Distinct categories of death stimuli require BAX and BAK at either mitochondria or ER control points (fig. S7). BAX and BAK at mitochondria are requisite for "BH3-only" proteins, whereas regulation of ER Ca^{2+} by BAX and BAK proved an obligate control point for lipid second messengers (*17–19*) and oxidative stimuli (*31*). A third category of stimuli is influenced by control points at both mitochondria and ER and includes intrinsic signals initiated by staurosporine, etoposide, and brefeldin-A. The coordinate roles for BAX and BAK at the ER and mitochondria illustrate the importance of Ca^{2+} dynamics between these organelles in apoptosis.

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Regulated Cycling of Mitochondrial Hsp70 at the Protein Import Channel

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Hsp70 of the mitochondrial matrix (mtHsp70) provides a critical driving force for the import of proteins into mitochondria. Tim44, a peripheral inner-membrane protein, tethers it to the import channel. Here, regulated interactions were found to maximize occupancy of the active, adenosine 5′-triphosphate (ATP)–bound mtHsp70 at the channel through its intrinsic high affinity for Tim44, as well as through release of adenosine diphosphate (ADP)–bound mtHsp70 from Tim44 by the cofactor Mge1. A model peptide substrate rapidly released mtHsp70 from Tim44, even in the absence of ATP hydrolysis. In vivo, the analogous interaction of translocating polypeptide would release mtHsp70 from the channel. Consistent with the ratchet model of translocation, subsequent hydrolysis of ATP would trap the polypeptide, driving import by preventing its movement back toward the cytosol.

Because only a few of the hundreds of proteins in mitochondria are encoded by mitochondrial DNA, mitochondrial function depends on efficient import of proteins translated on cytosolic ribosomes. Translocation of these proteins across the inner mitochondrial membrane through the inner membrane (TIM) channel is driven by an import motor. A critical component of this machine is the major Hsp70 molecular chaperone of mitochondria (mtHsp70; Ssc1 in yeast) (*1, 2*), which binds short hydrophobic segments of the incoming polypeptide chains. MtHsp70 is tethered to the TIM channel by interaction with the peripherally associated membrane protein Tim44 (*3–5*). How this interaction promotes the function of mtHsp70 in mitochondrial import is unresolved and remains controversial (*6–8*). Because the mechanism of mitochondrial import has been addressed primarily by using complex in organellar systems, we purified mtHsp70 and Tim44, thus allowing a dissection of interactions in vitro.

To assess the mtHsp70-Tim44 interaction in vitro, we monitored complex formation by the ability of Tim44 to be precipitated by mtHsp70-specific antibodies (*9*). mtHsp70, like other Hsp70s, is an adenosine triphosphatase (ATPase), whose chaperone activity is dependent upon alternating between ATP- and ADP-bound states (*10*, *11*). To mimic the in vivo situation, we carried out reactions in the presence of ATP or ADP. About 50% of the Tim44 was coimmunoprecipitated in the presence of either nucleotide (Fig. 1A; fig. S2). This lack of nucleotide dependence of complex formation was surprising, because coimmunoprecipitation analysis with mitochondrial lysates had indicated that the mtHsp70-Tim44 interaction was extremely ATP sensitive (*12–14*) (Fig. 1A and fig. S2). To ensure that the interaction of purified Tim44 and mtHsp70 observed in the presence of ATP was not due to hydrolysis during the course of the experiment, we tested the interaction of mtHsp70 with a peptide substrate, an interaction known to be sensitive to ATP both in vivo and in vitro. A model peptide tagged with fluorescein was used to monitor binding in the mtHsp70 peptide-binding cleft under the conditions used to analyze the mtHsp70-Tim44 interaction. As expected from previous results (*11*), mtHsp70 had a dissociation constant (K_d) of 0.3 μM for peptide in the presence of ADP, but a drastically reduced affinity (~50-fold less) in the presence of ATP or a nonhydrolyzable ATP analog, AMP-PNP (5′-adenylylimido diphosphate) (Fig. 1B). The reduced peptide affinity in the presence of ATP or AMP-PNP indicated that ATP hydrolysis was inconsequential under these assay conditions.

To assess more quantitatively the mtHsp70-Tim44 interaction, we incubated increasing concentrations of mtHsp70 (0.35 to 2.8 μM) in the presence of 0.09 μM Tim44 with ADP, ATP, or AMP-PNP (Fig. 1, C and D). Concentration-dependent binding was observed in all cases,

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with 50% of Tim44 binding occurring at mtHsp70 concentrations between 0.5 and 1.5 μM. Thus, mtHsp70 and Tim44 formed a complex in the presence of ATP, ADP, or AMP-PNP. Because the amount of mtHsp70 required to form a complex was similar in the presence of ATP and AMP-PNP, hydrolysis of ATP was not required for this interaction. In addition, the mtHsp70-Tim44 interaction was fundamentally distinct from a peptide substrate interaction, because mtHsp70 had a similar affinity for Tim44 regardless of its nucleotide state.

In vivo, the critical functional interaction is the formation of a ternary complex between Tim44-bound mtHsp70 and the translocating polypeptide chain at the import channel. MtHsp70(ATP) initiates this interaction because the on rate is very rapid (*10*). Subsequent hydrolysis of ATP to ADP, which is stimulated by polypeptide binding, stabilizes this interaction, because the off rate of polypeptide is slow in the ADP-bound state. To mimic this interaction in vitro, we added peptide to a preformed mtHsp70-Tim44 complex. The addition of peptide destabilized the mtHsp70(ATP)-Tim44 complex, but not the mtHsp70(ADP)-TIM44 complex (Fig. 2A). Thus, in vivo, the binding of polypeptide to Hsp70 may trigger the activation of the import machine, stimulating the release of mtHsp70 from Tim44.

The results of this in vitro analysis also provide an explanation for the sensitivity of the mtHsp70-Tim44 complex to ATP, but not to ADP, observed in mitochondrial lysates. Lysates contain a variety of polypeptides that may have exposed hydrophobic amino acids, providing mtHsp70 binding sites. The availability of such substrates would promote dissociation of the mtHsp70(ATP)-Tim44 complex, which otherwise would remain stable. On the other hand, the presence of such potential substrates would be expected to have no effect on the mtHsp70(ADP)-Tim44 complex.

Because polypeptide binding stimulated ATP hydrolysis, we examined whether peptide-induced release of mtHsp70 from Tim44 required hydrolysis by substituting AMP-PNP for ATP. Release still occurred (Fig. 2A). Because the order of Ssc1's release from Tim44 and ATP hydrolysis has mechanistic implications, we compared the rates of the two events (*9*). Fifteen seconds after addition of peptide, no detectable Tim44-mtHsp70 complex remained (Fig. 2B). The rate of hydrolysis of ATP in a preformed Tim44-mtHsp70(ATP) complex was stimulated by the addition of peptide. However, 15 s after addition of peptide, less than 5% of the ATP was hydrolyzed (Fig. 2B). Thus, ATP hydrolysis was not required for, nor did it occur prior to, release.

Although events subsequent to mtHsp70 binding to translocating polypeptide are critical, efficient import will only occur if occupancy of the productive, ATP-bound form of mtHsp70 at the import channel is maximized. Given that the estimated concentrations of mtHsp70 and Tim44 in mitochondria are in the range of 70 and 3 μM, respectively (*15, 16*), most of the Tim44 should be complexed with mtHsp70 in vivo. However, barring the effects of unknown regulatory mechanisms, mtHsp70(ATP) and mtHsp70(ADP) would bind with similar affinity. In addition, because mtHsp70s have an intrinsic ATPase activity, conversion of mtHsp70(ATP)-Tim44 to mtHsp70(ADP)-Tim44 could occur at the channel. Thus, we investigated whether other factors functioned to maximize occupancy by mtHsp70(ATP).

In organello studies indicated that Mge1, a mtHsp70 nucleotide release factor, promotes release of mtHsp70 from Tim44 (*17*). We examined the effect of Mge1 on the mtHsp70-Tim44 interaction in vitro. Consistent with previous results, Mge1 stimulated the release of mtHsp70(ADP) from Tim44 (Fig. 2; fig. S3). However, Mge1 had little effect on the mtHsp70(ATP)-Tim44 interaction. Thus, Mge1 may facilitate the dissociation of nonproductive mtHsp70(ADP)-Tim44 complexes without affecting the stability of productive mtHsp70(ATP)-Tim44 complexes. In vivo, Mge1 would function to maintain mtHsp70(ATP) at the import channel poised to bind an incoming polypeptide chain.

Next we tested whether a mtHsp70-polypeptide complex recently released from the import channel would compete with

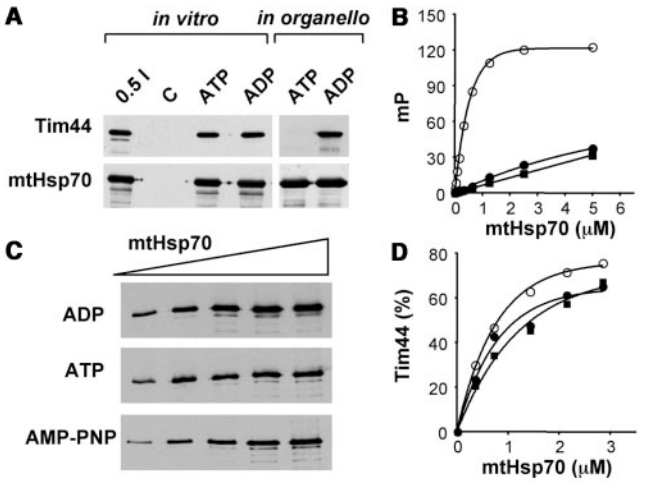

Fig. 1. Characterization of the mtHsp70-Tim44 interaction. (**A**) (Left) Coimmunoprecipitation of Tim44 with mtHsp70 in vitro: 0.6 μM mtHsp70 (Ssc1) and 0.09 μM Tim44 were incubated in the presence of 80 mM KCl, 10 mM $Mg(OAc)_2$, and either 1 mM ATP or 1 mM ADP at 23°C. Before the addition, Hsp70 was mixed with affinity-purified mtHsp70-specific antibodies cross-linked to protein A–Sepharose beads. After centrifugation and washing, beads were subjected to electrophoresis and immunoblot analysis with antibodies specific for mtHsp70 or Tim44. As a control (C), mtHsp70 was omitted. A sample containing one-half of the input served as a control for immunoprecipitation efficiency (0.5 I). (Right) In organello analysis: Mitochondria from wild-type *Saccharomyces cerevisiae* cells were lyzed in the presence of ATP or ADP. (**B**) Binding of mtHsp70 to peptide substrate. Fluorescein-labeled peptide CALLLSAPRR P5 (10 nM) was incubated in the presence of the indicated concentrations of mtHsp70 and ADP (open circles), ATP (filled circles), or AMP-PNP (filled squares) under the same conditions used for the immunoprecipitation assays in (A). Fluorescence anisotropy measurements were taken. After subtraction of background polarization, millipolarization (mP) was plotted as a function of increasing mtHsp70 concentration. (**C**) Coimmunoprecipitation of Tim44 with mtHsp70-specific antibodies in vitro using increasing concentrations of mtHsp70 in the presence of 0.09 μM Tim44. (**D**) Data from (C) were quantified. Symbols as in (B).

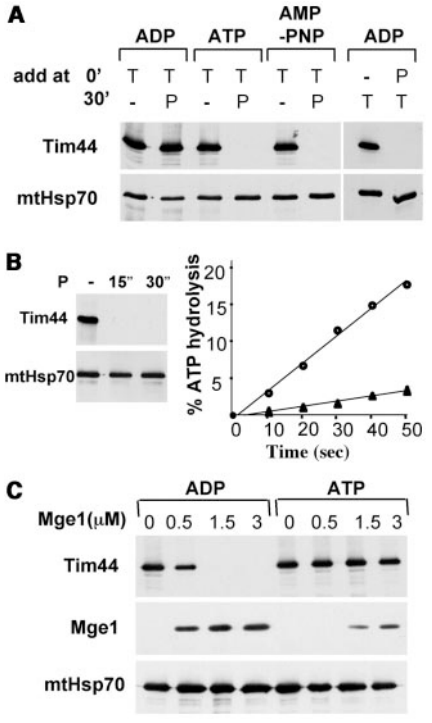

Fig. 2. Effect of peptide (**A** and **B**) or Mge1 (**C**) on the Hsp70-Tim44 interaction in vitro. Immunoprecipitation assays were performed as described for Fig. 1. (A) mtHsp70 was preincubated with Tim44 (T) or P5 peptide (P) for 30 min before addition of the other component; incubation was continued for 30 min before analysis. (B) (Left) Tim44 and mtHsp70 were preincubated and peptide was added for the indicated times. (Right) Preformed radiolabeled ATP-mtHsp70 complex was preincubated with Tim44; at time zero, incubation at 23°C was initiated in the presence (circles) or absence (triangles) of 20 μM peptide; after the reaction was stopped, conversion to ADP was measured (*9*). (C) Tim44 (0.09 μM) and mtHsp70 (0.6 μM) were preincubated in the presence of ADP or ATP. Then Mge1 was added and incubation was continued for 30 min before processing.

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mtHsp70(ATP) for binding to Tim44. Purified Tim44 was added to preformed mtHsp70(ADP)-peptide complex (Fig. 2A). No Hsp70(ADP)-Tim44 complex was detected. Likewise, preformed mtHsp70(ADP)-Tim44 complex was stable in the presence of peptide (Fig. 2A). To determine if a stable triple complex of mtHsp70, Tim44, and peptide could be detected, we incubated the two proteins and fluorescently labeled peptide in various combinations in the presence of ADP, and then separated the complexes by size exclusion chromatography. Whereas mtHsp70-peptide and mtHsp70-Tim44 complexes were detected by this method, no mtHsp70-Tim44-peptide complex was observed (Fig. 3A), suggesting that mtHsp70(ADP) did not bind Tim44 and polypeptide simultaneously.

The results reported here can be incorporated into a comprehensive model of mitochondrial protein import. The functional ATP-bound form of mtHsp70, which can rapidly bind to the incoming polypeptide chain, predominates at the import channel because of its inherent high affinity for Tim44, as well as the dissociation of any ADP-bound mtHsp70 by Mge1 (Fig. 3B, point 1). As the presequence of the polypeptide emerges into the matrix driven by the membrane potential, it interacts with mtHsp70. This binding of polypeptide stimulates the rapid release of mtHsp70 from Tim44 (point 2). Polypeptide binding also stimulates ATP hydrolysis (point 3), which stabilizes mtHsp70's interaction with the polypeptide, preventing retrograde movement of the polypeptide back toward the cytosol. The release of mtHsp70 not only allows diffusion of the polypeptide chain, with mtHsp70 attached, away from the import channel, it also frees Tim44 for binding to another mtHsp70, initiating another cycle of mtHsp70 interaction with the polypeptide and driving import of the chain. The recently released mtHsp70-polypeptide complex is not able to rebind to Tim44, and thus it does not compete with binding of free ATP-bound mtHsp70 (point 4). In time, Mge1, acting as a nucleotide release factor (*18, 19*), binds mtHsp70(ADP), stimulating release of ADP, and thus carrying out its second function in mitochondrial import. Subsequent binding of ATP causes release of mtHsp70 from the chain, owing to the rapid off rate in this nucleotide state, completing the cycle of mtHsp70-polypeptide interaction. The active, ATP-bound form of mtHsp70 is regenerated, and folding of the polypeptide chain proceeds.

A particularly controversial issue has been whether mtHsp70 functions as a ratchet, allowing movement of the polypeptide chain into the matrix but not back toward the cytosol (Brownian ratchet model), or whether mtHsp70 exerts a force on the chain "pulling" it into the matrix (power-stroke/pulling model) (*6–8*, *20*, *21*). According to the power-stroke model, mtHsp70 exerts a force on the polypeptide chain, using Tim44 as a fulcrum as it undergoes a conformational change associated with ATP hydrolysis, which also stabilizes the mtHsp70-polypeptide interaction. The power-stroke model is not compatible with the results presented here without evoking unknown components. Not only is ATP hydrolysis unnecessary for release of mtHsp70 from Tim44, but Tim44 release also is more rapid than the rate of ATP hydrolysis. Our data are completely consistent with the ratchet model.

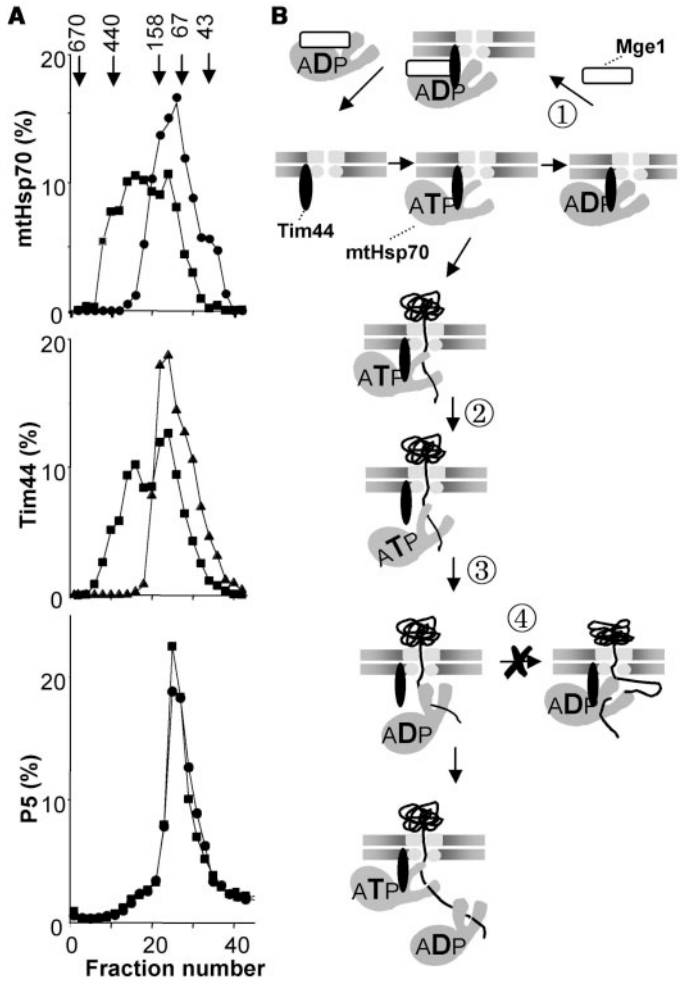

Fig. 3. Interaction of mtHsp70 with cofactors and substrate peptide. (**A**) Analysis of mtHsp70 complexes by size exclusion chromatography. Reactions: (1) 15 μg of Tim44 (triangles); (2) 30 μg of mtHsp70 and fluorescein-labeled peptide P5 (circles); (3) 30 μg of mtHsp70 incubated with 15 μg of Tim44 for 30 min, followed by the addition of fluorescein-labeled P5 and further incubation for 1 hour (squares). Reactions were subjected to chromatography on a Superdex 200HR-10/30 column. Fractions were subjected to electrophoresis and immunoblot analysis with mtHsp70- and Tim44-specific antibodies; signals were quantified by densitometry. P5 peptide in each fraction was monitored by fluorescence intensity. Tim44 forms a dimer (*16*), hence it shows an elution profile similar to that of mtHsp70. Note that when fluorescein-tagged peptide was added to mtHsp70 and Tim44, no fluorescence was detected in the fractions containing the faster moving Tim44-mtHsp70 complex. (**B**) Model of regulation of the interactions between mtHsp70 and Tim44, translocating polypeptide, and Mge1. Circled numbers refer to points of discussion in the text.

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