Identification of the SecA Protein Homolog in Pea Chloroplasts and Its Possible Involvement in Thylakoidal Protein Transport*

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Recently, we identified the SecA and SecY proteins in the cyanobacterium Synechococcus PCC7942. Antibodies raised against cyanobacterial SecA specifically reacted with a 110-kDa protein of pea chloroplasts, suggesting the presence of SecA in higher plant chloroplasts. A part of the pea SecA cDNA was polymerase chain reaction-amplified with degenerated oligonucleotide primers and with pea cDNA as a template. The deduced amino acid sequence shows 62% identity with cyanobacterial SecA and 52% identity with Escherichia coli SecA. Antibodies raised against the pea SecA fragment, which was expressed in E. coli cells from the obtained polymerase chain reaction-amplified cDNA, reacted with the 110-kDa chloroplast protein; the 110-kDa protein was mainly found in the stroma but partly in the thylakoid membrane. The anti-pea SecA IgG inhibited the in vitro import of the 33-kDa protein of the oxygen-evolving complex, but not of the 23-kDa protein of the oxygen-evolving complex, into thylakoids. These results suggest that SecA facilitates transport of a subset of thylakoid lumenal proteins including the 33-kDa protein into thylakoids. We propose that a bacterial-type Sec protein-dependent transport system operates for protein transport into thylakoids in higher plant chloroplasts.

Biogenesis of the photosynthetic apparatus in higher plant chloroplasts requires the assembly in a coordinated fashion of the thylakoid proteins encoded by the two distinct genetic systems, the plastid and the nuclear genomes. The nuclear encoded thylakoid proteins are synthesized in the cytosol as precursors with an amino-terminal extension called a transit peptide, imported into the chloroplast stroma and further directed across the thylakoid membrane (1, 2). The transit peptide of the thylakoidal protein transport.

The results of precursor competition experiments are consistent with the classification in energy and stromal factor requirements in thylakoidal protein transport. 23K competes for 16K transport, and 33K competes for PC transport, but the two groups do not compete with each other (5). Sorting between the two pathways for transport into the thylakoidal lumen is likely mediated by the thylakoid targeting domain of the transit peptides but not by passenger proteins (6, 7).

The bulk of evidence suggests a eubacterial or cyanobacterial origin for plastids; chloroplasts have most likely arisen from endosymbionts that resembled cyanobacteria and harbored in an originally non-photosynthetic host (8, 9). We previously cloned the secA and secY genes (whose counterparts in Escherichia coli encode components of the protein export machinery in the cytoplasmic membrane) from the genome of the cyanobacterium Synechococcus PCC7942 (10, 11). The cyanobacterial SecY protein and the membrane-bound form of the cyanobacterial SecA protein were found in the thylakoid membrane as well as the cytoplasmic membrane (11, 12). Possible involvement of the Sec proteins in protein translocation across the cyanobacterial thylakoid membrane prompted us to test if a similar Sec protein-dependent mechanism is in operation for the protein transport into thylakoids in higher plant chloroplasts. This possibility is further supported by the following facts. secA and secY genes have been identified in the plastid genomes of red algae and cyanophytes (13–16). Sodium azide, a potent inhibitor of SecA, specifically inhibits transport of 33K and PC into thylakoids (6, 17). However, there has been no direct evidence so far for involvement of the SecA or SecY proteins in protein translocation across the thylakoid membrane in higher plant chloroplasts.

In the present study, we have immunologically identified a SecA protein homolog in the pea chloroplasts. We have also cloned a part of the pea secA cDNA, which may encode the chloroplast SecA protein. Antibodies raised against the pea SecA protein inhibited translocation of 33K across the thylakoid membrane, suggesting participation of the SecA protein in thylakoidal protein transport.

EXPERIMENTAL PROCEDURES

Materials—Chloroplasts were isolated from 10–15-day-old pea seedlings (Pisum sativum, var. Alaska), which were grown under a 12-h photoperiod according to the published methods (18). Plasmids for the in vitro translocation were constructed with pOEM-4Z (Promega) as an

The abbreviations used are: PC, plastocyanin; PAGE, polyacrylamide gel electrophoresis; 16K, 23K, and 33K, the 16-, 23-, and 33-kDa proteins of the photosystem II oxygen-evolving complex, respectively; PCR, polymerase chain reaction.
expression vector; the coding sequence for the artificial intermediate form of the pea 33K protein was amplified by polymerase chain reaction (PCR) with the forward primer 5'-cgacttcaaatgggctgcttttgta-3' and the reverse primer 5'-cagtgcttacgccatcatggctga-3', using the cDNA synthesized from the pea polyA+ RNA as a template. The coding sequence for the artificial intermediate form of the wheat 33K protein was amplified by PCR with the forward primer 5'-cagtgcttacgccatcatggctga-3' and the universal sequencing primer, using pGEM-4Z: p23K (provided by Dr. Colin Robinson) as a template. In vitro transcription of the constructed genes was performed with the Megascript system (Ambion). The synthesized RNA was purified and then transcribed in the presence of [35S]methionine and [35S]cysteine in a wheat germ cell-free system (19).

In Vitro Import into Pea Thylakoids—Transport of radiolabeled proteins into thylakoids was conducted with chloroplast lysates. Briefly, chloroplast lysates were prepared from intact chloroplasts (2 mg of chlorophyll) by osmotic lysis in 1 ml of the import buffer (10 mM Hepes-KOH, pH 8.0, 27 mM MgCl2, 8 mM ATP, 0.1 mg/ml bovine serum albumin 50 mM Hepes-KOH, pH 8.0, and 330 mM sorbitol, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.

PCR Cloning, Sequencing, and Southern Analysis—Partially degenerated primers were designed on the basis of the reported amino acid sequences of the SecA proteins from various sources as described previously (11). With these primers, PCR was performed according to the methods by Compton (20) using random hexanucleotide-primed pea cDNA as a template. Reaction products of the expected size were isolated and subcloned into pUC119 and sequenced with the Taq dye primer cycle sequencing kit (Applied Biosystems). Southern hybridization was performed for the chromosomal DNA prepared from pea leaves with the fluorescein gene images system (Amersham Corp.).

Production of Antiserum and Preparation of the Anti-SecA Ig—Preparation of antisera against the cyanobacterial SecA protein fragment was described by Nakai et al. (11). The PCR-amplified peptide cDNA fragment was inserted into an E. coli expression vector, pET-21b (Novagen). The overexpressed SecA fragment was purified and used for generation of antibodies in rabbits. The anti-pea SecA IgG was further purified by affinity chromatography through the SecA-conjugated Sepharose 4B (Pharmacia Biotech Inc.) according to the instruction manual. The anti-pea SecA IgG was prepared from immunized sera with Protein A-conjugated Sepharose (Pharmacia).

Miscellaneous Methods—Published procedures were used for recombinant DNA technique (21), suborganellar fractionation of chloroplasts (18), SDS-PAGE, fluorography, and immunoblotting analyses (21). Gel filtration analyses of the chloroplast SecA protein were performed by applying the soluble stromal fraction to a Superose 6 column equilibrated with 20 mM Hepes-KOH (pH 7.4) and 150 mM KCl using a SMART system (Pharmacia). Fluorography and immunoblots were quantified with a PD110 laser densitometer (Molecular Dynamics).

RESULTS

Immunological Detection of a SecA Protein Homolog in the Chloroplast Stroma—We previously identified the SecA homolog in the cyanobacterium Synechococcus PCC7942 and raised antibodies against the cyanobacterial SecA fragment (11). We used these antibodies to test if the plant chloroplast SecA contains any SecA protein homolog. The anti-cyanobacterial SecA IgG, which was affinity-purified with the immobilized cyanobacterial SecA, reacted with SecA from cyanobacteria, as expected, and a 95-kDa protein of E. coli, which probably corresponds to E. coli SecA (Fig. 1, A and B, lanes 2 and 3). The anti-cyanobacterial SecA IgG also recognized a 110-kDa protein in the stromal fraction, which was prepared from the pea chloroplasts (Fig. 1, A and B, lanes 2). When the antibodies were affinity-purified with the immobilized stromal 110-kDa protein, they in turn recognized the cyanobacterial SecA protein (data not shown). These results suggest the presence of SecA homolog in the chloroplast stroma.

PCR Cloning of the secA cDNA Fragment—We designed partially degenerated oligonucleotides corresponding to the conserved amino acid stretches among the previously identified SecA proteins (11, 13, 22–24) and used them as primers in PCR with the pea cDNA as a template; the cDNA libraries were prepared from poly(A)+ RNA isolated from the leaves of pea plants. Subclones harboring the PCR products of the expected size were sequenced and found to encode a part of the secA homologous gene. Southern hybridization analysis confirmed that the obtained secA fragment was derived from the pea genome (data not shown).

The pea cDNA fragment encodes a polyepitope of 276 amino acids, which corresponds to residues 217–509 of the E. coli SecA protein of 901 amino acids. Fig. 2 shows the alignment of the pea SecA fragment with the corresponding regions of the cyanobacterial and E. coli SecA proteins (11, 22). Among the previously identified SecA proteins from various sources, cyanobacterial SecA shows the highest degree of identity (62%) with the pea SecA fragment, whereas E. coli SecA shows 52% identity. The pea SecA fragment is 53 and 51% identical with the plastid-encoded SecA proteins from red alga (16) and chlorophytic alga (13), respectively.

The identified secA cDNA Fragment May Encode a Part of the 110-kDa Chloroplast Protein—We next examined if the obtained secA cDNA fragment may encode a part of the chloroplast protein. The pea secA cDNA fragment was recloned into an E. coli expression vector pET-21b, and the overexpressed SecA fragment, which formed inclusion bodies in E. coli cells, was purified and used for injection into rabbits to raise antiserum. The anti-secA SecA IgG was prepared from the antiserum and affinity-purified with the immobilized pea SecA fragment. The anti-secA SecA IgG specifically reacted with a 110-kDa protein of the pea chloroplasts (Fig. 3A, lane 1), which was apparently identical from its size with the chloroplast protein recognized by the anti-cyanobacterial SecA IgG; the 110-kDa protein was enriched in the intact chloroplast fraction after subcellular fractionation (data not shown). Upon suborganellar fractionation of the chloroplasts, the 110-kDa protein was mainly recovered in the stroma and partly in the thylakoid membrane fractions (Fig. 3A, lanes 2–4). These results suggest that the obtained secA cDNA fragment most likely encodes the 110-kDa protein in chloroplasts.

Apparent molecular mass of the 110-kDa chloroplast protein was analyzed by gel filtration sizing chromatography. The 110-kDa protein was eluted at around the fractions where 232-kDa catalase was eluted, suggesting that the 110-kDa protein exists as a homodimer in the stroma (data not shown). This is consistent with the fact that the soluble cytosolic form of E. coli SecA forms a homodimer (23).

Quantification of the immunoblots allowed us to estimate the
The Anti-pea SecA Antibodies Block the in Vitro Import of the Lumenal Proteins into Thylakoids—Previous works have shown that the artificial intermediate forms of 33K and 23K of the thylakoid luminal proteins can be taken up in vitro by isolated thylakoids (24). The 33K and 23K intermediate forms were translated from the corresponding mRNA in a wheat germ cell-free system and incubated with chloroplast lysates containing thylakoids and the stromal fraction. The 33K and 23K intermediates were converted to mature sized forms that were protected from the externally added protease, indicating the efficient import into the thylakoid lumen (Fig. 4, A and B, lane 1). The presence of sodium azide, an inhibitor of SecA, impaired the import of the 33K intermediate, but not the 23K intermediate, into thylakoids (Fig. 4, A and B, lanes 2 and 3), as reported recently (6, 17).

To study the function of the chloroplast SecA protein directly, we examined the effects of the anti-pea SecA antibodies on the import of the 33K and 23K intermediates into thylakoids. The chloroplast lysates were preincubated with the anti-pea SecA IgG or control IgG and subjected to incubation with the 33K and 23K intermediates. Pretreatment of the chloroplast lysates, i.e. the thylakoids and stromal fraction, with increasing amounts of the anti-pea SecA IgG led to decreased translocation of the 33K intermediate across the thylakoid membrane (Fig. 4A, lanes 4-6). 18 μg of the anti-pea SecA IgG (Anti-SecA) (lanes 4-6, respectively) or control IgG (Preimmune) (lanes 7-9, respectively) for 15 min at 25°C. The artificial intermediates for wheat 23K (panel A) and pea 33K (panel B) were incubated with the chloroplast lysates for 20 min at 25°C in 150 μl of the import buffer. Then a treatment with thermolysin (0.4 mg/ml) was performed for 30 min at 0°C. The thylakoids were reisolated and analyzed by SDS-PAGE followed by fluorography. 23K, 33K, and 33K intermediates or mature form of 23K and 33K, respectively; T, translation product.

FIG. 4. Effects of the anti-pea SecA IgG on in vitro import of 23K and 33K into thylakoids. Chloroplast lysates (0.15 mg of chlorophyll, 75 μl) containing thylakoids and a stromal fraction were preincubated with 0, 10, or 20 μg sodium azide (Azide) (lanes 1-3) or with 18, 35, or 70 μg of the anti-pea SecA IgG (Anti-SecA) (lanes 4-6, respectively) or control IgG (Preimmune) (lanes 7-9, respectively) for 15 min at 25°C. The artificial intermediates for wheat 23K (panel A) and pea 33K (panel B) were incubated with the chloroplast lysates for 20 min at 25°C in 150 μl of the import buffer. Then a treatment with thermolysin (0.4 mg/ml) was performed for 30 min at 0°C. The thylakoids were reisolated and analyzed by SDS-PAGE followed by fluorography. 23K, 23K, 33K, and 33K intermediates or mature form of 23K and 33K, respectively; T, translation product.

To study the function of the chloroplast SecA protein directly, we examined the effects of the anti-pea SecA antibodies on the import of the 33K and 23K intermediates into thylakoids. The chloroplast lysates were preincubated with the anti-pea SecA antibodies, using the anti-pea SecA IgG and subjected to incubation with the 33K and 23K intermediates. Pretreatment of the chloroplast lysates, i.e. the thylakoids and stromal fraction, with increasing amounts of the anti-pea SecA IgG led to decreased translocation of the 33K intermediate across the thylakoid membrane (Fig. 4A, lanes 4-6). 18 μg of the anti-pea SecA IgG led to 60% inhibition of 33K transport and 70 μg of IgG to >90% inhibition. Control IgG prepared from preimmune sera in the same concentration range showed no significant effect on the import of the 33K intermediate into thylakoids (Fig. 4A, lanes 7-9). Interestingly, translocation of the 23K intermediate across the thylakoid membrane was not affected at all by the anti-pea SecA IgG even at the highest concentration (Fig. 4B, lanes 4-9).

In order to eliminate possible artifacts of the antibody inhibition experiments, we performed the following experiments. The stromal fraction was incubated with protein A-Sepharose carrying the anti-pea SecA IgG and subjected to centrifugation. This treatment removed about 75% of SecA from the stromal fraction (Fig. 5A) whereas it hardly affected the amounts of other stromal proteins by judging from the SDS-PAGE gels stained with Coomassie Brilliant Blue (not shown). The stromal fraction depleted of 75% of SecA showed less stimulation activity (about 35%) for the import of...
of the 33K intermediate into thylakoids than the control stromal fraction (Fig. 5B). The good correlation between the amount of SecA in the stromal fraction and the activity of the stromal fraction to stimulate the import of the 33K intermediate suggests that the observed IgG inhibition of the 33K intermediate import reflects the specific interaction of the IgG with the chloroplast SecA in the stroma. Therefore, involvement of the chloroplast SecA protein in the translocation of 33K, but not 23K, across the thylakoid membrane is strongly suggested.

**DISCUSSION**

In the present study, we have identified immunologically the 110-kDa SecA protein homolog in pea chloroplasts and cloned a part of the secA homologous cDNA, which likely encodes the 110-kDa chloroplast SecA protein. While the chloroplast SecA protein was mainly found as a soluble homodimer in the stroma, the remaining small but distinct fraction of SecA was associated with the thylakoid membrane. Since SecA plays a central role in protein transport across the cytoplasmic membrane in *E. coli* (25), the chloroplast SecA protein may also participate in protein transport within chloroplasts. Indeed in the present study, participation of the chloroplast SecA protein in the thylakoidal protein transport was strongly suggested by the inhibition of 33K transport into thylakoids by the anti-pea SecA antibodies. The SecA protein could therefore be the first protein that was found to mediate protein translocation across the thylakoid membrane.

The findings that the inhibitory effects of the anti-pea SecA antibodies were observed only for 33K transport but not for 23K transport suggest that SecA is responsible for only one of the two mechanisms for protein transport into thylakoids; the SecA protein facilitates transport of 33K and probably PC, which requires ATP, stromal factor(s), and the thylakoidal ΔpH and is inhibited by sodium azide (4, 6, 17). The target of sodium azide is probably the chloroplast SecA protein, and since the bacterial SecA protein has an ATPase activity, requirement for ATP in 33K transport may well reflect, at least partly, that for the chloroplast SecA protein. However, this does not rule out the possibility that other ATP-dependent chaperones may also be involved in 33K transport and partly responsible for the requirement for ATP.

Pathway specificity of the thylakoid luminal proteins appears to be determined by the thylakoid-targeting domain of the transit peptides (6, 7). Since genetic and biochemical studies suggested that the bacterial SecA protein interacts directly with signal peptides (25-27), the chloroplast SecA protein may well recognize the thylakoid-targeting domain of the transit peptides and mediate sorting between the two thylakoidal protein transport pathways.

The findings that the chloroplast SecA protein participates in 33K transport suggest that the bacterial-type Sec protein-dependent system for protein transport is in operation for a subset of thylakoid luminal proteins including 33K in higher plant chloroplasts. Since cyanobacteria as well as chloroplasts of plants and algae have 33K and PC, 33K (and probably PC) may have employed the Sec protein-dependent protein transport system inherited from the evolutionary progenitor, the cyanobacterial prokaryotes. On the other hand, 23K and 16K, which are found only in the chloroplasts but not in cyanobacteria, may have perhaps developed a new, non-conservative protein transport pathway (5). Cloning of the entire secA gene of pea chloroplasts and identification of other Sec proteins including SecY and SecE in higher plant chloroplasts are under way.

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**FIG. 5.** Depletion of SecA from the stromal fraction leads to the inhibition of the 33K import into thylakoids. Chloroplast lysates (0.15 mg of chlorophyll) were fractionated into the thylakoid and the stromal fractions by centrifugation (4,000 × g for 10 min at 4°C). The thylakoids were washed twice with the buffer containing 10 mM Hepes-KOH, pH 8.0, 10 mM MgCl₂, and kept on ice until its use. The stromal fraction was incubated for 30 min at 25°C with 15 μl of Protein A-Sepharose (Pharmacia) carrying 300 μg of the anti-pea SecA IgG (lane 1) or control IgG prepared from preimmune sera (lane 3) or with 15 μl of Protein A-Sepharose alone (lane 1). The Sepharose beads were then removed by centrifugation, and the supernatants were combined with the washed thylakoids. The resulting mixtures of the thylakoids and the stromal fractions were analyzed for its effects on in vitro import of 33K as described in Fig. 4 (panel B) and for immunoblotting with the anti-pea SecA antibody (panel A).