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Interaction of the protein import and folding machineries in the chloroplast

(chloroplast protein import/inner membrane/chaperonin)

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ABSTRACT **We report the molecular cloning of import intermediate associated protein (IAP) 100, a 100-kDa protein of the chloroplast protein import machinery of peas. IAP100 contains two potential α-helical transmembrane segments and also behaves like an integral membrane protein. It was localized to the inner chloroplast envelope membrane. Immunoprecipitation experiments using monospecific anti-IAP100 antibodies and a nonionic detergent-generated chloroplast lysate gave the following results. (*i*) The four integral membrane proteins of the outer chloroplast import machinery were not coprecipitated with IAP100 indicating that the inner and outer membrane import machineries are not coupled in isolated chloroplasts. (*ii*) the major protein that coprecipitated with IAP100 was identified as stromal chaperonin 60 (cpn60); the association of IAP100 and cpn60 was specific and was abolished when immunoprecipitation was carried out in the presence of ATP. (*iii*) In a lysate from chloroplasts that had been preincubated for various lengths of time in an import reaction with radiolabeled precursor (pS) of the small subunit of Rubisco, we detected coimmunoprecipitation of IAP100, cpn60, and the imported mature form (S) of precursor. Relative to the time course of import, coprecipitation of S first increased and then decreased, consistent with a transient association of the newly imported S with the chaperonin bound to IAP100. These data suggest that IAP100 serves in recruiting chaperonin for folding of newly imported proteins.**

Most chloroplast proteins are synthesized in the cytoplasm as larger precursors with a cleavable N-terminal transit sequence. Distinct regions of the transit sequence presumably function to consecutively engage the translocation machinery (1, 2), first of the outer and then of the inner chloroplast envelope membrane (3). The subsequent folding and assembly of imported proteins requires the chloroplast chaperonin 60 (cpn60) (4).

We have previously used translocation intermediates to isolate components of the chloroplast protein import machinery (5). An "early" translocation intermediate associated with four proteins (IAPs, for import intermediate associated protein) of the outer membrane, whereas a "late" translocation intermediate interacted with two additional IAPs (IAP36 and IAP100), likely of the inner membrane. Two of the early IAPs, IAP34 and IAP86, are exposed on the chloroplast surface and bind GTP (6). By analogy to the two GTP binding proteins that constitute the signal-recognition particle receptor of the endoplasmic reticulum, IAP36 and IAP84 might be involved in the targeting of precursor proteins to the outer chloroplast membrane translocation machinery. IAP75 is embedded in the outer membrane and is a candidate for a protein conducting channel (5). The fourth and last early IAP is an integral membrane *hsp70* homolog of the outer membrane and faces the intermembrane space.

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In this paper we report on the molecular cloning and characterization of IAP100, an integral membrane protein of the protein import machinery of the chloroplast envelope inner membrane. Immunoprecipitation experiments with monospecific anti-IAP100 antibodies suggest that IAP100 serves to recruit stromal chaperonin for folding of newly imported proteins.

MATERIALS AND METHODS

Isolation of Late IAPs. Isolation of late IAPs was from chloroplasts corresponding to 65 mg of chlorophyll incubated with 6.5 nmol of precursor form of the small subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco) (pS) fused to protein A (7) as described (5) except that the binding and re-isolation steps were omitted.

Cloning and Sequencing of IAP100. Based on sequence data of an internal peptide of IAP100, DDPDAAAVHMEIG (residues 207–219, see Fig. 1*B*), a degenerate oligonucleotide mixture, AA(CT) ATI CCI GAT GCI GCI GCI GTI CA(CT) ATG GAI ATI GG, was used to screen a λgtll cDNA library from pea (Clontech) by standard aqueous hybridization (8). The insert cDNA of a positive isolate was excised by *Eco*RI digestion and subcloned into pBluescript II vector, yielding pBS-IAP100-N. A 600-bp fragment of pBS-IAP100-N was amplified using the T3 primer and a primer corresponding to residues 61–68 (see Fig. 1*B*), TCG AGA TTC TAG TTG ATA AGT. The fragment was used to screen an unamplified λgtll cDNA library from pea (6) by the method of Church and Gilbert (9). The insert of a positive isolate was excised by *Not*I digestion and subcloned into pBluescript II yielding plasmid pBS-IAP100-C. The overlapping cDNAs contained the entire coding sequence of IAP100 as determined by automated DNA sequencing.

Antibody Production and Purification. A DNA fragment coding the mature N terminus of IAP100 (amino acid residues 38–237, see Fig. 1*B*) was amplified from pBS-IAP100-N with the primers TAT ATA CCA TGG CTA GCT CCG ACA CTA ACA ACC CTG CT (sense) and ATA TGG ATC CCG AGC GTC CGG GTC ATC AAG ACC (antisense) with incorporated *Nco*I and *Bam*HI sites, respectively. The fragment was cloned into pET21d, yielding pET21d-IAP100$_{38-237}$, which was transformed into *Escherichia coli* BL21(DE3) cells. For expression and purification of the His$_6$-tagged N-terminal fragment of IAP100 on Ni-nitrilo-triacetic acid resin, the protocols provided by Qiagen (Chatsworth, CA) were used. Antibodies against the purified fragment were generated in rabbits as described (10). IgG against the His$_6$-tagged N-terminal frag-

Abbreviations: IAPs, import intermediate associated proteins; cpn60, chaperonin 60; pS and S, precursor and mature form of the small subunit of Rubisco (ribulose 1,5-bisphosphate carboxylase).

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U56419).

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ment of IAP100 was purified using the recombinant antigen immobilized on Affi-Gel 10 (Bio-Rad).

Immunoprecipitation. Isolated chloroplasts (11) were lysed at a concentration of 0.3 mg of chlorophyll/ml in IP buffer [50 mM tricine, pH 7.5/5 mM EDTA/150 mM NaCl/0.5% (vol/vol) Triton X-100/10% glycerol (vol/vol)]. The lysate was clarified for 15 min at 45,000 rpm in a Beckman TLA45 rotor and incubated with 10 μg of affinity-purified anti-IAP100 IgG or unspecific IgG overnight at 4°C under rotation. For SDS denaturation, chloroplasts were incubated in 2% SDS, 50 mM tricine (pH 7.5), and 5 mM EDTA at 1 mg of chlorophyll/ml for 10 min at 95°C and diluted 30-fold in IP buffer prior to the addition of IgG. Five mM MgATP or 5 mM $MgCl_2$ plus 5 units/ml apyrase were added to the IP buffer where indicated. Immune complexes were collected by incubation with protein A-Sepharose (20 μl of a 50% slurry) for 1 h at 4°C.

Protein Import Experiments. The import of urea denatured [^{35}S]pS into intact chloroplasts was done as described (11). [^{35}S]pS was produced in *E. coli* BL21(DE3) cells containing the pET8c-pS construct (7). pS was sequestered in inclusion bodies. The inclusion body fraction was purified as described by Marston (12). For use in import experiments the [^{35}S]pS inclusion bodies were dissolved in 8 M urea for 30 min at 20°C. Insoluble material was removed by centrifugation at 100,000 × *g*. Urea denatured [^{35}S]pS was added to the import experiment at a final concentration of 90 nM. The final concentration of urea in the experiment did not exceed 100 mM. After import, chloroplasts were washed with 10 vol of ice-cold HS buffer (50 mM Hepes, pH 7.7/330 mM sorbitol) and centrifuged at 5000 rpm for 2 min in a microcentrifuge. The chloroplast pellet was lysed in IP buffer as described above. The lysate was analyzed directly by SDS/12.5% PAGE or subjected to immunoprecipitation using affinity-purified anti-IAP100 IgG.

Miscellaneous Methods. Chloroplast isolation from pea leafs (11), envelope membrane fractionation (7), membrane extractions (6), thermolysin (13), and trypsin (3) treatments were done as described and as detailed in the figure legends. Antibodies against GroEL (bacterial cpn60) were generated in rabbits using GroEL overexpressed from pOF-39 as an antigen. The ATPase deficient mutant "Trap" was prepared as described (14).

RESULTS

Identification and Molecular Cloning of IAP100. IAP100 (Fig. 1*A*, lane 2) comigrates with a 100-kDa protein of the chloroplast inner envelope membrane (lane 1). We obtained N-terminal and internal protein sequence data and used derived oligonucleotide probes to obtain cDNA clones. The cDNA deduced primary structure of the 100-kDa protein (Fig. 1*B*) had a calculated molecular mass of 109,994.3 daltons and an N-terminal extension of 37 residues when compared with the N-terminal sequence determined by protein sequencing. The preponderance of basic residues in this N-terminal extension suggested that it functions as a transit sequence for import into the chloroplast (15). Hydropathy analysis indicated two hydrophobic regions (residues 74–93 and 101–120) that are predicted to form α-helical transmembrane segments (16). Searches of the databases revealed significant homology only to three rice expressed sequence tags (RICR1877A, RICS11841A, and RICS11836A), suggesting that they represent segments of a rice homolog.

To verify that the 100-kDa protein of the inner chloroplast membrane was identical with IAP100, we prepared antibodies against an *E. coli*-expressed fragment of the protein (residues 38–237). Indeed, affinity-purified anti-IAP100 IgG reacted specifically with the 100-kDa band of the inner chloroplast membrane fraction (Fig. 1*A*, lane 3) and with IAP100 (lane 4) in immunoblots indicating that the two proteins are identical.

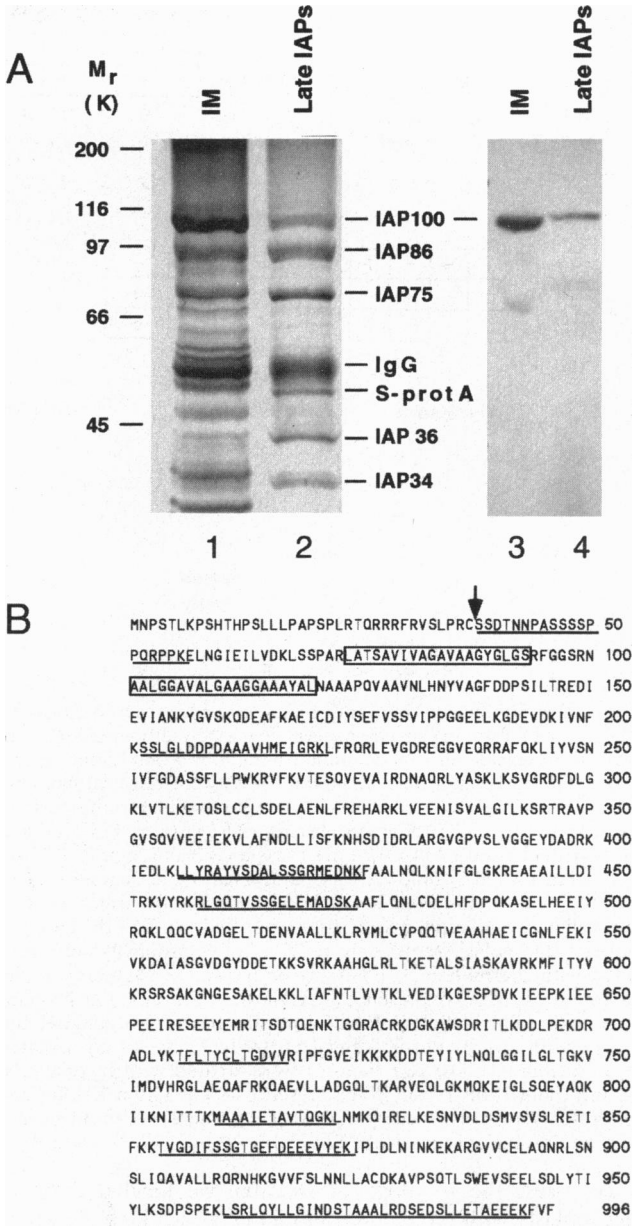

FIG. 1. Molecular cloning and sequencing of IAP100. (*A*) Twenty-microgram proteins of an isolated inner chloroplast membrane fraction (lanes 1 and 3) and proteins (IAPs) associated with late import intermediates (5) (lanes 2 and 4) were analyzed by SDS/PAGE and then either stained with Coomassie blue (lanes 1 and 2) or transferred to nitrocellulose and decorated with affinity-purified anti-IAP100 IgG (lanes 3 and 4). (*B*) cDNA-derived amino acid sequence of IAP100. The arrow indicates the start of the mature protein. The boxed sequences correspond to predicted transmembrane helices (15). The underlines indicate peptides obtained by protein sequencing.

Sublocalization and Biochemical Characterization of IAP100. Chloroplast envelope subfractions were isolated on a sucrose gradient and analyzed by immunoblotting with anti-IAP100 IgG (Fig. 2*A*). As expected, IAP100 was present primarily in the inner membrane (lane 3) and to a lesser extent in a mixed inner/outer membrane fraction (lane 2). It was absent from the outer membrane fraction (lane 1). We conclude that IAP100 is an inner membrane protein.

IAP100 behaved as an integral membrane protein because it could not be extracted by carbonate at pH 11.5 (Fig. 2*B*, compare lanes 2 and 3). It could be solubilized by Triton X-100 (compare lanes 4 and 5).

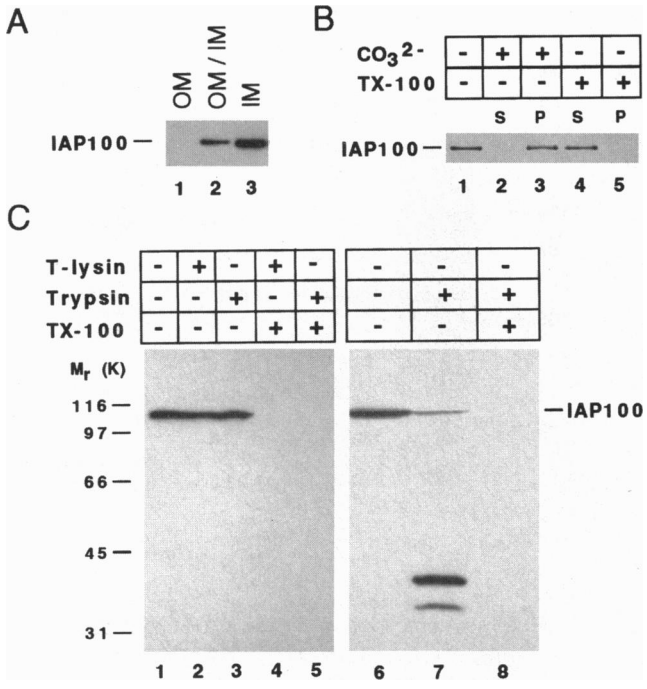

FIG. 2. Sublocalization and biochemical characterization of IAP100. (*A*) Proteins of chloroplast envelope subfractions (10 μg each) representing outer membrane (OM), mixed outer/inner membrane (OM/IM), and inner membrane (IM) were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-IAP100 IgG. (*B*) Inner membranes (20 μg) (lane 1) were incubated with either 0.1 M CO_3^{2-}, pH 11.5 (lanes 2 and 3) or 2% Triton X-100 (lanes 4 and 5), followed by separation into supernatants (S) and pellets (P). Proteins were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-IAP100 IgG. (*C*) Isolated chloroplasts (equivalent to 25 μg of chlorophyll) were incubated with thermolysin (+ T-lysin) or trypsin (+ Trypsin) in the absence (− TX-100) or presence (+ TX-100) of Triton X-100. Proteins were resolved by SDS/10% PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-IAP100 IgG (lanes 1–5). Isolated inner membranes (10 μg) (lane 6) were treated with trypsin (200 μg/ml) (lane 7) or trypsin in the presence of 1% Triton X-100 (lane 8). Proteins were analyzed by SDS/PAGE, transferred to nitrocellulose, and probed with anti-IAP100 IgG.

To assess the topology of IAP100, we incubated intact chloroplasts with either thermolysin (Fig. 2*C*, lane 2), which degrades surface-exposed proteins or with trypsin (lane 3), which is known to access the intermembrane space (13). IAP100 was resistant to both proteases. Resistance to trypsin predicted that IAP100 is not exposed on the intermembrane side of the inner membrane (13). Sensitivity to both proteases in the presence of Triton X-100 indicated that IAP100 is not *per se* protease resistant (Fig. 2*C*, lanes 4 and 5). To determine whether IAP100 would be accessible at the stromal side of the inner membrane, inner membrane vesicles were incubated with trypsin. Most of IAP100 was degraded to a membrane protected fragment of about 35 kDa (Fig. 2*C*, lane 7), which was detected by immunoblotting using the anti-IAP100 antibodies. As these antibodies are directed against residues 38–237, this fragment represented the N-terminal region of IAP100, which contains the two potential transmembrane segments.

Identification of Proteins Interacting with IAP100. To identify interacting proteins, IAP100 was immunoprecipitated from isolated chloroplasts that were solubilized in Triton X-100 in the presence of EDTA. Under these conditions IAP100 was quantitatively immunodepleted from the lysate (data not shown). Analysis of the immunoprecipitate by SDS/PAGE (Fig. 3 *A*, lane 1, compare also with *B*, lane 2) showed a major band at 100 kDa, a band at 60 kDa (arrowhead), and a minor band at 40 kDa (asterisk). Based on its comigration with IAP36 (data not shown), the 40-kDa band might be identical with IAP36. By immunoblotting the 100-kDa band was identified as IAP100 (data not shown) and the 60-kDa band as cpn60 (Fig. 3*A*, lane 4). The anti-cpn60 antibodies were monospecific as they recognized a single band of 60 kDa among total chloroplast proteins on an immunoblot (Fig. 3*A*, lane 6). After denaturation of chloroplast proteins with 2% SDS, cpn60 was not coimmunoprecipitated (Fig. 3*A*, lane 2). In a control immunoprecipitation experiment using nonspecific IgG cpn60 could not be detected (Fig. 3*A*, lanes 3 and 5).

The anti-IAP100 immunoprecipitate did not contain any outer membrane IAPs as assessed by SDS/PAGE followed by Coomassie blue staining (Fig. 3 *A*, lane 1, compare also with *B*, lanes 1 and 2) or immunoblotting with antibodies against IAP34, IAP75, and IAP86 (data not shown). This indicates that the translocation machineries of the outer and inner membrane are not coupled to each other in isolated chloroplasts.

Proteins of the cpn60 class are ATPases (17). To investigate a possible ATP dependency of the association of IAP100 with cpn60, the immunoprecipitation was done either in the presence of 5 mM MgATP (+ Mg-ATP) or Mg plus apyrase (− Mg-ATP). In the presence of Mg plus apyrase, cpn60 was coprecipitated as judged by SDS/PAGE followed by Coomassie blue staining (Fig. 3*B*, lane 2) or immunoblotting using anti-cpn60 antibodies (Fig. 3*B*, lane 4). In contrast, in the presence of MgATP [Fig. 3*B*, lane 1 (Coomassie blue) and lane 3 (immunoblotting)], cpn60 was not coprecipitated at a detectable level.

IAP100 Interacts Specifically with cpn60. The physiological function of cpn60 is to assist the folding of proteins in a process that requires ATP hydrolysis. Because the cpn60/IAP100 interaction may have resulted from partial unfolding of IAP100 during nonionic detergent solubilization, immunoprecipitation was performed in the presence of the mutant bacterial cpn60 GroEL 337/349 (Trap). Trap being unable to hydrolyze ATP, irreversibly "traps" unfolded proteins in its central cavity (18). Purified Trap was added to chloroplasts before the addition of Triton X-100 in an estimated molar excess over the endogenous cpn60. If IAP100 were unfolded during the solubilization procedure, Trap would bind to it irreversibly, in competition with endogenous cpn60, and would be coprecipitated, both in the absence or presence of ATP. However, no coprecipitation of Trap could be detected, either in the absence (Fig. 3*C*, lane 3) or presence (Fig. 3*C*, lane 4) of ATP. Purified Trap (equivalent to 4% of the Trap contained in the immunoprecipitation experiments) is shown in Fig. 3*C*, lane 5 (note that Trap migrated faster than endogenous chloroplast cpn60). The function of Trap was monitored by a binding assay using urea denatured [^{35}S]pS as a substrate. The binding experiment was analyzed by 6% nondenaturing PAGE and fluorography. Trap, as expected, bound urea denatured [^{35}S]pS equally well in the presence or absence of ATP (data not shown). These data suggest that the association of cpn60 with IAP100 is not due to unfolding of IAP100 by nonionic detergent during chloroplast solubilization.

Interaction of pS and S with the IAP100 Complex During Import. We carried out an import reaction of urea denatured [^{35}S]pS. At different times during the import reaction chloroplasts were re-isolated and lysed with Triton X-100. Aliquots of the lysate were either directly analyzed by SDS/PAGE and fluorography (Fig. 4*A*) or were first subjected to immunoprecipitation with anti-IAP100 antibodies and the precipitates analyzed by SDS/PAGE and fluorography (Fig. 4*B*). As expected, import of pS into the chloroplast was accompanied by conversion of pS to S, resulting from cleavage of the transit sequence in the chloroplast stroma (Fig. 4*A*). The corresponding anti-IAP100 immunoprecipitates showed coprecipitation of both pS and S (Fig. 4*B*; note that the amount of coprecipitated cpn60 is aproximately constant at all times). A quanti-

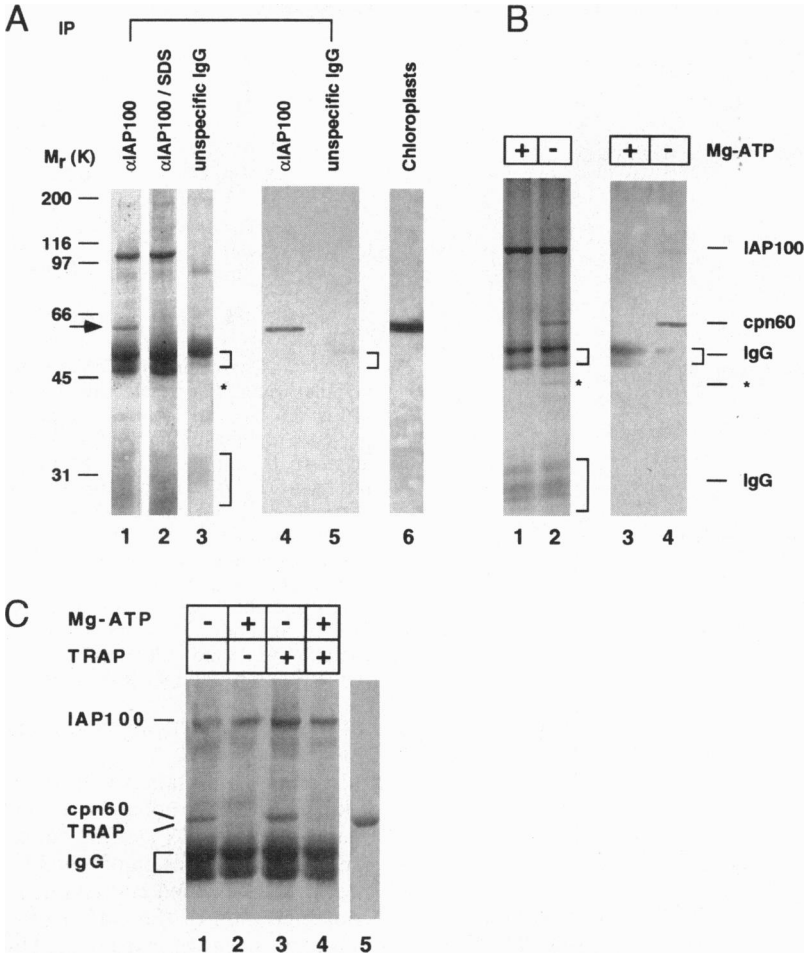

FIG. 3. IAP100 forms a complex with cpn60. (*A*) A chloroplast (200 μg of chlorophyll) lysate was subjected to native (lane 1) or denaturing (lane 2) immunoprecipitation with affinity-purified anti-IAP100 IgG or immunoprecipitation with unspecific (rabbit anti-mouse) affinity-purified IgG (lane 3). Proteins in the immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose, and either stained with amido black (lanes 1–3) or probed with anti-*E. coli* GroEL (anti-cpn60) antibodies (lanes 4 and 5). The anti-cpn60 antibodies recognized a single 60-kDa band among the SDS/PAGE resolved proteins of chloroplasts (25 μg of chlorophyll) (lane 6). Arrow indicates cpn60, the asterisk indicates a band that is likely to correspond to IAP36, brackets indicate heavy and light chains of IgG, respectively. Numbers to the left indicate positions of molecular weight standards. (*B*) ATP dissociates the complex of IAP100 and cpn60. Chloroplasts (200 μg of chlorophyll) were lysed by 0.5% Triton X-100 in the presence of 5 mM MgATP (+ Mg-ATP) or in the presence of 5 mM $MgCl_2$ and 5 units/ml of apyrase (− Mg-ATP). The lysates were immunoprecipitated with affinity-purified anti-IAP100 IgG. The precipitated proteins were resolved by SDS/PAGE and either stained with Coomassie blue (lanes 1 and 2) or probed with anti-cpn60 antibodies (lanes 3 and 4). (*C*) Anti-IAP100 immunoprecipitation in the presence of purified GroEL 337/349 (TRAP). Chloroplasts (50 μg of chlorophyll) were lysed in 150 μl of IP buffer containing either EDTA (− Mg-ATP, lanes 1 and 3) or MgATP (+ Mg-ATP, lanes 2 and 4). Twenty-five micrograms of purified Trap (0.2 μM oligomer) were added prior to the addition of Triton X-100 (+ TRAP, lanes 3 and 4). The proteins that were immunoprecipitated by anti-IAP100 IgG were analyzed by SDS/PAGE and Coomassie blue staining. One microgram of purified Trap is shown in lane 5.

tative analysis of the data for S (Fig. 4*C*) showed that import and conversion of pS to S was nearly complete at the 15-min time point of the import reaction (Fig. 4 *A* and *C*, ○). However, a peak of association of S with the IAP100–cpn60 complex is already evident at the 3- and 6-min time points of the import reaction; thereafter, this level of association declines by about 30% at the 60-min time point (Fig. 4*C*, ●). These data are consistent with the newly imported S being captured and released by or together with the cpn60 chaperone.

To further characterize the interaction of pS and S with the IAP100 complex we carried out additional controls of the immunoprecipitation reaction using the 6-min time point of the import reaction (Fig. 5). Nonimmune IgG (unspecific IgG) did not precipitate S, but did coprecipitate some pS (Fig. 5*A*). Moreover, digestion of chloroplasts with thermolysin prior to detergent lysis had no effect on the coprecipitation of S, but abolished the coprecipitation of pS (Fig. 5*B*). Finally, coprecipitation of S was abolished in the presence of ATP, whereas that of pS was only slightly reduced (Fig. 5*C*). These controls support the conclusion that the imported S associates with cpn60, which in turn associates with IAP100. The data on the coprecipitation of pS are less conclusive; some coprecipitation is likely to be unspecific (Fig. 5*A*); some may be specific and might result from a direct interaction of pS with IAP100.

DISCUSSION

We have molecularly cloned and characterized IAP100, a protein that was previously shown to be associated with a late translocation intermediate of protein import into chloroplasts (5). Consistent with a function at a late stage in the protein import process, we showed that IAP100 was a major protein in an isolated inner membrane fraction of the chloroplast envelope. Protein sequence data and the cDNA deduced primary structure of IAP100 showed that it contained a 37 amino acid long N-terminal extension that was rich in basic residues and thus resembled a transit sequence that is removed from the precursor during import into the chloroplast (15). Primary

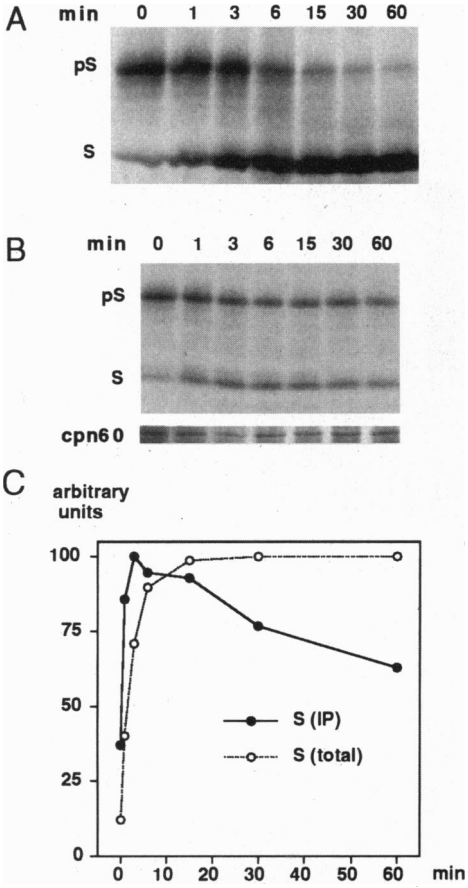

FIG. 4. Time course of pS import into chloroplasts and association of S with the IAP100 complex. Chloroplasts (1 mg of chlorophyll/ml) were incubated with urea denatured [^{35}S]pS in a standard import reaction. At the given times aliquots (equivalent to 100 μg of chlorophyll) were removed; chloroplasts were washed with ice-cold HS buffer and lysed in 300 μl of IP buffer containing EDTA. (*A*) Aliquots (equivalent to 5 μg of chlorophyll) from each time point were separated by SDS/12.5% PAGE and analyzed by fluorography. (*B*) Aliquots (equivalent to 95 μg of chlorophyll) from each time point were subjected to immunoprecipitation with anti-IAP100 IgG. The precipitates were analyzed by SDS/12.5% PAGE followed by fluorography. The section of the Coomassie blue stained gel containing cpn60 is shown at the bottom (cpn60). (*C*) Quantitation of S in Fig. 4*A* [S (total)] and *B* [S (IP)]. Quantitation was done with the National Institutes of Health Imager software package. The values were expressed in arbitrary units on a scale from 0 to 100.

structure analysis predicted the presence of two hydrophobic membrane-spanning segments (residues 74–93 and 101–120). Indeed, we found IAP100 to be resistant to carbonate extraction, consistent with it being an integral membrane protein. Incubation of chloroplasts with trypsin showed the protein to be inaccessible from the intermembrane space of the chloroplast. However, IAP100 was accessible to trypsin when isolated inner membrane vesicles were used, indicating that these vesicles are inverted with the stromal side exposed on the vesicle surface. Incubation of these vesicles with trypsin yielded an ≈35-kDa fragment that was detected by our IAP100 antibodies. As these antibodies were directed toward residues 38–327 of IAP100, the trypsin resistant 35-kDa fragment represents the membrane-integrated portion of IAP100 that contains the two putative α-helical transmembrane segments. It thus appears likely that the trypsin sensitive portion of IAP100 is soluble and exposed to the stroma. However, it cannot be excluded that there were other membrane protected fragments of IAP100. Such fragments may represent downstream integral transmembrane segments that are β-strands

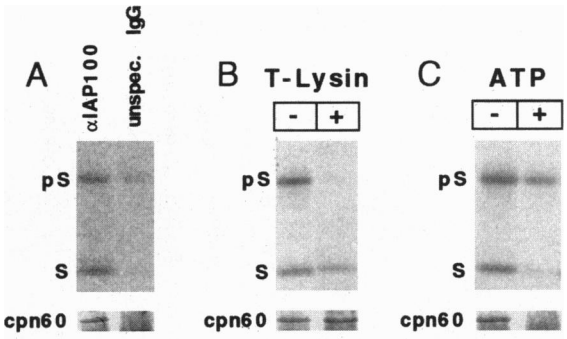

FIG. 5. Characterization of pS and S association with the IAP100 complex using chloroplasts from the 6-min time point during pS import in Fig. 4*A*. (*A*) Chloroplasts (95 μg of chlorophyll) were lysed in the presence of 5 mM EDTA and subjected to immunoprecipitation with anti-IAP100 antibodies (αIAP100) or unspecific IgG. (*B*) Chloroplasts (95 μg of chlorophyll) were incubated with 200 μg/ml of thermolysin (+ T-Lysin) for 20 min on ice and re-isolated prior to lysis in the presence of 5 mM EDTA and immunoprecipitation with anti-IAP100 antibodies. (*C*) Chloroplasts (95 μg of chlorophyll) were lysed in the presence of 5 mM EDTA (− ATP) or 5 mM MgATP (+ ATP) and the lysate subjected to immunoprecipitation with anti-IAP100 antibodies. The immunoprecipitated proteins were resolved by SDS/PAGE and stained with Coomassie blue. The Coomassie blue stained section of the gel containing cpn60 is shown at bottom (cpn60).

rather than α-helices and that would not have been detected by the antibodies.

Immunoprecipitation with monospecific anti-IAP100 antibodies using chloroplasts lysed by nonionic detergent in the presence of EDTA yielded coprecipitation of two proteins. One of these, a protein of ≈40 kDa, might be IAP36, another previously identified late IAP (5). Significantly, there was no coprecipitation of the early IAPs that form the outer membrane translocation machinery. This result was expected, as we previously showed that an early translocation intermediate engages only the translocation machinery of the outer membrane and that the translocation machinery of the inner membrane is consecutively coengaged by a late translocation intermediate (5). Thus, the two translocation machineries are separate functional units that appear to be linked only indirectly by the translocating chain. Alternatively, the translocating chain may trigger a direct interaction among the two translocation machineries.

The major protein coimmunoprecipitating with IAP100 was identified as cpn60, a stromal oligomeric ATPase that functions in folding newly translocated proteins in its central cavity. The association between IAP100 and cpn60 appeared to be specific and physiologically relevant based on several lines of evidence. First, cpn60 binding to IAP100 was sensitive to ATP and was only observed either by dissociating ATP by EDTA or by depleting ATP by apyrase. The complex was not observed when immunoprecipitation was carried out in the presence of MgATP. Second, the formation of the IAP100–cpn60 complex did not result from denaturation of IAP100 during chloroplast lysis by nonionic detergent. This was shown by using Trap, a recombinant bacterial mutant GroEL (i.e., an analog of the chloroplast cpn60) that is unable to hydrolyze ATP. Unfolded proteins are irreversibly trapped within Trap's central cavity (18). We observed that addition of Trap prior to chloroplast lysis by nonionic detergent did not result in Trap binding to IAP100 or in displacing cpn60 from IAP100. Competitive displacement of cpn60 and binding of Trap would occur if IAP100 were unfolded during detergent lysis of the chloroplast. Hence, the association of IAP100 with cpn60 is not by IAP100 being bound within the cpn60 central cavity. Third, we found a newly imported protein, the mature form of the small subunit (S) of Rubisco, to be associated with the

IAP100–cpn60 complex in an ATP sensitive manner. This association was observed to increase and then to decrease with time, consistent with an association of newly imported S with cpn60 transiently bound to IAP100. Together, these data suggest that IAP100, being a component of the inner membrane translocation machinery, can recruit cpn60 in an ATP sensitive manner and thereby functions to couple the chloroplast inner membrane's translocation machinery to the stromal chaperonin for folding newly imported proteins.

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