The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64

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Precursor protein targeting toward organellar surfaces is assisted by different cytosolic chaperones. We demonstrate that the chloroplast protein translocon subunit Toc64 is the docking site for Hsp90 affiliated preproteins. Thereby, Hsp90 is recognised by the clamp type TPR domain of Toc64. The subsequent transfer of the preprotein from Toc64 to the major receptor of the Toc complex, namely Toc34, is affinity driven and nucleotide dependent. We propose that Toc64 acts as an initial docking site for Hsp90 associated precursor proteins. We outline a mechanism in which chaperones are recruited for a specific targeting event by a membrane-inserted receptor.

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Introduction

Most chloroplast proteins are nuclear encoded, synthesised on cytosolic ribosomes as precursor proteins, and imported into the organelle via translocation complexes in the outer and inner envelope membrane of chloroplasts (Jarvis and Robinson, 2004; Kessler and Schnell, 2004; Soll and Schleiff, 2004). While the translocation process itself is understood in some molecular detail, the mechanism by which the preproteins are transferred from the cytosol to the Toc translocon remains still elusive. In case of chloroplasts, it is proposed that phosphorylation of some transit peptides (e.g. of the small subunit of RubisCO (pSSU)) enhances the import rate presumably through interaction with 14-3-3 proteins. It was postulated that 14-3-3 proteins together with Hsp70 form a guidance complex for targeting of phosphorylated preproteins to the chloroplast surface (May and Soll, 2000). Cytosolic Hsp70 is generally involved in the folding of newly synthesised proteins (Bukau *et al*, 2000; Young *et al*, 2004), and its association seems to be required to keep the preproteins in an import competent unfolded state (Soll and Schleiff, 2004). The translocon for preprotein transport across

the outer envelope consists of three precursor binding proteins with known or proposed functions, Toc34, Toc159 and Toc64 (translocon of the outer envelope of chloroplasts; for a review, see Kessler and Schnell, 2004; Soll and Schleiff, 2004). Toc159 and Toc34 are GTPases regulated by phosphorylation. Toc34 acts as initial receptor within the Toc core complex composed of Toc34, Toc159 and Toc75 (Schleiff *et al*, 2003; Becker *et al*, 2004), whereas Toc159 is crucial for the import process (Bauer *et al*, 2000) by facilitating the translocation event (Schleiff *et al*, 2003). In contrast to both GTPases, not much is known about Toc64. Toc64 is dynamically associated with the Toc core complex in pea (Schleiff *et al*, 2003b). It contains three tetratricopeptide repeats (TPR; Sohrt and Soll, 2000), which share some similarity to such motifs of proteins acting as cofactors of Hsp90 and Hsp70 chaperones or of the mitochondrial protein import receptor Tom70. The TPR domain of Tom70 mediates the association of chaperone affiliated preproteins (Young *et al*, 2003). The similarity of the TPR domains of Tom70 and Toc64 is in line with the observation that one member of the Toc64 protein family in *Arabidopsis thaliana* seems to replace Tom70 in plant mitochondria (Chew *et al*, 2004). Peptide regions composed of three TPRs are organised into a super-helical structure (Scheufler *et al*, 2000) and form a dicarboxylate clamp coordinating the C-terminal aspartate residue conserved in the C-terminal sequence of Hsp90 and/or Hsp70. The specificity for either Hsp70 or Hsp90 is determined by hydrophobic contacts with neighbouring residues (Scheufler *et al*, 2000). However, the function of the TPR domain in Toc64 is still elusive. Here, we demonstrate that the C-terminal TPR domain of Toc64 recognises precursor proteins via interaction with Hsp90. The preprotein is further transferred toward the Toc core complex. This transfer is achieved by a GTP-dependent association of Toc64 with Toc34.

Results

Interaction of precursor proteins with Toc64

To elucidate a role of Toc64 in preprotein translocation into chloroplasts, recombinant proteins were used to inhibit import of preproteins (Figure 1A). The translocation of wheat germ translated (wgt) ³⁵S-labelled precursor of the small subunit of RubisCO (pSSU) into isolated organelles was reduced by 80% in the presence of 10 µM of Toc64 (Figure 1B, Toc64 and Figure 1C, black circle, Supplementary data) or of Toc34ΔTM_{GTP} from *Pisum sativum* (Toc34, white circle), but not of cBag (the C-terminal domain of the Bcl2-associated anthanogene; Figure 1B and C, grey circle). cBag was found to inhibit the Hsp70 dependent translocation into mitochondria (Young *et al*, 2003). This finding indicates that Hsp70 might not be essential for targeting. The influence of the Toc proteins used is not restricted to pSSU since the translocation of the precursor form of the thylakoid lumen localised oxygen evolving complex subunit of 33 kDa (pOE33) containing a bipartite targeting signal was also reduced by more than 80%

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Figure 1 Toc64 is a preprotein receptor. **(A)** Toc34 and Toc64 constructs are shown. Domains are highlighted (HR, hydrophobic region). Line represents deleted TM section of Toc34. **(B)** Chloroplasts (20 µg chlorophyll) were incubated with wgt-³⁵S-labelled pSSU in the presence of increasing amounts (1–10 µM final, lanes 3–6) of cBAG (upper part), Toc64 (middle part) or Toc34ΔTM (lower part) at 25°C (lanes 2–6) for 10 min. A control was kept at 4°C (lane 1). Chloroplasts were re-isolated and import was visualised by phosphor-imaging (p, precursor; m, mature protein). **(C)** As in (B), chloroplasts (20 µg chlorophyll) were incubated with wgt-³⁵S-labelled pSSU (circle) or pOE33 (triangle) in the presence of increasing amounts of cBAG (grey), Toc64 (black) or Toc34ΔTM (white). Chloroplasts were re-isolated, import was visualised by phosphor-imaging, quantified and compared to import without competitor. The average of at least three independent experiments is shown. Error bars are omitted for clarity. **(D)** Wgt-³⁵S-labelled pOE33 (top), pSSU (middle) or mSSU (bottom) were incubated with a Toc64 (lanes 2 and 3), Toc34 (lanes 4 and 5) or BSA (lanes 6 and 7) affinity matrix. Proteins in final wash (W) and elution (E) are visualised. Lane 1 (TP) shows 20% of the protein loaded.

of the control in the presence of 10 µM Toc64 (black triangle), but significantly less in the presence of 10 µM Toc34ΔTM_{GTP} (white triangle). Furthermore, both wgt-preproteins bind to a matrix coated with either receptor (Figure 1D, lanes 3 and 5),

but only with background levels to a BSA coated matrix (lane 7). The recognition of preproteins is transit sequence dependent since no interaction of the mature form of SSU with either Toc34 or Toc64 above background level was observed (lanes 3 and 5). Summarising, we conclude that Toc64 and Toc34 act as receptors for preproteins.

To identify the domains of Toc64 acting as receptor for preproteins, two polypeptides representing either Toc64ΔTPR or Toc64TPR (Figure 2A) were used for binding studies. Toc64TPR inhibited the translocation of pOE33 into chloroplasts (Figure 2B, TPR, lanes 3–6 and Figure 1C, black triangle) with the same efficiency as full-length Toc64 (Figure 1C). Toc64ΔTPR competed with a five times lower efficiency when compared to the full-length receptor (Figure 2B, ΔTPR, lanes 3–6 and Figure 1C, white triangle). Surprisingly, the translocation of pSSU was not affected by addition of Toc64TPR (Figure 2C, black circle, Supplementary data), but by addition of Toc64ΔTPR (white circle). In line, we observed a strong association of pSSU with a Toc64ΔTPR affinity matrix (Figure 2D, lane 5, ΔTPR and Figure 2F), but only a weak interaction with a Toc64TPR coated matrix (Figure 2D, lane 7, TPR). In contrast, pOE33 was recognised by Toc64TPR (lane 7, TPR), but less efficient by Toc64ΔTPR (lane 5, ΔTPR). The latter result is in line with the low capacity of Toc64ΔTPR to compete for the translocation of pOE33 into chloroplasts. Analyzing the binding of reticulocyte lysate translated (rlt) pSSU and pOE33 to Toc64 showed a similar association of pSSU with Toc64 (Figure 2E, lane 3) as with Toc64ΔTPR. In contrast, the interaction of pOE33 with Toc64TPR is reduced to the level of Toc64ΔTPR when rlt-preprotein is used (lane 6, compare with Figure 2D, lanes 3, 5 and 7).

To confirm that the observed differential recognition by Toc64 is not limited to pOE33 and pSSU, the association of the nucleotide transport protein 1 (pNTT1), the precursor of the malate dehydrogenase (pMDH), ferredoxin (pFd) or plastocyanine 1 (pPC) with the different Toc64 polypeptides was analysed (Figure 2D and F). Like for pOE33, an association of wgt-pNTT1, pPC with the TPR was observed. In contrast, the stromal proteins pMDH and pFd behaved as pSSU when translated in wheat germ lysate. Therefore, distinct preproteins contain additional information, which results in targeting via Toc64.

The observed differential affinity of the two domains of Toc64 was further explored by their ability to compete for the interaction between wgt-pOE33 or wgt-pSSU and full-length Toc64 (Figure 3A, Supplementary data). Toc64TPR efficiently competed for the association between Toc64 and pOE33 (Figure 3B, triangle), but not for the association between Toc64 and pSSU (circle). In line, significant higher concentrations of ΔTPR than of TPR are required for competition of the pOE33–Toc64 interaction (Figure 3B and C triangles). The opposite result was obtained for pSSU (circles). Therefore, different domains of Toc64 facilitate the recognition of pSSU and pOE33. The recognition of preproteins by the TPR domain of Toc64 is translation system dependent, whereas preproteins are recognised by the N-terminal domain with lower affinity in a translation system independent manner (Figure 2E). To distinguish between a direct and an indirect interaction, a matrix charged with Toc64 was incubated with wgt-pOE33 in the presence of heterologous-expressed pOE33 as competitor. Here, only a weak competition was obtained

(Figure 3D, triangle) since about 90% of the wgt-pOE33 remained bound to Toc64 even in the presence of 0.8 μ M pOE33. In contrast, expressed pSSU competed efficiently for the binding of wgt-pSSU to Toc64 (circle). This indicates that a specificity factor present in the wheat germ lysate might mediate the interaction of wgt-pOE33 with the TPR region,

whereas the association between wgt-pSSU seems to be direct to the N-terminal domain of Toc64.

Toc64 recognises Hsp90 associated precursor proteins

Since our observations indicate a mediated interaction between wgt-pOE33 and Toc64, it was tested if ATP addition elutes wgt-pOE33 from a Toc64 or Toc34 charged matrix (Figure 4A). Indeed, ATP eluted the bound preprotein from Toc64 (lane 3), but not from Toc34. This suggests an interaction of Toc64 with ATP dependent factors, for example, chaperones affiliated pOE33 in the cytosol. In contrast, wgt-pSSU bound to Toc64 was not eluted in the presence of ATP supporting a differential mode of recognition by Toc64. Previously, it was speculated (Sohrt and Soll, 2000) that Toc64 might be the docking site for a cytosolic guidance complex for chloroplastic preproteins, which consist of at least 14-3-3 and Hsp70 (May and Soll, 2000). To test this notion, soluble leaf extract from *P. sativum* or wheat germ lysate was incubated with a Toc64, Toc34 or BSA matrix (Figure 4B and C). We obtained an interaction of the major guidance complex component 14-3-3 with Toc34, but not with Toc64 (Figure 4C). The interaction between 14-3-3 and import components were further confirmed by incubation of purified Toc34 and 14-3-3 in solution and subsequent co-immunoprecipitation by α 14-3-3 or α Toc34 antibodies (Figure 4D, lane 3). The precipitation observed was found to be specific since preimmunserum was not able to precipitate the complex (Figure 4E). When Toc64 was preincubated with 14-3-3, no complex could be precipitated by α 14-3-3 or α Toc64 antibodies (Figure 4F, lane 3). To control for an influence of Toc64 on the interaction of Toc34 with 14-3-3, both receptor proteins and 14-3-3 were mixed. Again, only Toc34, but not Toc64 was precipitated by the 14-3-3 antibodies (Figure 4G, lane 3). Taken these results, we suggest that Toc34 assembles the initial receptor for the guidance complex delivered preproteins.

So far we established that Toc64 interacts with wgt-pOE33 in an ATP-dependent manner (Figure 4A) but does not recognise the guidance complex (Figure 4B–G). To analyse, which chaperones are associated with the different pre-

Figure 2 Different preprotein recognition sites of Toc64. (A) Toc64 constructs are shown, lines represent deleted sections. (B) Chloroplasts (20 μ g chlorophyll) were incubated with wgt- 35 S-labelled pOE33 in the presence of 1–10 μ M (final, lanes 3–6) Toc64 Δ TPR (upper part) or Toc64TPR (lower part) at 25°C (lanes 2–5) for 10 min. A control was kept at 4°C (lane 1). Chloroplasts were re-isolated and import was visualised (p, precursor; i, stromal intermediate; m, mature protein). (C) As in (B), chloroplasts (20 μ g chlorophyll) were incubated with wgt- 35 S-labelled pOE33 (triangle) or pSSU (circle) in the presence of 1–10 μ M Toc64 Δ TPR (white) or Toc64TPR (black). Translocation efficiency was quantified after re-isolation of chloroplasts and compared to import without competitor. The average of three independent experiments is shown. (D) Wgt- 35 S-labelled pSSU (top), MDH (second), Ferredoxin (third), pOE33 (forth), NTT1 (fifth) or PC (bottom) were incubated with a Toc64 (lanes 2 and 3), Toc64 Δ TPR (lanes 4 and 5), Toc64TPR (lanes 6 and 7) or BSA (lanes 8 and 9) matrix. Proteins of wash (W) and elution fraction (E) are visualised. Lane 1 shows 20% of the labelled protein used (TP). (E) Rlt- 35 S-labelled pSSU (lane 1, 100% loading) or pOE33 (TP, lane 4, 100% loading) were incubated with a Toc64, Toc64 Δ TPR (Δ TPR), Toc64TPR (TPR) or BSA matrix. Proteins of wash (W) and elution (E) are visualised. (F) Quantification of multiple experiments as in (D) and (E) is provided. Values are given in percent input.

Figure 3 Preprotein recognition and transfer. (A) The experimental scheme including the Symbol legend is given. (B–D) Wgt ^{35}S -labelled pOE33 (triangle) or pSSU (circle) were incubated with a Toc64 matrix. After sufficient wash, bound proteins were eluted by increasing amounts of expressed Toc64TPR (B), Toc64 Δ TPR (C), pSSU (for pSSU, D) or pOE33 (for pOE33, D). The amount of bound preprotein was quantified and is shown as percent of initial bound protein. The average of at least three independent results is shown.

Figure 4 Toc64 recognises preproteins associated with Hsp90. (A) Wgt ^{35}S -labelled pOE33 or pSSU (lane 1, 20% of total) was bound to Toc34 or Toc64 affinity matrix and eluted by ATP. Proteins of wash (lane 2), elution (lane 3) and remaining on the column after ATP treatment (lane 4) were visualised. (B, C) Cytosolic extract from *P. sativum* (B) or wheat germ (C) was incubated with a Toc34, Toc64 or BSA matrix. Flow through (lane 1, B) or 10% loading (lane 1, C), wash (lane 2) and elution (lane 3) were immunodecorated using α 14-3-3 antibodies. (D) Expressed *at*14-3-3- ϵ was incubated with Toc34 Δ TM (10% shown in lane 1), precipitated by α 14-3-3 (top) or α Toc34 antibodies and loaded protein, the wash (lane 2) and elution (lane 3) immunodecorated with the complementary antibody. (E) As in (D) but precipitated with α 14-3-3 (lane 1) or preimmuneserum (lane 2) and the precipitate decorated with α Toc34 antibodies. (F) Expressed *at*14-3-3- ϵ was incubated with Toc64 (10% shown in lane 1), precipitated by α 14-3-3 (top) or α Toc64 antibodies and loaded protein, the wash (lane 2) and elution (lane 3) immunodecorated with the complementary antibody. (G) Expressed *at*14-3-3- ϵ was incubated with Toc64 and Toc34 (10% shown in lane 1), precipitated by α 14-3-3 and loaded protein, the wash (lane 2) and elution (lane 3) immunodecorated with the complementary antibody. (H) Rlt-pOE33 (–r), wgt-pOE33 (–wg) or wgt-pSSU (lane 1) was precipitated by preimmuneserum (lane 2), α Hsp70 (lane 3), α Hsp90 (lane 4) or α 14-3-3 antibodies (lane 5). The precipitated proteins were visualised by autoradiography. (I) Wgt pSSU (black bar) or pOE33 (grey bar) was incubated with Toc64 matrix in the presence of the C-terminal domain of Hsp90 (C90) or 14-3-3- ϵ . The subsequent binding of preproteins was quantified and compared to binding without competitor. (J) Translocation efficiency of pSSU (circle) or pOE33 (triangle) in the presence of increasing amounts of the C90 construct was quantified and compared to import without competitor. The average of three independent experiments is shown.

proteins, wgt-pSSU, wgt-pOE33 or rlt-pOE33 (pOE33-r, Figure 4H) were immunoprecipitated using antibodies against 14-3-3 (lane 5), Hsp70 (lane 3) or Hsp90 (lane 4). All proteins were precipitated by Hsp70 antibodies, but only pSSU by 14-3-3 antibodies. Here, wgt-pOE33, but not rlt-pOE33, was most efficiently precipitated by α Hsp90 (lane 4) even though Hsp90 is present in reticulocyte lysate (Young *et al*, 2003) and recognised by our antibodies (not shown).

Hence, wgt-pOE33 seems to be associated with a complex distinct from the previously identified guidance complex (May and Soll, 2000). When wgt-pSSU (Figure 4I, black bar) or wgt-pOE33 (grey bar) were incubated with a Toc64 charged matrix in the presence of the C-terminal domain of *human*Hsp90 (aa 566–732, Figure 4I, C90; Young *et al*, 2003), only the interaction of pOE33 with Toc64 was reduced by 60%, whereas the interaction between pSSU and Toc64 was

not affected. In line with this observation, the import of pOE33 into chloroplasts (Figure 4J, triangle), but not of pSSU is reduced in the presence of C90 (circle). This supports that pOE33 is directed to Toc64 via Hsp90 since C90 is known to compete for the recognition of Hsp90 by TPR domains (Young *et al*, 2003). In contrast, the association between pSSU and Toc64 was largely decreased in the presence of 14-3-3 (Figure 4I, black bar), most likely due to a direct competition for binding sites within the transit sequence, since 14-3-3 does not interact with Toc64 (Figure 4B, C, F and G, lane 3), but with the preprotein (Figure 4H, lane 5). 14-3-3 also reduced the association of pOE33 and Toc64 (Figure 4I, grey bar) even though the effect of 14-3-3 was not as pronounced as for pSSU. This might be explained by the similar fold of 14-3-3 proteins and TPR domains (Das *et al*, 1998). 14-3-3 would therefore account as competitor by recognition of chaperones *in vitro* when added in chemical amounts. Summarising, the association between Toc64 and wgt-pOE33 resembles the behaviour of a chaperone-mediated interaction.

The Hsp90 is recognised by the TPR domain

The association of Hsp70 and Hsp90 to pOE33 (Figure 4) leads to the question which chaperone is recognised by Toc64. Therefore, Toc64, TPR and Δ TPR (Figure 2A) were incubated with a matrix charged with a polypeptide reflecting the C-terminal portion of human Hsp70 (C70, aa 383–646, Figure 5A, lanes 1–3) or Hsp90 (C90, lanes 4–6). Toc64 and Toc64TPR bound to the C90 (lane 6), but not to the C70 matrix (lane 3). In addition, a molar excess of a peptide representing the 23 C-terminal amino acids of Hsp90 (P90, Figure 5B, lane 4), but not the peptide representing the 25 C-terminal amino acids of Hsp70 (P70, lane 3) is able to compete for the interaction between Toc64TPR and the C-terminal construct of Hsp90 (C90). In contrast, Toc64 recognises both, Hsp70 and Hsp90 present in wheat germ lysate as determined by their interaction with a matrix charged with Toc64 or Toc64TPR (Figure 5C, upper panel). Here, Toc64 recognises Hsp70 with higher efficiency than Hsp90 (Figure 5C, lane 2), whereas the TPR region revealed a stronger interaction with Hsp90 (lane 4) suggesting that the transmembrane region present in Toc64 acts as substrate for Hsp70. Silver staining of the fractions eluted from the affinity matrices revealed the association of three proteins. Hsp90 and Hsp70 were confirmed by immunodecoration and mass spectrometry (identified peptides matched to gi5123910 and gi2827002, not shown). The protein of 50 kDa was identified as tubulin (Figure 5C, square); however, the specificity of this interaction remains elusive. To analyse the specificity of the chaperone interaction, wheat germ lysate was supplemented by additional cytosolic *pea*Hsp70 prior to incubation with Toc64TPR. Again, no competition of Hsp90 recognition was observed (Figure 5D). Similarly, incubating increasing amounts of wheat germ lysate with a Toc64TPR matrix enhanced the interaction of Hsp90, but not of Hsp70 (Figure 5E). Further, incubating wheat germ lysate with the TPR matrix in the presence of the C90 construct reduced the interaction of Hsp90 but not of Hsp70 (Figure 5F). All results together confirm that Hsp90 is recognised with higher affinity than Hsp70. In line, incubating Toc64-TPR matrix with a soluble extract from pea, a more pronounced association of

Figure 5 Toc64 interacts with chaperones. (A) Toc64, Δ TPR, or TPR were incubated with a C70 (C-terminus of Hsp70; lanes 1–3) or C90-matrix (lanes 4–6). Flow through (FT), wash (W) and elution (E) was subjected onto SDS-PAGE and visualised by silver-staining. (B) A Toc64TPR matrix was incubated with a synthetic peptide representing the C-terminus of Hsp70 (lane 3) or Hsp90 (lane 4) followed by incubation with 10 times lower molar amount of the C90 fragment (lanes 2–4). For control, a Δ TPR charged matrix was incubated with C90 (lane 5). The loading (lane 1) and elutions are shown (lanes 2–5). (C) Wheat germ lysate was incubated with a Toc64 (lanes 1 and 2) or TPR (lanes 3 and 4) matrix. Proteins from wash (W) and elution (E) were either immunodecorated by α Hsp70 or α Hsp90 (WB) antibodies or silver stained (bottom). Square indicates tubulin. (D–F) Wheat germ lysate (L, lane 1 shows 25%) was incubated with a TPR matrix (lanes 2–7) in the presence of 0.4 μ M (D, lanes 4 and 5) or 0.8 μ M additional Hsp70 (D, lanes 6 and 7) or of 0.4 μ M (F, lanes 4 and 5) or 0.8 μ M of expressed C90 (F, lanes 6 and 7). In (E) the amount of wheat germ lysate used was 20 μ l (E, lanes 2 and 3), 40 μ l (E, lanes 4 and 5) or 100 μ l (E, lanes 6 and 7; L, lane 1 shows 5 μ l). Proteins from final wash (W) and elution (E) were immunodecorated by α Hsp70 (bottom) or α Hsp90 (top). (G) Lysate from *P. sativum* (L, lane 1 shows 25%) was incubated with a Toc64 matrix. Wash (lane 2) and elution (lane 3) were immunodecorated by α Hsp70 or α Hsp90 antibodies. (H) The binding efficiency of Hsp90 or Hsp70 (as in G) was quantified for at least three independent experiments and is given as percent of input.

Hsp90 (60% of total) in comparison to Hsp70 (15% of total) was obtained (Figure 5G, lane 1 versus 3, and Figure 5H).

The TPR domain of Toc64 assembles a clamp type fold as shown by its alignment with the TPR of Hop and Tom70 (Figure 6A). We therefore introduced point mutations specifically reducing the interaction with chaperones (Scheufler *et al*, 2000). Replacing arginine 550 facing the chaperone-binding pocket within Toc64TPR by alanine (Figure 6B, 5th helix, red) reduces the interaction of Toc64TPR with Hsp90 or Hsp70 from wheat germ lysate by 70–80% (Figure 6C, lanes 3, 5 and 7, and Figure 6D). In contrast, Toc64TPR_{N516A}

(Figure 6B, 3rd helix, red) still recognises Hsp70, but not Hsp90 (Figure 6C, lane 9 and Figure 6D). In line with this observation, Toc64TPR_{N516A} perturbs the import of pOE33 into chloroplasts only very little (Figure 6E) and recognises pOE33 only with a significantly reduced efficiency when compared to wild-type TPR (Figure 6F). In summary, the clamp type TPR motif of Toc64 interacts with Hsp90 but also with Hsp70 though with low efficiency. However, Hsp70 seems not to mediate the recognition of preproteins on the chloroplast surface since addition of C70 does not interfere with the recognition and subsequent import of pOE33 (data not shown). Furthermore, pOE33 import into isolated chloro-

plasts was not affected by the presence of cBag (Figure 1). This C-terminal domain of Bag-1 binds to the ATPase domain of Hsp70 to promote the exchange of ADP to ATP (Brehmer *et al*, 2001), which leads to the dissociation of the polypeptide from Hsp70. In contrast, cBag does not affect the Hsp90 substrate interaction (Young and Hartl, 2000). Therefore, in case of an Hsp70-mediated interaction with Toc64, the presence of cBag should have reduced the recognition and subsequent translocation of the preprotein. All results together suggest a receptor function of the TPR domain of Toc64 preferentially for Hsp90 bound preproteins.

The pOE33 guiding complex

After demonstrating that pOE33 is associated with cytosolic chaperones (Figure 4), which are recognised by Toc64 (Figures 5 and 6), we analysed the complex itself. Subjecting wgt-pOE33 in the presence of ADP to size exclusion chromatography revealed an average size of about 350 kDa for the complex (Figure 7A, filled circles). Shifting the experimental conditions from 4 to 25°C significantly decreased the amount of complex while purification (not shown). When ATP is added, which stimulates the dissociation of the chaperones, almost no complex was obtained (open circle). Comparing the distribution of pSSU translated in wheat germ (triangle), it becomes obvious that both proteins assemble different complexes.

The association of pOE33 with Hsp90 was subsequently probed by immunoprecipitating the protein from fractions of the gradient (Figure 7B). The amount of protein precipitated was highest in the peak fraction. However, a minor amount of protein was still precipitated from the fraction of higher and lower molecular weight. This might reflect the portion of complex assembled pOE33 in these fractions considering normal distributions of complexes in a size exclusion experiment (indicated in Figure 7A, bottom). In line pOE33 of the peak fractions binds to Toc64TPR (Figure 7B, bottom). Therefore, we conclude that pOE33 is assembled in a complex distinct from that targeting pSSU.

Figure 6 Toc64 interaction with chaperones is specific. **(A)** An alignment of psToc64TPR (aa 477–593), hsStyl1 (aa 4–120, TPR1, aa 225–247, TPR2A) and scTom70 (aa 99–213) is shown. Circles indicate positions of electrostatic interaction of TPR2A with the C-terminus of Hsp90 and squares positions of hydrophobic interactions. Closed symbols mark amino acids mutated in Toc64. **(B)** A Toc64TPR model based on the TPR1 domain of Hop (Scheufler *et al*, 2000) is shown as ribbon representation. Conserved amino acids interacting with the peptide electrostatically (yellow, red) or by hydrophobic and van der Waals forces (green; Scheufler *et al*, 2000) are highlighted. Red indicates mutated amino acids. **(C)** Wheat germ lysate (L, lane 1 shows 25%) was incubated with a Toc64 (lanes 2 and 3), TPR (lanes 4 and 5), TPR_{R550A} (lanes 6 and 7) or TPR_{N516A} (lanes 8 and 9) matrix. Proteins from final wash (W) and elution (E) were immunodecorated with αHsp70 (bottom) or αHsp90 (top) antibodies. **(D)** At least three experiments as in **(C)** were quantified and binding efficiency of Hsp90 (black) or Hsp70 (grey) normalised to interaction of the chaperones to Toc64. **(E)** Chloroplasts (20 μg chlorophyll) were incubated with wgt-³⁵S-labelled pOE33 in the presence of indicated amounts of Toc64TPR (solid line) or Toc64TPR_{N516A} (dashed line) at 25°C (lanes 2–5) for 10 min. Translocation efficiency of pOE33 (triangle) was quantified and compared to import without competitor. The average of at least three independent experiments is shown. **(F)** Wgt ³⁵S-labelled pOE33 was incubated with a Toc64TPR or Toc64TPR_{N516A} matrix. The elutions were collected, the amount of ³⁵S-labelled pOE33 quantified and normalised to association of pOE33 to Toc64TPR.

Figure 7 The pOE33 guiding complex. (A) Wgt ^{35}S -labelled pOE33 (circle) or pSSU (triangle) was incubated with ADP (closed symbol) or ATP (open symbol) and subjected onto Superdex 200 at 4°C. The amount of pOE33 in indicated fractions was quantified. The column was calibrated with standard molecules for size exclusion chromatography (white triangle). On the bottom, the distribution of the two peaks is indicated by a gaussian distribution (lines). (B) Indicated fractions of the size exclusion separation (in A), 10% shown in lane 1) was incubated with an affinity matrix charged with Toc34 (top) or Toc64 (bottom) after addition of ADP (lane 2) or ATP (lane 3). The latter lanes show the eluted protein. (C) Fraction II of the size exclusion (see A, 10% shown in lane 1) was incubated with an affinity matrix charged with Toc34 (top) or Toc64 (bottom) after addition of ADP (lane 2) or ATP (lane 3). The latter lanes show the eluted protein. (D) Wgt ^{35}S -labelled pOE33 was subjected onto glycerol gradient centrifugation. The density direction is given on the bottom. The grey area indicates fraction where RubisCO was detected. Four fractions (I–IV) were incubated with a TPR-matrix (top panel, right). Flow through (F) and elution (E) are shown. pOE33 was subsequently immunoprecipitated from fraction III by αHsp90 antibodies (bottom). (E) Wgt ^{35}S -labelled pOE33 (–) was incubated with apyrase (AP), 1 mM ADP (ADP) or geldanamycin (GA) and subjected to a glycerol gradient centrifugation. After fractionation, the amount of pOE33 in high molecular weight complexes was quantified and normalised to loading. The amount of complexed pOE33 is shown in relation to treatment with geldanamycin. (F) Wgt ^{35}S -labelled pOE33 (shown are 10%, L) was incubated with ADP (lanes 3–8) or TPR (lanes 6–8) prior to immunoprecipitation by αToc12 (lanes 3 and 6) or αHsp90 antibodies (lanes 4, 5, 7 and 8). The precipitate of Hsp90 antibodies were further immunoprecipitated by antisera against Toc64 (αHsp90 → αToc64). Shown are one representative wash step (lane 2) and elutions.

To probe, if this complex can also be recognised by Toc34, the complex was incubated with ADP (Figure 7C, lane 2) or ATP (lane 3) and with Toc34 or Toc64, respectively. As before, Toc64 was able to interact with the complex in the presence of ADP, but this binding was drastically reduced in the presence of ATP, which leads to a disassembly of the complex (Figure 7A). In contrast, Toc34 only interacts with the pre-protein after the complex is dissociated (Figure 7C, lane 2 versus 3). To confirm the presence of the complex observed by size exclusion, wgt-pOE33 was subjected to a glycerol

gradient centrifugation in the presence of geldanamycin (Figure 7D). The peak fractions co-migrating with the RubisCO complex (II, III, grey region) contain the recognised form of pOE33 since only the preprotein present in these fractions reveals a high affinity for a Toc64TPR matrix (right, top). In addition, an association of pOE33 present in fraction III of the glycerol gradient with Hsp90 could be demonstrated (III, bottom). However, this method is limited by the extended experimental time explaining the lower abundance of the complex in comparison to the size exclusion purification. To

test the stability of the complex, wgt-pOE33 was treated with apyrase, ADP or geldanamycin (GD), which is known to stabilise the substrate binding of Hsp90 specifically (Young and Hartl, 2000). After separation of wgt-pOE33 by a glycerol gradient we obtained the largest amount of complex in the presence of geldanamycin (Figure 7E), and the smallest without addition (–). We conclude that the complex formation is dependent on precursor recognition by Hsp90.

So far we demonstrated that pOE33 is part of complex containing Hsp90, which is recognised by Toc64. To further demonstrate that the entire complex is indeed recognised by the TPR domain, wgt-pOE33 was co-precipitated by α Hsp90 and α Toc12 antibodies (latter for control) in the absence or presence of Toc64TPR (Figure 7F). As seen before, α Hsp90 antibodies efficiently precipitated pOE33 (lanes 4 and 7) independent of the presence of the TPR. When the eluted proteins were precipitated by α Toc64 antibodies, complex formation could be confirmed (lane 8). Hence, we can conclude that Toc64 indeed recognises the cytosolic complex containing pOE33 and Hsp90.

To demonstrate the participation of Hsp90 in targeting of preproteins to Toc64, wgt pSSU and pOE33 were imported into chloroplasts *in vitro* (Figure 8A) or GFP fusions of the preproteins *in vivo* (Figure 8B) in the absence or presence of geldanamycin. In the presence of geldanamycin, a reduced translocation of pOE33, but not of pSSU, was obtained in both experiments. *In vitro* the translocation was reduced by 40%. The inhibition efficiency resembles the previously reported reduction of translocation of Tom70 dependent precursor into mitochondria in the presence of geldanamycin (Young *et al*, 2003). To further investigate the role of Toc64, a loss of function mutant for Toc64-III (At3g17970) was analysed (Figure 8C). Plants of selected line 1 contain a homozygote T-DNA insertion since no gene specific PCR

product could be obtained (Figure 8D, lane 2). Furthermore, these plants do not contain Toc64-III protein (Figure 8E), nor *toc64-III* mRNA (Figure 8F). The transcript level of *toc64-I* or *toc64-V* is not altered. However, these two proteins can not replace Toc64 in the outer envelope of chloroplasts since Toc64-V is a mitochondrial receptor, and Toc64-I does not contain a TPR domain (Chew *et al*, 2004). In line with the previous report, the plants did not show a visible growth phenotype (Rosenbaum-Hofmann and Theg, 2005). However, in contrast to the previous report, where translocation of not Hsp90 associated rti-pOE33 (Figure 4H) into chloroplasts of mutants was not altered when compared to wild type, the initial rate of translocation of wgt-pOE33 associated with Hsp90 (Figure 8G and H) into chloroplasts from the knock out line was reduced (about 40%, see inset). A similar reduction was reported for the translocation of proteins dependent on the TPR domain containing Tom70 in the deletion strain of this receptor (Hines *et al*, 1990; Young *et al*, 2003). Summarising, Toc64 is the receptor for preproteins delivered by a complex including the cytosolic Hsp90.

The functional association of Toc64 with the Toc core complex

Toc64 represents the docking site for Hsp90 delivered preproteins. Hence, we investigated the association of Toc64 with the Toc complex. The Toc core components co-precipitated by α Toc64 antibodies in the presence of GMP-PNP, a nonhydrolysable analogue of GTP (Figure 9A, lane 2), but not in the presence of GDP (lane 3) using solubilised OEV's. This finding was confirmed by precipitation using α Toc34 antibodies (Figure 9B). The OE protein Oep24 was not precipitated (Figure 9A, lanes 2 and 3), suggesting a specific interaction between the Toc core components and Toc64. The GTP dependence of the interaction points toward an

Figure 8 Protein translocation depends on recognition of Hsp90 by Toc64. (A) Wgt-pSSU (upper panel, white bar) or pOE33 (lower panel, black) were imported into isolated chloroplasts (20 μ g chlorophyll) after mock (lane 2) or geldanamycin treatment (lanes 3 and 4) of translation product (lane 1 shows 10 %). Proteins of the re-isolated chloroplasts were separated by SDS-PAGE, visualised by phosphorimaging and import efficiency (appearance of mature protein) quantified using AIDA software. The relation of import of geldanamycin treated to mock treated translation products is depicted. (B) Tobacco protoplasts were transformed with plasmids encoding pSSU-GFP or pOE33-GFP. At 16 h after transfection radioactive labelled methionine was added and cells incubated for 2 h in the absence (lane 1) or presence (lane 2) of geldanamycin. The mature protein was immunoprecipitated by GFP antibodies. (C–F) Plants of salk line 087087 (T-DNA insertion model in C) were grown on soil. T-DNA insertion was analysed by PCR using UTR specific and UTR/T-DNA specific primer pairs (D). Chloroplasts (10 μ g chlorophyll, E) of wild type (lane 1) or knock out plants (lane 2) were separated on SDS-PAGE, immunodecorated using indicated antibodies (top) or stained by coomassie blue (CB). Isolated mRNA was used for RT-PCR for indicated cycles amplifying the indicated genes (F, actin is used for loading control). (G) Wgt-pOE33 (lane 1, 10% translation product) was incubated with chloroplasts isolated from wild type (lanes 2–4, closed circles) or knock out line 1 (lanes 5 and 6, open circles) plants for the indicated times. (H) The import efficiency (G) was quantified for three independent experiments. The reduction of pOE33 import into line 1 chloroplasts compared to wild-type chloroplasts is shown as inset.

association of Toc64 with either Toc159 or Toc34. Using purified proteins, an interaction of Toc64 with Toc34_{GMP-PNP}, but not with Toc159_{GMP-PNP} (Figure 9C, lane 2) could be established. This interaction is mediated by the cytosolic exposed regions of the receptors since Toc34 Δ TM, which lacks the C-terminal membrane anchor was recognised by a matrix charged with Toc64 (Figure 9D, lane 2) or Toc64TPR (lane 3), but not with Toc64 Δ TPR (lane 4) after loading with GMP-PNP. This interaction also occurs in the presence of Hsp90 since Toc34_{GMP-PNP} can be recovered from a C90 charged matrix (Figure 9E) in the presence of Toc64TPR (lanes 3 and 5). In turn, association of Toc64TPR with C90 was not affected by the presence of Toc34_{GMP-PNP} (lanes 3 and 4). We conclude that Toc64TPR associated with Hsp90 interacts with the cytosolic domain of Toc34 in a GTP dependent manner.

To reconstitute preprotein transfer, wgt-pOE33 was incubated with a matrix charged with Toc64 (Figure 9F) or Toc34 Δ TM in the presence of GMP-PNP (Figure 9G). Subsequently, the binding of the precursor protein was competed

for by Toc64 (black), Toc34 in the absence (white) or presence of GMP-PNP (grey). Soluble Toc64 efficiently competes for the interaction between Toc64 and pOE33 (Figure 9F, black triangle). In contrast, Toc34 competed for the interaction between Toc64 and pOE33 only with low efficiency (white triangle) even in the presence of GMP-PNP (grey triangle). However, Toc34_{GMP-PNP} recognises preproteins like pOE33 with high affinity (Figure 1D), but only after disassembly of the chaperone complex (Figure 7C). Therefore, the interaction might be targeted toward the free preprotein, since the high affinity interaction was reconstituted *in vitro* as well (Schleiff *et al*, 2002). Hence, the competition experiment was performed with Toc34 as bait. The competition of the complex between pOE33 and Toc34 by Toc34 in the absence or presence of GMP-PNP revealed a similar efficiency as found for the self-competition by Toc64 (not shown, Figure 9G, grey triangle). Toc64 competed with a low efficiency for the interaction between Toc34_{GMP-PNP} and pOE33 (black triangle). This finding supports that Toc34 recognises the precursor after dissociation from Hsp90, since Toc64 only recognises the chaperone associated precursor protein.

The results suggest that ATP is indeed required for the release of pOE33 from Toc64 to Toc34. To test this notion, a wgt-pOE33 pre-incubated Toc64TPR matrix was incubated with Toc34_{GMP-PNP} in the presence (Figure 9H, lane 5) or absence of ATP (lane 6) followed by recovery of Toc34 by Ni-NTA matrix. The recovery of pOE33 by this procedure was five-fold higher in the presence of ATP than in the

Figure 9 Toc64 association with the Toc core complex. (A, B) OEV's were solubilised and complexes precipitated by α Toc64 (A, lanes 2 and 3), α Toc34 antibodies (B) or preimmuniserum (lane 4) in the presence of 1 mM GDP (lane 3) or 1 mM GMP-PNP (lane 2). Elution fractions (lanes 2–4) were immunodecorated by indicated antibodies. Lane 1 shows 10% of used proteins. (C) Isolated Toc159 or Toc34 Δ TM were incubated with a Toc64 affinity matrix in the presence of GMP-PNP. 10% of the used protein (lane 1) and the eluted protein (lane 2) were immunodecorated with indicated antibodies. (D) Toc34 Δ TM was incubated with a Toc64 (lane 2), TPR (lane 3), Δ TPR (lane 4) or BSA (lane 5) matrix in the presence of GMP-PNP (up) or GDP (down). The eluted protein was visualised by immunodecoration with α Toc34 antibodies. Lane 1 shows 10% of used proteins. (E) Toc34 preloaded with GMP-PNP (lanes 2, 3 and 5) and Toc64TPR (lanes 1, 3 and 4) were incubated with a matrix charged with the C90 construct. A silver stained gel of the protein bound to the column is shown. Lanes 1 and 2 represent 100% loading. (F, G) Wgt ³⁵S-labelled pOE33 was incubated with a Toc64 (F) or Toc34 Δ TM coated matrix in the presence of 0.5 mM MgCl₂ and 1 mM GMP-PNP (G) followed by addition of increasing amounts of Toc64 (black) or Toc34 Δ TM (white) loaded with GMP-PNP (grey). The amount of bound preprotein was quantified and is shown as percent of total bound protein. The average of at least three independent results is shown. (H) Wgt-pOE33 (lane 1 shows 5%) was incubated with a matrix charged with Toc64TPR. The flow through was incubated with a matrix charged with Toc34_{GMP-PNP}. Flow through (lane 2), wash (lane 3) and eluate (lane 4) of this column is shown. The Toc64TPR matrix was washed followed by addition of Toc34_{GMP-PNP} in the presence (lane 5) or absence of ATP (lane 6). The Toc34 was captured by incubation with Ni-NTA coated matrix and the eluted pOE33 is shown. The graph shows the average of at least three independent experiments. Top shows the ratio of pOE33 eluted from Toc64TPR and the bottom the fraction of eluted pOE33 bound to Toc34_{GMP-PNP} affinity matrix. (I) OEV's were solubilised and complexes precipitated by α Toc64 antibodies in the presence of 1 mM GMP-PNP and of expressed pOE33 (lanes 4–6). Flow through (lanes 1 and 4), wash (lanes 2 and 5) and elution fraction (lanes 3 and 6) were immunodecorated by indicated antibodies.

absence of ATP (upper half of diagram), where the binding efficiency of eluted pOE33 to Toc34 is not ATP dependent (lower half of diagram). Therefore, the chaperone activation by ATP might be the initial trigger for precursor release. Using the flow through from the Toc64TPR matrix generated prior to addition of Toc34 (lanes 2–4), most of the preprotein could be precipitated by subsequent addition of the receptor (lane 4). This is in line with the presented results (Figure 7D) that most of the precursor protein is not chaperone associated when *in vitro* wheat germ lysate is incubated at 25°C for prolonged times. To finalise the cycle of precursor protein delivery, the association between Toc64 and the Toc core complex within OE membranes was analysed in the presence of heterologous expressed pOE33. In the absence of pOE33, interaction between Toc64, Toc159 and Toc34 can be detected (Figure 9I, lane 3). In the presence of pOE33 (lane 6), the interaction between Toc64 and the Toc core complex was reduced even in the presence of GMP-PNP (Figure 9I, lanes 3 and 6). Since the precursor protein does not directly interact with Toc64 (Figure 3) but with Toc34 (Schleiff *et al*, 2002), we conclude that Toc64 interacts with the core complex only when Toc34 is in a precursor free state.

Discussion

Many proteins have to traverse at least one membrane to reach their place of function. They have to be recognised by membrane localised receptors. The translocon at the outer envelope of chloroplasts contains at least two proteins discussed as receptors for chloroplastic precursor proteins, namely Toc34 and Toc64 (Soll and Schleiff, 2004). In contrast to Toc34, Toc64 is only dynamically associated with the core complex (Figure 9, Schleiff *et al*, 2003b). Toc34 acts as an initial receptor for preproteins either in monomeric form or delivered by the guidance complex (Figures 5, 9 and 10; Becker *et al*, 2004). Toc34 recognises both, the transit sequence (Schleiff *et al*, 2002) and the 14-3-3 protein of the guidance complex (Figure 4) via a GTP regulated cytosolic domain (Schleiff *et al*, 2003). In contrast, Toc64 can recognise a subset of preproteins associated with chaperones distinct from the previously identified guidance complex (May and Soll, 2000; Figures 1, 4, 5, 8 and 9). This interaction is

mediated by its cytosolic exposed clamp type TPR domain (Figures 2 and 9), which interacts with Hsp90 chaperone (Figures 5–8) but not directly with the preprotein (Figure 3). Reticulocyte lysate synthesised preproteins are not recognised by Toc64 since they do not interact with the mammalian Hsp90 (Figure 4). This might point to a different architecture of the substrate-binding domain of the plant and mammalian Hsp90 proteins, respectively. The Hsp90 interaction with Toc64 is in line with the observation that clamp type TPR domains recognise the C-terminus of the chaperones (Scheufler *et al*, 2000). Here, the domains recognising Hsp90 molecules reveal the highest affinity for the last four amino acids, which are highly conserved among all Hsp70 and Hsp90 molecules. Furthermore, TPR domains recognising Hsp90 reveal also a higher affinity for Hsp70 molecules than TPR domains recognising Hsp70 proteins for Hsp90 (Scheufler *et al*, 2000; Brinker *et al*, 2002). Therefore, in *in vitro* pull down experiments, a basal recognition of Hsp70 was expected. However, Toc64 has a pronounced selection for Hsp90 because Toc64 was not able to compete for translocation of Hsp70 associated pSSU (Figures 1 and 4). Additionally, only the C-terminus of Hsp90, but not of Hsp70 was able to compete for translocation of wgt-pOE33 (Figure 6, not shown). Furthermore, Hsp90 interaction with Toc64TPR was not disrupted by addition of increasing amounts of Hsp70 (Figure 5). Finally, it could be demonstrated that Toc64TPR preprotein interaction is not direct but that complex formation proceeds indirectly via Hsp90 (Figure 7). Depletion of Toc64 reduces the translocation rate *in vitro* and *in vivo* (Figures 6 and 8). Therefore, the clamp type TPR domain of Toc64 builds an Hsp90 docking site receiving chaperone complexed preproteins.

Toc64 itself associates with the GTP-charged Toc complex by interaction of its TPR domain with the cytosolic exposed region of Toc34 (Figure 9). Loading of the TPR domain with Hsp90 does not interfere with the association to Toc34 (Figure 9). Therefore, a region distinct from the chaperone recognition site of the Toc64TPR motif must be postulated to act as a docking site for Toc34. In line with this notion, structural analysis of the protein phosphatase 5 revealed an interaction between TPR and the phosphatase domain involving the loop 3 and 5 of the clamp type fold (Yang *et al*, 2005). Dissociation of the preprotein from the chaperone (Figures 4 and 9) initiates its recognition by the second receptor of this pathway, Toc34 (Figures 1, 4, 9 and 10). Finally, delivery of the preprotein from Toc64 to the core complex leads to the dissociation of Toc64 (Figure 9). Hence, Toc34 acts as a general entrance receptor of the Toc core complex for incoming preproteins (Figure 10). This observation is in line with the reduction of pOE33 import into chloroplasts from Δ Toc33 knock out plants (Kubis *et al*, 2003). Beside the recognition of cytosolic precursor containing complexes, Toc64 contains a further binding site downstream of the TPR domain. As the topology of Toc64 is not established at present, this region might be a part of the previously identified intermembrane space complex (Becker *et al*, 2004b). However, this function has to be explored in the future.

When compared to the translocon of mitochondria, the function of Toc64 parallels the action of Tom70 (Rehling *et al*, 2004). Both receptors are not essential as determined by knock out analysis (Young *et al*, 2003; Rosenbaum-Hofmann and Theg, 2005; Figure 8). Furthermore, a homo-

Figure 10 Functional model of the Toc complex. For details see Discussion.

logue of Toc64, but no homologue of Tom70, is found in the translocon of plant mitochondria (Chew *et al*, 2004). In turn, Toc34 takes over the function of two mitochondrial translocon subunits, namely Tom20, the initial receptor for mitochondrial preproteins, and Tom22, the initial docking site for all preproteins within the core complex (Rehling *et al*, 2004).

Both receptors, Tom70 and Toc64, contain a clamp type TPR domain (Figure 5A, Young *et al*, 2003). Like in Tom70 (Young *et al*, 2003), two point mutations within the Toc64TPR region at position 516 or position 550 (Figure 6) altered the affinity of Toc64 for chaperones (Figure 5). Interestingly, yeast Tom70 interacts with Hsp70, whereas human Tom70 recognises both, Hsp70 and Hsp90, even though Hsp90 with higher affinity (Young *et al*, 2003). Similarly, Toc64 recognises Hsp90 from plants with higher efficiency than Hsp70. Hsp70 from mammals or yeast is not recognised by Toc64TPR (Figure 5 and data not shown). The different selectivity might be explained by the conserved lysine within the C-terminal portion of the plant Hsp70 and Hsp90 chaperones. Hsp70 of other species have a branched threonine at this position. In our model, the lysine would interact with arginine 580 within the Toc64TPR via hydrophobic interaction (Figure 6D). At the same position of the TPR fold of Hsp70 recognising proteins, an alanine is found in yeast Tom70 and a glutamine in the TPR1 of Hop (Figure 5A).

In the past, protein import experiments were often performed in a heterologous approach using either denatured or rlt preproteins. Even taking the homologous approach, one has to take care that the precursor is indeed assembled with chaperones *in vitro* since nonassembled preprotein can bypass regulatory events on the chloroplast surface. In our experience, different precursor populations occur simultaneously in a translation mixture, that is, chaperone associated, monomeric soluble and aggregated preproteins, depending on the experimental conditions used (e.g. Figure 7). Hence, differences in import behaviour can only be determined in the linear range of the reaction (e.g. Figure 8) meaning, it is important to measure kinetic differences in the presence of an appropriate number of organelles. Therefore, the function of the Toc64 receptor could only be explored using preproteins translated in a homologous system (e.g. Figure 2), performing import kinetics in the linear range (e.g. Figures 1 and 8) and isolating the population of preprotein associated with the chaperone complex after *in vitro* translation (Figure 7). Summarising, our results indicate a specificity of Toc64TPR for Hsp90 associated preproteins, which are associated via a direct interaction of the TPR region with the C-terminus of Hsp90. Toc64 is therefore a preprotein receptor and parallels the action of Tom70.

Materials and methods

General

RNAse free DNase, RNAse I, apyrase and wheat germ lysate were from Roche (Penzberg, Germany); ³⁵S-labelled methionine, stan-

dard molecules for size exclusion chromatography, Q-Sepharose and Percoll from Amersham-Biosciences (Freiburg, Germany) and geldanamycin from Sigma Aldrich (München, Germany). Data are presented using Adobe Photoshop 6.0 (Adobe Systems Inc., USA), Corel Draw 12 (Eastman Kodak Company, USA) and Sigma Plot 7.0 (SPSS Inc., Chicago, USA). Generation, translation or expression of pOE33, pSSU, NTT1, MDC, PC1, Fd, C70, C90, 14-3-3, cBag, Toc64 or Toc34ATM and standard techniques like immunoprecipitation, SDS-PAGE analysis and others are previously described (Sohrt and Soll, 2000; Schleiff *et al*, 2002; Young *et al*, 2003; Becker *et al*, 2004). Toc64 from *P. sativum* was used as template for construct generation (Sohrt and Soll, 2000; Figures 1A and 2A). Constructs were cloned into pET21d (Novagen, Madison, USA) and controlled by sequencing.

In vitro binding assay and *in vitro* import into chloroplasts

The postribosomal supernatant of radioactive-labelled proteins after centrifugation (10 min/250 000 g/4°C) was used for experiments. Chloroplasts isolation from pea (Schleiff *et al*, 2003) or *A. thaliana* (Aronsson and Jarvis, 2002), *in vitro* import (Schleiff *et al*, 2003) and binding experiments (Becker *et al*, 2004) were performed as described. For competition, Hsp70 was purified from the supernatant of lysed plant cells supplemented with buffer to a final composition of 10 mM KoAc, 2 mM DTT, 2 mM MgCl₂, 20 mM HEPES/KOH pH 7.6 (buffer A) followed by incubation with ATP agarose (16 h/4°C). The beads were washed twice with two column volumes of buffer A, one column volume of buffer A containing 1 M KoAc and one column volume buffer A before elution of Hsp70 by 10 mM ATP in binding buffer. The purity of the chaperone was controlled by SDS-PAGE analysis.

Size exclusion and glycerol gradient

Wgt ³⁵S-labelled pOE33 was preincubated as indicated and subjected onto a Superdex 200 (at 4°C) using 100 mM KoAc, 20 mM HEPES/KOH, pH 7.6, 5 mM MgCl₂ as running buffer or a 4 ml glycerol step gradient of 20 mM Tris/HCl pH 7.6, 50 mM KoAc and 2 mM MgCl₂ containing 10–60% glycerol. The gradient was fractionated after centrifugation (83 000 g/4°C/over night). Fractions were analysed by separation on SDS-PAGE and autoradiography.

Homology modelling

The TPR1 of Hop (pdb:1EWL) revealed a gapless alignment with Toc64TPR. Thus, 1EWL was used as template for protein and peptide. The homology model of psToc64-TPR was built using WHAT IF (Vriend, 1990).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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