Toc12, a Novel Subunit of the Intermembrane Space Preprotein Translocon of Chloroplasts

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Translocation of proteins across membranes is essential for the biogenesis of each cell and is achieved by proteinaceous complexes. We analyzed the translocation complex of the intermembrane space from chloroplasts and identified a 12-kDa protein associated with the Toc machinery. Toc12 is an outer envelope protein exposing a soluble domain into the intermembrane space. Toc12 contains a J-domain and stimulates the ATPase activity of DnaK. The conformational stability and the ability to stimulate Hsp70 are dependent on a disulfide bridge within the loop region of the J-domain, suggesting a redox-regulated activation of the chaperone. Toc12 is associated with Toc64 and Tic22. Its J-domain recruits the Hsp70 of outer envelope membrane to the intermembrane space translocon and facilitates its interaction to the preprotein.

INTRODUCTION

Chloroplasts import most of their protein complement post-translationally from the cytosol. The N-terminal cleavable transit sequence is essential and sufficient for targeting of preproteins toward chloroplasts. Proteinaceous machineries will be engaged for the translocation of the preproteins across the two membranes of the plastid and hydrolysis of ATP and GTP is required for this process (Soll and Schleiff, 2004). In the past the molecular composition of the transloci at the outer and inner envelope has been characterized, but almost nothing is known about the transport across the intermembrane space. Even though it was suggested that the translocation machineries might form contact sites during translocation (Akita *et al.*, 1997; Nielsen *et al.*, 1997), details for this junction formation are not available.

At least 11 envelope proteins are involved in protein translocation and its regulation. Four proteins assist the translocation across the outer envelope. Toc75 (translocon at the outer envelope of chloroplasts of 75 kDa; Schnell *et al.*, 1997) forms the translocation channel (Hinnah *et al.*, 1997). Toc34 and Toc159 are two GTPases with receptor function (Hirsch *et al.*, 1994; Kessler *et al.*, 1994; Chen *et al.*, 2000; Sveshnikova *et al.*, 2000; Jelic *et al.*, 2002) and Toc64 is discussed as docking site for the guidance complex (Sohr and Soll, 2000). At the inner envelope seven components were identified, Tic110, Tic55, Tic62, Tic32, Tic20, Tic22, and Tic40 (Hörmann *et al.*, 2004; Soll and Schleiff, 2004). Tic110 assem-

bles the protein translocation pore in the inner envelope and Tic55 and Tic62 are involved in redox regulation of the translocation process (Soll and Schleiff, 2004). The function of Tic40, Tic32, Tic22, and Tic20 remains elusive. However, Tic40 shares homology to heat shock-associated proteins (Stahl *et al.*, 1999; Chou *et al.*, 2003), Tic32 shows similarity to small short-chain dehydrogenases and faces the stroma (Hörmann *et al.*, 2004), Tic22 might function as a receptor for preproteins in the intermembrane space, and Tic20 might form a part of the translocation channel (Kouranov *et al.*, 1998). In the stroma, the transit sequence is removed by the signal peptidase, and a chaperone system assists in folding of the mature protein (Jackson-Constan *et al.*, 2001).

Especially the integration of the chaperone system into the translocation process is not yet understood. Cpn60 (chaperonin of 60 kDa) was found to be associated with the Tic machinery (Kessler and Blobel, 1996). In contrast, additional data suggest that the chaperonin acts downstream of the stromal Hsp70 (Tsugeki and Nishimura, 1993). Further, it was postulated that Hsp100 (ClpC, caseinolytic protease, subunit C) is associated with the translocation machinery (Akita *et al.*, 1997; Nielsen *et al.*, 1997). Hsp100 is also associated with the protease ClpP and therefore is involved in intraorganellar protein degradation (Sokolenko *et al.*, 1998). Furthermore, two Hsp70 type chaperones, one located at the cytosolic site (Wu *et al.*, 1994) and one at the intermembrane space site of the outer envelope membrane (Marshall *et al.*, 1990; Waegemann and Soll, 1991; Schnell *et al.*, 1994), assist protein translocation across the outer envelope. However, nothing is known about the regulation of the activity of these chaperones or about their recruitment to the translocon.

In general, Hsp70 proteins are activated by cochaperones like DnaJ proteins. DnaJ proteins share the signature J-domain required for cooperation with Hsp70 partner proteins. This J-domain stimulates ATP-hydrolysis of Hsp70 proteins, which results in substrate binding of the chaperone. (Bukau and Horwich, 1998). Hsp70 proteins have a high affinity and

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a low exchange rate for substrates in the ADP-bound form (Schmid *et al.*, 1994). Such DnaJ homologues are also known to activate and to recruit Hsp70s to protein translocation systems, for example, the J-domain of Sec63 recruits the BiP protein to the Sec translocon (Corsi and Schekman, 1997).

Here we present a novel Toc component with an intermembrane space located J-domain as a component of the intermembrane space localized translocation complex. This complex is formed by multiple interactions of proteins of the outer envelope and intermembrane space to Toc64. Toc12 itself is able to stimulate the ATPase activity of Hsp70. Further, the activation of Hsp70 is dependent on a disulfide bridge, which is able to stabilize the loop structure of the J-domain.

MATERIALS AND METHODS

General

Outer envelope vesicles (OEVs) were purified as described (Schleiff *et al.*, 2003). Antibodies against Tic110, Toc75, Hsp70, Toc64, Toc34, OEP24, Tic22, OEP21, and OEP16 were used as earlier described (Vojta *et al.*, 2004). Antibodies against DnaJ were purchased from StressGen (Victoria, BC, Canada) and SPA820 from BioMol (Hamburg, Germany). POE33, pSSU, Toc34ΔTM, Tic32, Tic22, and Toc64 were expressed and purified as described (Waegemann and Soll, 1996; Sohr and Soll, 2000; Heins *et al.*, 2002; Jelic *et al.*, 2002; Hörmann *et al.*, 2004). The isolation of mRNA out of 5- and 10-d-old plant tissues and the reverse transcription-polymerase chain reaction (RT-PCR) were performed according to the manufacturer's recommendations (Invitrogen, Karlsruhe, Germany). Extraction of associated proteins was accomplished by the incubation of OEVs (30 μg protein) with 1 M NaCl, 0.1 M Na₂CO₃ or 4 M urea for 30 min at 2°C. After reisolation of the membrane vesicles by centrifugation (256,000 × g, 10 min, 4°C) the soluble and membrane fractions were subjected to SDS-PAGE analysis and immunoblotting.

Isolation of a Toc12 cDNA Clone and Subsequent Cloning

The screening of the λgt11-cDNA library with DIG (digoxigenin)-labeled DNA-probes, and the isolation of the phage-DNA was performed as described (Stahl *et al.*, 1999). Flanking *Xho*I and *Nco*I restriction sites were added to the Toc12 cDNA clone via PCR, and the construct was cloned into the expression vector pET21d, providing a C-terminal His-tag (Novagen, Schwalbach, Germany). Subsequently, point mutations in the J-domain of the Toc12 construct were introduced by recombinant PCR. Sequencing confirmed the cDNA of all constructs.

Expression and Raising of Antiserum against Toc12

For expression BL21(DE3) cells (Novagen) were transformed with the constructs containing plasmid, and the expression was induced by addition of IPTG (1 mM final). After incubation for 3 h at 37°C bacterial cells were harvested and lysed by 1200 psi pressure in a french press. The Toc12 proteins were purified over Ni-NTA (Qiagen, Hilden, Germany) affinity chromatography according to the manufacturer's recommendations. For raising antiserum against Toc12 the isolated recombinant protein was injected into rabbits.

Cell Fractionation and Isolation of Chloroplasts

Cell fractionation was performed by differential centrifugation of a cell extract from 10–12-d-old pea seedlings (*Pisum sativum*). After removing chloroplasts and nuclei (1600 × g, 1 min, 4°C) the mitochondria-containing fraction was pelleted (8000 × g, 10 min, 4°C). Subsequently, the supernatant was cleaned from residual organelles (46,000 × g, 10 min, 4°C), and cellular membranes were separated from a cytosolic fraction by a final centrifugation step (100,000 × g, 1 h, 4°C). Intact chloroplasts from 10–12-d-old pea seedlings were purified as described (Schleiff *et al.*, 2003).

Transformation of Tobacco Protoplasts with Toc12-GFP and Chloroplast Isolation

The isolation of protoplasts of tobacco leaves and the transformation with Toc12-GFP tag proceeds as described (Dovzhenko *et al.*, 1998). The intactness of the protoplasts was controlled by fluorescence microscopy using the chlorophyll fluorescence as an indicator. Subsequently, chloroplasts were isolated as described (Fitzpatrick and Keegstra, 2001) and subjected to a trypsin and thermolysin treatment. After separation through SDS-PAGE and transfer to nitrocellulose membrane, an immunostaining with αGFP (Roche, Mannheim, Germany) was performed.

Transcription, Translation, and Protein Import into Chloroplasts

The transcription and translation are described elsewhere (Schleiff *et al.*, 2001). The translation mixture was centrifuged for 1 h at 260,000 × g at 4°C and the postribosomal supernatant was used for import. Chloroplasts from *P. sativum* were isolated by standard procedures and further purified on Percoll gradients (Schleiff *et al.*, 2001). The chlorophyll concentration was determined to standardize import results. Standard import into chloroplasts equivalent to 40 μg chlorophyll was performed in 100 μl import buffer (10 mM methionine [or leucine], 20 mM potassium gluconate, 10 mM NaHCO₃, 3 mM MgSO₄, 330 mM sorbitol, 50 mM HEPES/KOH, pH 7.6) containing 10% of in vitro-translated ³⁵S-labeled protein. Import was initiated by addition of organelles to import mixture and stopped after 15 min. Intact chloroplast were reisolated through a Percoll cushion (40% Percoll in 330 mM sorbitol, 50 mM HEPES/KOH, pH 7.6), washed once in 330 mM sorbitol, 50 mM HEPES/KOH, pH 7.6, 3 mM MgCl₂, and used for further treatments as described (Schleiff *et al.*, 2001).

Protease Treatment

Purified chloroplasts were incubated with 0.5 μg/μl trypsin or 5 μg/μl thermolysin in 330 mM sorbitol, 50 mM HEPES/KOH, pH 7.6, 0.5 mM CaCl₂ at 25°C. The reactions were stopped by addition of 10 times excess of trypsin inhibitor or 25 mM EDTA/EGTA at the indicated times points. Intact chloroplasts were reisolated after centrifugation (3025 × g, 5 min, 4°C) on a 40% Percoll cushion. OEVs were incubated with 0.25 μg/μl trypsin, and the reaction was stopped by addition of a 10 times excess of trypsin inhibitor at the indicated time points. The intact membrane vesicles were recovered by centrifugation (256,000 × g, 10 min, 4°C). For analysis the samples were subjected to SDS-PAGE and subsequent immunoblotting.

Binding Assays, Coimmunoprecipitation, and Complex Isolation

For affinity chromatography OEVs (75 μg protein) were solubilized by 1.5% *n*-decylmaltoside for 5 min at room temperature (RT) and subsequently centrifuged at 100,000 × g for 10 min at 4°C. The supernatant was diluted 10 times in binding buffer (20 mM HEPES/KOH, pH 7.6, 50 mM KCl, 0.2% *n*-decylmaltoside) and incubated with 50 μl Ni-NTA coated with 200 μg Toc12, Toc64, or Tic22 for 1 h at RT. After sufficient washing with binding buffer, the bound proteins were eluted by 250 mM imidazol in the same buffer. The flow-through, the wash and the eluted fractions were subjected to SDS-PAGE analysis followed by immunoblotting. The coupling of pSSU and the binding to the preprotein was performed as described (Schleiff *et al.*, 2003). For binding assays using isolated proteins, the same procedure was used. The chaperone binding assay to immobilized pSSU was performed as described (Brychzy *et al.*, 2003).

For coimmunoprecipitation 1 ml antiserum was coupled to 250 mg Toyopearl AF-Tresyl 650M (TosoHaas, Tokyo, Japan) as described in the manufacturer's manual. OEVs according to 750 μg protein content were solubilized by 1.5% *n*-decylmaltoside and diluted 10 times in IP buffer (50 mM aminocaproic acid, 20 mM HEPES/KOH, pH 7.6, 0.2% *n*-decylmaltoside) for incubation with the column material at 4°C for 12 h. After sufficient washing with IP buffer, the bound proteins were eluted by 0.1 M glycine pH 2.5. The flow-through, the wash, and the eluted fractions were subjected to SDS-PAGE analysis and immunoblotting.

The complex was isolated by linear sucrose density centrifugation following the published protocol (Schleiff *et al.*, 2003). Cross-linking was performed as described (Akita *et al.*, 1997).

Zinc-releasing Assay

The release of bound zinc by PMB (*p*-hydroxy-mercuribenzoic acid) was measured as described (Hunt *et al.*, 1984). For this assay 2 μl of 4 mM PMB was stepwise added to 1 ml 40 mM KP_i, 20 mM Tris/HCl, pH 7, 100 μM PAR (4-(2-pyridylazo)-resorcinol) containing 6 μM Toc12. After each addition the amount of released zinc atoms was determined as an increment of absorption at 500 nm.

Measurements of the ATP Hydrolysis by DnaK

The purification of DnaK and DnaJ was performed as described (Zylicz *et al.*, 1985; Buchberger *et al.*, 1994). For measurement of the ATP hydrolysis by DnaK the reaction was started by the addition of DnaK to a final concentration of 6.5 μM in 50 μl reaction mix (25 mM HEPES/KOH, pH 7.6, 5 mM MgCl₂, 50 mM KCl, 100 μM ATP, 50 μCi [α -³²P]ATP). At the indicated times 2 μl of the reaction mix was spotted on a TLC plate (Merck, Darmstadt, Germany), and the separation of ATP and ADP was accomplished by incubation of the plate in 0.6 M NaH₂PO₃. The relation between radiolabeled ATP and ADP was determined by phosphorimager, and the evaluation was performed using the AIDA program. The assay was performed in the presence or absence of Toc12 constructs and substrate proteins.

Protein Modeling

Toc12 protein consists of a defined membrane part and soluble part (amino acids 44–102). Before modeling of the C-terminal domain of Toc12, the sequence was analyzed by several prediction server in order to confirm the template used. The C-terminal amino acids (48–103) were submitted to the following prediction server: UCLA/DOE Fold Server (<http://fold.doe-mbi.ucla.edu/>; Mallik *et al.*, 2002); 123D+ (<http://123d.ncicrf.gov/run123D+.html>; Alexandrov *et al.*, 1995); UCSC HMM Applications (<http://www.cse.ucsc.edu/research/compbio/HMM-apps/>; Karplus *et al.*, 1998); FFAS-PDB (<http://bioinformatics.burnham-inst.org/FFAS/index.html>; Rychlewski *et al.*, 2000) and 3d-psm (<http://www.sbg.bio.ic.ac.uk/~3dpsm/>; Kelley *et al.*, 2000). In our simulations we constructed a 3D structure of the 44–102 region (in the following renumbered to 1–59) of Toc12 (next only Toc12 structure) on the basis of the 3D structure of human Hsp40 J-domain (deposited in PDB database as 1HDJ) with differing amino acids taken from the backbone–side-chain–dependent library (Dunbrack and Karplus, 1993). Rotamer conformation was adjusted to minimize backbone–side-chain and side-chain–side-chain clashes. For peptide extended-atom GROMOS 96 force field (Hermans *et al.*, 1984; VanGunsteren *et al.*, 1996) was used, as implemented in GROMACS MD simulation package (Berendsen *et al.*, 1995; Lindahl *et al.*, 2001). The water was modeled as simple point charges (SPC; Berendsen *et al.*, 1981). Counterions to charged amino acids were added at the positions with the most favorable electrostatic potential to obtain an electroneutral system.

All molecular dynamics simulations were carried out using the Gromacs MD simulation package (Version 3.1.4; Berendsen *et al.*, 1995; Lindahl *et al.*, 2001) running on a Linux cluster. The Verlet integration scheme (leapfrog) was used with time step 2 fs. Periodic boundary conditions with a rectangular box were applied to avoid edge effects. LINCS algorithm (Hess *et al.*, 1997) was used to constrain all covalent bonds in nonwater molecules. The SETTLE algorithm was used to constrain bond lengths and angles in the water molecules. The temperature was controlled using weak coupling to a bath (Berendsen *et al.*, 1984) of 300 K with a time constant of 0.1 ps. Solvent (i.e., water and ions) and peptide were independently coupled to the heat bath. Initial velocities were randomly generated from a Maxwell distribution at 300 K, in accordance with the masses assigned to the atoms. The pressure was also controlled using weak coupling (Berendsen *et al.*, 1984) to atmospheric pressure (isotropic scaling) with time constant of 1.0 ps. The van der Waals interactions were modeled using a 6–12 Lennard-Jones potential, cut off at 14 Å. Long-range electrostatic was treated by Particle Mesh Ewald algorithm (Essmann *et al.*, 1995), with a 9-Å cutoff for the direct space calculation. The reciprocal space calculation was performed using a fast Fourier transformation algorithm. Molecular dynamic trajectories as well as static structures were visualized using VMD molecular visualizer (Humphrey *et al.*, 1996) and POV-Ray renderer (<http://www.povray.org>).

RESULTS

Identification of a Novel Component of the Outer Envelope of Chloroplasts of *P. sativum*

While analyzing the proteome of the outer envelope of chloroplasts (Schleiff *et al.*, 2003a), we identified a peptide not matching any so far known sequence. Subsequent screening of a cDNA library generated from *P. sativum* revealed a cDNA encoding a 12-kDa protein (acc. no. AY357119, Figure 1A). The identified in frame stop codon in front of the initiation atg and the stop codon at the 3' end of the coding region of the cDNA indicate that the complete coding region was found. Sequence analysis revealed a relation to DnaJ homologues by the presence of the characteristic HPD motif in the C-terminal portion of the p12 protein (Figure 1A, underlined). This C-terminal portion revealed a homology of 55% to the J-domain of *Escherichia coli* DnaJ (Figure 1B). Many amino acids essential for the interaction between DnaJ and DnaK (Greene *et al.*, 1998) are conserved in the newly identified protein (Figure 1B).

Antibodies raised against p12 recognize a single protein in purified OEVs of chloroplasts (Figure 1C) and in isolated chloroplasts (Figure 1D, lane 4). The purity of the organelles was tested by chlorophyll content (unpublished data) and by identification of the outer envelope protein Toc34, the thylakoid protein LHCP (light harvesting complex protein II; lane 4), or the outer mitochondrial membrane voltage-dependent anion channel (VDAC; Figure 1D). In contrast, the antibodies did not recognize a protein of expected size in mitochondria (lane 3), other cellular membranes (lane 2), or the cytosol (lane 1). Further chloroplast fractionation shows

Figure 1. A novel DnaJ homologue in the outer envelope of chloroplasts of *P. sativum*. (A) The identified cDNA (acc. number AY357119) and amino acid sequence is shown. The peptide identified by amino acid sequencing is framed. Arrowheads point to the four cysteines. The HPD motive is underlined. (B) An alignment of the amino acid sequence of the C-terminal portion of the identified p12 to the J domain of the *E. coli* DnaJ (acc. number P08622) using clustal X is shown. Essential amino acids for the function of the J-domain are marked on top. (C) OEVs were separated by SDS-PAGE followed by immunodecoration using p12 (Toc12) antisera. (D) Leaves from 10-d-old plants (*P. sativum*) were harvested, lysed, and fractionated. Cytosol (lane 1), microsomal (lane 2), mitochondrial and chloroplast proteins (30 μg each) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with antiserum against Toc34, VDAC, LHCP, and p12 (Toc12). (E) Chloroplasts from *P. sativum* were fractionated into stroma (lane 1), outer envelope (lane 2), inner envelope (lane 3), and thylakoids (lane 4). Fractions were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with indicated antisera. (F) The transcript level of p12 (Toc12, top panel) was tested in roots (lanes 1 and 4), stems (lanes 2 and 5), and leaves (lanes 3 and 6) of 5- (lanes 1–3) and 10-d-old (lanes 4–6) plants (*P. sativum*). For control, the amount of 18S RNA was analyzed (bottom panel).

a localization of p12 in outer envelope vesicles exclusively (Figure 1E, lane 2). The purity of the fractions was judged by the presence of the inner envelope protein Tic110 (lane 3), the large subunit of RubisCO (ribulose 1,5-bisphosphate carboxylase-oxygenase; lane 1), Toc34 (lane 2), or LHCP

Figure 2. p12 is an integral outer membrane protein of chloroplasts containing a soluble region facing the intermembrane space. (A) Isolated OEVs were incubated with 1 M NaCl (lanes 1 and 2), 100 mM Na_2CO_3 , pH 11.4 (lanes 3 and 4) or 4 M urea (lanes 5 and 6). The pellet (lanes 1, 3, and 5) and supernatant (lanes 2, 4, and 6) were separated and subjected to SDS-PAGE followed by immunodecoration using antisera against Toc75 (top panel), Tic22 (middle panel), and p12 (Toc12, bottom panel). (B and C) Isolated chloroplasts (40 μg chlorophyll, lane 1) were incubated with thermolysin (250 μg , lane 2, B) or trypsin (25 μg , C) after (lane 2) or before addition of trypsin inhibitor (lane 2, C). After inhibition of the protease (lane 2, B; lane 3, C) chloroplasts were reisolated and proteins separated by SDS-PAGE followed by immunodecoration by indicated antisera. (D) Isolated OEVs, 30 μg (lanes 1 and 4), were incubated with 12.5 μg trypsin for 0.5 (lanes 2 and 5) or 4 min (lanes 3 and 6) without (lanes 1–3) or with sonication (lanes 4–6). After inhibition of the protease, the envelope was subjected to SDS-PAGE, transferred to nitrocellulose, and decorated with antibodies against Toc75 (top panel) or p12 (Toc12, bottom panel). (E) Outer envelope vesicles treated as described in B were subjected to SDS-PAGE analysis, transferred to nitrocellulose, and immunodecorated with antibodies against DnaJ.

(lane 4). The tissue-specific expression of the gene was investigated by RT-PCR (Figure 1F). mRNA was observed in all tissues tested, but the level of the PCR products suggests a more pronounced expression in nongreen tissues compared with leaves (compare lanes 1, 3, 4, and 6). The presence of p12 in root plastids was verified by immunoblotting (unpublished data). We conclude that the identified 12-kDa protein is localized in the outer envelope of plastids and not restricted to chloroplasts.

To confirm the membrane insertion of p12, isolated OEVs were extracted using salt (Figure 2A, lane 1 and 2), carbonate (lane 3 and 4), or a chaotropic reagent (lane 5 and 6). In case of a loose association with the membrane, the protein should be removed from the surface (Breyton *et al.*, 1994) as seen for the peripheral protein Tic22 (lanes 2, 4, and 6). However, p12 behaves like the integral membrane protein Toc75 and was only detectable in the membrane fraction (lanes 2, 4, and 6), even though minor amounts of Toc75 were extractable by carbonate (lane 4). The soluble region seemed to be intermembrane space located because thermolysin treatment of chloroplasts did not result in p12 proteolysis (Figure 2B, lane 2). In contrast the cytosol facing Toc34 was degraded by thermolysin (lane 2). In line with this notion, the 12-kDa protein becomes degraded by high concentrations of trypsin (Figure 2C, lane 3), which penetrates the outer membrane as monitored by Tic110 digestion (lane 3). The degradation of p12 and Tic110 is not the result of postlysis digestion of inner envelope or stromal proteins, because the used amount of trypsin inhibitor effectively blocks the protease (lane 2) and Tic62 facing the stroma is not proteolysed (lane 3). In a second approach a low concentration of trypsin was added to right side out OEVs (Waegemann *et al.*, 1992; Figure 2D, lanes 1–3) solubilized by Triton X-100 (unpublished data) or sonication (lanes 4–6). As before, Toc34 was degraded even without solubilization (unpublished data). In contrast, the intermembrane space side exposed region of Toc75 (Schleiff *et al.* 2003a) became accessible only after

sonication (lanes 5 and 6). Membrane penetration by trypsin under the used conditions started only at the second time point, as seen by the appearance of the degradation product of Toc75 (lane 3). Similar to Toc75, p12 was stable against proteolysis before (lanes 2 and 3) but not after (lanes 5 and 6) lysis of the OEVs, as determined by immunoblotting using either p12 or commercially available DnaJ antibodies (Figure 2E, lanes 5 and 6). Similar to Toc75, p12 becomes slightly degraded after 4 min even without solubilization, which can clearly be seen using DnaJ antibodies (lane 3). The observed degradation of the p12 J-domain only after solubilization of the OEVs by using DnaJ antibody suggests an exposure of the p12 J-domain to the intermembrane space.

P12 Is Targeted to Chloroplasts In Vitro and In Vivo

The analysis of the deduced protein sequence revealed no classical cleavable transit peptide (Figure 1A). To further analyze the localization of p12, in vitro-translated protein was incubated with isolated chloroplasts (Figure 3A, lane 1), followed by treatment with sodium carbonate (lanes 2 and 3) or urea (lanes 4 and 5). As expected for a membrane-inserted protein, p12 remained in the membrane fraction (lanes 2 and 4). Lysis of chloroplasts by detergent removed p12 from the pellet (lane 7), demonstrating that the result observed was not due to protein aggregation. When the plastids were posttreated with thermolysin (lane 8) p12 remained stable, whereas trypsin posttreatment (lane 9) resulted in a loss of detectable protein.

To demonstrate that the identified protein is able to insert into the outer envelope of chloroplasts, a hybrid between p12 and a C-terminal-fused GFP was transformed into tobacco protoplasts. Subsequently, chloroplasts were isolated from transformed protoplasts. We identified the GFP fusion protein within the chloroplast fraction using commercially available GFP antibodies (Figure 3B, lane 1). Therefore, the 12-kDa protein was able to target GFP to chloroplasts. To

Figure 3. p12 is targeted in vitro and in vivo to chloroplasts. (A) Isolated chloroplasts (40 μ g) from *P. sativum* were incubated with in vitro-translated and ³⁵S-labeled p12 for 15 min (lane 1) followed by reisolation of chloroplasts and subsequent incubation with 100 mM Na₂CO₃, pH 11.4 (lanes 2 and 3), 8 M urea (lanes 4 and 5), or 0.5% Triton X-100, followed by separation of the pellet (lanes 2, 4, and 6) and supernatant (lanes 3, 5, and 7). Further, chloroplasts were treated with thermolysin (250 μ g, lane 8) or trypsin (25 μ g, lane 9). Samples were separated and subjected to SDS-PAGE followed by visualization by autoradiography. (B) Chloroplasts isolated from tobacco protoplasts expressing the p12-GFP fusion (lane 1) were incubated with 100 μ g thermolysin (lane 2) or 10 μ g trypsin (lane 3) for 5 min at 25°C. After inhibition of the proteases chloroplasts were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies against the inner envelope protein Tic110 (top lane), GFP (panel 2), LHCPII (panel 3), or SSU (bottom panel). (C) In vitro-translated GFP (lane 1) was incubated with 2 μ g thermolysin for 5 min at 25°C (lane 2), subjected to SDS-PAGE, and visualized by autoradiography.

determine the orientation of the protein the chloroplasts were incubated with thermolysin. This protease does not penetrate the membrane under the conditions used as seen from the protection of the inner envelope protein Tic110 (lane 2). After incubation with thermolysin, Toc34 (unpublished data), but not the p12-GFP fusion protein (lane 2), was degraded. However, when isolated GFP was incubated with thermolysin under similar conditions, the protein was not protease resistant (Figure 3C, lane 2), suggesting that p12-GFP must have crossed the outer membrane. To analyze whether GFP was localized in the intermembrane space, isolated chloroplasts were incubated with trypsin. The conditions used resulted in a membrane penetration by trypsin as determined by the degradation of the inner envelope protein Tic110 (Figure 3B, lane 3). Under these conditions p12-GFP became protease sensitive (lane 3). In contrast, the stroma protein SSU and the thylakoid protein LHCPII remained protease resistant (lane 3). All results indicate that the GFP domain was exposed to the intermembrane space. Furthermore, the p12-GFP fusion protein remained in the membrane even after incubation with salt or a pH shift (unpublished data). We conclude, that the p12-GFP hybrid was inserted

Figure 4. Transmembrane prediction of Toc12. (A) The hydrophobicity distribution for the whole sequence using a window of 9 amino acids was calculated using the Kyte & Doolittle scale. (B) The average energy for the transfer of a 9-amino acid stretch into an octanol layer was calculated according to Wimley and White (2000). (C) The average alternating energy for the transfer of a β -barrel segment of 9 amino acids into the octanol layer was calculated. (D) The exact β -barrel score (Schleif *et al.*, 2003a) was calculated using a window of 10 amino acids.

into the outer envelope of chloroplasts. Furthermore, the protease accessibility of GFP suggests that the C-terminal region of p12 represents the soluble intermembrane space region. In the other case, the p12 should represent a C_{out}-N_{in} topology, which would expose the GFP to the chloroplast surface. GFP would then have been accessible to thermolysin treatment (Figure 3C).

The N-terminus Anchors p12 by a β -barrel-type Domain into the Outer Membrane

Because in vivo and in vitro analysis indicates the presence of a transmembrane anchor, an in silico approach was used to analyze the protein. A hydrophobicity plot reveals two hydrophobic regions at the N-terminus of the protein (amino acid 1–15 and amino acid 38–48) and one hydrophobic cluster at the extreme C-terminus (Figure 4A). The C-terminal portion reveals high sequence similarity to the J-domain of DnaJ (Figure 1B) and can be proteolysed after vesicle opening by detergent or by sonication (Figure 2, D and E). Thus, the N-terminus was used to identify the architecture of the transmembrane domains. When the transfer energy from water to membrane phase was analyzed in a 9-amino acid window, only the extreme N-terminus revealed enough energy to possess a helical transmembrane region (amino acid 10–24, Figure 4B). To distinguish between a putative α -helical and a β -sheet conformation of the potential transmembrane segments the alternating transfer energy was calculated (Figure 4C). For helical regions, a clear plateau is expected, whereas a β -sheet segment should reveal a clear

Figure 5. The cysteines of p12 do not promote zinc binding but might stabilize the secondary structure. (A) A chelate column (lanes 5–7) was coated with zinc (lanes 2–4) and incubated with purified p12 (Toc12, top panel) or ALDH (bottom panel). Lane 1 shows the amount of protein loaded. The flow-through (lanes 2 and 5), wash (lanes 3 and 6), and elution (lanes 4 and 7) were subjected to SDS-PAGE followed by immunodecoration using p12 antibodies (top panel) or silver staining (bottom panel). (B) The release of zinc from p12 (○) or ALDH (●) after incubation with indicated amounts of PMB was determined. A representative result is shown. (C) The alignment of the C-terminus of the 12-kDa protein to the J-domain of Hsp40 is shown. The structural features of Hsp40 are indicated; H represents a helical structure and T a turn structure. The proline in Hsp40 is marked by an arrow. (D) The average structure of the last two nanoseconds of the molecular dynamic simulation of the p12 J-domain structure is shown. The disulfide bridge between cysteine 81 and cysteine 86 is shown in gray and the localization of the HPD motif in dark gray. (E) The RMSD during molecular dynamic simulation in comparison to the initial structure derived by amino acid replacement using the amino acids of Toc12 and the structural model of Hsp40 (hdj) is given. (F) As in E with the substitution of the his and asp amino acid in the HPD domain by gln and ala. (G) As in E with substitution of the both cys by ser.

alternating behavior (Wimley and White, 2000). Indeed, such behavior was found in two regions at amino acids 10–25 and 35–48 (Figure 4C). Especially the later segment shows such typical profile defined by alternating peaks for hydrophobic and hydrophilic clusters as found in membrane inserted β -sheet segments. To confirm this observation, the exact β -barrel score (Schleiff *et al.*, 2003a) for this region was calculated. Again, as seen for the alternating transfer energy, both regions account for a membrane inserted β -sheet conformation (Figure 4D). Remarkably, the value for both segments was found to be larger than 2, the cutoff value for selecting β -sheets in membrane proteins (Schleiff *et al.*, 2003a). Summarizing, we could experimentally demonstrate that the 12-kDa protein has a C-terminal J-domain, which is exposed to the intermembrane space. Furthermore, our analysis of the primary sequence of the N-terminal part suggests a β -barrel-type membrane anchor.

The Cysteines within the Loop Region Seem to Stabilize the Structure

Small proteins of the intermembrane space involved in protein translocation are also known from mitochondria. Remarkably, these proteins contain cysteines, which were postulated to be either involved in zinc binding or in complex formation (Rehling *et al.*, 2004). To get insights into the function of the cysteines of p12, OEVs were solubilized and incubated with a zinc-coated affinity matrix (Figure 5A). We did not observe any bound p12 (lane 4) but alcohol dehydrogenase (ALDH) known to bind zinc (Pietruszko, 1975; bottom panel, lanes 4 and 7). In parallel, facilitating a zinc-releasing assay zinc could be detected bound to ALDH (●), but not to p12 (○). From this we conclude that the cysteines in the 12-kDa protein are not used for zinc binding.

A further function of the cysteines might be the stabilization of the tertiary structure of the J-domain. To test the

influence of the cysteines on the structure of the J-domain of p12, the J-domain was modeled using a known J-domain structure as template. To identify a J-domain with similar predicted fold to the p12 J-domain, the amino acid sequence was analyzed by five different threading programs (Table 1). Thereby, four programs predicted the domain to be similar to the J-domain of human *huHsp40* (Table 1, hdj, Figure 5C) and only one ranked DnaJ from *E. coli* highest (Table 1, xbl, UCSC server, Figure 1B). Therefore, the J-domain of *huHsp40* was used as template for modeling of p12, and its amino acids were replaced by p12 ones. Subsequently, the stability of the initial 3D structure was challenged during 20-ns molecular dynamic simulation. After an initial adoption, the 3D structure reached an energetic stable area (Figure 5E; ESB), indicating the validity of the fold prediction. This analysis showed that the structure obtained for p12 is stable without significant conformational changes after 1 ns. The average structure of the stable conformation of p12 represents a J-domain with an exposed HPD motif (Figure 5D). It comprises a flexible part from amino acids 44–60 and a predicted highly structured and rigid region between amino acids 63 and 103 (Figure 5D). This region is build up by two helices that are connected by a loop. The proline present in the loop of *huHsp40*, connecting helix one and two, does not exist in p12 (Figure 5C). However, in p12 two cysteines at both ends of the loop can replace the structural stabilizing features of the proline by disulfide formation. To test this idea the structure of two mutated p12 constructs, one with the exchange of the histidine and aspartic acid of the HPD motif to glutamine and alanine, respectively, and a second with an exchange of the two cysteines to serines were modeled (Figure 5, F and G). The features of the conformational stable region aa 63–103 of p12 was taken to assess the effect of the mutations (Figure 5E). No drastic alteration during molecular dynamic simulations could be seen for the first mutant after initial adoption of the structure (Figure 5F). In contrast, the disruption of the disulfide bond in the second mutant destabilized the structure as seen from a sharp jump of the root mean square deviation (RMSD) value reflecting a conformational change for p12 with a cysteine to serine exchange (Figure 5G). From this observation we expect that the disulfide bridge formation contributes to the structural stability and functional integrity of the J-domain of p12.

P12 Interacts with the Intermembrane Space Facing Hsp70 of the Outer Envelope Membrane and Induces ATPase Activity

P12 contains a J-domain localized in the intermembrane spaces. As shown for many J-proteins such region regulates

the Hsp70 function (Kelley, 1998). In the following we address the question whether p12 J-domain is also regulating Hsp70. Hence, we investigated the association of p12 with the outer envelope Hsp70 (Marshall *et al.*, 1990), which is resistant to treatments with high salt (Figure 6A, up, lanes 1 and 2), carbonate (middle, lanes 1 and 2), or chaotropic reagents (low, lanes 1 and 2). As before Toc75 and p12, Hsp70 becomes only degraded after prolonged trypsin treatment, suggesting an intermembrane space exposition (Figure 6B, lane 3). This notion was supported by a rapid degradation of the protein upon membrane lysis (lane 3). When solubilized proteins of the outer membranes were incubated with an affinity matrix (Figure 6C, lanes 1–3) coated with the intermembrane space-localized J-domain of p12, an interaction with Hsp70 was observed (lane 3). The specificity of our antibodies for the intermembrane space Hsp70 was confirmed by commercial antibodies SPA 820 (Figure 6D) previously found to specifically recognize this protein (Schnell *et al.*, 1994).

To test if p12 can stimulate ATP hydrolysis, isolated DnaK from *E. coli* was preincubated with ATP at 4°C. Shifting the reaction to RT initiated ATP hydrolysis. DnaK has a slow intrinsic hydrolysis rate (Figure 6E, ●), which can be stimulated by addition of the purified DnaJ (○). Addition of the soluble J domain of p12 also increases the ATP hydrolysis rate of DnaK, but not as pronounced as found for DnaJ. This can either be explained by DnaJ-DnaK being a homologous system, whereas p12-DnaK is representing a heterologous system. It was previously described that substrate binding by Hsp70 homologous stimulates the ATPase activity of the chaperone (Bukau and Horwich, 1998). Therefore, another explanation of the observed ATPase stimulation by p12 J-domain is that p12 acts as a substrate of DnaK. To exclude this possibility, DnaK was incubated with the denatured form of the outer envelope protein of 16 kDa, Oep16 (Δ). Because Oep16 as integral membrane protein contains hydrophobic regions, it serves most likely as a substrate of DnaK because Hsp70 homologous bind preferentially to hydrophobic stretches (Bukau and Horwich, 1998). In line with this considerations the addition of Oep16 increased the hydrolysis rate, but not as drastically as found for p12Δ48 (Δ). In general the stimulatory effect of substrates is much less pronounced as for the J-proteins and can be therefore distinguished from cochaperone activity (Bukau and Horwich, 1998). Further, we used a p12Δ48 mutant bearing an aspartic acid-to-alanine and histidine-to-glutamine exchange in the HPD motif, which inhibits the action of a DnaJ protein (Scidmore *et al.*, 1993; Wall *et al.*, 1995). When this mutant was added to DnaK (□), only a substrate dependent stimulation was observed. We conclude that p12 can specif-

Table 1. Fold prediction by different software packages

UCLA		123+		UCSC		FFAS-PDB		3d-pssm	
hit ^a	S ^b	Hit	S	hit	S	hit	S	hit	S
1hdj00 ^c	27	1hdj ^c	9.41	1xbl	49.38	1hdj	35.9	1hdj	0.0001
1b8cA0	23	1xbl	8.82	1hdj	42.74	1xbl	31.0	1xbl	0.0027
256bA0	22	1ebdC	1.63	1bqz	42.21	1fafA	28.8	1fafA	0.0765
1vnc01	21	1bbl	1.25	1bq0	42.00	1fpoA	20.4	1fpoA	1.71
1qcqA0	20	1aqt1	1.03	1fpoA	26.78	1hcrA	4.51	256bA	3.49

^a Detected fold.

^b Score defined by the program.

^c Nomenclature according to the PDB identification code.

Figure 6. p12 interacts with the membrane inserted intermembrane space facing Hsp70 and stimulates ATPase activity of DnaK. (A) OEVs were incubated with 1 M NaCl (top), 100 mM Na_2CO_3 , pH 11.4 (middle), or 4 M urea (bottom). The pellet (lane 1) and the supernatant (lane 2) were separated and subjected to SDS-PAGE followed by immunodecoration using Hsp70-antiserum. (B) Isolated OEVs, 30 μg (lane 1), were incubated with 12.5 μg trypsin for 0.5 (lane 2) or 4 min (lane 3) in the absence (top panel) or presence (bottom panel) of 1% Triton X-100. After inhibition of the protease, the envelope was subjected to SDS-PAGE, transferred to nitrocellulose, and decorated with Hsp70-antibodies. (C) A Ni^{2+} -NTA matrix (lanes 4–6) was coated with p12 Δ 48 fused to a C-terminal hexa-histidine extension (Toc12 Δ 48, lanes 1–3), and incubated with solubilized OEVs (75 μg). The flow-through (5%, lanes 1 and 4), wash (5%, lanes 2 and 5), and eluted fractions (100%, lanes 3 and 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using Hsp70 antibodies. For controls see Figure 7C. (D) As in C, but elution fractions of the p12 Δ 48 (Toc12 Δ 48, lane 1) or BSA-coated column (lane 2) were immunodecorated by SPA820 antibodies. For comparison OEVs (7.5 μg) are shown in lane 3. (E) DnaK, 6.5 μM (●, solid line), was preloaded with ATP followed by incubation with 1 μM of DnaJ (○, solid line) or 5 μM of p12 (■, solid lines), p12 HD/QA (□, solid lines), p12 C/S (▲, solid line), or denatured Oep16 (△, dashed line). ATP hydrolysis was determined and quantified as described. Data reflect the average of at least three independent measurements.

ically stimulate the ATPase activity of an Hsp70-related chaperone. We hypothesized that the cysteines might play a role for the stability of the J-domain (Figure 5). To disturb a disulfide bridge formation, one of the cysteines was replaced by a serine. When this point-mutated p12 was added to the DnaK-ATP complex, again only a substrate-dependent stimulation of the hydrolysis rate was detected (Figure 6E, ▲). We therefore conclude, that the disruption of the disulfide bridge alters the structural stability of the protein and results in the loss of cochaperone activity.

P12 Is Associated with the Toc Complex

The 12-kDa protein is localized in the outer envelope of plastids (Figure 1) and activates Hsp70 proteins (Figure 6). Because an outer envelope Hsp70 was found in association with arrested preproteins (Waegemann and Soll, 1991), we analyzed whether p12 is involved in protein translocation. Using p12 antibodies for coimmunoprecipitation, we observed an interaction of p12 with Toc64, Toc34, and Toc75 (Figure 7A, lane 3) and Toc159 (unpublished data). In contrast, we did not observe an interaction with Tic110 or the outer envelope channel Oep21 (Figure 7A, lane 3). We conclude that p12 is a member of the translocation ma-

chinery and will be named Toc12. To confirm the interaction between Toc64 and Toc12, Toc64 was immunoprecipitated. The precipitate contained Toc64, Toc159, Toc75, Toc34, Tic22, Toc12, and Hsp70 (Figure 7B, lanes 3 and 6). Again, this complex did not contain Tic110 or a typical pore of the outer envelope Oep24 (lane 3). To further analyze the interacting domain of Toc12, solubilized OEVs were incubated with an affinity matrix coated with the C-terminal J-domain of Toc12. Again, we observed an interaction between Toc12, Toc64, Toc34, Tic22 (Figure 7C, lanes 3 and 6), Toc75, and Toc159 (unpublished data), but not with the outer envelope pore forming proteins Oep24 and Oep16 (lane 3). Furthermore, a silver-stained gel of the elution fraction of the Toc12 Δ 48 affinity matrix confirms the specificity of this interactions because beside Toc159, Toc75, Toc64, Toc34, and Tic22, only one unknown protein was detected (Figure 7D). The interaction between Toc12 and Toc64 is not mediated by other components, because purified Toc64 binds to a Toc12 affinity matrix (Figure 7E, lane 1). This result indicates that Toc12 is directly interacting with Toc64 via its C-terminal intermembrane space localized region.

Figure 7. p12 is a Toc component and involved in formation of the intermembrane space translocon. (A) Solubilized OEVs (400 μ g) were incubated with Toyopearl material coated with p12 antibodies (Toc12, lanes 1–3) or preimmune serum (Pis, lanes 4–6). The flow-through (5%, lanes 1 and 4), wash (5%, lanes 2 and 5) and eluted fractions (100%, lanes 3 and 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. (B) Solubilized OEVs (400 μ g) were incubated with Toyopearl coated with Toc64-antibodies (α Toc64, lanes 1–3) or Pis (lanes 4–6). The flow-through (5%, lanes 1 and 4), wash (5%, lanes 2 and 5) and eluate (100%, lanes 3 and 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. (C) Ni²⁺-NTA coated with either Toc12 Δ 48 (lanes 1–3) or BSA (lanes 4–6) and incubated with solubilized OEVs (75 μ g). The flow-through (5%, lanes 1 and 4), wash (5%, lanes 2 and 5), and eluted fractions (100%, lanes 3 and 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. (D) Ni²⁺-NTA coated with Toc12 Δ 48 (lanes 1–3) was incubated with solubilized OEVs (75 μ g). Fifty percent of the elution fraction was subjected to SDS-PAGE followed by silver-staining. The major protein band was marked according to immunodecoration results from C. Unspecifically bound large subunit of the RubisCo is marked by asterisk. (E) Expressed Toc64 was incubated with Toyopearl matrix (lane 2) coated with 50 μ g Toc12 Δ 48 (lane 1). The bound protein was eluted and subjected to SDS-PAGE followed by immunodecoration using Toc64 antibodies. (F) Expressed and purified Toc64, 100 μ g (lane 1), Toc34 Δ TM (lane 2), or Tic22 (lane 3) were coupled to a Ni-NTA affinity matrix (lane 4) followed by incubation with solubilized OEVs. The bound proteins were eluted and subjected to SDS-PAGE followed by immunodecoration using indicated antibodies. (G) Tic22 was extracted from inner envelope membranes by treatment with 0.1 M sodium carbonate and dialyzed. Aggregated proteins were removed by centrifugation (2 h, 300,000 $\times$ g). Subsequently, purified Tic22 was incubated with an affinity matrix (lane 1) coated with 100 μ g Toc12 Δ 48 (lane 2) or Toc64 (lane 3). The bound protein was eluted and subjected to SDS-PAGE followed by immunodecoration using Tic22 antibodies. (H) OEVs (150 μ g protein) were solubilized with 1.5% decylmaltoside and subjected to sucrose density centrifugation in the absence (left panel) or in the presence (right panel) of 1 mM ATP. The outer membrane proteins were separated by SDS-PAGE, transferred to nitrocellulose-membrane, and immunodecorated with the indicated antisera. The intermembrane space complex containing fraction and the Toc core complex fraction are marked by box 1 or box 2, respectively.

Figure 8. The intermembrane space translocon interacts with pSSU. (A) OEVs (75 μ g protein) were solubilized and incubated with a Ni-NTA coated with 100 μ g pSSU (lanes 1–3), mSSU (lanes 4–6), pOE33 (lanes 7–9), or Tic32 (lanes 10–12). The flow-through (5%, lanes 1, 4, 7, and 10), wash (5%, lanes 2, 5, 8, and 11), and eluate (100%, lanes 3, 6, 9, and 12) was collected and subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. (B) As in A, in the absence (lane 1) or presence of 1 mM ATP (lane 2) or ADP (lane 3). (C) OEVs (150 μ g protein) were incubated with 0.5 mM $MgCl_2$ (lanes 1–5) and 1 mM ATP (lanes 2, 4, and 6), 1 mM ADP (lanes 3, 5, 7), 10 μ g pSSU (lanes 4 and 5), 10 μ g pOE33 (lanes 6 and 7) solubilized and subjected on top of a sucrose gradient as in Figure 7G (25–70%). A fraction containing the intermembrane space complex (compare Figure 7G) were collected and subjected to SDS-PAGE analysis followed by blotting and incubation with indicated antibodies. (D) Expressed and purified Toc64, Tic22, or Toc12, 5 μ g, was incubated with a Toyopearl matrix (lanes 3 and 4) coated with 6 μ g pSSU (lanes 1 and 2) or mSSU (lanes 3 and 4). The flow-through (5%, lanes 1 and 3) and eluate (100%, lanes 2 and 4) were subjected to SDS-PAGE followed by blotting and immunodecoration by indicated antibodies. (E) Isolated chloroplasts were incubated with pSSU (top panel) or pOE33 (bottom panel) for 10 min at 4°C (lanes 2) in the presence of chemical cross-linker DSP (lanes 4–8) followed by lysis of the chloroplasts and incubation with indicated antisera. After immunoprecipitation, the cross-linker was cleaved by DTT, the proteins were separated on SDS-PAGE, and pSSU or mSSU was visualized by phosphorimaging. Lane 3 shows an import at 25°C. In lane 1, 1% of translation product (TP) is loaded.

Surprising was the identification of the interaction of Toc64 and Toc12 with Tic22. Tic22 is the only soluble intermembrane space protein involved in protein translocation described so far. To test the specificity of the interaction, Toc64, Tic22, and the cytosolic domain of Toc34 were expressed, purified, and bound to an affinity matrix. Solubilized OEVs were incubated with the affinity matrix, and proteins specifically bound were eluted by imidazol. None of the proteins were found to interact with a bovine serum albumin (BSA)-coated matrix (Figure 7F, lane 4). Only Toc64, but not Toc12 was found to interact with the cytosolic domain of Toc34 and vice versa (lanes 2 and 1, respectively). Hsp70 was found to interact with Toc64 and with Tic22 affinity matrix (lanes 1 and 3, respectively). We further observed an interaction between Toc64 and Toc12 (lane 1) as well as between Toc64 and Tic22 and vice versa (lanes 3 and 1, respectively). Interestingly, only a weak interaction between Toc12 and Tic22 was observed (lane 3). However, Toc64 mediated this interaction since purified endogenous Tic22 did not bind the C-terminus of Toc12 (Figure 7G, lane 2) but Toc64 protein (lane 3). We therefore conclude, that the intermembrane space domain of Toc64 (Schleiff, unpublished results) and Toc12 interact with each other and that Toc64 recruits Tic22 to the complex.

To verify the presence of such intermembrane space complex outer membrane proteins were separated by sucrose density centrifugation either in the absence (Figure 7H, left panel) or in the presence of ATP (Figure 7H, right panel). This experimental approach was previously demonstrated to be suitable for Toc core complex isolation (Schleiff *et al.*,

2003). In both cases Toc64 was present in fractions of lower sucrose concentration compared with the Toc core components Toc75 and Toc34 (Figure 7H, box 1 vs. box 2). In the absence of ATP only minor amounts of Hsp70, Tic22, and Toc12 were detected in the Toc64 fractions of middle sucrose concentration (Figure 7H, left panel, box 1), whereas in the presence of ATP Toc64 comigrates with Tic22, Tic22, and Hsp70 (Figure 7H, right panel, box 1). This observation suggests an assembly of the intermembrane space complex in the presence of ATP. This is in line with the ATP-dependent association between Hsp70 proteins and DnaJ proteins. In contrast ATP does not affect the formation of the Toc core complex as judged by appearance of Toc34 and Toc75 (Figure 7H, right and left panel, box 2). The detection of all outer envelope proteins tested at the top of both gradients is due to the presence of nonsolubilized OEVs (Figure 7H, left and right panel, fractions 1–3). Therefore, we concluded that Toc64, Toc22, Toc12, and Hsp70 forms an intermembrane space complex in an ATP-dependent manner, which might be involved in preprotein translocation across the intermembrane space.

To test this hypothesis, we coated a matrix with a set of preproteins, of the small subunit of the RubisCO (pSSU), of the 33-kDa subunit of the oxygen evolving complex (pOE33) or of Tic32. The matrices were incubated with solubilized OEVs. Toc64, Hsp70, Tic22, and Toc12 were in the bound fraction as determined by immunodecoration (Figure 8, B, lane 1, and A, lanes 3, 9, and 12), whereas most of the Toc34 was in the flow-through as expected in the absence of GTP (lanes 1, 7, and 10). Interestingly, no binding of Toc34 to

Tic32, an inner membrane protein without cleavable transit peptide (Hörmann *et al.*, 2005), was observed, whereas components of the intermembrane space translocon bind efficiently to this protein (lane 12). This observation indicates a substrate recognition of the intermembrane space translocon components independent of Toc34. The interaction was specific for the targeting sequence, because no binding to an mSSU column was observed (lane 6). Nevertheless, a significant amount of Toc12 and Hsp70 was also detected in the flow-through, indicating a dynamic complex assembly. In the presence of ATP, Hsp70 but not Toc12 dissociated from the complex (Figure 8B, lane 2), whereas in the presence of ADP, the complex was stabilized compared with the absence of nucleotides (lane 3). This is in line with a tight substrate association of Hsp70. In a complementary approach we investigated the effect of pSSU and of pOE33 on the nucleotide-dependent complex formation. Therefore, the complex was isolated by sucrose density centrifugation without (Figure 8C, lane 1) or with addition of either ATP (lanes 2, 4, and 6) or ADP (lanes 3, 5, and 7) in the absence (lanes 1–3) and presence of either pSSU (lanes 4 and 5) or pOE33 (lanes 6 and 7). One fraction containing the intermembrane space components (Figure 7H, box 1) is shown for analysis of nucleotide or preprotein influence on the complex (Figure 8C). As before, addition of ATP during solubilization revealed a complex formation composed of Toc64, Tic22, Toc12, and Hsp70 (lane 2), which is not destroyed by the incoming preprotein (lanes 4 and 6). When the OEVs were solubilized in the absence of nucleotides or in the presence of ADP, the complex could not be detected in the gradient (lanes 1 and 3), even though minor amounts of Hsp70 were recovered in the same fraction as Toc64. However, addition of a substrate like pSSU or pOE33 in the presence of ADP leads to detection of the complex (lanes 5 and 7). Similar results were obtained by the addition of Tic32 (unpublished data). We assume that at this stage the components of the complex interact with the preprotein. For Hsp70 the substrate recognition in the ADP bound state was expected. To manifest the direct interaction of the other components with the preprotein, isolated Toc64, Tic22, and Toc12 were incubated with the pSSU affinity matrix (Figure 8D). Both, Toc64 and Tic22 showed a stable interaction to pSSU (lane 2) but not to mSSU (lane 4), whereas Toc12 did not interact with the precursor protein (lane 2). However, a close proximity of Toc12 to an early translocating intermediate of a preprotein can be proposed because Toc12 is cross-linked to the unprocessed form of pSSU and pOE33 in the presence of low ATP concentration (Figure 8E, lane 6). In line with previous results (Figure 8A) no cross-link to mature form of either preprotein could be detected (Figure 8E, lane 6). The same was observed for the Toc components, Toc159 (lane 4) and Toc64 (lane 5), but not for the outer envelope protein Oep24 (lane 7). For comparison, preimmunserum did not precipitate either the precursor or mature form of the preproteins (lane 8). From the observed results we conclude, that the components of the intermembrane space translocon interact with incoming preproteins in early import intermediates. Moreover, ATP assembles a stable complex in the absence of precursor protein, which disassembles in the presence of ADP. However, the association with a precursor leads to a stabilization of the complex in the ADP-bound state.

Toc12 Recruits the Hsp70 to the Intermembrane Space Translocon

After establishing Toc12 as component of the intermembrane space translocon we analyzed the function of the small J-domain in the ATP-dependent assembly of this translocon

(Figure 8C). Considering the observations that the interaction between Hsp70 proteins and their respective J-proteins is regulated by the nucleotide bound stage of the chaperone (Corsi and Schekman, 1997), we tested how different adenine nucleotides affect association of the Hsp70 and the other components of the intermembrane space translocon to a Toc12Δ48 affinity matrix (Figure 9A). Although ATP has a little stimulating effect on the association of Hsp70 to Toc12Δ48 (lane 6), the addition of ADP induces dissociation of Hsp70 from the J-protein (lane 9). This observation is in line with the previously characterized interaction between Sec63p and BiP of the translocation apparatus in the endoplasmic reticulum (Corsi and Schekman, 1997). Interestingly, the interaction of the other intermembrane space translocon components was not affected by nucleotide addition, suggesting a Hsp70-independent interaction of these components (lanes 3, 6, and 9). To confirm these results, we took advantage of two Toc12 mutants, which were not able to stimulate ATPase activity of DnaK (Figure 6E). These proteins were coupled to the matrix and incubated with solubilized OEVs. The classical mutation in the HPD motif of Toc12Δ48, which abolished the interaction of J-domains with its chaperones (Bukau and Horwich, 1998), results in a lack of binding of Hsp70 to the Toc12Δ48QPA affinity matrix (Figure 9, B, lane 9, and C). Furthermore, we also analyzed the interaction of Hsp70 with Toc12Δ48S81, a Toc12 construct not able to stimulate ATPase activity of DnaK (Figure 6C). Again the binding of Hsp70 was drastically reduced in comparison to Toc12Δ48 (Figure 9, B, lanes 1 and 6, and C). However, the association of Toc64, Toc34, and Tic22 to both constructs were not affected (lanes 6 and 9). These observations indicate an interaction of Toc64 and Tic22 to Toc12Δ48 independent of a functional J-domain, whereas the association of the Hsp70 with the intermembrane space translocon relies on the presence of a functional Toc12 J-domain.

It was previously described that the targeting sequence of preproteins contains binding sites for DnaK (Rial *et al.*, 2000). Thus, we asked whether the interaction of Toc12 with DnaK in the presence of ATP could stimulate the binding of the Hsp70 protein to a preprotein. Therefore, we preincubated Toc12Δ48 constructs with DnaK and subsequently performed an *in vitro* chaperone-binding assay with immobilized pSSU (Figure 9C). In line with previous observations (Figures 6C and 9, A and B) the stimulating effect was restricted to a functional J-domain (Figure 9C, lane 2), whereas the two mutations Toc12Δ48QPA and Toc12Δ48S81 were unable to induce binding of DnaK to its substrate above background level (compare lane 1 and lanes 3 and 4).

DISCUSSION

Translocation of chloroplastic proteins is assisted by a translocon combining proteins of the inner and outer envelope membranes. In contrast to the translocation across the outer membrane, almost nothing is known about the mechanism of translocation through the intermembrane space. The only putative component is Tic22, which is peripherally associated with the intermembrane space facing leaflet of the inner envelope (Kouranov *et al.*, 1998). However, nothing is known about the function of this protein.

A second protein discussed to be involved in protein translocation across the intermembrane space is an outer envelope Hsp70 facing the intermembrane space (Marshall *et al.*, 1990; Waegemann and Soll 1991; Schnell *et al.*, 1994). In here we present the link between the chaperone and protein translocation through the newly identified component of the Toc complex, namely Toc12 (Figure 6). Toc12 is integrated in

Figure 9. Toc12 recruits Hsp70 to the intermembrane space translocon. (A) Ni²⁺-NTA coated with 200 μ g Toc12Δ48 and incubated with solubilized OEVs (75 μ g) in the absence (lanes 1–3) or presence of 0.5 mM ATP (lanes 4–6) or 0.5 mM ADP (lanes 7–9). The flow-through (5%, lanes 1, 4, and 7), wash (5%, lanes 2, 5, and 8) and eluted fractions (100%, lanes 3, 6, and 9) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. (B) Ni²⁺-NTA coated with 200 μ g of either Toc12Δ48 (lanes 1–3), Toc12Δ48S81 (lanes 4–6), or Toc12Δ48QPA (lanes 7–9) were incubated with solubilized OEVs (75 μ g) in the presence of 0.5 mM ATP. The flow-through (5%, lanes 1, 4, and 7), wash (5%, lanes 2, 5, and 8), and eluted fractions (100%, lanes 3, 6, and 9) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. One representative result of three experiments is shown. (C) Quantification of the results shown in B. The relative amount of bound Hsp70 to Toc12Δ48 constructs is depicted in percentage of the binding to Toc12Δ48 wild type. (D) DnaK, 1 μ M, was incubated in the absence (lane 1) or presence of either 2 μ M Toc12Δ48 (lane 2), Toc12Δ48S81 (lane 3), or Toc12Δ48QPA (lane 4) with on Toyopearl column material immobilized pSSU as described (Brychzy *et al.*, 2003). After sufficient washing, bound proteins were eluted by 8 M urea, subjected to SDS-PAGE, and subsequently silver-stained. The amount of bound DnaK was quantified using AIDA software. (E) Dynamic model of the intermembrane space complex action. For detailed description see *Discussion*.

the outer envelope and its catalytic C-terminal domain is facing the intermembrane space (Figures 1–3). Toc12 does not contain a typical targeting sequence like most of the outer envelope proteins (Figure 1; Schleiff and Klösgen, 2001) but is targeted to the outer envelope *in vitro* and *in vivo* (Figure 3). Interestingly, *in silico* analysis revealed a possible β -barrel conformation of the N-terminal transmembrane domain (Figure 4). The RNA level of Toc12 in leaves is lower than in roots or stems. A similar result was observed for at Toc33, where a higher RNA level was found in stems and roots than in leaves (Gutensohn *et al.*, 2000). Recently it was demonstrated that preproteins are imported by a different mechanism, depending on the developmental state of the plastid (Kim and Apel, 2004). Therefore, the differential expression of Toc12 might point to a more pronounced function during chloroplast development.

The C-terminal region of Toc12 forms a J-domain (Figure 5). This J-domain stimulates the ATPase activity of

Hsp70-type chaperones (Figure 6) and interacts with the intermembrane space localized Hsp70 (Figure 6). The close association of Toc12 to Toc64, the protein discussed to be the docking side for a cytosolic guidance complex (Sohr and Soll, 2000), suggests the following model. The guidance complex is recognized by Toc64. This leads on one hand to the release of the preprotein and subsequent recognition by the receptor complex of Toc34 and Toc159 and on the other hand to the activation of Toc12. Although the translocation across the outer envelope occurs in a GTP-driven manner by Toc159 (Schleiff *et al.*, 2003b) Toc12 is recruiting Hsp70 to the complex (Figure 9D). The interaction between Hsp70 in the ATP-bound stage and the intermembrane space translocon is dependent on a functional J-domain (Figure 9). On ATP hydrolysis induced by Toc12 the chaperone is transferred to an incoming preprotein. This explains the preprotein-dependent association of the translocon in the presence of ADP (Figure

8C). After nucleotide exchange the chaperone is transferred back in its ATP-bound form, and the complex is ready for a new round of preprotein uptake (Figure 9D). The preprotein is released to later steps in protein translocation involving the Tic translocon.

Such translocation systems involving a Hsp70 and a J-protein are already described for the Sec translocon of the endoplasmic reticulum, BiP and Sec63, and for the translocation across the inner mitochondrial membrane mediated by the Pam complex, Tim14/Pam18 and mtHsp70 (Corsi and Schekman, 1997; Rehling *et al.*, 2004). Like Toc12 also Sec63 and Tim14/Pam18 lack a zinc-binding region for substrate recognition in the J-protein, as was found for DnaJ (Sadler *et al.*, 1989; Mokranjac *et al.*, 2003; Truscott *et al.*, 2003). DnaJ delivers substrates to DnaK via its zinc-binding region (Bukau and Horwich, 1998). In all three transport machines the J-proteins recruits the chaperone to a proteinaceous complex, which provides a substrate in close proximity. Thus, no direct interaction of the J-domain to the substrate is required explaining the lack of a substrate-binding region in J-proteins involved in preprotein translocation. The driving force for translocation across the inner mitochondrial membrane is subsequently provided by an ATP-consuming cycle of preprotein binding and releasing by mtHsp70. This action is regulated by its cochaperones such as the nucleotide-exchange factor Mge1 and Tim14/Pam18 (Rehling *et al.*, 2004). For the here-described chloroplast outer envelope system the mode of Hsp70 action and the identity of potential further cochaperones remains to be investigated.

For the translocation of proteins across the intermembrane space distinct complexes composed of small Tim proteins exist in mitochondria (Rehling *et al.*, 2004). Tim9/Tim10 interact with the transmembrane domains of proteins of the inner mitochondrial membrane (Curran *et al.*, 2002), whereas Tim8/Tim13 interact specifically with the N-terminal domain of Tim23 during its translocation (Paschen *et al.*, 2000). Both complexes keep proteins crossing the intermembrane space in a translocation-competent state. When we compare the chloroplast system with the mitochondrial system, we cannot exclude that we have isolated an intermembrane space complex similar to the small Tim complexes that are restricted to a certain class of proteins. This would be in line with the observation that Hsp70, Toc64, Toc12, and Tic22 are less abundant in comparison to Toc159, Toc75, and Toc34 in isolated OEVs. For example, Toc34 is present in a 4–5-fold higher amount than Toc64 (Vojta *et al.*, 2004). Thus, the Hsp70 association will keep the protein partly unfolded, whereas Tic22 could transport the protein toward the Tic complex.

The presence of two cysteines in the loop region of Toc12 following the HPD motif is unique. The structure of the loop region of other J-domains is stabilized by proline in the loop region in a large number of J-proteins (for example, in Hsp40, Ydj1p, Sis1p, Sec63p, and more, Figure 6A) or a salt bridge (for example, in DnaJ or SV40; Kelley 1998). Such structure was not found in Toc12. In contrast, the disulfide bridge formation between both cysteines was found to stabilize the structure (Figure 5). Furthermore, the replacement of one of the cysteines resulted in a loss of the protein function even though a nonessential amino acid was replaced (Greene *et al.*, 1998). Therefore, the disulfide bridge might also be important for the regulation of the translocation event, because reversible disulfide bridge formation is a widespread mechanism to alter chloroplast enzyme function (Pfannschmidt, 2003). This disulfide bridge would be sensitive to the redox state, a signal that alters the capability of protein translocation of the inner envelope (Soll and Schleiff, 2004). Furthermore, it was demonstrated that certain pro-

teins will be mis-targeted to the intermembrane space under high light conditions inducing an alteration of the redox state (Hirohashi *et al.*, 2001). This might now be explained by the sensitivity of the folding machinery in the intermembrane space to the redox state of the chloroplast, because folding of the preprotein in the intermembrane space leads to its arrest.

Summarizing, we have identified a new component of the Toc complex with unique features in regard to its size, the membrane anchor, and the localization of its active domain. This protein represents a missing link between the transport machinery of the intermembrane space and the protein translocon. It reveals features that would allow a tight regulation of protein translocation across the intermembrane space.

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