Preprotein recognition and translocation by the Toc complex

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Motus animi continuus

Contents:

Abbreviations:

BiP	immunoglobulin heavy chain binding protein
BN-PAGE	blue native-polyacrylamide gel electrophoresis
Bag1	Bcl-2-associated athanogene
ClpC	caseinolytic protein C
Cpn60	Chaperonin of 60 kDa
Com70	chloroplast outer membrane protein of 70 kDa
Cim37	chloroplast inner membrane protein of 37 kDa
Css1	major stromal Hsp70 of *P. sativum*
DnaJ	J-domain containing protein of *E.coli*
DnaK	major Hsp70 of *E. coli*
GMP-PNP	5'-guanylylimidodiphosphate
Hsp70	heat shock protein of 70kDa
Hsp90	heat shock protein of 90kDa
isHsp70	Hsp70 of the intermembrane space
LHCPII	light harvesting complex protein II
NTA agarose	Nitrilotriacetic acid agarose
Oep	outer envelope protein
Pam	presequence translocase-associated motor
mSSU	mature form of pSSU
pOE33	preprotein form of the 33 kDa subunit of the oxygen evolving complex;
pSSU	preprotein form of the small subunit of ribulose-1,5-biphosphate carboxylase-oxygenase (rubisco)
TPR	tetratricopeptide repeat
Toc/Tic	Translocon of the outer/inner envelope of chloroplast;
Tom/Tim	Translocon of the outer/inner membrane of mitochondria
Toc34ΔTM	Toc34 lacking the transmembrane domain
Toc64ΔTPR	Toc64 lacking the TPR domain
Toc64-TPR	the TPR domain of Toc64
Toc12Δ48	J-domain of Toc12
VDAC	voltage dependent anion channel

1. Abstract

Preproptein recognition and translocation across the outer envelope membrane of plastids is catalysed by a proteinaceous machinery, called Toc translocon. The Toc core complex is composed of the pore forming Toc75 and the two GTP-regulated receptors Toc159 and Toc34. A main issue of this work is the nature of preprotein recognition and transfer by this Toc apparatus. It is still under debate, whether Toc34 or Toc159 is the initial receptor. Here, using proteoliposomes with reconstituted either Toc core complex or Toc159 and Toc75 and several *in vitro* binding analysis Toc34 was shown to act upstream of Toc159. Moreover, a certain set of preproteins engages Toc64 before passing the Toc core complex. The receptor function and the dynamic association with the Toc core complex of this protein are established. Toc64 is the central component of the intermembrane space translocon. This complex migrates at approximately 700 kDa in a BN-PAGE and contains Toc64, Tic22, Toc12 and isHsp70. Toc12 is a novel identified Toc component, which exposes a J-domain toward the intermembrane space. This domain recruits isHsp70 to the intermembrane space translocon in an ATP and preprotein dependent manner. Finally, the work addresses the molecular identity of this isHsp70. Therefore, isHsp70 was purified by chromatographic approaches and analysed by mass spectroscopy. Several peptide masses were obtained, which reveal high similarity of *P. sativum* isHsp70 to *S. oleracea* Com70.

2. Zusammenfassung

Die Erkennung und die Translokation von Vorstufenproteinen über die äußere plastidäre Hüllmembran werden durch eine Proteinmaschinerie katalysiert, die Toc Translokon genannt wird. Der Toc Kernkomplex besteht aus der Pore Toc75 und den zwei GTP-regulierten Rezeptoren Toc159 und Toc34. Eine Hauptfragestellung dieser Arbeit ist die Art und Weise der Erkennung von Vorstufenproteinen durch den Toc Komplex. Dabei wird analysiert, ob Toc34 oder Toc159 als initialer Rezeptor des Toc Kernkomplexes fungiert. Durch Verwendung von Proteoliposomen mit entweder rekonstituierten Toc Kernkomplex oder rekonstituierten Toc159 und Toc75 sowie durch *in vitro* Anbindungsanalysen konnte in dieser Arbeit Toc34 als Eingangsrezeptor charakterisiert werden. Darüber hinaus konnte für einige Vorstufenproteine Toc64 als dem Toc Kernkomlex vorgeschalteter Rezeptor gezeigt werden. Es wurden die Rezeptorfunktion und die dynamische Interaktion zum Toc Kernkomplex dieses Proteins etabliert. Toc64 ist die zentrale Komponente des Intermembranraumkomplexes. Dieser Komplex läuft mit einem Molekulargewicht von etwa 700 kDa in einem BN-PAGE und enthält Toc64, Tic22, Toc12 und isHsp70. Toc12 ist eine neu identifizierte Toc Komponente und exponiert eine J-Domäne in den Intermembranraum. Diese Domäne rekrutiert isHsp70 in einer ATP- und Vorstufenprotein abhängigen Art und Weise. Weiter wird die molekulare Identität des isHsp70 thematisiert. Dazu wurde das Protein durch chromatographische Techniken angereichert und durch Massenspektroskopie analysiert. Die daraus identifizierten Peptidemassen weisen eine hohe Sequenzähnlichkeit zu Com70 aus Spinatchloroplasten auf.

3. Introduction

The double membrane surrounded plastids originate from the engulfment of an ancestral cyanobacterium by a heterotrophic host cell that already contained mitochondria, a process commonly referred to as endosymbiosis (Weeden, 1981). During the course of evolution, most of the genomic information of the endosymbiont were transferred to the nuclear genome of the host (Martin et al., 1998; Blanchard and Lynch; 2000, Timmes et al., 2004). Thus, the semi-autonomous genome of the modern plastids contains about 60 - 200 open reading frames, implying a drastic reduction in the genetic information compared to the genome of a modern cyanobacterium encoding several thousands of genes (Leister, 2003). In order to maintain various biochemical functions, such as photosynthesis or amino acid- and lipid synthesis, more than 95% of all plastid proteins are translated by cytosolic ribosomes and are posttranslationally imported into the organelle. The import comprises a complex system of cytosolic targeting, translocation through the two envelope membranes and a subsequent intra-organellar sorting. Proteins destined for plastid import can be divided at least into two classes (Soll and Schleiff, 2004). The first class includes proteins, which are translated on cytosolic ribosomes as so called precursor proteins with a cleavable N-terminal transit peptide. The transit peptide provides essential and sufficient information for subsequent targeting towards and translocation into the plastid. This class encloses the majority of plastid proteins (Soll and Schleiff, 2004). The proteins that belong to the second class do not contain any cleavable transit peptide. Members of this class are most of the outer envelope proteins and two inner envelope membrane proteins (Schleiff and Klösgen, 2001; Miras et al., 2002; Nada and Soll, 2004). Furthermore, proteomic analysis of the plastid protein content suggests the existence of at least 140 proteins of this class (Kleffmann et al., 2004). In general cytosolic chaperones guide preproteins to the chloroplast surface. This interaction keeps the preproteins in an unfolded stage that is suitable for subsequent translocation through a channel. The association of chaperones also prevents that the enzymatic functions of these proteins take place in the wrong cellular compartment. For some preproteins with a cleavable transit peptide a phosphorylation by an unknown cytosolic kinase was reported (Waegemann and Soll, 1996). The phosphorylation site in the transit peptide can be recognised by cytosolic 14-3-3 proteins, which form a so called guidance complex together with Hsp70 and potential other factors (May and Soll, 2000). The import of pSSU into chloroplasts of *P. sativum* is enhanced four fold when associated with the guidance complex (May and Soll, 2000). Thus, phosphorylation of preproteins is not essential for import into chloroplasts, but was suggested to accelerate the targeting and not the import process of a specific set of preproteins (Soll and Schleiff, 2004). Prior to translocation across the envelope membranes dephosphorylation has to occur (Waegemann and Soll, 1996). However, it remains unclear whether dephosphorylation occurs at the outer or inner envelope membrane.

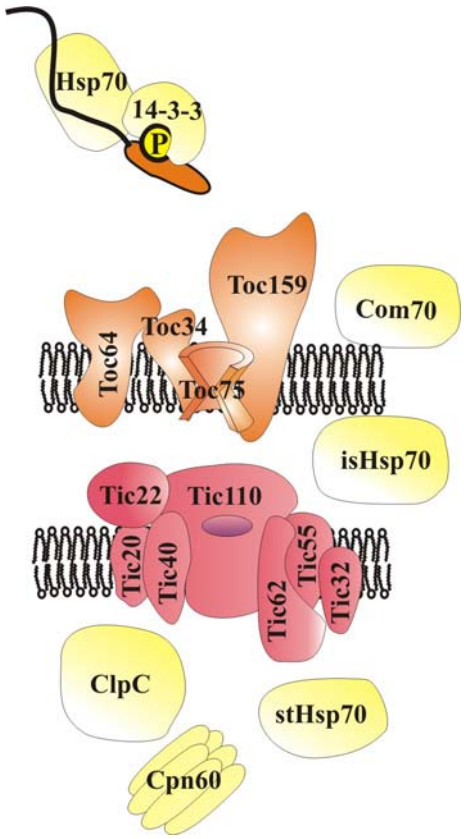

Figure 1. A model summarising the current knowledge about the molecular composition of the Toc machinery of the outer (orange) and of the Tic complex of the inner (red) envelope membrane is depicted. Furthermore, chaperones involved in preprotein import are shown in yellow.

On the chloroplast surface preproteins are recognised by a proteinaceous machinery, called Toc translocon (translocon of the outer envelope membrane of chloroplasts, suffices indicate the molecular weight of the components; Schnell et al., 1997). This complex is composed of the pore forming Toc75 and three receptors: the two GTP regulated Toc34 and Toc159, and the TPR domain (tetratricopeptide repeat) containing Toc64 (Fig. 1; Soll and Schleiff, 2004). Cross-linking approaches indicate an involvement of an intermembrane facing Hsp70 (isHsp70) in the import process (Fig. 1; Marshall et al., 1990; Waegemann and Soll, 1991). The passage of the preprotein across the inner envelope membrane involves a second protein complex, termed Tic translocon (translocon of the inner envelope membrane of chloroplasts). So far seven Tic components are described and biochemically characterised in chloroplasts of *P. sativum* (Fig. 1). The central component is Tic110. An association with Tic20 and the intermembrane space localised Tic22 was found via chemical cross-linking (Kouranov and Schnell, 1997). In a BN-PAGE Tic110 migrates in a complex together with Tic62 and Tic55, but neither with Tic22 nor Tic20 (Küchler et al., 2002), which might indicate the presence of multiple Tic complexes. An association of Tic110 with two further components, namely Tic40 and Tic32 was shown by chemical cross-linking or co-immunoprecipitation, respectively (Stahl et al., 1999, Hörmann et al., 2004). Electrophysiological data of reconstituted Tic110 suggest that this

protein forms a pore *in vitro* (Heins et al., 2002), whereas chemical cross-linking results point towards a role as a docking site for stromal chaperones (Kessler and Blobel, 1996; Nielsen et al., 1997). The central function of Tic110 in *A. thaliana* (*at*Tic110) is underlined by the embryonal lethality of a Tic110 knockout line (Koucheva et al., 2005). Much less is known about the other Tic components. Tic62, Tic55 and Tic32 are proposed to be involved in redox-regulation of the Tic machinery, whereas Tic40 might be an anchor for stromal chaperones (Soll and Schleiff, 2004; Hörmann et al., 2004). Tic20 was proposed to be part of the channel, since *A. thaliana* antisense plants reveal a severe phenotype (Chen et al., 2002). However, biochemical data supporting this notion are still missing. The driving force for preprotein translocation across the inner envelope membrane might be provided by stromal chaperones like ClpC (caseinolytic protein C), Cpn60 (chaperonin of 60 kDa) or Hsp70 (heat shock protein of 70 kDa; Soll and Schleiff, 2004). Finally, after reaching the stroma the preprotein is maturated by a signal peptidase (Richter and Lamppa, 1998) and folded by the chloroplast chaperone system.

To understand the function of the Toc complex the molecular basis of preprotein recognition is essential. The Toc core complex of *P. sativum* chloroplasts was purified by sucrose density centrifugation and revealed a stoichiometry of 1:4:4-5 of Toc159, Toc75 and Toc34 (Schleiff et al., 2003a). Structural studies of the Toc complex by electron microscopy revealed a solid ring with less dense interior, which is divided into four ring-like parts with a central finger (Schleiff et al., 2003a). Based on the stoichiometry it was suggested that Toc159 represents the central finger-like domain, whereas the four Toc75 proteins form the surrounding channels (Schleiff et al., 2003a). Due to its high protease sensitivity Toc159 was first characterised as its 86 kDa breakdown product (Schnell et al., 1994; Hirsch et al., 1994). The protein comprises an acidic A-domain, a central G-domain and a C-terminal M-domain, which embeds the protein into the envelope membrane (Chen et al., 2000). Toc34 is anchored in the outer envelope membrane by a C-terminal transmembrane domain and exposes a G-domain towards the cytosol (Seedorf et al., 1995). Several *in vitro* and *in vivo* studies were undertaken to elucidate the mode of Toc action. Indeed, two contradicting models of Toc action are proposed so far (Fig. 2; Schnell and Kessler, 2004). A central unsolved question is the identity of the initial receptor of the Toc core complex. For both receptor proteins a direct interaction with preproteins was demonstrated *in vitro* (Jelic et al., 2002, Smith et al., 2004). One model proposed Toc159 as the initial receptor due to a soluble cytosolic pool of Toc159, which was suggested by Toc159-GFP localisation and by biochemical purification (Hiltbrunner et al., 2001; Smith et al., 2002). According to one hypothesis Toc159 is shuttling between a soluble and a membrane bound form and thereby delivering preproteins to the translocation channel (Fig. 2A; Schnell and Kessler, 2004). Here, Toc34 acts as a docking site for soluble Toc159. This interaction is mediated by the two G-domains (Smith et al., 2002). However, a preprotein binding by the cytosolic Toc159 was not yet shown. Furthermore, the presence of Toc159 in a soluble extract (Hiltbrunner et al., 2001) might be due to contamination of the extract by lipid shreds from the chloroplast envelope membranes (Vojta, personal communication). In

line with this conclusion, Toc159 is not extractable from the membrane either by pH shift (Hiltbrunner et al., 2001), or by urea treatment and it spans the envelope membrane (Schleiff, personal communication). Thus, the shuttle between the soluble and membranous form of Toc159 would demand high energy costs, which would be a highly unfavourable event for preprotein import.

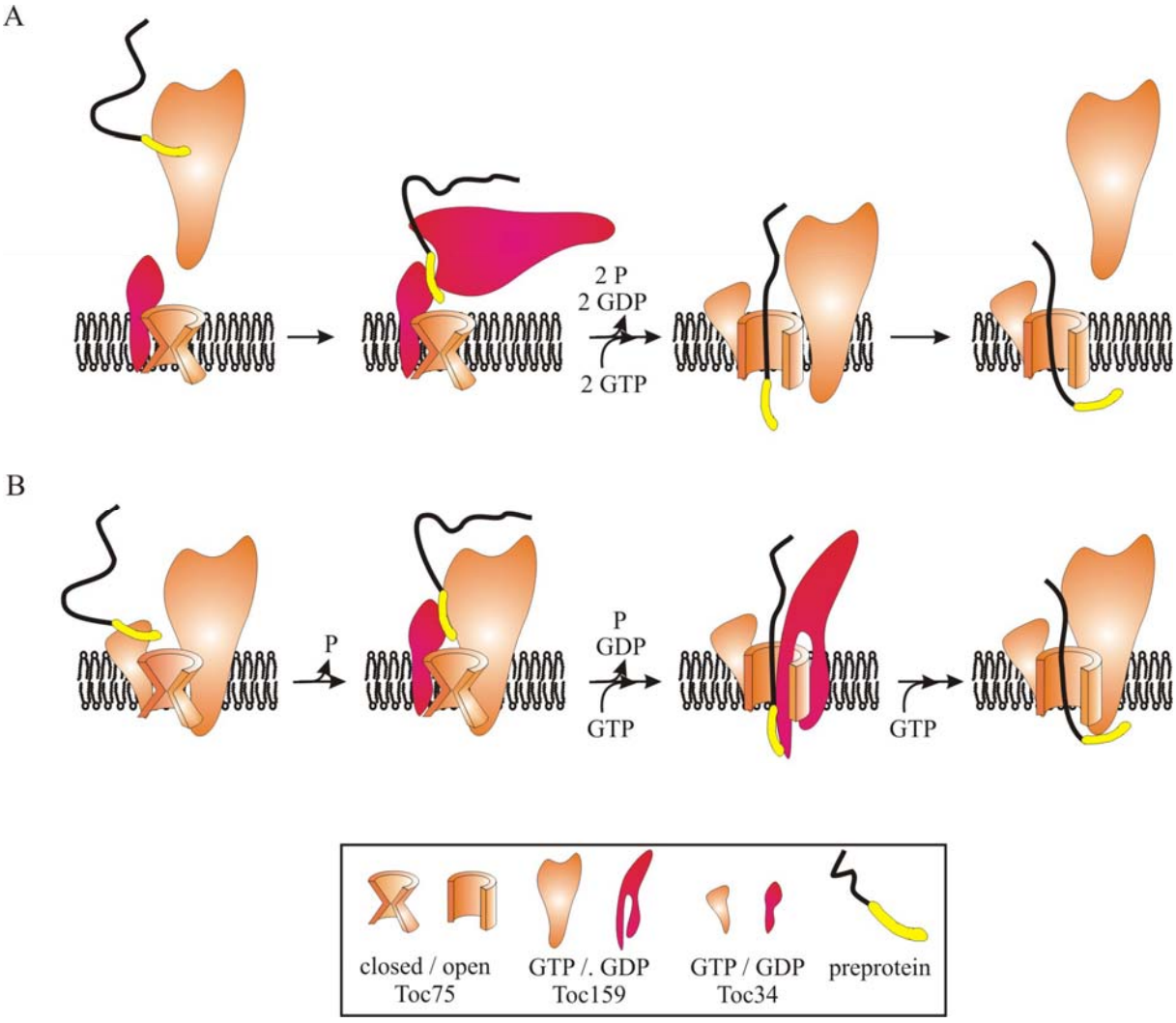

Figure 2. Two models of preprotein recognition and translocation by the Toc complex. **(A)** The shuttle model is shown, which includes a soluble pool of Toc159 as the primary receptor and Toc34 as a docking site for soluble Toc159. **(B)** The motor model comprises a membrane bound Toc159, which acts as molecular sewing machine after receiving the preprotein from the initial receptor Toc34.

Opposing this view another model focuses on a membrane bound Toc complex (Fig. 2B). Here, Toc34 is proposed as initial receptor for preproteins, which is switched off upon phosphorylation by an unknown kinase (Sveshnikova et al., 2000). This regulation of Toc34 underlines its role in early steps of import. This conclusion is supported by the observation that Toc34 can be cross-linked to preproteins during energy independent binding (Kouranov et al., 1997). Import studies in proteoliposomes with reconstituted single proteins or Toc core complex reveal that the translocation is GTP dependent and that the minimal set up for a successful translocation is Toc75 and Toc159

(Schleiff et al., 2003b). Therefore, the GTPase Toc159 was proposed to push the preprotein through the translocation channel by acting like a "sewing machine" (Fig. 2B; Schleiff et al., 2003b). This essential role of Toc159 is underlined by a lethal phenotype of a T-DNA insertion into *at*Toc159 gene (Bauer et. al., 2000). However, the severe phenotype was not complemented by the other three Toc159 isoforms, which are present in *A. thaliana* (Bauer et al., 2000). In line with this finding, the Toc159 paralogs in *A. thaliana* exhibit different substrate specificity (Ivanova et al., 2004, Kubis et al., 2003). Also for the two Toc34 homologues of *P. sativum* Toc34 in *A. thaliana*, namely *at*Toc33 and *at*Toc34, recognise different subsets of preproteins. *at*Toc33 mainly recognises photosynthetic preproteins, whereas *at*Toc34 is important for plastid biogenesis in roots (Kubis et al., 2003, Jelic et al., 2003, Constant et al., 2004). In contrast to Toc159 knockouts, the pale phenotype of *at*Toc33 knockout plants can be recovered by *at*Toc34 expression indicating partial functional overlap of both receptors (Jarvis et al., 1998). Altogether, those *in vivo* data were taken as support for the model of Toc159 as initial receptor (Schnell and Kessler, 2004). To solve the question for the first Toc receptor several biochemical analyses were conducted in this work. Based on the data a model of preprotein recognition and transfer on the cytosolic site of the outer envelope membrane by the Toc machinery is postulated and discussed.

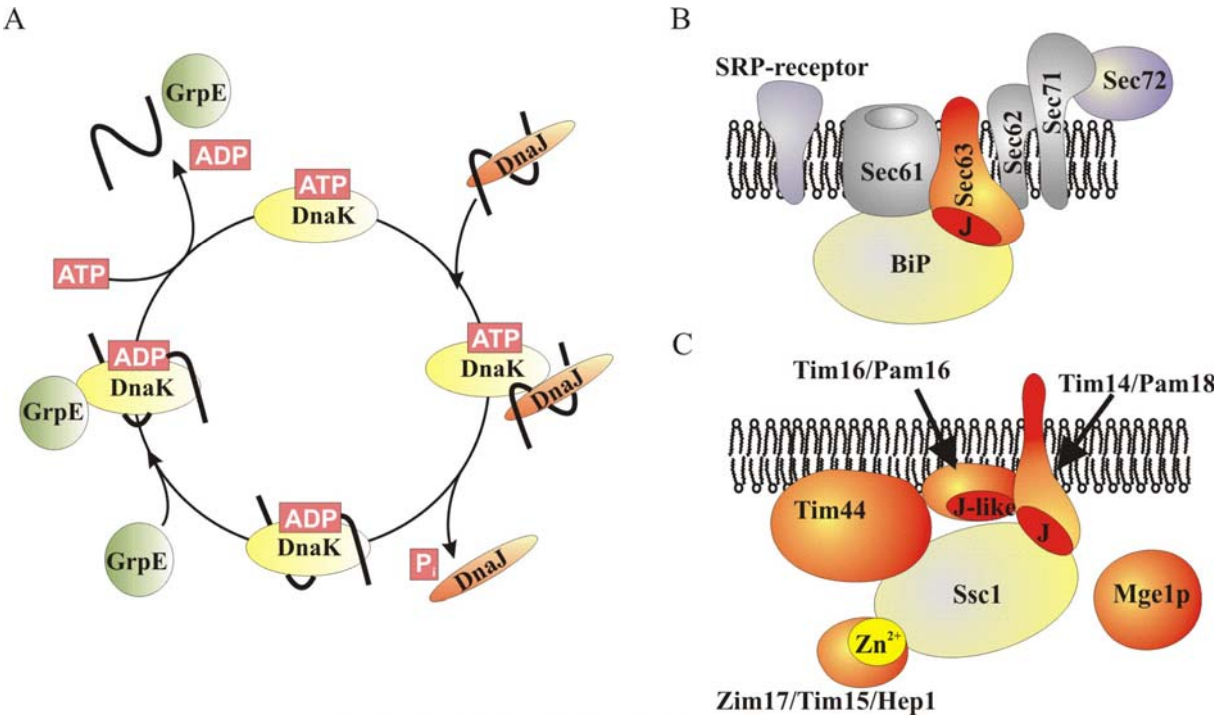

Figure 3. (A) The reaction cycle of *E. coli* DnaK and its regulation by the co-chaperones DnaJ and GrpE is shown. **(B+C)** J-proteins are involved in preprotein translocation into the ER-lumen (B) and into the mitochondrial matrix (C). Highlighted in yellow are the Hsp70 proteins and in orange their interacting partners. J-domains are shown in red. The components of the Sec translocon are depicted in violet. For further details see text.

In order to complete passage through the Toc channel further energy consumption on the intermembrane site of the plastid outer envelope membrane might be necessary, probably due to action of isHsp70 (Fig. 1; Soll and Schleiff, 2004). This isHsp70 is tightly associated with the outer envelope membrane of *P. sativum* chloroplasts (Marshall et al., 1990, Waegemann and Soll, 1991, Schnell et al., 1994). As judged by thermolysin insensitivity the chaperone faces the intermembrane space (Marshall et al., 1990; Schnell et al., 1994). In contrast, a second described Hsp70 of the outer envelope membrane, namely Com70 (chloroplasts outer membrane protein of 70 kDa), was reported to be thermolysin sensitive, which indicates a cytosolic exposure (Fig. 1; Ko et al., 1992). Both Hsp70 proteins were cross-linked to arrested preproteins and, therefore, were suggested to be involved in preprotein import (Waegemann and Soll, 1991; Kourtz et al., 1997). However, the function of isHsp70 during preprotein import remains elusive. In general, Hsp70 proteins are known to fulfil a variety of essential functions in the living cell. They facilitate folding reactions of freshly translated proteins, protein degradation, and maturation of steroid receptors, disassembly of oligomeric protein structures and protein import (Bukau and Horwich, 1998). These diverse functions are performed by Hsp70 proteins via a cycle of ATP regulated substrate binding and releasing (Fig. 3A). This reaction is highly stimulated by two co-chaperones. DnaJ proteins stimulate the ATPase activity of the Hsp70 and deliver substrate proteins to the chaperone. The Hps70 protein is converted from its ATP loaded stage with low affinity for its substrate proteins to its ADP bound stage with high affinity (Bukau and Horwich, 1998). This stimulating effect is based on the interaction of a J-domain with the ATPase domain of Hsp70 proteins. Thus, the substrate is transferred from the zinc domain of DnaJ to the peptide domain of DnaK. The completion of the Hsp70 reaction cycle is achieved by the ADP to ATP exchange and by its connected substrate release. This process is catalysed by a nucleotide exchange factor (Bukau and Horwich, 1998). Such Hsp70 chaperone system is already described for two different protein import machineries. For both, co- and post-translational import into the ER lumen, the action of the J-domain containing Sec63 and the luminal Hsp70 BiP (immunoglobulin heavy-chain-binding protein) is essential (Fig. 3B; Corsi and Schekman, 1997, Young et al., 2001). For the Pam (presequence translocase-associated motor) complex of the inner mitochondrial membrane Tim14/Pam18 was described as J-protein for Ssc1, the Hsp70 of the mitochondrial matrix (Fig. 3C; Mokranjac et al. 2003; Trucott et al., 2003). Interestingly, two further components were described to interact with Ssc1, namely Tim44 as a docking site and Tim16/Pam16 as a regulatory component of the ATPase cycle (Fig. 3C; Kozany et al., 2004, Li et al., 2004). In addition the zinc finger domain containing Tim15/Zim17/Hep1 was described (Burri et al., 2004, Yamamoto et al., 2005). It was shown that it prevents Ssc1 from aggregation in its nucleotide free state (Sichting et al., 2005). Therefore, such molecular motors for preprotein import involving Hsp70 are widely distributed. Regarding the outer envelope translocon system the docking site of isHsp70 at the Toc machinery and the molecular identity of this protein remain elusive (Fig. 1). The presented work provides the first view for the recruitment of isHsp70 during translocation of preproteins across the intermembrane

space via a novel J-domain containing Toc12 protein. This chaperone system forms an intermembrane space complex together with Toc64 and Tic22, which is dynamically associated with the Toc core machinery. Moreover, mass spectroscopy results and several biochemical analyses point to an identity of isHsp70 and the previously described Com70. Thus, chloroplast translocation across the outer envelope membrane involves a Hsp70/DnaJ system similar to the transport systems of the endoplasmatic membrane and of the mitochondrial inner membrane.

4. Materials

4.1. Chemicals and plants

All used chemicals were purchased in high purity from Sigma Aldrich (München, Germany), Roth (Karlsruhe, Germany), Roche (Penzberg, Germany) and Merck (Darmstadt, Germany). N-decyl-maltoside was supplied from Glycon GmbH (Luckenwalde, Germany); n-dodecyl-maltoside was obtained from Biomol (Hamburg, Germany). Radio-labelled amino acids and nucleotides were obtained from Amersham Biosciences (Freiburg, Germany). Pea (*P. sativum*) seeds of the sort "Arvica" (Praha, Czech Rebublik) were used for plant growing.

4.2. Enzymes, peptides and kits

Enzymes for cloning, such as restriction-endonucleases, T4-DNA-ligases and Tag-polymerases, were obtained from Roche (Penzberg, Germany), MBI Fermentas (St. Leon-Rot, Germany) and Quiagen (Hilden, Germany). Thermolysin was purchased from Roche (Penzberg, Germany), trypsin and lipase VII from Sigma Aldrich (München, Germany), RNase from Amersham Biosciences (Freiburg, Germany) and lysozyme from Serva (Heidelberg, Germany). For DNA purification with high yield the Plasmid Midi Kit from Macherey and Nagel (Düren, Germany) was used, whereas for DNA purification in a smaller scale the Silica Spin Kit from Biometra (Göttingen, Germany) was utilised. RT-PCR was performed with the Super ScriptII One Step Kit from Invitrogen (Karlsruhe, Germany). The peptides A1, B2 and E2 of pSSU and C90 of human Hsp90 protein were synthesised at the Department of Peptide and Protein Chemistry at the Charite (Berlin, Germany). *In vitro* translation was performed with the uncoupled wheat germ or reticulocyte lysate kit from Promega (Madison, USA).

4.3. Strains, vectors, clones and oligonucleotides

For DNA amplification the *E.coli* strain DH5α (GibcoBRL, Eggenstein) was used. The expression of Toc12 constructs was performed in the *E. coli* strain Omega (Novagen, Madison, USA). Other constructs were expressed in Bl21 (DE3) cells (Novagen, Madison, USA). Constructs for expression were cloned in pET21d vector (Novagen, Madison, USA), whereas RACE products were cloned into TOPO vector (Invitrogen, Karlsruhe, Germany). For GFP fusion, Toc12 constructs were inserted into GFP-2 vector (Promega, Madison, USA). Constructs for *in vitro* transcription and translation were inserted into either SP6-vector (Promega, Madison, USA) under the control of SP6-Polymerase like pSSU and pOE33 or into pBluescript under the control of T7 Polymerase like Toc64 (Stratagene, La Jolla, USA). The used oligonucleotides were purchased from MWG AF Biotech (Ebersberg, Germany). Com70 was cloned into pGEM4 for both expression and *in vitro* translation and was kindly provided by Prof. Dr. K. Ko.

4.4. Membranes

Nitrocellulose-membranes (Protran BA-S83, 0.2µm) were purchased from Schleicher and Schüll (Dassel, Germany).

4.5. Antibodies

In general primary antibodies were produced by injection of recombinantly expressed proteins into rabbits (Vojta et al., 2004). Commercial primary antibody against DnaJ from *E.coli*, SPA820 against cytosolic Hsp70 from human helper cells and an antibody against GFP from *A. victoria* were purchased from StressGen (Victoria, BC, Canada) and Roche (Penzburg, Germany), respectively. Antibodies against eukaryotic and prokaryotic Hsp70 proteins were kindly provided by Prof. Dr. L. Nover and Prof. Dr. F.U. Hartl, respectively. Secondary antibodies against rabbit-, mouse- and chicken IgG conjugates from goat were purchased from Sigma (München, Germany).

4.6. Columns and column materials

The Ni-NTA^{2+} column material was supplied by Qiagen (Hilden, Germany), the AF-Tresyl Toyopearl 650M for N-terminal protein coupling was obtained from Tosoh cooperation (Tokyo, Japan). ATP-Agarose and the thiol-activated propyl-Sepharose column material were obtained from Sigma (München, Germany). Protein-A Sepharose was supplied by Amersham Biosciences (Freiburg, Germany). Prepacked MonoQ HR5/5 and Sephadex75 were purchased from Amersham Biosciences (Freiburg, Germany).

5. Methods

5.1. Molecular biological methods

5.1.1. General molecular biological methods

General molecular biological methods like growing conditions of the bacteria, DNA precipitation, determination of DNA solutions and transformation were performed as described (Sambrook et al., 1989). The preparation of transformation competent cells was conducted according to the protocol published by Hanahan and co-worker (Hanahan et al., 1985). Restrictions, ligations and agarose gel electrophoresis were performed as described (Sambrook et al., 1989). Thereby, the reaction conditions were adjusted to the manufacturer's recommendations. EcoRI and HindIII digested λ-phage-DNA was used as molecular weight standard for gel electrophoresis.

Table 1. The constructs used in this study are listed. Given are the name (column 1), the used vector (column 2), purpose for cloning (column 3) and the biological (column 4) and scientific source (column 5) of the constructs.

Construct	Vector	Purpose	Organism	Origin
Toc12ΔTM	pET21d	Expression	*P. sativum*	This work
Toc12ΔTMS80	pET21d	Expression	*P. sativum*	This work
Toc12ΔTMQPA	pET21d	Expression	*P. sativum*	This work
Toc12GFP	GFPvector	Protoplast transformation	*P. sativum*	This work
Toc64	pET21d	Expression	*P. sativum*	Sohrt and Soll, 2000
Toc64ΔTPR	pET21d	Expression	*P. sativum*	Sohrt and Soll, 2000
Toc64-TPR	pET21d	Expression	*P. sativum*	Sohrt and Soll, 2000
Toc64	pBluescript	Transcription/ Translation	*P. sativum*	Sohrt and Soll, 2000
Toc34ΔTM	pET21d	Expression	*P. sativum*	Jelic et al., 2002
pOE33	pGEM42	Transcription/ Translation	*P. sativum*	Waegemann and Soll, 1996
pOE33	pET3c	Expression	*P. sativum*	Waegemann and Soll, 1996
pSSU	pSP65	Transcription/ Translation	*P. sativum*	Waegemann and Soll, 1996
pSSU	pET21d	Expression	*N. tabacum*	Waegemann and Soll, 1996
Tic22	pET21d	Expression	*A. thaliana*	Heins et al., 2002
Tic32	pET21d	Expression	*P. sativum*	Hörmann et al., 2004
Com70	pGEM4	Expression	*S. oleracea*	Ko et al., 1992
Com70	pGEM4	Transcription/ Translation	*S. oleracea*	Ko et al., 1992

5.1.2. Cloning strategies

Flanking XhoI and NcoI restriction sites were added to the Toc12 cDNA-clone (Caliebe, 1998) by PCR and the construct was cloned into the expression vector pET21d providing a C-terminal His-tag (Novagen, Madison, USA). Point mutations in the J-domain of the Toc12 construct were introduced by recombinant PCR. Truncations of Toc12 comprising solely the J-domain were created by using a 5`primer aligning an internal sequence of Toc12 with an artificial AUG start codon. All constructs were confirmed by sequencing. Truncated versions of Toc12 wild type clone from *P. sativum*, Toc12 QPA and Toc12 S80, each construct containing only the J-domain, were used for recombinant

expression (Tab. 1). Toc64, Toc64ΔTPR and Toc64-TPR from *P. sativum* were created previously (Sohrt and Soll, 2000). Toc34ΔTM from *P. sativum* (Jelic et al., 2002), Com70 from *S. oleracea* (Ko et al., 1992), Tic22 from *P. sativum* (Heins et al., 2002), pSSU from *N. tabacum* and pOE33 from *P. sativum* (Waegemann and Soll, 1996) were used for recombinant expression (Tab. 1).

5.1.3. RT- and RACE-PCR

The isolation of mRNA out of five and ten day's old plant tissues and the RT-PCR and RACE-PCR were performed according to the manufacturer's recommendations (Invitrogen, Karlsruhe, Germany). For amplification purposes the RACE products were cloned into TOPO vector (Invitrogen, Karlsruhe, Germany) and, subsequently, into a vector with a C-terminal fused GFP-tag (GFP-2, Promega, Madison, USA; Tab. 1).

5.2. Biochemical methods

5.2.1. General biochemical methods

Protein concentrations were determined by using the Biorad reagent (München, Germany) according to manufacturer's recommendations. Precipitations of proteins out of solution by acetone or tri-chlor-acetic acid were performed according to published procedures (Sambrook et al., 1989). SDS-PAGE was performed according to Laemmli (1970). BN-PAGE was conducted as described previously (Schägger et al., 1994). Gels were stained either by Coomassie Brilliant Blue R250 or silver-stained as outlined elsewhere (Sambrook et al., 1989). For immunodetection proteins were transferred on a nitrocellulose membrane by semi-dry blotting (Towbin et al., 1979). Immunodecoration and detection by using the alkaline phosphatase system were performed as described (Sambrook et al., 1979).

5.2.2. Protein expression and purification

pOE33, pSSU, Toc34ΔTM, Tic32 and Tic22 were expressed and purified as described (Waegemann and Soll, 1996, Jelic et al. 2002, Sohrt and Soll, 2000, Heins et al., 2002, Hörmann et al., 2004). Com70 expression in BL21(DE3) cells was induced by 1 mM IPTG for 3 h at 37°C. The soluble protein was purified under native condition according to the manufacturer's recommendations.

For expression of Toc12 constructs BL21(DE3) cells (Novagen, Madison, USA) were transformed with the respective plasmids and grown in 2YT/A media to an OD_{600} of 0.6-0.8. Subsequently, the expression was induced by addition of IPTG (1 mM final). After incubation for 3 h at 37°C bacterial cells were harvested, resuspended in resuspension buffer (100 mM Tris/HCl pH 8, 200 mM NaCl, 10 mM β-mercaptoethanol) and lysed by 1200 psi pressure in a french press. Large molecules like DNA or membrane shreds were destroyed by ten seconds of sonication. Subsequently, insoluble inclusion bodies were separated by centrifugation (20 000 x g, 30 min, 4°C). Toc12 constructs present in inclusion bodies were resolubilised by 6 M Guanidine/HCL, 50 mM NaP_i pH 6.8, 150 mM NaCl, 10 mM β-Mercaptoethanol and the binding to Ni-NTA affinity chromatography (Quiagen, Hilden,

Germany) was allowed for 30 min at 20°C. Subsequently, the column was washed with fifteen column volumes 6 M Urea, 50 mM NaP_i pH 8, 150 mM NaCl, 10 mM β-mercaptoethanol and five column volumes of 6 M Urea, 50 mM NaP_i pH 8, 1 M NaCl, 10 mM β-mercaptoethanol. Bound proteins were eluted by 500 mM imidazol, 6 M Urea, 50 mM NaP_i pH 8, 150 mM NaCl, 10 mM β-mercaptoethanol. Finally, the proteins were dialysed in the buffer used for the experiments. After dialysis soluble Toc12 proteins were gained either by separation of the soluble pool from the precipitated proteins via centrifugation (25 000 x g, 10 min, 4°C) and used directly for biochemical studies or precipitated Toc12 constructs were refolded on a Ni-NTA column before usage for affinity matrices (Rogl et al., 1998).

For soluble expression of Toc64 constructs BL21(DE3) cells (Novagen, Madison, USA) were transformed with the respective plasmids and grown in 2YT/A media to an OD_{600} of 0.4 at 37°C. Subsequently, the culture was cooled to 12°C before the expression was induced by addition of IPTG (1 mM final). Cell harvest and breakage were performed as described for Toc12 constructs. The Toc64 proteins were coupled to Ni-NTA affinity chromatography for 45 min at 20°C (Quiagen, Hilden, Germany). Subsequently, the column was washed with fifteen column volumes 50 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM β-mercaptoethanol and five column volumes of 50 mM Tris/HCl pH 8, 1 M NaCl, 1 mM β-Mercaptoethanol. Bound proteins were eluted by 250 mM imidazol, 50 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM β-mercaptoethanol. The eluted proteins were pooled and directly dialysed against 5 mM Hepes KOH pH 7.6, 50 mM Acetate, 4.3% Glycerol and 0.7 mM β-mercaptoethanol over night at 4°C. Precipitated proteins were removed by centrifugation (25 000 x g, 5 min, 4°C).

5.2.3. Purification of endogenous isHsp70

5.2.3.1. Isolation of Hsp70 proteins by ATP-Agarose

70 mg ATP-Agarose beads were incubated with ten column volumes of water for 1 h at 4°C. The beads were washed sufficiently with water and equilibrated with 10 mM KAcetate, 2 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6. Intact chloroplasts corresponding to 100 mg chlorophyll were isolated as described (5.4.2.) and solubilised in 1% Triton X-100, 10 mM KAcetate, 2 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6 for 30 min on 4°C. Non-solubilised membranes were removed (17 000 x g, 15 min, 4°C) and the supernatant was diluted in 10 mM KAcetate, 2 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6 to 0.2% Triton X-100 final concentration. The soluble extract was incubated with prepared 70 mg ATP-Agarose over night at 4°C while constantly shaking. The flow through was discarded and the beads were washed with twenty column volumes of 10 mM KAcetate, 2 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6 and ten column volumes of 1 M KAcetate, 2 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6. Remaining proteins were eluted from the ATP-Agarose by ten column volumes of 10 mM KAcetate, 10 mM $MgCl_2$, 2 mM DTT, 20 mM Hepes/KOH pH 7.6, 10 mM ATP. The purity of the elution sample was controlled by SDS-PAGE and Coomassie Brilliant Blue R250 staining. When outer envelope vesicles according to 4 mg protein

content were used the membranes were solubilised with 1% n-decyl-maltoside and the procedure was performed with 20 mg ATP-Agarose as outlined above.

5.2.3.2. Separation of the chloroplast Hsp70 proteins by anion-exchange chromatography

One strategy for Hsp70 purification was the separation of the ATP-agarose elution samples by other chromatographic methods. Therefore, the Hsp70 containing fractions from the ATP-Agarose were pooled and subjected to anion-exchange chromatography (MonoQ HR_5/5, Amersham Biosciences). A twenty column volume comprising linear KCl gradient from 50 mM to 1 M in 20 mM Hepes/KOH pH 7.6, 10 mM β-mercaptoethanol was applied. The protein content of 500 µl fraction was analysed by SDS-PAGE.

Alternatively, isolated outer envelope vesicles corresponding to 5 mg protein content were extracted by 8 M Urea. After removal of non-solubilised particles (256 000 x g, 10 min, 4°C) the soluble extract was directly subjected to anion-exchange chromatography (MonoQ HR_5/5, Amersham Biosciences). A twenty column volume comprising linear KCl gradient from 50 mM to 1 M in 20 mM Hepes/KOH pH 7.6, 10 mM β-mercaptoethanol and 6 M Urea was applied. The protein content of 500 µl fraction was analysed by SDS-PAGE. Hsp70 containing fractions were pooled, diluted four times in 6 M Urea, 20 mM Hepes/KOH pH 7.6 and 10 mM β-mercaptoethanol and applied for a second time on a MonoQ HR_5/5 anion-exchange column. The linear gradient was performed under the same conditions as described before.

5.2.4. Isolation of Toc159 from outer envelope vesicles

For Toc159 isolation outer envelope vesicles according to 450 µg protein content were separated over SDS-PAGE and, subsequently, the protein band corresponding to the 86 kDa fragment of Toc159 was gel eluted for 18 h at 20°C in SDS-PAGE running buffer (Laemmli 1970). Finally, the solution was concentrated and the yield was controlled by SDS-PAGE analysis and immunoblotting.

5.2.5. Affinity chromatography

5.2.5.1 Affinity chromatography with Ni-NTA column matrix

For interaction analysis of Toc and intermembrane space components outer envelope vesicles (75µg protein) were solubilised with 1.5% n-decylmaltoside for 30 min at 4°C and, subsequently, centrifuged at 100 000 x g for 10 min, at 4°C. The supernatant was diluted ten times in binding buffer (20 mM Hepes/KOH pH 7.6, 50 mM KCl, 0.2% n-decylmaltoside) and incubated with50 µl Ni-NTA coated with 200 µg of target proteins for 1 h at RT. After sufficient washing with binding buffer the bound proteins were eluted by 250 mM imidazol in the same buffer. Flow through, wash and eluted fractions were subjected to SDS-PAGE analysis followed by immunoblotting.

For *in vitro* interaction analysis of isolated proteins the target protein was coupled to Ni-NTA to a final concentration of 0.1 mg/ml column resin. Each assay was performed with 20 µl with target

protein coated column material, which was preequilibrated with binding buffer (20 mM Tricine pH 7.6; 50 mM NaCl, 0.5% Triton X-100, 0.1% BSA, 0.1 mM DTT, 1 mM $MgCl_2$) before incubation with the analysed proteins for 30 min at RT. The bound proteins were eluted by 250 mM imidazol in binding buffer. The eluted samples were subjected to SDS-PAGE analysis and immunoblotting.

5.2.5.2. Affinity chromatography with to Toyopearl matrix coupled target proteins

Preproteins and Toc64 constructs were coupled to Toyopearl affinity matrix as described (Schleiff et al., 2003a). The concentration of bound target proteins on Toyopearl column material was 1 mg/ml. For interaction analysis outer envelope vesicles (75µg protein) were solubilised with 1.5% n-decylmaltoside for 30 min at 4°C and, subsequently, centrifuged at 100 000 x g for 10 min, at 4°C. The supernatant was diluted ten times in binding buffer (20 mM Hepes/KOH pH 7.6, 50 mM KCl, 0.2% n-decylmaltoside) and incubated with the column material. After sufficient washing bound proteins were eluted by 8 M urea from Toyopearl column material.

The chaperone binding assay to immobilised pSSU was performed according to a published procedure (Brychzy et al., 2003). The elution samples were separated by SDS-PAGE and silver-stained. The amount of bound chaperones was determined by AIDA software.

5.2.5.3. Affinity chromatography with peptide matrices

For binding site analysis peptides of transit sequence of pSSU or C90 peptide were covalently coupled via a C-terminal cysteine to thiol-activated propyl-Sepharose (Sigma, München, Germany) to a final concentration of 0.6 mg/ml column resin in buffer (100 mM Tri/HCL pH 7,5, 500 mM NaCl, 1 mM EDTA) over night at 4°C. The matrix was incubated with isolated receptor proteins in binding buffer (20 mM Tricine/KOH pH 7.6, 100 µM DTT, 5% glycerol, 0.05% TX-100, 50 mM NaCl, 2 mM $MgCl_2$, 0.1% BSA) for 45 min at 20°C. After binding the column was washed sufficiently with binding buffer and bound proteins were eluted with 8 M urea. The eluted samples were subjected to SDS-PAGE analysis and immunoblotting.

5.2.6. ATP-hydrolysis assay

The purification of DnaK and DnaJ was performed as described (Buchberger et al. 1994, Zylicz et al. 1985). 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 $MgCl_2$, 50 mM KCl, 100 µM ATP, 50 µCi ^{32}P-αATP). At the indicated times 2 µl of the reaction mix were spotted on a TLC-plate (Merck, Darmstadt, Germany) and the separation of ATP and ADP was accomplished by incubation of the plate in 600 mM NaH_2PO_3. The relation between radio-labelled ATP and ADP was determined by phosphor-imager and the evaluation was performed using the AIDA program. The assay was performed in the presence or absence of Toc12 constructs and substrate proteins.

5.2.7. Protein quantification

5.2.7.1. Protein quantification by western blotting

Chloroplast outer envelope vesicles were prepared from *P. sativum* after 1 h of light treatment as described (5.4.2.). Defined amounts of purified proteins or proteins present in the outer envelope were separated by SDS-PAGE, blotted and incubated with primary antibodies. Proteins were visualised by alkaline phosphatase staining and proteins were quantified after scanning of blots using AIDA image analysis software. Expressed protein dilution series served as a standard for comparison with outer envelope bands thus enabling protein amount determination in those samples.

5.2.7.2. Quantification of the isHsp70 by chemical cross-linking of ^{32}P-αATP and ^{32}P-αGTP to outer envelope proteins and DnaK

The binding of ^{32}P-αATP and ^{32}P-αGTP to different amounts of outer envelope proteins and to DnaK was performed as described (Sveshnikova et al., 2000). Proteins were separated by SDS-PAGE and the nucleotide binding was analysed by phosphor-imager. The molecular ratio between the GTP loaded Toc34 and the ATP loaded isHsp70 was quantified using AIDA software.

5.3. Immunological Methods

5.3.1. Affinity purification of antibodies

Specific antibodies were purified from the sera using antigen-affinity columns. Recombinantly expressed proteins were coupled to CNBr-Sepharose (Amersham Biosciences, Freiburg, Germany) in 100 mM boric acid pH 8.4, 500 mM NaCl over night at 4°C. Subsequently, empty binding site were blocked by incubation with 1M Tris/HCl pH 8 at 20°C for 4h. After alternating washing with 100 mM NaAcetate pH 4, 500 mM NaCl and with 100 mM boric acid pH 8.4, 500 mM NaCl the coupling of the antibodies to their antigen was performed for 1.5 h at 20°C in 100 mM boric acid pH 8.4, 500 mM NaCl. Bound antibodies were eluted by 100 mM glycine pH 2.5.

5.3.2. Co-immunoprecipitation

5.3.2.1. Co-immunoprecipiation using ProteinA-Sepharose column matrix

Purified outer envelope vesicles were solubilised by incubation with 1,5% n-decylmaltoside, 25 mM Hepes/KOH pH7.6, 150 mM NaCl for 5 min at 20°C. Unsolved particles were removed (100 000 x g, 10 min, 4°C) and the supernatant was diluted ten times in IP-buffer (25 mM Hepes/KOH pH 7.6, 150 mM NaCl, 0.2% n-decylmaltoside) including 0.05% oval albumin. After addition of 10 µl antiserum the sample was incubated for 1 h at 20°C. For isolation of the antiserum and its bound proteins 50 µl of with IP-buffer preequilibrated Protein-A Sepharose (Amersham Bioscience, Freiburg, Germany) was added and incubated for another 1 h at 20°C. After sufficient washing with IP-buffer the bound

proteins were eluted by boiling in SDS-PAGE loading buffer. Eluted fractions were separated by SDS-PAGE and transferred to nitrocellulose membrane and immunodecorated with the indicated antisera.

5.3.2.2. Co-immunoprecipiation using Toyopearl AF650M column matrix

For co-immunoprecipitation 1 ml antiserum was coupled to 250 mg Toyopearl AF-Tresyl 650M (TosoHaas, Tokyo, Japan) as described in the manufacturers manual. Outer envelope vesicles according to 750 µg protein content were solubilised with 1.5% n-decylmaltoside and diluted ten times in IP-buffer (50 mM aminocapronic 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 100 mM glycine pH 2.5. After concentration the flow through, the wash and the eluted fractions were subjected to SDS-PAGE analysis and immunoblotting.

5.4. Cell biological methods

5.4.1. Cell fractionation

Cell fractionation was performed by differential centrifugation of a cell extract from ten to twelve days old *P. sativum* seedlings. A cell extract was obtained by homogenisation with a blender and by filtration through four layers of mull and one layer of gaze. After removing chloroplasts and nuclei (1 600 x g, 1 min, 4°C) the mitochondria containing fraction was pelleted (8 000 x g, 10 min, 4°C). The supernatant was cleaned from residual organelles (46 000 x g, 10 min, 4°C) and cellular membranes were separated from a cytosolic fraction by a final centrifugation step (100 000 x g, 1 h, 4°C).

5.4.2. Chloroplast isolation, subfractionation and complex isolation

Intact chloroplasts and chloroplast fractions from ten to twelve days old *P. sativum* seedlings were purified as described (Schleiff et al. 2003a). The Toc core complex was isolated from outer envelope vesicles by sucrose density centrifugation according to a published protocol (Schleiff et al., 2003a). The synthesis of the liposomes, the reconstitution of the Toc components and the binding/import assay was performed as described (Schleiff *et al.*, 2003b). For import competition analysis 5 µM of pSSU presequence peptides as indicated were added to the reaction mix.

5.4.3. BN-PAGE with isolated chloroplasts

For BN-PAGE analysis intact chloroplasts equivalent to 100µg chlorophyll were solubilised with 1% n-dodecyl-maltoside for 30 min on ice. Insoluble material was removed by centrifugation (256 000 x g, 10 min, 4°C). BN-PAGE loading buffer (Schägger et al., 1994) was added before the sample was loaded on the gel. The running was performed as outlined elsewhere (Schägger et al., 1994).

5.4.4. Extraction of outer envelope vesicles and chloroplasts

Extraction of associated proteins was accomplished by the incubation of isolated chloroplasts (20 µg chlorophyll content) or outer envelope vesicles (20 µg protein) with either 1 M NaCl or 100 mM Na_2CO_3 pH 11.4 or 4 M urea for 30 min at 2°C. After re-isolation of the membrane vesicles by centrifugation (256 000 x g, 10 min, 4°C) the soluble and membrane fractions were subjected to SDS-PAGE analysis and immunoblotting.

5.4.5. Protease treatment of chloroplasts and outer envelope vesicles

Purified chloroplasts were incubated with 0.5 µg/µg chlorophyll trypsin or 5 µg/µg chlorophyll thermolysin in 330 mM sorbit, 50 mM Hepes/KOH pH 7.6, 500 µM $CaCl_2$ at 25°C. The reactions were stopped by addition of ten fold excess of trypsin inhibitor or by EDTA/EGTA to a final concentration of 5mM at the indicated times points. Intact chloroplasts were re-isolated after centrifugation (3 025 x g, 5 min, 4°C) through a 40% Percoll cushion (40% Percoll in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6). Outer envelope vesicles were incubated with 0.25 µg/µg chlorophyll trypsin and the reaction was stopped by addition of a ten fold excess of trypsin inhibitor at the indicated time points. The intact membrane vesicles were recovered by centrifugation (256 000 x g, 10 min, 4°C). For analysis the samples were subjected to SDS-PAGE and subsequent immunoblotting.

5.4.6. Import assay into isolated chloroplasts

The translation mixture was centrifuged for 10 min at 256 000 x g at 4°C and the post ribosomal supernatant was used for import. After chloroplast purification the chlorophyll concentration was determined according to Arnon (1949). A standard import assay into chloroplasts equivalent to 20 µg chlorophyll was performed in 100 µl import buffer (10 mM methionine 10 mM cysteine, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 3 mM $MgSO_4$, 330 mM sorbitol, 50 mM Hepes/KOH pH 7.6) containing 10% of *in vitro* translated radio-labelled protein. Import was initiated by addition of organelles to import mixture and was transferred to 25°C. The reaction was stopped after 15 min. Intact chloroplast were re-isolated 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 $MgCl_2$, and used for further treatments as described (Schleiff et al., 2003b). Cross-linking of arrested preproteins was performed as described (Akita et al. 1997).

5.4.7. Transformation of *N. tabacum* protoplasts with Toc12-GFP and chloroplast isolation

The isolation of protoplasts from *N. tabacum* leaves and the transformation with Toc12-GFP fusion construct was proceeded as described (Dovzhenko et al. 1998). The intactness of the protoplasts was controlled by bright–field and by fluorescence-microscopy using the chlorophyll fluorescence as an indicator. Chloroplasts were isolated as described (3.4.2.1) and subjected to a trypsin and thermolysin treatment. After separation through SDS-PAGE and transfer to nitrocellulose membrane an immunostaining with αGFP (Roche, Penzberg, Germany) was performed.

5.5. Structural analysis

5.5.1. CD-Spectroscopy

For CD-spectroscopy recombinantly expressed Toc64 was purified as described (3.2.2.3). Low molecular contaminations were removed by size exclusion chromatography using a Superdex 75 16/30 column (Amersham, Freiburg, Germany) in 150 mM NaCl, 50 mM NaP_i pH 8. The purity of the elution samples was controlled by SDS-PAGE and subsequent Coomassie R250 Blue staining. Ten CD-spectra were recorded in 150 mM NaCl, 50 mM NaP_i pH 8 with a protein concentration of 100 nM and the mean values were calculated. The values were corrected by the absorption of the buffer. A CD-spectrometer Jobin Yvon CD 6 (Division Instruments, USA) was used. The graphic output was generated by usage of Sigma Plot 7.0.

6. Results

6.1. Preprotein recognition and stability of the Toc core complex

6.1.1. Preprotein recognition by Toc159

The Toc core complex is composed of the pore forming Toc75 and the two GTP regulated receptors Toc159 and Toc34. For Toc34 the GTP dependent receptor function was described (Sveshnikova et al., 2000, Jelic et al., 2002). Based on chemical cross-linking to an arrested preprotein and import inhibition by Toc159 antiserum Toc159 is also thought to function as a receptor during preprotein translocation (Kessler et al., 1994; Hirsch et al,. 1994). However, the exact mechanism of preprotein recognition remains elusive. To put some light into the mode of recognition of the incoming preprotein by Toc159, the interaction with the model precursor pSSU was investigated. Toc159 was isolated from outer envelope vesicles from *P. sativum* chloroplasts by SDS-PAGE and gel elution (Fig. 4A). The receptor was eluted as a 86 kDa fragment covering G- and M-domain ($Toc159_f$) and was used for an *in vitro* binding assay to immobilised pSSU in the absence or presence of various guanidine (Fig. 4B) and adenine nucleotides (Fig. 4C). The strongest binding of Toc159 to pSSU was shown in the presence of GTP and its non-hydrolysable analogue GMP-PNP (Fig. 4B, lane 2 and 4). The binding of Toc159 to pSSU in the presence of GTP is less pronounced as in the presence of GMP-PNP (Fig. 4B, lane 2 and 4). This might be due to a partial hydrolysis of GTP, which transfers the GTPases to the GDP bound stage with a reduced affinity to the precursor protein (Fig. 4B, lane 3). No effect on the binding efficiency of Toc159 to pSSU by adenine nucleotides was observed (Fig. 4C). Altogether, the interaction between Toc159 and pSSU is GTP-dependent.

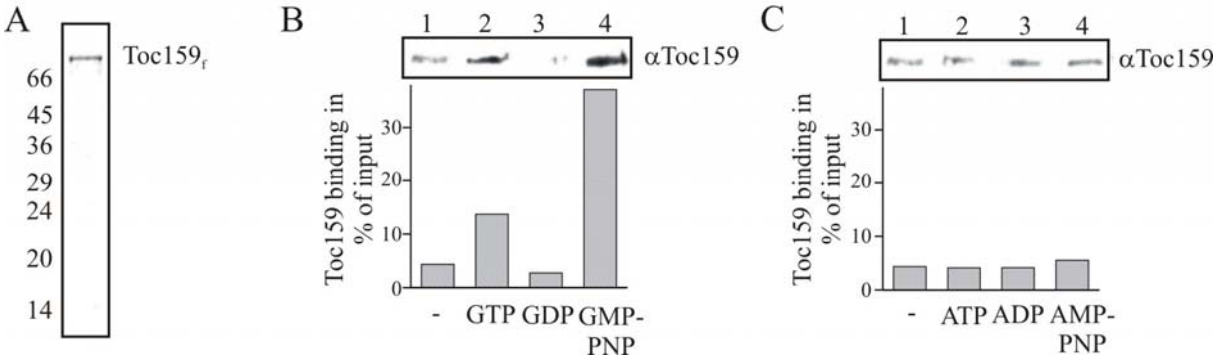

Figure 4. Recognition of pSSU by Toc159 is GTP dependent. **(A)** $Toc159_f$ was purified out of outer envelope vesicles by gel elution. The purity of the sample was controlled by SDS-PAGE and subsequent Coomassie R250 staining. **(B, C)** 20 µg pSSU were coupled to 20 µl Toyopearl AF-tresyl 650M column material and, subsequently, incubated with 250 ng $Toc159_f$. The incubation was performed in absence or presence of guanine (B) or adenine nucleotides (C). The amount of bound Toc159 in percentage of input was quantified after immunostaining using AIDA software. One representative result of 3 experiments is depicted.

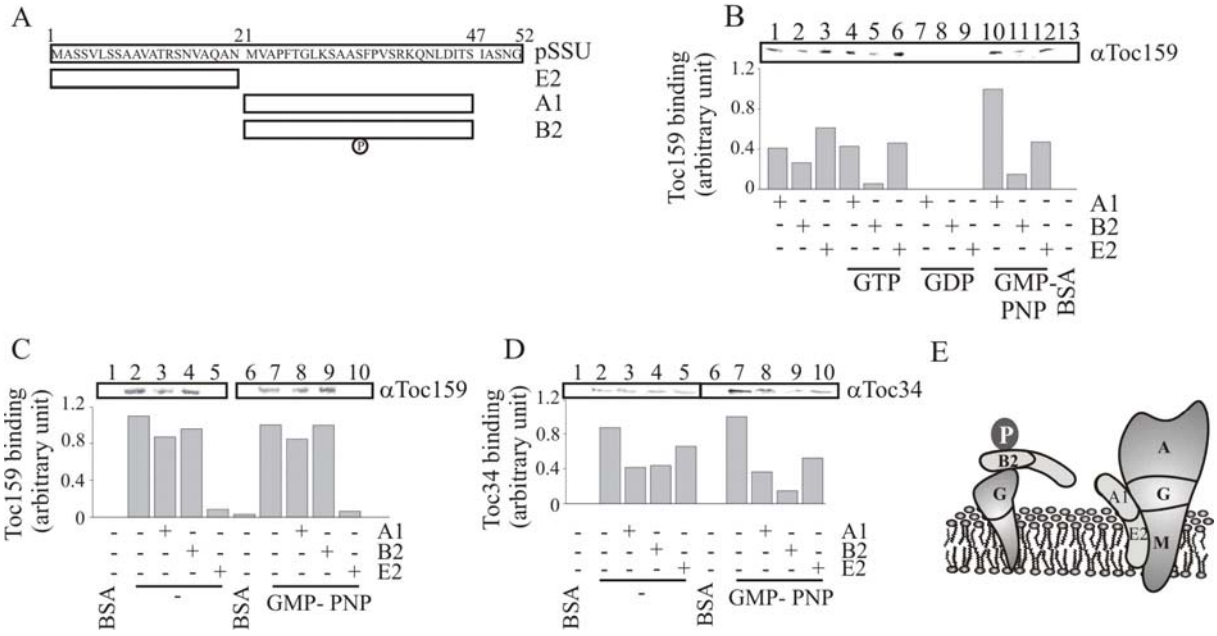

Figure 5. Toc159 recognises the N-terminal part of pSSU transit peptide with high affinity. **(A)** An outline of pSSU presequence from *P. sativum* and its peptides A1, B2 and E2 used in the assays is presented. **(B)** 20 µl thiol-activated propyl-Sepharose with bound peptides (0.6 mg/ml) was incubated with 250 ng $Toc159_f$ without (lane 1-3) or with GTP (lane 4-6), GDP (lane 7-9) or GMP-PNP (lane 10-12). A BSA coated column was used as control (lane 13). The quantification of one representative example of three independent experiments is shown. The Toc159 binding to A1 in the presence of GMP-PNP was set to 1. **(C)** 20 µl of Toyopearl material coated with pSSU (1 mg/ml) was incubated with 250 ng Toc159 in the absence (lane 2-5) or presence of 500 µM GMP-PNP (lane 7-10). The binding was competed for by addition of peptides to a final concentration of 5 µM. A BSA coated column was used as a control (lane 1, 6). The quantification of one representative example of three independent experiments is shown. The Toc159 binding to pSSU in the presence of GMP-PNP was set to 1. **(D)** The same experiment as in C but with expressed Toc34ΔTM. **(E)** A model of interaction of the presequence of pSSU is depicted.

To localise the section of the transit sequence interacting with Toc159, the transit sequence of pSSU was dissected into two parts. Peptides, each covering one of the two parts, were synthesised (Fig. 5A). One peptide is covering the N-terminal half (E2), whereas two others encompass the C-terminal part either phosphorylated (B2) or non-phosphorylated (A1). For *in vitro* binding assays peptides were coupled via a C-terminal cysteine to a thiol-activated propyl-Sepharose column material (Fig. 5B). Gel eluted Toc159 was incubated with no nucleotide (Fig. 5B, lane 1-3), GTP (Fig. 5B, lane 4-6), GDP (Fig. 5B, lane 7-9) and GMP-PNP (Fig. 5B, lane 10-12) before the binding of the receptor to the coated column material was analysed. Thereby, a GMP-PNP induced binding to A1 was observed (Fig. 5B, lane 10). The association of Toc159 to E1 coated column material in the absence or presence of GMP-PNP or GTP was comparable (Fig. 5B, lane 3, 6 and 12). The phosphorylated C-terminal part (B2) of pSSU transit peptide binds less Toc159 than the non-phosphorylated C-terminal or N-terminal part (Fig. 5B, lane 2, 5 and 11), which is consistent with the missing interaction of Toc159 to phosphorylated pSSU (Jelic, personal communication). GDP loaded Toc159 does not recognise any part of pSSU transit peptide (Fig. 5B, lane 7-9), which is in line with the reduction of the receptor binding to pSSU after GDP loading (Fig. 4B, lane 3). To analyse the affinities of the receptor to the

peptides, the competition for Toc159 binding to immobilised pSSU by the indicated peptides was determined. Gel eluted Toc159 preincubated with or without different nucleotides was incubated with the pSSU column in absence or presence of pSSU transit sequence peptides (Fig. 5C). A strong GMP-PNP independent competition effect of E2 binding to pSSU was obtained (Fig. 5C, lane 5 and 10). Interestingly, A1 shows no competition of pSSU binding to Toc159 indicating a lower affinity of Toc159 for this peptide in comparison to pSSU (Fig. 5C, lane 3 and 8). As expected from the direct binding a competition for the B2 peptide is not detectable (Fig. 5C, lane 4 and 9). An unspecific binding to column material can be ruled out by the lack of Toc159 binding to the unloaded column matrix (Fig. 5C, lane 1 and 6). To prove the selected experimental setup the competition assay was also performed with isolated Toc34ΔTM, which revealed the strongest inhibition of pSSU binding to Toc34 ΔTM by B2 in the presence of GMP-PNP (Fig. 5D, lane 9). This result is consistent with previously published data (Sveshnikova et al., 2000, Schleiff et al., 2002). Therefore, a strong and non GMP-PNP induced interaction of Toc159 to the N-terminal part of pSSU transit peptide is concluded. The non-phosphorylated C-terminal A1 peptide is preferentially recognised by $Toc159_{GMPPNP}$ albeit with reduced affinity compared to the N-terminal part (Fig. 5E).

6.1.2. Preprotein recognition and translocation by the Toc core complex

In addition to the strong binding of Toc159 to the N-terminal peptide it was shown that the C-terminal non-phosphorylated peptide induces the GTPase activity of the receptor (Jelic, personal communication). The impact of the observed interactions on protein import across the Toc translocon was further analysed. Isolated chloroplasts were preincubated with the different peptides and the import was initiated by addition of radio-labelled pSSU (Fig. 6A). Preincubation with B2 and E2 lead to a 50% decrease in import, whereas A1 had no effect (Fig. 6A, lane 2-4). These findings support the earlier result that B2 and E2 strongly bind to Toc34 or Toc159, respectively, whereas A1 reveals a low affinity for Toc159 (Fig. 5C and Fig. 5D). After preincubation with expressed full-length pSSU the import of radio-labelled pSSU was almost completely abolished (Fig. 6A, lane 5).

To manifest an effect of the peptides on the Toc core translocon, isolated Toc complex was reconstituted into liposomes as previously described (Schleiff et al., 2003b). Proteoliposomes were incubated with radio-labelled pSSU in the presence of GMP-PNP to stimulate binding (Fig. 6B, lane 15 and 16) or GTP to promote import (Fig. 6B, lane 23 and 24). To distinguish bound and pSSU imported into proteoliposomes a trypsin treatment was performed in order to digest surface bound preproteins, whereas imported pSSU remains unaffected (Schleiff et al., 2003b). In the presence of GMP-PNP the precursor remains protease sensitive indicating its presence on the surface of the liposomes (Fig. 6B, lane 16), while in the presence of GTP pSSU was protease resistant indicating its translocation into the proteoliposomes (Fig. 6B, lane 24). The binding of the preprotein in the presence of GMP-PNP or GTP was enhanced compared to the control conditions with no nucleotides (Fig. 6B, lane 7, 15 and 23). Proteoliposomes were subsequently preincubated with the peptides A1, B2 or E2

before addition of precursor protein (Fig. 6B, lane 1-6, 9-14 and 17-22). A1 slightly reduces the binding of pSSU in the absence or presence of GMP-PNP in comparison to the amount of bound pSSU in the absence of peptides (Fig. 6B, lane 3 and 11). However, A1 does not impair import of pSSU into proteoliposomes since the protease protected form of pSSU was detected (Fig. 6B, lane 20). In contrast, E2 reduces pSSU binding in the absence of nucleotides (Fig. 6B, lane 5) and like B2 inhibits import of pSSU (Fig. 6B, lane 18 and 22). Under binding conditions in the presence of GMP-PNP B2 is the only peptide, which leads to a reduction of pSSU association to the proteoliposomes (Fig. 6B, lane 9). This observation can be explained by a preceded binding of B2 to Toc34 while the interaction of Toc159 with E2 takes place at a later stage and inhibits import.

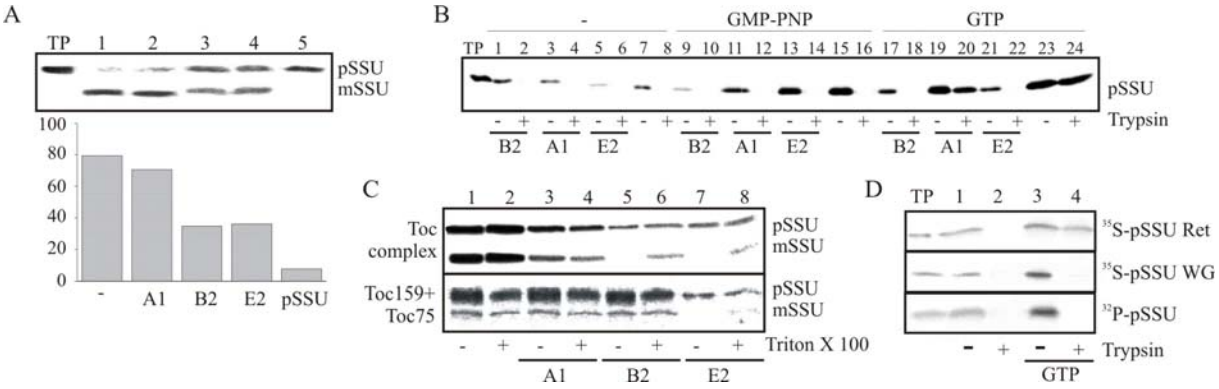

Figure 6. Import of pSSU is reduced by the N-terminal and phosphorylated C-terminal part of pSSU. **(A)** Isolated chloroplasts were incubated with radio-labelled pSSU translated in reticulocyte lysate for 10min at 20°C in the absence (lane 1) or presence of 5 µM peptides of pSSU presequence (lane 2-4) or pSSU (lane 5). Samples were separated via SDS-PAGE and visualised by phosphor-imager. The import rate into isolated chloroplasts is given in percentage of input. **(B)** Proteoliposomes with reconstituted Toc complex were incubated with radio-labelled in reticulocyte lysate translated pSSU in the absence (lane 1-8) or presence of GMP-PNP (lane 9-16) or GTP (lane 17-24). The binding and import reaction was performed in the absence (lane 7-8, 15-16, 23-24) or presence of peptides of pSSU presequence (lane 1-6, 9-14, 17-22). Surface bound preproteins were digested by trypsin treatment (even lanes). **(C)** Import of radio-labelled pSSU translated in reticulocyte lysate into proteoliposomes with enclosed stromal fraction containing either reconstituted Toc complex (upper panel) or co-reconstituted Toc159 and Toc75 (lower panel) was performed in the absence (lane 1-2) or presence of peptides of the pSSU presequence (lane 3-8). **(D)** Toc complex proteoliposomes were incubated with *in vitro* translated pSSU using reticulocyte lysate (^{35}S-pSSU-Ret, up) or wheat germ lysate (^{35}S-pSSU-WG, middle) or with *in vitro* phosphorylated pSSU (^{32}P-pSSU, down), in the absence (lane 1, 2) or presence of GTP (lane 3, 4). Surface bound preproteins were digested by trypsin treatment (lane 2, 4).

In a parallel approach proteoliposomes were loaded with stromal extract containing the precursor processing peptidase (Schleiff et al., 2003b). In this system a processing event of pSSU to mSSU (mature form of pSSU) indicates a successful import reaction (Fig. 6C, lane 1). To reveal the specificity of the peptides the assays were conducted with proteoliposomes containing the Toc complex (Toc159, Toc75 and Toc34; upper panel) or Toc159 and Toc75 as a minimal import competent unit (lower panel, Schleiff et al., 2003b). The proteoliposomes were now incubated with A1, B2 or E2 before adding the full-length precursor. The B2 and E2 peptides are able to inhibit translocation of pSSU in proteoliposomes containing the Toc complex (Fig. 6C, lane 5 and 7, upper panel), whereas only E2 prevents pSSU import into proteoliposomes containing Toc159 and Toc75

(Fig. 6C, lane 7-8, lower panel). This result supports the observation that B2 is strongly bound by Toc34 but not by Toc159, whereas E2 is only recognised by Toc159 with high affinity (Fig. 5C and 5D). In contrast, import of pSSU into proteoliposomes with the reconstituted Toc complex is only slightly reduced by the presence of A1 (Fig. 6C, lane 4, upper part). This can be explained by the low affinity binding of A1 to Toc34 (Schleiff et al., 2002). Preincubation with A1 does not affect import into proteoliposomes with reconstituted Toc159 and Toc75. This result indicates a transient interaction of Toc159 to A1 since it is capable to stimulate GTP-hydrolysis of Toc159 but it does not block the import event. To reveal the activity of the signal peptidase in all cases membranes were solubilised with the detergent Triton X-100 to release the signal peptidase, which results in processing of pSSU (Fig. 6C, lane 2, 4, 6 and 8).

It was shown that Toc34 recognises the C-terminal phosphorylated peptide, where Toc159 interacts with the non-phosphorylated C-terminal part. This indicates that dephosphorylation before translocation across the envelope membranes occurs already at the Toc complex. To support this conclusion, pSSU was translated either in reticulocyte lysate (resulting in no phosphorylation) or in wheat germ lysate (resulting in phosphorylation (Waegemann and Soll, 1996)). In addition purified pSSU was phosphorylated *in vitro*. All three different kinds of pSSU were used for import assays into proteoliposomes with reconstituted Toc complex (Fig. 6D). In line with previous results translocation of pSSU that was translated in reticulocyte lysate was only achieved in the presence of GTP (Fig. 6D, lane 4, Schleiff et al., 2003b) as judged by the protease resistant form. When *in vitro* phosphorylated or in wheat germ lysate translated pSSU was used (Fig. 6D, middle, lower panel) no protease resistant pSSU could be observed. The results indicate that phosphorylated pSSU cannot be translocated across the membrane.

6.1.3. Guanine nucleotides affect the stability of the Toc core complex

The receptor function of both Toc159 and Toc34 is GTP regulated (Fig. 4B and 5D). For understanding the preprotein transfer between the two receptors one central question still remained: how is the association of the two receptor proteins regulated? Since both proteins are GTPases it is intriguing to ask whether GTP or GDP affect the stability of the Toc complex. Therefore, solubilised outer envelope vesicles were incubated with GDP or GMP-PNP. Antisera against Toc34 or Toc159 were used for immunoprecipitation (Fig. 7A). All three Toc core components were co-immunoprecipitated with the same efficiency in the absence of nucleotides or in the presence of GMP-PNP by either Toc34 (Fig. 7A, lane 1 and 3) or Toc159 antiserum (Fig. 7A, lane 5 and 7). In contrast, incubation with GDP leads to a less efficient co-immunoprecipitation of Toc159 by Toc34 antiserum and vice versa (Fig. 7A, lane 2 and 6). Interestingly, the amount of co-immunoprecipitated Toc75 by Toc34 antiserum in the presence of GDP is reduced as well (Fig. 7A, lane 2), whereas Toc159 antiserum co-immunoprecipitates Toc75 in a similar manner regardless of added nucleotides (Fig. 7A, lane 5-7). This observation indicates a nucleotide independent association of Toc159 and Toc75. The

specificity of the reaction was confirmed by using preimmunserum (Fig. 7A, lane 4 and 8) and by investigating the presence of Oep24 (outer envelope protein of 24 kDa) as a control protein. Only a minor amount of Oep24 was co-immunoprecipitated by Toc34 antiserum (Fig. 7A, lane 1-3). Furthermore, Toc75, Toc159 and Toc34 were not co-immunoprecipitated by Oep24 antibodies regardless of the added nucleotides (Fig 7B. lane 3-5). Therefore, the precipitation of Oep24 by Toc34 antiserum was considered as an unspecific reaction.

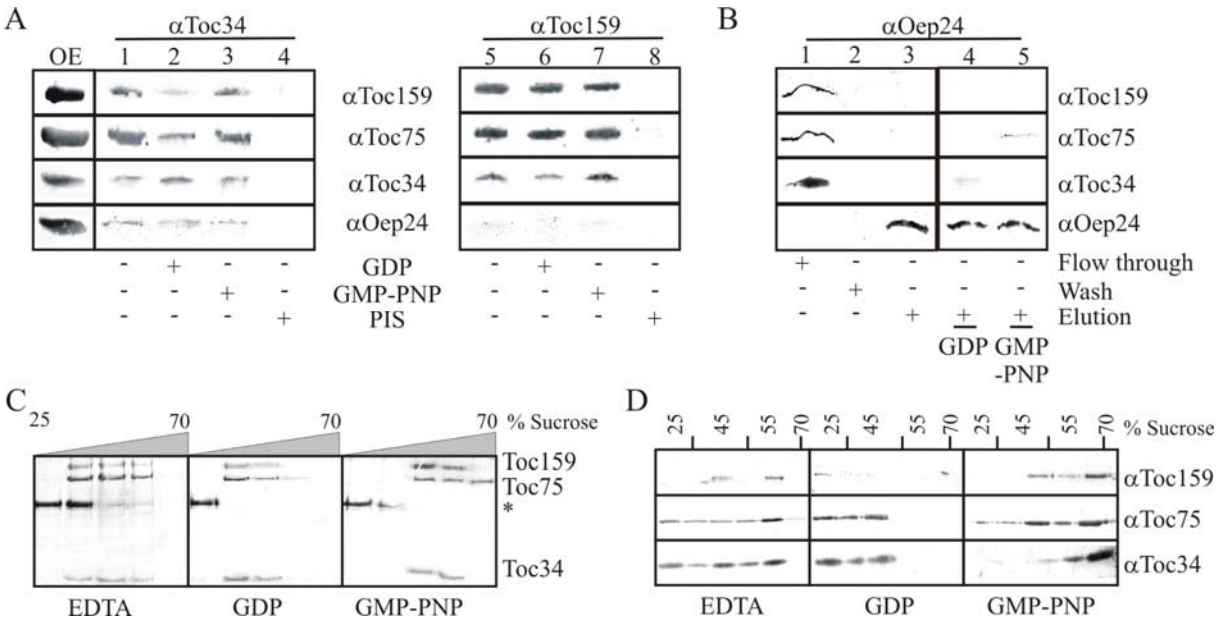

Figure 7. The stability of the Toc complex is guanine nucleotide dependent. **(A)** Co-immunoprecipitation of purified outer envelope vesicles (150 µg protein) with antisera against Toc34 (A, left), Toc159 (A, right), preimmunsera (A, lane 4, 8) or Oep24 **(B)** were performed in the absence (A, lane 1, 4, 5, 8; B, lane 1-3) and presence (A, lane 2-3, 6-7; B, lane 4, 5) of guanine nucleotides. In B, the flow through (lane 1) and wash (lane 2) of the immunoprecipitation using Oep24 antibodies is shown. The bound proteins were identified by immunoblotting. **(C)** Outer envelope vesicles (150 µg protein) were incubated with EDTA (left panel), GDP (middle panel) or GMP-PNP (right panel), solubilised with 1.5% n-decylmaltoside as well as with lipase treatment and separated by linear sucrose gradient centrifugation. Fractions of the gradient were collected and the protein content was analysed by silver staining. The identified Toc components and the lipase (*) are marked. **(D)** The Toc complex from a linear gradient in the absence of nucleotides was incubated with EDTA (left panel), GDP (middle panel) or GMP-PNP (right panel) and subjected to a sucrose step gradient centrifugation. The protein content of the fractions was analysed by immunoblotting with the indicated antisera.

To confirm this observation, the stability of the Toc complex was analysed during isolation by sucrose gradient centrifugation. Outer envelope vesicles were incubated with EDTA, GDP or GMP-PNP before solubilisation and subjected to linear sucrose gradient centrifugation. In the presence of GMP-PNP, the Toc components are enriched in higher density fractions as when no nucleotides were added (Fig. 7C, right and left panel). In the presence of GDP, Toc components migrate only to lower density fractions (Fig. 7C, middle panel). In the absence of nucleotides Toc complex containing fractions (Fig. 7C, left panel) were subsequently pooled and incubated in the presence and absence of GMP-PNP or GDP. These samples were subjected to a sucrose step gradient centrifugation. In the presence of GMP-

PNP the amount of Toc components, especially Toc34, is enriched in fractions of higher density in comparison to the amount in the absence of nucleotides (Fig. 7D, left and middle panel). In the presence of GDP no Toc components were found in fractions with high sucrose concentrations (Fig. 7D, middle panel). The results from linear and step gradient analysis of the Toc complex suggest that GMP-PNP leads to a stabilisation of the Toc complex, whereas GDP weakens the interaction between the Toc components (Fig. 7C and 7D).

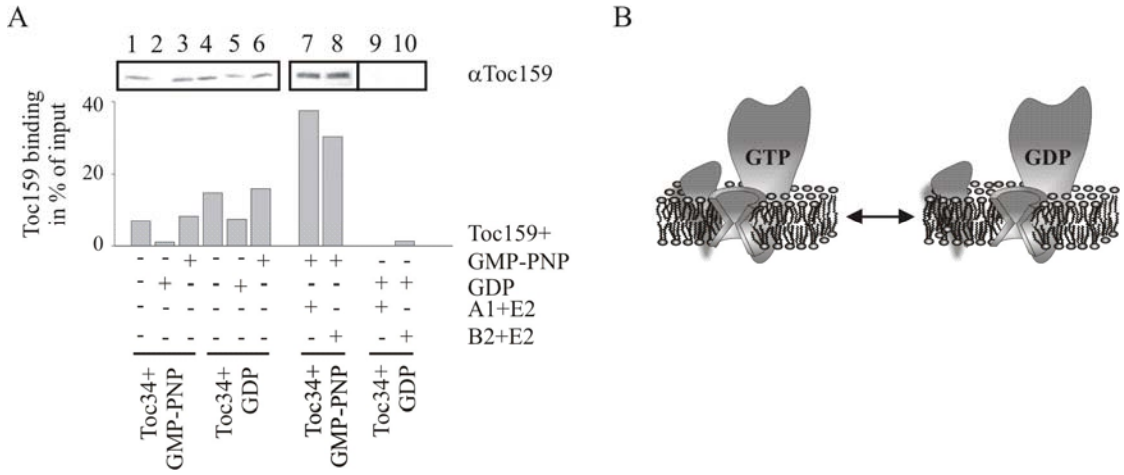

Figure 8. Toc159 dissociates from Toc34 upon GDP loading. **(A)** 2 µg Toc34 was preincubated with GMP-PNP (lane 1-3, 7, 8) or GDP (lane 4-6, 9, 10) followed by incubation with 250 ng $Toc159_f$ (lane 1, 4) preincubated with GMP-PNP (lane 3, 6-8) or GDP (lane 4-6, 9, 10) and pSSU presequence peptides (lane 7-10). The amount of bound Toc159 is depicted as percentage of total input. **(B)** A model of GTP-dependent association of Toc34 to Toc159/Toc75 is presented.

To further underline the role of the two GTPases in Toc stability the interaction between Toc159 and Toc34 was analysed *in vitro*. Gel eluted Toc159 (Fig. 4A) was used, which comprises G- and M-domain. It is assumed that the A-domain might not affect this interaction drastically since truncated Toc159 lacking the A-domain is capable to promote protein translocation in protoplasts (Lee *et al.*, 2003). His-tagged Toc34ΔTM (Toc34 lacking the C-terminal transmembrane region) was bound to Ni-NTA column and incubated with GDP (Fig. 8A, lane 4-6, 9 and 10) or GMP-PNP (Fig. 8A, lane 1-3, 7 and 8). The Toc34ΔTM affinity matrix was incubated with Toc159 (Fig. 8A, lane 1 and 4) preloaded with GMP-PNP (Fig. 8A, lane 3 and 6-8) or GDP (Fig. 8A, lane 2, 5, 9 and 10). This approach allows the analysis of a different nucleotide loading stage of both GTPases. The amount of bound Toc159 was determined. The binding of Toc159 to $Toc34\Delta TM_{GDP}$ (Fig. 8A, lane 4-6) was slightly increased in comparison to $Toc34\Delta TM_{GMP\text{-}PNP}$ (Fig. 8A, lane 1-3). $Toc159_{GMP\text{-}PNP}$ binds to Toc34ΔTM to the same extend as nucleotide free Toc159 (Fig. 8A, lane 1, 3, 4 and 6). In contrast, $Toc159_{GDP}$ shows reduced binding to Toc34ΔTM independent of its nucleotide loaded stage compared to $Toc159_{GMP\text{-}PNP}$ (Fig. 8A, lane 2, 5). Since $Toc34\Delta TM_{GMP\text{-}PNP}$ recognises pSSU with higher affinity compared to the nucleotide free receptor (Schleiff et al., 2002), the effect of the pSSU transit peptide on the interaction between Toc159 and Toc34 was investigated. Both receptors were incubated with peptides covering parts of pSSU presequence (Fig. 5A) in the presence of GMP-PNP. The N-terminal

peptide (E2) was combined either with peptides covering the non-phosphorylated (A1) or phosphorylated C-terminal part (B2). Both peptide mixtures showed a strong stimulation of the interaction between the Toc GTPases in GMP-PNP stage in comparison to peptide free conditions (Fig. 8A, lane 3, 7 and 8). In contrast, the interaction between both receptors loaded with GDP was drastically reduced (Fig. 8A, lane 5, 9 and 10). Taken together, the Toc core complex is stabilised by GMP-PNP. This stabilising effect is due to a pronounced association of $Toc159_{GMP\text{-}PNP}$ to Toc34ΔTM. In contrast, $Toc159_{GDP}$ favours Toc34ΔTM dissociation (Fig. 8B). Binding of the transit peptide further strengthened the interaction between the two GTPases in the GMP-PNP loaded stage of both receptors (Fig. 8B).

6.2. The dynamically associated preprotein receptor Toc64

6.2.1. Toc64 acts as preprotein receptor

After characterising preprotein recognition by Toc34 and Toc159, the two receptors of the Toc core complex, the function of Toc64 was addressed. Toc64 was not isolated as a part of the Toc core complex (Schleiff et al., 2003a), but its function in preprotein translocation was proposed (Sohrt and Soll, 2000). It is still an open issue whether Toc64 acts as a preprotein receptor.

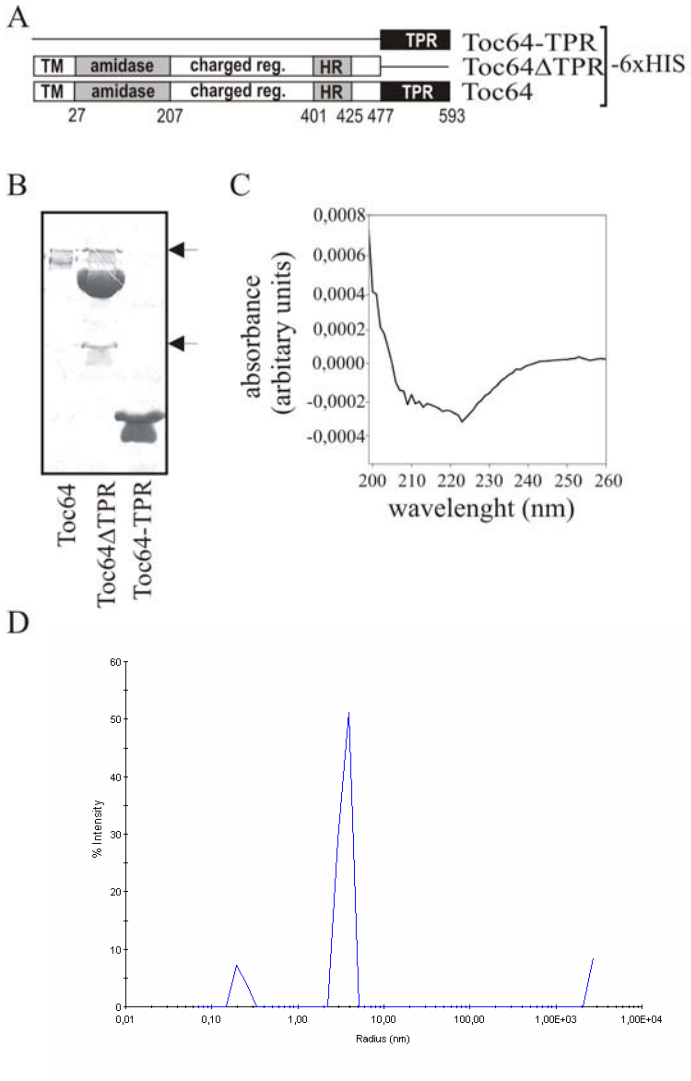

Figure 9. Toc64 constructs are soluble expressed **(A)** A schematic picture of the expressed Toc64 constructs. Toc64ΔTPR covers the transmembrane region (TM), the amidase, charged region and the hydrophobic region (HR). Full length Toc64 further contains a C-terminal TPR domain, which was exclusively covered by third construct (Toc64-TPR). **(B)** Toc64 (lane 1), Toc64ΔTPR (lane 2) and Toc64-TPR (lane 3) were expressed and purified facilitating their C-terminal His-tag. The purity was controlled by SDS-PAGE and subsequent Coomassie Blue staining. Arrows mark unspecific contaminants from *E. coli* **(C)** 100 nm Toc64 was subjected CD-spectroscopy. The average of ten measurements is shown. **(D)** 1mg/ml Toc64 was subjected dynamic light scattering analysis. Depicted is one peak with high intensity at 3.5 nm radius representing Toc64 monomer. Two further peaks are due to the used buffer. The data were kindly provided by Dr. I. Tews.

To test this idea Toc64 and the two truncated constructs Toc64ΔTPR and Toc64-TPR motif were soluble expressed and purified via their C-terminal His-tag (Fig. 9A, B). The identity of the expressed protein was confirmed by mass spectroscopy (Tews, personal communication). Surprisingly, the membranous full length Toc64 embedded protein (Sohrt and Soll, 2000) was soluble expressed. To exclude any artificial structures the secondary structure content was analysed by CD-spectroscopy (Fig. 9C). Thereby, α-helical and β-sheets content was observed. Furthermore, light scattering measurements showed that the expressed Toc64 forms a monomer in solution as judged by the presence of a single peak with calculated mass of 65kDa (Fig. 9D). The results were confirmed by size exclusion analysis (Tews, personal communication). The two peaks with low intensity are due to the buffer and not to protein content (Fig. 9D). Therefore, the expressed Toc64 does not resemble a randomly arranged structural composition and was used for *in vitro* analysis.

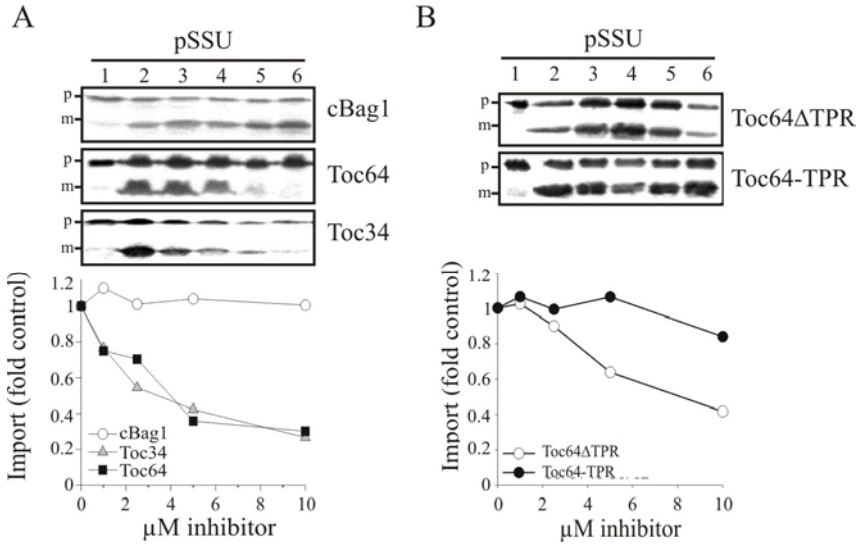

Figure 10. Toc64 acts as preprotein receptor. **(A, B)** Radio-labelled pSSU was imported into isolated chloroplasts in the absence (lane 2) or presence of different expressed proteins (lane 2-7). Increasing amounts of Toc64, Toc34 and cBag1 (A) as well as Toc64-TPR and Toc64ΔTPR (B) were used for import inhibition. As control the import on ice is depicted (lane 1).

In order to determine a receptor function increasing amounts of Toc64 were added to an import assay of radio-labelled pSSU into isolated chloroplasts. A Toc64 concentration dependent inhibition of preprotein translocation was demonstrated (Fig. 10A, middle panel). A similar inhibitory effect was observed by addition of Toc34 (Fig. 10A, lower panel), which was previously established as a preprotein receptor (Sveshnikova et al., 2000). The inhibition is not due to the presence of a His-tag since an increment of the unrelated His-tagged protein cBag1 (C-terminus of Bcl-2-associated athanogene (Bag) domain containing protein 1) does not reduce pSSU import (Fig. 10A, upper panel). Furthermore, the import of pOE33 (precursor of the 33kDa subunit of the oxygen evolving complex) was inhibited by Toc64 addition to a similar extent (Qbadou, personal communication). Therefore, Toc64 is acting as a receptor for both tested preproteins. Toc64 exposes a TPR-domain into the

cytosol, which is a docking site for cytosolic chaperones like Hsp70 or Hsp90, which affiliates preproteins such as pOE33 (Qbadou, personal communication). In addition, the receptor contains a large domain facing the intermembrane space of the plastid envelope membrane (Qbadou, personal communication). To dissect whether only the TPR-domain or also other regions of Toc64 are involved in preprotein recognition, the inhibitory effect of two truncated constructs, Toc64-TPR and Toc64ΔTPR (Fig. 9A), on pSSU import into isolated chloroplasts were analysed (Fig. 10B). Toc64-TPR reduces the pSSU import slightly (Fig. 10B, lower panel), whereas Toc64ΔTPR inhibits pSSU translocation stronger than the TPR-domain (Fig. 10B, upper panel). This observation indicates the presence of a second region beside the TPR-domain of Toc64 that is involved in preprotein recognition. Furthermore, recent studies showed that the second preprotein recognition site of Toc64 is exposed to the intermembrane space (Qbadou, personal communication). In conclusion, Toc64 is a preprotein receptor with two different binding sites for preproteins on either site of the outer envelope membrane.

6.2.2. Toc64 is dynamically associated with the Toc core translocon

By chemical cross-linking Toc64 was found in association with Toc core complex (Sohrt and Soll, 2000). However, Toc64 was not co-purified with the Toc core components in a linear sucrose gradient. Hence, a dynamic association of Toc64 with the Toc core translocon was suggested (Schleiff et al., 2003a).

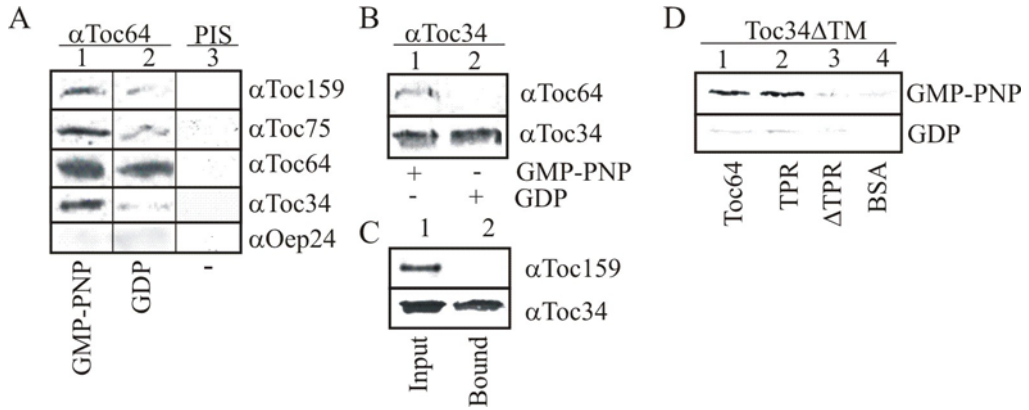

Figure 11. Toc64 is dynamically associated with the Toc core translocon **(A, B)** Outer envelope vesicles were solubilised and incubated with Toc64 (A), Toc34 (B) antiserum or preimmunserum (A, lane 3). The co-immunoprecipitation was performed in the presence of either GMP-PNP (lane 1) or GDP (lane 2). The precipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunodecorated with the indicated antisera. **(C)** 200 ng gel eluted Toc159 (upper panel) or recombinantly expressed Toc34ΔTM (lower panel) were incubated with Toc64 affinity matrix. 10% of the input is shown (lane 1). Bound Toc159 or Toc34ΔTM were eluted and analysed by SDS-PAGE with subsequent immunodecoration. **(D)** 1 µg Toc34ΔTM was incubated in presence of GMP-PNP (upper panel) or GDP (lower panel) with Toc64 (lane 1), Toc64–TPR (lane 2), Toc64ΔTPR (lane 3) or BSA (lane 4) Toyopearl affinity matrices. The bound Toc34ΔTM was detected as under (C).

To test this hypothesis solubilised outer envelope vesicles were used for a co-immunoprecipitation approach with Toc64 antiserum in presence of either GMP-PNP or GDP (Fig. 11A). Under both conditions the efficiency of Toc64 immunoprecipitation was comparable (Fig. 11A, lane 1 and 2). However, the amount of Toc34 and Toc159 co-immunoprecipitated by Toc64 was reduced in the presence of GDP in comparison to GMP-PNP (Fig. 11A, lane 1 and 2). Oep24 was not found in the elution samples (Fig. 11A, lane 1 and 2). Moreover, preimmunserum did not precipitate either protein that was detected here (Fig. 11A, lane 3). Thus, both controls underline the specificity of the reaction. Using Toc34 antiserum for co-immunoprecipiation provides a similar observation (Fig. 11B). Under both conditions the Toc34 immunoprecipitation was comparable, whereas less Toc64 was co-immunoprecipitated in the presence of GDP compared to GMP-PNP (Fig. 11B, lane 1 and 2). Since the association of Toc64 with the Toc core complex is favoured upon GMP-PNP addition its interaction to the two GTP regulated receptors was examined *in vitro*. Therefore, isolated Toc34 or Toc159 were incubated with a Toc64 affinity matrix and the amount of bound proteins were analysed (Fig. 11C). Thereby, Toc34 but not Toc159 was bound to Toc64 affinity matrix (Fig. 11C, lane 2). To determine the site of Toc64 recognised by Toc34 the binding of Toc34ΔTM to Toc64, Toc64-TPR and Toc64ΔTPR affinity matrices was investigated (Fig. 11D). It was shown that the association of Toc34 to Toc64 (Fig. 11D, lane 1) is mediated by the cytosolic exposed TPR domain of Toc64 (Fig. 11D, lane 2) and not by Toc64ΔTPR (Fig. 11D, lane 3). No Toc34ΔTM binding to a BSA coated control column was observed (Fig. 11D, lane 4). Moreover, the interaction of Toc34ΔTM to Toc64 (Fig. 11D, lane 1) and to Toc64-TPR (Fig. 11D, lane 2) is more intensive to $Toc34\Delta TM_{GMP\text{-}PNP}$ (Fig. 11D, upper panel) than to $Toc34\Delta TM_{GDP}$ (Fig. 11D, lower panel). The specificity of the assay was confirmed by the absence of Toc34 association with the BSA coated column (Fig. 11D, lane 4). In conclusion, $Toc34_{GMP\text{-}PNP}$ is the docking site for the dynamically associated Toc64 in the Toc core complex. This interaction is mediated by the cytosolic exposed TPR domain of Toc64.

6.2.3. Preprotein transfer from Toc64 to Toc34

In the previous sections it was shown that Toc64 acts as preprotein receptor and interacts with Toc34, which is the initial receptor of the Toc core translocon. In a parallel study it could be demonstrated that the TPR domain of Toc64 is a docking site for cytosolic chaperones like Hsp90, which affiliate a subset of preproteins (Qbadou, personal communication). Two open questions remain: First, is the preprotein bound to Toc64-TPR transferred to Toc34? Second, how does the preprotein or chaperone binding influence the interaction between both receptors? To mimic an Hsp90 loading of Toc64 the recombinantly expressed TPR domain (Fig. 12A, lane 2) was bound to a C90 peptide column (Fig. 12A, lane 4). C90 comprises the C-terminal 23 residues of human Hsp90 and was shown to bind specifically to Toc64-TPR (Qbadou, personal communication). Expressed Toc34ΔTM (Fig. 12A, lane 1) does not bind to the column material (Fig. 12A, lane 5). However, in the presence of Toc64-TPR a binding of Toc34ΔTM to the column material was detected (Fig. 12A, lane 3). This result indicates

that Toc64-TPR can bind simultaneously Toc34ΔTM and C90. In order to test whether this eluted preproteins can be recognised by Toc34 an *in vitro* approach was utilised (Fig. 12B). Wheat germ translated pOE33 was allowed to couple to a Toc64-TPR affinity matrix. POE33 that is not bound to Toc64-TPR was efficiently recognised by Toc34ΔTM$_{GMP-PNP}$ (Fig. 12B, lane 4). Toc64-TPR with bound pOE33 was incubated with Toc34ΔTM$_{GMP-PNP}$ in the absence (Fig. 12B, lane 6) or presence of ATP (Fig. 12B, lane 5). It was shown in a parallel study that Hsp90 guided preproteins that are bound to Toc64-TPR can be eluted by ATP (Qbadou, personal communication). Thus, in the presence of ATP five fold higher amounts of pOE33 were eluted from Toc64-TPR and were bound by Toc34ΔTM$_{GMP-PNP}$ (Fig. 12B, upper graph). However, the fraction of the eluted pOE33 recognised by Toc34ΔTM$_{GMP-PNP}$ is in both cases comparable (Fig. 12B, lower graph). Therefore, pOE33 that is eluted from Toc64-TPR can be recognised by Toc34.

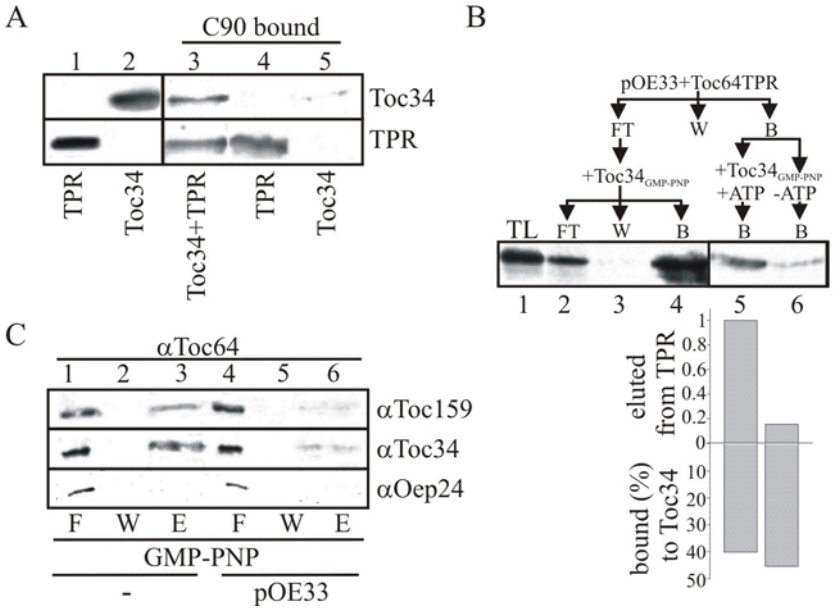

Figure 12: Preprotein transfer between Toc64 and Toc34. **(A)** Expressed and purified Toc64-TPR (lane 1) and Toc34ΔTM (lane 2) were incubated either together (lane 3) or separately (lane 4, 5) with C90 peptide coated thiol-propyl Sepharose in equimolar amounts. After sufficient washing bound proteins were eluted, separated by SDS-PAGE and silver-stained. **(B)** In wheat germ translated pOE33 (lane 1) was incubated with a Toc64-TPR coated Toyopearl matrix. Non bound pOE33 was subsequently incubated with a Toc34ΔTM affinity matrix (lane 2-4). 5% of the flow through and wash fraction and 100% of the bound protein is shown. Toc64-TPR columns with bound pOE33 were incubated with 7.5 µg Toc34ΔTM, which was preincubated with GMP-PNP, in the presence (lane 5) or absence of ATP (lane 6). The eluted pOE33 was quantified (upper graph). After separation from the Toc64-TPR affinity matrix Toc34ΔTM was purified facilitating its C-terminal Hi-tag. Bound pOE33 was eluted and quantified (lower graph). A scheme of the reaction flow is depicted. **(C)** Outer envelope vesicles (150 µg protein content) were solubilised by 1.5% n-decyl-maltoside and 500 µM GMP-PNP in the absence (lane 1-3) or presence of 5 µM expressed and purified pOE33 (lane 4-6). Subsequently, a co-immunoprecipitation with Toc64 antiserum was performed. 5% of flow through (lane 1, 4), wash (lane 2, 5) and 100% of the bound fraction was separated by SDS-PAGE and analysed by immunodecoration with the indicated antisera.

Finally, the impact of preproteins loading of Toc34 on the interaction between Toc34 and Toc64 has to be considered. To address this issue co-immunoprecipitation with Toc64 antiserum in the presence of GMP-PNP was performed before (Fig. 12C, lane 1-3) or after incubation of outer envelope vesicles

with recombinant expressed pOE33 (Fig. 12C, lane 4-6). It was shown that recombinantly expressed pOE33 does not bind to Toc64-TPR, but to Toc34 (Qbadou, personal communication). Therefore, pOE33 incubation leads to a preprotein loading of the Toc core complex receptors like Toc34, but not of Toc64. The binding of the preprotein to the receptors was performed at 2°C and without hydrolysable nucleotides to prevent further translocation. Interestingly, the presence of pOE33 destabilises the association of Toc64 to the Toc core complex (Fig. 12C, lane 3 and 6). The reduced association of Toc64 is probably due to the preprotein loading of Toc34. Thus, the interaction of Toc64 with Toc34 is different than the interaction of Toc159 and Toc34, which is enhanced upon preprotein recognition in the presence of GMP-PNP (Fig. 8A). In conclusion, these results suggest a preprotein loaded Toc64 interacts with $Toc34_{GMP\text{-}PNP}$. Upon ATP induced release from Toc64-TPR the preprotein is transferred to $Toc34_{GMP\text{-}PNP}$, which in turn does not associate with Toc64 in the preprotein loaded state, when it is in a preprotein loaded stage.

6.2.4. Toc64 forms a complex with Tic22, isHsp70 and Toc12 in the presence of ATP

Since Toc64 is not a stable associated component of the Toc core translocon it was tested whether the receptor can be co-purified with Toc components under specific conditions in a linear sucrose gradient. Nucleotides affect the association of Toc64 to the Toc core machinery (Fig. 11). Thus, the complex purification was performed in the absence (Fig. 13, left panel) or presence of ATP (Fig. 13, right panel).

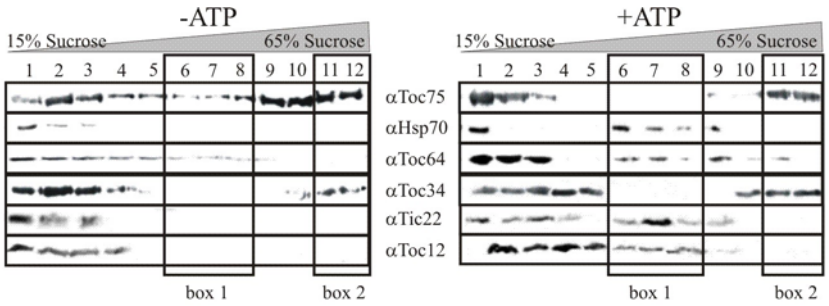

Figure 13. Toc64 is associated with isHsp70, Tic22 and the novel Toc12. Outer envelope vesicles (150 µg protein) were solubilised with 1.5% decylmaltoside and subjected to sucrose density centrifugation in the absence (left panel) or in the presence (right panel) of 1 mM ATP. Outer membrane proteins 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 box1 and box2, respectively.

In line with earlier observations, in both cases Toc64 was present in fractions of lower sucrose concentration compared to the Toc core components Toc75 and Toc34 (Fig. 13, box 1 versus box 2). Therefore, Toc64 was not co-purified with the Toc core complex under the tested condition. However, in the presence of ATP Toc64 was detected in sucrose fractions containing the intermembrane space localised isHsp70, Tic22, and a 12kDa protein (Fig. 13, right panel, box 1). The latter protein was identified as a Toc component (see below) and was termed Toc12. In the absence of ATP Toc64 does not co-migrate with isHsp70, Tic22 and Toc12 (Fig. 13, left panel, box 1). This observation suggests

that the association of these proteins is ATP dependent, which might be due to the ATP dependent association of Hsp70 proteins and DnaJ proteins. In contrast ATP does not affect the formation of the Toc core complex as judged by the appearance of Toc34 and Toc75 (Fig. 13, 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 non-solubilised outer envelope vesicles (Fig. 13, left and right panel, fractions 1-3). In conclusion, Toc64 could not be co-purified with Toc core components in a linear sucrose gradient, but upon ATP addition it pellets together with Tic22, isHsp70 and the novel Toc12 in sucrose fractions of lower density than Toc34/Toc75 containing fractions.

6.3. Toc12, a novel component of the Toc translocon

6.3.1. Identification of a novel component of the outer envelope of chloroplasts of *P. sativum*

The observation that Toc64, isHsp70 and Tic22 co-purify with Toc12 in a linear sucrose gradient (Fig. 13), leads to the question about the molecular and functional features of the novel Toc12. A peptide of Toc12 was found while analysing the proteome of the outer envelope of chloroplasts (Fig. 14A, framed sequence). Screening of a cDNA library generated from *P. sativum* with degenerative primers revealed a cDNA clone encoding for a protein of 12kDa (acc. No. AY357129, Fig. 14A; Caliebe 1998), which was subsequently termed Toc12 based on the results presented below. An in frame stop codon in front of the initiation atg was identified. The 5´ end was further confirmed by 5´RACE- PCR, which does not reveal any coding sequence for an additional start methionine in front of the coding region (data not shown). Another stop codon at the 3´ end of the coding region of the cDNA was found. Therefore, the complete coding region of Toc12 was elucidated.

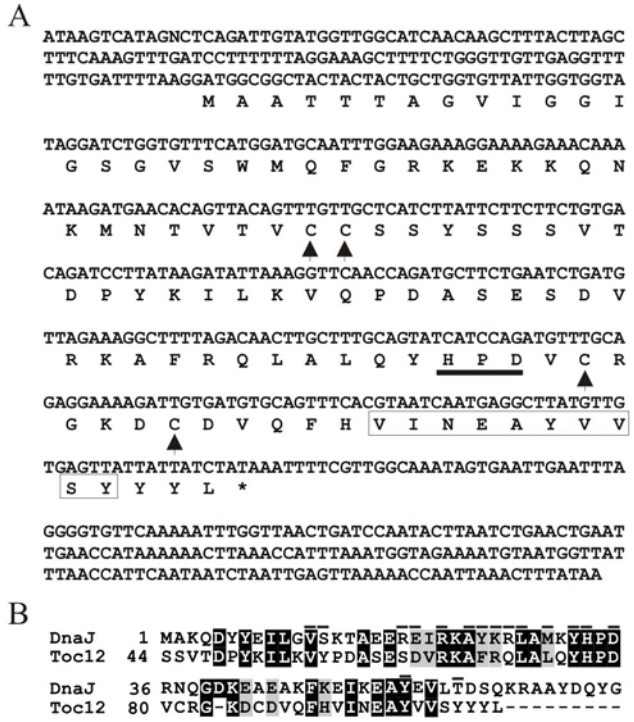

Figure 14. A novel DnaJ homologue in the outer envelope of chloroplasts of *P. sativum.* **(A)** The identified cDNA (Acc. number AY357119) and amino acid sequence is shown. The peptide identified by amino acid sequencing is framed. Arrowheads point to the four cysteines. The HPD motive is underlined. **(B)** An alignment of the amino acid sequence of the C-terminal portion of the identified Toc12 to the J domain of the *E.coli* DnaJ (Acc. number P08622) using clustal w is shown. Essential amino acids for the function of the J domain are marked on top.

The investigated protein does not contain any predicted targeting signal as most of the outer envelope membrane proteins (Schleiff and Klösgen, 2001). Sequence analysis revealed the presence of a for J-proteins characteristic HPD motif in the C-terminal portion of the Toc12 protein (Fig. 14A, underlined). This C-terminal portion shows a homology of 55 % to the J domain of *E.coli* DnaJ (Fig. 14B). Many amino acids essential for the interaction between DnaJ and DnaK (Greene et al. 1998) are conserved in the newly identified protein (Fig. 14B, marks). Antibodies raised against Toc12 recognise a single protein in purified outer envelope vesicles of chloroplasts (Fig 15A). For subcellular localisation a cell fraction by differential centrifugation was performed. A cytosolic fraction (Fig. 15B, lane 1), a cellular membrane pellet comprising peroxisomal, endoplasmatic and plasma membranes (Fig. 15B, lane 2) as well as purified mitochondria (Fig. 15B, lane 3) and chloroplasts (Fig. 15B, lane 4) were tested for the presence of Toc12. The protein was solely detected in chloroplasts (Fig. 15B, lane 4), whereas no protein was detected by Toc12 antiserum in mitochondria, cellular membranes and in the cytosol (Fig. 15B, lane 1-3). The purity of the organelles was tested by chlorophyll content (not shown) and by identification of the outer envelope protein Toc34, the thylakoid protein LHCPII (light harvesting complex protein II) (Fig. 15B, lane 4) or the outer mitochondrial membrane VDAC (voltage dependent anion channel; Fig. 15B, lane 3).

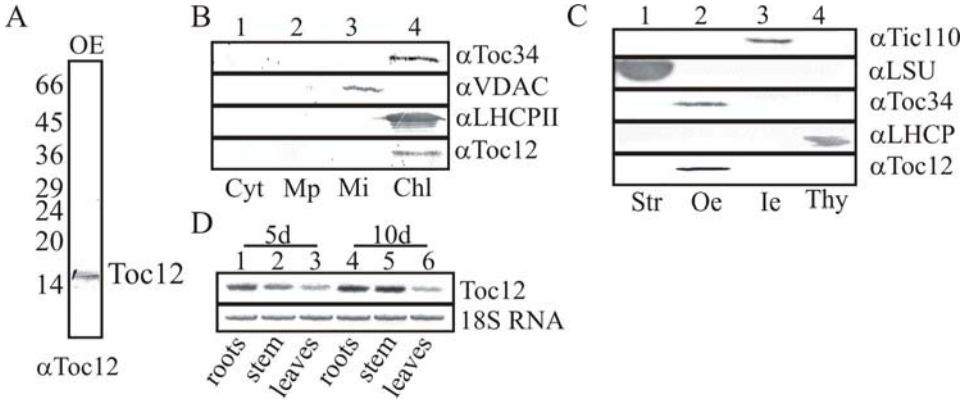

Figure 15. Toc12 is an outer envelope protein of chloroplasts. **(A)** Outer envelope vesicles were separated by SDS-PAGE followed by immunodecoration using Toc12 antisera. **(B)** Leaves from 10-day-old plants (*P. sativum*) were harvested, lysed and fractionated. Cytosol (lane 1), cellular membrane (lane 2), mitochondrial and chloroplast proteins (30 µg each) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with antiserum against Toc34, VDAC, LHCPII and Toc12. **(C)** 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 the indicated antisera. **(D)** The transcript level of Toc12 (Toc12, upper panel) was tested in roots (lane 1, 4), stems (lane 2, 5) and leaves (lane 3, 6) of 5 (lane 1-3) and 10 days (lane 4-6) old plants (*P. sativum*). For control, the amount of 18S RNA was analysed (lower panel).

To determine the localisation of Toc12 within purified chloroplasts fractionation of the cell organelle was performed. Thereby, an exclusive localisation of Toc12 in outer envelope vesicles was demonstrated (Fig. 15C, lane 2). The purity of the fractions was judged by the presence of the inner envelope protein Tic110 (Fig. 15C, lane 3), the large subunit of RubisCO of the stroma (Fig. 15C, lane 1), the outer envelope Toc34 (Fig. 15C, lane 2) or thylakoid protein LHCPII (Fig. 15C, lane 4). Higher plants contain different kind of plastids in their tissues. The expression of the gene in roots, stems and

leaves was investigated by RT-PCR (Fig. 15D). Toc12 mRNA was observed in all tissues tested, but the level of the PCR products suggest a more pronounced expression in roots compared to leaves (Fig. 15D, comp. lane 1, 3, 4 and 6). The presence of Toc12 in root plastids was verified by immunoblotting of isolated envelope vesicles (data not shown). Therefore, Toc12 is localised in the outer envelope of plastids and is not restricted to chloroplasts. To confirm the membrane insertion of Toc12, isolated outer envelope vesicles were extracted using high salt concentrations (Fig. 16A, lane 1 and 2), carbonate (Fig. 16A, lane 3 and 4) or a chaotropic reagent (Fig. 16A, lane 5 and 6). In the 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 (Fig. 16A lane 2, 4 and 6). However, Toc12 like the integral membrane protein Toc75 was only detectable in the membrane fraction (Fig. 16A, lane 2, 4 and 6), even though minor amounts of Toc75 were extractable by carbonate (Fig. 16A, lane 4).

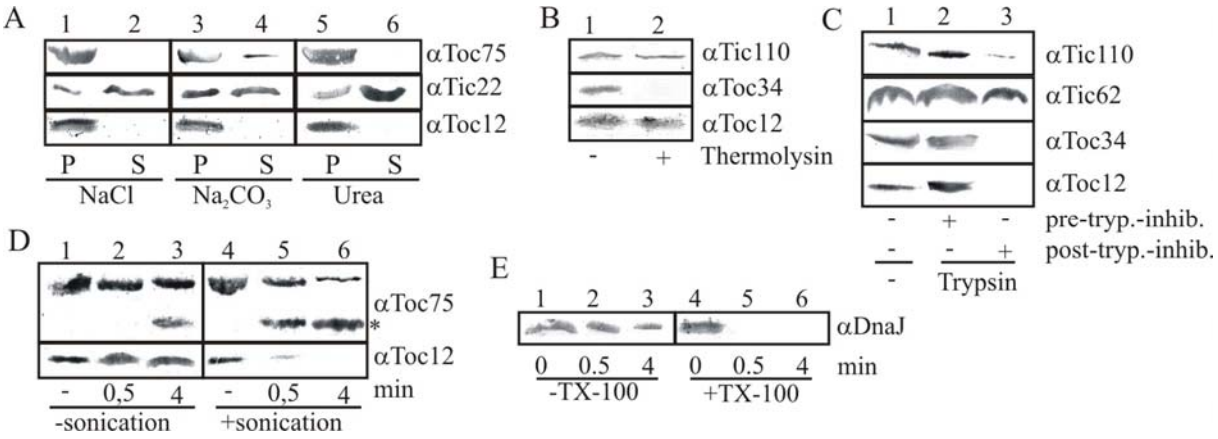

Figure 16. Toc12 is an integral outer membrane protein of chloroplasts that contains a soluble region facing the intermembrane space. **(A)** Isolated outer envelope vesicles were incubated with 1 M NaCl (lane 1, 2), 100 mM Na_2CO_3 pH11.4 (lane 3, 4) or 4 M Urea (lane 5, 6). The pellet (1, 3, 5) and supernatant (lane 2, 4, 6) were separated and subjected to SDS-PAGE followed by immunodecoration using antisera against Toc75 (upper panel), Tic22 (middle panel) and Toc12 (lower panel). **(B, 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, C) or before addition of trypsin inhibitor (lane 2, C). After inhibition of the protease (lane 2, B; lane 3, C) chloroplasts were re-isolated and the proteins were separated by SDS-PAGE followed by immunodecoration with the indicated antisera. **(D)** 30 µg isolated outer envelope vesicles (lane 1, 4) were incubated with 12.5 µg trypsin for 0.5 (lane 2, 5) or 4 min. (lane 3, 6) without (lane 1-3) or with sonication (lane 4-6). After inhibition of the protease, the envelope was subjected to SDS-PAGE, transferred to nitrocellulose and decorated with antibodies against Toc75 (upper panel) or Toc12 (lower 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 from *E. coli*.

After establishing Toc12 as integral outer envelope membrane protein the topology of the protein was addressed. The soluble region seemed to be intermembrane space located since thermolysin treatment of chloroplasts did not result in Toc12 proteolysis (Fig. 16B, lane 2). In contrast the cytosol facing Toc34 was degraded by thermolysin (Fig. 16B, lane 2). In line with this notion, Toc12 becomes degraded by high concentrations of trypsin (Fig 16C, lane 3), which penetrates the outer membrane as monitored by Tic110 digestion (Fig. 16C, lane 3). The degradation of Toc12 and Tic110 is not the result of post-lysis digestion of inner envelope or stromal proteins, since the used amount of trypsin inhibitor effectively blocks the protease (Fig. 16C, lane 2) and Tic62 facing the stroma is not

proteolysed (Fig. 16C, lane 3). In a second approach low concentrations of trypsin were added to right side out outer envelope vesicles (Waegemann et al. 1992; Fig. 16D and Fig. 16E, lane 1-3), which were either solubilised with Triton X-100 (Fig. 16E, lane 4-6) or disrupted by sonication (Fig. 16D, lane 4-6). As before, Toc34 was degraded even without solubilisation (data not shown). In contrast, the intermembrane space side exposed region of Toc75 (Sveshnikova et al., 2000) became accessible only after sonication (Fig. 16D, lane 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 (Fig. 16D, lane 3). Similar to Toc75, Toc12 was stable against proteolysis before (Fig. 16D, lane 2 and 3) but not after (Fig. 16D, lane 5 and 6) lysis of the outer envelope vesicles as determined by immunoblotting using either Toc12 (Fig. 16D, lane 5 and 6) or commercially available DnaJ antibodies (Fig. 16E, lane 5, 6). Similar to Toc75, Toc12 is slightly degraded after 4 min even without solubilisation as detected by DnaJ antibodies (Fig. 16E, lane 3). This observed degradation of the Toc12 J-domain only after solubilisation of the outer envelope vesicles suggests its exposure to the intermembrane space.

6.3.2. Toc12 is targeted to chloroplasts *in vivo*

The analysis of the deduced protein sequence of Toc12 did not reveal a classical cleavable transit peptide (Fig. 14A). In order to demonstrate that the identified protein is able to insert into the outer envelope of chloroplasts, a hybrid between Toc12 and a C-terminal fused GFP (green fluorescence protein) was transformed into *N. tabacum* protoplasts.

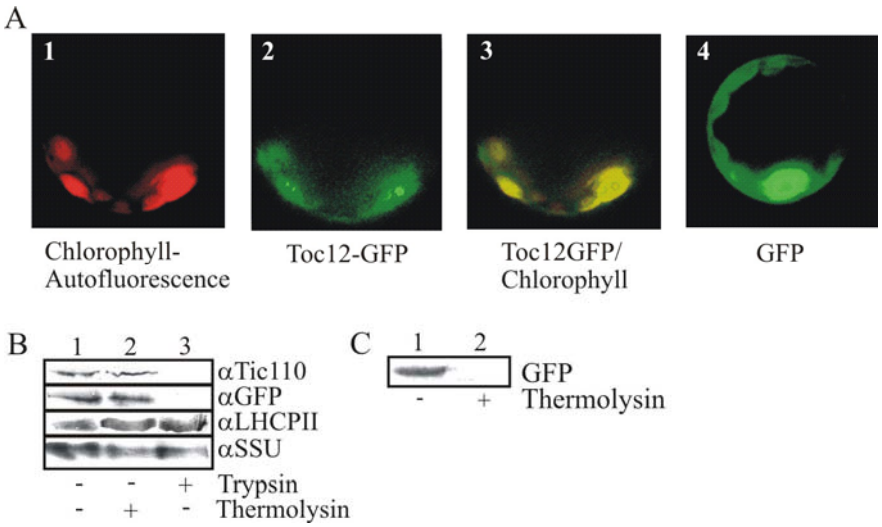

Figure 17. Toc12 is targeted *in vivo* to chloroplasts. **(A)** Protoplasts from *N. tabacum* were transformed with a Toc12-GFP fusion construct. The protoplasts were analysed by fluorescence microscopy. The red auto fluorescence (first panel), the green fluorescence of the Toc12-GFP construct (second panel), a merge of both fluorescence (third panel) and the appearance of GFP expressed alone in protoplasts (fourth panel) are shown. **(B)** Chloroplasts isolated from *N. tabacum* protoplasts expressing the Toc12-GFP fusion (lane 1) were incubated with 100 µg thermolysin (lane 2) or 10 µg trypsin (lane 3) for 5 min at 25°C. After inhibition of the proteases chloroplasts were subjected to SDS-PAGE, transferred to nitrocellulose and immundecorated with the indicated antisera **(C)** *In vitro* translated GFP (lane 1) was incubated with 2 µg thermolysin for 5 min at 25°C (lane 2), subjected to SDS-PAGE and visualised by autoradiography.

The intactness of the protoplasts was controlled by bright field microscopy (data not shown). The plastid localisation was shown by the red auto fluorescence of the chlorophyll (Fig. 17A, first panel). Both, auto fluorescence of the chloroplast and the green fluorescence of the GFP construct (Fig. 17A, second panel) can be merged (Fig. 17A, third panel), which shows a chloroplast localisation of the construct. GFP alone is localised to the cytosol and to the nucleus (Fig. 17A, fourth panel). Furthermore, after protoplast fractionation the construct was detected in the chloroplast fraction using commercially available GFP antibodies (Fig. 17B, lane 1). Therefore, Toc12 was able to target GFP to chloroplasts *in vivo*. To determine the orientation of the fusion-protein the purified *N. tabacum* 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 (Fig. 17B, lane 2). After incubation with thermolysin Toc12-GFP fusion protein was not degraded (Fig. 17B, lane 2). To exclude high protease stability isolated GFP was incubated with thermolysin under similar conditions. The protein was not thermolysin resistant (Fig. 17C, lane 2) suggesting that Toc12-GFP must have crossed the outer membrane completely. In order to analyse whether GFP was localised 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 (Fig. 17B, lane 3). Under these conditions Toc12-GFP became protease sensitive (Fig. 17B, lane 3). In contrast, the stroma protein SSU and the thylakoid protein LHCPII remained protease resistant, which reveals the intactness of the inner envelope membrane (Fig. 17B, lane 3). All results indicate that the GFP domain was exposed to the intermembrane space. Furthermore, the protease accessibility of GFP suggests that the C-terminal region of Toc12 represents the soluble intermembrane space region. In the other case, the Toc12 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 (Fig. 17C). These results are supported by *in vitro* import experiments using radio-labelled Toc12 (data not shown). Here, Toc12 is integrated in the membranes and was resistant to thermolysin, but not to trypsin (data not shown). Therefore, Toc12 integrates into the plastid envelope membranes in the topology as determined before (Fig.16).

6.3.3. Toc12 interacts with the intermembrane space facing isHsp70 of the outer envelope membrane and induces ATPase activity

Topology studies reveal that Toc12 contains a J-domain that is localised in the intermembrane space. As shown for many J-proteins such region regulates the Hsp70 function (Kelley, 1998). Therefore, the ability of Toc12 J-domain to interact with Hsp70 proteins was analysed. A likely candidate for an interaction partner of Toc12 is isHsp70 of the outer envelope membrane (Marshall et al. 1990), which is resistant to treatments with high salt (Fig. 18A, upper panel, lane 1 and 2), carbonate (Fig. 18A. middle panel, lane 1 and 2) or chaotropic reagents (Fig. 18A, lower panel, lane 1 and 2). Topology studies suggest an intermembrane space exposition since isHsp70 becomes only degraded after

prolonged trypsin treatment (Fig. 18B, lane 2 and 3). This notion was supported by a rapid degradation of the protein upon membrane lysis (Fig. 18B, lane 2 and 3) and is in line with earlier findings (Marshall et al., 1990, Soll and Waegemann, 1991, Schnell et al., 1994). In order to demonstrate an interaction of isHsp70 to Toc12, a Toc12 J-domain coated affinity matrix (Fig. 18C, lane 1-3) was incubated with solubilised outer membranes vesicles. Indeed, an interaction with isHsp70 was observed (Fig. 18C, lane 3). The specificity of our antibodies for the intermembrane space Hsp70 was confirmed by the commercial antibody SPA820 (Fig. 18D) that specifically recognises this protein (Schnell et al. 1994).

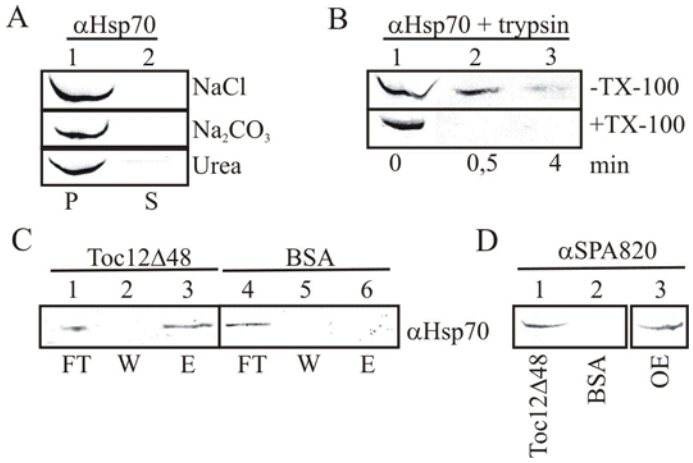

Figure 18. Toc12 interacts with the membrane inserted intermembrane space facing Hsp70 **(A)** Outer envelope vesicles were incubated with 1 M NaCl (up), 100 mM Na_2CO_3 pH11.4 (middle) or 4 M Urea (low). The pellet (lane 1) and the supernatant (lane 2) were separated and subjected to SDS-PAGE followed by immunodecoration using Hsp70-antiserum. **(B)** 30 µg isolated outer envelope vesicles (lane 1) were incubated with 12.5 µg trypsin for 0.5 (lane 2) or 4 min. (lane 3) in the absence (upper panel) or presence (lower 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 (lane 4-6) was coated with Toc12Δ48 fused to a C-terminal hexa-histidine extension (Toc12Δ48, lane 1-3) and incubated with solubilised outer envelope vesicles (75 µg). The flow through (5%, lane 1, 4), wash (5%, lane 2, 5) and eluted fractions (100%, lane 3, 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using Hsp70 antibodies. For controls see Fig. 20C. **(D)** As in C, elution fractions of the Toc12Δ48 (Toc12Δ48, lane 1) or BSA coated column (lane 2) were immunodecorated by SPA820 antibodies. For comparison outer envelope vesicles (7.5 µg) are shown in lane 3.

A function of J-proteins is their stimulation of the ATPase activity of Hsp70 proteins via a direct interaction of the J-domain with the ATPase domain of the chaperone (Bukau and Horwich, 1998). Toc12 J-domain exhibits high primary structure conservation to the J-domain of human Hsp40. Therefore, huHsp40 was used as a template for dynamic molecular simulation analysis (Hritz, personal communication). The modelled structure shows that the C-terminal part of Toc12 has a J-domain fold. Interestingly, the J-domain of Toc12 is stabilised by a disulfide bridge (Fig. 19A, yellow residues). In order to test if the predicted J-domain can stimulate ATP hydrolysis of Hsp70 proteins an *in vitro* ATPase assay was performed. Isolated DnaK from *E.coli* was preincubated with radio-labelled ATP at 4°C. Shifting the reaction to 20°C initiated ATP hydrolysis. DnaK has a slow intrinsic hydrolysis rate (Fig. 19C, closed circles), which can be stimulated by addition of the purified DnaJ (Fig. 19C, open circles). Addition of the soluble J-domain of Toc12 (Fig. 19B) increases the ATP

hydrolysis rate of DnaK (Fig. 19C, open squares), but not as pronounced as found for DnaJ (Fig. 19C, open circles). This can be explained by the fact that DnaJ-DnaK is a homologous system, whereas Toc12-DnaK represents a heterologous system. However, it was previously described that substrate binding of Hsp70 homologues also stimulates the ATPase activity of the chaperone (Bukau and Horwich, 1998). Therefore, another explanation of the observed ATPase stimulation by Toc12 J-domain is that Toc12Δ48 might act as a substrate of DnaK. To exclude this, DnaK was incubated with the denatured form of Oep16 (Fig. 19B, open triangle). Since the integral membrane protein Oep16 contains hydrophobic regions it serves most likely as a substrate of DnaK because Hsp70 homologues bind predominantly to hydrophobic stretches (Bukau and Horwich, 1998).

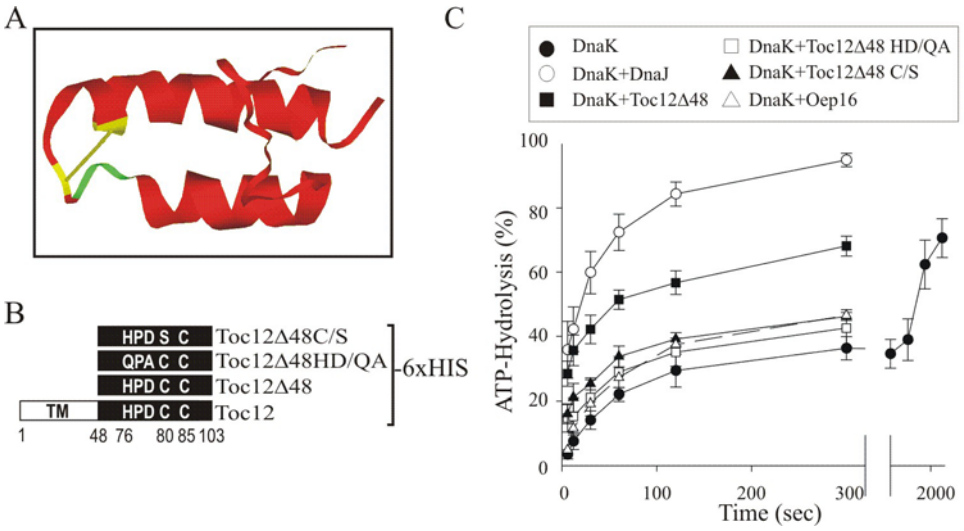

Figure 19. Toc12 stimulates ATPase activity of DnaK. **(A)** The average structure of the last two nano-seconds of the molecular dynamic simulation of Toc12 J-domain structure is shown. The disulfide bridge between cysteine 80 and cysteine 85 is shown in yellow, the localisation of the HPD motif in green. The modelled structure was kindly provided by J. Hritz. **(B)** A schematic presentation of the used Toc12 constructs is depicted. **(C)** 6.5 µM of DnaK (filled circles, solid line) was preloaded with ATP followed by incubation with 1 µM of DnaJ (open circles, solid line) or 5 µM of Toc12 (closed squares, solid lines), Toc12 HD/QA (open squares, solid lines), Toc12 C/S (closed triangle, solid line) or denatured Oep16 (open triangle, dashed line). ATP hydrolysis was determined and quantified as described. The data reflect the average of at least three independent measurements.

In line with this considerations the addition of Oep16 increased the hydrolysis rate, but not as drastic as Toc12Δ48 (Fig. 19C, open triangle). In general the stimulatory effect of substrates is much less pronounced as of the J-proteins and can be therefore distinguished from co-chaperone activity (Bukau and Horwich, 1998). Further, a Toc12Δ48 mutant bearing an aspartic acid to alanine and histidine to glutamine exchange in the HPD motif was used in this assay (Fig. 19A, green residues and Fig. 19B). It was reported that mutations in the HPD motif of the J-domain abolishes the stimulatory function of J-proteins on Hsp70 ATPase activity (Scidmore et al. 1993, Wall et al. 1995). This mutant exhibits only a substrate dependent stimulation on DnaK like it was observed for Oep16 (Fig. 19C, open squares), Therefore, Toc12 comprises a J-domain, which specifically stimulates the ATPase activity of an Hsp70 chaperone. Interestingly, according to the modelled structure of the Toc12 J-domain the cysteines might play a role for the stability of this domain (Fig. 19A, yellow residues). In order to

disturb a disulfide bridge formation, a construct was generated, in which one of the cysteines was mutated to a serine (Fig. 19B). When this point-mutated Toc12 was added to the DnaK-ATP complex, again only a substrate dependent stimulation of the hydrolysis rate was detected (Fig. 19C, closed triangle). This result indicates that the disruption of the disulfide-bridge alters the structural stability of the protein and results in the loss of co-chaperone activity.

6.3.4. Toc12 is a component of the intermembrane space translocon

It had been shown before that Toc12 is localised in the outer envelope of plastids (Fig. 15) and activates Hsp70 proteins (Fig. 19). Moreover, the intermembrane space facing J-domain of Toc12 was shown to interact with isHsp70 (Fig. 18). Another indication for the interaction between Toc12 and isHsp70 derives from co-purification in a linear sucrose gradient upon ATP addition (Fig. 13).

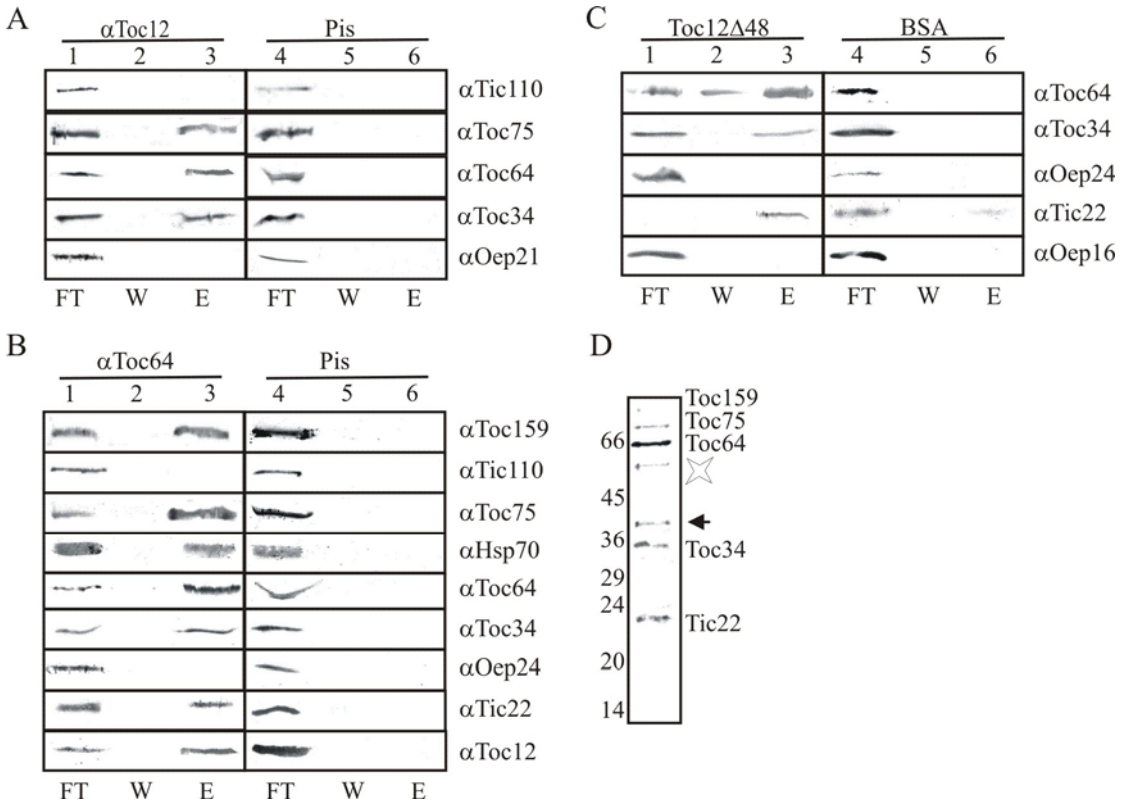

Figure 20. Toc12 is a Toc component and part of the intermembrane space translocon. **(A)** Solubilised outer envelope vesicles (400 µg) were incubated with Toyopearl material coated with Toc12-antibodies (lane 1-3) or preimmunserum (Pis, lane 4-6). The flow through (5%, lane 1, 4), wash (5%, lane 2, 5) and eluted fractions (100%, lane 3, 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. **(B)** Solubilised outer envelope vesicles (400 µg) were incubated with Toyopearl coated with Toc64-antibodies (lane 1-3) or preimmunserum (4-6). The flow through (5%, lane 1, 4), wash (5%, lane 2, 5) and eluate (100%, lane 3, 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using the indicated antibodies. **(C)** Ni^{2+}-NTA coated with either Toc12Δ48 (lane 1-3) or BSA (lane 4-6) and incubated with solubilised outer envelope vesicles (75 µg). The flow through (5%, lane 1, 4), wash (5%, lane 2, 5) and eluted fractions (100%, lane 3, 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using the indicated antibodies. **(D)** Ni^{2+}-NTA column material coated with Toc12Δ48 (lane 1-3) was incubated with solubilised outer envelope vesicles (75 µg). 50% of the elution fraction was subjected to SDS-PAGE followed by silver-staining. The major protein bands were marked according to immunodecoration results from (C). Unspecifically bound large subunit of the RubisCo is marked by asterisk. An unknown protein is marked by an arrow.

Interestingly, two components known to be involved in preprotein translocation, namely Toc64 and Tic22, were also found in the same sucrose fraction (Fig. 13). Therefore, the question whether Toc12 is part of the preprotein translocation machinery was analysed. Using Toc12 antibodies for co-immunoprecipitation an interaction of Toc12 with Toc64, Toc34 and Toc75 (Fig. 20A, lane 3) and Toc159 (not shown) was observed. In contrast, neither Tic110 nor the outer envelope channel Oep21 was co-immunoprecipitated by Toc12 antiserum (Fig. 20A, lane 3). To confirm the interaction between Toc64 and Toc12, Toc64 was immunoprecipitated. The precipitate contained Toc64, Toc159, Toc75, Toc34, Tic22, Toc12 and Hsp70 (Fig. 20B, lane 3). Again, the elution sample did not contain Tic110 and Oep24 (Fig. 20B, lane 3). The precipitate of preimmunserum contains none of the detected outer envelope proteins (Fig. 20B, lane 6), which reveals the specificity of the reaction. To further analyse the interacting domain of Toc12, solubilised outer envelope vesicles were incubated with an affinity matrix coated with the C-terminal J-domain of Toc12. Again, an interaction between Toc12, Toc64, Toc34, Tic22 (Fig. 20C, lane 3 and 6), Toc75 and Toc159 (not shown), but not with the outer envelope proteins Oep24 and Oep16 (Fig. 20C, lane 3) was detected. Furthermore, a silver-stained gel of the elution fraction of the Toc12Δ48 affinity matrix confirms the specificity of these interactions since beside Toc159, Toc75, Toc64, Toc34 and Tic22 only one unknown protein was detected (Fig. 20D). Based on its association with several Toc and Tic components Toc12 is a member of the Toc translocon of the envelope membrane.

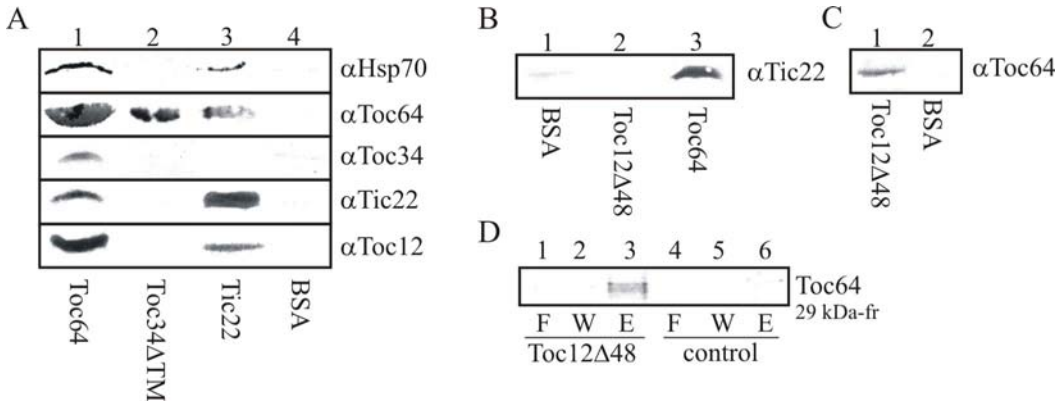

Figure 21. Toc64 is the central component of the intermembrane space translocon. **(A)** 100 µg of expressed and purified Toc64 (lane 1), Toc34ΔTM (lane 2) or Tic22 (lane 3) were coupled to a Ni-NTA affinity matrix (lane 4) followed by incubation with solubilised outer envelope vesicles. The bound proteins were eluted, subjected to SDS-PAGE followed by immunodecoration using indicated antibodies. **(B)** Tic22 was extracted from inner envelope membranes by treatment with 100 mM Na_2CO_3 pH11.4 and dialysed. Aggregated proteins were removed by centrifugation (256 000 x g, 1 h, 4°C). Subsequently, purified Tic22 was incubated with an affinity matrix (lane 1) coated with 100 µg Toc12Δ48 (lane 2) or Toc64 (lane 3). The bound protein was eluted, subjected to SDS-PAGE followed by immunodecoration using Tic22 antibodies. **(C)** Expressed Toc64 was incubated with Toyopearl matrix (lane 2) coated with 50 µg Toc12Δ48 (lane 1). The bound protein was eluted, subjected to SDS-PAGE followed by immunodecoration using Toc64 antibodies. **(D)** Isolated outer envelope vesicles were thermolysin digested, re-isolated and solubilised. Subsequently, the proteins were incubated with a Toc12Δ48 affinity matrix (lane 1-3) or with BSA coated Ni-NTA column as control (lane 4-6). The flow through (5%, lane 1, 4), wash (5%, lane 2, 5) and eluted fractions (100%, lane 3, 6) were subjected to SDS-PAGE followed by blotting and immunodecoration using Toc64 antiserum. The intermembrane space facing 29 kDa fragment (Qbadou, personal communication) is shown.

Surprisingly, an interaction of Toc64 and Toc12 with Tic22 was identified. 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. Solubilised outer envelope vesicles were incubated with the affinity matrix and specifically bound proteins were eluted by imidazol. None of the proteins found interacted with a BSA coated matrix (Fig. 21A, lane 4). Hsp70 was found to interact with Toc64 and with Tic22 affinity matrix (Fig. 21A, lane 1 and 3). Moreover, an interaction between Toc64 and Toc12 (Fig. 21A, lane 1) as well as between Toc64 and Tic22 and vice versa was observed (Fig. 21A, lane 1 and 3). Interestingly, only a weak interaction between Toc12 and Tic22 was observed (Fig. 21A, lane 3). However, this interaction was mediated by Toc64 since purified endogenous Tic22 did not bind the C-terminus of Toc12 (Fig 21B, lane 2) but Toc64 protein (Fig. 21B, lane 3). Moreover, only Toc64, but not Toc12 was found to interact with the cytosolic domain of Toc34 and vice versa (Fig. 21A, lane 1-2). Thus, the presence of Toc core components in samples eluted of a Toc12 affinity matrix is due to the association between Toc64 and Toc34 (Fig. 11). The interaction between Toc12 and Toc64 is not mediated by any other components, since purified Toc64 binds directly to a Toc12Δ48 affinity matrix (Fig. 21C, lane 1). Furthermore, this interaction is mediated by the intermembrane space exposed domain of Toc64 (Fig. 20D, lane 3; Qbadou, personal communication), which mediates an assembly of the intermembrane space complex by separate interactions to Toc12 and Tic22. In conclusion, Toc64, Toc12, Tic22 and Hsp70 are arranged in a protein complex which is subsequently called intermembrane space translocon.

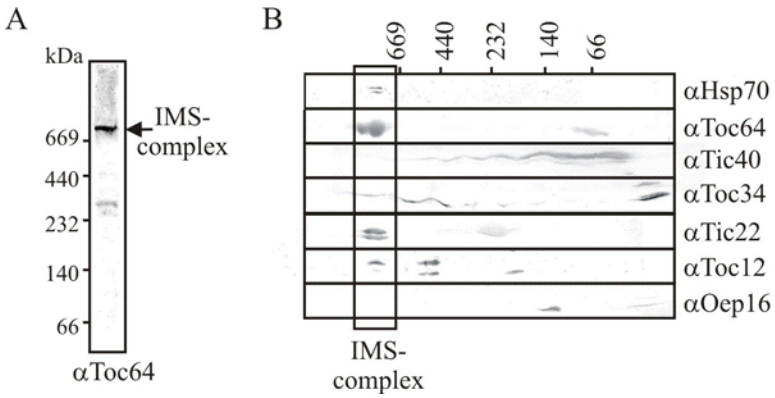

Figure 22. The intermembrane space translocon has a size of 700kDa in a BN-PAGE. **(A)** Isolated chloroplasts (100 µg chlorophyll) were solubilised with 1% dodcylmaltoside and protein complexes were separated over a 5-16% BN-PAGE. Subsequently, after transfer on a nitrocellulose membrane the first dimension was immunodecorated with Toc64 antiserum. The intermembrane space translocon complex is marked. **(B)** Protein complexes separated by a BN-PAGE were denaturised by incubation in SDS-PAGE running buffer and subsequently, the single proteins were separated in a second dimension by a SDS-PAGE and detected after transfer on a nitrocellulose membrane by the indicated antisera. Given is the molecular weight from the BN-PAGE for determination of complex sizes.

To get an idea of the size of the intermembrane space complex, the translocon was analysed by separation of isolated and solubilised chloroplasts over a BN-PAGE (Fig. 22). Immunodecoration of the first dimension with antiserum against Toc64 reveals a prominent band around 700kDa (Fig. 22A). The presence of all further intermembrane space translocon components was confirmed by immunodecoration of the second dimension (Fig. 22B). The reduced presence of isHsp70 in the intermembrane space complex might reveal the dynamic association of isHsp70 with the other components (see below). A second minor band detected by Toc64 antiserum in the first dimension was not found in the second dimension (Fig. 22). The Toc core complex was not detected in a BN-PAGE as shown by Toc34, which does not reveal a distinct complex band (Fig. 22B). The amino acid transporter Oep16 was detected in monomeric as well as in oligomeric structure different from the intermembrane space translocon (Fig. 22B). Furthermore, Tic40 was not found as component of the intermembrane space complex since it was detected as lower molecular masses (Fig. 22B). In conclusion, the molecular composition of the intermembrane space translocon was analysed by different biochemical approaches, which all confirm Toc64, Tic22, Toc12 and isHsp70 as components of the intermembrane space translocon.

6.3.5. The intermembrane space translocon binds specifically to preproteins

Previously, a direct preprotein recognition by Toc159, Toc64 and Toc34 was shown (Fig. 5, Fig. 10). To test the involvement of the intermembrane space complex in preprotein translocation, different matrices with a set of preproteins like pSSU, pOE33 or Tic32 were incubated with solubilised outer envelope vesicles. Toc64, Hsp70, Tic22 and Toc12 were found in the bound fraction of all tested preproteins as determined by immunodecoration (Fig. 23A, lanes 3, 9 and 12), while most of the Toc34 did not bind as expected in the absence of GMP-PNP (Fig. 23A, lane 1, 7 and 10). Interestingly, no binding of Toc34 to Tic32, an inner membrane protein without a cleavable transit peptide (Hörmann et al., 2004), was observed, whereas components of the intermembrane space translocon bind efficiently to this protein (Fig. 23A, lane, 12). This observation indicates Toc34 independent substrate recognition of the intermembrane space translocon. The interaction depends on the targeting sequence, since no binding to a mSSU column was observed (Fig. 23A, lane 6). In order to investigate the direct interaction of the intermembrane space translocon components with the preprotein, isolated Toc64, Tic22 and Toc12 were incubated with a pSSU affinity matrix (Fig. 23B). Both, Toc64 and Tic22 showed a stable interaction to pSSU (Fig. 23B, lane 2) but not to mSSU (Fig. 23B, lane 4), whereas Toc12 interact with neither the mature nor the precursor form of the protein (Fig. 23B, lane 4). Thus, Toc12 is a Toc component not directly interacting with preproteins. However, a close proximity of Toc12 to an early translocating intermediate of a preprotein can be proposed since Toc12 cross-links to the unprocessed form of pSSU and pOE33 in the presence of low ATP concentration (Fig. 23C, lane 6). In line with previous results (Fig. 22A) cross-linking to the mature form of either preprotein could not be detected (Fig. 23C, lane 6). The same was observed for Toc159 (Fig. 23C, lane 4) and Toc64 (Fig. 23C, lane 5), but not for Oep24 (Fig. 23C, lane 7).

Preimmunserum precipitate neither the precursor nor the mature form of the preproteins (Fig. 23C, lane 8). In conclusion, these results indicate that the components of the intermembrane space translocon interact with the incoming preproteins at early import stages.

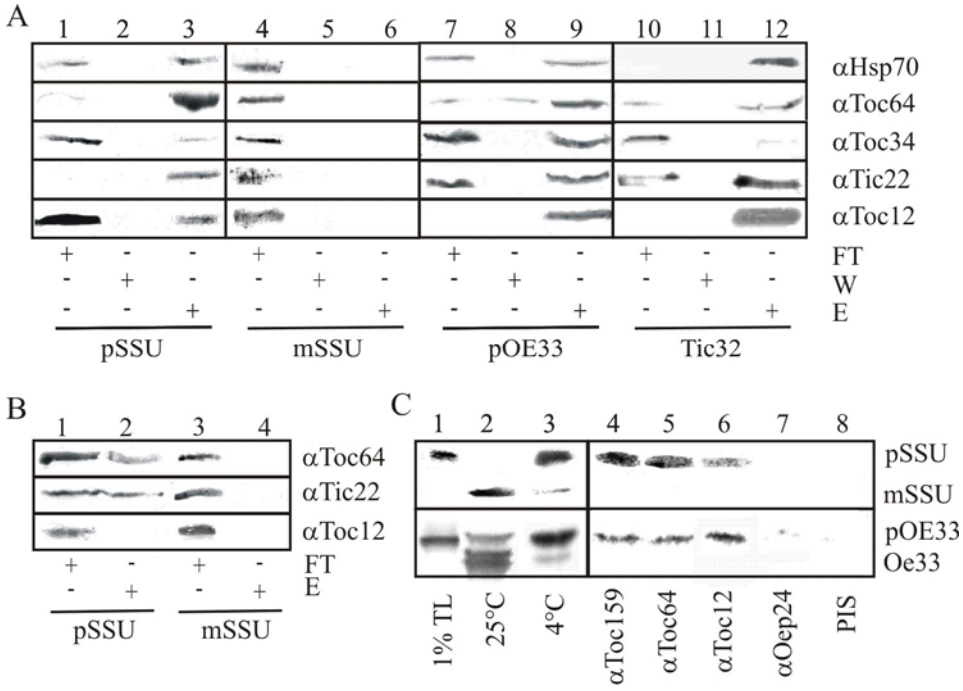

Figure 23. The intermembrane space translocon interacts with preproteins. **(A)** Outer envelope vesicles (75 µg protein) were solubilised and incubated with a Ni-NTA coated with 100 µg pSSU (lane 1-3), mSSU (lane 4-6), pOE33 (lane 7-9) or Tic32 (10-12). The flow through (5%, lane 1, 4, 7, 10), wash (5%, lane 2, 5, 8, 11) and eluate (100%, lane 3, 6, 9, 12) was collected, subjected to SDS-PAGE followed by blotting and immunodecoration using the indicated antibodies. **(B)** 5 µg of expressed and purified Toc64, Tic22 or Toc12 were incubated with a Toyopearl matrix coated with 6 µg pSSU (lane 1-2) or mSSU (lane 3-4). The flow through (5%, lane 1, 3) and eluate (100%, lane 2, 4) were subjected to SDS-PAGE followed by blotting and immunodecoration with the indicated antibodies. **(C)** Isolated Chloroplasts were incubated with pSSU (lane 1, upper panel) or pOE33 (lane 1, lower panel) for 10 min at 4°C (lane 3) in the presence of chemical cross linker DSP (lane 4-8) followed by lysis of the chloroplasts and incubation with the indicated antisera. After immunoprecipitation, the cross linker was cleaved by DTT, the proteins were separated on SDS-Page and pSSU or mSSU were visualised by phosphor-imaging. Lane 2 shows an import at 25°C. In lane 1 1% of translation product (TP) is loaded.

The substrate recognition by Hsp70 proteins is ATP dependent. Therefore, it was further tested whether adenine nucleotide affects preprotein binding of the intermembrane space translocon (Fig. 24A). Indeed, isHsp70 binds to pSSU affinity matrix in a nucleotide dependent manner (Fig. 24A). In the presence of ATP no isHsp70 was bound to pSSU (Fig. 24A, lane 2), whereas in the presence of ADP a strong binding of isHsp70 to pSSU was observed (Fig. 24A, lane 3). The nucleotide dependent association reflects a tight substrate association of Hsp70. Interestingly, the preprotein recognition by Toc64 and its associated Toc12 is not affected by either ATP or ADP (Fig. 24A, lane 1-3). This observation indicates a dynamic association of isHsp70 to the other components of intermembrane space translocon. In a complementary approach the effect of pSSU and of pOE33 on the nucleotide dependent complex formation was investigated. Therefore, the complex was isolated by sucrose

density centrifugation without (Fig. 24B, lane 1) or with addition of either ATP (Fig. 24B, lanes 2, 4 and 6) or ADP (Fig. 24B, lanes 3, 5 and 7) in the absence (Fig. 24B, lanes 1-3) and presence of either pSSU (Fig. 24B, lanes 4 and 5) or pOE33 (Fig. 24B, lanes 6 and 7). One representative intermembrane space components containing fraction (Fig. 13, box 1) is shown for analysis of nucleotide or preprotein influence on the complex stability (Fig 24B).

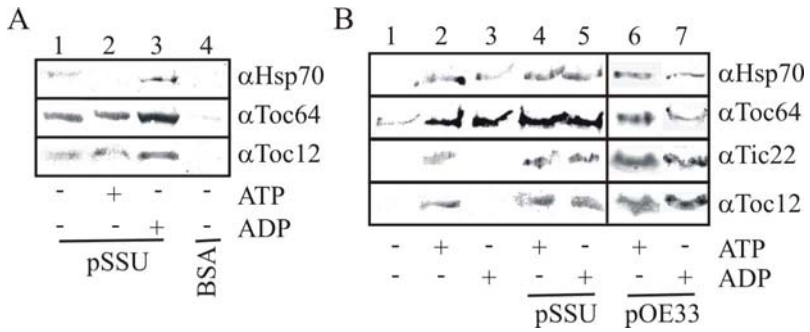

Figure 24. The recruitment of isHsp70 to the intermembrane space translocon is nucleotide and preprotein dependent **(A)** The binding of Toc64, Toc12 and isHsp70 to a pSSU affinity matrix was analysed as described in Fig. 23A, in the absence (lane 1) or presence of 1 mM ATP (lane 2) or 1 mM ADP (lane 3). A BSA coated column was used as control (lane 4). **(B)** Outer envelope vesicles (150 µg protein) were incubated with 500 µM $MgCl_2$, (lane 1-7) and either with 1 mM ATP (lane 2, 4, 6) 1 mM ADP (lane 3, 5, 7), 10 µg pSSU (lane 4, 5) or with 10 µg pOE33 (lane 6, 7) solubilised and subjected on top of a sucrose gradient as in Fig. 13 (25-70%). A fraction containing the intermembrane space complex (compare Fig. 13) were collected and subjected to SDS-PAGE analysis followed by blotting and incubation with indicated antibodies.

As before ATP addition during solubilisation induced the formation of a complex composed of Toc64, Tic22, Toc12 and Hsp70 (Fig. 22C, lane 2), which is not destroyed by the incoming preprotein (Fig. 24B, lane 4 and 6). When the outer envelope vesicles were solubilised in the absence of nucleotides or in the presence of ADP the intermembrane space translocon was not purified in the analysed fractions (Fig. 24B, lane 1 and 3). However, in the presence of ADP addition of a substrate like pSSU or pOE33 leads to complex formation (Fig. 24B, lanes 5 and 7). Similar results were obtained by the addition of Tic32 (data not shown). In conclusion, in the absence of a precursor a stable complex forms upon addition of ATP, which disassembles in the presence of ADP. The association with a precursor leads to a stabilisation of the complex in the ADP-bound state.

6.3.6. Toc12 recruits isHsp70 to the intermembrane space translocon

The association of isHsp70 with the intermembrane space translocon is adenine nucleotide and preprotein dependent. Since the Toc12 J-domain is the interacting partner of isHsp70 its role in the ATP dependent assembly of this translocon was analysed (Fig. 25). The interaction between Hsp70 proteins and their respective J-proteins is regulated by the nucleotide bound stage of the chaperone (Corsi and Schekmann, 1997). Therefore, the effect of different adenine nucleotides on the association of isHsp70 and the other components of the intermembrane space translocon to a Toc12Δ48 affinity matrix was tested (Fig. 25A). Whereas ATP has weak stimulating effect on the association of isHsp70 to Toc12Δ48 (Fig. 25, lane 6), the addition of ADP induces the dissociation of isHsp70 from the J-protein (Fig. 25A, lane 9). This observation is in line with the previously characterised interaction

between Sec63p and BiP of the translocation apparatus in the endoplasmatic reticulum (Corsi and Schekman, 1997). Interestingly, the interaction of the other intermembrane space translocon components was not affected by nucleotide addition suggesting an isHsp70 independent interaction of these components (Fig. 25A, lanes 3, 6 and 9).

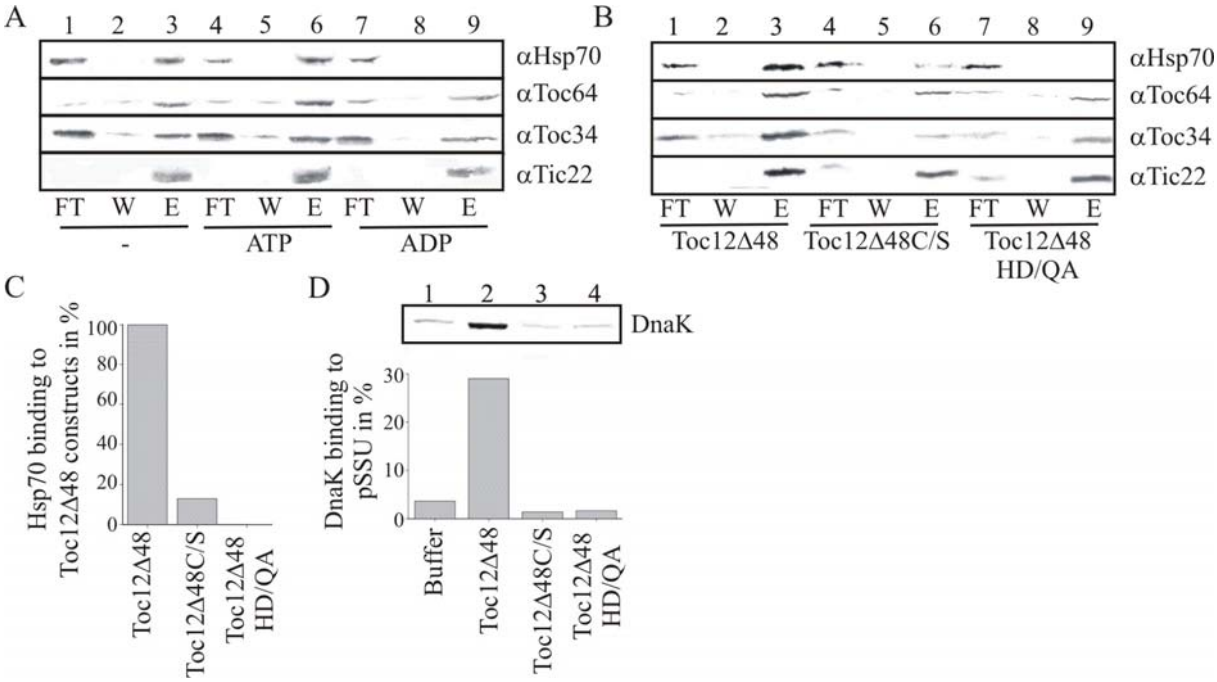

Figure 25. Toc12 recruits Hsp70 to the intermembrane space translocon. (A) A Toc12Δ48 affinity matrix was incubated with solubilised outer envelope vesicles (75 µg) in the absence (lane 1-3) or presence of 500 µM ATP (lane 4-6) or 500 µM ADP (lane 7-9). The flow through (5%, lane 1, 4, 7), wash (5%, lane 2, 5, 8) and eluted fractions (100%, lane 3, 6, 9) were subjected to SDS-PAGE followed by blotting and immunodecoration using indicated antibodies. **(B)** Ni^{2+}-NTA coated with 200 µg of either Toc12Δ48 (lane 1-3), Toc12ΔC/S (lane 4-6) or Toc12Δ48HD/QA (lane 7-9) were incubated with solubilised outer envelope vesicles (75 µg) in the presence of 500 µM ATP. The flow through (5%, lane 1, 4, 7), wash (5%, lane 2, 5, 8) and eluted fractions (100%, lane 3, 6, 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)** 1 µM DnaK was incubated in the absence (lane 1) or presence of either 2 µM Toc12Δ48 (lane 2), Toc12ΔC/S (lane 3) or Toc12Δ48HD/QA (lane 4) with on Toyopearl column material immobilised 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.

Furthermore, the two Toc12 mutants, which were not able to stimulate ATPase activity of DnaK (Fig. 19C) were coupled to the matrix and incubated with solubilised outer envelope vesicles. A mutation in the HPD motif of Toc12Δ48 results in a lack of binding of isHsp70 to the Toc12Δ48HD/QA affinity matrix (Fig. 25B, lane 9, Fig. 25C). Furthermore, the interaction of isHsp70 with Toc12ΔC/S was analysed. Again the binding of isHsp70 was drastically reduced in comparison to Toc12Δ48 (Fig. 25B, lanes 1 and 6, Fig. 25C). However, the association of Toc64, Toc34 and Tic22 was not affected by both constructs (Fig. 25C, 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 isHsp70 with the

intermembrane space translocon relies on the presence of a functional Toc12 J-domain. Previously, it was described that the targeting sequence of preproteins contains binding sites for DnaK (Rial et al., 2000). Thus, it was analysed whether the interaction of Toc12 with DnaK in the presence of ATP could stimulate the binding of the Hsp70 protein to a preprotein. Therefore, Toc12Δ48 constructs were preincubated with DnaK in an *in vitro* chaperone-binding assay with immobilised pSSU was performed (Fig. 25D). In line with previous observations (Fig. 19C, Fig. 25A and B) the stimulating effect was dependent on a functional J-domain (Fig. 25D, lane 2), whereas the two mutations Toc12Δ48QPA and Toc12Δ48S81 were unable to induce binding of DnaK to its substrate above background level (Fig. 25C, compare lane 1 and lanes 3 and 4).

6.3.7. Abundance and stoichiometry of the intermembrane space translocon

Protein levels might indicate a general or more specialised function of the analysed proteins. In order to analyse an involvement of the intermembrane space translocon in the general import pathway across the Toc core complex the protein level of Toc64 and Toc12 were compared to that of Toc34 in isolated outer envelope vesicles.

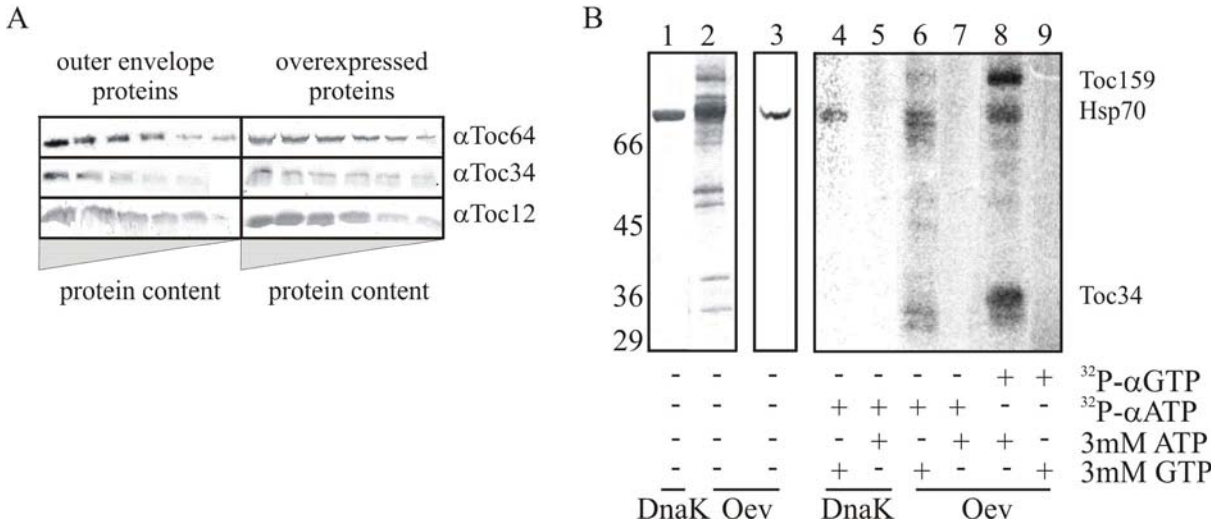

Figure 26. The intermembrane space translocon components are less abundant than Toc core complex components in outer envelope vesicles **(A)** Increasing amounts of isolated outer envelope vesicles or recombinantly expressed Toc64, Toc34 and Toc12 were separated by SDS-PAGE, blotted and immunodecorated with the indicated antisera. Subsequently, the intensity of the protein bands were quantified using AIDA software and the amount of proteins in outer envelope vesicles were calculated proportional to the expressed proteins. The determined ratios between Toc64, Toc34 and Toc12 are described in the text. **(B)** Purified outer envelope vesicles (Oev, 20 µg protein) and 0.5 µg DnaK were separated by SDS-PAGE and stained with Coomassie (lane 1, 2) or immunodecorated with SPA820 antibody after transfer to nitrocellulose (lane 3). ^{32}P-ATP (lanes 4-7) or ^{32}P-GTP (lane 8, 9) was cross-linked to outer envelope proteins and DnaK in the presence of either cold ATP (lane 5, 7, 8) or GTP (lane 4, 6, 9). Radioactivity was visualised by a phosphor-imager. Toc159, Toc34 and Hsp70 are marked. Molecular weight markers are shown.

Serial dilutions of expressed proteins were used for quantification (Fig. 26A). The protein level was normalised to the expression level of Toc34 of the Toc core complex (Fig. 26). The amount of isHsp70 in comparison to Toc34 was determined by chemical cross-linking with radioactive ATP and

GTP (Fig. 26B, lane 6, 8). Toc64, Toc12 and isHsp70 are less abundant than the other components. Toc64 is the most abundant component of the intermembrane space translocon since it is at six to eight folds higher amounts present than Toc12 and exhibits one to two higher protein level than isHsp70. In addition to these three low abundant proteins Tic22 could be detected in low amounts in the outer envelope, which were not quantified. Toc64 is present in a four to five fold lower amount than Toc34. Since four to five Toc34 molecules are involved in the core complex formation (Schleiff et al., 2003a), the association of one intermembrane space translocon with one core complex might be assumed. This leads to the assumption that the intermembrane space complex might assist the translocation of a subset of preproteins, whereas Toc34 fulfils a more general function in preprotein import.

6.3.8. A potential Toc12 homologue in *A. thaliana*

The low amount of Toc12 in isolated outer envelope vesicles of chloroplasts from *P. sativum* might indicate a specialised or assisting function during preprotein translocation. To confirm the presence of Toc12 in other plant species, purified chloroplasts from *A. thaliana* were separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane and immunodecorated with antibodies against Toc12 and Hsp70 (Fig. 27A).

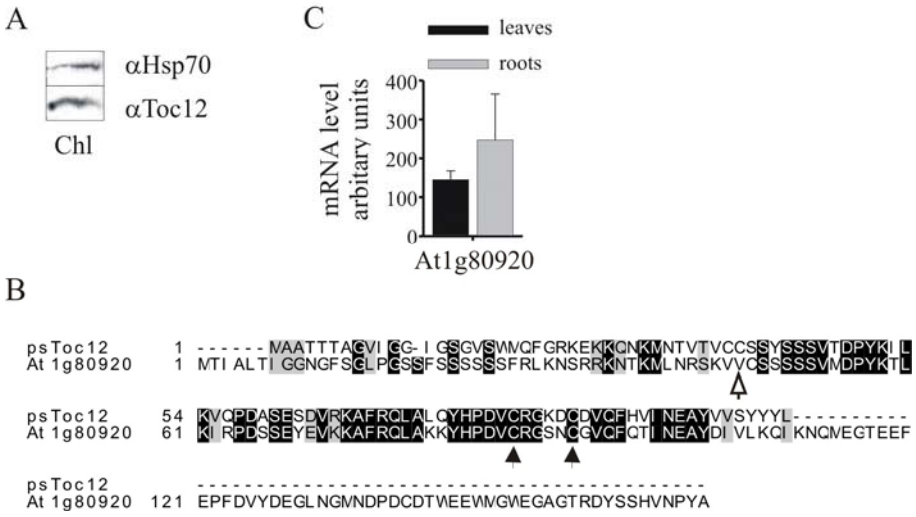

Figure 27. The intermembrane space chaperone system in *A. thaliana*. **(A)** Isolated chloroplasts according to 30µg chlorophyll content were separated over a SDS-PAGE, blotted and immunodecorated with the indicated antisera. **(B)** An alignment between Toc12 and At1g80920 using clustal w is shown. Identical amino acids are boxed in black, homologues in grey. The cysteine residues in the J-domain are marked by black arrows, whereas the cleavage site of the predicted transit peptide of At1g80920 is marked by a white arrow. **(C)** Transcript levels of At1g80920 were analysed by Affymetrix micro array analysis (kindly provided by Vojta A.). The expression level in leaves and roots are depicted.

One protein is recognised by Toc12 antibodies (Fig. 27A) and one Hsp70 was detected in this fraction (Fig. 27A). Therefore, chloroplasts from *A. thaliana* most likely contain an intermembrane space chaperone system as well. Screening the *A. thaliana* database for a potential homologue of Toc12 one sequence could be identified, which encompasses a C-terminal J-domain with two cysteine residues

like described for Toc12 (Fig. 27B, black arrows). In contrast to Toc12 from *P. sativum*, the *A. thaliana* protein encoded by At1g80920 comprises a predicted N-terminal transit peptide suggesting a translocation towards the interior of chloroplasts (Fig. 27B, white arrow). The expression analysis of At1g80920 by Affymetrix micro array (kindly provided by Vojta, A.) reveals higher mRNA levels in roots than in leaves (Fig. 27C).

Table 2. The expression profiles of J-proteins with predicted chloroplasts localisation (column 1) were correlated with the expression profile of *toc64-III* (column 2) and *tic22-III* (column 3). High values indicating a correlation are depicted in bold.

	toc64-III	*tic22-III*
At1g80920	**0.172**	**0.089**
At4g36040	0.063	0.060
At5g23240	0.046	0.047
At2g42750	0.013	0.028
At2g17880	0.087	0.021
At3g17830	**0.101**	**0.191**
At1g77930	0.027	0.047
At1g80030	0.018	0.000
At5g05750	0.073	**0.122**

Analysing Affymetrix data from 1804 experiments available on the internet (https://www.genevestigator.ethz.ch) allows the correlation of the mRNA level of different genes (Alavi-Khorassani and Schleiff, 2005). Therefore, it was tested whether the mRNA levels of At1g80920 correlates with that one of Tic22 and Toc64. Two genes encoding for Tic22 and three genes encoding for Toc64 isoforms are described in *A. thaliana* (Jackson-Constan and Keegstra, 2001). For further analysis *toc64-III* and *tic22-III* were selected as homologues of *P. sativum toc64* and *tic22* (Tab. 2). *tic22-IV* was not considered because it reveals a lower correlation to *toc64-III* than *tic22-III* (data not shown). The gene product of *toc64-V* was previously localised in purified mitochondria (Chew et al., 2004) and was not selected here. Moreover, the amino acid sequence of *toc64-I* is less similar to *P. sativum* Toc64 as that one of *toc64-III* (Chew et al., 2004). The gene expression of At1g80922 correlates with that one of *toc64-III* and *tic22-III*. The other J-proteins with predicted chloroplasts localisation (data not shown) reveal different expression patterns than *toc64-III* and *tic22-III* (Tab. 2). The only exception is At3g17830. The expression of this specific gene also correlates with the expression of *toc64-III* and *tic22-III* based on Affymetrix data (Tab. 2). However, the resulting protein of 517 amino acids is much larger as At1g80920 and Toc12 and does not contain the two cysteine residues in the J-domain (data not shown). Indeed, At1g80920 is the only J-protein in *A. thaliana* containing two cysteines within the J-domain (data not shown) and is a most likely candidate for the Toc12 homologue.

6.4. Molecular characterisation of isHsp70

6.4.1. The isHsp70 is from eukaryotic origin

In the previous section an interaction of Toc12 to isHsp70 via its J-domain was demonstrated (Fig. 22 and Fig. 25). This chaperone is part of the intermembrane space translocon (Fig. 13, Fig. 21 and Fig. 22) and its presence in the outer envelope membrane of plastids was detected by immunodecoration with polyclonal antisera (Fig. 18; Marshall et al., 1990). However, the molecular identity of isHsp70 remains elusive. Difficulties of the molecular identification derive from the high degree of sequence conservation among Hsp70 homologues (Bukau and Horwich, 1998). Moreover, in eukaryotic organisms several Hsp70 proteins are in different cellular compartments like cytosol, endoplasmatic reticulum, mitochondria and plastids. Analysing the *A. thaliana* genome 18 genes encoding for Hsp70 proteins were found (Tab. 3). To identify the Hsp70 of the intermembrane space of plastids Hsp70 proteins of different function had to be ruled out.

Table 3. The Hsp70 set of *A. thaliana*. Listed are all annotated Hsp70s of *A. thaliana* with their Atg number (column 1), name (column 2), type (column 3), predicted localisation (column 4) and length in amino acids (column 5). Hsp70 proteins of prokaryotic origin are marked in italics. The nomenclature was made according to the suggestions by Sung and colleagues (2001) and by Li and colleagues (2001). It is further indicated whether the Hsp70 protein were detected (+) or not detected (-) in a proteomic analysis of *A. thaliana* envelope preparation (column 6). The proteomic data were obtained from Kleffmann and colleagues (2004) and from Soll, J. (personal communication).

Atg number	Name	type	localisation	length	Envelope
At5g02500	Hsc70-1	DnaK type	Cytosol	651	+
At5g02490	Hsc70-2	DnaK type	Cytosol	653	-
At3g09440	Hsc70-3	DnaK type	Cytosol	649	-
At3g12580	Hsp70	DnaK type	Cytosol	650	-
At1g16030	Hsp70b	DnaK type	Cytosol	646	+
At5g28540	BiP-1	DnaK type	ER lumen	669	-
At5g42040	BiP-2	DnaK type	ER lumen	668	-
At1g09080	BiP-3	DnaK type	ER lumen	678	-
At4g37910	mtHsc70-1	DnaK type	Mitochondria	666	-
At5g09590	mtHsc70-2	DnaK type	Mitochondria	682	-
At4g24280	cpHsc70-1	DnaK type	Plastid	718	+
At5g49910	cpHsc70-2	DnaK type	Plastid	718	+
At1g56410	Hsp70t-1	DnaK type	Unknown	617	+
At2g32120	Hsp70t-2	DnaK type	Plastid	563	-
At1g79920	Hsp70.15	Hsp110/SSE	Cytosol	736	-
At1g79930	Hsp70.14	Hsp110/SSE	Cytosol	831	-
At1g11660	Hsp70.16	Hsp110/SSE	Cytosol	773	-
At4g16650	Hsp70.17	Hsp110/SSE	ER lumen	867	-

As a first approach it was analysed whether the isHsp70 is from prokaryotic or eukaryotic origin. Hsp70 proteins can be dissected into an ATPase (Fig. 28A, AD), peptide binding (Fig. 28A, PBD) and C-terminal domain of unknown function (Fig. 28A, UFD). An alignment of two typical eukaryotic Hsp70s, namely Hsc71 from *P. sativum* and Ssa1p from yeast cytosol, with the prokaryotic Css1 from *P. sativum* plastid stroma and with the *E. coli* DnaK reveals high amino acid conservation in the ATPase and peptide binding domain (Fig. 28B, black line). Furthermore, beside their close similarity to DnaK Hsp70 proteins of the mitochondrial matrix or the plastid stroma comprises an N-terminal

transit peptide (Fig. 28B, grey lane; data not shown). However, sequence differences between Hsp70 from either prokaryotic or eukaryotic origin appear more pronounced in the C-terminal region, which is of unknown function (Fig. 28B).

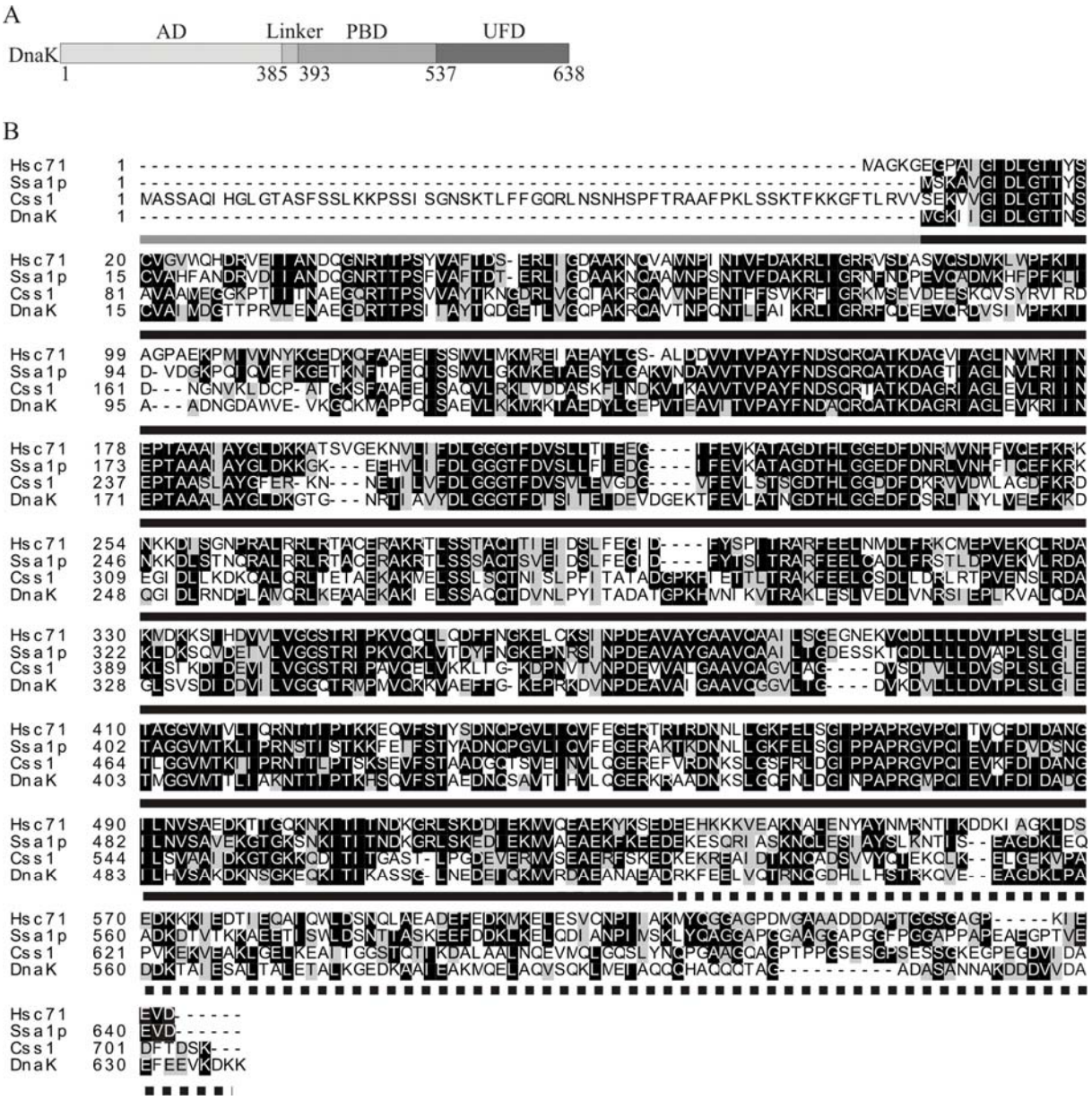

Figure 28. Hsp70 proteins are highly conserved between eukaryotic and prokaryotic organism. **(A)** A schematic model of DnaK is depicted. The protein can be dissected in the ATPase (AD), linker, peptide binding (PBD) and a domain of unknown function (UFD). **(B)** The eukaryotic Hsc71 from *P. sativum* and Ssa1p from *S. cereviseae* cytosol were aligned with Css1 from *P. sativum* plastid stroma and DnaK from *E. coli* using clustal w. Identical amino acids are shown in a black box and homologous one in grey box. Regions of high homology are marked by black lane, whereas regions with low homology are underlined with dashed lane. The transit peptide of Css1 is underlined in grey.

Based on that antibodies specific for either eukaryotic or prokaryotic Hsp70 can be generated. The specificity of the used antibodies was demonstrated by detection of the stromal Css1 by antiserum against prokaryotic (Fig. 29A, lower panel) but not antiserum against eukaryotic Hsp70 (Fig. 29A, upper panel). The other way around, Hsp70 in the cytosolic extract was exclusively detected by the

antiserum against eukaryotic (Fig. 29A, upper panel) but not by the antiserum against prokaryotic Hsp70 (Fig. 29A, lower panel). Both antisera were used to detect Hsp70 proteins in plastid fractions (Fig. 29B). It was shown that the Hsp70 of outer envelope vesicles is from eukaryotic origin since it was detected by an antibody against eukaryotic (Fig. 29B, lane 1) but not from one against prokaryotic Hsp70 (Fig. 29B, lane 1). In contrast Css1 in the stromal fraction is solely detected by antiserum against prokaryotic Hsp70 (Fig. 29B, lane 3). The purity of the plastid fractions was controlled by detection of Toc34 in outer envelope (Fig. 29B, lane 1), Tic40 in inner envelope vesicles (Fig. 29B, lane 2), SSU in the stromal (Fig. 29B, lane 3) and LHCP in the thylakoid fraction (Fig. 29B, lane 4). Therefore, isHsp70 most likely reveals sequence similarity to Hsp70 proteins of eukaryotic origin that are present in the cytosol or in the endoplasmatic reticulum.

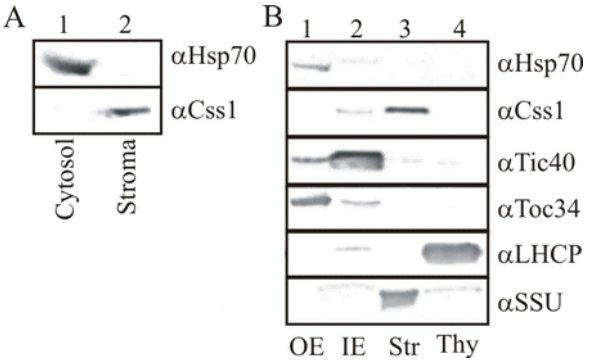

Figure 29. The isHsp70 is from eukaryotic origin. **(A)** Stromal and cytosolic proteins from *P. sativum* corresponding to 30 µg protein content were separated by SDS-PAGE, blotted and immunodecorated by antiserum rose either against eukaryotic (upper panel) or prokaryotic Hsp70 (lower panel). **(B)** An outer (lane 1) and inner envelope (lane 2), stromal (lane 3) and thylakoid fraction (lane 4) from *P. sativum* chloroplasts according to 20 µg protein content were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunodecorated by the indicated antisera.

Taking this observation into account five Hsp70 proteins with high homology to DnaK from *E. coli* from *A. thaliana* can be ruled out, which are present in the endosymbiotic derived mitochondria and plastids (Tab. 3, italics). Moreover, considering the size of the isHsp70 of 70kDa in a SDS-PAGE members of the Hsp110/SSE family are also no potential candidates for the isHsp70 (Tab. 3). After reducing the list of Hsp70 proteins two main groups remain: Three Hsp70 proteins with predicted endoplasmatic reticulum localisation and six potential cytosolic chaperones. Considering the strong membrane association of isHsp70 (Fig. 18A) primary structure analysis like hydrophobicity plots were utilised. This kind of *in silico* analysis and signal peptide prediction did not point out any clear difference of the remaining Hsp70 proteins (data not shown).

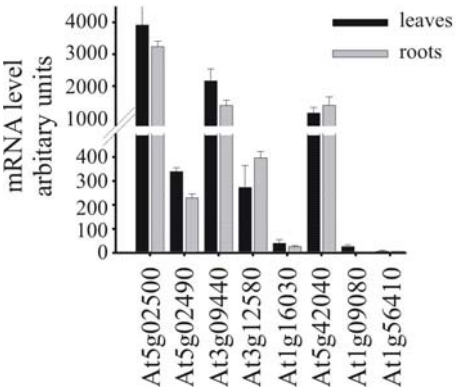

Figure 30. Eukaryotic Hsp70 proteins in *A. thaliana* reveal different expression levels. Transcript levels of potential candidates for isHsp70 from *A. thaliana* were analysed by Affymetrix micro array analysis (kindly provided by Vojta A.). The expression level in leaves and roots are depicted. For previous selection strategies compare text.

As a further approach mRNA levels in root and leaves were analysed by Affymetrix analysis (Fig. 30; kindly provided Vojta, A.). Based on their high expression level three Hsp70 (At5g02500, At3g09440, At5g42240) are most likely not candidates for isHsp70 (Fig. 30) since they largely exceeds that one of *toc64-III* (110 arbitrary units), *tic22-III* (130 arbitrary units; Vojta, personal communication) and of At1g80922 (140 arbitrary units; Fig. 27C). However, it has to be kept in mind that gene expression level does not necessarily correlates with protein levels.

Table 4. The expression profiles of selected candidates for the homologue of *P. sativum* isHsp70 in the *A. thaliana* genome base (column 1) that are depicted with their corresponding names (column 2) were correlated with those of *toc64-III* (column 3) and *tic22-III* (column 4). High values indicating a correlation are depicted in bold. For the selection strategies compare text.

	Name	*toc64-III*	*tic22-III*
At3g12580	Hsp70	0.005	0.002
At1g16030	Hsp70b	**0.022**	**0.076**
At1g56410	Hsp70t-1	0.000	0.005
At5g02490	Hsc70-2	0.000	0.000
At1g09080	BiP-3	0.004	**0.032**

Based on Affymetrix data of 1804 experiments available on the internet (https://www.genevestigator.ethz.ch) the expression profiles of the remaining Hsp70 proteins were compared to those of *toc64-III* and of *tic22-III* (Tab. 4). All Hsp70 tested show low values for correlation with *toc64-III* and *tic22-III* (Tab. 4). However, expression profile analyses of At1g16030 and At1g09080 yield the highest correlation values (Tab. 4). Interestingly, the gene products of At1g16030 and At1g56410 were also found in proteomic approaches (Tab. 3) using purified outer envelope vesicles (Kleffmann et al., 2004; Soll, J. personal communication). The detection of other Hsp70 might be due to cytosolic or stromal contamination since some of them represent those Hsp70 proteins with high expression levels (Fig. 30, data not shown). For all J-proteins tested, a significant correlation to any Hsp70 protein could not be observed (data not shown). A possible reason might be that one Hsp70 protein can interact with different J- proteins (Bukau and Horwich, 1998) and that At1g80920 is solely a potential homologue of Toc12. Therefore, based on the presented data isHsp70 is from eukaryotic origin. In line with immunodetection results, all three selected Hsp70 proteins are from eukaryotic origin. Moreover, proteomic and mRNA level analysis of Hsp70 proteins in *A. thaliana* leads to the selection of three potential candidates for isHsp70, namely At1g09080, At1g16030 and At1g56410.

6.4.2. Purification of isHsp70

To reveal the molecular identity of isHsp70, the biochemical purification was initiated to analyse the protein by mass spectroscopy. IsHsp70 migrates in a SDS-PAGE directly beneath Toc75, the most abundant outer envelope protein (data not shown). Therefore, different approaches were utilised to separate isHsp70 from Toc75 to enable the gel excision of the protein.

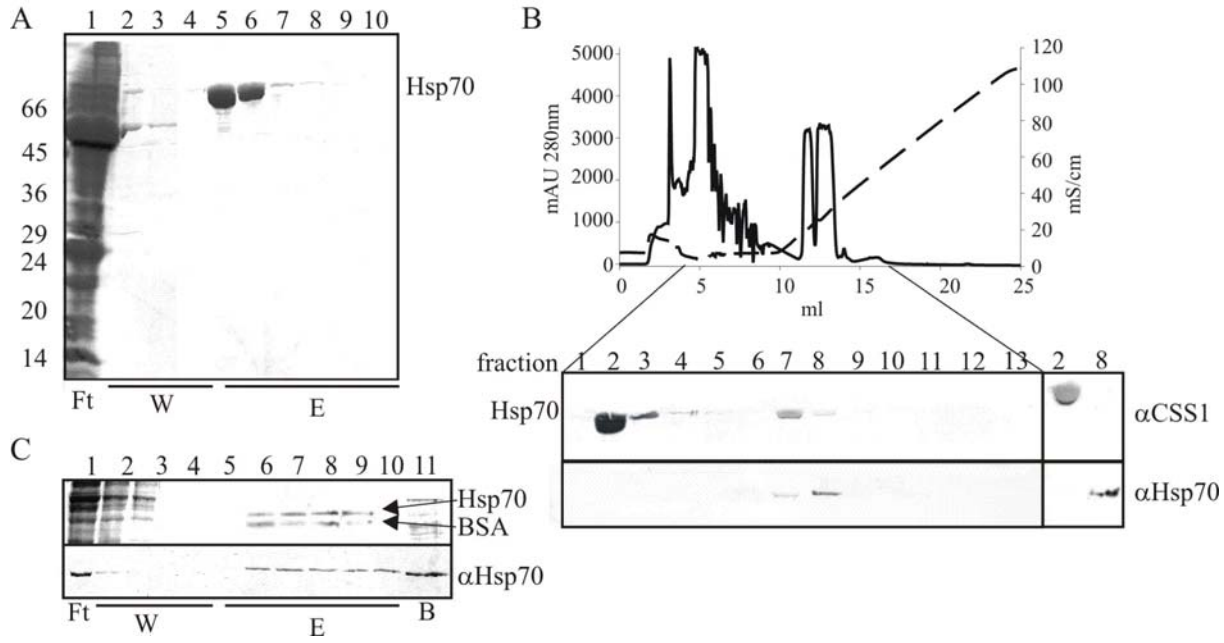

Figure 31. The stromal Css1 binds efficiently to ATP-Agarose. **(A)** Isolated chloroplasts according to 100 mg chlorophyll content were solubilised and incubated with ATP-Agarose. Bound proteins were eluted by 10 mM ATP. 0.1% of the flow though (lane 1), 1% of the washing steps (lane 2-4) and 5% of the elution sample were subjected to a SDS-PAGE and stained with Coomassie. The Hsp70 proteins are marked. **(B)** The elution samples from (A) were pooled and loaded on a MonoQ anion-exchanger. Subsequently, the proteins were eluted by a linear potassium gradient (dashed line) as measured by 280nm absorbance (solid line). Proteins from 16% of the fractions were separated by SDS-PAGE and either stained with Coomassie (upper panel) or blotted and immunodecorated with Hsp70 antiserum (lower panel). In addition proteins from fraction 2 and 8 were immunodecorated with αCss1 and αHsp70 (left panel). **(C)** Outer envelope vesicles according to 2 mg protein content were solubilised and incubated with ATP-Agarose. Bound proteins were eluted by 10 mM ATP. 1% of the flow through (lane 1) and of the wash fraction (lane 2-4) and 50% of the elution sample were subjected to a SDS-PAGE. Subsequently, the protein content was either stained with Coomassie (upper panel) or blotted and immunodecorated with Hsp70 antiserum (lower panel).

Isolated chloroplasts were solubilised and incubated with ATP-Agarose, which is a widely used method for Hsp70 purification (Hartman et al., 1992). Due to their ATPase domain Hsp70 proteins bind to the column material and can subsequently be eluted by ATP (Fig. 31A, lane 5-10). To separate the isHsp70 from the more abundant stromal Hsp70, Css1, the elution fractions were pooled and separated on a MonoQ anion-exchange chromatography (Fig. 31) applying a linear potassium gradient. The vast majority of the Hsp70 proteins are in the flow through (Fig. 31B, left panel, fraction 2) and identified by immunodecoration using prokaryotic antibodies (Fig. 31B, right panel, fraction 2). Another portion of the Hsp70 proteins was eluted at a salt concentration of 250-300 mM KCl (Fig. 31B, left panel, fraction 8). This fraction contains the isHsp70 but no detectable amounts of Css1 as shown by immunodecoration (Fig. 31B, right and left panel, fraction 8). However, subsequent mass spectroscopy analysis reveals the presence of Css1 in this fraction, but only minor amounts of a eukaryotic Hsp70 (data not shown). A similar result was obtained by using solubilised outer envelope vesicles for isHsp70 purification by ATP-Agarose (Fig. 31C). Again Coomassie stainable amounts were eluted from the ATP-Agarose (Fig. 31C, upper panel, lane 6-10) and the presence of isHsp70

was shown by immunodecoration (Fig. 31C, lower panel, lane 6-10). However, mass spectroscopy analysis showed again the presence of Css1 (data not shown). Thus, Css1 co-purified and only minor amounts of the stromal chaperone were sufficient to cover the isHsp70 signal in mass spectroscopy.

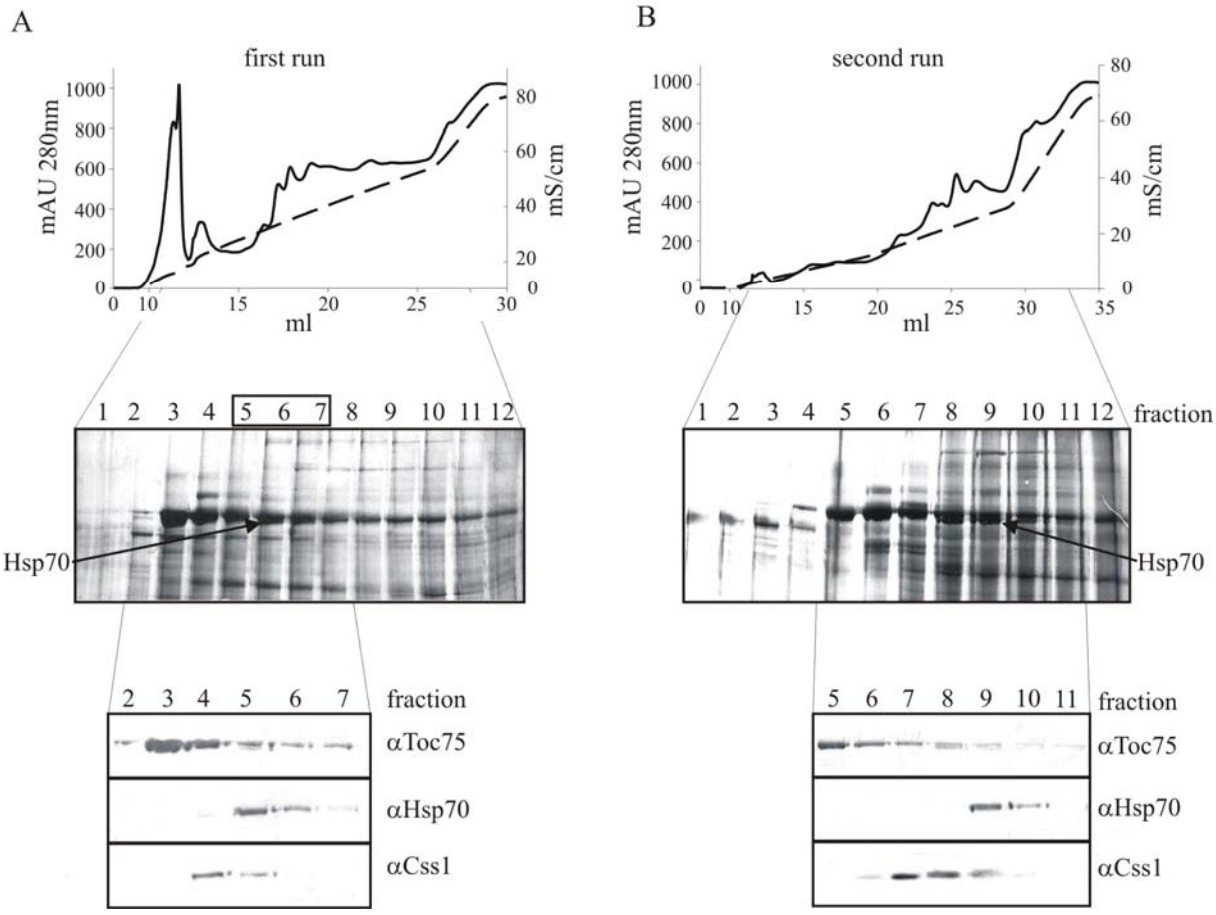

Figure 32. IsHsp70 was purified by anion exchange chromatography. **(A)** Outer envelope vesicles according to 4 mg protein content were extracted by a vast excess of 8 M urea and subjected to MonoQ anion-exchange chromatography. Bound proteins were eluted by a linear potassium gradient (dashed line) as measured by absorbance at 280 nm (solid line). The protein content of 16% of the elution fraction was analysed either by silver-staining (middle panel) or by western blotting and immunodecoration with the indicated antisera (lower panel). **(B)** The isHsp70 containing fraction were pooled, diluted and treated as described under (A). The isHsp70 in the silver-stained gel is marked by arrow.

In parallel, outer envelope vesicle proteins were extracted by incubation with 8M urea. Under this condition a large portion of outer envelope proteins were solubilised (data not shown). The soluble extract was applied on a MonoQ anion exchange chromatography. Running a linear potassium gradient a separation of isHsp70 (Fig. 32A, lower panel, fraction 5-7) from the bulk of Toc75 (Fig. 32A, lower panel, fraction 3 and 4) and Css1 (Fig. 32A, lower panel, fraction 4) was achieved.

Table 5. Peptide masses of the purified isHsp70 are listed. The sequences of the peptide masses yielded by mass spectroscopy (column 1), their localization within the protein (column 2) and their corresponding chaperone (column 3) are listed below. Hsp70 proteins are divided into the ATPase (AD), linker, peptide binding (PBD) and into a domain of unknown function (UFD). Several peptides were found in many Hsp70 proteins, which are indicated as conserved under the protein name (column 3).

Peptide sequence	**Region**	**Protein**
MAGKGEGPAIGIDLGTTYSR	AD	*S. oleracea*, Com70
VEIIANDQGNR	AD	Conserved
TTPSYVAFTDS(T)ER	AD	Conserved
NQVAMSPVNTVFDAKR	AD	*A. thaliana*, Hsc70.1
NQVAMNPI(V)NTVFDAKR	AD	Conserved
VIAGPGDKPMIVVS(Q)YK	AD	*C. maxima*, Hsp70, *O. sativa*, Hsp70
VIPGAGDKPMIVVSYK	AD	*S. oleracea*, Com70
VISGPGDKPMIVVNYK	AD	*N. tabacum*, Hsp70-3
IIAGPAEKPMIVVNYK	AD	*P. sativum*, Hsp71 *B. napus*, Hsc70
HWPFK	AD	*S. oleracea*, Hsc70.1 *M. domesticus*, Hsp70
EFAAEEISSMVLIK	AD	Conserved
QFS(A)AEEISSMVLIKMK	AD	Conserved
NAVVTVPAYFNDSQR	AD	Conserved
EIAEAYLGTTI(V)K	AD	Conserved
EIAEAYLGSNIK	AD	*O. sativa*, putative Hsp70
MKEIAEAF(Y)LGSTVK	AD	*N. tabacum*, Hsp70-3, *S. oleracea*, Com70
NAVVTVPAYFNDSQR	AD	Conserved
QATKDAGVIS(A)GLNVM(R)RIINEPTAAAIAYGLDK	AD	Conserved
IINEPTAAAIAYGLDKK	AD	Conserved
VGEKNVLIFDLGGGTFDVSLLTIEEGIFEVK	AD	Conserved
ATAGDTHLGGEDFDNR	AD	Conserved
KDIMETPGHIR	AD	*S. oleracea*, Com70
M(L)VNHFVQEFKR	AD	Conserved
MVNHSLQEFK	AD	*S. oleracea*, Com70
TLSSTAQTTIEIDSLYEGIDFYSTITR	AD	Conserved
TLSSTAQTTIEIDSLFEGIDFTPR	AD	*Z. mays*, Hsp70
ARFEELNMDLFR	AD	Conserved
FEELNMDLFR	AD	Conserved
MDKST(S)V(I)HDVVLVGGSTR	AD	Conserved
VQQLLQDFFN(D)GK	AD	Conserved
SINPDEAVAYGAAVQAAILSGEGNEK	AD/Linker	Conserved
EQVFSTYSDNQPGVLIQVY(F)EGERTR	PBD	Conserved
FELSGIPPGPR	PBD	*S. oleracea*, Com70
FELTGIPPAPR	PBD	*L. esculentum*, Hsc70
MVQEAEKYK	PBD	Conserved
NA(S)LENYAYNMR	UFD	Conserved
FSSKLDPADK	UFD	*C. sativus*, Hsp70, *C. maxima*, Hsc70.2
LSADDRTK	UFD	*A. thaliana*, Hsp70.2
ELESICNPIIAK	UFD	Conserved
IDDAIEQSIQWLDN(A)NQLAEADEFEDKMK	UFD	*P. hybrida*, Hsp70, *C. sativus*, Hsp70
MKELESVWSTIITK	UFD	*A. thaliana*, Hsp70t-1
MYQGAGGDMDDEGPAPSGGGAGPKIEEVD	UFD	*L. esculentum*, Hsc70

Since the isHsp70 was not sufficiently separated from Toc75 at this stage (Fig. 32A, middle panel, fraction 5-6) the isHsp70 containing fractions (Fig. 32A, box) were pooled and the proteins were separated by a second anion exchange chromatography (Fig. 32B). Again the majority of Toc75 (Fig. 32B, lower panel, fraction 7 and 8) and Css1 (Fig. 32B, lower panel, fraction 6-8) was eluted under lower salt concentrations than isHsp70 (Fig. 32B, lower panel, fraction 9 and 10). Due to less Toc75 the isHsp70 band could be separated (Fig. 32B, middle panel, arrow) and was used for mass spectroscopy analysis. Peptide masses with high similarity to those of eukaryotic Hsp70 proteins were determined (Tab. 5). The peptide masses indicate a eukaryotic identity of isHsp70 and reveals highest sequence similarity to cytosolic chaperones. No peptide mass that matches Hsp70 proteins from the endoplasmatic reticulum was found (Tab. 5). Interestingly, peptide masses of isolated *P. sativum* isHsp70 gave six peptide matching solely Com70 (chloroplast outer membrane protein of 70 kDa; Tab. 5) and a sequence coverage of 39.5% this protein (Tab. 6, Fig. 33, frames sequences).

Table 6. Peptide masses of the purified isHsp70 matching Com70 amino acid sequence are listed. The sequences of the peptide masses yielded by mass spectroscopy (column 1) and their localization within the protein are listed as amino acid position (column 2) and as region in the chaperon (column 3). Hsp70 proteins are divided into the ATPase (AD), linker, peptide binding (PBD) and into a domain of unknown function (UFD).

Peptide sequence	Amino acids	Region
MAGKGEGPAIGIDLGTTYSR	1-20	AD
VEIIANDQGNR	29-39	AD
TTPSYVAFTDSER	40-52	AD
NQVAMNPINTVFDAK	60-74	AD
EIAEAYLGSTVK	133-144	AD
NAVVTVPAYFNDSQR	145-159	AD
QATKDAGVISGLNVMR	160-175	AD
IINEPTAAAIAYGLDKK	176-192	AD
ATAGDTHLGGEDFDNR	227-242	AD
MVNHSLQEFK	243-252	AD
KDIMETPGHIR	257-267	AD
MDKSTVHDVVLVGGSTR	332-348	AD
VQQLLQDFFNGK	352-363	AD
SINPDEAVAYGAAVQAAILSGEG	368-393	AD/Linker
EQVFSTYSDNQPGVLIQVYEGERTR	431-455	PBD
FELSGIPPGPR	465-475	PBD
MVQEAEKYK	524-532	PBD
NALENYAYNMR	546-556	UFD
ELESICNPIIAK	604-615	UFD

Furthermore, an alignment of Com70 to *A. thaliana* cytosolic Hsp70 proteins reveals highest homology to At3g12580 and At1g16030 (Fig. 33), from which At1g16030 was selected before by mRNA and proteomic analysis (Tab. 3; Fig. 30). The identified peptide masses matches to Com70 sequences throughout the entire protein (Fig. 33, framed sequences). In conclusion, the purified Hsp70 from chloroplasts reveals high similarity to cytosolic Hsp70 proteins and biochemical approaches and mass spectroscopy lead to Com70, a protein of the outer envelope membrane of chloroplasts (Ko et al., 1992).

Figure 33. Com70 exhibits high sequence similarity to cytosolic Hsp70 from *A. thaliana*. An alignment of Com 70 with two cytosolic Hsp70 of *A. thaliana* showing highest sequence homology is shown using clustal w. Identical amino acids are shown in a black box and homologues one in grey box. Marked in white boxes are the peptides obtained by mass spectroscopy.

6.4.3 Com70 is most likely identical to isHsp70

The observation that the purified protein comprises peptide masses fitting to Com70 leads to the question whether Com70 is identical to isHsp70. Interestingly, all existing manuscripts investigating either Com70 or isHsp70 have in common that only one Hsp70 was detected in the outer envelope membrane (Marshall et al., 1990, Waegemann and Soll, 1991, Schnell et al., 1994, Kourtz and Ko, 1997). Both chaperones cannot be extracted from the outer envelope membrane of chloroplasts by carbonate treatment and they can be found in association with arrested preproteins (Waegemann and Soll, 1991, Kourtz and Ko, 1997). In contrast to isHsp70, Com70 was described to be thermolysin sensitive (Ko et al., 1992, Kourtz and Ko, 1997). Antisera against a purified Hsp70 from *P. sativum* plastid envelopes were used to screen a *S. oleracea* cDNA library and thereby Com70 was found (Ko et al., 1992). However, all biochemical characterisations of Com70 were performed with *P. sativum* chloroplasts (Ko et al., 1992, Kourtz and Ko, 1997). Since isHsp70 was also described for *P. sativum* plastids (Fig. 18; Marshall et al., 1990) one should expect the detection of two Hsp70 proteins in outer envelope vesicles. Hence, the presence of multiple Hsp70 proteins in isolated chloroplasts or envelope membrane of *P. sativum* was re-investigated by using polyclonal antisera against eukaryotic Hsp70 proteins (Fig. 34A, left panel). In the chloroplast (Fig. 34A, left panel, lane 1) as well as in an outer envelope fraction (Fig. 34A, left panel, lane 2) only one Hsp70 was detected.

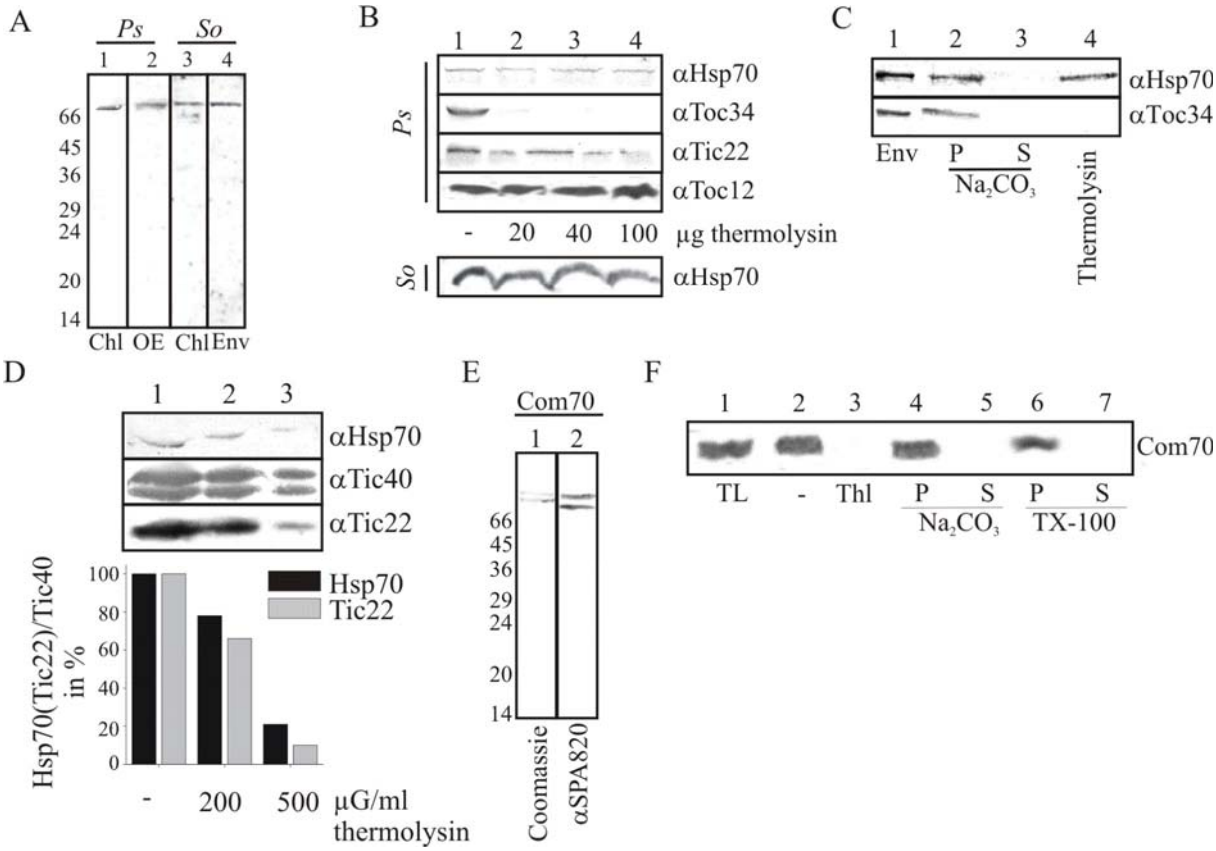

Figure 34. Com70 is likely candidate for isHsp70 **(A)** Isolated chloroplasts (lane 1, 2) and purified outer (lane 3) and mixed envelope fraction (lane 4) according to 20 µg protein content from *P. sativum* (lane 1, 2; *ps*) or *S. oleracea* (lane 3, 4, *so*) were tested for the Hsp70 presence by immunodecoration. **(B)** Isolated *P. sativum* (upper panel, *ps*) or *S. oleracea* chloroplasts (lower panel, *so*) according to 20 µg chlorophyll content (lane 1) were treated with increasing amounts of thermolysin (lane 2-4). After blotting the proteins were immunodecorated with the indicated antisera. **(C)** *S. oleracea* mixed envelope vesicles (20 µg) were either extracted with 100 mM Na_2CO_3 pH 11.4 (lane 2 and 3) or digested with 20 µg thermolysin (lane 4). After extraction proteins in the supernatant were precipitated by tri-chlor-acetic acid treatment. Total protein content were loaded on SDS-PAGE and immunodecorated with antisera against either Hsp70 (upper panel) or Toc34 (lower panel). **(D)** Mixed envelope vesicles were isolated from chloroplasts from *P. sativum* according to 10 mg chlorophyll content that were either non-treated (lane 1) or digested with 200 (lane 2) or 500 µg/ml thermolysin (lane 4). The ratio between Hsp70/Tic40 and Tic22/Tic40 was determined by using AIDA software. **(E)** Com70 was recombinantly expressed, purified and separated by SDS-PAGE. Proteins were either stained with Coomassie (lane 1) or immunodecorated with SPA820 antiserum (lane 2). **(F)** Radio-labelled Com70 (lane 1) was imported into isolated chloroplasts (20 µg) from *P. sativum* (lane 2) and, subsequently, treated with 20 µg thermolysin (lane 3). Further, chloroplasts were incubated with 100mM Na_2CO_3 pH11.4 (lane 4, 5), or 1% Triton X-100 (lane 6, 7) followed by separation of the pellet (4, 6) and supernatant (lane 5, 7). Proteins of the supernatant fractions were precipitated by tri-chloric-acid. Samples were separated and subjected to SDS-PAGE followed by visualisation via autoradiography.

To exclude any specific feature of *S. oleracea* plastid the Hsp70 presence was also tested for isolated chloroplasts and mixed envelopes of *S. oleracea* (Fig. 34A, right panel, lane 3 and 4). In line with *P. sativum* chloroplasts solely one Hsp70 was detected (Fig. 34A, left panel, lane 3 and 4). Furthermore, for both plastids no digestion of Hsp70 was detected (Fig. 34B), whereas the cytosolic exposed Toc34 from *P. sativum* is already digested at low thermolysin concentrations (Fig. 34B, upper panel). Proteins of the intermembrane space like Tic22 and Toc12 remain unaffected (Fig. 34B, upper panel). Similar to *P. sativum* outer envelope vesicles (Fig. 18B) the strongly membrane attached Hsp70 (Fig.

34C, lane 2) of mixed *S. oleracea* envelopes is not degraded upon thermolysin treatment (Fig. 34C, lane 4). The topology of these vesicles is right-side-out since Toc34 is digested by thermolysin (Fig. 34C, lane 4). Kourtz and Ko (1997) based their topology on the observation that the Com70 content in mixed envelopes isolated from thermolysin treated *P. sativum* chloroplasts, decrease in comparison to the inner membrane protein Cim37 (chloroplasts inner membrane protein of 37 kDa). Performing the same procedure a drastic decrease of isHsp70 in comparison to the inner envelope protein Tic40 was observed (Fig. 34D). Moreover, for Tic22 (Fig. 34D) and for Toc12 (data not shown) similar ratios as for Hsp70 to Tic40 were detected. Therefore, the thermolysin treatment of intact chloroplasts with subsequent subfractionation leads to a reduced amount of an intermembrane space component. All the presented data support the conclusion that the detected Hsp70 is not exposed to the cytosol. To confirm that Com70 as well as isHsp70 are detected by the same antibodies Com70 was expressed, purified (Fig. 34E, lane 1) and subsequently immunodecorated with SPA820 (Fig. 34E, lane 2). The isHsp70 specific antiserum (Schnell et al., 1994) recognises also Com70 (Fig. 34E). Finally, it was reported that *in vitro* translated Com70 is thermolysin sensitive after integration into the outer envelope membrane of *P. sativum* chloroplasts (Wu et al., 1994). However, the authors did not perform any carbonate extraction to show membrane integration (Wu et al., 1994). To test whether translated Com70 (Fig. 34F, lane 1) is integrated into the outer envelope membrane an import experiment into isolated chloroplasts was performed. As described before the chloroplast associated Com70 (Fig. 34F, lane 2) was thermolysin sensitive (Fig. 34F, lane 3; Wu et al., 1994). Performing carbonate extraction Com70 was found in the membrane fraction and not in the soluble supernatant (Fig. 34F, lane 4 and 5). To elucidate whether the observed pellet fraction of Com70 might be due to precipitation of the translated protein the chloroplasts were solubilised with detergent after Com70 was imported (Fig. 34F, lane 6 and 7). Under these conditions membrane inserted proteins like Toc12 were found in the supernatant (data not shown). Com70 was found in the pellet fraction indicating a precipitation of the protein (Fig. 34F, lane 6). Therefore, the protein was not integrated into the outer chloroplasts membrane and can not be used for topology determination, although stated differently (Wu et al., 1994). Summarising all presented results, Com70 exhibits the same features as isHsp70 of *P. sativum* and no additional cytosol exposed Hsp70 was detected (Fig. 34B). Thus, Com70 is a most likely the isHsp70. However, the presented data are not sufficient to elucidate the molecular identity of isHsp70. Future work has to be done to give a final answer on this issue.

7. Discussion

7.1. Preprotein recognition and translocation by the Toc core translocon

According to existing (Jelic et al., 2002, Schleiff et al., 2003b) and to the presented data Toc34 acts as an initial receptor (Fig. 35). This conclusion is based on multiple observations. First, Toc34 interacts with phosphorylated preprotein whereas Toc159 recognises only non-phosphorylated one (Jelic, personal communication, Sveshnikova et al., 2000). Previously, it was shown that prior to complementation of translocation across the envelope membrane dephosphorylation has to occur (Waegemann and Soll, 1996). According to the presented results dephosphorylation takes place when the preprotein is transferred from Toc34 to Toc159 (Fig. 6). Second, addition of the N-terminal peptide of pSSU transit sequence to the reconstituted Toc complex affects binding of pSSU in the absence of GMP-PNP (Fig. 6B). In contrast, the C-terminal phosphorylated peptide reduces the amount of bound pSSU only in the presence of GMP-PNP (Fig. 6B), which is required for recognition by Toc34 (Fig. 5C). Under this condition the N-terminal part of pSSU presequence does not inhibit pSSU binding to proteoliposomes (Fig. 6B). These observations indicate that the recognition of the phosphorylated C-terminal peptide by Toc34 (Fig. 5D) precedes that one of the N-terminal part of the presequence by Toc159 (Fig. 5B). Third, Toc34 was predominantly found in association with cross-linked preprotein at early import stages, whereas Toc159 interaction was more pronounced at later stages (Kouranov and Schnell, 1997). Fourth, Toc159 was found to provide the driving force for translocation by GTP-hydrolysis (Schleiff et al., 2003b). Fifth, the stoichiometry of Toc159: Toc75: Toc34 in the Toc complex is 1:4:4-5 (Schleiff et al., 2003a) suggesting Toc34 to function as a receptor and Toc159 as a central catalytic motor for translocation. Sixth, the receptor function of Toc34 can be switched of by phosphorylation (Jelic et al., 2002), which occurs most likely during early import stages. Seventh, in contrast to Toc159, Toc34 is not essential for the translocation process (Fig. 6C) and probably serves as a gate to regulate advancement to the channel. This notion is supported by the observation that the *at*Toc33 knock out pale phenotype but not the Toc159 knock out can be recovered during development (Jarvis et al., 1998). It can be speculated that Toc34 is part of a timing mechanism and defines the targeting fidelity such that the precursor has to stay bound to Toc34 long enough for the phosphate to be removed in order for it to be passed to the motor and the channel. Alternatively to the presented model Toc159 was suggested as initial receptor (Schnell and Kessler, 2004). This hypothesis is based on the presence of a soluble pool of Toc159 (Hiltbrunner et al., 2001). Here, Toc34 is thought to act docking site for the preprotein loaded Toc159 (Kessler and Schnell, 2004). The existence of such soluble pool is still under debate. Further analysis of the soluble Toc159 reveals that it is present in lipid shreds, which derived from partial chloroplasts envelope disruption (Vojta, personal communication). Another argument for the soluble Toc159 was the detection of a Toc159 GFP-fusion protein in the cytosol (Smith et al., 2002). The construct was expressed in isolated *A. thaliana* protoplasts under the control of cauliflower mosaic virus 35S promoter (Bauer et al., 2002). However, even mild expression of tagged proteins can lead to mis-sorting to the cytosol and to the

endoplasmatic reticulum as shown for several mitochondrial proteins (Sickmann et al., 2003). The relevance of the proposed soluble Toc159 in preprotein targeting was not demonstrated. Finally, it was not shown how an integral membrane protein like Toc159 (Hiltbrunner et al., 2001) can shuttle between a soluble and membrane bound pool. Another argument for Toc159 as initial receptor is the presence of four isoforms in *A. thaliana*, which recognise different subsets of preproteins (Kubis et al., 2004, Ivanova et al., 2004). For Toc34 two isoforms in *A. thaliana* are described (Soll and Schleiff, 2004). The homologue of *ps*Toc34, *at*Toc33, was found in a complex with *at*Toc159 and seems to catalyse the import of photosynthetic preproteins (Ivanova et al., 2004). It was further suggested that *at*Toc90 may be a component of the same import pathways (Hiltbrunner et al., 2004). This notion is based on the observation that a double knock out plant of *at*Toc159 and *at*Toc90 reveals a more drastic reduction of accumulation of photosynthetic proteins in chloroplasts than *at*Toc159 knock out plant alone (Hiltbrunner et al., 2004). In contrast, *at*Toc34 is associated with *at*Toc120 and *at*Toc132, which are important for import of housekeeping preproteins (Ivanova et al., 2004). In conclusion, both suggested import pathways involve a different Toc34 isoform, whereas two Toc159 isoforms might act in the same pathway (Ivanova et al., 2004). In line with a crucial role of Toc34 proteins a homozygous double knock out of *at*Toc33 and *at*Toc34 is not viable (Constan et al., 2004). Hence, despite of the reduced number of Toc34 isoforms in comparison to Toc159 paralogs each import pathway involves one Toc34 isoform, which might define preprotein specificity. Regarding this aspect different substrate specificities for *at*Toc33 and *at*Toc34 were already reported (Jelic et al., 2003, Ivanova et al., 2004) Therefore, the presence of four Toc159 isoforms does not contract the model of Toc34 as initial receptor.

In addition to the receptor function, the effect of GTP-binding and hydrolysis of Toc159 and Toc34 on Toc core complex stability was addressed. Thereby, Toc159 seems to play a central role. The Toc core translocon is stabilised upon GMP-PNP loading (Fig. 7). The observed lower complex stability upon GDP loading was attributed to dissociation of Toc34 from $Toc159_{GDP}$ *in vitro* (Fig. 8). Toc159 acts as a GTP-dependent motor during translocation across a membrane (Schleiff et al., 2003b). A conformational change upon GTP-hydrolysis by Toc159 is proposed in order to fulfil the pushing mechanism. Therefore, GDP loaded Toc159 might provide an unfavourable conformation for Toc34 interaction. However, the observed association of Toc159 and Toc34 in the presence of GMP-PNP and the dissociation in the presence of GDP is not consistent with previously proposed GDP enhanced interaction between the G-domains of *at*Toc33 and *at*Toc159 (Smith et al., 2002). There, *in vitro* translated *at*Toc33 was used. The presence of soluble factors such as molecular chaperones influencing the interaction between the two receptors in the translated system can not be excluded. In the here shown experiments the presence of such factors can be precluded since the purity of expressed Toc34ΔTM (data not shown) and of the gel-eluted $Toc159_f$ was controlled (Fig. 4A). In addition, the interaction of the G-domains of both proteins was investigated (Smith et al., 2002), whereas here $Toc159_f$ was used comprising G- and M-domain. An effect of the M-domain on the G-

domain activity cannot be excluded. The GTP-induced association of the receptors is further strengthened by the presence of the transit peptide of pSSU (Fig. 8), suggesting that a GMP-PNP loaded Toc complex is open for preprotein uptake. Interestingly, such a formation of trimeric intermediates between two GTP loaded receptor proteins, namely SRP and SRP receptor, and a ribosome nascent chain complex was also found in the co-translational import into the endoplasmatic reticulum (Keenan et al., 2001). Here, GTP hydrolysis by SRP and SRP receptor leads to a dissociation of the complex and the ribosome nascent chain complex is transferred to the translocation channel (Keenan et al., 2001).

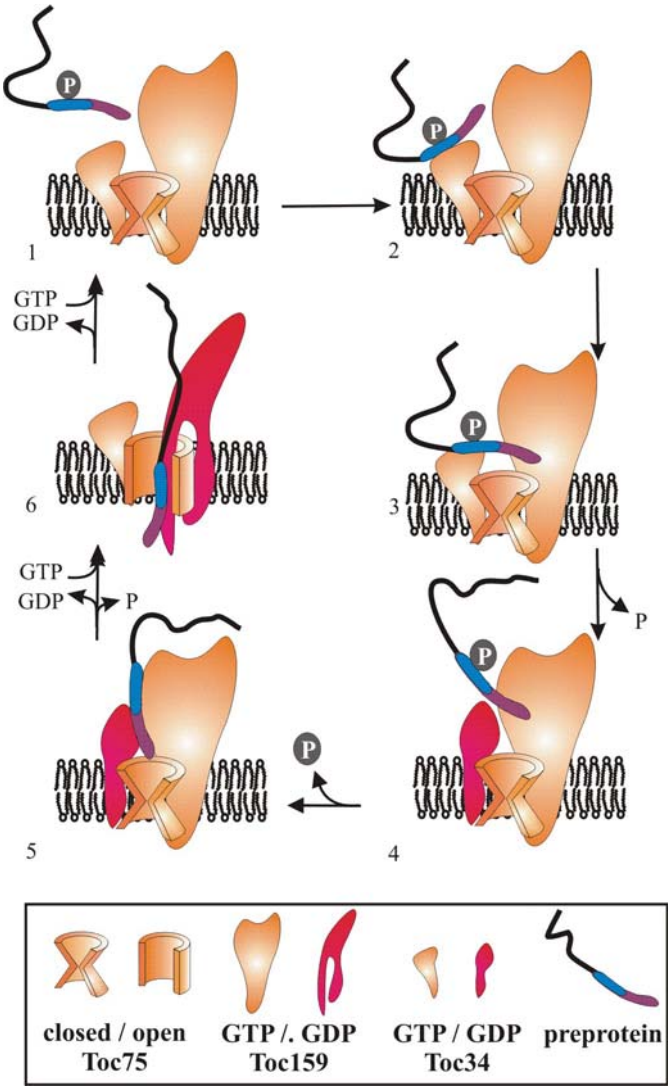

Figure 35. A proposed model of Toc core complex action during preprotein recognition and translocation is shown. For further details see text.

Based on the results a model of pSSU recognition and translocation across the Toc complex was proposed (Fig. 35). The phosphorylated C-terminal part of pSSU presequence is first recognised by $Toc34_{GTP}$ (Schleiff et al., 2002; Fig. 35, stage 2). Subsequently, by recognition of the N-terminal part of the pSSU presequence by Toc159 an intermediary trimeric complex is formed, consisting of Toc159, Toc34 and the preprotein (Fig. 35, stage 4). The interaction of the preprotein with Toc34

stimulates GTPase activity of the receptor leading to a dissociation of Toc34 from the preprotein (Jelic et al., 2002; Fig. 35, stage 3 and 4). After dephosphorylation by an unidentified phosphatase, the C-terminal part induces GTP-hydrolysis of Toc159 (Fig. 35, stage 5) by which the receptor pushes the preprotein through the translocation channel (Fig. 35, stage 6; Schleiff et al., 2003b). Upon GDP to GTP exchange the Toc complex is transferred to its GTP loaded state and it is prepared for the next round of preprotein uptake (Fig. 35, stage 1).

Therefore, the transport across the outer envelope membrane of chloroplasts is a highly regulated event. The process is catalysed by GTP hydrolysis as the driving force for the initial steps. In contrast, protein import across the mitochondrial outer membrane does not depend on any nucleotides (Rehling et al., 2004). It is a primarily forced by the increasing affinities of the preproteins to the receptor closer to the translocation channel than to the more distant one. This view is based on the interaction of the positively charged preprotein with acidic receptor domains and called the acid chain hypothesis (Komiya et al., 1998). Regulatory elements remain unknown. Although plastid and mitochondria arose from endosymbiotic event the translocation across the Toc core translocon differs in demands of energy and regulation drastically from the import via the Tom core complex.

7.2. The dynamically associated Toc64

Previously, another receptor of the Toc machinery, namely Toc64, was identified (Sohrt and Soll, 2000). Toc64 was linked to the Toc translocon by chemical cross-linking (Sohrt and Soll, 2000), but it was not be co-purified with the Toc core complex during sucrose density centrifugation (Schleiff et al., 2003a). Here, a dynamic association of this component with the Toc machinery was shown (Fig. 11). Based on co-immunoprecipiation and *in vitro* binding assays (Fig. 11) Toc34 was identified as interacting partner of Toc64 in the Toc core complex. This association is mediated by the cytosolic exposed TPR-domain of Toc64 (Fig. 11D). Furthermore, the receptor function of Toc64 was established (Fig. 10). The clamp-type TPR-domain of Toc64 recognises the C-terminal MEEVD motif of Hsp90 proteins, which guides preproteins to the chloroplasts surface (Qbadou, personal communication). Based on the presented data (Fig. 12) a model of the preprotein transfer between the Toc64 and Toc34 is proposed (Fig. 36). Preproteins guided by Hsp90 (Fig. 36, stage 1) are recognised by Toc64 (Fig. 36, stage 2). The chaperone loaded Toc64 receptor associates with the GMP-PNP loaded Toc34 (Fig. 36, stage 3). Thereby, the TPR-domain can bind Hsp90 and Toc34 simultaneously (Fig. 12, Fig. 36, stage 3). Upon addition of ATP the preprotein is released from Toc64 (Fig. 12B) and can now be recognised by Toc34 (Fig. 12B, Fig. 36, stage 4). It remains to be clarified whether the chaperone also dissociates from the receptor at this step. Preprotein loaded Toc34 does not interact with Toc64 (Fig. 12C). Therefore, Toc64 dissociates from preprotein loaded Toc34 and is open for new uptake of Hsp90 guided preproteins (Fig. 36, stage 4).

This receptor function of Toc64 might be restricted to a specific set of preproteins since the amount of Toc64 in outer envelope vesicles is four fold reduced in comparison to Toc34 (Fig. 26). Interestingly,

Tom70 of the mitochondrial outer membrane import apparatus shares several features of Toc64. First, it dynamically associates with the Tom core complex (Künkele et al., 1998). However, this interaction has not been studied in detail. Second, it exposes a TPR-domain towards the cytosol, which was shown to interact with chaperones affiliating preproteins (Young et al., 2003). Third, the amount of Tom70 is two to five fold reduced in comparison to the receptor components and Tom40 in the purified Tom translocon (Künkele et al., 1998). Fourth, Tom70 is not essential for preprotein import (Rehling et al., 2004). Also for Toc64 a triple knock out in *A. thaliana* is viable (Aronsson, H., personal communication). Supporting the functional proximity of these two proteins Tom70 of *A. thaliana* mitochondria might be replaced by Toc64-V (Chew et al., 2004).

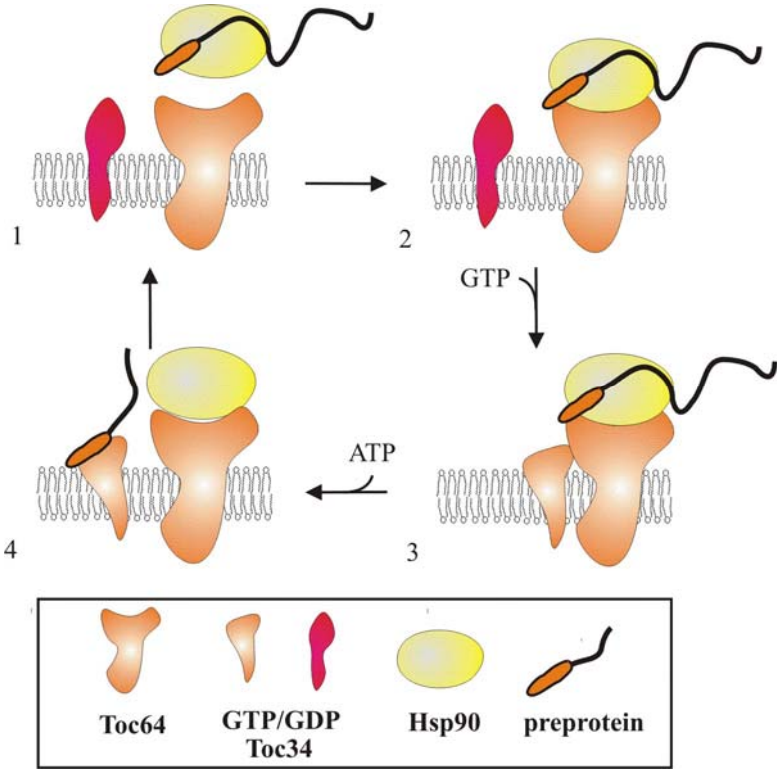

Figure 36. A proposed model of preprotein transfer between Toc64 and Toc34 is shown. For further detail compare text.

Moreover, it was shown that Tom70 mediates import of a specific set of preproteins. This set encompasses primarily hydrophobic proteins from the inner membrane (Brix et al., 1997), whereas matrix proteins circumvent Tom70 and interacts directly with the central receptor Tom20 of the Tom complex. Consistent with the mitochondrial system the thylakoid protein pOE33 engage the TPR domain of Toc64 during import, whereas the stromal pSSU not (Qbadou, personal communication). Future work has to be done in order to characterise the preproteins interacting with Toc64. As for Toc64 and Toc34 an ATP dependent transfer of preproteins from Tom70 to Tom20 was demonstrated *in vitro* (Komiya et al., 1997). In this system an intermediate trimeric complex between Tom70, Tom20 and the preprotein was described (Komiya et al., 1997), which is consistent with observed trimeric complex between Toc64, Toc34 and Hsp90 (Fig. 12A). Moreover, an interaction between

Tom20 and Tom70 was reported by chemical cross-linking and co-immunoprecipiation *in situ* (Haucke et al., 1996). Similar to Toc34/Toc64 the association of Tom70 and Tom20 is mediated by a TPR domain of Tom20 (Haucke et al., 1996). For Tom70 a direct interaction to preproteins was shown (Brix et al., 1997). As for Toc64 a second preprotein recognition site different from the clamp-type TPR domain in a 25kDa core domain was reported (Brix et al., 2000). In contrast to Tom70, Toc64 exposes a large portion towards the intermembrane space (Qbadou, personal communication), which directly binds preproteins (Fig. 10).

Interestingly, the recognition of preprotein guided by chaperones via TPR-domains is similar between the mitochondrial and the plastid import apparatus. This is somewhat surprising since the Toc core complex action provides more similarity to the Sec machinery than to the mitochondrial system. But TPR domain containing proteins seems to be widely used in preprotein import in eukaryotic cells. Sec72 of the posttranslational import into the endoplasmatic reticulum and Pex5 of the peroxisomal protein translocation machinery are two receptor proteins with TPR domains (Gatto et al., 2000, Ponting 2000). However, an interaction to cytosolic chaperones of Pex5 and Sec72 remain to be investigated (Ponting 2000, Harper et al., 2003). A common feature of the mitochondria and plastid import systems is therefore the action of a TPR-domain for a subset of preproteins, which coordinates the interplay with the cytosolic unfolding and targeting action of chaperones with the translocation event.

7.3. The intermembrane space translocon

Translocation of chloroplastic proteins is assisted by a translocon combining proteins of the inner and outer envelope membranes. The initial steps of preprotein translocation by the Toc complex were discussed above and presented in two models of the proposed mechanisms (Fig. 35 and Fig. 36). In contrast to the initial steps of 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, the function of this protein remains elusive. A second protein discussed to be involved in protein translocation across the intermembrane space is an outer envelope isHsp70 facing the intermembrane space (Marshall et al. 1990, Waegemann and Soll 1991, Schnell et al. 1994). In here, the link between the chaperone and the protein translocation machinery through the newly identified component of the Toc complex, namely Toc12, was presented (Fig. 18). Toc12 is integrated in the outer envelope and forms a complex with Toc64, Tic22 and isHsp70 (Fig. 13, Fig 20 and Fig. 22). Its intermembrane space exposed J-domain stimulates the ATPase activity of Hsp70 proteins and recruits isHsp70 in an ATP dependent manner to the intermembrane space translocon (Fig.19 and Fig. 25). The close association of Toc12 to Toc64, which interacts with Hsp90 guiding a preprotein (Fig. 36), suggests the following model. The guided preprotein is recognised by Toc64 via association of the chaperones with the TPR-domain (Fig. 36). This leads on one hand to the

release of the preprotein and subsequent transfer via Toc34 to the Toc core complex (Fig. 36) and on the other hand to the activation of Toc12. While the translocation across the outer envelope occurs in a GTP driven manner by Toc159 (Schleiff et al. 2003b) Toc12 recruits isHsp70 to the complex (Fig. 37, step 1). The interaction between Hsp70 in the ATP bound stage and the intermembrane space translocon is dependent on a functional J-domain (Fig. 25). Upon ATP-hydrolysis induced by Toc12 the chaperone is transferred to an incoming preprotein (Fig. 37, step 2). This explains the preprotein dependent stabilisation of the translocon in the presence of ADP (Fig. 24B). 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 (Fig. 37, step 3 and 4). The preprotein is released to later steps in protein translocation involving the Tic translocon.

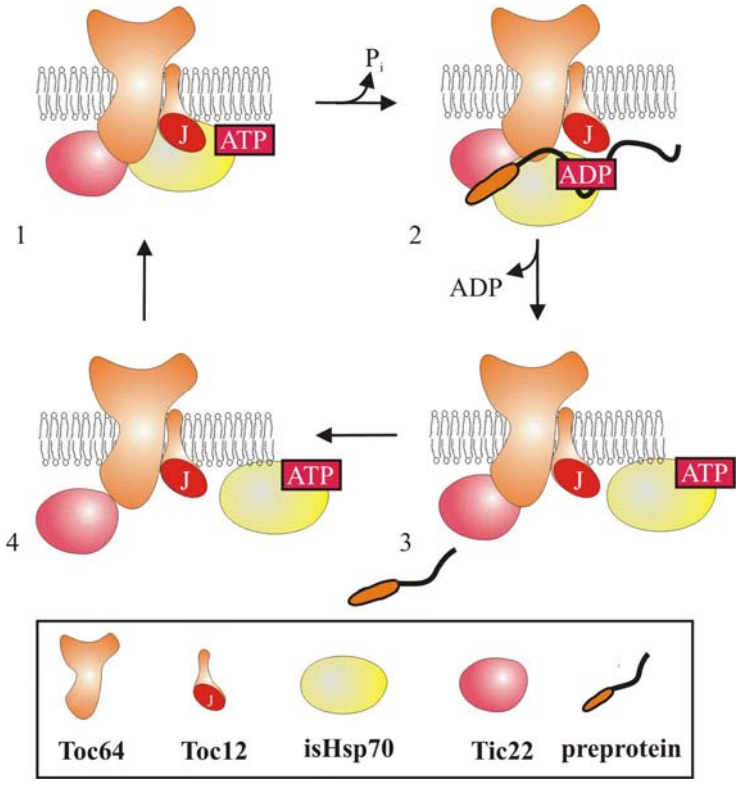

Figure 37. A proposed model of isHsp70 recruitment to the intermembrane space translocon by Toc12 action is depicted. For a detailed description see text.

Such translocation systems involving a Hsp70 and a J-protein are already described for the Sec translocon of the endoplasmatic reticulum, BiP and Sec63, and for the translocation across the inner mitochondrial membrane mediated by the Pam complex, Tim14/Pam18 and mtHsp70 (Fig. 3B and C; Corsi and Schekman, 1997, Rehling et al., 2004). Like Toc12 also Sec63 and Tim14/Pam18 lack a zinc binding region and belong to the type III class of J-proteins (Sadler et al. 1989, Kelley, 1998, Mokranjac et al. 2003, Truscott et al. 2003). The zinc binding domain of the *E. coli* DnaJ interacts to substrates of DnaK and delivers this cargo to the chaperone (Bukau and Horwich, 1998). In all three transport machines the J-proteins recruit 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 line with this interpretation no preprotein interaction of expressed Toc12 was observed (Fig. 23B). Interestingly, in the mitochondrial matrix Hep1/Zim17/Tim14 was identified (Buri et al., 2004, Yamamoto et al., 2005, Sichting et al., 2005). This protein comprises a zinc binding region. An involvement in preprotein import was suggested based on reduced import efficiencies in the Hep1/Tim17/Tim14 knock out strain (Buri et al., 2004, Yamamoto et al., 2005). However, further studies reveal a function in mtHsp70 stabilisation in its nucleotide free state (Sichting et al., 2005). Therefore, the impact on preprotein import in the Hep1/Zim17/Tim14 knock out strain is most likely an indirect effect. J-domains of the translocation systems seem to be specific for their Hsp70 protein. The J-domain of Tim14/Pam18 cannot be replaced neither by the J-domain of the matrix Mdj1p nor by that one of Toc12 using the yeast *in vivo* system (Hell, personal communication). This observation implicates the involvement of further structural elements in the J-protein Hsp70 interaction *in vivo*. Such features might be provided by other translocon components since *in vitro* Toc12 J-domain was capable to stimulate the ATPase activity of the heterelogous DnaK (Fig. 19). Indeed, in the Pam complex the involvement of Tim44, Tim16/Pam16 and the nucleotide-exchange factor Mge1 influence mtHsp70 action (Rehling et al., 2004). In the case of Tim16/Pam16 a regulatory impact on Tim14/Pam18 interaction to mtHsp70 was reported (Li et al., 2004). The driving force for translocation across the inner mitochondrial membrane is provided by an ATP consuming cycle of preprotein binding and releasing by mtHsp70. A similar function was shown for BiP and Sec63 of the endoplasmatic reticulum (Corsi and Schekman, 1997). The here presented model suggests a completion of the transverse of preprotein across the Toc complex by isHsp70 action. However, to substantiate this notion the mode of isHsp70 action and the identity of potential further co-chaperones remain to be investigated.

Alternatively, a comparison of the chloroplast with the mitochondrial system another functional model of the intermembrane space translocon seems reasonable. For the translocation of proteins across the intermembrane space of mitochondria 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. Therefore, it can not be ruled out that intermembrane translocon exhibits similar function as the small Tim complexes that are restricted to a certain class of proteins. In this model the isHsp70 association will keep the protein partly unfolded, whereas Tic22 could transport the protein toward the Tic complex. Moreover, hydrophobic preproteins recognised by Tom70 are preferentially delivered by Tim9/Tim10 action to the Tim22 complex, where the integration into the inner membrane takes place (Rehling et al., 2004). This would be in line with the observation that isHsp70 and Toc12 were found in close association to Toc64 (Fig. 21). It further implicates a divergence of two import pathways after passing Toc75 like it was shown for the mitochondrial system (Rehling et al., 2004). In line with this consideration Tic110

was found in association with Tic55 and Tic62 on the one hand and Tic20 and Tic22 on the other hand (Küchler et al., 2002, Kouranov et al., 1998). However, the presence of two Tic complexes is not shown yet and remains speculative. The idea of a specific set of preproteins utilising the intermembrane space translocon is supported by the fact that Toc64, Toc12, Tic22 and isHsp70 are less abundant in comparison to Toc159, Toc75 and Toc34 in isolated outer envelope vesicles. For example, Toc34 is present in a four to five fold higher amount than Toc64 (Fig. 26). In line with a specialised function is the observation that the mRNA level of Toc12 in leaves is lower than in roots or stems (Fig. 15D). A similar result was observed for *at*Toc34, where a higher RNA level was found in stems and roots compared to 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 specific function during chloroplast development.

Another interesting feature of Toc12 is the stabilisation of the J-domain fold by two cysteines in the loop region (Fig. 18). The relevance of a disulfide-bridge between the two cysteines was demonstrated by its missing interaction to isHsp70 after exchanging one cysteine by point mutation (Fig. 25). The structure of the loop region of other J-domains is stabilised by proline in the loop region in a large number of J-proteins (for example in Hsp40, Ydj1p, Sis1p, Sec63p and more, Fig. 14) 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 stabilise the structure (Hritz, personal communication). The disulfide-bridge might also be important for the regulation of the translocation event, since reversible disulfide-bridge formation is a wide spread 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 proteins 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, since folding of the preprotein in the intermembrane space leads to its arrest.

Summarising, a new component of the Toc complex was identified with unique features in regard to its size, the membrane anchor and the localisation 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.

8. Conclusion

In the presented work the translocation process of preproteins across the outer chloroplast envelope membrane facilitating the Toc complex was analysed. Based on the obtained results three mechanistic models were presented. A subset of preproteins guided by Hsp90 is recognised by the clamp-type TPR of Toc64 and the subsequent transfer to Toc34 is analysed. This process resembles the preprotein delivery from Tom70 to Tom 20 in the mitochondria import system. Further transport to the translocation channel Toc75 involves the concerted action of the two GTPases Toc34 and Toc159. Dephosphorylation of the preprotein has to occur before the passage of the translocation pore via Toc159. In order to complete the transport across the outer envelope membrane an isHsp70 action might be involved. Therefore, the translocation process across the outer chloroplast envelope membrane requires energy at different stages. In summary, the Toc complex seems more regulated than the Tom complex. Here, the driving force is provided by increasing affinities of the receptor domains towards the preprotein. In contrast to the detailed characterisation of the import events on the cytosolic site the understanding of the intermembrane space translocon and the mode of transfer to the Tic complex is at the beginning. For further elucidations the identity of isHsp70 has to be clarified. This work suggests Com70 as one candidate for isHsp70, which has to be clarified further. The unambiguous identification of Toc12 and isHsp70 in the *A. thaliana* genome enables knock out analysis, which might reflect the importance of such chaperone system in protein import. Moreover, using the proteoliposome system with reconstituted Toc core and intermembrane space complex might provide further view in side the requirements of the latter translocon. Therefore, the presented work not only gives answers to several discussed issues but also opens new questions, which should be addressed in future work.

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10. Publications:

Original papers:

Schleiff, E., Eichacker, L.A., Eckart, K., **Becker, T.**, Mirus, O., Stahl, T., and Soll, J. (2003) Prediction of the plant beta-barrel proteome: a case study of the chloroplast outer envelope. Protein Sci. 12, 748-759.

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Reviews:

Becker T., Qbadou S., Jelic, M. and Schleiff E. (2005) Let´s talk about…chloroplast import. Plant Biol. 7, 1-14.

Becker T., Soll, J., and Schleiff E. (2005) Chaperone systems in chloroplasts. In Handbook of protein folding. Edited by Kiefhaber, T. and Buchner J. , 1019-1064.

Poster and Conferences:

Small in size but complex in function – the novel Toc12. **Becker, T.,** Soll, J. and Schleiff E. on Predocsymposium Heidelberg (2002)

Nobelprice winner meeting in Lindau (2003). Invited by Bayrische Chemieverbände und by Verband Bayerischer Metall- und Elektroindustrie.

Small in size but complex in function – the novel Toc12. **Becker, T.** Schleiff, E. on ESF – Meeting Protein Targeting Spa, Belgium (2003)

Toc12 – A novel component of the chloroplast outer envelope translocon with unique features. **Becker T.** and Schleiff E. on SFB-Meeting Molecular Machines, Munich, Germany (2003)

Toc12 regulates isHsp70. **Becker T.**, Soll, J. and Schleiff, E. on Proteinsorting, Freiburg, Germany (2005)

Toc12 – A novel component of the chloroplast outer envelope translocon with unique features. **Becker, T.**, Soll J. and Schleiff, E. on Gordon Conferences Protein transport across membranes. New London , USA (2005).

Talks

Sitting in the VIPP lounge: Tocs, Tics and Oeps. (2003) MÜPF-Meeting, Freising, Germany.

Proteinimport in Chloroplasten. ÄKTA User Club (2003), Breisach, Germany.

Protein translocation into chloroplasts Structure and function of the Toc complex. (2004) SFB594-Meeting, Munich, Germany

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Curriculum vitae

4.10.1977	Birth in Marienhospital in Osnabrück
1984	Begin of the primary school at the Elisabethschule in Osnabrück
1988	Begin of the intermediate school at the Orientierungsstufe Dom in Osnabrück
1990	Begin of the high school at the Ursulaschule in Osnabrück
10.6.1997	End of school time with passing the exame
24.9.1997	Exclusion from military service
1.10.1997	Start of the biology study at the Christian-Albrechts University in Kiel
25.10.1999	Passing the prediploma exame with the mark “sehr gut“ (1.0)
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17.7.2001-11.3.2002	Diploma thesis in cell biology in the working group of Prof. Dr. J. Soll at the Institut of Botany of the Christian-Albrechts-University of Kiel
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1.5.2002	Start of the PhD- thesis in the working group of Prof. Dr. J. Soll at the Department of Botany at the Ludwig-Maximilians University of Munich

Ehrenwörtliche Versicherung

Die vorliegende Dissertation wurde von Dipl. Biol. Thomas Becker selbständig und ohne unerlaubte Hilfe angefertigt. Der Verfasser hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommision weder in Teilen noch als Ganzes vorgelegt.

Thomas Becker, München 20.5.05