the exported protein is threaded (29). SecA is thought to act like a piston, pushing the protein through the membrane channel (30, 31). Bacteria also contain an SRP, and recently it was shown that polytopic membrane proteins are dependent on this complex for insertion into the cytoplasmic membrane (32). Furthermore, it was shown that the Sec translocase is utilized in the bacterial SRP pathway. Thus, the SRP- and SecY-dependent pathways converge at SecY (33).

Bacteria also contain a pathway for exporting proteins across the inner membrane independently of SecY (34). Proteins that utilize this pathway have a twin arginine motif at the N terminus (34, 35), as do proteins that utilize the ΔpH pathway in chloroplasts (36). After Hcf106 was identified as a membrane component of the ΔpH pathway (13, 21), it became clear that two bacterial homologues, now designated TatA and TatE, also exist (13, 37, 38). Deletion of tatA/E inactivates transport of proteins containing a twin arginine motif but has no effect on Sec-dependent proteins (37). Thus, it was shown that the ΔpH pathway first described in chloroplasts also exists in bacteria (13, 34, 37, 38).

In addition to SecA, chloroplasts contain a thylakoid protein related to SecY (39). However, the cpSec translocase is largely uncharacterized. It has been assumed, although it remains to be shown, that cpSecY is part of the translocase that translo-

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§ The abbreviations used are: SRP, signal recognition particle; cpSRP, chloroplast SRP; OE33, intermediate form of the 33-kDa oxygen evolving protein; pOE23, precursor of the 23-kDa oxygen evolving protein; chl, chlorophyll; HM buffer, 10 mM Hepes-KOH, pH 8.0, 5 mM MgCl₂.

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cates SecA-dependent substrates. Furthermore, it is not known whether there is convergence of the SRP- and ΔH-dependent targeting pathways at the level of SecY. Genetic experiments with maize mutants support the idea of pathway convergence, as SecY mutants have a more severe phenotype than SecA/Hcf106 double mutants (40). In this report, we have characterized a putative cSpSecE homologue, and we establish that this protein indeed is a chloroplast protein that forms a complex with cSpSecY. Furthermore, we have raised antibodies against cSpSecY and have used these antibodies to address whether SecA and ΔH-dependent proteins both utilize SecY for their translocation across thylakoid membranes.

EXPERIMENTAL PROCEDURES

Arabidopsis thaliana (ecotype Columbia) was grown in a growth chamber in a 16 h light/22 °C temperature versus 8 h dark/18 °C temperature cycle. The light intensity during the light period was 60 μE m⁻² s⁻¹. Digitonin (Calbiochem, high purity) was solubilized as a 10% stock solution in boiling ddH₂O and kept at 95 °C for 15 min. After cooling the solution was spun for 10 min in a microcentrifuge, and the supernatant was used as stock solution.

Radiolabeled Arabidopsis cSpSecE precursor (cSpSecE), the intermediate form of wheat OE33 (iOE33), OE23 precursor (pOE23) were prepared by in vitro transcription and translation by using SP6 polymerase and [³⁵S]methionine as described (41).

CpSecE Cloning—The forward primer 5′-CCACATGCTACTAACCAGCGCACAATTCT-3′ and the reverse primer 5′-CCCAAGCTTCCATCACATGCTGAAGATCTTGAAAC-3′ were used to amplify the gene encoding cpSecE (Cse) from Arabidopsis genomic DNA by PCR using Pfu polymerase (Stratagene). To enhance radiolabeling, the amplified Cse PCR product was designed to contain two additional methionine residues at the C terminus. The PCR product was digested with A III and HindIII and cloned into the Nool-HindIII site of the translation vector pGem43S6.NcolI (17), resulting in the plasmid pGem44SS6.NcolIcpSecE.

For overexpression of cSpSecE, a N-terminal hexahistidine-tagged version was constructed. cSse was amplified from pGem43S6.SNcolEpSecE by using the forward primer 5′-CCACATGCTACTAACCAGCGCACAATTCT-3′ and the reverse primer 5′-CCGATGCCATATGGTACACTACGCACAATTCT-3′. The PCR product was digested with HindIII and BamHI and cloned into the HindIII-BamHI site of the expression vector pQE30 (Qiagen). The resulting plasmid (pQEm30cpSecE) was transformed into S GI3009 cells.

Antibodies and Immunoblot Analysis—SG13009 cells containing pQEm30cpSecE were grown to an A₅₆₀ of 0.6 and incubated with 1 mM isopropyl-β-D-thiogalactoside overnight. Cells were harvested, frozen and lyzed in Buffer B (8 mM urea, 100 mM Na₂HPO₄/NaH₂PO₄, 10 mM Tris, pH 8.0) + 1 mM phenylmethylsulfonyl fluoride. The histidine-tagged cSpSecE was bound to Ni²⁺-NTA agarose; the column was washed three times with Buffer B and one time with Buffer C (8 mM urea, 100 mM Na₂HPO₄/NaH₂PO₄, 10 mM Tris, pH 6.3) and eluted with Buffer C + 250 mM imidazole. The eluted cSpSecE was further purified by SDS-polyacrylamide gel electrophoresis. The major 26-kDa band was excised from acrylamide gels and used to raise antibodies in chicken (Cocalico Biologicals, Inc., Reamstown, PA).

Antibodies against cSpSecE were raised in rabbits injected with the synthetic peptide CCKNIEFYELDKYDP, corresponding to the C terminus of Arabidopsis cSpSecE, fused to a keyhole limpet hemocyanin.

Immunoblot analysis was done as described in Ref. 42. For cpSecE detection, crude antiserum was used at a dilution of 1:750. For cpSecY detection, the IgG fraction purified by ammonium sulfate precipitation, the IgG fraction purified by ammonium sulfate precipitation, the IgG fraction purified by ammonium sulfate precipitation, the IgG fraction purified by ammonium sulfate precipitation.

RESULTS

Arabidopsis Contains a Homologue of Bacterial SecE—SecE is an essential protein in bacteria (47, 48). Most forms of SecE contain a single transmembrane domain at the C terminus, unlike Escherichia coli, which contains three transmembrane domains (49). The highest sequence conservation between homologues occurs at the cytoplasmic domain just preceding the transmembrane domain (49, 50). Mutational analysis in E. coli has revealed that the conserved region followed by a generic transmembrane domain is essential for SecE function (50, 51).

Recently, Bevan et al. (52) deposited into the GenBank data base, 1.9 mB of contiguous sequence from chromosome 4 of Arabidopsis. They noted that one of the hypothetical open reading frames has similarity to SecE preprotein translocase (GenBank accession number, Z97337). Fig. 1 shows an alignment of the Arabidopsis hypothetical protein and bacterial SecE sequences. The putative protein most resembles SecE from Thermotoga maritimus, in which the overall similarity is 28%, and similarity is 69% between residues 111 and 173. Like other SecE proteins, the Arabidopsis sequence predicts a protein with a single transmembrane domain at the C terminus with type II topology.

Arabidopsis SecE Is a Chloroplast Protein—The putative SecE protein is predicted to have a chloroplast transit peptide with a processing site between residues 39 and 39 based on the ChloroP transit peptide prediction program (53). To test this prediction, radiolabeled putative SecE protein (Fig. 2, lane 1) was incubated with isolated pea chloroplasts for 30 min. Non-imported protein was degraded by protease treatment, and chloroplasts were fractionated into stroma and thylakoids. As shown in Fig. 2, lane 3, a smaller, protease-resistant 16-kDa protein was present in the stroma fraction consistent with the size of the product predicted by ChloroP (15 kDa).

That the putative SecE clone encodes a chloroplast protein was further established by immunoblot analysis. Antibodies that were raised against recombinant protein expressed from the putative SecE clone co-reacted with a 16-kDa thylakoid protein that had the same apparent molecular weight as the
Fig. 1. Alignment of *A. thaliana* cpSecE with bacterial SecE sequences. Sequences of SecE homologues from *Thermotoga maritimus*, *Bacillus subtilis*, *Synechocystis* sp. strain PCC 6803, and *Staphylococcus aureus* were aligned to *A. thaliana* cpSecE sequence using Clustal W 1.7 and shaded with Boxshade. Residues conserved in all sequences are shaded in black, and residues conserved in four or five sequences are shaded in gray. Predicted transmembrane domains are underlined. The putative cleavage site for the stromal processing peptidase is indicated by an arrow.

| A.th. | S.coli | B.s. | S.au. | Th.mar. | S.ep. |
|-------|--------|------|-------|---------|-------|
| 91    | 47     | 1    | 1     | 1       | 1     |
| ISAIQGIEIKKANQUSRTABEDFGHPQAIGKVEIADFQGALNITIFNHIQAVF | NAQIGIAKQISRTAEESQPEQMCHIGIAKVEIADFQGALNITIFNHIQAVF | NAIKQGIEIKKANQUSRTABEDFGHPQAIGKVEIADFQGALNITIFNHIQAVF | NAQIGIAKQISRTAEQPEQMCHIGIAKVEIADFQGALNITIFNHIQAVF | NAQIGIAKQISRTAEQPEQMCHIGIAKVEIADFQGALNITIFNHIQAVF | NAIKQGIEIKKANQUSRTABEDFGHPQAIGKVEIADFQGALNITIFNHIQAVF |

Fig. 2. CpSecE is an integral membrane protein of the thylakoid membrane. *A*, in vitro translated [35S]methionine-labeled cpSecE precursor (pcpSecE) (lane 1) was imported into isolated pea chloroplasts. After the import reaction, chloroplasts were thermoslysin-treated, repurified by centrifugation over a Percoll cushion, and separated into the stromal compartment (str.) (lane 2) and thylakoid membranes (thyl.) (lane 3). *B*, Arabidopsis thylakoid membrane proteins (equivalent to 20 μg of chl) were separated by SDS-polyacrylamide gel electrophoresis (15% acrylamide) and subjected to immunoblot analysis with anti-cpSecE antibodies (lanes 4–6) and preimmune serum (PI-serum) (lanes 7–9). Integral membrane proteins (P) (lanes 5 and 8) were separated from peripheral membrane proteins (S) (lanes 6 and 9) by incubating thylakoid membranes with 0.1 N NaOH for 15 min at 4 °C followed by a 5 min centrifugation in a microcentrifuge.

The imported protein (Fig. 2, lanes 3 and 4). The protein could not be extracted from the thylakoid membrane by incubation of the membranes with 0.1 N NaOH (Fig. 2, lanes 5 and 6), indicating that cpSecE is an integral membrane protein, as predicted from the sequence analysis (Fig. 1). Together, these experiments indicate that the putative SecE protein is encoded as a precursor containing a functional chloroplast transit peptide and the mature protein is localized in the thylakoid membrane.

*cpSecE Is Bound to cpSecY*—To test whether cpSecE forms a complex with cpSecY, we examined whether the two proteins co-chromatographed and co-immunoprecipitated after detergent solubilization of thylakoid membranes. To facilitate this analysis, polyclonal antibodies were raised against a C-terminal peptide of Arabidopsis cpSecE. These antibodies reacted with a single protein in wheat germ translation extracts containing cpSecY precursor (Fig. 3) but did not cross-react with any proteins in wheat germ extract (data not shown) and reacted with a single 44-kDa protein found in the thylakoid membrane fraction after alkali extraction (Fig. 3), consistent with the fact that SecY is an integral membrane protein with 10 putative transmembrane helices (54).

Thylakoid membrane proteins were solubilized with 2% digitonin, at approximately 70% efficiency, and the extracted proteins were separated by gel filtration chromatography. The relative amount of cpSecY and cpSecE in the various fractions was determined by immunoblot analysis using antibodies against cpSecY and cpSecE, respectively. As shown in Fig. 4, both proteins co-eluted in a single peak as higher molecular mass species of approximately 180 kDa. These data suggest that either other subunits are present or multiple copies of SecY and SecE are present in each complex. Furthermore, these data indicate that most, if not all, cpSecY and cpSecE are associated.

A similar conclusion is reached by the co-immunoprecipitation experiment. Antibodies raised against cpSecY were used to immunoprecipitate the digitonin-solubilized complex, and cpSecY and cpSecE in the supernatant and precipitate were detected by immunoblot analysis. As shown in Fig. 5, cpSecY and cpSecE were quantitatively removed from the solubilized thylakoid proteins by the anti-cpSecY antibody and were recovered in the immunoprecipitate, whereas none of the proteins were precipitated by an irrelevant antiserum. cpSecY/E complex was not stable in 1% octylglucoside/dodecylmaltoside (1:1) or 1% Triton X-100 (data not shown). Where reconstitution of the translocase has been successful, digitonin has also been the detergent of choice (25, 55).

*cpSecY Is Sensitive to Trypsin*—It is well established that trypsin treatment of thylakoid membranes inhibits the transproteolytic activity of both SecY and SecE, and this inhibition is prevented by subsequent treatment with 0.1 N NaOH (Fig. 3). That trypsin treatment also inhibits import of precursor proteins is demonstrated in Fig. 6. The protein import reaction was performed with thylakoid membranes in the absence or presence of trypsin, and import activity was determined by immunoblot analysis using antibodies against cpSecY or cpSecE. The results show that trypsin treatment inhibits import of both cpSecY and cpSecE precursors, and that NaOH treatment of the trypsin-treated membranes restores import activity to control levels. These data suggest that trypsin treatment inhibits import of both cpSecY and cpSecE precursors by cleaving a single 44-kDa protein found in the thylakoid membrane.
CpSecY Translocates OE33 but Not OE23 across the Thylakoid Membrane. To address whether cpSecY is a component of the translocation machinery, we examined the ability of cpSecY to mediate the translocation of substrates on the Sec or ΔpH pathways. Fig. 6 reveals that the presence of cpSecY in the translocation assay is indeed required for translocation. The results indicate that cpSecY is an essential component of the translocation machinery.

FIG. 6. CpSecY is sensitive to trypsin digestion. Thylakoid membranes (2.5 μg of chlorophyll) were digested with the indicated amounts of trypsin for 30 min on ice in 50 mM Hepes-KOH, pH 8.0, 330 mM sorbitol. After stopping the reaction by the addition of 2 mM phenylmethylsulfonyl fluoride, the membranes were washed in 50 mM Hepes-KOH, pH 8.0, 330 mM sorbitol, 25 mM EDTA. The membrane proteins were solubilized in 4× sample buffer for 30 min at 37 °C and analyzed by immunoblot using antibodies against cpSecY.

This work clearly establishes the existence of a chloroplast localized SecE protein that is tightly associated with cpSecY. Thus, we can conclude that at a minimum, chloroplasts contain all the core elements of the Sec translocon: SecY, SecA, and SecE. The core elements of the Sec related translocase of the ER include Sec61 and Sec61β (49). Three to four of these heterotrimers form a pore-like structure in the ER (57, 58) that remains stable after solubilization with digitonin. Solubilization of the thylakoid membrane using the same detergent releases a 180-kDa complex that contains SecY and SecE. This complex may consist of multiple copies of SecY/E dimers forming a ring-like structure, like those seen after purification of the ER complex, and may also include additional subunits, e.g., a SecG homologue. An important goal for future work will be to establish the subunit composition and stoichiometry of the proteins in the complex.

Several lines of evidence have indicated that there are mul-
multiple pathways for targeting proteins to the thylakoid membrane (reviewed in Refs. 1, 59, and 60). First, in vitro studies indicated that substrates fall into distinct classes with regard to their ability to act as competitors of protein targeting to the thylakoid membrane (11). Second, each of these classes has distinct energetic requirements for protein targeting (10, 16). Third, genetic studies largely corroborate the in vitro studies; loss of Hcf106, SecA, or cpSRP43 resulted in selective reductions in the proteins shown to be substrates for the ΔpH, Sec, and cpSRP pathways, respectively (12, 20, 61, 62). However, these studies did not exclude the possibility that SecY was common to all pathways. It has been observed that the targeting information specifying the ΔpH versus the Sec pathway is present in the transit peptide (36, 63–66), and when a Sec transit peptide is used to direct a ΔpH protein to the Sec translocase, the protein fails to be translocated across the thylakoid membrane (63, 65). Based on these findings, it has been postulated that substrates using the ΔpH pathway are unable to translocate through the Sec system, and hence a distinct translocase may be employed by the ΔpH pathway (65). The results from this paper clearly establish the validity of this hypothesis, as convergence at the level of the Sec translocase does not occur for the ΔpH pathway.

To test whether convergence occurs for the cpSRP pathway, considerable effort was made to reconstitute LHCP integration in Arabidopsis thylakoids supplemented with pea stroma. Unfortunately, thylakoids that translocated OE33 and OE23 failed to integrate LHCP. Arabidopsis thylakoids added to pea stroma efficiently inhibited LHCP integration into the pea thylakoids, and the inhibition could be overcome by treatment of the Arabidopsis thylakoids with alkylating agents. Thus, it appears that the Arabidopsis thylakoids possess an inhibitory activity that may act on either the pea stroma or thylakoids to prevent LHCP integration.

Plants that lack cpSRP are viable and contain elevated levels of cpSecY, suggesting the possibility that the increases observed in the mutant compensate for the loss of targeting efficiency resulting from the absence of cpSecY. Alternatively, the elevated level of cpSecY could indicate that cpSecY forms an alternative pathway for the cpSRP-dependent substrates. However, if the cpSRP delivers its substrate to cpSecY, it must use the translocase independently of SecA, as LHCP integration is not inhibited by azide, which inhibits SecA activity (11). LHCP integration is not competed by SecA-dependent substrates (11), and LHCP levels are not reduced in SecA mutants (12). These observations suggest the possibility that the SecY/E core has activity in the absence of SecA. Whereas loss of either SecA and SecY is lethal, the phenotype of the SecY mutant is more severe than the SecA mutant or even the SecA/Hcf106 double mutant (40). This observation is also consistent with the notion that cpSecY has a residual activity in the absence of SecA.

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