IAP34 is a 34-kDa component of the outer membrane complex that mediates the initial stages of protein import into chloroplasts (Seedorf, M., Waegemann, K., and Soll, J. (1995) Plant J. 7, 401–411; Kessler, F., Blobel, G., Patel, H. A., and Schnell, D. J. (1994) Science 266, 1035–1039). We have investigated the targeting and insertion of IAP34 at the outer envelope membrane. The analyses of IAP34 deletion mutants and hybrid proteins (consisting of regions of IAP34 fused to the soluble IgG-binding domain of staphylococcal protein A) suggest that the transmembrane domain and C-terminal tail of IAP34 contain information essential but not sufficient for targeting to the outer membrane. Treatment of chloroplasts with exogenous proteases does not affect IAP34 insertion, indicating that targeting does not require surface-exposed receptors at the envelope. GTP or GDP is required for maximal integration of IAP34 into the outer membrane. The GTP/GDP requirement is attributed to the intrinsic GTP binding activity of IAP34 because GTP/GDP binding-deficient mutants are defective in outer membrane insertion. On the basis of these observations, we propose that IAP34 is targeted to the chloroplast by a C-terminal signal and efficiently integrated into the outer membrane by conformation-induced insertion upon GTP/GDP binding.

The import of nuclear-encoded preproteins into chloroplasts is mediated by the coordinate interaction of protein conducting machineries in the outer and inner membranes of the chloroplast envelope (3). Initial recognition of the precursor is mediated by the interaction of its N-terminal targeting signal (transit sequence) with a trimeric complex in the chloroplasm outer membrane (4–6). This complex consists of two structurally related GTP-binding proteins, IAP34 (1, 2) and IAP86 (2, 7), and IAP75 (4, 8), a unique polypeptide that is deeply embedded in the lipid bilayer of the outer membrane.

All three components of the outer membrane import complex are nuclear-encoded. Both IAP75 and IAP86 are unusual for outer membrane proteins in that they possess N-terminal presequences (2, 4, 7, 8). The presequence of IAP75 is bipartite, consisting of two functional targeting domains (23). The N-terminal domain of the presequence can target a soluble fusion protein to the stroma and, therefore, is functionally equivalent to a typical transit sequence. The C-terminal domain of the IAP75 presequence is necessary to prevent complete translocation of preIAP75 into the stroma. On the basis of these results, it has been proposed that preIAP75 initially engages the general protein import machinery via the N-terminal stromal targeting domain, but the C-terminal domain prevents translocation across the inner membrane and triggers insertion of IAP75 into the outer membrane (23).

The presequence and membrane domain of IAP86 have been shown to be necessary for insertion of the polypeptide into the outer membrane in an ATP-dependent reaction (24). However, unlike preIAP75, the insertion of preIAP86 does not appear to involve the general protein import machinery for stromal proteins, but relies on other protease-sensitive components of the outer membrane. IAP34 is not synthesized as a precursor polypeptide, and therefore contains outer membrane targeting information within its mature sequence. In this respect, IAP34 is similar to the previously characterized outer membrane proteins, Omp24 (9), Om14 (10), and Om7 (11). All three of these proteins insert into the outer membrane independent of surface-exposed receptors, but Omp24 is the only protein whose insertion is stimulated by ATP. The targeting signals and mechanism of insertion of these outer membrane proteins remain to be investigated.

Two groups have come to different conclusions as to whether IAP34 insertion is dependent on energy and proteinaceous components of the outer membrane. Both groups studied the insertion of IAP34 into isolated chloroplasts using in vitro assays; however, they used different methods to assess membrane insertion of the polypeptide. Seedorf et al. (1) assayed the insertion of IAP34 by observing the specific proteolytic peptide map of IAP34 generated following the insertion reaction. Using this assay, they concluded that the insertion of IAP34 was dependent on ATP and proteinaceous components of the outer membrane. Kessler et al. (2) assayed IAP34 insertion by measuring the insensitivity of chloroplast-associated IAP34 to alkaline extraction, a common method for distinguishing peripheral from integral membrane proteins. Using this method, IAP34 insertion was shown to be unaffected by the addition of exogenous ATP or the treatment of chloroplasts with thermoslysin prior to the insertion assay.

We have studied the requirements for the insertion of IAP34 with the chloroplast outer membrane in an effort to define the mechanism of outer membrane targeting and integration. Our results suggest a unique mechanism for targeting to the outer membrane that is mediated by a C-terminal targeting signal and is driven by the intrinsic GTP binding activity of the protein.

EXPERIMENTAL PROCEDURES

Chloroplast Isolation and Subfractionation—Intact chloroplasts were isolated from 10–14-day-old pea seedlings (Pisum sativum var.
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Green Arrow) by homogenization and Percoll silica gel gradient centrifugation as described previously (12). For insertion assays, chloroplasts were resuspended in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) to a concentration equivalent to 2–3 mg of chlorophyll/ml. Thermolysin treatment of intact chloroplasts was performed by the method of Diner (13). Trypsin and proteinase K treatments were performed as described previously (12). All protease treatments were performed for 30 min on ice at a protease concentration of 200 μg/ml and a chloroplast concentration of 1 mg of chlorophyll/ml.

To prepare chloroplast envelope membranes, intact chloroplasts were lysed under hypertonie conditions and separated into soluble and membrane fractions by differential centrifugation as described by Keegstra and Yousif (14).

IAP34 Mutations and Protein A Fusion Proteins—pET21d-IAP34-N218K/D219L and pET21d-IAP34-G48R/K52N/S53R encoding the N218K/D219L and pET21d-IAP34-G48R/K52N/S53R mutants, respectively, were generated by polymerase chain reaction (PCR)1 mutagenesis using the coding sequence of pBS-IAP34 (2) as a template. For IAP34-G48R/K52N/S53R, the primers for PCR introduced the following point mutations: codon GCT changed to CGT resulting in a Gly to Arg mutation at amino acid 53. For N218K/D219L, the primers for PCR introduced the following point mutations; codon AAT changed to CTC resulting in an Asp to Leu mutation at amino acid 218, and codon GAC changed to CTT resulting in a Asp to Lys mutation at amino acid 48. The primers for PCR were designed and synthesized by Operon Technologies, Inc. (Alameda, CA) and targeted to the coding regions of the plasmids, a segment of the staphylococcal protein A expression in Escherichia coli. The G48R/K52N/S53R and N218K/D219L mutant coding regions were inserted into pET21d for coupled transcription-translation and expression in E. coli.

To construct the pET21b-protA-IAP34(237–310) plasmids, two DNA fragments were generated by PCR using primers that replaced Leu-288 and Asn-245 with stop codons, respectively. pBS-IAP34 (2) was used as the template for PCR. The resulting DNA fragments were inserted into the NcoI/BamHI sites of pET21d (Novagen, Inc., Madison, WI) for coupled transcription-translation.

To construct the pET21b-protA-IAP34(237–310) and pET21b-protA-IAP34(237–287) plasmids, a segment of the staphylococcal protein A gene from nucleotides 272–1104 was amplified by PCR using primers that incorporated an in-frame EcoRI site at the 5′ end and NcoI and XhoI sites at the 3′ end. pET8c-protAIAP34 served as the template (15). This fragment was inserted into the EcoRI/XhoI site of pET21b to generate pET21b-protA. DNA fragments corresponding to amino acids 237–310 or 237–287 of IAP34 were amplified by PCR with primers that generated 5′ NcoI sites and 3′ stop codons followed by XhoI sites. The fragments were then inserted into the NcoI/XhoI sites of plasmid pET21b-protA to generate pET21b-protA-IAP34(237–310) and pET21b-protA-IAP34(237–287), and they were used for coupled transcription and translation reactions. All mutations and fusion constructs were confirmed by the dideoxy sequencing method using an automated ABI 373 DNA Sequencer (Applied Biosystems, Foster City, CA). The nitrocellulose was washed three times with cold 50 mM Hepes-KOH, pH 7.5, 5 mM MgOAc. Slices of nitrocellulose corresponding to the bound proteins were excised, and bound [35S]GTP was quantitated using a scintillation counter or a PhosphorImager SI (Molecular Dynamics, Inc., Sunnyvale, CA) with the IPLab Gel Scientific Image Processing version 1.5c program (Signal Analytics, Vienna, VA).

For assay of the insertion of E. coli synthesized substrates, the purified inclusion body fractions from extracts of induced E. coli cultures were dissolved in 6 M urea, 50 mM Tris-HCl, pH 7.5 to a concentration of 0.2 mg of protein/ml and diluted 10-fold with import buffer at room temperature. After 30 min, the samples were centrifuged at 30,000 × g for 30 min to remove insoluble aggregates, and the supernatant was added to the insertion reaction to a final concentration of 50 μM. After 30 min, the aliquots were analyzed for identical to insertion reactions performed with reticulocyte lysate PRS.

GTP Binding Activity of IAP34 and IAP34 Mutants—IAP34 and the IAP34-N218K/D219L and IAP34-G48R/K52N/S53R mutants containing the C-terminal six histidine residues were expressed in E. coli from pET21d (Novagen, Inc., Madison, WI). The six-histidine fusions were generated by deleting the normal stop codons with PCR mutagenesis resulting in a coding region in frame with the six-histidine coding region of pET21d. The expressed proteins were dissolved in inclusion bodies in 6 M urea, 50 mM Tris-HCl, pH 7.5 and purified on a nickel-chelate matrix as recommended by the supplier (Novagen, Inc., Madison, WI). Refolding of the purified proteins was performed as given for the preparation of the import substrates.

For the assay of GTP binding to soluble proteins, 100 ng of each protein was incubated with 50 μM [35S]GTP (3000 Ci/mmol, DuPont NEN), 100 mM ATP, 50 mM Hepes-KOH, pH 7.5, 5 mM MgOAc in a final volume of 100 μl at 26 °C for 15 min. The mixture was chilled on ice and filtered through nitrocellulose using a slot blot apparatus (Hoefer, Inc., San Francisco, CA). The nitrocellulose was washed three times with cold 50 mM Hepes-KOH, pH 7.5, 5 mM MgOAc. Slices of nitrocellulose corresponding to the bound proteins were excised, and bound [35S]GTP was quantitated using a scintillation counter or a PhosphorImager SI (Molecular Dynamics, Inc.).

Immunoblot Analysis—Antibodies to IAP34, IAP75, and IAP86 were prepared as described previously (6). Antibody detection in immunoblots was performed using a chemiluminescence detection system (DuPont NEN).

RESULTS

Energy Requirements for IAP34 Insertion—We define insertion of IAP34 as correct targeting and integration of the protein at the lipid bilayer of the outer envelope membrane. We assay outer membrane targeting by determining the extent of association of IAP34 in vitro synthesized 35S-IAP34 with isolated chloroplasts. Integration of IAP34 into the membrane is assayed as the fraction of membrane-associated IAP34 that is resistant to extraction by alkaline buffer (i.e. 0.2 M Na2CO3, pH 11.5). To insure the correct localization of IAP34 to the outer membrane, we assess the sensitivity of insertcd IAP34 to thermolysin, a protease that digests surface-exposed proteins, but does not permeate the outer membrane (13).

As a first step in defining the mechanism of IAP34 insertion, we investigated the energy requirements for targeting and integration. Seedorf et al. (14) previously had shown that the addition of ATP to an in vitro insertion assay containing isolated chloroplasts stimulated the association of IAP34 with the outer envelope membrane, whereas we had observed no effect of exogenous ATP on insertion (2). We wished to reexamine the energetics of IAP34 insertion with precautions to eliminate any free nucleoside triphosphates (NTPs) from the in vitro transla-

1 The abbreviations used are: PCR, polymerase chain reaction; PRS, post-ribosomal supernatant; PAGE, polyacrylamide gel electrophoresis.

In Vitro Translation and Expression in E. coli—All in vitro synthesized substrates for the insertion assays were generated in a coupled transcription-translation system containing reticulocyte lysate according to the supplier’s recommendations (Promega Corp., Madison, WI). After the translation reaction, the translation mixture was centrifuged for 22 min at 150,000 × g at 4 °C to yield a postribosomal supernatant (PRS). The PRS was treated with 20 μl EDTA for 5 min at 26 °C and gel filtered using Sephadex G-25 (Pharmacia Biotech Inc.) to remove free nucleotides. pET21d-IAP34, pET21d-IAP34-N218K/D219L, and pET21d-IAP34-G48R/K52N/S53R were transformed into E. coli BL21 (DE3), and the fraction of membrane-associated IAP34 that is resistant to extraction by alkaline buffer (i.e. 0.2 M Na2CO3, pH 11.5). To insure the correct localization of IAP34 to the outer membrane, we assess the sensitivity of insertcd IAP34 to thermolysin, a protease that digests surface-exposed proteins, but does not permeate the outer membrane (13).

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chloroplasts were incubated in the presence or absence of 200 mM EDTA and then gel-filtered to remove free NTPs prior to the insertion reaction. The membrane fractions were analyzed for associated IAP34.

Experimentally, energy-depleted chloroplasts in an insertion reaction were preincubated at 26°C in the dark in the presence of apyrase to deplete both endogenous and exogenous NTPs. The filtered lysates were incubated with iso-reticulocyte lysate PRS containing 35S-IAP34 or the isolated chloroplasts. ATP (Fig. 1A, lanes 3 and 4 and GMP (Fig. 1A, lanes 9 and 10) also stimulate insertion, but only approximately 2-fold over the basal level (Fig. 1B). The addition of both ATP and GTP to the reaction (Fig. 1A, lanes 11 and 12) had no additional effect over GTP alone (Fig. 1B). Approximately 80–90% of membrane-associated IAP34 was recovered in the membrane fractions after alkaline extraction, indicating that the polypeptide was integrated into the lipid bilayer in each reaction (Fig. 1A, lanes 4, 6, 8, 10, and 12). The integrated IAP34 from insertion reactions performed in the presence of apyrase or GTP were sensitive to exogenous thermolysin confirming their correct localization in the outer membrane (Fig. 1A, compare lanes 13 and 14, and lanes 15 and 16).

To completely eliminate the possibility that the low levels of IAP34 insertion observed in the absence of added NTP was due to residual free NTP from the translation lysate, we tested the effects of NTPs on the insertion of purified IAP34. IAP34 was expressed in the presence of [35S]methionine in E. coli and tested for its ability to insert into the outer membrane. E. coli-expressed [35S]IAP34 was purified in the presence of 6 M urea. Prior to the insertion assay, the protein was diluted 10-fold into import buffer lacking urea. At low concentrations, IAP34 remained soluble in import buffer upon dilution from urea (Fig. 2A). The soluble protein was added to the standard insertion reaction containing isolated chloroplasts. The results presented in Fig. 2B indicate that E. coli-expressed IAP34 inserts into the outer membrane of isolated chloroplasts. Insertion is stimulated 5-fold by the addition of GTP (Fig. 2, B, compare lanes 6 and 7; and C) or GDP (Fig. 2, B, compare lanes 6 and 9; and C) to the reaction. ATP stimulates insertion by 2-fold (Fig. 2, B, compare lanes 6 and 8; and C). In all cases, the membrane-associated IAP34 is resistant to alkali extraction, indicating that the protein has integrated into the lipid bilayer (Fig. 2B, compare lanes 2–5 with lanes 6–9). The results with purified E. coli-expressed IAP34 are indistinguishable from the results obtained with in vitro synthesized protein. These data confirm that IAP34 insertion into the outer membrane is maximally stimulated by GTP or GDP.

Mutations That Decrease GTP Binding Decrease IAP34 Membrane Insertion—The observation that GTP and GDP are most effective in promoting insertion of IAP34 into the outer membrane suggests that the protein’s endogenous GTP/GDP binding may play a role in chloroplast targeting and membrane integration. To test this possibility, we generated two mutant forms of IAP34 that are altered in their ability to bind GTP and tested their competence for insertion into the outer envelope. The nature of the IAP34 mutations is shown in Fig. 3. The G48R/K52N/S53R contains three point mutations within the predicted G1 motif (18) of the consensus GTP-binding site of the protein (Fig. 3A). The N218K/D219L contains two adjacent point mutations within the predicted G4 motif (18) of the GTP-binding site (Fig. 3B).

The relative GTP binding capacities of the mutant and wild type forms of IAP34 were tested using purified proteins from E. coli expression systems. The expressed forms of wild type and mutant IAP34 contained the C-terminal six-histidine tags, and were purified on a nickel-chelate matrix in the presence of 6 M urea. Upon rapid dilution from urea, the proteins remained soluble at concentrations at or below 0.1 mg/ml (data not shown). The results in Fig. 4 indicate that these soluble forms of IAP34 regain GTP-binding after dilution from urea. The mutations in N218K/D219L had a moderate affect on GTP binding, reducing the apparent binding capability by 40% rel-
The relative efficiency of insertion of the two mutants correlated with the presence or absence of 0.2 M Na$_2$CO$_3$, pH 11.5. GTP binding by G48R/K52N/S53R and N218K/D219L were synthesized in a reticulocyte lysate and their insertion was compared to wild type IAP34. The insertion of N218K/D219L is reduced to 75% of wild type IAP34 (Fig. 5, compare lanes 4 and 5; and B), whereas G48R/K52N/S53R inserts at levels that are approximately 15% of the levels of wild type IAP34 (Fig. 5B, compare lanes 1 and 3 and lanes 4 and 6; and Fig. 1C). The relative efficiency of insertion of the two mutants correlated well with their reduced level of GTP binding (compare Figs. 4 and 5B). In fact, the levels of insertion observed with G48R/K52N/S53R, the mutant with very low relative GTP binding, are nearly identical to the levels of insertion for wild type IAP34 in the absence of added NTP (compare Figs. 1B and 5B). These results indicate that the intrinsic GTP binding of IAP34 is required for proper insertion into the outer envelope, and that this requirement accounts for the stimulation of IAP34 insertion by GTP or GDP.

**IAP34 Contains a C-terminal Targeting Signal—Protease sensitivity mapping and primary sequence analyses predict that IAP34 contains a single helical transmembrane domain (amino acids 267–283) with its N-terminal GTP-binding domain located in the intermembrane space of the envelope. The low levels of IAP34 outer membrane insertion observed in the absence of added energy (Fig. 1B) and with the GTP-binding mutants (Fig. 5B) suggest that targeting of IAP34 to the outer membrane may be directed by a signal independent of GTP binding, but that GTP binding may be necessary for efficient membrane insertion. As a first step to identify possible targeting signals, deletion mutants lacking one or more regions of the C-terminal portion of IAP34 were tested for their ability to insert into the outer membrane. We focused our deletion mutation analysis on the C-terminal tail and transmembrane domain because comparable domains have been shown to be important in targeting and anchoring proteins to the mitochondrial outer membrane and the endoplasmic reticulum membrane. In addition, by restricting the mutations to the C-terminal portions of IAP34, we attempted to avoid introducing mutations in the GTP-binding site that would affect insertion. A schematic representation of the deletions is shown in Fig. 6A. These deletions cover the C-terminal tail of IAP34 (amino acids 288–310)
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or the C-terminal tail plus the putative transmembrane helix (Δ245–310). The results of insertion experiments shown in Fig. 6B indicate that deletion of the C-terminal tail and the putative transmembrane domain (Δ245–310) eliminates the ability of IAP34 to associate with the outer membrane (Fig. 6, B, lane 5; and C). This result is consistent with a previous study (1) and is not surprising because deletion of the transmembrane helix presumably eliminates the membrane anchor for IAP34. Deletion of the C-terminal tail alone reduces IAP34 insertion to 11% of the level of wild type IAP34 (Fig. 6, B, lane 3; and C). The decrease in insertion observed with Δ288–310 was not due to a disruption of GTP binding because this deletion mutant bound GTP at levels comparable to wild type IAP34 (data not shown).

The protease sensitivity of the Δ288–310 indicates that the deletion did not affect localization of IAP34 to the outer membrane (Fig. 6B, lane 4).

On the basis of the results in Fig. 6, it appears that the C-terminal domain of IAP34 contains information necessary for targeting and insertion to the outer membrane. To further dissect the role of these sequences in targeting, we tested the ability of the C-terminal tail and the putative transmembrane domain to target a soluble protein to the outer membrane. The first fusion protein, protA-34(237–310), consisted of the protein A IgG-binding domains fused to the C-terminal 74 amino acids of IAP34 including the transmembrane domain and C-terminal tail (Fig. 7A). The second fusion protein, protA-34(288–310), consisted of the IgG-binding domain of staphylococcal protein A fused at its C terminus to a segment of IAP34 (amino acids 237–287) that encompasses only the putative transmembrane domain (Fig. 7A). Fig. 7 shows that protA-34(237–310) is targeted to the envelope with an efficiency equal to 17% of IAP34 (Fig. 7, B, lane 3; and C). The chloroplast-associated protA-34(237–310) is membrane-integrated because it is not extracted with alkali, and it is correctly targeted to the outer membrane because it remains sensitive to exogenous thermolysin (Fig. 7B, lane 4). The levels of protA-34(237–310) insertion are comparable to those observed with IAP34 in the absence of GTP (Fig. 1B) or with the N218K/D219L GTP-binding mutant (Fig. 5B), providing additional evidence that the C-terminal 25 amino acids of IAP34 contain information for targeting to the outer membrane. Prota-34(237–287) does not associate with isolated chloroplasts, indicating that the transmembrane domain alone is insufficient to act as a targeting signal (Fig. 7B, lanes 5; and C).

IAP34 Insertion Is Independent of Surface Receptors—Previous studies have shown that the insertion of three outer membrane proteins, Om14 (10), Om7 (11), and Omp24 (9), occurs independent of surface-exposed receptors. We previously showed that IAP34 insertion, assessed by resistance to alkali extraction, is not affected by pretreating chloroplasts with thermolysin (2), a protease that digests cytoplasmically exposed outer membrane proteins (13). A second group showed that IAP34 that associated with the outer membrane following thermolysin treatment of the chloroplasts is more sensitive to subsequent thermolysin treatment (1). Based on these results, this

**Fig. 5. Insertion of IAP34 GTP-binding mutants into the outer membrane of isolated chloroplasts.** *In vitro* synthesized 35S-IAP34, 35S-G48R/K52N/S53R, or 35S-N218K/D219L was incubated with isolated chloroplasts (50 μg of chlorophyll) in a standard insertion reaction (see legend to Fig. 1) in the presence of 0.1 mM GTP. Each reaction contained an equimolar amount of 35S-IAP34, 35S-G48R/K52N/S53R, or 35S-N218K/D219L. After the insertion reaction, the chloroplasts were reisolated, lysed, and the total membrane fraction was incubated in the presence or absence of 0.2 mM Na2CO3, pH 11.5 (+/− pH 11.5), for 10 min on ice. The membrane fractions were collected and analyzed by SDS-PAGE and fluorography. A, fluorograph of SDS-PAGE-resolved membrane proteins from the insertion reactions. B, quantitation of lanes 4–6 from A.

**Fig. 6. Insertion of IAP34 deletion mutants into isolated chloroplasts.** *In vitro* synthesized 35S-IAP34, 35S-Δ288–310, and 35S-Δ245–310 were incubated with isolated chloroplasts (50 μg of chlorophyll) in a standard insertion reaction (see legend to Fig. 1) in the presence of 0.1 mM GTP. After the insertion reaction, chloroplasts were incubated in the presence or absence of 200 μg/ml thermolysin (+/− Protease) for 30 min on ice. The chloroplasts were reisolated and lysed, and the total membrane fraction was incubated in the presence of 0.2 mM Na2CO3, pH 11.5, for 10 min on ice. The membrane fractions were collected and analyzed by SDS-PAGE and fluorography. A, diagrammatic representation of IAP34, Δ288–310, and Δ245–310 indicating the GTP-binding, transmembrane, and C-terminal tail domains. B, fluorograph of SDS-PAGE-resolved membrane proteins from the insertion reactions. C, quantitation of lanes 1, 3, and 5 of B.
group concluded that IAP34 insertion requires other outer membrane proteins. To clarify whether surface-exposed outer membrane proteins are involved in IAP34 insertion, isolated chloroplasts were treated with three different proteases prior to the insertion reaction. Trypsin, proteinase K, and thermolysin were chosen for these treatments. The outer membrane is not permeable to thermolysin, but is permeable to trypsin and proteinase K (13). The results of IAP34 insertion into protease-treated chloroplasts is shown in Fig. 8. None of the three protease treatments had a significant effect on IAP34 insertion (Fig. 8A). However, all three proteases were active because endogenous IAP34, IAP75, and IAP86 were all degraded to various extents by the protease treatments (Fig. 8B). Our results make it unlikely that IAP34 insertion is dependent on one of the components of the outer membrane import complex or another unidentified surface-exposed receptor.

DISCUSSION

IAP34 is a 34-kDa component of an outer membrane complex, which mediates the recognition and initial translocation of nuclear-encoded precursor proteins at the chloroplast envelope. It is integrated into the membrane in a N_\text{out}-C_\text{in} orientation with a 29-kDa GTP-binding domain exposed to the cytoplasm, a single transmembrane domain, and a predicted 3-kDa C-terminal tail exposed to the intermembrane space of the envelope (1, 2). We have investigated the requirements for the targeting and insertion of IAP34 into the chloroplast outer envelope membrane. Our results suggest a mechanism of IAP34 insertion that requires both intrinsic targeting information and the intrinsic GTP-binding ability of the protein.

A role for GTP binding was revealed when we investigated the energy requirements for IAP34 insertion. Our results indicated that GTP or GDP was necessary for maximal integration of IAP34 into the outer membrane (Figs. 1 and 2). The GTP/GDP requirement was apparently due to the intrinsic GTP/GDP binding of IAP34, because mutants deficient in binding were deficient in integration (Fig. 5). These results suggest that the integration of IAP34 into the outer membrane requires a stable conformation that is induced by the binding of GTP or GDP. Like other small GTP-binding proteins, IAP34 is capable of binding to both GDP and GTP with relatively high affinity (1, 2). It is unlikely that GTP hydrolysis plays a role in insertion because GDP is as effective as GTP in stimulating insertion. Rather, GTP or GDP binding at the cytoplasmic domain may

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2 D. Chen and D. J. Schnell, unpublished observations.
induce or stabilize an insertion-competent conformation in IAP34.

Seedorf et al. (1) previously observed that ATP stimulated IAP34 association with the outer membrane 2-fold. The effect of other NTPs was not tested. We obtained similar results with ATP. However, we believe that the moderate stimulation of ATP on insertion is not due to an independent role for ATP in insertion, but could be explained by low affinity binding of ATP to IAP34 in the absence of GTP/GDP. This interpretation is supported by the observation that GTP and ATP together have no additional stimulatory effect on IAP34 insertion, and that GMP also was able to stimulate insertion at levels comparable to ATP (Figs. 1 and 2).

In addition to GTP/GDP binding, the C-terminal 23 amino acids of IAP34 appear to be necessary to target and/or integrate the protein into the membrane. Deletion of the C-terminal region reduces insertion by 89% compared to the wild type protein (Fig. 6). A role for the C terminus in targeting is supported by the observation that this C-terminal segment along with the IAP34 transmembrane domain targets a soluble domain of staphylococcal protein A to the outer membrane, albeit the efficiency of targeting of the fusion protein is only 17% of that observed with IAP34 (Fig. 7). Both the transmembrane domain and the C-terminal 23 amino acids of IAP34 appear to be necessary, but insufficient elements for IAP34 insertion (Fig. 7).

Surface-exposed outer membrane proteins do not appear to play a role in IAP34 insertion, and therefore it is unlikely that the C-terminal tail binds to a surface receptor. Treatment of isolated chloroplasts with three different proteases had little effect on IAP34 insertion (Fig. 8). However, we cannot completely rule out the possibility that the C-terminal domain interacts with a protease-insensitive component of the outer membrane. This conclusion is in contrast to that of a previous study by Seedorf et al. (1), who proposed that protease-sensitive outer membrane components were necessary for IAP34 insertion. The discrepancy in the conclusions of these two studies is most likely due to the different methods used to assess the integration of IAP34 into the outer membrane. We assessed integration by testing the resistance of membrane-associated IAP34 to extraction with alkaline buffer (i.e. 0.2 m Na$_2$CO$_3$, pH 11.5). This protocol is a standard method for defining integral versus peripherally associated membrane proteins (17). Seedorf et al. (1) assayed IAP34 integration by treating chloroplasts with thermolysin following the insertion reaction and assessing the appearance of an 8-kDa protease-insensitive C-terminal fragment of IAP34. The sensitivity of membrane-associated IAP34 to extraction by alkaline buffer in untreated or protease-treated chloroplasts was not tested. IAP34 is known to form a tight complex with at least two other outer membrane components, IAP75 and IAP86, suggesting that part of the 8-kDa fragment may be protected from digestion due to its association with one of these components and not due to its insertion across the outer membrane. Supporting this possibility is the fact that the 8-kDa proteolytic fragment is significantly larger than the 5-kDa fragment encompassing the transmembrane domain and C-terminal tail that is predicted to be protected by the lipid bilayer (1, 2). Therefore, we believe that the protease-sensitivity assay of Seedorf et al. may have measured a step other than membrane integration such as assembly or binding with other outer membrane components.

Several elements of IAP34 insertion are similar to those observed for other outer membrane proteins. Three proteins, Om7 (11), Om14 (10), and Omp24 (9), also insert into the outer membrane independently of a cleavable targeting signal or surface-exposed receptors. The targeting signals for these proteins have not been determined, and it is not clear whether the integration of these proteins requires a folded tertiary structure. Omp24 is the only protein of this group whose insertion is stimulated by energy in the form of ATP. It is unlikely that this stimulation is due to direct binding of ATP to Omp24, because its primary structure lacks typical ATP binding motifs.

The requirement of a folded tertiary conformation for membrane insertion of IAP34 is unique among known membrane targeting reactions of cellular proteins (19). For example, the integration of mitochondrial outer membrane proteins that do not contain cleavable targeting signals appears to depend only on internal signals contained within a transmembrane domain (20–22). Although the mechanism of IAP34 insertion remains to be investigated, a likely hypothesis is that the C-terminal tail acts to specifically target the protein to the chloroplast outer membrane via an interaction with a unique component of the outer membrane surface. One candidate for this component is the unique lipid composition of the outer membrane. Integration of the protein into the outer membrane would require a stable tertiary structure that would position the transmembrane domain in the correct conformation for insertion. This conformation would be provided by the binding of GTP or GDP at the cytoplasmic domain. Whether the conformation induced by GDP or GTP binding serves only to position the C-terminal tail for insertion or whether binding provides the energy to drive the C terminus across the outer membrane remains to be determined.

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