Chloroplast Oxa1p Homolog Albino3 Is Required for Post-translational Integration of the Light Harvesting Chlorophyll-binding Protein into Thylakoid Membranes*

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Multiple sorting pathways operate in chloroplasts to localize proteins to the thylakoid membrane. The signal recognition particle (SRP) pathway in chloroplasts employs the function of a signal recognition particle (cpSRP) to target light harvesting chlorophyll-binding protein (LHCP) to the thylakoid membrane. In assays that reconstitute stroma-dependent LHCP integration in vitro, the stroma is replaceable by the addition of GTP, cpSRP, and an SRP receptor homolog, cpFtsY. Still lacking is an understanding of events that take place at the thylakoid membrane including the identification of membrane proteins that may function at the level of cpFtsY binding or LHCP integration. The identification of Oxa1p in mitochondria, an inner membrane translocase component homologous to predicted proteins in bacteria and to the albino3 (ALB3) protein in thylakoids, led us to investigate the potential role of ALB3 in LHCP integration. Antibody raised against a 50-amino acid region of ALB3 (ALB3-50aa) identified a single 45-kDa thylakoid protein. Treatment of thylakoids with antibody to ALB3-50aa inhibited LHCP integration, whereas the same antibody treatment performed in the presence of antigen reversed the inhibition. In contrast, transport by the thylakoid Sec or Delta pH pathways was unaffected. These data support a model whereby a distinct translocase containing ALB3 is used to integrate LHCP into thylakoid membranes.

Prokaryote-derived protein export systems are used to transport polypeptides into or across the endoplasmic reticulum, the mitochondrial inner membrane, and the chloroplast thylakoid membrane (1). Chloroplasts possess several different export systems (pathways). Each pathway exhibits unique energy and stromal protein requirements to localize a distinct subset of thylakoid proteins, both chloroplast-synthesized as well as nuclear-encoded proteins imported from the cytosol through the action of cleavable organelle-targeting sequences (2–5). The Sec pathway requires ATP and cpSecA, a homolog of bacterial SecA, to transport plastocyanin, OE33,1 PSI-F, and the chloroplast-encoded cytochrome f. In contrast, the Delta pH pathway uses a trans-thylakoid pH gradient as its sole energy source to transport OE23, OE17, PSI-T, and PSI-N by a mechanism that requires no soluble protein factors. An SRP pathway targets a family of nuclear-encoded integral thylakoid proteins, LHCPs. Similar to SRP-based systems used to target precursors to the endoplasmic reticulum and bacterial cytoplasmic membrane (6, 7), LHCP targeting requires GTP (8) and chloroplast homologs of SRP and its receptor, cpSRP and cpFtsY, respectively (9–12).

Considerably less is known about the thylakoid membrane components that are used for polypeptide translocation. Genomic and EST sequence data were used to identify cpSecY and cpSecE (13, 14). Their homologs, SecY and SecE, are components of the well studied SecYEG translocation pore (translocon) in bacteria (15). A maize mutant exhibiting a selective reduction in the level of proteins transported by the Delta pH system led to the identification of Hcf106 (16). The similarity between Hcf106 and predicted proteins in Escherichia coli revealed a homologous Delta pH system in bacteria, the TAT pathway (3). A recent study by Mori et al. (17) demonstrated that antibodies to cpSecY inhibited transport of Sec pathway precursors, whereas antibodies to Hcf106 and its ortholog, Tha4, blocked transport of precursors that use the Delta pH pathway. In no instance was integration of SRP-targeted LHCP affected by these antibodies, which raises the possibility that LHCP does not require a translocon for integration. Alternatively, LHCP may utilize a translocon that does not employ the function of Hcf106, Tha4, or cpSecY.

Studies of protein export in mitochondria have established that the inner membrane protein, Oxa1p, is a translocase component used to integrate a subset of inner membrane proteins encoded by both mitochondrial and nuclear DNA (18–20). A homolog of Oxa1p in thylakoids, the albino3 protein (ALB3), was identified in Arabidopsis by transposon tagging (21). Mutants failing to synthesize ALB3 exhibited an albino phenotype, indicating that ALB3 is required for thylakoid biogenesis. Taken together, these observations led to the suggestion that ALB3 is a candidate for the LHCP translocon (17). Here we show that antibody to ALB3 specifically inhibits LHCP integration without affecting transport of precursors localized by the Sec or Delta pH pathways. Our data support a model wherein the Oxa1p homolog, ALB3, is part of a thylakoid translocase distinct from the Sec or Delta pH transporters.

EXPERIMENTAL PROCEDURES

Materials

All reagents and enzymes used were purchased commercially. Previously described plasmids were used for in vitro transcription/translation of pLHCP (psAB80XD/4) (22), OE23 (23), and OE33 (24). Antis

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‡ This abbreviation used is: OE33/OE23/OE17, the 33-, 23-, and 17-kDa components of the oxygen evolving complex; i and t, intermediate and truncated precursor forms; SRP, signal recognition particle; cpSRP, chloroplast SRP; LHCP, light harvesting chlorophyll-binding protein; pLHCP, precursor to LHCP; ALB3, Albino3 protein; ALB3-50aa, 50 amino acid peptide from ALB3; GST, glutathione S-transferase; GST-ALB3-50aa, GST fused to ALB3-50aa; IB, import buffer; TM, transmembrane; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS, photosystem; SE, stromal extract.
body to SecY has been described (17) and was generously provided by Dr. Kenneth Cline, University of Florida.

**Methods**

**Plasmid Construction, Protein Expression, and Purification—**
A cDNA clone for ALB3 was obtained by reverse transcription-polymerase chain reaction (PCR) using total RNA from Arabidopsis thaliana. Forward and reverse primers (Integrated DNA Technologies) matching the published sequence for ALB3 (21) were designed to include EcoRI and XhoI sites, respectively, for ligation into pGEM 4Z. The coding sequence for ALB3-50aa, a 50-amino acid segment of ALB3 beginning at PLTKQ and ending at GVNL, was amplified by PCR and inserted in-frame behind the coding sequence for glutathione S-transferase (GST) in pGEX-6p-2 (Amersham Pharmacia Biotech) to produce pGEX-ALB3-50aa, which codes for the fusion protein GST-ALB3-50aa. All cloned constructs were sequenced (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR) to verify the fidelity of the PCR reaction.

GST and GST-ALB3-50aa fusion protein were expressed in E. coli strain BL21 and purified by affinity to glutathione-Sepharose (Amersham Pharmacia Biotech). GST-ALB3-50aa was subsequently used as antigen to prepare polyclonal antibodies (Cocalico Biologicals, Reamstown, PA); ALB3-50aa peptide was isolated by on-column cleavage of the GST-ALB3-50aa fusion protein with PreScission protease (Amersham Pharmacia Biotech) as described in the manufacturer’s manual, except phosphate-buffered saline (pH 8), 10 mM dithiothreitol was used as cleavage buffer. IgG was purified as described (25) using protein A-Sepharose and stored at 4 °C in 0.5 M phosphate, pH 7.0.

**Preparation of Radiolabeled Precursor, Chloroplasts, Thylakoids, and Stromal Extract—**
Capped RNA was translated in the presence of [3H]leucine using a wheat germ system to produce radiolabeled precursor proteins (26). Translation products were diluted 3-fold and adjusted to import buffer (IB; 50 mM Hepes/KOH, pH 8.0, 0.33 M sorbitol) containing 30 mM unlabeled leucine. Intact chloroplasts were isolated from 9–10-day-old pea seedlings (Laxton’s Progress) and used to prepare thylakoids and stromal extract (SE) (23, 26). Chlorophyll content was determined according to Arnon (27).

**Assays for Antibody Inhibition of Protein Transport into Thylakoids—**
Antibodies were used to inhibit protein transport essentially as described in Mori et al. (17), except that thylakoids were suspended to 1 mg/ml chlorophyll in 10 mM Hepes-KOH, pH 8.0, 10 mM MgCl2 before dilution to 0.33 mg/ml chlorophyll by the addition of 0.54 M phosphate (pH 7) containing IgG or sera. Alternatively, thylakoids were diluted with IgG that had been incubated with ALB3-50aa peptide (~30 mol/mol IgG) for 30 min on ice. The ratio of ALB3-50aa peptide:IgG was based on the amount of ALB3-50aa peptide required to block antibody binding to pea thylakoids on Western blots. Briefly, diluted thylakoids were incubated (1 h, 4 °C) with buffer alone, sera (10% of final volume), IgG, or IgG plus ALB3-50aa peptide and washed once with IB (10 mM MgCl2 in IB) to remove unbound antibodies before resuspension in IB to 1 mg/ml chlorophyll. Transport assays (150 μl final), conducted at 25 °C for 30 min in light, were initiated by adding radiolabeled precursor protein (25 μl) to antibody-treated thylakoids (50 μg of chlorophyll) mixed with SE (~0.5 mg of protein), 60 mM MgATP in IB (12.5 μl), and 25 mM NaGTP in IB (6 μl). Recovered thylakoids, post-treated with thermolysin, were dissociated with 25 μl of SDS-PAGE sample buffer (23).

**Analysis of Samples—**
A portion of each assay (10 μl) for thylakoid transport/integration was analyzed by SDS-PAGE followed by fluorography. Quantification was by scintillation counting of radiolabeled proteins extracted from excised gel bands (28).

**Miscellaneous—**
IgGs were quantified using absorbance at 280 nm (25). IgG binding to immunobots was visualized by staining with bromochloroindolyl phosphate/nitro blue tetrazolium (25). The concentration of ALB3 in pea chloroplasts was estimated from immunoblots using antibody binding to ALB3-50aa peptide as a standard. The concentration of purified ALB3-50aa peptide was determined by Noninterfering Protein Assay (Genotech). Low range prestained SDS-PAGE standards (Bio-Rad) were used to calculate molecular weights.

**RESULTS**

Based on the sequence similarity between ALB3 and Oxa1p and the topology of Oxa1p in the inner mitochondrial membrane (20, 29), we predicted that ALB3 in thylakoids would similarly span the membrane 5 times in an N out–C in orientation, in which the N terminus extends into the thylakoid lumen. This model of Arabidopsis ALB3 suggests that residues 157–206 of the full-length ALB3 precursor (ALB3-50aa), located between the first and second transmembrane (TM) domains, would be exposed on the stromal thylakoid face. Antibodies directed to this region of ALB3, if added to isolated thylakoids, could bind ALB3 and act as potent inhibitors of its function in protein transport assays. Because our assays for protein transport use thylakoid membranes from pea, we could not be certain that antibodies against ALB3-50aa from Arabidopsis would cross-react with ALB3 in pea. Therefore, it was necessary to identify pea ALB3. A BLAST search indicated that the ALB3 homolog in pea is PPF-1, the product of a cDNA isolated based on its up-regulation in apical buds following treatment with gibberelzin (30). In addition to exhibiting a high level of sequence identity to Arabidopsis ALB3, radiolabeled PPF-1 was localized to the thylakoid membrane fraction following its import into isolated chloroplasts, which supports the assertion that PPF-1 is ALB3 in pea. The mature protein migrated in denaturing gels at a Mr of ~45,000. In the ALB3-50aa region, PPF-1 differs from Arabidopsis ALB3 by only two amino acids.

ALB3-50aa, expressed as a GST-fusion protein in E. coli, was purified and used as antigen (Fig. 1A). Western blots demonstrated that the resulting antibodies recognized GST, the GST-ALB3-50aa fusion protein, and the ALB3-50aa fragment purified away from GST (Fig. 1B, compare lanes 1–3). In addition, a single ~45-kDa thylakoid protein was recognized in pea chloroplasts (compare lanes 4–6). Antibody binding to the 45-kDa thylakoid protein was prevented when blots were probed in the presence of purified ALB3-50aa peptide (Fig. 1C). Hence, detection of the 45-kDa polypeptide resulted from binding of antibody to ALB3-50aa and not from binding of antibody against GST. Because mature PPF-1 is a 45-kDa protein in thylakoids, and ALB3 and PPF-1 are 96% identical in the ALB3-50aa region, we conclude that the immunoreactive protein is PPF-1. Using ALB3-50aa as a standard, we calculated that there are 650,000 molecules of PPF-1 per pea chloroplast.

Serum directed against the GST-ALB3-50aa fusion (hereafter referred to as anti-ALB3-50aa) was used to investigate the involvement of ALB3 in thylakoid protein transport. Fig. 2 shows that preincubation of thylakoids with anti-ALB3-50aa specifically inhibited LHCP integration. Relative to assays conducted with preimmune or anti-SecY treated thylakoids, anti-ALB3-50aa reduced LHCP integration by ~60%. In contrast,
FIG. 2. GST-ALB3-50aa immune serum inhibits LHCP integration. Thylakoids were incubated with preimmune (α ALB3 Plm) or immune serum directed against GST-ALB3-50aa (α ALB3 Im) or cpSecY (α cpSecY Im) and subsequently examined for the ability to transport the radiolabeled precursors, indicated to the left of each fluorogram (see "Experimental Procedures"). Numbers below each lane represent the relative efficiency of transport for each precursor compared with preimmune-treated thylakoids. The translation product (TP) is shown for comparison with mature (m) OE23 and OE33. DP is an LHCP degradation product produced by protease treatment of thylakoids and is characteristic of properly integrated LHCP.

Anti-ALB3-50aa had little effect on OE23 or OE33 transport when compared with preimmune-treated thylakoids. As previously reported for Sec pathway precursors, OE33 transport was sensitive to incubation of thylakoids with anti-SecY serum (14, 17), which resulted in ∼70% reduction of OE33 transport relative to thylakoids incubated with preimmune or immune ALB3-50aa sera. The level of OE23 transport, a precursor localized by the Delta pH pathway, did not differ among the three sera.

IgG isolated from anti-ALB3-50aa serum was used to confirm the results obtained with sera. As shown in Fig. 3A, the efficiency of LHCP integration dropped in an antibody concentration-dependent manner, reduced to 16% at the highest concentration of IgG examined (1.0 mg/ml). Thylakoid pretreatment with anti-ALB3-50aa IgG over a wide concentration range (0.1–1.0 mg/ml IgG) had little influence on OE23 and OE33 transport, demonstrating that the inhibition was specific for LHCP. Inhibition of LHCP integration was reversed by including ALB3-50aa peptide with immune IgG during thylakoid preincubation (Fig. 3A), raising the efficiency of LHCP integration from 16% in the absence of peptide to ∼70% in the presence of peptide. A separate assay showed that when ALB3-50aa peptide was replaced with GST during thylakoid preincubation with immune IgG, inhibition of LHCP integration was not reversed (Fig. 3B). Taken together, our data suggest that ALB3 is a thylakoid translocon component used to integrate LHCP.

DISCUSSION

In this report we have used antibody to a chloroplast homolog of mitochondrial Oxa1p, ALB3, to study its potential role in thylakoid protein transport. Our results suggest a model whereby a novel post-translational SRP-based targeting system in chloroplasts is used to direct protein precursors, e.g. LHCP, to a translocon composed of or containing ALB3. Based on the mechanism of LHCP targeting to thylakoids, the exact nature of antibody inhibition is not clear. It is believed that imported LHCP, bound to cpSRP in a soluble transit complex, is the LHCP form targeted to thylakoids (9, 10). LHCP integration requires the function of an SRP receptor homolog, cpFtsY (11, 12), which is partitioned between the stroma and thylakoid membrane (11). It is possible that recruitment of cpFtsY to thylakoids relies on a cpFtsY-binding protein at the membrane, possibly ALB3 or a protein closely associated with ALB3. In this context, anti-ALB3-50aa could act to block cpFtsY binding to the thylakoid, resulting in a loss of LHCP integration. However, preliminary data indicate that ALB3 antibody does not inhibit cpFtsY binding to thylakoids. Therefore, anti-ALB3-50aa most likely blocks LHCP binding to an ALB3 translocon or prevents ALB3 function in the LHCP integration reaction.

The involvement of ALB3 in LHCP integration suggests that integration of LHCP into thylakoids may closely resemble Oxa1p-dependent integration of proteins into the inner mitochondrial membrane. In mitochondria, Oxa1p is used to transport N- and C-terminal tails from the matrix to the intermembrane space and also to mediate the insertion of TM domains into the membrane in a pairwise fashion, which results in the export of connecting regions between TM domains (18–20, 29). LHCP contains three TM domains in an N-in-C-out topology (31). Integration requires transport of a short C-terminal tail and an 33-amino acid loop between TM domains 1 and 2. By analogy to Oxa1p function (29), the thylakoid ALB3 translocon may insert TM 1 and TM 2 in a pairwise manner and transport the C-terminal tail as a separate translocation event, a model that we are currently testing.

In addition to the post-translational SRP targeting system used to route LHCP, cpSRP also appears to function in cotranslational targeting of integral thylakoid proteins, e.g. the

3 M. Moore, E. Peterson, and R. Henry, unpublished observation.
D1 protein of photosystem II (32). The identity of translocation components used to integrate proteins targeted by the co-translational SRP pathway is unknown. In *E. coli*, where SRP-based targeting is exclusively co-translational (6), SRP dependence is not correlated with the use of a specific translocon. For example, SRP-targeted FtsQ (33) and mannitol permease (34) require the function of a SecYEG translocon for integration, whereas integration of a ProW derivative, which proceeds by an N-terminal tail transport mechanism, takes place in the absence of a functional Sec translocase (35). These results indicate that translocon usage in *E. coli* is not linked to SRP dependence. Rather, elements in the SRP-targeted protein are used to specify the translocon. If cpSRP is similarly used to localize proteins to other translocons in the thylakoid, *e.g.* the Sec or Delta pH transporters, then it is likely that elements in LHCP are required to target LHCP to the ALB3 translocon in thylakoids.

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