SecA Homolog in Protein Transport Within Chloroplasts: Evidence for Endosymbiont-Derived Sorting

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The SecA protein is an essential, azide-sensitive component of the bacterial protein translocation machinery. A SecA protein homolog (CPSecA) now identified in pea chloroplasts was purified to homogeneity. CPSecA supported protein transport into thylakoids, the chloroplast internal membrane network, in an azide-sensitive fashion. Only one of three pathways for protein transport into thylakoids uses the CPSecA mechanism. The use of a bacteria-homologous mechanism in intrachloroplast protein transport provides evidence for conservative sorting of proteins within chloroplasts.

Nuclear-encoded proteins destined for the thylakoid lumen are made in the cytosol as larger precursors with bipartite NH_2-terminal extensions that coordinate a two-step localization process (*1*). Stroma-targeting domains govern import across the chloroplast envelope membranes and are removed by a protease in the stroma; lumen-targeting domains further direct the resulting intermediates across the thylakoid membrane. Because of the endosymbiotic origin of chloroplasts from an ancestral cyanobacterium, it has been speculated that protein transport into plant thylakoids resembles protein export across the cytoplasmic membrane of prokaryotes (*2*). Evidence suggesting a thylakoid system homologous to the general pathway for bacterial protein export (*3*) has accumulated. First, the lumen-targeting domains of thylakoid lumenal precursor proteins are similar to prokaryotic signal sequences (*4*) and function as such in *Escherichia coli* (*5*). Second, the cleavage specificity of the thylakoidal peptidase that removes lumen-targeting signals is identical to that of the *E. coli* signal peptidase (*6*). Third, *secA* and *secY* gene homologs are present in the plastid genomes of various algae (*7, 8*).

Azide is an inhibitor of bacterial protein export owing to its interference with the SecA translocation adenosine triphosphatase (*9*). It was previously reported (*10–12*) that azide inhibits thylakoid transport of certain lumenal proteins. When plastocyanin (PC) and the 33-kD subunit of the oxygen-evolving complex (OE33) were imported into chloroplasts in the presence of sodium azide, intermediates accumulated, indicating that thylakoid transport was inhibited (Fig. 1A). In contrast, the thylakoid localization of the 23-kD and 17-kD subunits of the oxygen-evolving complex (OE23 and OE17) was unaffected by azide; only mature forms of the proteins accumulated. Sodium azide had similar effects on protein transport when conducted with isolated thylakoid membranes (Fig. 1B). Integration of a membrane protein, the light-harvesting chlorophyll a/b protein (LHCP), was also unaffected by sodium azide (Fig. 1B). These results confirm and extend previous studies (*10–12*) of proteins that exhibit azide-sensitive transport.

There are at least three pathways for protein transport into thylakoids (*10*); PC and OE33 are transported on one pathway. Azide inhibition of transport of PC, OE33, and a chimeral protein that has the OE33 lumen-targeting sequence and the OE17 mature sequence (*12*) indicates that azide sensitivity is a pathway characteristic and suggests that this pathway uses a mechanism similar to that of the SecA-dependent system in bacteria. PC and OE33 transport also exhibit similar energy requirements to those of SecA-dependent transport in *E. coli* (*13*).

To identify a chloroplast SecA homolog, we prepared an antibody to a peptide that is conserved among bacterial SecA proteins and deduced algal SecA protein sequences. This peptide, from residues 95 to 222 of the deduced *Pavlova lutherii* gene product (*7*), contains sites identified in *E. coli* SecA for adenosine triphosphate (ATP) binding and azide sensitivity suppression (*14*) and a site in *Bacillus subtilis* SecA necessary for functional complementation of *E. coli* SecA mutants (*15*). Antibody to this peptide reacted specifically with the 102-kD (*3*) SecA protein from *E. coli* (Fig. 2A) and with a protein of ~110 kD from pea chloroplasts. The chloroplast SecA (CPSecA) was mainly in the stromal fraction, but was also present in trace amounts in thylakoid and envelope membrane fractions.

CPSecA was purified from stromal extract (SE) by a combination of conventional and high-performance chromatography steps (*16*) with immunoblotting as an assay. Purified CPSecA was homogeneous as assessed by Coomassie blue staining of the SDS-polyacrylamide gel (Fig. 2B). From an immunoblotting dilution series it was estimated that about 0.4% of the stromal protein was CPSecA, that is, there was a 250-fold purification. This amount of CPSecA in chloroplasts is comparable to that of SecA in *E. coli*, in which SecA represents 0.25 to 0.5% of total cellular protein (*17*). Purified CPSecA eluted from a Superose 6 gel filtration column as a homodimer with a size of 200 to 250 kD (*18*). *Escherichia coli* SecA has been shown to function as a homodimer (*19*).

CPSecA was tested for its ability to support protein transport into isolated thylakoids. Transport of PC and OE33 requires SE and does not occur in its absence (*13*,

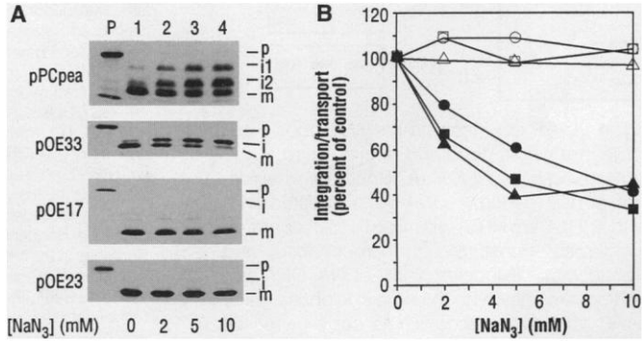

Fig. 1. Effect of azide on protein transport into thylakoids assessed with intact chloroplasts during import (**A**) or with isolated thylakoids during transport (**B**). Radiolabeled precursors (p) of PC, OE33, OE23, and OE17 from pea were assayed for import and subsequent localization with intact chloroplasts (*25*) in the absence or presence of increasing concentrations of sodium azide. Lane P contains the radiolabeled precursor proteins added to each set of assays (lanes 1 to 4). Accumulation of the mature forms (m) of the proteins indicates that the imported proteins have been transported into thylakoids. Accumulation of intermediate species (i) indicates that thylakoid transport of the imported proteins was inhibited. The intermediates i1 and i2 accumulate when thylakoid localization of PC from pea is inhibited (*10*). (B) Transport of PC (■), OE33 (▲), OE23 (□), and OE17 (△) from pea and PC (●) from *Arabidopsis thaliana* and integration of pLHCP (○) from pea into thylakoids (*25*) were carried out with chloroplast lysates in the absence or presence of increasing concentrations of sodium azide.

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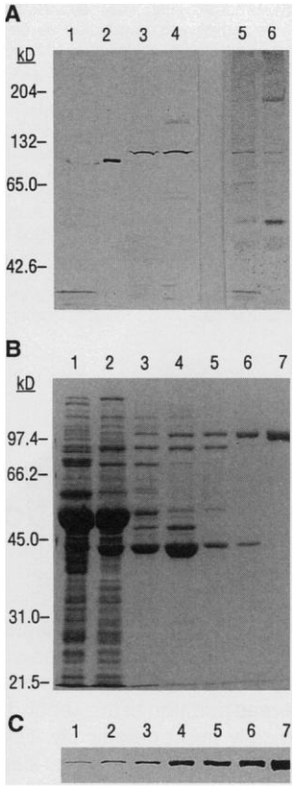

Fig. 2. Detection and purification of a SecA homolog from pea chloroplasts. (**A**) Immunoblotting of *E. coli* and chloroplast protein with an antibody to a conserved peptide deduced from an algal *secA* gene (*26*). Lane 1, total *E. coli* protein from strain TB1 (60 μg); lane 2, soluble extract of *E. coli* cells BL21(λDE3) harboring the SecA-overexpression plasmid pT7-secA (2 μg) (*27*); lane 3, total chloroplast protein (25 μg); lane 4, stromal protein (11 μg); lane 5, thylakoid protein (11 μg); and lane 6, total envelope protein (14 μg). (**B**) Protein profile of the Coomassie blue–stained gel of the CPSecA-containing fraction from each purification step. Lane 1, total stromal protein (12.5 μg); lane 2, ammonium sulfate precipitation (11.5 μg); lane 3, DEAE-Sepharose ion exchange (3.5 μg); lane 4, hydroxylapatite chromatography (5 μg); lane 5, Sephacryl S-300 gel filtration (1.75 μg); lane 6, Mono-Q ion exchange (1 μg); and lane 7, Hydropore hydrophobic interaction chromatography (2 μg). (**C**) An immunoblot of the samples shown in (B).

20) (Fig. 3). CPSecA could replace SE for PC and OE33 transport (Fig. 3). A Sephacryl S-300 gel filtration fraction that contained more stromal protein but was devoid of CPSecA could not support transport (Fig. 3), which demonstrates the specificity for CPSecA. Integration of LHCP also requires the presence of stromal protein (or proteins) (*21*), but CPSecA was unable to replace SE for LHCP integration (Fig. 3), consistent with evidence that LHCP uses a separate pathway for integration (*10*). CPSecA had no effect on OE23 and OE17 transport. Transport of these proteins into the lumen occurs by a different pathway that is both nucleotide- and stromal protein–independent (*10*, *22*).

Transport of PC and OE33 increased with increasing concentrations of CPSecA (Fig. 4A). Comparable amounts of transport were obtained in a 75-μl assay with either 0.5 μg of purified CPSecA or 125 μg of stromal protein (estimated to contain 0.5 μg of CPSecA) (Fig. 4A). Purified CPSecA-supported transport of PC and OE33 was inhibited by sodium azide and gave a similar azide inhibition profile as that of SE-supported transport (Fig. 4B).

Thus, one of at least three pathways for protein transport across or into the thylakoid membrane (*10*) uses a CPSecA-dependent translocation mechanism. CPSecA displays all of the hallmarks of bacterial SecA: It reacts with SecA-specific antiserum and is approximately the same size as the *E. coli* SecA; it exists in solution as a homodimer and supports transport of signal peptide–bearing precursors; it mediates transport of proteins previously shown to require ATP (*13*, *20*); and its activity is sensitive to inhibition by azide. Although only PC and OE33 have been confirmed to be on the CPSecA-dependent pathway, the concentration of CPSecA in chloroplasts is as high as that of SecA in *E. coli*, in which virtually all of the periplasmic and outer-membrane proteins are probably transported by the SecA-dependent mechanism. This suggests that other chloroplast proteins may rely on CPSecA for membrane transport. There are approximately 60 major polypeptides identified for the thylakoids (*23*), many of which are encoded and synthesized within the plastid. Little is known about the assembly pathways for these proteins. It is possible that they rely on CPSecA for membrane transport, that plastid envelope proteins require CPSecA for transport, or both.

The identification of a SecA homolog in chloroplasts and the demonstration that it is necessary for transport of a subset of thylakoid proteins provide direct evidence for conservative sorting of proteins inside the chloroplast. According to the conservative sorting hypothesis (*2*), nuclear-encoded proteins destined for the inside of the chloroplast are imported into the organelle by a common translocator in the chloroplast envelope membranes (*10*) and then delivered to routing pathways derived from the ancestral endosymbiont.

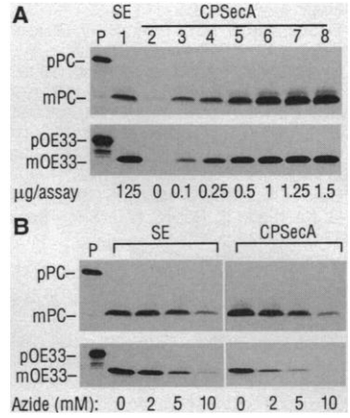

Fig. 4. (**A**) Transport of PC and OE33 in the presence of increasing concentrations of purified CPSecA. Radiolabeled pPC and pOE33 were assayed for transport into isolated thylakoids in the presence of total stromal protein (SE, 125 μg per assay) or varying amounts of CPSecA (micrograms per assay). Lane P contains the radiolabeled precursor proteins added to each set of assays. The precursors were pPC from *A. thaliana* and pOE33 from wheat. All assay volumes were 75 μl. (**B**) Inhibition of SE-supported or CPSecA-supported transport of PC and OE33 by sodium azide. Sodium azide at concentrations of 2, 5, and 10 mM was included in transport assays (75 μl) supported by SE protein (315 μg per assay) or CPSecA (1.2 μg per assay) as described (*25*).

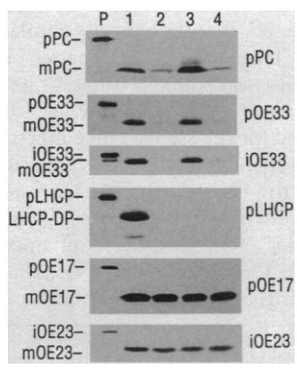

Fig. 3. Reconstitution of protein transport with purified CPSecA and isolated thylakoids. Radiolabeled full-length or intermediate-sized precursor proteins were mixed with thylakoids and assayed for transport or integration with total stromal protein (315 μg per assay, lane 1), in the absence of soluble protein (lane 2), with purified CPSecA (1.2 μg per assay, lane 3), or with a stromal fraction from gel filtration that lacked CPSecA (26 μg per assay, lane 4). Lane P contains the radiolabeled precursor proteins added to each set of assays. Radiolabeled proteins used in this experiment were pPC from *A. thaliana*, pOE33 and iOE33 from wheat (*20*), and pLHCP, pOE17, and iOE23 (*10*) from pea. Purified CPSecA was prepared for assay by buffer exchange on a Superose 6 gel filtration column. Assays were done as described (*25*) except that each received 4 mM ATP and 4 mM guanosine triphosphate. All assay volumes were 75 μl and contained the same buffer composition.

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16. Stromal extract (260 ml, 1.3 g of protein) was fractionated by ammonium sulfate precipitation (20 to 40% saturation). Precipitated proteins were dissolved in 120 ml of buffer A [25 mM Hepes-KOH (pH 8), 50 mM KCl, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride] and applied to a 90-ml DEAE-Sepharose column. The column was washed with 200 ml of buffer A and eluted with a 50 to 350 mM KCl gradient (500 ml). CpSecA-containing fractions at ~170 mM KCl were mixed with an equal volume of buffer A and applied to a 45-ml hydroxylapatite column. After washing with 100 ml of 10 mM potassium phosphate (pH 7) and 100 ml of 1 M KCl (unbuffered), the column was eluted with a 10 to 300 mM potassium phosphate gradient (300 ml). CPSecA-containing fractions at ~150 mM potassium phosphate were pooled and concentrated to 1 ml on a Centriprep-10 (Amicon) and then applied to an 80-ml Sephacryl S-300 gel filtration column and eluted with 20 mM Hepes-KOH (pH 8), 65 mM KCl, 1 mM dithiothreitol, 1% ethylene glycol. Fractions with CPSecA were applied to a 1-ml Mono Q column. After washing with 10 ml of 20 mM Hepes-KOH (pH 8), 50 mM KCl, the column was eluted with 30 ml of a 50 to 300 mM KCl gradient. CPSecA-containing fractions (~225 mM KCl) were adjusted to 50 mM potassium phosphate (pH 7), 1.5 M ammonium sulfate and applied to a 7.85-ml polyethylene glycol Hydropore-HIC column (Rainin). After washing with 20 ml of the same buffer, the column was eluted with 50 ml of 1.5 to 0 M ammonium sulfate in a descending gradient. CPSecA eluted at ~0.6 M ammonium sulfate.
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25. Radiolabeled precursors were prepared by in vitro transcription and translation (*10*). Intact chloroplasts were isolated from 9- to 10-day-old pea (Laxton's Progress 9) seedlings (*24*). Chloroplast lysate, thylakoids, and SE were prepared as described (*13*). Assays for import into chloroplasts or for transport or integration into thylakoids were carried out for 10 min and 30 min, respectively, in white light and 4 mM MgATP as described (*10*, *13*). Import assays were terminated with $HgCl_2$ [J. E. Reed *et al.*, *Eur. J. Biochem.* **194**, 33 (1990)]. Sodium azide was added to chloroplasts and lysates from a stock of 0.6 M in import buffer, and the mixture was incubated for 10 min at 25°C in light (70 $\mu Em^{-2}\ s^{-1}$) before the addition of precursor. Samples were analyzed by SDS–polyacrylamide gel electrophoresis–fluorography and quantification carried out by extraction of radiolabeled proteins from gel slices and scintillation counting (*24*). Chlorophyll concentrations were determined according to D. I. Arnon [*Plant Physiol.* **24**, 1 (1949)].
26. A highly conserved region was identified by comparison of *secA* sequences from *P. lutherii*, *Antithamnion spp*, *E. coli*, and *B. subtilis*. This region of the *P. lutherii* chloroplast *secA* gene, from base pair 1043 to 1458, was amplified by polymerase chain reaction with primer 5′-GCTCCACCATATGAAA-ATCGCCGAGATGAAGACAGG-3′ containing an in-frame Nde I site and reverse primer 5′- GGAA-TGTTTCAAGCTTTCGGGAGATTATTAGTGG-3′ containing a Hind III site with pMAQ805 (*7*) as template and was cloned into an appropriately digested pET24b (Novagen) to allow in-frame fusion with the His-6 tag. The resulting clone was introduced into BL21(λDE3) and the protein expressed and purified from inclusion bodies as described (*10*). Antibody to the SDS-denatured peptide was prepared in rabbits by Cocalico Biologicals (Reamstown, PA). Protein assays were performed with bovine serum albumin as standard as described [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. Immunoblotting was conducted as described [L. Payan and K. Cline, *J. Cell Biol.* **112**, 603 (1991)].
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28. We thank A. Lewin, G. Moore, D. McCarty, and E. Vallejos for critically reading the manuscript; H. W. Stokes (Macquarie University, Australia) and C. D. Scaramuzzi (University of Syndey, Australia) for providing pMAQ805; D. Oliver (Wesleyan University) for BL21(λDE3)/pT7-secA; C. Robinson (University of Warwick, UK) for the pOE33 and iOE33 wheat clones; and C. Li for technical assistance. Supported in part by National Institutes of Health grant 1 R01 GM46951 to K.C. This paper is Florida Agricultural Experiment Station Journal Series No. R-04057.

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Experimental Evidence That Competition Promotes Divergence in Adaptive Radiation

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Interspecific competition driving divergence in adaptive radiation has not previously been tested experimentally. Natural selection on a morphologically variable species of stickleback fish was contrasted in the presence and absence of a close relative. Selection was nondirectional when the target species was alone, whereas addition of the second species favored individuals most different from it morphologically and ecologically. Disproportionately severe competition between similar phenotypes indicates frequency-dependent selection, verifying a crucial element of theory of competition and character divergence. The findings help resolve outstanding debates on the ecological causes of diversification and the evolutionary consequences of competitive interactions.

The ecological causes of adaptive radiation are poorly understood. Especially contentious is the issue of whether rates and patterns of speciation and morphological divergence have been greatly affected by resource competition between species (*1*). This debate mirrors a long-standing issue in ecological research: whether differences between coexisting species are commonly the outcome of ecological character displacement (evolutionary change resulting from interspecific competition) (*2*–*4*). Conflicting views have been difficult to resolve because evidence is scarce and entirely correlative. I addressed the problem experimentally by measuring the strength of divergent natural selection between closely related, morphologically similar species.

Threespine sticklebacks (*Gasterosteus aculeatus* complex) inhabiting small lakes of coastal British Columbia, Canada, were used for the study. The collection of species diversified very recently, mainly at the end of the Pleistocene (≤13,000 years ago) (*4*, *5*). Earlier work suggested that coexisting pairs of species were character-displaced (*4*): One species (the "benthic") feeds on benthic invertebrates in the littoral zone and is large and deep-bodied with few, short gill rakers and a wide gape; the other species (the "limnetic") feeds on plankton, is small and slender, and has long, numerous gill rakers and a narrow gape. Species occurring alone in lakes are intermediate in body form and exploit both benthic and plankton habitats. These morphological differences are strongly associated with feeding efficiency and growth rate in the two habitats (*6*, *7*). The pattern is replicated over several watersheds and is a general characteristic of radiations of fish taxa that inhabit low-diversity post-Pleistocene lakes (*8*).

The experiment was carried out in summer 1993 in two divided 23 m by 23 m ponds on the University of British Columbia campus (*9*). The solitary species from Cranby Lake, Texada Island, was the target of the experiment; it is morphologically intermediate between benthic and limnetic species (*4*). The limnetic species from nearby Paxton Lake was the potential competitor. This species is morphologically and ecologically most similar to one extreme of the range of phenotypes in the Cranby species (*4*) (Table 1). The goal of the experiment was to test the prediction from theory that individuals at this extreme should suffer disproportionately when the limnetic species is added, generating natural selec-

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