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Regulated interactions of mtHsp70 with Tim44 at the translocon in the mitochondrial inner membrane

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Preproteins synthesized on cytosolic ribosomes, but destined for the mitochondrial matrix, pass through the presequence translocase of the inner membrane. Translocation is driven by the import motor, having at its core the essential chaperone mtHsp70 (Ssc1 in yeast). MtHsp70 is tethered to the translocon channel at the matrix side of the inner membrane by the peripheral membrane protein Tim44. A key question in mitochondrial import is how the mtHsp70-Tim44 interaction is regulated. Here we report that Tim44 interacts with both the ATPase and peptide-binding domains of mtHsp70. Disruption of these interactions upon binding of polypeptide substrates requires concerted conformational changes involving both domains of mtHsp70. Our results fit a model in which regulated interactions between Tim44 and mtHsp70, controlled by polypeptide binding, are required for efficient translocation across the mitochondrial inner membrane *in vivo*.

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Because only a handful of the hundreds of proteins present in mitochondria are encoded by mitochondrial DNA, mitochondrial function depends on the efficient import of proteins translated on cytosolic ribosomes[1]. Proteins destined for the matrix must pass through two proteinaceous channels, first through the outer membrane (TOM) channel, and then through the inner membrane (TIM) channel[1,2]. Translocation of the positively charged presequence through the TIM channel, which is composed of three integral membrane proteins, Tim17, Tim23 and Tim50, is dependent on the membrane potential across the inner membrane[1–6]. However, translocation of the remainder of the polypeptide requires function of the import motor, having at its core mtHsp70 (Ssc1 in yeast) (refs. 1,7–13). The efficient function of the motor is dependent on three essential peripheral membrane proteins that are part of the translocase: Tim44, which tethers mtHsp70 to the TIM channel; Pam18, which serves as the J-protein cochaperone of mtHsp70; and Pam16, a degenerate J protein that forms a heterodimer with Pam18 (refs. 1,11–20).

Although the exact mechanism by which mtHsp70 drives protein import is controversial, it is well established that mtHsp70 interacts with translocating polypeptides in a manner expected of a member of the highly conserved Hsp70 family[13,21]. Hsp70s have two domains, an N-terminal ATPase domain and a C-terminal domain that binds short hydrophobic segments of polypeptide (PBD)[22,23]. Interaction with polypeptide substrates is regulated by cycles of ATP binding and hydrolysis that modulate the affinity of Hsp70 for substrate. It is believed that Hsp70-ATP initiates interaction with substrate *in vivo* because of its very fast on-rate. However, the off-rate is very rapid. The Hsp70-substrate interaction is stabilized by the hydrolysis of ATP, as Hsp70-ADP has a much slower off-rate for substrates as compared with Hsp70-ATP[24–26]. ATP hydrolysis is stimulated by both the binding of substrate polypeptide and by the action of a J-protein cochaperone[26,27].

On the basis of these conserved properties of Hsp70s, mtHsp70 in complex with ATP is the form expected to be tethered to the import channel and initiate contact with the incoming polypeptide chain[1,11,13]. In line with this idea, Tim44 forms a stable complex with mtHsp70 in the presence of ATP, as well as ADP, *in vitro*[13]. This mtHsp70-ATP-Tim44 interaction is destabilized upon binding a model peptide, consistent with the unstable interaction observed when ATP is added to mitochondrial extracts that probably contain many potential Hsp70 substrates. *In vivo*, this destabilization would be important for forward movement of the translocating polypeptide: the leading segment of the polypeptide chain disengaged from the channel could allow binding of a free mtHsp70-ATP to Tim44, thus enabling initiation of the next cycle of mtHsp70 interaction with polypeptide[13].

Despite the central role of the mtHsp70-Tim44 interaction in mitochondrial import, little is understood about the nature and regulation of this interaction. Reported results from *in organellar* analyses have often yielded seemingly contradictory results, even as to which domain(s) of mtHsp70 is (are) important for the interaction[28–30]. Therefore, we undertook an analysis of the mtHsp70-Tim44 interaction using purified components of the yeast system. We found that both domains of Ssc1 are capable of independently interacting with Tim44, but the full-length protein is required for release by a peptide substrate. Point mutations were identified that led to loss of this regulated dissociation. Cells containing these mutations were compromised in protein translocation, indicating that regulated interaction between Ssc1 and Tim44 is required *in vivo* for efficient translocation.

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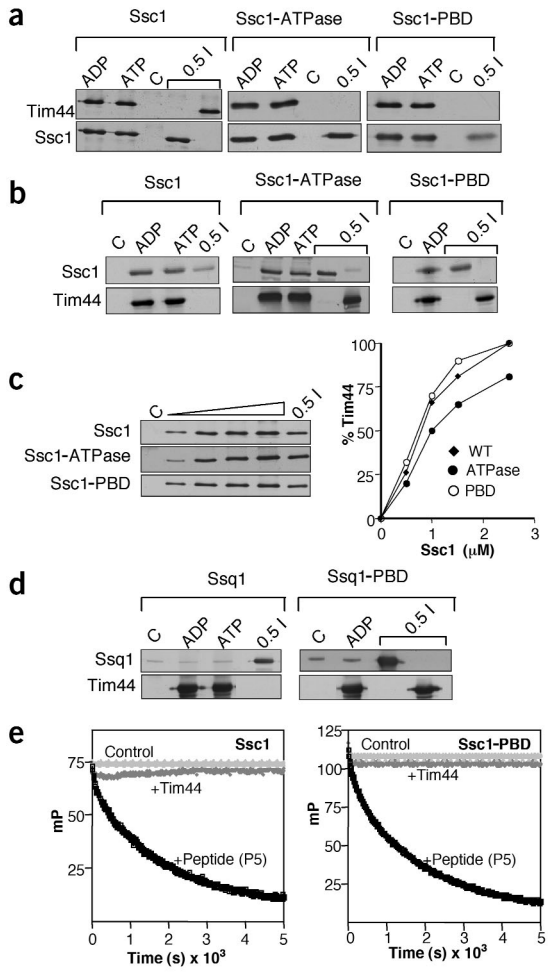

Figure 1 Interaction of Tim44 with the ATPase domain and PBD of Ssc1. (**a**) Coimmunoprecipitation of Tim44 with Ssc1 using Ssc1-specific antibodies. Full-length, ATPase domain or PBD Ssc1 (0.6 μM) and Tim44 (0.09 μM) were incubated in the presence of 1 mM ATP or ADP for 30 min before immunoprecipitation. Tim44 and Ssc1 in the precipitate were assessed by immunoblotting using antibodies specific for the respective proteins. As a control (C), Ssc1 was omitted. A sample containing one-half of input served as a control for immunoprecipitation efficiency (0.5 I). (**b**) Coimmunoprecipitation of full-length Ssc1 or its domains with Tim44 using Tim44-specific antibodies. Purified Tim44 (1 μM) was incubated with either 0.05 μM full-length Ssc1 or domains in the presence of 1 mM ATP or ADP for 30 min before immunoprecipitation. Analysis was as described in **a**. (**c**) Comparison of interaction of full-length Ssc1 and its domains with Tim44. Tim44 (0.09 μM) was incubated with increasing concentrations of Ssc1 in the presence of 1 mM ADP. Mixtures were subjected to immunoprecipitation as described above (left). Immunoblot signal was quantified (right). WT, wild type. (**d**) Coimmunoprecipitation of full-length Ssq1 or its domains with Tim44 using Tim44-specific antibodies. Conditions were as described in **b** except that full-length Ssq1 or domains was used. Analysis was as described in **a**. (**e**) Peptide binding and release measured by fluorescence anisotropy. Full-length or PBD Ssc1 (2 μM) was incubated with fluorescein-labeled P5 (10 nM) in the presence of 0.5 mM ADP. Unlabeled P5 (300-fold molar excess), Tim44 (1,500-fold molar excess), or buffer alone (control) was added and anisotropy measurements carried out.

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RESULTS

ATPase domain and PBD of mtHsp70 bind Tim44

Using purified components, formation of complex between the mtHsp70 Ssc1 and Tim44 can be assessed by the ability of Ssc1-specific antibodies to precipitate Tim44 (ref. 13). To directly test which domain(s) of Ssc1 interacts with Tim44, we purified the ATPase domain (residues 24–411) and the PBD (residues 412–654). As was the case with full-length mature Ssc1 (residues 24–654), ~50% of Tim44 coprecipitated with either domain regardless of whether ADP or ATP was present (**Fig. 1a**). Similar results were obtained when interaction was assessed by the ability of Tim44-specific antibody to precipitate the individual domains of Ssc1 (**Fig. 1b**).

The fact that both domains of Ssc1 interact with Tim44 indicates that there are at least two sites of interaction of Tim44 with Ssc1. To assess more quantitatively the interactions of the two domains of Ssc1 with Tim44, increasing concentrations (0.5–2.5 μM) of full-length, ATPase domain or PBD were incubated in the presence of 0.09 μM Tim44 (**Fig. 1c**). Similar concentration-dependent binding was observed in the three reactions, with 50% binding occurring between concentrations of 0.8 and 1.3 μM.

Tim44 could possibly be interacting as a substrate with Ssc1-PBD, perhaps owing to the presence of some partially folded molecules in our preparation. To eliminate this possibility, we carried out several experiments. First we tested whether Tim44 formed a complex with another Hsp70 of the mitochondrial matrix, Ssq1, which is involved in the biogenesis of Fe-S cluster biogenesis, rather than mitochondrial import[31]. Neither full-length Ssq1 nor the Ssq1 peptide-binding domain coimmunoprecipitated with Tim44 (**Fig. 1d**).

Secondly, we reasoned that if Tim44 was interacting as a substrate it should compete for binding with a peptide substrate. We used a peptide, CALLLSAPRR (P5), a portion of the mitochondrial targeting sequence of chicken aspartate aminotransferase, as a model substrate. As expected from previous results[21], both full-length Ssc1 and the PBD had a K_d of ~0.3 μM in the presence of ADP or in the absence of nucleotide, as measured by fluorescence anisotropy (data not shown). Addition of an excess of untagged peptide led to a decrease in anisotropy, indicating a dynamic interaction with a k_{off} of ~0.0024 s^{-1} (**Fig. 1e**). Addition of Tim44 did not alter anisotropy substantially, even when added at a concentration of 15 μM, a 1,500-fold excess over the concentration of P5. These results indicated that Tim44 was not acting as a substrate and did not interfere with binding of peptide.

PBD lid required for release from Tim44

A critical interaction for protein translocation *in vivo* is the formation of a ternary complex between the Ssc1–ATP–Tim44 complex and the translocating polypeptide at the import channel. Binding of a peptide substrate (P5) destabilized the full-length Ssc1-Tim44 interaction[13] (**Fig. 2a**), as did a peptide having the sequence of a portion of the presequence of yeast mitochondrial CoxIV (**Supplementary Fig. 1** online). To test whether binding of peptide to the PBD destabilizes interaction with Tim44, peptide (P5) was added to a preformed Ssc1-PBD–Tim44 complex. After addition of peptide, Tim44 immunoprecipitated with Ssc1-PBD, but not with full-length Ssc1 (**Fig. 2a**). Thus the ATPase domain is required for peptide-stimulated release of Tim44, presumably to foster a change in conformation required for destabilization.

In addition to the ATPase domain and the peptide-binding cleft formed by a series of β-sheets, Hsp70s have an extended α-helical C-terminal region that in part seems to serve as a lid over the peptide-binding cleft[25,32] (**Fig. 2b**). To further define sequences important for interaction with Tim44 we made truncated constructs ending at residue 561 or 536. The longer constructs lacked the portion of the α-helical region predicted to cover the peptide-binding cleft, whereas

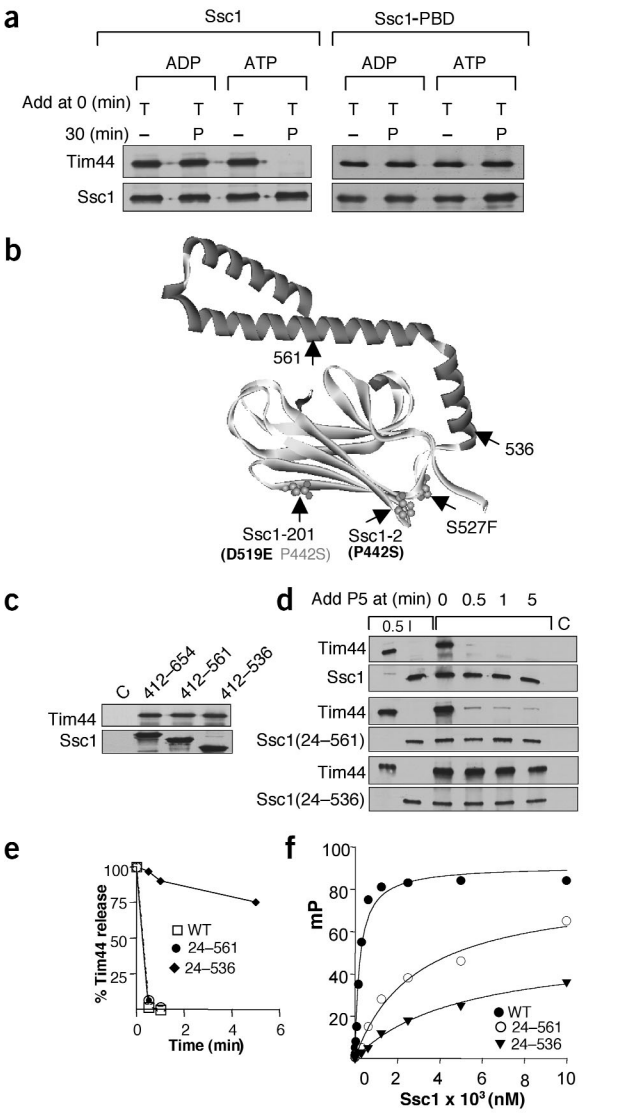

Figure 2 Effect of peptide on the Ssc1-Tim44 interaction *in vitro*. (**a**) Interaction with PBD. Full-length Ssc1 or the PBD (0.6 μM) was preincubated with 0.09 μM Tim44 (T) for 30 min; peptide P5 (P) at a concentration of 50 μM was added and incubation continued for 30 min. Immunoprecipitations were carried out using Ssc1-specific antibodies as described in the legend to **Figure 1a** and immunoblotted with the indicated antibodies. (**b**) The Ssc1 backbone sequence (residues 412–654) was modeled from the *E. coli* DnaK structure[25,32]. The positions of point and truncation mutants are highlighted. (**c**) Interaction of Ssc1 truncation mutants. The immunoprecipitations of Ssc1(412–654), Ssc1(412–561) and Ssc1(412–536) were carried out using Ssc1-specific antibodies as described in **Figure 1a**. (**d**) Interaction of truncations of mature full-length Ssc1. Ssc1(24–654), Ssc1(24–561) and Ssc1(24–536) were individually preincubated with Tim44 as described in **a**. Peptide P5 (50 μM) was then added and incubation continued for the indicated times. Immunoprecipitations were carried out with the indicated antibodies. (**e**) Immunoblot signal from **d** was quantified. The amount of Ssc1–Tim44 complex at 0 time is set to 100%. WT, wild type. (**f**) Peptide binding of Ssc1 truncations as measured by fluorescence anisotropy. Fluorescein-labeled P5 peptide (10 nM) was incubated in the presence of indicated concentrations of full-length Ssc1, Ssc1(24–561) and Ssc1(24–536).

the shorter construct lacked the entire α-helical region. The purified truncated PBD fragments (residues 412–561 and 412–536) co-immunoprecipitated with Tim44 as well as the intact C-terminal domain (residues 412–654) (**Fig. 2c**). Similarly, truncated constructs containing the ATPase domain also interacted with Tim44 as well as full-length wild type (**Fig. 2d**).

To assess whether truncated Ssc1 was released from Tim44 upon peptide binding, we preformed Ssc1–ATP–Tim44 complexes and then added an excess of peptide P5. After only a 30-s incubation with peptide, Tim44 no longer immunoprecipitated with wild type and the Ssc1(24–561) truncation (**Fig. 2d**). However, even after 5 min of incubation, the efficiency of coimmunoprecipitation of Tim44 with Ssc1(24–536) was 75% of that observed when no peptide was added, suggesting that the 25-residue segment between residues 536 and 561 is important in regulating the interaction with Tim44 (**Fig. 2e**).

Alterations in the α-helical lid region of DnaK have been shown to greatly increase the off-rate of peptide[25]. To test whether the inability of the peptide to destabilize the interaction of Tim44 with Ssc1(24–536) was due to altered interaction with the peptide rather than a direct affect on the Ssc1-Tim44 interaction, we monitored the binding of peptide to Ssc1(24–536) or Ssc1(24–561). Even though these two truncations showed different interactions with Tim44 in response to peptide addition, they interacted similarly with the peptide. Both showed a ~20× increase in off-rate (data not shown) and a 20–35× increase in K_d (**Fig. 2f**) compared with full-length wild-type protein. Therefore, even though Ssc1(24–561) had a decreased affinity for peptide, Tim44 release was triggered at the high concentration of peptide (50 μM) used in these experiments. We conclude that amino acids between 537 and 561 play a role in regulated interaction of Ssc1 with Tim44.

SSC1 mutations affecting release from Tim44

To better understand the Ssc1-Tim44 interaction, we screened through several conditional and slow-growing *SSC1* mutants to identify those showing defects in interaction with Tim44. As expected, wild-type mitochondrial extracts showed coimmunoprecipitation of both Tim44 and Mge1 with Ssc1 in the absence of ATP, but no detectable coprecipitation in the presence of ATP (**Fig. 3a**). The absence of the Tim44-Ssc1 interaction in the presence of ATP could be due to partially unfolded proteins in the lysate that have exposed hydrophobic sequences and serve as substrates for Ssc1, triggering release of Tim44.

No candidates showed obvious differences from wild type in regard to Tim44 binding in the absence of ATP. However, three coimmunoprecipitated with Tim44 in the presence of ATP, albeit at a reduced level compared with that in the absence of ATP, even though no interaction with Mge1 was detected under these conditions (**Fig. 3a**). These three mutants all grew more slowly than wild type at the optimal yeast growth temperature of 30 °C (**Fig. 3b**). Two encoded amino acid alterations in the ATPase domain, S66F and L37S; one encoded an amino acid alteration, S527F, in the peptide-binding domain (**Fig. 2b**).

To assess whether these mutants had a defect in mitochondrial protein import *in vivo*, accumulation of the precursor form of a nuclear-encoded mitochondrial protein, Hsp60, was monitored. As is the case with most nuclear-encoded matrix proteins, the presequence of Hsp60 is cleaved by the matrix processing protease immediately upon traversing the inner membrane, and accumulation of the normally undetectable precursor form has been used as an indicator of the malfunction of the import process[7,9]. *ssc1-2*, which has been shown to have a defect in protein import and accumulate Hsp60 precursor[7,9], was used as a control. Mutant cells were grown at 30 °C, a temperature

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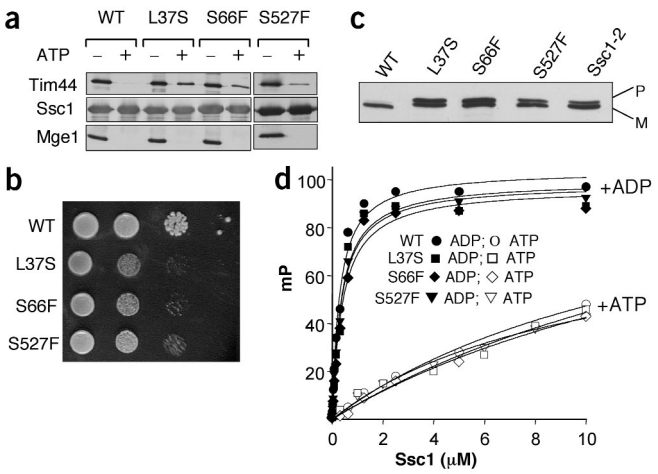

Figure 3 Analysis of *SSC1* mutants defective in interaction with Tim44. (**a**) Analysis of Ssc1 and Tim44 interaction *in organello*. Mitochondria (200 μg) were incubated in mitochondrial lysis buffer with either 5 mM EDTA or 10 mM Mg-acetate plus 1 mM ATP and immunoprecipitated using Ssc1-specific antibody. (**b**) Growth phenotypes of *SSC1* mutants. Ten-fold serial dilutions of yeast cells expressing wild-type (WT), L37S, S66F or S527F Ssc1 were spotted onto minimal media and incubated at 30 °C for 2 d. (**c**) *In vivo* precursor accumulation. Wild-type, S66F, L37S and S527F Ssc1 were grown at 28 °C in rich media to early log phase. Cell extracts (10 μg) were analyzed by SDS-PAGE, followed by immunoblot analysis using Hsp60-specific antibodies. (**d**) Peptide binding measured by fluorescence anisotropy. Fluorescein-labeled P5 peptide (10 nM) was incubated in the presence of the increasing concentrations of wild-type, L37S, S66F and S527F Ssc1 in the presence of ATP or ADP and fluorescence anisotropy measurements were taken. After background subtractions the polarization values were fitted to a one-site binding hyperbola equation to determine the K_d using Prism (GraphPad).

at which all three mutant strains showed a growth defect. The precursor form of Hsp60 accumulated in the three mutant strains, indicating a defect in protein translocation *in vivo* (**Fig. 3c**).

We purified the three mutant Hsp70 proteins and characterized their biochemical properties. The mutant proteins interacted with the model peptide P5 indistinguishably from wild-type protein, having K_d values in ADP of ~300 nM, and estimated K_d values of 12–15 μM in ATP (**Fig. 3d**). Basal ATPase activity, and the effectiveness of peptide P5 and J-protein Mdj1 in stimulating it, were assessed by measuring the rates of hydrolysis using preformed Ssc1–ATP complexes. The basal ATPase activities were similar to that of wild type, with wild type, S66F, L37F and S527F having basal ATPase activities of approximately 0.065, 0.065, 0.085 and 0.11 min^{-1}, respectively. All the mutants were stimulated to a similar extent as wild type by both peptide P5 and the J-protein Mdj1 (**Fig. 4**). Similar results were obtained using the J-protein Pam18/Tim14 (data not shown). Thus, the peptide binding and ATPase activity were not obviously affected in the mutant proteins.

All three proteins also formed a complex with Tim44 in the presence of ADP, ATP or AMPPNP (**Fig. 5a**,**b**). However, consistent with the *in organellar* observation, preformed Ssc1–ATP–Tim44 or Ssc1–AMPPNP–Tim44 complexes were not destabilized by addition of peptide, even though peptide-binding was normal in these mutants (**Fig. 5a**,**b**). In the case of wild type, Ssc1–ADP–peptide complexes do not stably interact with Tim44 (ref. 13). To test such binding of the mutant proteins, Tim44 was added to preformed Ssc1–peptide complexes. Unlike the wild-type protein, Tim44 coprecipitated with all three mutant proteins, indicating that these proteins were able to form a ternary Tim44–Ssc1–peptide complex (**Fig. 5c**). To directly test this hypothesis we carried out size-exclusion chromatography. Tim44 alone eluted similarly to Ssc1 (**Fig. 5d**), consistent with it being a homodimer[13]. Incubation of either wild-type Ssc1 or Ssc1 S527F led to the formation of a complex accounting for ~50% of Ssc1 and Tim44 that eluted at a position consistent with a Tim44–Ssc1 complex, peak fractions 12–16 (ref. 13; data not shown). As previously reported[13], when wild type was preincubated with fluorescently labeled peptide, and then added to Tim44, Ssc1–peptide and Ssc1–Tim44 complexes were detected, but no Ssc1–Tim44–peptide complex was observed, as indicated by the lack of fluorescence in peak fractions 12–16 (**Fig. 5d**, bottom left panel). On the other hand, a ternary complex containing the S527F mutant was easily detected, as demonstrated by a peak of fluorescence that coeluted with S527F Ssc1 and Tim44 (**Fig. 5d**, bottom right panel). These results confirm the idea that Ssc1 S527F, unlike wild-type Ssc1, interacts with Tim44 and a peptide substrate simultaneously.

Defects of Ssc1-2 in Tim44-regulated interactions

The mtHsp70 mutant *ssc1-2* (P442S) has been extensively used in *in vivo* and *in organellar* studies to understand the mechanism of protein translocation across the inner mitochondrial membrane[6,11,12,33–36]. Ssc1-2 mitochondria are defective in both import of proteins and their folding. However, two intragenic suppressors, *ssc1-201* (P442S D519E) and *ssc1-202* (P442S V524I), which reverse the import, but not the folding defect, have been isolated[12]. Reduced coimmunoprecipitation of Tim44 with Ssc1-2 has been observed in extracts of mitochondria incubated at 37 °C. This decreased interaction with Tim44, which is restored to the normal level by the suppressor mutations[12], has been proposed to be the cause of the import defect of *ssc1-2*.

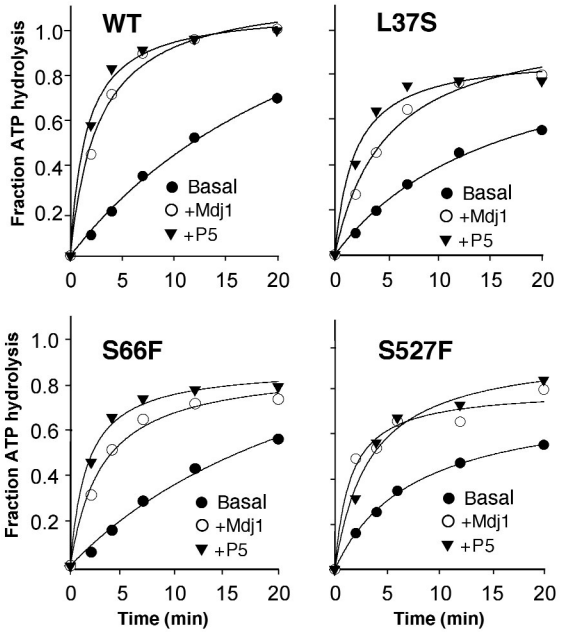

Figure 4 Stimulation of ATPase activity of Ssc1 by a J protein and peptide. Wild-type (WT), L37S, S66F and S527F Ssc1–[^{32}P]ATP complexes (~0.4 μM) were prepared; the rate of ATP hydrolysis was measured at 23 °C in the presence or absence of 2 μM Mdj1 or 50 μM peptide P5. The rate of conversion to ADP was measured and plotted against time of incubation.

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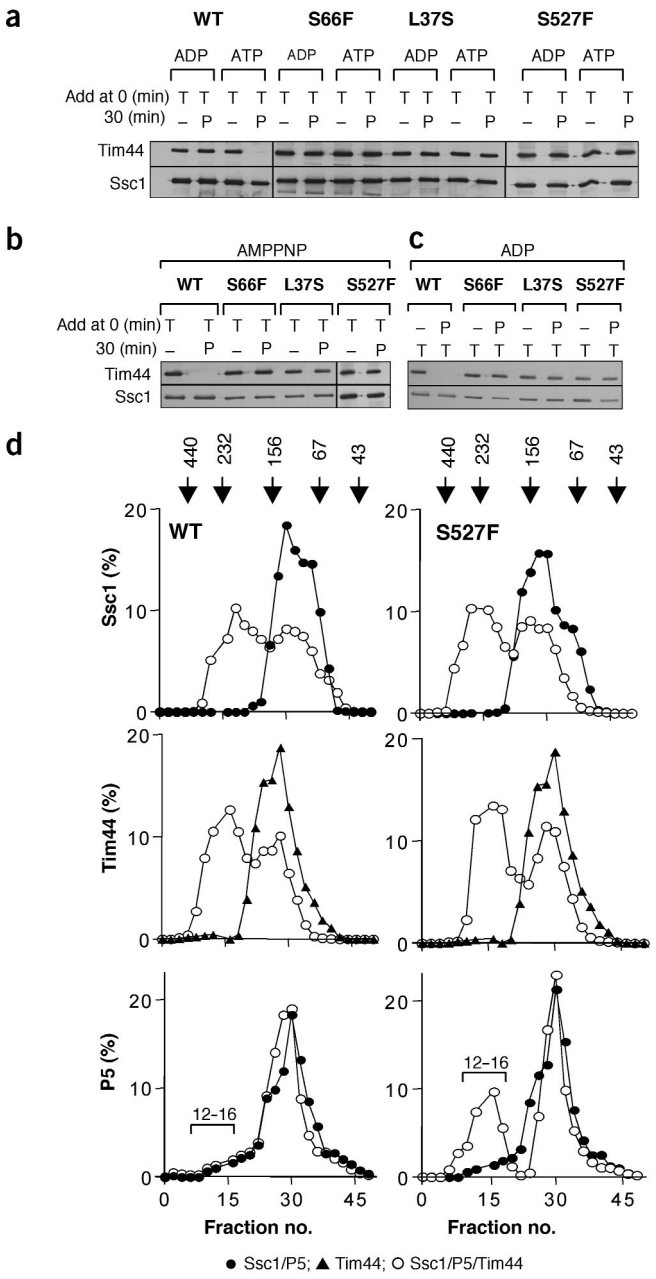

Figure 5 Effect of peptide on mutant Ssc1-Tim44 interactions *in vitro*. (**a**,**b**) Ssc1 (0.6 μM; wild type (WT), L37S, S66F and S527F) and 0.09 μM Tim44 (T) were incubated in the presence of 1 mM ADP, ATP or AMPPNP in mitochondrial lysis buffer at 23 °C for 30 min. Peptide P5 (P; 50 μM) was added and incubation continued for 30 min. The Ssc1–Tim44 complex was immunoprecipitated using Ssc1-specific antibodies and subjected to immunoblot analysis using the indicated antibodies. (**c**) Experiment was done as in **a**,**b** except peptide was incubated with Ssc1 in the presence of ADP to preformed Ssc1–ADP–P5 complexes of Ssc1 WT, L37S, S66F and S527F, which were then incubated with Tim44 (T) for 30 min, followed by immunoprecipitation and immunoblot analysis. (**d**) Analysis of Ssc1–peptide–Tim44 complexes by size-exclusion chromatography. Three different reactions were carried out: (i) 50 μg of Tim44 alone; (ii) 100 μg of Ssc1 (WT and S527F) and fluorescein-labeled P5; (iii) 100 μg of Ssc1 (WT and S527F) incubated with fluorescein-labeled P5 for 1 h, followed by the addition of 50 μg of Tim44 and further incubated for 30 min at 23 °C. Reaction mixtures were subjected to chromatography. Fractions were subjected to immunoblot analysis using Ssc1- and Tim44-specific antibodies; signals were quantified by densitometry. P5 peptide in each fraction was monitored by fluorescence intensity.

Because of the extensive use of these mutants to probe the import process, we characterized the interactions of the purified Ssc1-2 and Ssc1-201 proteins with Tim44. Consistent with the results in isolated mitochondria, Ssc1-2 showed decreased interaction with Tim44 in both ADP and ATP, whereas Ssc1-201 coprecipitated nearly as well as wild type (**Fig. 6a**). To assess quantitatively the Tim44-Ssc1 interaction, increasing concentrations of Ssc1-2, Ssc1-201 and wild-type Ssc1 (0.2 to ~3 μM) were incubated in the presence of 0.09 μM Tim44. Ssc1 and Ssc1-201 interacted similarly; Ssc1-2 had a reduced apparent affinity, estimated to be about four-fold lower than that of wild-type (**Fig. 6b**). Because Ssc1-2 is a temperature-sensitive mutant *in vivo*, we tested whether incubation of the purified protein at 37 °C for 10 min affected its ability to interact with Tim44; interaction was very similar to that seen with protein incubated on ice (data not shown).

Although this difference in affinity between wild-type and mutant protein could affect the interaction of the two proteins in extracts that are substantially less concentrated than the mitochondrial matrix, it is unlikely to affect the amount of Ssc1 tethered to the import channel. The concentrations of Ssc1 and Tim44 in mitochondria are ~200 and 30 μM (~1–2% and 0.25% of mitochondrial protein)[37], respectively. Thus, even with this lowered affinity, Tim44 should be fully occupied by Ssc1-2. Previously we reported that Ssc1-2-ATP on-rates and Ssc1-2–ADP off-rates for peptide substrates were very similar to those of wild-type protein[21], and thus could not account for the defect in protein translocation. For this reason, we extended our analysis of the Tim44 interaction, testing its ability to be regulated by peptide substrates. Peptide did not destabilize the Ssc1-2–ATP–Tim44 or Ssc1-2–AMPPNP–Tim44 interaction (**Fig. 6c**), even though Ssc1-2 has normal peptide interaction when bound to ATP[21]. In addition, when prebound to peptide, Ssc1-2 formed a complex with Tim44, whereas Ssc1-201, like wild type, did not (**Fig. 6d**). Therefore, Ssc1-2 and S527F behave similarly in regard to the effect of peptide binding on their interaction with Tim44.

DISCUSSION

Our analysis of the interaction between mitochondrial Hsp70 Ssc1 and its translocon tether Tim44 establishes two important points concerning the function of the mitochondrial import motor: (i) interaction of both domains of Ssc1 with Tim44 is required for regulated interactions governed by binding of polypeptide substrates and (ii) these regulated interactions are biologically important for driving protein translocation at the rates required for normal growth.

The results reported here using purified components demonstrate that the ATPase domain and the peptide-binding domain of mtHsp70 have affinities for Tim44 similar to that of the full-length protein. However, reports in the literature have often been at odds with each other; some reports indicate that the critical interaction is with the ATPase domain, and others that it is with the peptide-binding domain[28–30]. These apparent inconsistencies are probably due to the inherent difficulties in stringently assaying interactions in mitochondrial lysates, particularly because Ssc1 is essential and therefore truncations and chimeric genes must be assayed in the presence of functional Ssc1.

Together our biochemical and genetic data indicate that both domains of Ssc1 are important for the conformational change occurring upon binding of a peptide substrate that leads to destabilization of

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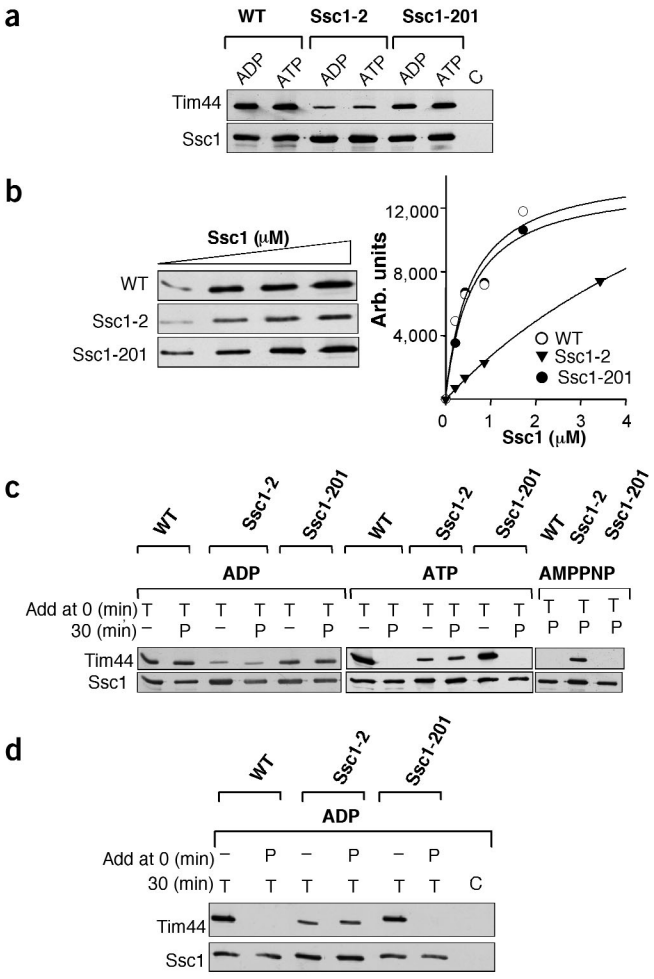

Figure 6 *In vitro* interaction of Ssc1-2 and Ssc1-201 with Tim44. (**a**) Purified Ssc1 (0.6 μM; wild type (WT), Ssc1-2 and Ssc1-201) and 0.09 μM Tim44 were incubated in the presence of 1 mM ATP or ADP in mitochondrial lysis buffer for 30 min. Mixtures were subjected to immunoprecipitation using Ssc1-specific antibodies. As a control (C), Ssc1 was omitted. (**b**) Increasing concentrations of WT Ssc1, Ssc1-2 and Ssc1-201 were incubated with Tim44 (0.09 μM) in the presence of ADP. The mixtures were subjected to immunoprecipitation and immunoblot analysis (left). The data were quantified (right). (**c**) Effect of peptide on the interaction of Ssc1-2 and Ssc1-201 with Tim44 *in vitro*. WT Ssc1 or Ssc1-201 (0.6 μM), 1.2 μM Ssc1-2 and 0.09 μM Tim44 (T) were incubated in the presence of 1 mM ADP, ATP or AMPPNP, as indicated, at 23 °C for 30 min. Peptide P5 (P; 50 μM) was added and incubation continued for 30 min. The Ssc1–Tim44 complex was immunoprecipitated using Ssc1-specific antibodies and immunoblotted with indicated antibodies. (**d**) Preformed Ssc1–ADP–P5 complexes of Ssc1, Ssc1-2 and Ssc1-201 were incubated with Tim44 (T) for 30 min in the presence of 1 mM ADP, followed by immunoprecipitation and immunoblot analysis as described in the legend to **Figure 1**.

the Tim44-Ssc1 interaction. Notably, the single-amino acid change in the peptide-binding domain affecting the regulation of the Tim44-Ssc1 interaction, S527F, is predicted to be near the juncture of the α-helical region and the β-sheet core containing the peptide-binding cleft. Our *in vitro* analysis of truncations suggests that the first 25 amino acids of this helical region are required for this destabilization as well. In mitochondrial extracts, Ssc1 truncations lacking these amino acids are not destabilized by the addition of ATP, unlike wild-type protein[29,38]. As we believe that the destabilization seen under these circumstances is the result of Ssc1 binding to protein substrates in the extracts, these *in organellar* results are actually consistent with the *in vitro* data.

The fact that both domains are required for the change in conformation that destabilizes the interaction is not completely surprising. There is ample evidence that peptide binding results in conformational changes not only in the peptide-binding domain, but also in the ATPase domain. It has been appreciated for many years that binding of peptide by an Hsp70 results in stimulation of ATPase activity[21,39], and that the presence of the C-terminal domain suppresses the inherent ATPase activity of the N terminus[40,41]. Yet, the complexities of the conformational change(s) of an Hsp70 that can occur upon binding of peptide substrates or interacting proteins are just beginning to be understood.

However, recent analysis of DnaK indicates that peptide binding leads to a concerted conformational change in the ATPase domain even in the absence of ATP hydrolysis[42,43]. Studies using a fluorescently tagged ATP analog detected an almost instantaneous change in fluorescence upon peptide binding[43]. This change was >4,000-fold faster than the resulting stimulation of ATP hydrolysis[43]. These results are consistent with a hypothesis that entrance of peptide into the peptide-binding site can initiate a conformational change that may not lead to ATP hydrolysis. Particularly in the absence of other stimulatory factors such as a J protein, multiple on-off cycles of peptide interaction may occur before ATP hydrolysis. This idea is consistent with the notion that upon addition of peptide to a Ssc1–ATP–Tim44 complex, Tim44 release can occur much more rapidly than ATP hydrolysis. The biochemical properties of the S527F mutant described here also suggest that identical conformational changes are not required for Tim44 release as for ATP hydrolysis, because this mutant protein is defective in Tim44 destabilization, but not stimulation of ATP hydrolysis.

However, although Ssc1 can be released from Tim44 upon peptide binding in the absence of ATP hydrolysis, we favor a model in which, *in vivo*, these conformational changes of Ssc1 are coupled with peptide binding initiating concerted conformational changes such that ATP hydrolysis and release from Tim44 are simultaneous. Such coupling, probably involving the J-protein Pam18 and the J-related protein Pam16, would lead to trapping of the incoming polypeptide chain by Ssc1 owing to the slow off-rate of substrate when in the ADP-bound state, as well as release from Tim44. This release from Tim44 would allow movement of the polypeptide chain into the matrix, away from the import channel, as well as binding of another Ssc1-ATP to Tim44. The Ssc1–ADP–peptide complex also has a very low affinity for Tim44 and, therefore, would not compete with binding of the productive free Ssc1-ATP free of substrate. These two aspects of the Tim44-Ssc1 interaction are closely related, and may well be the result of the same local conformational change(s), as the mutants we isolated are defective in both regulatory functions.

The protein encoded by the well-studied *ssc1-2* mutant is also defective in these regulated interactions with Tim44. These defects are corrected by the amino acid alteration present in the suppressor Ssc1-201, which also substantially corrects the *ssc1-2* translocation defect[12]. These results provide evidence that the regulated interactions between Tim44 and Ssc1 play an important role in the efficiency of translocation *in vivo*. The defects observed in *ssc1-2* mitochondria are particularly apparent in the case of tightly folded substrates[12,33,36], as is also the case with S527F mitochondria (data not shown). Thus these regulatory mechanisms are crucial for maintaining a high rate of import

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that is required *in vivo*. In the case of a folded protein it is probably critical that the import channel be primed for action with Ssc1 in the ATP state bound to Tim44. Either a slow release of Ssc1 from Tim44 once it has bound the incoming polypeptide, or rebinding of a recently released Ssc1 still bound to the polypeptide, will decrease import efficiency and have severe consequences in living cells. It is likely that other regulated interactions within the translocation machinery of the inner membrane have evolved to make the import motor such an efficient machine. A more complete understanding of how the functions of all the components of the import motor, Ssc1, Tim44, Pam18 and Pam16, are coupled with each other and with the inner membrane translocon awaits further analysis.

METHODS

Strains, plasmids and genetic techniques. A library of *SSC1* mutants in pRS314-*SSC1* was created by random mutagenesis using Taq1 polymerase. The library was transformed into DG252: *(trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2^{+} met2-Δ1 lys2-Δ2 ssc1ΔClaI:LEU2 pGAL1:SSC1 (URA3))* in which viability was maintained on galactose-based media by the plasmid containing the wild-type *SSC1* gene under control of the *GAL1* promoter. Transformants were patched onto galactose-based media and replica-plated onto glucose media at a variety of temperatures. Cells that grew slowly at any temperature were selected for further analysis.

The yeast strain QL1 (ref. 44) and *Escherichia coli* strain C41 (ref. 45) were used for protein purification. Six histidine codons were present at the 3′ end of all genes. If mutant *SSC1* genes could maintain viability, protein was purified from yeast lacking the wild-type protein. The C terminus of *SSC1* (residues 412–654) and its truncations (residues 412–561 and 412–536), as well as the C terminus of *SSQ1* (residues 424–657), were cloned into the *E. coli* plasmid pET3a. Expression was induced by addition of IPTG.

Protein purification. *Escherichia coli* cell pellets were resuspended in buffer A (20 mM Tris buffer, pH 8.0, 100 mM KCl, 10% (v/v) glycerol, 1 mM DTT, 10 mM imidazole, EDTA-free protease inhibitors (Roche), or in the case of Ssq1 C terminus (residues 424–657), 250 mM KCl and 0.05% (v/v) Triton X-100 in all buffers, and subjected to treatment in a French Press twice at 4 °C. Extracts were clarified by centrifugation at 12,000*g* for 30 min at 4 °C. The clear supernatant was subjected to affinity chromatography using Ni-NTA agarose, which was subsequently washed 10 times with the buffer A, then twice with buffer B (20 mM Tris buffer, pH 8.0, 100 mM KCl, 10% (v/v) glycerol, 60 mM imidazole, 5 mM ATP), twice with buffer C (20 mM Tris buffer, pH 8.0, 1 M KCl, 10% (v/v) glycerol, 60 mM imidazole), twice with buffer D (20 mM Tris buffer, pH 8.0, 100 mM KCl, 10% (v/v) glycerol, 60 mM imidazole). Bound proteins were eluted with buffer E (20 mM Tris buffer, pH 8.0, 100 mM KCl, 10% (v/v) glycerol, 500 mM imidazole) and dialyzed against buffer appropriate for use in particular experiments. Pam18/Tim14 (ref. 18) and His-tagged Mdj1 were purified from *E. coli* as described[46].

Ssc1 C-terminal truncations were purified from mitochondrial lysates in the presence of 1 M KCl in 25 mM HEPES-KOH, pH 7.4, 0.15 M KCl, 10% (v/v) glycerol, 20 mM imidazole, 0.5% (v/v) Triton X-100, 1 mM PMSF (IMAC-20) buffer. Ni-NTA agarose was washed with 5 mM ATP in IMAC-20 buffer before elution. His-tagged Tim44 (ref. 13) and His-tagged Ssq1 (ref. 47) were purified from yeast as described.

ATPase assay. Isolation of Hsp70–ATP complexes and ATPase assays were carried out essentially as described[13,21], using preformed [^{32}P]ATP-Ssc1 complexes. Aliquots of complexes were thawed on ice; time 0 was the time of shift to 23 °C. Hydrolysis at time 0 (~15–25%) was subtracted from all time points.

***In vitro* coimmunoprecipitation assay.** The Ssc1 coimmunoprecipitation assay was carried out essentially as described[13]. Affinity-purified antibodies against full-length Ssc1 were used for coimmunoprecipitation of N- and C-terminal domains and truncations (residues 24–561 and 24–536), whereas affinity-purified antibodies against the C-terminal fragment of Ssc1 (residues 349–654) were used for L37S, S66F, S527F, Ssc1(412–561) and Ssc1(412–536). Tim44-specific antibodies were affinity-purified using full-length Tim44 (ref. 13). Ssc1 (6 μg) was incubated with Ssc1 antibody–protein A–Sepharose beads at 4 °C for 1 h (or 7 μg of Tim44 was incubated with Tim44 antibody beads). Under these conditions, ~90% of Ssc1 or Tim44 was typically immunoprecipitated. After three washes, Ssc1 (0.5 μg) or Tim44 (0.6 μg) was added. The mixture was incubated in a final volume of 150 μl at 23 °C for 30 min, at which point, in some cases, 1.5 μl of a 1 mg ml^{-1} solution of peptide P5 or the yeast mitochondrial CoxIV presequence, MLSLRQSIRFFKPATRRLC, was added and incubation continued for an additional 30 min.

Peptide-binding assay. Fluorescence anisotropy assays for binding of peptide were carried out as described[13,21]. Various concentrations of Ssc1 were incubated with 10 nM fluorescein-labeled P5 (F-P5) at 23 °C; after binding reached equilibrium, anisotropy measurements were made and the K_d calculated by fitting to a one-site binding hyperbola equation. k_{off} data were fitted to a one-phase exponential decay equation.

Miscellaneous. Mitochondria were purified as described[21]. Size-exclusion chromatography, *in organellar* coimmunoprecipitation assays and purification of nucleotides were carried out as described[13].

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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