Insertion of atToc34 into the Chloroplastic Outer Membrane Is Assisted by at Least Two Proteinaceous Components in the Import System

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Toc34 is a member of the outer membrane translocon complex that mediates the initial stage of protein import into chloroplasts. Toc34, like most outer membrane proteins, is synthesized in the cytosol at its mature size without a cleavable transit peptide. The majority of outer membrane proteins do not require thermolysin-sensitive components on the chloroplastic surface or ATP for their insertion into the outer membrane. However, different results have been obtained concerning the factors required for Toc34 insertion into the outer membrane. Using an Arabidopsis homologue of pea Toc34, atToc34, we show that the insertion of atToc34 was greatly reduced by thermolysin pretreatment of chloroplasts as assayed either by protease digestion or by alkaline extraction. The insertion was also dependent on the presence of ATP or GTP. A mutant of atToc34 with the GTP-binding domain deleted still required ATP for optimal insertion, indicating that ATP was used by other protein components in the import system. The ATP-supported insertion was observed even in thermolysin-pretreated chloroplasts, suggesting that the protein component responsible for ATP-stimulated insertion is a different protein from the thermolysin-sensitive component that assists atToc34 insertion.

Most proteins in chloroplasts are encoded by nuclear genes and post-translationally imported from the cytosol. There appear to be at least two classes of nucleus-encoded chloroplastic proteins, distinguished by the presence or absence of cleavable targeting signals. The first class of proteins are synthesized in the cytosol as higher molecular weight precursor proteins with N-terminal extensions called transit peptides. This class of proteins consists of proteins targeted to the interior of chloroplasts (the inner envelope membrane, the stroma, the thylakoid membrane, and the thylakoid lumen) and two outer membrane proteins (1–4). Transit peptides are necessary and sufficient for the import of these precursor proteins into chloroplasts. The import process is initiated by a binding step that involves specific interaction between the transit peptide and a thermolysin-sensitive protein complex on the chloroplastic surface, followed by translocation of the precursor protein across the envelope. The binding step requires ATP in the 100 μM range, and translocation across the envelope requires about 1 mM ATP (5). Several envelope proteins that are associated with precursor proteins in the binding step have been identified (6, 7). These proteins are likely to be components of the machinery responsible for the import of precursor proteins into chloroplasts (8). These proteins are collectively named Toc (translocon of the outer membrane of chloroplasts) or Tic (translocon of the inner membrane of chloroplasts) proteins (9).

Members of the second class of nucleus-encoded chloroplastic proteins are synthesized in the cytosol at their mature size without cleavable transit peptides. This class of proteins includes most chloroplastic outer membrane proteins. Much less is known about the import mechanism of these outer membrane proteins. Most studies indicate that their insertion into the outer membrane does not require ATP or any thermolysin-sensitive proteins on the chloroplastic surface (10–13). One exception to this rule is the outer membrane protein Toc34.

Toc34 was identified as a member of the translocon complex at the outer membrane (1, 14). It is a GTP-binding protein and contains an outer membrane-targeting/insertion signal located at the C terminus of the protein (14–16). Using the appearance of a protease-resistant fragment corresponding to the C-terminal membrane anchor to assess insertion, one research group reported that insertion of Toc34 into the outer membrane is sensitive to thermolysin pretreatment of chloroplasts and also reduced by glucose/hexokinase treatment to remove ATP (14). It is not clear whether the ATP is used by Toc34 itself or by other ATP-requiring protein components in the import system. In contrast, using resistance to alkaline extraction to assess insertion, a second group showed that ATP removal by apyrase or thermolysin pretreatment of chloroplasts has no effect on the insertion of Toc34 into the outer membrane (1). However, the second group later showed that Toc34 insertion is indeed stimulated by ATP or GTP, but the NTP is most likely bound only by Toc34 itself to acquire an insertion-competent conformation (15).

Because Toc34 is a member of the translocon for protein import into chloroplasts, a clear understanding of the mechanism used for its insertion into the outer membrane will shed light on the question of how components in the translocon are transported to chloroplasts themselves. We have therefore made a more detailed analysis of the import characteristics of Toc34. We investigated Toc34 import kinetics and developed a new and cleaner method of removing NTP from the import system. We then assayed the insertion of Toc34 both by protease treatment and by alkaline extraction. We further investigated the NTP requirement using a mutant form of Toc34 that no longer binds NTP. Our results indicate that insertion of Toc34 into the chloroplastic outer membrane is assisted by at

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least one thermolysin-sensitive chloroplastic protein and one ATP-utilizing protein in the import system.

**EXPERIMENTAL PROCEDURES**

**Conjugation of Apyrase—Apyrase (Sigma, Grade VIII) was conjugated to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s suggestions. Briefly, beads were swollen in 1 M HCl, incubated with apyrase in a coupling buffer (100 mM NaHCO₃, pH 8.3, and 500 mM NaCl) at room temperature for 4 h, transferred to a blocking buffer (200 mM glycine in 50 mM Tris-HCl, pH 8.0) at room temperature for 2 h, and washed alternately in the coupling buffer and an acetate buffer (100 mM NaAc, pH 4.0, and 500 mM NaCl) four times. After a final additional wash in the coupling buffer, a small portion of the beads were removed and used to determine the ATPase activity of each batch, and the rest of the beads were stored in a sterile MES buffer (1 M MES, pH 6.3, and 5 M NaCl) at 4 °C until use. Before use, the conjugated apyrase beads were pelleted, washed in the import buffer (50 mM Hepes/KOH, pH 8.0, and 300 mM sorbitol) used for dilution of in vitro translation products and chloroplast import experiments, and diluted to 1 unit/µl with the import buffer.

**ATPase Activity Assay**—Fifty µl of 100 mM ATP were mixed with 1 µl of [γ-32P]ATP (5000 Ci/mmol). 2.6 µl of this ATP solution was added to 197.4 µl of the import buffer used for chloroplast import reactions. 0.3 µl of the mixture was spotted on a TLC plate as the 0-min sample. 4 µl of Sepharose-conjugated apyrase was then added to the remaining mixture, and 0.3 µl of the mixture was removed at the indicated time and spotted on the TLC plate. After all samples were taken, the TLC plate was resolved with a solution containing LiCl and formic acid. The TLC plate was then air-dried and exposed to x-ray films or quantified with a PhosphorImager SP (Molecular Dynamics, Inc., Sunnyvale, CA).

ATP concentrations in the wheat germ extracts before and after apyrase treatment were measured using the ATP bioluminescent assay kit (Sigma) and a Lumat luminometer (Berthold LB9501).

**Protein Import and Postimport Treatments—**[35S]Methionine-labeled proteins were synthesized by in vitro transcription (17) and in vitro translation with wheat germ extracts (Promega, Madison, WI) or rabbit reticulocyte lysate (Promega) according to the manufacturer’s specifications. After synthesis, the translation products were diluted with equal volumes of 2X import buffer containing 50 mM cold methionine. Isolation of chloroplasts from 9–11-day-old pea (Pisum sativum cv. Little Marvel or Dark-skinned Perfection) seedlings and import of proteins into chloroplasts were performed as described (17), except import reactions were terminated by adding HgCl₂ to 3.3 mM as described by Reed et al. (18).

To deplete NTP from import reactions, the in vitro translation products were incubated with the import buffer-washed apyrase beads at a ratio of 0.5 unit apyrase to 1 µl translation product at room temperature for 15 min with constant rotation. The apyrase was then removed by passing the in vitro translation product through a piece of 3MM paper. For most experiments, chloroplasts were depleted of internal ATP by incubating the isolated chloroplasts in the dark at 4 °C for 2 h. For the experiment with the deletion mutant SN206 (18) shown in Fig. 5, after a 1-h dark incubation at 4 °C, chloroplasts were further incubated with Sepharose-conjugated apyrase for 60 min in the dark at room temperature and passed through one layer of Miracloth (Calbiochem) to remove the apyrase beads. All import experiments investigating NTP requirements were conducted under a dim green safelight. NTPs were purchased from Amersham Pharmacia Biotech as 100 mM solutions, pH 7.0.

Thermolysin pretreatment of chloroplasts was performed by resuspending chloroplasts (equivalent to 200 mg of chlorophyll) in 1 ml of import buffer containing 200 µg/ml thermolysin and 1 mM CaCl₂. The digestion was performed for 30 min on ice and terminated by adding 200 µl of 50 mM EDTA in import buffer. Intact chloroplasts were repurified through a 40% Percoll cushion and resuspended to a concentration approximately equivalent to 1 mg/ml chlorophyll and equal to the concentration (number of plastids/ml) of control chloroplasts that have gone through the same procedure but were treated with import buffer that did not contain thermolysin.

The pea leaf soluble extracts were prepared essentially as described by Waegemann et al. (19). In short, 5-day-old seedlings were homogenized in a grinding buffer containing 20 mM Hepes-KOH (pH 7.6), 100 mM KAc, 1 mM Mg(OAc)₂, 2 mM CaCl₂, and a mixture of protease inhibitors. Dithiothreitol was added to 1 mM before use. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged first at 3000 × g for 3 min, at 23,000 × g for 10 min, and then at 100,000 × g for 45 min. The supernatant fraction was aliquoted, frozen in liquid nitrogen, and stored at −80 °C until use. When added to import reactions, the extracts were diluted with one volume of 2X import buffer to maintain the osmotic pressure of the import reactions.

Thermolysin digestion (20) and alkaline extraction (4, 21) of chloroplasts after import was performed as described previously. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on 10% NuPAGE gels with MES running buffer purchased from Novex (San Diego, CA). Quantification of samples was performed using the PhosphorImager with dried gels. Pictures of gels were taken from x-ray films generated by exposing the dried gels to Eastman Kodak Co. MS film with a BioMax TranScreen-LE intensifying screen.

**RESULTS**

**Import of atToc34 to the Outer Membrane Is Time- and Temperature-dependent**—We used an Arabidopsis homologue of the pea Toc34, atToc34, to perform chloroplast import studies. Both pea Toc34 and atToc34 use their C-terminal targeting/insertion signal to insert into the outer membrane (14–16). When treated with thermolysin after insertion, both proteins are cleaved to a specific 6-kDa membrane-protected fragment corresponding to the C-terminal membrane anchor (14, 16). The reason for using the Arabidopsis protein is that atToc34, but not pea Toc34, contains two methionine residues in the 6-kDa fragment. When [35S]methionine is used to label atToc34 for import experiments, the 6-kDa fragment can be visualized by autoradiography and quantified by PhosphorImager. Throughout this report, we will refer to the amount of full-length atToc34 observed on chloroplasts after an import reaction before thermolysin treatments as “total association.” This includes two groups of atToc34 molecules: those bound to the surface of chloroplasts and those that have truly inserted into the outer membrane. We will use the full-length atToc34 remaining after alkaline extraction or the amount of the 6-kDa membrane-protected fragment after thermolysin digestion to represent the amount of atToc34 that was truly inserted into the outer membrane.

To perform experiments investigating factors that may affect import, it is better to inspect the results at a time point when the rate of import is at or close to its maximum so that any difference can be better observed. We therefore first determined the import time course of atToc34. We used HgCl₂ to rapidly terminate import at defined time points. HgCl₂ has been used as an effective agent to rapidly stop the import of precursor proteins into the interior of chloroplasts (18). We also have data indicating that HgCl₂, added before or during import, could rapidly stop further association of atToc34 with chloroplasts (data not shown).

As shown in Fig. 1A, association of atToc34 with chloroplasts increased almost linearly for about 16 min and then gradually slowed down (see also Fig. 1C; filled circles). After treating the chloroplasts with thermolysin to reveal the amounts of protein inserted into the outer membrane (Fig. 1B), the kinetics was similar although the amounts were much lower (see also Fig. 1D, filled circles). This result indicated that many of the atToc34 molecules associated with chloroplasts did not insert into the outer membrane. Further characterization of atToc34 import were all conducted for 16 min or less when the amount of atToc34 associated with chloroplasts still increased linearly.

The association and insertion of atToc34 to the outer membrane were also temperature-dependent. At 4 °C, atToc34 had reduced rates of import, and the amount of protein imported reached a plateau at about 50% of the amount at 25 °C (Fig. 1, C–F).

**Association of atToc34 with the Outer Membrane Required**
Some Thermolysin-sensitive Components—We then tested if association and insertion of atToc34 to chloroplasts were sensitive to thermolysin pretreatment of chloroplasts. We assayed the amount of protein inserted both by thermolysin post-treatment and by alkaline extraction. We also used the import of two other proteins as functional controls for the effectiveness of the thermolysin pretreatment. The first protein we used is the precursor to the chlorophyll a/b-binding protein of photosystem II (CAB), whose import is known to be insen-
sitive to the pretreatment (11). As shown in Fig. 2A, the thermolysin pretreatment of chloroplasts reduced the amount of CAB that was imported into chloroplasts (compare lanes 2 and 3). The same treatment also reduced the amount of atToc34 associated (compare lanes 13 and 14) and inserted into the chloroplasts as assayed either by the amounts of alkaline-resistant protein (compare lanes 15 and 19) or the thermolysin-digested fragment (compare lanes 14 and 15). A 25-kDa fragment and a 5-kDa fragment (lane 14, asterisks) sometimes were also observed after thermolysin digestion of atToc34 and pea Toc34 (14, 16). However, because they did not always appear, only the amount of the 6-kDa fragment (lane 14, arrow) was quantified as the amount of protein inserted. In thermolysin-pretreated chloroplasts, the amount of the 6-kDa fragment was reduced to 41 ± 6% (n = 3) of that in untreated chloroplasts (Fig. 2B). The results were the same whether the translation product was made with wheat germ extracts or rabbit reticulocyte lysates (data not shown).

The insertion of OEP14 can be assayed by the appearance of a specific 4-kDa membrane-protected fragment after thermolysin digestion (21) or by alkaline extraction. As shown in Fig. 2A, lanes 5–12, the thermolysin pretreatment of chloroplasts had almost no effect on the association (lanes 5 and 9) or insertion of OEP14 as assayed either by the appearance of the 4-kDa thermolysin-digested fragment (lanes 6 and 10) or by alkaline extraction (lanes 7 and 11).

The results shown in Fig. 2, A and B, indicate that there is a thermolysin-sensitive component that is required for the association and insertion of atToc34 into the outer membrane. To distinguish whether the thermolysin-sensitive component is a
chloroplast protein or a soluble cytosolic protein adhering to chloroplasts during chloroplast isolation, soluble pea leaf extracts were supplemented to the thermolysin-pretreated chloroplasts. As shown in Fig. 2C, the association and insertion of atToc34 were still sensitive to thermolysin pretreatment of chloroplasts even when soluble leaf extracts were present (lanes 7 and 8). In fact, the leaf extracts seemed to reduce the association of atToc34 with chloroplasts. We also observed similar effects with the addition of wheat germ extracts (data not shown). In any event, the result suggests that the thermolysin-sensitive component is most likely a surface-exposed chloroplastic protein that is not present in the soluble fraction of leaf extracts or in the wheat germ extracts.

**NTP Requirement for atToc34 Insertion into the Outer Membrane**—To carry out experiments investigating NTP requirement of atToc34 import, a method that can thoroughly remove NTPs from the import system is critical. We have decided to use apyrase. Apyrase is more powerful than most ATP-hydrolyzing enzymes and can hydrolyze all NTPs to NMPs, preventing the possible conversion of NDPs back to NTPs by the energy regeneration system present in the in vitro translation system.

In order to remove apyrase after treatment, we conjugated apyrase to agarose beads. We then tested the conjugated apyrase and showed that it could still effectively hydrolyze ATP (Fig. 3). When the conjugated apyrase was used to treat the wheat germ extracts used for in vitro translation, the ATP concentration in the extracts was reduced from 400 μM to 60 nM (data not shown).

When the apyrase-treated *in vitro* translation product of atToc34 was used in an import experiment, very little atToc34 could insert into the outer membrane (Fig. 4, lane 6). Adding back ATP or GTP could restore the insertion. This result is similar to what has been observed with pea Toc34 (14, 15). We further tested the effect of other NTPs. CTP could also stimulate insertion of atToc34 but to a lesser extent (Fig. 4A, lane 10, and Fig. 4B). UTP did not have an effect. As for the total amount of atToc34 associated with chloroplasts before thermolysin treatment, we did not observe a consistent stimulation effect by NTP addition (Fig. 4B).

**Mutant with the GTP-binding Domain Deleted Still Required ATP to Support Insertion**—The ATP and GTP stimulation of Toc34 insertion has been attributed to the intrinsic GTP-binding activity of Toc34 (15). The conclusion was made based on experiments showing that Toc34 mutants with the GTP-binding site mutated have a much lower insertion efficiency in the presence of GTP compared with the wild type. It was therefore proposed that GTP was bound only by Toc34 itself to acquire an insertion-competent conformation, not by other proteins in the import system (15). However, the insertion efficiency of the mutants in the absence of GTP was never analyzed. It is possible that in the absence of ATP or GTP, the insertion efficiency of the mutants would be even lower. The insertion efficiencies observed in the presence of ATP or GTP might be still supported by ATP or GTP. Because the mutants have very low affinities for GTP or ATP, this may indicate that there are other protein components in the import system that use the ATP or GTP to support the insertion of the mutants.

To investigate if ATP or GTP is used by atToc34 alone or also by other components in the import system, we studied the energy requirement for the import of an atToc34 mutant, ΔN206 (16). In ΔN206, the first 206 amino acids of atToc34, including the GTP-binding motifs, have been deleted. We have shown previously that ΔN206 could insert into the outer membrane with the same orientation as the full-length atToc34 and produce the same 6-kDa thermolysin-resistant fragment after...
insertion (16). If GTP or ATP is only required by atToc34 to maintain its tertiary structure, the removal or addition of NTP in the import system should have no effect on the insertion of ΔN206, since ΔN206 should no longer bind GTP. We first treated the in vitro translation product of ΔN206 with apyrase to remove NTP. We observed a reduction in the insertion efficiency but a substantial amount of ΔN206 could still insert (data not shown). To thoroughly remove NTP from the import system, we then also treated the chloroplasts (see "Experimental Procedures") with apyrase before import. As shown in Fig. 5, like the full-length atToc34, very little ΔN206 could insert into the outer membrane after removal of NTP from the import system. Adding back ATP could stimulate the insertion (Fig. 5, lane 5). Interestingly, in contrast to full-length atToc34, adding back GTP had very little stimulation effect on the insertion of ΔN206 (lane 6). These results indicated that there is at least one additional component in the import system that requires ATP, but not GTP, to assist the insertion of ΔN206 into the outer membrane.

ATP Still Stimulated atToc34 Insertion in Thermolysin-pretreated Chloroplasts—We further asked whether the ATP-utilizing component is the same protein as the thermolysin-sensitive chloroplastic protein that assists the insertion of atToc34. We pretreated chloroplasts with thermolysin to destroy the thermolysin-sensitive component and then checked if ATP could still stimulate insertion under these conditions. If the ATP-utilizing component is the same protein as the thermolysin-sensitive component, then ATP should no longer stimulate insertion once the ATP-utilizing component is destroyed by thermolysin pretreatment. On the other hand, if the ATP-utilizing component is a different protein from the thermolysin-sensitive component (e.g. if the ATP-utilizing component is a thermolysin-resistant chloroplastic protein or a cytosolic protein), then ATP should still stimulate insertion even after thermolysin pretreatment of chloroplasts. As shown in Fig. 6, thermolysin pretreatment reduced the amount of ΔN206 that could associate with (compare lanes 1 and 5) or insert into (compare lanes 3 and 7) the outer membrane. Furthermore, ATP still greatly stimulated the insertion of ΔN206 even in thermolysin-pretreated chloroplasts (compare lanes 7 and 8). The same result was also observed with atToc34 (compare lanes 15 and 16). These results suggest that the component responsible for ATP-stimulated insertion is most likely a different protein from the thermolysin-sensitive components that assists atToc34 insertion.

**FIG. 5.** NTP requirement for the insertion of ΔN206 into the chloroplastic outer membrane. Apyrase-treated ΔN206 and chloroplasts were incubated under a dim green safelight at room temperature for 16 min either without any NTP (−) or with 1 mM ATP (A) or GTP (G) as indicated at the top of the gel. The import reactions were terminated with HgCl₂. Half of the chloroplasts from each sample were further treated with thermolysin to reveal the amounts of protein inserted (lanes 4–6). The positions of full-length ΔN206 in lanes 1–3 and the 6-kDa thermolysin-resistant fragment generated after insertion in lanes 4–6 are marked by arrows. The faster migrating band underneath the full-length ΔN206 in lanes 1–3 is a product from internal initiation of in vitro translation. Because this internal initiation product still contains the C-terminal outer membrane targeting signal, it still associates with chloroplasts (see Ref. 16).

**FIG. 6.** ATP stimulates insertion in thermolysin-pretreated chloroplasts. Import experiments were conducted with NTP-depleted in vitro translation products and chloroplasts that had (+ in the row labeled ther-pretreat) or had not been pretreated with thermolysin. 1 mM ATP was added to half of the samples (+ in the row labeled ATP). The import reactions were conducted for 10 min. After the import reaction, chloroplasts were repurified. Half of the chloroplasts from each sample were further treated with thermolysin (+ in the row labeled ther-post) to reveal the amounts of proteins inserted. The full-length proteins used for the import experiments are marked with open circles, and the thermolysin-resistant fragments generated after insertion are indicated with filled circles.

**DISCUSSION**

We have tried to resolve the ambiguities of, and have also carried out further analysis on, the insertion mechanism of atToc34 into the chloroplastic outer membrane. Using an Arabidopsis homologue of pea Toc34, our results suggest that insertion of atToc34 is assisted by at least two proteinaceous components in the import system: one being thermolysin-sensitive and one requiring ATP for its function. Our previous results have also shown that the targeting signal of atToc34, when fused in front of a passenger protein, can translocate a passenger protein of 26 kDa across the chloroplastic outer membrane (16). This result also suggests a mechanism of protein-assisted insertion rather than an insertion mechanism based solely on direct interactions with membrane lipids.

We have assayed atToc34 insertion both by alkaline extraction and by thermolysin digestion. We showed that insertion of atToc34 is indeed reduced by thermolysin pretreatment of chloroplasts. It is not clear why conflicting results were reported concerning this issue (1, 14, 15). It is possible that differences in the protease treatment conditions may cause different levels of reduction in import. We have therefore used the import of CAB, which is one of the first proteins used to demonstrate the thermolysin sensitivity of chloroplast protein import, as a control for the effectiveness of our thermolysin treatment. We showed that under conditions when the import of CAB was reduced, the association and insertion of atToc34 was also reduced. These conditions may be harsher than conditions used by other investigators. Furthermore, it may be important to inspect the effect in the linear range of import, before import reaches a plateau, so that any reduction can be more readily detected.

We have reinvestigated the NTP requirement for atToc34 import by using an NTP removal system that can fully hydrolyze all NTP to NMP. Our results are consistent with previous findings that insertion of atToc34 is stimulated by ATP and GTP (14, 15). GTP is most likely used by atToc34 itself to acquire an import-competent conformation (15). Our data further extend these results by showing that atToc34 was not the only protein in the import system that required NTP. An atToc34 mutant with the GTP-binding domain deleted still required ATP for insertion, indicating that some other component in the system also required ATP. We propose that, in import reactions with full-length atToc34, the small amount of ATP remaining on the surface of chloroplasts after dark depletion was enough to support atToc34 insertion when only GTP was added. The
stimulation we observed after adding GTP was mostly due to atToc34 itself changing to an import-competent conformation after binding GTP. The ATP requirement was made more evident when using a substrate that does not bind GTP (ΔN206) and pretreating the chloroplasts with apyrase in addition to dark incubation. We have no data to indicate where the ATP-utilizing component may reside. It could be an ATP-binding protein at the chloroplastic envelope, or it could be a chaperone protein in the in vitro translation system we used to prepare the import substrates. However, it has been shown that E. coli overexpressed and purified Toc34 is fully competent in inserting into the outer membrane (15). We also have data indicating that either pea leaf soluble extracts or wheat germ extracts could not increase the insertion efficiency of purified atToc34 even in NaCl and EDTA-prewashed chloroplasts (data not shown). Therefore, at least in the in vitro import system, a cytosolic chaperone protein may not be a necessary component for Toc34 import.

The ATP-requiring component that assists atToc34 insertion was present even after thermolysin pretreatment of chloroplasts. This result suggests that the ATP-requiring component is a different protein from the thermolysin-sensitive component that is also required for atToc34 insertion. However, we cannot exclude the possibility that they are two domains of the same protein. Further investigations are necessary to reveal the identity of these components and the mechanism used to target Toc34 to the chloroplastic outer membrane.

There are four integral chloroplastic outer membrane proteins identified so far that do not contain cleavable transit peptides (1, 10–12, 14). Among them, the import of OEP14 and OEP6.7 is resistant to thermolysin pretreatment of chloroplasts and also does not require ATP (11, 12). The import of OEP24, on the other hand, is resistant to thermolysin pretreatment of chloroplasts, but its insertion into the outer membrane is stimulated by ATP (10). Our data indicate that the import of Toc34 is sensitive to thermolysin pretreatment of chloroplasts and also requires ATP. These features seems to make the import of Toc34 more similar to the import of precursor proteins into the interior of chloroplasts. However, the location and composition of the outer membrane targeting signal of Toc34 (a C-terminal hydrophobic sequence) makes it unlikely that Toc34 uses the Tic/Toc translocon used by precursor proteins imported into the interior of chloroplasts. It has also been mentioned that import of Toc34 was not competed by excess amounts of a precursor protein imported through the Tic/Toc translocon (14). It therefore seems there are several different pathways for protein import into chloroplasts. However, it is still possible that these different import characteristics we observed among different groups of proteins represent small branches in the same pathway. For example, each group of proteins may have some unique translocon components that they use in their import pathway, and these different import pathways may converge at various points in the import process.

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