Isolation of Components of the Chloroplast Protein Import Machinery

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Components of the protein import machinery of the chloroplast were isolated by a procedure in which the import machinery was engaged in vitro with a tagged import substrate under conditions that yielded largely chloroplast envelope-bound import intermediates. Subsequent detergent solubilization of envelope membranes showed that six envelope polypeptides copurified specifically and, apparently, stoichiometrically with the import intermediates. Four of these polypeptides are components of the outer membrane import machinery and are associated with early import intermediates. Two of these polypeptides have been characterized. One is a homolog of the heat shock protein hsp70; the other one is a channel-protein candidate.

Many chloroplast proteins are synthesized in the cytosol as larger precursors with an amino-terminal transit sequence that functions in translocating these precursors across the two membranes of the chloroplast envelope. During or shortly after translocation, the transit sequence is removed by a specific soluble endoprotease (transit peptidase) that is located in the chloroplast stroma.

Little is known about the machinery that allows protein translocation across the two chloroplast envelope membranes. It has been proposed that translocation proceeds through two separate protein conducting channels in the outer and inner membranes of the chloroplast, respectively, and that two separate domains of the transit sequence each serve as a distinct ligand to consecutively open these channels (*1*, *2*), thereby allowing translocation of a protein from the cytosol into the chloroplast stroma, presumably by a thermal ratchet mechanism (*3*).

Although various potential candidate proteins for the chloroplast envelope import machinery have been reported (*4*–*9*), their role in the import process, if any, remains to be determined. We isolated components of the protein import machinery of the chloroplast envelope by a procedure based on our previous demonstration of the existence of distinct import intermediates that arise during the course of an in vitro import reaction and remain associated with the chloroplast envelope membranes (*1*). These envelope-bound import intermediates remained associated with components of the import machinery even after detergent solubilization of the membrane and, therefore, could be used as a bait for the isolation of this machinery. We obtained six distinct envelope proteins that were specifically associated with the envelope-bound import intermediates. We refer to these proteins as IAPs (for import intermediate associated proteins). A subset of four of the six IAPs copurified with early import intermediates. These four "early" IAPs represent components of the import machinery of the outer chloroplast membrane. Two additional IAPs copurified with a late import intermediate that had gained partial access to the chloroplast stroma. These two "late" IAPs are likely to represent components of the import machinery of the inner chloroplast envelope. We characterized two of these four early IAPs, both of an apparent molecular size of 75 kD. One of these was characterized immunologically and shown to be a member of the heat shock protein (hsp) 70 family. This protein is an integral protein of the outer membrane that is inaccessible on the chloroplast surface to externally added thermolysin (*10*). The other protein, referred to as IAP75, was molecularly cloned and sequenced. Because it is largely embedded in the outer membrane, IAP75 is a candidate for a channel protein. The characterization of the other two early IAPs is reported in the accompanying paper (*11*). These two proteins, IAP34 and IAP85, are guanosine triphosphate (GTP)–binding proteins.

Isolation of import machinery. As an easily retrievable import substrate, we used a chimeric protein, referred to as pS-protA, consisting of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pS) fused at its COOH-terminus to staphylococcal A protein (protA) (*1*). This protein was expressed in *Escherichia coli*, purified, and urea-denatured before being incubated at 26°C in an import reaction containing isolated chloroplasts (*1*). The import reaction was stopped after 2.5 minutes by cooling on ice. At this time the two previously characterized envelope-bound import intermediates were present in their highest amounts (*1*), with the early import intermediate being the uncleaved pS-protA and the late import intermediate being the cleaved S-protA (see below) (the latter having gained access to the stromal transit peptidase). Chloroplasts from the 2.5-minute reaction point were reisolated, rapidly disrupted, and processed to yield a crude envelope fraction. This fraction was further separated by flotation on a sucrose gradient to obtain a light outer membrane fraction (OM), an intermediate density fraction (OM-IM), and a slightly denser inner membrane (IM) fraction (Fig. 1) (*1*). Immunoblots showed that most of an outer membrane marker protein (OM37) (*12*) was present in the light outer membrane fraction, and most of an inner membrane marker protein (IM35) (*12*) was present in the inner membrane fraction (Fig. 1B). The intermediate density fraction contained detectable amounts of OM37 and IM35 and therefore consisted of remnants of both outer and inner membranes, presumably held together by contact sites (*13*). A large proportion of each of the two import intermediates was found in the intermediate density fraction (Fig. 1A) (*1*). None were detectable in the outer membrane fraction (Fig. 1A).

The intermediate density fraction (OM-IM) was solubilized in a buffer containing 2 percent Triton X-100, 250 mM NaCl and 10 percent glycerol (by volume) (*14*). The solubilized import intermediates (^{35}S-labeled) were bound to immunoglobulin G (IgG)–Sepharose, extensively washed, and eluted by SDS. They were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining (Fig. 2A) and fluorography (Fig. 2B). The eluted proteins consisted of the two import intermediates (pS-protA and S-protA), some IgG heavy chain that leached from the IgG-Sepharose column, and five polypeptide bands of 34, 36, 75, 86, and 100 kD (Fig. 2A). These five polypeptide bands copurified with the two import intermediates in approximately stoichiometric amounts (as judged from their staining intensity in Figs. 2 and 3), suggesting a specific association.

Several control experiments indicated that these five copurifying polypeptide bands were indeed specifically associated with the two import intermediates. First, neither import intermediates nor copurifying envelope polypeptides were detected when the chloroplasts were incubated with thermolysin before the import reaction (Fig. 2A). Incubation of chloroplasts with ther-

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molysin abolishes their ability to import proteins (*15*). Second, omission of adenosine triphosphate (ATP) in the import reaction yielded neither import intermediates nor copurifying envelope polypeptides (Fig. 2A), a result consistent with the known requirement of import for ATP (*16–18*). Third, the use of mature S-protA as a substrate in the import reaction yielded neither import intermediates nor copurifying envelope polypeptides (Fig. 2A) as would be expected from the known dependence of import on the transit sequence (*19*). Fourth, an import reaction in the absence of import substrate did not yield envelope polypeptides that bound to IgG-Sepharose (Fig. 2A). This control excluded the possibility that envelope peptides bound nonspecifically to IgG-Sepharose. Finally, no envelope polypeptide copurified with import substrate when it was added during the solubilization of the OM-IM fraction of the envelope (Fig. 2, A and B). This control excluded the possibility that pS-protA interacted with envelope polypeptides in an import-independent reaction. Thus, we conclude that the five polypeptide bands that copurify with the import intermediates are specifically associated with them. They are therefore referred to as IAPs.

Precursors to be imported into the chloroplast stroma are known to compete for import, presumably by utilizing the same import machinery (*20–22*). Therefore, import intermediates of another chimeric protein, in which pS was replaced by preferredoxin (pFd), were expected to bind to identical envelope proteins. Indeed, envelope-bound import intermediates, pFd-protA and Fd-protA, copurified with five polypeptide bands of electrophoretic mobility similar to those associated with pS-protA and S-protA (Fig. 3, A and B). A comparison of the copurifying polypeptide bands with comigrating bands in the isolated OM fraction or in the OM-IM fraction suggests that the 75-kD and 86-kD bands are major components of the OM and OM-IM fraction (Fig. 3A). A 100-kD comigrating band was absent in the OM fraction but present in the OM-IM fraction (Fig. 3A) suggesting that it is derived from the import machinery of the inner membrane.

If the import machinery of the outer membrane is engaged first and independently of that in the inner membrane, it might be possible to isolate the outer membrane's import machinery separately with the use of early import intermediates. Indeed, if pS-protA was incubated with chloroplasts in the presence of low concentrations of ATP, the envelope-bound import intermediates consisted of only the precursor form and lacked the cleaved form (Fig. 3B). Only the 34-, 75- and 86-kD polypeptide bands copurified with pS-protA at this early stage of import, whereas the 100-kD and 36-kD bands did not (Fig. 3A). This suggested that the latter two polypeptide bands are components of the import machinery in the inner membrane, whereas the 34-, 75-, and 86-kD bands are components of the import machinery in the outer membrane.

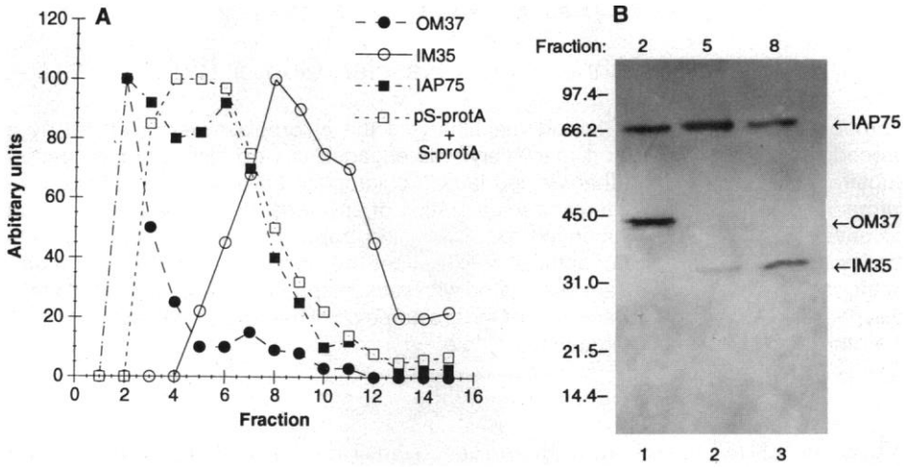

Fig. 1. Distribution of import intermediates and IAP75 in chloroplast envelope membranes fractionated on a sucrose gradient. Isolated pea chloroplasts (equivalent to 50 mg of chlorophyll) (*17*) were incubated with urea-denatured [^{35}S]pS-protA (*33*) in the presence of 100 μM MgATP in the dark to maximize precursor binding but to preclude import (*1*). After the binding reaction, the chloroplasts were again isolated, and import of the bound precursor was initiated by the addition of 2 mM MgATP (*1*). Two and one-half minutes after MgATP was added, the import reaction was stopped by dilution of the mixture with ice-cold import buffer (*1*). The chloroplasts were reisolated and lysed under hypertonic conditions (*34*), and the total membrane fraction was subfractionated by flotation into linear sucrose gradients (20 to 38 percent) (*35*). The gradients were fractionated, and the proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and subjected directly to fluorography to detect pS-protA and S-protA or immunoblotted with antiserum to OM37, IM35, or IAP75 (*12*). (**A**) Distribution of OM37, IM35, IAP75, and pS-protA and S-protA in the sucrose gradient profile. Each fraction analyzed contained 10 μg of protein. (**B**) Immunoblot of fraction 2 (lane 1), fraction 5 (lane 2), and fraction 8 (lane 3) from (A) with anti-OM37, anti-IM35, and anti-IAP75 (*12*). All lanes contained 2 μg of protein.

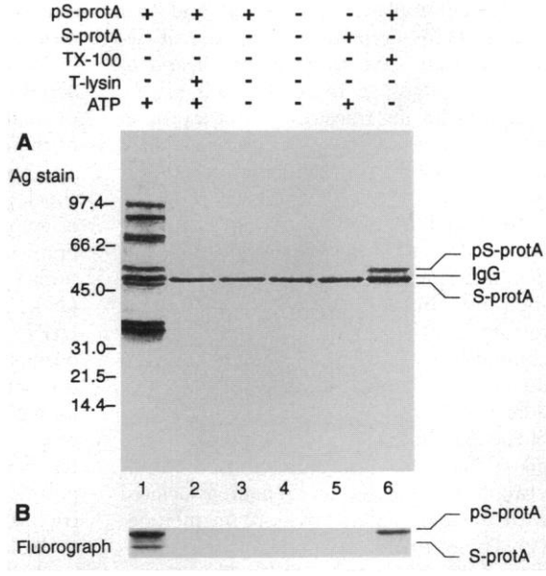

Fig. 2. Affinity purification on IgG-Sepharose of IAPs from detergent solubilized envelope membranes. (Lane 1) Urea-denatured [^{35}S]pS-protA (*33*) was imported into isolated chloroplasts (equivalent to 50 mg of chlorophyll) from the envelope-bound state for 2.5 minutes as described (Fig. 1). After the import reaction, the chloroplasts were reisolated, lysed, and fractionated by flotation into linear sucrose gradients (20 to 38 percent) (*35*). Fractions from the sucrose gradient containing OM-IM with associated pS-protA or S-protA were collected, pooled, and concentrated by centrifugation. Portions of the OM-IM (1 mg of protein) were solubilized (*14*) and applied to rabbit IgG-Sepharose. Bound proteins were eluted (*14*) and analyzed by SDS-PAGE and silver staining (**A**) or fluorography (**B**). (Lane 2), Chloroplasts were treated with thermolysin (T-lysin) (200 μg/ml) on ice for 30 minutes before the precursor binding reaction (*25*). (Lane 3) ATP was omitted from the binding and import reaction. (Lane 4) [^{35}S]S-protA was omitted from the binding and import reactions. (Lane 5) The import substrate was [^{35}S]S-protA instead of [^{35}S]pS-protA. (Lane 6) Envelope membranes (1 mg of protein) from chloroplasts that had not undergone a prior import reaction were solubilized in Triton X-100 (TX-100) (*14*) before the addition of pS-protA. The positions of precursor (pS-protA) and mature (S-protA) forms of the import substrate and the heavy chain of IgG (IgG) are indicated to the right. The molecular sizes of standard proteins in kilodaltons are indicated at the left.

We previously identified a 36-kD envelope polypeptide (p36) as a receptor for protein import into chloroplasts (*7*, *8*). However, other studies have indicated that p36 functions as the triose phosphate-3-phosphoglycerate-phosphate translocator of the chloroplast inner membrane (*23*). An antiserum to p36 (*7*, *8*) did not recognize any polypeptides in the IAP fraction (*24*). This argues against the participation of p36 in protein import, although it remains possible that p36 was dissociated during isolation of the IAPs.

Presence of an integral outer membrane hsp70 homolog among the early IAPs. The chloroplast envelope contains an hsp70 homolog that is distinct from hsp70 homologs in the cytosol and in the chloroplast stroma (*10*). This envelope hsp70 is an integral outer membrane protein that is resistant to digestion when intact chloroplasts are treated with thermolysin (*10*), suggesting that it is largely exposed to the intermembrane space of the envelope. During import such an integral outer membrane hsp70 homolog might be recruited to the outer membrane's import machinery and therefore become an IAP. Indeed, we found that a commercially available monoclonal antibody (mAb SPA-820) with a broad cross-reactivity with a number of mammalian cytosolic hsp70 homologs (*12*) reacted strongly with the 75-kD band in the IAP fraction and with a band of identical mobility in the OM fraction and the OM-IM fraction (Fig. 4A). There were no cross-reactive bands in the thylakoid and stromal fractions of the chloroplast (*24*). Moreover, the mAb SPA-820–reactive 75-kD band is not a contaminating cytosolic hsp70 homolog because antibodies to a cytosolic hsp70 homolog (*12*) did not react with proteins in the OM and OM-IM subfractions of the envelope or with any of the IAPs (Fig. 4B).

The mAb SPA-820-reactive band was resistant to digestion when intact chloroplasts were treated with thermolysin and was not extracted from outer membranes by pH 11.5 or high concentrations of salt (*24*). The partial amino acid sequence of several peptides derived from purified hsp70 IAP confirmed that it is similar but not identical to known hsp70 proteins (*24*). Thus, an integral outer membrane hsp70 homolog is part of the outer membrane's import machinery and is likely identical to the previously detected envelope bound hsp70 homolog (*10*). An hsp70 homolog has also been shown to cosediment with a detergent-extracted outer membrane fraction that contains bound pS (9). Whether this protein is identical to the hsp70 IAP is not yet known.

Characterization of IAP75. To further characterize the 75-kD band of the IAP fraction, we obtained a partial amino acid sequence from a peptide derived from IAPs separated by SDS-PAGE (Fig. 3A). Comparison of this peptide sequence with protein sequences in data banks did not reveal any sequence similarity to known hsp70 proteins, suggesting that the 75-kD band also contained another polypeptide that comigrated with the hsp70 homolog. To further characterize this protein, referred to as IAP75, we obtained antibodies to a synthetic peptide corresponding to the peptide sequence (*12*). These antibodies reacted specifically with a 75-kD protein in the OM-IM subfraction of the chloroplast envelope, but they did not react with proteins in the thylakoid or stromal fraction or a whole leaf soluble fraction (Fig. 5A). The antibodies (anti-IAP75) also immunoprecipitated a 75-kD polypeptide from among the SDS-denatured proteins of the OM-IM envelope subfraction. This separated the hsp70 from the IAP75, because when the proteins of the anti-IAP75 immunoprecipitate were separated by SDS-PAGE, IAP75 reacted only with the anti-IAP75, but not with the mAb SPA-820 to hsp70 (Fig. 5, B and C). IAP75 and hsp70 IAP were also separated by two-dimensional gel electrophoresis (*24*).

Both the hsp70 IAP and IAP75 were associated with import intermediates at early and later times in the import reaction (Fig. 5, D and E). IAP75 was found in the OM and the OM-IM fraction (Fig. 1, A and B). Moreover, IAP75 is an integral membrane protein that was not extracted by high concentrations of salt or at pH 11.5 (Fig. 6A). Incubation of chloroplasts with thermolysin showed that IAP75 was resistant to digestion (Fig. 6B), suggesting that there are no thermolysin-sensitive sites of IAP75 exposed on the chloroplast surface. However, incubation of chloroplasts with trypsin caused degradation of IAP75 to a 52-kD membrane-associated fragment that still reacted with anti-IAP75 (Fig. 6B). Trypsin damages the outer chloroplast membrane and gains access to the intermembrane space between the outer and inner membrane (but not to the stroma) (*25*). Thus, the 52-kD fragment might have been generated by trypsin cleavage of IAP75 on the side of the outer membrane

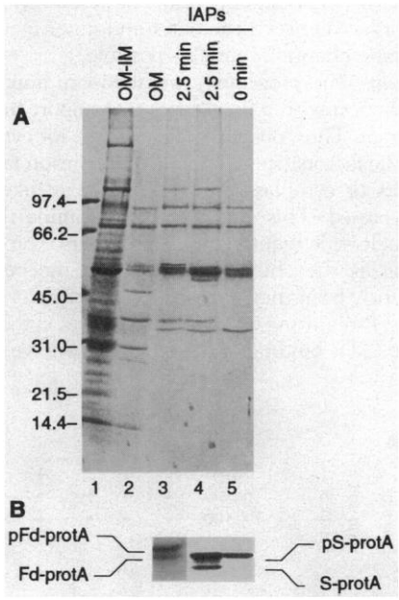

Fig. 3. Association of two distinct import substrates with identical IAPs and association of an early import intermediate with a subset of IAPs. Urea-denatured [^{35}S]pS-protA (lane 4) or [^{3}H]pFd-protA (*33*) (lane 3) were imported into isolated chloroplasts from the bound state for 2.5 minutes (Fig. 1). The 0-minute time point (lane 5) refers to chloroplasts that had been incubated with [^{35}S]pS-protA under binding conditions, but did not undergo the subsequent import reaction. The chloroplasts were reisolated and separated into OM, OM-IM, and IM fractions (Fig. 1). IAPs were purified (*35*) from OM-IM fractions containing 1 mg of protein (*14*). The IAPs and the OM (5 μg of protein) and OM-IM (10 μg of protein) fractions were analyzed by SDS-PAGE and Coomassie blue staining (**A**) or fluorography (**B**). The positions of precursor (pS-protA and pFd-protA) and mature (S-protA and Fd-protA) forms of the import substrates are indicated (**B**). The molecular sizes of standard proteins in kilodaltons are indicated at the left.

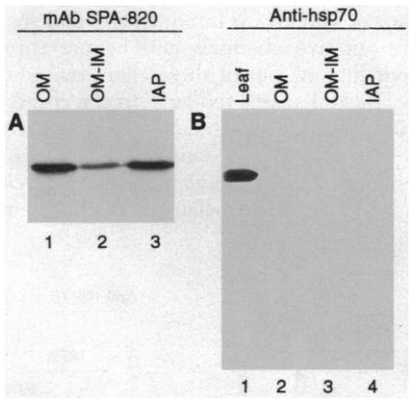

Fig. 4. A chloroplast outer membrane hsp70 homolog is among the IAPs. (**A**) Proteins of the OM, OM-IM, and the IAPs from the 2.5-minute time point in pS-protA import were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal antibody to human cytosolic hsp72 (*12*) (mAb SPA 820). Lanes 1 and 2 contained 10 μg of protein. The IAPs in lane 3 were derived from OM-IM containing 1 mg of protein (Fig. 2). (**B**) The proteins of a whole leaf soluble fraction (Leaf), OM, OM-IM, and the IAPs from the 2.5-minute time point in pS-protA import (IAP) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antiserum to cytosolic hsp70 from pea (*12*). The leaf soluble fraction corresponds to the supernatant of a pea leaf homogenate that had been centrifuged at 200,000*g* for 30 minutes. Lanes 1, 2, and 3 contained 10 μg of protein. Lane 4 contained IAPs derived from OM-IM containing 1 mg of protein.

facing the intermembrane space. Taken together, the proteolysis data suggest that IAP75 might be largely embedded in the outer membrane.

An outer membrane–associated 75-kD protein also has been identified by crosslinking of chloroplast bound pS (*4*). IAP75 has a similar mobility on two-dimensional gel electrophoresis as the crosslinked product (*4*, *24*). Therefore, it is likely that IAP75 and the crosslinked 75-kD protein are identical.

A pea complementary DNA (cDNA) library was screened with a degenerate oligonucleotide mixture that was synthesized on the basis of one of the internal peptide sequences that we obtained from IAP75 (*26*). A cDNA clone was isolated and sequenced. The primary structure of IAP75 was derived from the cDNA (Fig. 7). Comparison of the primary structure with that of other proteins in the data bank showed no similarity. All five of the internal peptide sequences obtained from IAP75 were found in the deduced primary structure (Fig. 7). The calculated molecular size of 88,280 daltons for IAP75 is considerably larger than that (75 kD) estimated from its electrophoretic mobility in SDS-PAGE (*26*). It is possible that the primary translation product is proteolytically processed at one or both termini to yield mature IAP75.

Although IAP75 appears to be embedded in the envelope outer membrane, standard hydrophilicity plots do not identify a segment of sufficient length and hydrophobicity to span the membrane as an alpha helix (*27*). However, according to a procedure for the identification of beta turns (*28*), there could be as many as 26 transmembrane segments in the primary translation product, most of them too short to be membrane spanning alpha helices but long enough to span the membrane as beta strands (*28*). Thus, if IAP75 is a component of the protein conducting channel in the membrane, it may, like other channel proteins, contain membrane traversing beta strands (*29*).

Implications for protein import into chloroplasts. The pivotal components of the chloroplast import machinery are envisaged as being two distinct protein-conducting channels, one in the outer membrane and one in the inner membrane (*1*) (Fig. 8). We propose that these channels, like other protein conducting channels (*2*, *30*), are ligand-gated. We speculate that the COOH-terminal portion of the transit sequence might serve as a ligand to open the channel in the outer membrane (*1*, *22*), whereas the NH_2-terminal portion might serve as a ligand to open the channel in the inner membrane. We further envisage that the channel in each of the two membranes is associated with other proteins, both integral and peripheral membrane proteins, and that these proteins serve regulatory functions. Together these components constitute the import machinery in the outer and inner chloroplast membrane.

Whether the six IAPs that we have isolated constitute all of the components of the two import machineries is not yet known. The import intermediates may span the putative channels and be prevented from slipping out of these channels either by folded domains too large to traverse the channel or by direct binding to one or more of the IAPs. Some channel-associated proteins that are components of the import machinery may dissociate during the membrane solubilization procedure and therefore not be among the isolated IAPs.

The import machinery of the outer membrane consists of at least four proteins. Two of these proteins, IAP34 and IAP86, are GTP-binding proteins. The characterization of these two IAPs is described in the accompanying report (*11*). We suggest that IAP34 and IAP86 take part in regulating presentation of the transit sequence to the protein conducting channel. This would be analogous to the presentation of the signal sequence to the endoplasmic reticulum's protein conducting channel that appears to be mediated by the GTP-binding subunits of the signal recognition particle (SRP) and the SRP receptor (*31*, *32*). IAP75 might constitute the channel, and the hsp70 IAP may bind to the portion of the import substrate that first appears in the intermembrane space thereby preventing its backward fluctuation out of the channel. Additional functions for the IAPs, such as regulating channel opening or establishing contact between the outer and inner membrane channels, are also possible.

In our procedure, we used an import reaction with a urea-denatured import substrate. Thus, possible requirements for cytosolic factors (specific signal recognition factors or cytoplasmic chaperones) are likely bypassed. This allowed us to examine the nucleotide requirements for precursor binding at the chloroplast envelope independently from those in the cytosol (such as an ATP-requiring cytosolic hsp70 or a cytosolic GTP binding protein that may serve in

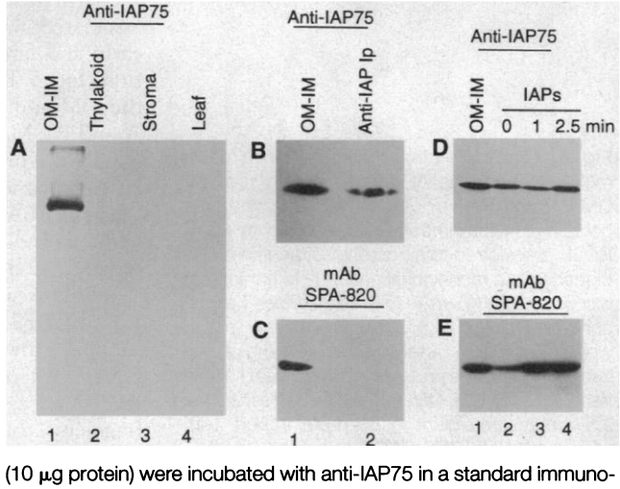

Fig. 5. Identification of IAP75 as a component of the outer membrane import machinery. (**A**) The proteins of a whole leaf soluble fraction (Leaf), OM-IM, a stromal extract (Stroma), and a thylakoid membrane fraction (Thylakoid) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-IAP75 (*12*). The whole leaf soluble extract was prepared as described (Fig. 4). The OM-IM, thylakoid membranes, and stromal extracts were prepared as described (Fig. 1). All lanes contained 10 μg of protein. (**B** and **C**) SDS-solubilized outer envelope membranes (10 μg protein) were incubated with anti-IAP75 in a standard immunoprecipitation reaction (*12*). The proteins of the immunoprecipitate (Anti-IAP Ip) and OM-IM (10 μg of protein) were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-IAP75 or mAb to hsp70 (mAb SPA-820) (*12*). (**D** and **E**) IAPs from 0, 1, and 2.5 minutes after the initiation of pS-protA import (IAP) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-IAP75 or mAb SPA 820 (*12*). The IAPs were derived from OM-IM containing 1 mg of protein (Fig. 2). (Lane 1) OM-IM contains 10 μg of protein.

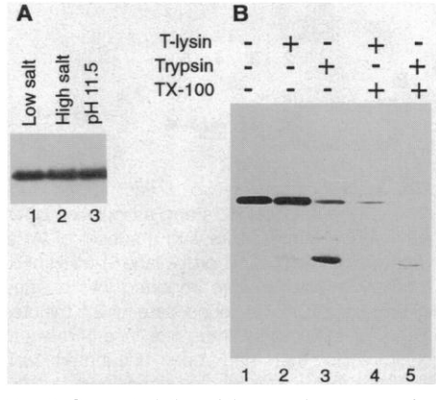

Fig. 6. Characteristics of the membrane association of IAP75. (**A**) OM (10 μg of protein) were resuspended in TE buffer (low salt), TE buffer containing 1 M NaCl (high salt), or 0.1 M Na_2CO_3 (pH 11.5) (*36*). The membranes were collected, and associated proteins were resolved by SDS-PAGE and immunoblotted with anti-IAP75 (*12*). (**B**) Isolated chloroplasts (equivalent to 25 μg of chlorophyll) were incubated with thermolysin (+ T-lysin) or trypsin (+ trypsin) in the presence (+) or absence (−) of Triton X-100 (TX-100) (*36*). Thereafter, the proteins were resolved by SDS-PAGE and immunoblotted with anti-IAP75 (*12*).

signal recognition). In this simplified cytosol-independent system there was nonetheless a requirement for ATP (*15*) and GTP in the binding of the precursor to the chloroplast (*11*). GTP (and its hydrolysis) may be required on the cis side of the channel and take part in the targeting and presentation of the transit sequence to the outer membrane channel and thereby participate in regulating opening of the channel.

In contrast, ATP would be required by the hsp70 IAP on the trans side of the outer membrane channel to keep the import intermediate anchored in the outer membrane channel. In the absence of ATP, the precursor might open the channel (requiring GTP hydrolysis) but might slip out of it resulting in channel closure and in a lack of "binding" to the chloroplast. In this view, the ATP- and GTP-dependent "binding" of the precursor to the chloroplast is the result of not one, but several, reactions that yield an early import intermediate whose NH_2-terminus has traversed the outer membrane channel and is retained in it by the hsp70 IAP (Fig. 8).

We believe that it is this channel-spanning import intermediate that allowed isolation of the outer membrane's import machinery. Because the import machinery of the inner membrane was not yet engaged, the outer membrane import machinery could be isolated separately from that of the inner membrane. Indeed, all of the early IAPs that we have characterized are integral proteins of the outer membrane. The separate isolation of the outer membrane import machinery suggests that it is not linked a priori to that of the inner membrane. Such a linkage appears to be accomplished, at least in part, by later import intermediates. If the four early IAPs make up a functional import complex, it may be possible to reconstitute the outer membrane machinery in whole or part and study their function in the translocation process by biochemical and electrophysiological methods.

The method of using channel-spanning translocation intermediates to isolate protein conducting channels and associated proteins may be productive for the isolation of the protein translocation machineries of other cellular membranes. The advantage of this method is that it appears to identify components of the translocation apparatus in apparently stoichiometric amounts relative to the translocation intermediates.

M	R	T	S	V	I	P	N	R	L	T	P	T	L	T	T	H	P	S	R	R	R	N	D	H	25
I	T	T	R	T	S	S	L	K	C	H	L	S	P	S	S	G	D	N	N	D	S	F	N	S	50
S	L	L	K	T	I	S	T	T	V	A	V	S	S	A	A	A	S	A	F	F	L	T	G	S	75
L	H	S	P	F	P	N	F	S	G	L	N	A	A	A	G	G	A	G	G	G	G	G	G	G	100
S	S	S	S	G	G	G	G	G	G	W	F	N	G	D	E	G	S	F	W	S	R	I	L	S	125
P	A	R	A	I	A	D	E	P	K	S	E	D	W	D	S	H	E	L	P	A	D	I	T	V	150
L	L	G	R	L	S	G	F	K	K	Y	K	I	S	D	I	L	F	F	D	R	N	K	K	S	175
K	V	E	T	Q	D	S	F	L	D	M	V	S	L	K	P	G	G	V	Y	T	K	A	Q	L	200
Q	K	E	L	E	S	L	A	T	C	G	M	F	E	K	V	D	M	E	G	K	T	N	A	D	225
G	S	L	G	L	T	I	S	F	A	E	S	M	W	E	R	A	D	R	F	R	C	I	N	V	250
G	L	M	G	Q	S	K	P	V	E	M	D	P	D	M	S	E	K	E	K	I	E	F	F	R	275
R	Q	E	R	E	Y	K	R	R	I	S	S	A	R	P	C	L	L	P	T	S	V	H	E	E	300
I	K	D	M	L	A	E	Q	G	R	V	S	A	R	L	L	Q	K	I	R	D	R	V	Q	S	325
W	Y	H	E	E	G	Y	A	C	A	Q	V	V	N	F	G	N	L	N	T	R	E	V	V	C	350
E	V	V	E	G	D	I	T	K	L	S	I	Q	Y	L	D	K	L	G	N	V	V	E	G	N	375
T	E	G	P	V	V	Q	R	E	L	P	K	Q	L	L	P	G	H	T	F	N	I	E	A	G	400
K	Q	A	L	R	N	I	N	S	L	A	L	F	S	N	I	E	V	N	P	R	P	D	E	M	425
N	E	G	S	I	I	V	E	I	K	L	K	E	L	E	Q	K	S	A	E	V	S	T	E	W	450
S	I	V	P	G	R	G	G	R	P	T	L	A	S	L	Q	P	G	G	T	I	T	F	E	H	475
R	N	L	Q	G	L	N	R	S	L	T	G	S	V	T	T	S	N	F	L	N	P	Q	D	D	500
L	A	F	K	M	E	Y	A	H	P	Y	L	D	G	V	D	N	P	R	N	R	T	L	R	V	525
S	C	F	N	S	R	K	L	S	P	V	F	T	G	G	P	G	V	D	E	V	P	S	I	W	550
V	D	R	A	G	V	K	A	N	I	T	E	N	F	S	R	Q	S	K	F	T	Y	G	L	V	575
M	E	E	I	I	T	R	D	E	S	N	H	I	C	S	N	G	Q	R	V	L	P	N	G	A	600
I	S	A	D	G	P	P	T	T	L	S	G	T	G	I	D	R	M	A	F	L	Q	A	N	I	625
T	R	D	N	T	R	F	V	N	G	T	I	V	G	S	R	N	M	F	Q	V	D	Q	G	L	650
G	V	G	S	N	F	P	F	F	N	R	H	Q	L	T	V	T	K	F	L	Q	L	M	S	V	675
E	E	G	A	G	K	S	P	P	P	V	L	V	L	H	G	H	Y	G	G	C	V	G	D	L	700
P	S	Y	D	A	F	T	L	G	G	P	Y	S	V	R	G	Y	N	M	G	E	I	G	A	A	725
R	N	I	L	E	L	A	A	E	I	R	I	P	I	K	G	T	H	V	Y	A	F	A	E	H	750
G	T	D	L	G	S	S	K	D	V	K	G	N	P	T	V	V	Y	R	R	M	G	Q	G	S	775
S	Y	G	A	G	M	K	L	G	L	V	R	A	E	Y	A	V	D	H	N	S	G	T	G	A	800
V	F	F	R	F	G	E	R	F																	809

Fig. 7. Deduced amino acid sequence of IAP75 cDNA (*26*). The amino acid residues, represented by their single letter code, are numbered at the right of the figure. Residues corresponding to peptide sequences obtained from IAP75 are underlined. The GenBank accession number for the IAP75 cDNA sequence is L36858.

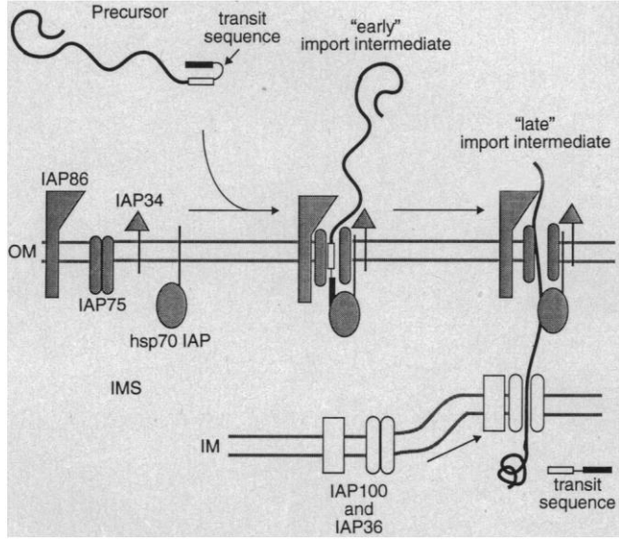

Fig. 8. Model for protein import into chloroplasts. OM, outer membrane; IMS, intermembrane space; and IM, inner membrane.

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12. Antiserum to the 37-kD outer membrane marker polypeptide (OM37) and the 35-kD inner membrane marker polypeptide (IM35) were prepared (*1*). MAb SPA-820 was from StressGen Biotechnologies Corp. (Victoria, B.C., Canada). Antiserum to pea cytosolic hsp70 was a gift of E. Vierling, University of Arizona, Tucson, AZ. Antibodies to IAP75 were produced from a synthetic peptide corresponding to amino acid residues 369 to 387 of the deduced sequence (Fig. 7) (*37*). SDS-PAGE, immunoblotting, and immunoprecipitation were performed as described (*7*, *38*).
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14. OM-IM (*35*) was resuspended in 50 mM tricine-KOH (pH 7.5), 2 mM EDTA, 10 percent glycerol, 250 mM NaCl (TEGS buffer) to a protein concentration of 1 mg/ml. Membranes (1 mg of protein) were diluted with an equal volume of TEGS buffer containing triton X-100 (4 percent by volume). The mixture was incubated on ice for 10 minutes and centrifuged at 100,000*g* for 15 minutes at 4°C to remove large aggregates. Approximately 75 percent of the envelope-associated pS-protA or S-protA remained in the supernatant after solubilization and centrifugation of the membranes. The supernatant was incubated with rabbit IgG-Sepharose (50-μl, packed-bed volume) (Cappel Organon Teknika, Inc.) for 2

hours at 4°C. The IgG-Sepharose was washed five times with 5 ml of 1 percent Triton X-100 in TEGS buffer and twice with 1 ml of TEGS buffer. Bound proteins were eluted from the gel with 150 μl of 1 percent SDS, 50 mM tris-HCl (pH 7.5). The recovery of [^{35}S]pS-protA and [^{35}S]S-protA on IgG-Sepharose from the solubilized membranes was close to 100 percent.
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26. Import intermediate-associated proteins from the 2.5-minute time point in pS-protA import were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and visualized with 0.1 percent amido black in 0.1 percent acetic acid. The protein band corresponding to 75 kD was excised and cleaved with endopeptidase Lys-C as described (*39*). Several internal peptides were subjected to NH_2-terminal sequence determination. A degenerate oligonucleotide mixture (TCIGTICCATGT/CTCIGCAAAIGCATAIACATGIGT), corresponding to the antisense strand encoding one of these peptides (residues 742 to 753 of the deduced sequence, Fig. 7), was synthesized and used to screen an unamplified λ gt11 cDNA library from pea by standard aqueous hybridization (*40*). The cDNA library was constructed as described (*41*) with polyadenylated RNA from pea seedlings grown in light. The 2.74-kb IAP75 cDNA was isolated and subcloned into pBluescript II (Stratagene). The resulting plasmid is referred to as pBS-IAP75. The nucleotide sequences of both strands of the cDNA were determined with Sequenase Version 2.0 (United States Biochemical).
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33. The pS-protA and S-protA hybrid proteins, as encoded by plasmid pET8c-pS-protA and pET8c-S-protA, respectively, were identical to those described (*1*). The plasmid pET8c-pFd-protA encoding the pFd-protA hybrid protein was constructed by digestion of pET8c-pS-protA with Nco I and Nsi I and replacing the pS coding sequence with nucleotides 1 to 375 of the coding sequence of the pFd cDNA (*42*). The pFd fragment for insertion in the plasmid was generated by amplifying the pFd sequence of pSPFD22 (*43*) with the polymerase chain reaction with primers that incorporated in-frame Nco I and Nsi I sites at the 5′ and 3′ ends of the DNA, respectively. The resulting pET8c-pFd-protA plasmid encoded amino acids −48 to 77 of pFd fused to amino acids −10 to 271 of the protein A polypeptide. The pS-protA, S-protA, and pFd-protA precursors were expressed in *E. coli* and purified from extracts by affinity chromatography on rabbit IgG-Sepharose (Cappel, Organon Teknika Corp.) as described (*1*). The pS-protA was labeled with a mixture of [^{35}S]methionine-cysteine, and pFd-protA was labeled with [^{3}H]leucine (DuPont New England Nuclear, Inc.). The binding and import of urea-denatured pS-protA or pFd-protA was assayed under substrate saturating conditions (*1*). Although the import reaction volumes varied depending on the amount of chloroplast added, each assay contained chloroplasts at a chlorophyll concentration equivalent to 0.5 to 1 mg/ml.
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35. Lysed chloroplasts were separated into soluble (stroma) and membrane fractions by differential centrifugation (*34*). The membrane pellet was resuspended in 50 mM tricine-KOH (pH 7.5), 2 mM EDTA (TE buffer) containing 42 percent sucrose and separated by flotation into linear sucrose gradients (20 to 38 percent) in a Beckman SW28 rotor (*1*). After centrifugation, the 20 to 38 percent sucrose layer that contained the bulk of the envelope membranes was separated into 1.5-ml fractions. Each fraction was diluted with 3 volumes of TE buffer, and the membranes were recovered by centrifugation at 200,000*g* for 2 hours. The 42 percent sucrose cushion containing the bulk of thylakoid membranes and residual stromal contents was discarded. OM, OM-IM, and IM refer to fractions 1 and 2, 3 to 9, and 10 to 13 in Fig. 1A, respectively.
36. OM (10 μg of protein) were resuspended in 2 ml of the buffer indicated and each sample was homogenized by sonication in a water bath for 30 seconds. The extracted membranes were collected by centrifugation at 200,000*g* for 30 minutes. The supernatant fraction was discarded and the proteins associated with the sedimented membranes were resolved by SDS-PAGE and immunoblotted with anti-IAP75 (*12*). Thermolysin and trypsin treatments of intact chloroplasts were performed as described (*22*, *25*).
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44. Supported by a Busch Memorial Fund Award (D.J.S.), NSF grant MCB-9404611 (D.J.S.), and an International Human Frontier Science Program Fellowship (F.K.). We thank The Rockefeller Protein Sequencing Facility for protein sequence determination.

28 July 1994; accepted 13 October 1994