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Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane

(*Saccharomyces cerevisiae*/chaperones/GrpEp/protein translocation/histidine tagging)

NAFSIKA G. KRONIDOU, WOLFGANG OPPLIGER, LUCA BOLLIGER, KEVIN HANNAVY, BENJAMIN S. GLICK, GOTTFRIED SCHATZ, AND MARTIN HORST*

Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Contributed by Gottfried Schatz, September 8, 1994

ABSTRACT **The protein import system of the yeast mitochondrial inner membrane includes at least three membrane proteins that presumably form a transmembrane channel as well as several chaperone proteins that mediate the import and refolding of precursor proteins. We show that one of the membrane proteins, Isp45, spans the mitochondrial inner membrane yet is extracted from this membrane at high pH. Solubilization of mitochondria with a nonionic detergent releases Isp45 as a complex with the chaperones mitochondrial hsp70 (mhsp70) and GrpEp. Both chaperones reversibly dissociate from Isp45 upon addition of ATP or adenosine 5′-[γ-thio]triphosphate, suggesting that dissociation requires the binding of ATP. Control experiments indicate that the interaction between mhsp70 and Isp45 occurs in the intact mitochondria. We propose that Isp45 lines the inside of a proteinaceous channel across the inner membrane and that it is the membrane anchor for an ATP-driven "import motor" composed of mhsp70 and GrpEp. This arrangement is reminiscent of the protein transport systems of the yeast endoplasmic reticulum and the bacterial plasma membrane.**

The mitochondrial inner and outer membranes both have their own protein import system (1, 2). Although these two systems can act independently, they cooperate during the import of precursor proteins into the matrix. Insertion of a matrix-targeting presequence into the inner membrane is driven by the electrochemical potential across that membrane; the remainder of the polypeptide chain is then apparently pulled into the matrix by the ATP-dependent action of mitochondrial hsp70 (mhsp70) and its cochaperone, GrpEp (3, 4).

Biochemical and genetic studies in yeast have identified three proteins that are putative subunits of a heterooligomeric protein import channel in the mitochondrial inner membrane (ref. 5; for review, see ref. 3). Isp45 (also termed Mpi1p or MIM44), the best-characterized of these proteins, can be crosslinked to a precursor arrested during its translocation across the inner membrane. Furthermore, antibodies against Isp45 inhibit protein import into inner membrane vesicles or mitoplasts (6, 7), and a mutation in *MPI1* (the gene encoding Isp45) inhibits mitochondrial protein import in intact yeast cells (8).

In this paper, we show that Isp45 is an unusual membrane protein; it spans the inner membrane, even though it can be extracted at alkaline pH. Upon solubilization of mitochondria with a nonionic detergent, Isp45 is released as a complex with mhsp70 and GrpEp. This complex reversibly dissociates upon addition of ATP. We suggest that the dynamic complex composed of Isp45, mhsp70, and GrpEp represents a force-generating motor that mediates the energy-dependent transport of precursors across the mitochondrial inner membrane.

MATERIALS AND METHODS

General Methods. Mitochondria were prepared from wild-type *Saccharomyces cerevisiae* strain D273-10B or YZIM6 and purified on a Nycodenz gradient (B.S.G. and L. Pon, unpublished data). Radiolabeled mitochondria (specific activity, 4×10^8 cpm/mg) were isolated from cells grown overnight to an A_{600} of 0.7 in low-sulfate medium supplemented with 2% lactate and 50 μCi of $Na_2{}^{35}SO_4$ per ml (1 Ci = 37 GBq) and were mixed with a 10-fold excess of unlabeled mitochondria that had been purified on a Nycodenz gradient. Published methods were used for generating mitoplasts by osmotic shock (9) and for treating mitochondria or mitoplasts for 20 min on ice with 50–100 μg of proteinase K per ml (10). mhsp70 was prepared as described (11). Hexahistidine-tagged mhsp70 was isolated from mitochondria purified on a Nycodenz gradient (B.S.G. and L. Pon, unpublished data).

DNA Constructs. A yeast strain expressing the hexahistidine-tagged mhsp70 has been described (4). Hexahistidine-tagged Isp45 was constructed by PCR as follows. Oligonucleotide HISTAG (5′-GCTCTAGACAATTCACCCAC-CACCACCACCACCACTGATTGGTTTCGATGTGC-3′) containing the final codon of the *MPI1* open reading frame followed by 6 histidine codons, a stop codon, and downstream yeast chromosomal sequence and oligonucleotide PEEWEE7 (5′-CCCCGAGCTCAAGCTTGTCCGTCAAG-3′) corresponding to positions 2356–2341 of the chromosomal *MPI1* gene (8) were used as primers. The template DNA was plasmid pWEE12, containing the 3′ end of the *MPI1* gene. The PCR product was subcloned into pCRII (Invitrogen) to yield pHIS1. pHIS2 was constructed by subcloning a 240-bp *Xba* I/*Sca* I fragment from pHIS1 into pWEE10 by cutting with *Hin*dIII, end-filling, and cutting with *Xba* I. pWEE10 is a yeast expression vector based on YCplac22 (12) containing *MPI1*. pHIS2 was transformed into the diploid yeast strain YZIM1 (*mpi1*::*URA3*/*MPI1*; derived from JK9-3d; ref. 13). Trp^+ transformants were sporulated, and the asci were dissected onto yeast extract/peptone/dextrose plates at 30°C. All spores that grew were either ura^+ or ura^- and trp^+ (data not shown). The *mpi1*::*URA3* disruption was thus complemented by the histidine-tagged Isp45. The haploid strain YZIM6 (*mpi1*::*URA3*/*pHIS2*) is a ura^-, trp^+ product of dissection.

Immunoprecipitations. Immunoprecipitations were performed essentially as described (14) except that the solubilization buffer contained 10% (wt/vol) glycerol. IgGs were affinity-purified on the appropriate antigen coupled to CNBr-Sepharose.

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Abbreviations: mhsp70, mitochondrial hsp70; ATP[γS], adenosine 5′-[γ-thio]triphosphate.

*To whom reprint requests should be addressed.

RESULTS

mhsp70 Coimmunoprecipitates with Isp45. To identify proteins associated with Isp45, ^{35}S-labeled yeast mitochondria were solubilized with 1% Triton X-100 and subjected to immunoprecipitation with affinity-purified anti-Isp45 IgGs. Isp45 and three additional proteins of apparent molecular masses 70, 50, and 48 kDa were immunoprecipitated (Fig. 1*A*, left lane). Essentially the same result was obtained when mitochondria were solubilized with other nonionic detergents; upon solubilization with SDS, however, only Isp45 was immunoprecipitated, confirming the monospecificity of the IgGs (data not shown). Freeze–thawing followed by sonication did not dissociate the complex between Isp45 and the 70-kDa protein but did release some of the 50-kDa protein from Isp45 (Fig. 1*A*, right lane). When the immunoprecipitated complex was analyzed by SDS/PAGE and immunoblotting, the 70-kDa band was identified as mhsp70 (Fig. 1*B*) and the 50-kDa band was identified as Mdj1p, a mitochondrial homologue of bacterial DnaJ (15) (data not shown). The 48-kDa band (Fig. 1*A*, X) has not yet been identified.

Isp45 Copurifies with Histidine-Tagged mhsp70. To determine whether the same Isp45 partner proteins could be detected by a method other than coimmunoprecipitation, we used a yeast strain in which all of the mhsp70 molecules carry a C-terminal hexahistidine tag (4). When mitochondria from this strain were solubilized with Triton X-100 and incubated with Ni-NTA-agarose beads, the beads bound not only the tagged mhsp70 but also two proteins of apparent molecular masses 45 and 24 kDa (Fig. 1*C*, lane 1). None of these proteins was bound when the experiment was performed with wild-type yeast expressing only untagged mhsp70 (data not shown). Immunoblotting identified the 70-kDa band as mhsp70 (Fig. 1*C*, lane 4), the 45-kDa band as Isp45, and the 24-kDa band as GrpEp, the mitochondrial homolog of bacterial GrpE (Fig. 1*C*, lanes 3 and 2, respectively; see also lane 4). Conversely, when a Triton X-100 extract of mitochondria carrying only hexahistidine-tagged Isp45 was incubated with Ni-NTA-agarose beads, binding of the tagged Isp45 to the beads was accompanied by binding of GrpEp and mhsp70; binding of these chaperones to Isp45 was prevented by ATP (Fig. 1*D*).

The Interaction Between Isp45 and mhsp70 Occurs in Intact Mitochondria. The association of mhsp70 with Isp45 could reflect nonspecific binding of a promiscuous chaperone to Isp45 molecules that had been partly denatured by solubilization. To exclude this possibility, we mixed radiolabeled wild-type mitochondria with a 10-fold weight excess of unlabeled mitochondria that contained only histidine-tagged mhsp70. The mixture was solubilized with Triton X-100 and two aliquots were then either immunoprecipitated with anti-Isp45 antiserum (Fig. 2, lane 1) or incubated with Ni-NTA-agarose beads (lane 2). Immunoprecipitates and proteins bound by the Ni-NTA-agarose beads were analyzed by SDS/PAGE and fluorography or immunoblotting. No radiolabeled Isp45 was bound by the Ni-NTA-agarose beads (lane 2). However, antibodies against Isp45 still immunoprecipitated both mhsp70 and Isp45 from the supernatant after sedimentation of the Ni-NTA beads, indicating that the complex had remained intact (lane 3). Also, the Ni-NTA beads had bound unlabeled Isp45 via unlabeled mhsp70, indicating that the mhsp70–Isp45 complex from the unlabeled mitochondria had remained intact (lane 4). As the great excess of histidine-tagged mhsp70 from the unlabeled mitochondria had not associated with Isp45 from the admixed radiolabeled mitochondria, the complex between mhsp70 and Isp45 must have already existed before solubilization.

Binding of ATP Disrupts the Complex Between Isp45 and mhsp70. To examine the effect of nucleotides on the interaction between Isp45 and mhsp70, we solubilized radiolabeled mitochondria with Triton X-100 and subjected the extract to immunoprecipitation with anti-Isp45 antibodies in the presence of ATP, ADP, or the nonhydrolyzable ATP analog adenosine 5′-[γ-thio]triphosphate (ATP[γS]). ATP or ATP[γS] abolished the Isp45–mhsp70 interaction, whereas ADP had no effect (Fig. 3*A*). Coimmunoprecipitation of Mdj1p and the 48-kDa protein with Isp45 was not affected by any of the nucleotides tested (data not shown). The complex

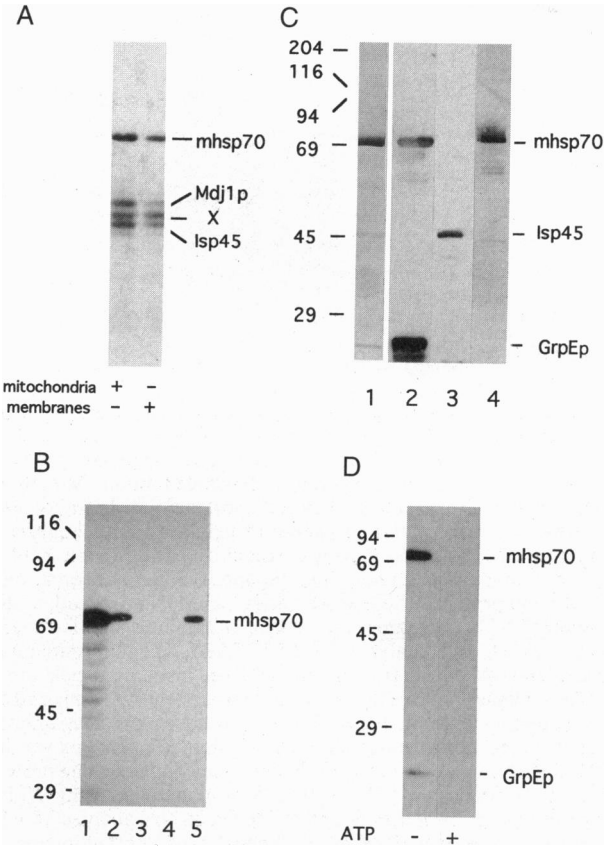

FIG. 1. mhsp70 copurifies with Isp45p. (*A*) Mitochondria were isolated from yeast cells grown in the presence of $^{35}SO_4^{2-}$. Half of the mitochondrial sample (200 μg of protein) was subjected to repeated cycles of freeze–thawing followed by sonication, and membranes were collected by centrifugation. Both samples were solubilized with 1% Triton X-100 and subjected to immunoprecipitation with affinity-purified IgGs against Isp45. Samples were analyzed by SDS/PAGE and fluorography. X, unidentified protein coimmunoprecipitating with Isp45. (*B*) Extract from unlabeled mitochondria (200 μg) was subjected to immunoprecipitation with nonimmune serum (lane 3), antiserum against cytochrome b_2 (lane 4), or affinity-purified IgGs against Isp45 (lane 5), and the immunoprecipitates were analyzed for mhsp70 by SDS/PAGE and immunoblotting. Immune complexes were visualized with ^{125}I-labeled protein A and fluorography. Lanes: 1, extract used for immunoprecipitation, subjected directly to SDS/PAGE; 2, 10% of the supernatant after sedimentation of protein A-Sepharose beads from the sample that had received preimmune serum. (*C*) Mitochondria (1 mg) containing histidine-tagged mhsp70 were solubilized with Triton X-100; extract was incubated with Ni-NTA-agarose beads, the beads were washed, and proteins bound to the beads were eluted and subjected to SDS/PAGE. Lane 1, Coomassie blue-stained gel showing mhsp70, Isp45, and GrpEp. These proteins were identified by immunoblotting with antisera against GrpEp (lane 2), against Isp45p (lane 3), or against mhsp70 (lane 4). Antiserum against GrpEp also contained antibodies against mhsp70. Numbers on left indicate size (kDa). (*D*) Mitochondria (1 mg) containing histidine-tagged Isp45 were solubilized with Triton X-100, and the extract was incubated with Ni-NTA-agarose beads in the absence (lane −) and presence (lane +) of 2 mM ATP. Beads were washed and bound proteins were eluted and analyzed for GrpEp by SDS/PAGE followed by immunoblotting. Immune complexes were visualized with ^{125}I-labeled protein A and fluorography. Antiserum against GrpE also contains antibodies against mhsp70.

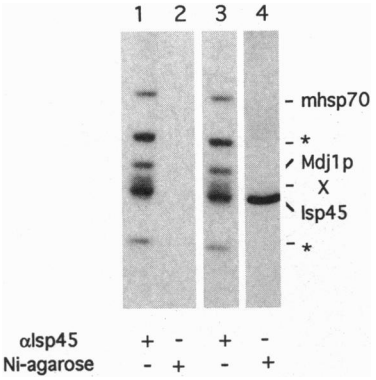

FIG. 2. Complex between Isp45 and mhsp70 exists in intact mitochondria. Cells expressing only histidine-tagged mhsp70 were grown in unlabeled medium, and cells expressing wild-type mhsp70 were grown in medium containing radiolabeled sulfate. Mitochondria were isolated from each batch of cells, and radiolabeled mitochondria were mixed with a 10-fold excess of unlabeled mitochondria. The mixture (0.4 mg of protein) was solubilized with Triton X-100 and divided into two aliquots. One aliquot received antiserum against Isp45 and protein A-Sepharose beads (lane 1), and the other aliquot received Ni-NTA-agarose beads (lane 2). Proteins bound to the beads were eluted and analyzed by SDS/PAGE and fluorography. No radiolabeled Isp45 cofractionated with the histidine-tagged mhsp70. After sedimentation of the Ni-NTA-agarose beads, the mhsp70 could be coimmunoprecipitated from the supernatant by using antibodies against Isp45, confirming that the Isp45–mhsp70 complex was intact (lane 3). After precipitation of the immune complex using protein A, the histidine-tagged mhsp70 was precipitated by using Ni-NTA-agarose beads. Material bound to the beads was analyzed for Isp45 by immunoblotting (lane 4). Unlabeled Isp45 cofractionated with histidine-tagged mhsp70. Asterisks, two proteins that are precipitated when total anti-Isp45 antiserum is used instead of affinity-purified anti-Isp45 IgGs. X, see Fig. 1.

between Isp45 and mhsp70 is thus disrupted by the binding of ATP; hydrolysis of ATP may not be required.

Interaction of Isp45 with mhsp70 Is Reversible. To test whether the binding of Isp45 to mhsp70 is reversible, mitochondria containing only histidine-tagged Isp45 were solubilized with Triton X-100 and the extract was incubated with Ni-NTA-agarose beads in the presence or absence of ATP. In the absence of ATP, both Isp45 and mhsp70 were bound to the beads, whereas in the presence of ATP only Isp45 was bound (Fig. 3*B*, columns 1 and 2; see also above). With extracts from wild-type mitochondria, neither Isp45 nor mhsp70 was associated with the beads, as expected (data not shown). When the beads that had bound histidine-tagged Isp45 in the presence of ATP were washed and then incubated with purified mhsp70 together with apyrase (to hydrolyze any remaining ATP), mhsp70 reassociated with the immobilized Isp45 to 75% of the original value (Fig. 3, column 3). No such binding of added mhsp70 was seen in the presence of ATP (Fig. 3*B*, column 4).

Isp45 Spans the Inner Membrane. Because Isp45 interacts with the matrix-localized mhsp70, at least a portion of Isp45 must be present at the inner face of the inner membrane. Isp45 also appears to be exposed to the outer face of the inner membrane because an epitope tag attached to its C terminus is accessible to protease in mitoplasts (mitochondria whose outer membrane had been selectively disrupted; ref. 8) and because antibodies monospecific for Isp45 block protein translocation into inner membrane vesicles (6, 7). These observations strongly suggest that Isp45 spans the inner membrane. However, transmembrane proteins are generally integral membrane proteins that cannot be solubilized at pH 11.5, whereas Isp45 is solubilized at that pH (6, 16). We therefore decided to reinvestigate the membrane topology of Isp45.

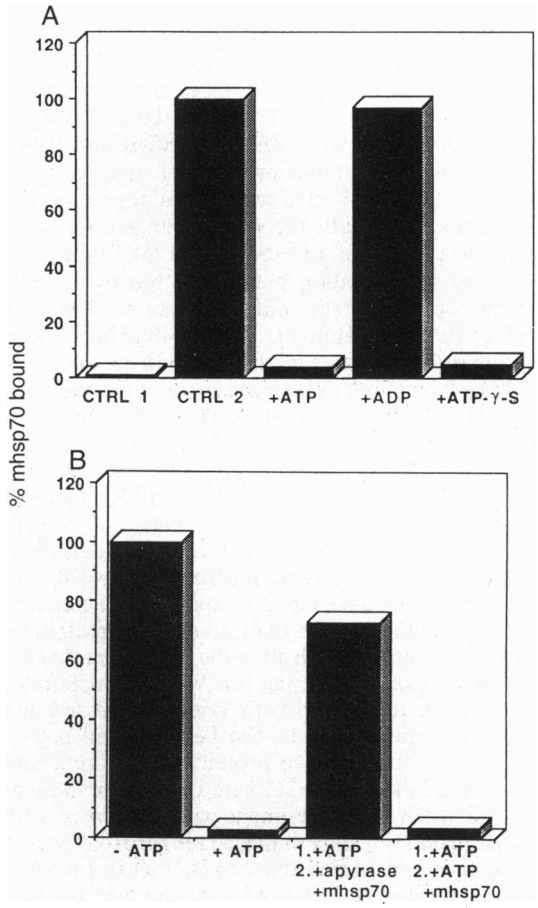

FIG. 3. Effect of nucleotides on interaction between Isp45 and mhsp70. (*A*) The Isp45–mhsp70 interaction is disrupted by ATP and ATP[γS]. A Triton extract of radiolabeled mitochondria was divided into five aliquots. The first aliquot was subjected to mock immunoprecipitation with preimmune serum (CTRL 1), the second was subjected to immunoprecipitation with antiserum against Isp45 in the absence of added nucleotides (CTRL 2), and the others were subjected to immunoprecipitation with antiserum against Isp45 in the presence of 2 mM ATP, ADP, or ATP[γS]. (*B*) Purified mhsp70 associates with Isp45. Mitochondria (0.5 mg) containing only histidine-tagged Isp45 were solubilized with Triton X-100, and the extract was incubated with Ni-NTA-agarose beads either in the absence of ATP (− ATP) or in the presence of 2 mM ATP (+ ATP). An aliquot of the beads that had been incubated with the extract in the presence of ATP was washed and incubated for 1 h at 4°C in the presence of purified mhsp70 (25 μg) either with apyrase (20 units/ml) (column 3) or with ATP (2 mM) (column 4). Beads were then washed and bound material was analyzed for mhsp70 by SDS/PAGE and immunoblotting. Immune complexes were visualized with ^{125}I-labeled protein A and fluorography, and results were quantified by densitometry of the fluorograms. The amount of mhsp70 coimmunoprecipitating with Isp45 in the absence of ATP was set at 100%.

Mitoplasts were incubated with IgGs monospecific for Isp45, sedimented through a sucrose cushion to remove unbound IgGs, and solubilized with Triton X-100. The extract was incubated with protein A-Sepharose beads, and immune complexes bound to the beads were analyzed for Isp45 by SDS/PAGE and immunoblotting. Isp45 was immunoprecipitated under these conditions (Fig. 4, lane 5), showing that at least one epitope of Isp45 faces the intermembrane space. To estimate the efficiency of the immunoprecipitation, we compared the amount of Isp45 recovered in this experiment with the amount that could be immunoprecipitated by adding IgGs to Triton-solubilized mitochondria. Quantification of the bands shown in Fig. 4*A* (lanes 4 and 5) showed that at least 70% of the total Isp45 molecules were accessible to IgGs in

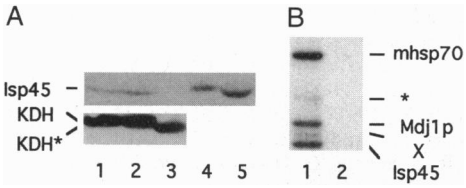

FIG. 4. Isp45 is a transmembrane protein. Mitoplasts (20 μg in 100 μl 0.6 M sorbitol/20 mM Hepes·KOH, pH 7.4) were incubated for 1 h at 4°C with 50 μg of affinity-purified IgGs against Isp45, washed by sedimention through a 2-ml cushion of 1.2 M sucrose, and solubilized with Triton X-100. The extract (0.2 ml) was incubated for 4 h at 4°C with 20 μl of a 1:1 slurry of protein A-Sepharose beads. Beads were reisolated and washed, and bound proteins were eluted and analyzed for Isp45 by SDS/PAGE and immunoblotting. Blots were developed with ^{125}I-labeled protein A. An aliquot of the mitoplasts was treated with proteinase K and analyzed by SDS/PAGE and immunoblotting for the matrix marker α-ketoglutarate dehydrogenase (KDH) (10). (*A*) Fluorograph of immunoblots. Upper lanes, antiserum against Isp45; lower lanes, antiserum against KDH. Lanes: 1, mitochondria (equivalent to the amount of mitoplasts used) were analyzed directly by SDS/PAGE and immunoblotting; 2, mitoplasts treated with proteinase K; 3, same as lane 2 except mitoplasts were protease treated in the presence of 1% Triton X-100; 4, Isp45 directly immunoprecipitated from Triton-solubilized mitochondria (corresponding to 1/3rd the amount of mitoplasts used); 5, immunoprecipitate from mitoplasts that had been incubated with Isp45 antiserum, washed, solubilized, and then incubated with protein A-Sepharose (see above). Bound antibodies were visualized with ^{125}I-labeled protein A and fluorography. KDH*, fragment of KDH generated by protease treatment (10). (*B*) Lanes: 1, Isp45 was immunoprecipitated from 0.2 mg of radiolabeled mitochondria; 2, 0.2 mg of radiolabeled mitochondria was added to 0.2 mg of unlabeled mitoplasts that had been incubated with antibodies against Isp45. Mixture was solubilized and subjected to immunoprecipitation with antiserum against Isp45; immunoprecipitate was then analyzed by SDS/PAGE and fluorography.

mitoplasts. The inner membrane of the mitoplasts was intact, as the matrix enzyme α-ketoglutarate dehydrogenase remained inaccessible to added protease (Fig. 4*A*, lane 2). To exclude the possibility that the IgGs against Isp45 had bound nonspecifically to the mitoplast surface and then associated with Isp45 upon solubilization, mitoplasts were incubated with IgGs, washed, and mixed with radiolabeled mitochondria that had not been incubated with IgGs. The mixture was then solubilized and subjected to incubation with protein A-Sepharose, SDS/PAGE, and fluorography as described above. No radiolabeled Isp45 was detected in the immunoprecipitate (Fig. 4*B*, lane 2). The IgG-treated and washed mitoplasts were thus devoid of IgGs capable of binding to Isp45 after solubilization. We conclude that Isp45 spans the inner membrane but that it is not firmly embedded in the lipid bilayer.

DISCUSSION

We have isolated a complex between Isp45, mhsp70, and GrpEp. These three proteins did not cofractionate with the other two known components of the inner membrane protein transport system, MIM17/Sms1p and Mas6p/MIM23 (ref. 5; for review, see ref. 3), even though we tried different detergents and isolation conditions. The complex described here was isolated by three different methods: coimmunoprecipitation with Isp45, coisolation with histidine-tagged mhsp70, and coisolation with histidine-tagged Isp45. Immunoprecipitation yielded two additional proteins, one of which was Mdj1p. It is unclear whether Mdj1p and the other unidentified protein are genuine components of the protein import system. This caution is underscored by the fact that Mdj1p is not required for protein translocation into mitochondria (15).

Does the complex of mhsp70 with Isp45 merely reflect the binding of the chaperone to a protein that had been partly denatured by solubilization? This concern was heightened by the observation that the complex is dissociated by ATP and thus resembles complexes between 70-kDa chaperones and unfolded polypeptides (17). However, the control experiment shown in Fig. 2 makes it unlikely that the complex is generated upon solubilization. This conclusion is supported by our finding that the membrane-permeable crosslinker dithiobis(succinimidyl propionate) crosslinks mhsp70 to Isp45 in intact mitochondria (data not shown). This latter result also suggests that Isp45 and mhsp70 interact directly and not via GrpEp or another protein that may have escaped detection by our methods.

Although the complex described here must be characterized further, we propose that it forms part of the protein import system in the mitochondrial inner membrane. Biochemical fractionation of heterooligomeric membrane complexes frequently yields subcomplexes that retain some, but not all, of the functions of the holocomplex (18). The complex consisting of Isp45, mhsp70, and GrpEp may represent the "motor" that uses the energy of ATP hydrolysis to pull precursors across the inner membrane. Both Isp45 and mhsp70 are in direct contact with a translocating precursor (6, 7, 11, 16, 19), and there is strong circumstantial evidence that mhsp70 functions as part of an ATP-dependent import motor (B.S.G., unpublished data). Because Isp45 spans the inner membrane and yet is solubilized at alkaline pH, this protein probably is not embedded in the lipid bilayer but instead may be anchored in the membrane by other proteins. These other proteins are most likely subunits of a heterooligomeric protein transport channel. Candidates for these channel subunits are Mas6p/MIM23 and Sms1p/MIM17, both of which appear to be true integral membrane proteins (ref. 5; for review, see ref. 3).

Our results suggest a model in which the energy-converting chaperone mhsp70 attaches to a component of the protein transport channel (Isp45) and pulls a precursor chain across the inner membrane, with GrpEp acting to stimulate mhsp70 function (Fig. 5). This model is strikingly similar to others that have been proposed for the protein transport systems of the yeast endoplasmic reticulum and the bacterial plasma membrane (3). BiP, the mhsp70 homolog in the lumen of the endoplasmic reticulum, promotes the translocation of proteins by interacting physically and functionally with Sec63, a transmembrane protein of the transport machinery. Upon solubilization of the endoplasmic reticulum membrane, BiP and Sec63 copurify as a complex that can be dissociated by ATP (20). In bacteria, the peripheral ATPase SecA reversibly binds to the transmembrane protein SecY in a process that is controlled by ATP (21). The dynamic complex between Isp45, mhsp70, and GrpEp may thus represent the mitochon-

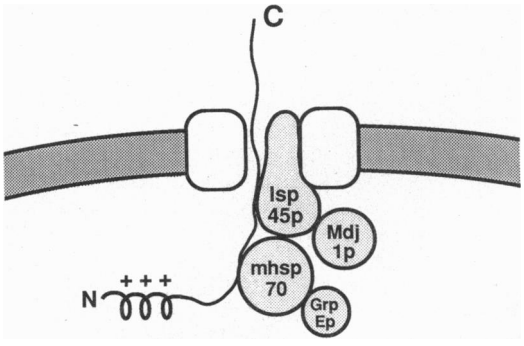

FIG. 5. Model for interaction of mhsp70 with inner membrane import machinery. See text for details.

drial variation on a common theme by which proteins are transported across biological membranes.

Note Added in Proof. The association of mhsp70 with Isp45 (MIM44) has recently been reported by Schneider *et al.* (22).

This work is dedicated to Peter Sitte on the occasion of his 65th birthday. We thank Dr. W. Neupert for the antiserum against Mdj1p. We are indebted to the members of our laboratory for helpful discussions; to H. Brütsch for technical assistance; and to M. Jäggi, V. Grieder, and L. Müller for the artwork. This study was supported by grants from the Swiss National Science Foundation (3-26189.89), the Human Frontier Science Program Organization, and the Human Capital and Mobility Program of the European Community, as well as by postdoctoral fellowships from the European Molecular Biology Organization (to M.H. and K.H.) and from the Human Frontier Science Program Organization and the Boehringer Ingelheim Fonds (to N.G.K.).

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