Tic22 Is Targeted to the Intermembrane Space of Chloroplasts by a Novel Pathway*

(Received for publication, May 6, 1999, and in revised form, June 13, 1999)

Andrei Kouranov, Huan Wang, and Danny J. Schnell‡

From the Department of Biological Sciences, Rutgers, The State University of New Jersey, Newark, New Jersey 07102

The chloroplast is a complex organelle that is subdivided into at least six suborganellar compartments by a double-membrane envelope and an internal thylakoid membrane. These three membranes segregate and organize a number of essential biochemical processes, including the light and dark reactions of photosynthesis and aspects of amino acid and fatty acid synthesis. The biogenesis and maintenance of this elaborate architecture requires the selective targeting of numerous nuclear-encoded proteins from their site of synthesis on cytoplasmic ribosomes to their proper suborganellar compartment. Targeting is mediated by a set of hierarchical targeting signals that are intrinsic to the nuclear-encoded protein. These signals act as a typical transit sequence to direct Toc75 to the general import machinery during synthesis. The biogenesis and maintenance of this elaborate architecture requires the selective targeting of numerous nuclear-encoded proteins from their site of synthesis on cytoplasmic ribosomes to their proper suborganellar compartment. Targeting is mediated by a set of hierarchical targeting signals that are intrinsic to the nuclear-encoded protein. These signals act

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1 The abbreviations used are: preTic22, precursor of Tic22; SSU, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; preSSU, precursor of SSU; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
as a preprotein containing an N-terminal extension with similarity to transit sequences. This raises the possibility that preTic22 may utilize the general import machinery en route to the intermembrane space. We used chimeric proteins in which the preTic22 presequence and the transit sequence of preSSU were reciprocally exchanged to investigate the functional role of the Tic22 presequence and mature sequence in targeting to the intermembrane space. Our experiments suggest that the presequence of preTic22 does not function as a stromal transit sequence but directs import into the intermembrane space by a previously unidentified mechanism.

**EXPERIMENTAL PROCEDURES**

**Chloroplast Isolation and Subfractionation**—Intact chloroplasts were isolated from 14-day-old pea seedlings (*Pisum sativum* variant Green Arrow) by homogenization and Percoll silica gel gradient centrifugation as described previously (16). A protease inhibitor mixture (P9599, Sigma) was included at all stages of chloroplast isolation according to the manufacturer's recommendations. Isolated chloroplasts were resuspended in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) to a concentration equivalent to 2–3 μg chlorophyll/mL. To separate the total membrane and soluble fractions, intact chloroplasts were lysed under hypertonic conditions (17), and total membrane and soluble fractions were separated by centrifugation at 40000 × g for 20 min in a TLA100.3 rotor (Beckman Instruments, Palo Alto, CA).

**Preparation of Fusion Proteins**—Plasmid pET21d-pTic22 for the in vitro expression of preTic22 was constructed as follows. The 759-base pair open reading frame of preTic22 cDNA (15) including the stop codon was amplified by PCR to incorporate NcoI and NotI sites at the 5′ and 3′ ends, respectively. The fragment was inserted into the NcoI and NotI sites of pET21d (Novagen, Madison, WI). Plasmid pET21d-Tic22MS, encoding the mature sequence of Tic22, was constructed by amplifying the 608-base pair fragment of preTic22 cDNA from nucleotide 163 to 771 by PCR to incorporate NheI and NotI sites at the 5′ and 3′ ends, respectively. The fragment was subcloned into the NheI and NotI sites of pET21d (Novagen).

Plasmid pET8c-pTic22-SSU, encoding the presequence of preTic22 fused to mature SSU, was constructed as follows. A region of pET21d-pTic22 was amplified by PCR with a sense primer specific for the 5′ promoter of the vector and an antisense NotI adaptor primer (5′-GAGTCCATTCGCCATCTTACTCC-3′) annealing to nucleotides 150–174 of the preTic22 cDNA. The DNA fragment was digested and inserted into the XbaI and NotI sites of pET8c-S (18). The resulting open reading frame consisted of the N-terminal 54 amino acids of preTic22 fused to the complete mature sequence of SSU.

Plasmid pET21d-pSSU-Tic22, encoding the transit sequence of preSSU fused to the mature sequence of preTic22, was constructed as follows. A region of pET8c-pSSU (18) was amplified by PCR with a sense primer specific for the 3′ promoter of the vector and an antisense NheI adaptor primer (5′-GAATTCCATTCGCCATCTTACTCC-3′) annealing to nucleotides 157–171 of the preSSU cDNA. The DNA fragment was digested and inserted into the XbaI and NheI sites of pET21d-Tic22MS. The resulting open reading frame consisted of the N-terminal 57 amino acids of preSSU fused to the complete mature sequence of preTic22.

In vitro synthesized preTic22 was associated with intact chloroplasts, and a portion of the preprotein was processed to its mature form, as indicated by the appearance of a radioactive band with a mobility identical to Tic22 (Fig. 1A, lane 1).

**RESULTS**

**Newly Imported Tic22 Is Localized to the Outer Surface of the Inner Membrane**—To investigate the import characteristics of Tic22, we performed in vitro import experiments using [35S]preTic22 synthesized in a reticulocyte lysate. PreTic22 was incubated with isolated chloroplasts in the presence of 2 mM ATP in a standard import assay. To assess the localization of imported preTic22, we treated samples of chloroplasts from the import reactions with thermolysin or trypsin. Thermolysin is an outer membrane impermeable protease that selectively degrades proteins exposed to the cytoplasm (20), whereas trypsin is capable of accessing the intermembrane space and degrading proteins that are exposed to the intermembrane space (19, 20). We previously demonstrated that endogenous Tic22 was resistant to thermolysin but sensitive to trypsin treatment, consistent with its localization in the intermembrane space (15).

In vitro synthesized preTic22 associated with intact chloroplasts, and a portion of the preprotein was processed to its mature form, as indicated by the appearance of a radioactive band with a mobility identical to Tic22 (Fig. 1A, lane 1). The...
mature Tic22 was resistant to thermolysin treatment (Fig. 1A, lane 2), but 50% of Tic22 was digested by trypsin (Fig. 1A, lane 3). The resistance of Tic22 to thermolysin was not due to inherent protease resistance because disruption of the envelope with detergent prior to thermolysin treatment resulted in complete degradation of the protein (Fig. 1C). Furthermore, neither protease digested a newly imported stromal protein, preSSU, eliminating the possibility that the degradation of Tic22 resulted from inadequate inhibition of the enzymes prior to disruption of the chloroplasts (Fig. 1B). The pattern of protease sensitivity for newly imported Tic22 is indistinguishable from that observed for endogenous Tic22 (15), indicating that a portion of preTic22 was translocated into the intermembrane space of the envelope and processed to its mature form.

Remarkably, a significant portion of bound preTic22 was insensitive to thermolysin digestion (Fig. 1A, lane 2). This observation suggests that the thermolysin-insensitive preTic22 might be translocated across the outer membrane but incompletely processed to mature Tic22. The insensitivity of bound preTic22 to thermolysin was not a kinetic effect because increasing the concentration of thermolysin to 500 μg/ml did not increase the degradation of the bound precursor (data not shown). To investigate the possibility that a portion of bound preTic22 was imported to the intermembrane space, we performed a time course of preTic22 import. At various time points after the addition of preTic22 to a standard import reaction, samples were removed from the reaction, and the chloroplasts were reisolated by sedimentation through 40% Percoll silica gel. The chloroplasts from each time point were split into two equal samples, and one sample was treated with thermolysin to remove surface bound precursor. Fig. 2 demonstrates that preTic22 associated with the chloroplast and was processed to Tic22 in a time-dependent manner. Quantitative analysis of the import reaction indicates that the binding of preTic22 to chloroplasts reaches a maximum of 16% of added translation product after 5 min of incubation (Fig. 2, lane 6). Although the binding of preTic22 reached a maximum at 5 min, the proportion of bound preTic22 that was insensitive to protease increased from 24% at 5 min to 56% at 30 min (Fig. 2, compare lanes 7 and 13). These data indicate that preTic22 binds to the chloroplast surface and is translocated across the outer membrane in a time-dependent manner. The accumulation of mature Tic22 increased up to 30 min of incubation, reaching a maximum of 4% of the precursor added to the import reaction (Fig. 2, compare lanes 2 and 12). The processed form of the Tic22 showed a pattern of thermolysin sensitivity similar to that of preTic22 over the course of the import reaction. The processed form of Tic22 was almost completely degraded by thermolysin at the early time points (Fig. 2, lanes 3 and 5); however, the sensitivity to protease decreased with time, and 90% of mature Tic22 was resistant to protease by 30 min (Fig. 2, lane 13). These data suggest that processing can occur prior to complete translocation across the outer membrane. Furthermore, the maturation of Tic22 does not appear to be tightly coupled to translocation because the major fraction of preTic22 is translocated to a thermolysin-sensitive compartment without being processed to its mature form. We attempted to test whether the imported preTic22 could be chased to the mature form by reisolating chloroplasts from the 20 min time point and subjecting them to a second 20-min incubation in the absence of added preTic22. No significant difference between the relative pattern of bound preTic22 or processed Tic22 before or after the second incubation was observed (data not shown). Therefore, the processing of preTic22 appears to be very slow.

Our import experiments suggest that the in vitro import assay reproduced proper targeting and translocation of Tic22 to the intermembrane space. However, it was not clear whether newly imported preTic22 or Tic22 associated with the inner membrane in a manner similar to endogenous Tic22. To test whether both forms of newly imported Tic22 associated with the inner membrane, we examined their distributions within various chloroplast subfractions. After a 30-min in vitro import reaction with preTic22, intact chloroplasts were reisolated, lysed, and fractionated to yield stroma and fractions enriched in outer and inner envelope membranes. Analysis of the distribution of the newly imported protein demonstrates that the majority of preTic22 and Tic22 are associated with the fraction enriched in inner membranes (Fig. 3, lane 1). Minor amounts of preTic22 and Tic22 were detected in the outer membrane fraction (Fig. 3, lane 2), and no significant amounts of either form were detected in the stromal fraction (Fig. 3, lane 3). We conclude that both preTic22 and mature Tic22 associate with the inner membrane upon entering the intermembrane space in a manner indistinguishable from endogenous Tic22.

Energy Dependence of Tic22 Import—Translocation of stromal or integral inner membrane proteins across the envelope strictly requires ATP hydrolysis, whereas the insertion of most integral outer membrane proteins is not dependent on an energy source. We tested the effects of added ATP on the import of preTic22 to determine the energy requirements for translocation to the intermembrane space. To eliminate the interference from nucleoside triphosphates in the reticulocyte lysate, the preTic22 in vitro translation system was gel filtered before addition to the import reaction. As an added precaution, chloroplasts were preincubated in the presence of 400 nM nigericin and apyrase in the dark to deplete exogenous and endogenous nucleoside triphosphates. As controls for the energy state of chloroplasts in the import reaction, we monitored the binding and import of preSSU. As expected, 100 μM ATP stimulated the binding of preSSU to chloroplasts (Fig. 4B, lane 3). At 2 mM
ATP, preSSU was imported into the chloroplast stroma and processed to its mature form (Fig. 4B, lane 7). The results shown in Fig. 4 demonstrate that preTic22 binds to isolated chloroplasts and is processed to Tic22 in the absence or presence of the externally added ATP (Fig. 4A, compare lanes 2, 4, and 5). However, 50% of Tic22 is susceptible to externally added thermolysin in the absence of ATP (Fig. 4A, lane 3), whereas Tic22 formed in the presence of low (100 mM) or high (2 mM) concentrations of ATP is not degraded by thermolysin (Fig. 4A, lanes 6 and 7, asterisks). These data suggest that the binding of preTic22 to chloroplasts does not require ATP hydrolysis. However, complete translocation to the intermembrane space is stimulated by ATP hydrolysis.

Two proteolytic degradation products of 24 and 18 kDa were observed in chloroplasts incubated with thermolysin (Fig. 4A, lanes 6 and 7). These fragments appear to represent protease-protected regions of preTic22 that result from partial translocation of the prep protein across the outer membrane. The degradation products are more pronounced when Tic22 is imported in the presence of ATP, providing additional evidence that ATP stimulates insertion of preTic22 across the outer membrane.

**Import of PreTic22**

**Isolated Chloroplasts**

Isolated chloroplasts were incubated in the presence (+) or absence (−) of 100 μg/ml thermolysin on ice for 30 min. Chloroplasts were reisolated in the presence of protease inhibitors and incubated with [35S]preTic22 or [35S]preSSU in a standard import reaction containing 2 mM ATP at 26 °C for 30 min. Chloroplasts were reisolated and analyzed by SDS-PAGE and phosphorimaging. Tr, 1/20 of the translation product added to the import assay. The positions of preTic22 (pTic22) and Tic22 are indicated to the right of each panel.

**Protein Targeting to the Chloroplast Intermembrane Space**

Fig. 4. Energy dependence of preTic22 import. Gel-filtered rabbit reticulocyte lysate containing in vitro synthesized [35S]preTic22 or [35S]preSSU was incubated with isolated chloroplasts at 26 °C in the presence (+) or absence (−) of apyrase or the indicated concentrations of ATP. A, chloroplasts (100 μg of chlorophyll) from the [35S]preTic22 import reactions were divided into equal samples and treated in the presence or absence of 200 μg/ml thermolysin on ice for 30 min. Chloroplasts were reisolated in the presence of protease inhibitors and analyzed directly by SDS-PAGE and phosphorimaging. B, chloroplasts (50 μg of chlorophyll) from the [35S]preSSU import reactions were lysed and separated into stroma and membrane fractions by differential centrifugation. Proteins from these fractions were resolved by SDS-PAGE and analyzed by phosphorimaging. Tr, 1/20 of the translation product added to the import assay. The positions of preTic22 (pTic22), Tic22, preSSU (pSSU), and SSU are indicated to the right of each panel.

Binding and import was not due to the degradation of preTic22 by residual thermolysin because no significant proteolysis of the unbound preTic22 from the import reaction was observed (data not shown). These data suggest that import of preTic22 into chloroplasts is mediated by proteinaceous components in the outer membrane.

**Fig. 5.** Import of preTic22 requires the presence of the thermolysin-sensitive components in the chloroplast outer membrane. Isolated chloroplasts were incubated in the presence (+) or absence (−) of 100 μg/ml thermolysin on ice for 30 min. Chloroplasts were reisolated in the presence of protease inhibitors and incubated with [35S]preTic22 or [35S]preSSU in a standard import reaction containing 2 mM ATP at 26 °C for 30 min. Chloroplasts were reisolated and analyzed by SDS-PAGE and phosphorimaging. Tr, 1/20 of the translation product added to the import assay. The positions of preTic22 (pTic22) and Tic22 are indicated to the right of each panel.
in vitro synthesized [35S]preTic22-SSU or [35S]preSSU-Tic22 was incubated with isolated chloroplasts (50 μg of chlorophyll) at 26 °C in a standard import reaction containing 2 mM ATP. After the import reaction, the chloroplasts were incubated in the absence (−) or presence (+) of 200 μg/ml thermolysin (T-lysin) on ice for 30 min. Chloroplasts were reisolated in the presence of protease inhibitors and analyzed by SDS-PAGE and phosphorimaging. Tr, % of the translation product added to the import reaction.

FIG. 6. Import of mature Tic22 into isolated chloroplasts. Rabbit reticulocyte lysate containing in vitro synthesized [35S]Tic22 was incubated with isolated chloroplasts (50 μg of chlorophyll) at 26 °C in a standard import reaction containing 2 mM ATP. After the import reaction, the chloroplasts were incubated in the absence (−) or presence (+) of 200 μg/ml thermolysin (T-lysin) on ice for 30 min. Chloroplasts were reisolated in the presence of protease inhibitors, lysed, and separated into membrane and soluble fractions by differential centrifugation. A, phosphorimage of SDS-PAGE-resolved proteins from soluble and membrane fractions of chloroplasts from an [35S]preTic22 import reaction. B, phosphorimage of SDS-PAGE-resolved proteins from soluble and membrane fractions of chloroplasts from an [35S]preTic22-SSU import reaction. Tr, % of the translation product added to the import assay. The positions of preTic22-SSU ([35S]preTic22-SSU), SSU, preTic22-Tic22 ([35S]preSSU-Tic22), and Tic22 are indicated to the left of the each panel.

The most likely explanation is that SSU cannot properly fold in the intermembrane space and may aggregate in an insoluble form that associates with the membrane. Only 25% of chloroplast bound preTic22-SSU remains intact following thermolysin treatment, and the majority is associated with the chloroplast membrane fraction (Fig. 7A, lane 3). However, 23% of protease-protected preTic22-SSU is observed in the soluble fraction of lyzed chloroplasts (Fig. 7A, lane 6). This observation suggests that imported preTic22-SSU might remain associated with the translocation machinery, but disruption of the machinery by proteolysis releases the bound preprotein. Taken together, the membrane-bound and soluble preTic22-SSU present after thermolysin treatment correspond to 23% of the preTic22-SSU added to the import reaction. The resistance of the mature and precursor forms to thermolysin indicates that both forms were imported across the outer membrane. Two partial degradation products of 16 and 12 kDa are apparent after treatment of the import reaction with thermolysin (Fig. 7A, lane 3, asterisks), suggesting that a portion of bound preTic22-SSU is partially inserted across the outer membrane and thereby protected from complete digestion. These proteolytic products are comparable to those observed in thermolysin treated preTic22 import reactions (Fig. 4A). Both the processed and precursor forms of the chimera were completely digested with trypsin (Fig. 7A, lane 4), indicating that the thermolysin-insensitive proteins were not translocated across the inner envelope membrane. Based on these observations, we conclude that the presequence of preTic22 is sufficient to target a passenger protein to the intermembrane space of the envelope.

The preSSU-Tic22 chimera is imported into the chloroplast and efficiently processed to mature SSU (Fig. 7B). The imported chimera is insensitive to both thermolysin (Fig. 7B, lane 3) and trypsin (Fig. 7B, lane 4) in intact chloroplasts and is found in the soluble fraction after chloroplast lysis (Fig. 7B, compare lanes 2–4 to lanes 5–7). These data are consistent with import to the stroma. A small fraction of preSSU-Tic22 remains bound to the chloroplast membrane fraction (Fig. 7B, lane 5), but it is sensitive to thermolysin digestion (Fig. 7B, lane 6), suggesting that it is bound to the outer surface of the envelope. In addition, a small portion of the imported mature protein is associated with the membrane fraction (Fig. 7B, lane 5). This mature Tic22 is not sensitive to either protease in intact chloroplasts (Fig. 7B, lanes 6 and 7), suggesting that it represents newly imported stromal Tic22 that contaminates the membrane fraction. The import properties of preSSU-Tic22 are indistinguishable from authentic preSSU, indicating that targeting is not detectably influenced by the mature sequences of Tic22.

PreTic22 Does Not Compete with preSSU for Import into Isolated Chloroplasts—The similarities between the import characteristics and presequences of preTic22 and stromal pre-
proteins raised the possibility that targeting to the intermembrane space may involve components of the general import machinery. To explore this possibility, we performed a competition experiment in which import of radioactively labeled preTic22 was carried out in the presence of excess unlabeled preSSU. Unlabeled preSSU efficiently competed for the import of radiolabeled preSSU (Fig. 8A). Inhibition of import was dose-dependent, reaching a maximum of 70% inhibition at 2 μM unlabeled preSSU (Fig. 8B). An equivalent concentration of mature SSU had no detectable effect on import. In contrast, the same concentrations of unlabeled preSSU had no detectable effect on the import and processing of preTic22 (Fig. 8, A, lane 6, and B). These results make it unlikely that the import pathways of preTic22 and preSSU share common components.

**DISCUSSION**

In this report, we investigated the import of preTic22 into isolated chloroplasts to define the signals and pathway for targeting to the chloroplast intermembrane space. PreTic22 was imported into isolated chloroplasts, and the pattern of protease sensitivity of the newly imported protein was similar to endogenous Tic22, indicating that the protein was accurately targeted to the intermembrane space in vitro. Our results show that the import of preTic22 is directed by the N-terminal presequence of the protein. The prerequisite is required for translocation across the outer membrane (Fig. 6), and it can target a soluble passenger protein to the intermembrane space (Fig. 7A). Therefore, it appears to be necessary and sufficient for targeting to the intermembrane space. Recognition of the presequence appears to be mediated by a proteinaceous receptor system at the surface of the outer membrane because proteolytic treatment of chloroplasts significantly reduces preTic22 binding and translocation (Fig. 5).

Approximately 20% of radiolabeled preTic22 added to the import assay was imported across the outer membrane, and the majority of the imported product associated with the inner membrane. Although import of the protein across the outer membrane was relatively efficient, processing of imported preTic22 was inefficient. Only 20% of the total imported protein was cleaved to mature Tic22 over the time course of our import assays (Fig. 2). We were unable to detect a significant conversion of imported preTic22 to mature Tic22 when chloroplasts from a preTic22 import reaction were reisolated and subjected to a second incubation under import conditions in the absence of added preprotein (data not shown). Processing of imported preTic22 therefore appears to be a slow event in the import process. The high proportion of imported preTic22 that remains unprocessed and the ability of preTic22 to associate with the inner membrane suggest that cleavage of the presequence is not tightly coupled to translocation across the outer membrane or binding to the inner membrane.

PreTic22 binds to chloroplasts and is processed with similar efficiency in the presence or absence of ATP (Fig. 4). However, the amount of imported protein that was insensitive to thermolysin degradation doubled in the presence of ATP. Therefore, it appears that ATP promotes efficient translocation across the outer membrane. It remains to be established whether ATP is utilized for translocation or for stabilizing Tic22 in the intermembrane space. The fact that a fraction of preTic22 is imported to a protease-insensitive compartment in the absence of exogenous ATP suggests that translocation can occur at reduced levels without energy. This observation favors the hypothesis that ATP may be necessary to stabilize Tic22 during or shortly after translocation. This interpretation is supported by the observation that thermolysin generates dis-

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