Membrane integration and assembly of MOM72 from Neurospora crassa and its yeast homolog MAS70 was studied with isolated mitochondria. After synthesis in vitro, the precursors of MOM72/MAS70 are tightly folded and expose only their N-terminal amino acid residues comprising the targeting and the membrane anchor domain. Insertion of the protein into the mitochondrial outer membrane (MOM) occurs in a time- and temperature-dependent manner and is stimulated by ATP. MOM72/MAS70 is then assembled into the outer membrane MOM complex. Whereas membrane insertion occurred independently of the presence of protease-sensitive surface components, the assembly reaction depended on such components. In the MOM complex, MOM72 and MAS70 were found in the neighborhood of different components in yeast and N. crassa mitochondria. MOM72 was found in association with MOM22 in N. crassa mitochondria, whereas MAS70 was in proximity to a 37-kDa component in yeast outer mitochondrial membrane. The interaction with the 37-kDa protein is important for integration of MAS70 into the MOM complex. Thus, the 37-kDa protein plays an important role in the biogenesis of MAS70.

The protein transport machinery of the mitochondrial outer membrane (MOM)1 consists of at least seven different protein components. The MOM complex facilitates insertion and membrane translocation of nuclear encoded mitochondrial proteins during their import into the various subcompartments of the mitochondrion. The proteins present in the MOM complex fulfill distinct functions in the transport process. In Neurospora and yeast, precursor proteins interact through targeting signals with mitochondrial import receptors MOM72, MOM19, and MOM22 at the cis-side of the outer membrane (1-5). Most of the precursors become inserted into a so far only poorly characterized pore that contains MOM38 and at least two other components, MOM8 and MOM7 (6, 7). The targeting signals then interact with a component at the trans-side of the outer membrane (5). Further translocation of the precursor protein across the outer membrane or integration into the outer membrane follows.

The proteins of the MOM complex are all encoded in the nucleus, synthesized on cytoplasmic polysemes, and then post-translationally targeted to the mitochondrial outer membrane.

More than one pathway exists for the targeting of the different MOM proteins to the mitochondrial outer membrane. The MOM19 preprotein requires for its import the pore component MOM38 but no protease-sensitive surface receptors (8). In contrast, the import of MOM22 and MOM38/ISP42 preproteins is dependent on the surface receptors MOM19 and MOM72 (9, 10). After their import the MOM proteins are assembled into the MOM complex. It is unclear so far how these proteins are sorted and assembled into this complex and how they interact with each other.

In this study we have analyzed insertion and assembly of Neurospora crassa MOM72 and its yeast-homolog MAS70 into the outer membrane and the MOM complex. Their insertion into the mitochondrial outer membrane was found to be stimulated by cytosolic ATP and independent of trypsin-sensitive components of the outer membrane. On the other hand, the assembly of MAS70 into the MOM complex was dependent on trypsin-sensitive surface components. Assembled MAS70 interacts in the mitochondrial outer membrane of yeast with a 37-kDa protein, whereas MOM72 interacts in N. crassa mitochondria with MOM22. MAS70 appears to be integrated by the 37-kDa protein into the MOM complex.

**Experimental Procedures**

Protein Import into Isolated Mitochondria—Yeast and N. crassa were grown, and mitochondria were isolated as described before (11, 12) and suspended in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2). Prior to import, mitochondria were diluted at a final concentration of 1 mg/ml into import buffer (250 mM sucrose, 3% (w/v) bovine serum albumin, 5 mM MgCl2, 80 mM KCl, 10 mM MOPS/KOH, pH 7.2) containing 1 mM ATP. 35S-labeled precursor proteins in reticulocyte lysate were added, and import was performed at 25 °C for the times indicated. The mitochondria were reisolated and then analyzed by SDS-PAGE and fluorography.

Immunoprecipitation and Coimmunoprecipitation after Import of Precursor Proteins into Mitochondria—Precursor proteins were first imported into mitochondria. Mitochondria were reisolated and then lysed in Triton X-100 buffer (1% Triton X-100 (w/v), 300 mM NaCl, 10 mM Tris/HCl, pH 7.5) containing 3% (w/v) bovine serum albumin. Unlysed material was removed by centrifugation for 12 min at 20,000 × g. The supernatant was then incubated with antibodies that were coupled to protein A-Sepharose beads. Afterwards the beads were washed with buffer. Bound proteins were analyzed by SDS-PAGE and fluorography.

Cross-linking with 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide—After import of precursor proteins, mitochondria (100 µg) were reisolated and suspended in SEM (20 µl). EDC was added at a concentration of 0.5–3 mM, and cross-linking was performed for 30 min at 0 °C. Excess EDC cross-linker was quenched by the addition of 50 mM Tris/HCl, pH 7.5 (final concentration).

Miscellaneous Procedures—The following procedures were carried out as described previously: alkaline treatment of mitochondria (13); in vitro synthesis of precursor proteins in rabbit reticulocyte lysate and labeling with [35S]methionine (14); determination of protein concentrations (15); SDS-PAGE (16); protein transfer to nitrocellulose by semi-dry blotting and subsequent immunodecoration with antibodies and detection by the ECL reagent (Amersham Corp.) (17, 18); and quantitation of fluorographs using the Image Master densitometer (5).
RESULTS

Insertion of MAS70 and MOM72 into Outer Mitochondrial Membranes—To investigate the biogenesis of yeast MAS70 and N. crassa MOM72, we first characterized the requirements of the insertion into the outer membrane. Precursors of MOM72 and MAS70 were synthesized in reticulocyte lysate and incubated with mitochondria from Neurospora and yeast. Import kinetics were linear for up to 30 min at 25°C. About 80% of input precursor were found in association with the mitochondria was performed for 30 min at 25°C. Then mitochondria were resolated and afterwards subjected to alkaline treatment with Na₂CO₃. Soluble and insoluble material were separated by centrifugation for 30 min at 50,000 × g. As control, reticulocyte lysate containing ³⁵S-labeled MAS70 was treated with Na₂CO₃. Total imported precursor (Total), supernatants (Sup), and pellets (Pel) were then analyzed as in A. Mitochondria, the amount of import to mitochondria was set to 100%; Lysate, the total input of MAS70 precursor corresponds to 100%.

ATP Stimulation of MAS70 Import and Interaction of MAS70 with HSP70—The import of the MAS70 precursor was stimulated by the addition of ATP but not GTP (Fig. 3A). This stimulatory effect of ATP may reflect an interaction of MAS70 with cytosolic HSP70. To test for this, coimmunoprecipitation of MAS70 precursor with antibodies against cytosolic HSP70 was carried out. MAS70 precursor was found to be coimmunoprecipitated with cytosolic HSP70, provided that ATP was depleted prior to antibody addition (Fig. 3B). Because the 60-kDa domain was in a folded, protease-resistant conformation in the imported into mitochondria, and these were subsequently treated with trypsin. The 60-kDa domain was cleaved off from the imported proteins and released into the supernatant (Fig. 2). The cytosolic precursor protein in the reticulocyte lysate was almost completely resistant to proteolytic attack under these conditions (Fig. 2). Only at higher trypsin concentrations (above 20 μg/ml) was the 60-kDa domain quantitatively formed.

ATP Stimulation of MAS70 Import and Interaction of MAS70 with HSP70—The import of the MAS70 precursor was stimulated by the addition of ATP but not GTP (Fig. 3A). This stimulatory effect of ATP may reflect a conformational change and/or release of factors that influence the protease sensitivity of MAS70/MOM72. From these results we conclude that insertion into the outer membrane is accompanied by a conformational change and/or release of factors that influence the protease sensitivity of MAS70/MOM72.
cytosolic precursor form (see Fig. 2) and was not found in contact with cytosolic HSP70 upon communoprecipitation (not shown), it is likely the N-terminal segment of 12 kDa bearing the membrane anchor sequence that interacts with cytosolic HSP70.

Dependence of MAS70 and MOM72 Import on Protease-sensitive Surface Components in the Outer Membrane—When mitochondria were pretreated with increasing concentrations of trypsin (Fig. 4) or elastase (not shown), the import of MAS70 was not affected. Likewise, the import of MOM72 into N. crassa mitochondria was not influenced by a pretreatment of the mitochondria with either trypsin or proteinase K (Fig. 4). Therefore import of MAS70/MOM72 appears to be independent on protease-sensitive receptor components. On the other hand, import of MAS70 was diminished when yeast mitochondria were pretreated with proteinase K (Fig. 4), and similarly, the MOM72 import was reduced after pretreatment of N. crassa mitochondria with elastase (not shown). MOM38 from N. crassa and ISP42 from yeast are insensitive to trypsin but cleaved into stable fragments by elastase and proteinase K, respectively (7). Thus, it is possible that MOM38/ISP42 are needed for the MOM72/MAS70 membrane insertion.

Assembly of Imported MAS70 into the MOM Complex—A characteristic property of endogenous MAS70 is its association with the MOM complex (22). Imported MAS70 was found to assemble into the MOM complex because it could be communoprecipitated with anti-ISP42 antibodies under conditions where the MOM complex is stable (Fig. 5). However, when the yeast mitochondria were pretreated with more than 2 μg/ml trypsin, the assembly of imported MAS70 into the complex was not observed. Similarly, trypsin pretreatment of mitochondria isolated from a ΔMAS70 yeast strain abolished the assembly of imported MAS70, excluding the possibility that endogenous MAS70 was causing this effect. In contrast, MOM70 membrane insertion was not affected by trypsin pretreatment of mitochondria. Therefore we conclude that a trypsin-sensitive mitochondrial surface component is essential for the assembly but not for the membrane insertion of MAS70.

Proximity of MAS70 to an Outer Membrane Protein with a Molecular Mass of 37 kDa—We attempted to determine direct neighbors of MAS70 after its import into the outer membrane complex of yeast mitochondria. For this purpose MAS70 was imported into yeast mitochondria, which was then reisolated and reacted with the cross-linker EDC. After lysis of mitochondria under conditions that lead to dissociation of the MOM complex, communoprecipitation with anti-MAS70 antibodies...
Interaction of MAS70 with components in the mitochondrial outer membrane. A, interaction of MAS70 in yeast mitochondria with a protein of 37 kDa. Import of MAS70 precursor into mitochondria was performed for 30 min at 25 °C. Then mitochondria were reisolated and suspended in SEM. The samples were incubated with or without EDC under the same conditions (reactions 1 and 2). In addition reticulocyte lysates containing MAS70 precursor were incubated with or without EDC under the same conditions (reactions 3 and 4). Then Laemmli buffer was added to the reactions, and SDS-PAGE and fluorography were performed (reactions 1–4 correspond to lanes 1–4). B, cross-link of endogenous MAS70 with the 37-kDa component. Yeast mitochondria (200 μg) were incubated with different concentrations of EDC (0.5–3 mM). Then the mitochondria were reisolated and analyzed by SDS-PAGE. Proteins were blotted on nitrocellulose and immunodecorated with antibodies directed against MAS70. C, prevention of cross-linking of MAS70 with the 37-kDa protein by trypsin pretreatment of yeast mitochondria. Mitochondria were treated with the indicated concentrations of trypsin. Then import of MAS70 precursor into mitochondria was performed as in A followed by cross-linking with EDC (1 mM). Proteins were analyzed by SDS-PAGE, fluorography, and laser densitometry. D, distinction of the cross-linked 37-kDa protein from ISP42. Import of MAS70 precursor into yeast mitochondria and reactions without and with EDC (0.5 mM) were performed as described in A (lanes 1 and 2). Then mitochondria were lysed in Triton X-100 buffer and immunoprecipitated with preimmune anti-MAS70 antibodies (Pre) or antibodies directed against ISP42 or MAS70. Immunoprecipitates were analyzed by SDS-PAGE and fluorography (lanes 3–8). x, cross-link of MAS70.

Cross-linking of MAS70 to the 37-kDa protein was abolished when mitochondria were pretreated with a concentration of 2 μg of trypsin/ml or higher (Fig. 6C). Thus, it appears that the 37-kDa protein either is a protease-sensitive component or assembly of MAS70 into the MOM complex is blocked in trypsin-treated mitochondria due to degradation of another component involved in assembly.

In order to exclude the possibility that the cross-linked protein was ISP42, immunoprecipitation with antibodies directed against ISP42 was performed, which quantitatively precipitated the ISP42 protein. The 37 kDa/MAS70 cross-linked species was not precipitated by these antibodies (Fig. 6D). We conclude that the 37-kDa protein is a protein in the yeast mitochondrial outer membrane, which is present in the immediate neighborhood of MAS70 and probably forms a complex with MAS70.

To determine whether also the cross-linked product MAS70-37 kDa is associated with the MOM complex, we used the coimmunoprecipitation procedure. Mitochondria in which MAS70 had been imported were treated with EDC and detergent-lysed, and then coimmunoprecipitation was performed with ISP42-specific antibodies (Fig. 7). The cross-linked product MAS70-37 kDa was precipitated by these antibodies. Interestingly, anti-ISP42 lead to a much more efficient precipitation of the MAS70-37 kDa cross-linked species than non-cross-linked MAS70. This enhanced coimmunoprecipitation of MAS70 indicates that ISP42 and the 37-kDa protein interact with each other (directly or indirectly) and that the 37-kDa protein recruits MAS70 into the MOM complex.

Interaction of MOM72 with MOM22 in N. crassa Mitochondria—To identify the nearest neighbor of MOM72, the cross-linking approach was also performed for MOM72 imported into N. crassa mitochondria. The predominant cross-linked protein had a molecular mass of about 20 kDa (Fig. 8A, I). Candidate proteins were MOM22 and MOM19. Antibodies against MOM22 but not against MOM19 could immunoprecipitate the cross-linked product (Fig. 8B). From these experiments we conclude that MOM22 is in the vicinity of MOM72 in the N. crassa mitochondrial outer membrane.

Finally we asked whether MOM72 could be cross-linked to the yeast 37-kDa protein or vice versa if MAS70 could be cross-linked with EDC (1 mM). Proteins were analyzed by SDS-PAGE, fluorography, and laser densitometry. D, distinction of the cross-linked 37-kDa protein from ISP42. Import of MAS70 precursor into yeast mitochondria and reactions without and with EDC (0.5 mM) were performed as described in A (lanes 1 and 2). Then mitochondria were lysed in Triton X-100 buffer and immunoprecipitated with preimmune anti-MAS70 antibodies (Pre) or antibodies directed against ISP42 or MAS70. Immunoprecipitates were analyzed by SDS-PAGE and fluorography (lanes 3–8). x, cross-link of MAS70.

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Finally we asked whether MOM72 could be cross-linked to the yeast 37-kDa protein or vice versa if MAS70 could be cross-linked with EDC (1 mM). Proteins were analyzed by SDS-PAGE, fluorography, and laser densitometry. D, distinction of the cross-linked 37-kDa protein from ISP42. Import of MAS70 precursor into yeast mitochondria and reactions without and with EDC (0.5 mM) were performed as described in A (lanes 1 and 2). Then mitochondria were lysed in Triton X-100 buffer and immunoprecipitated with preimmune anti-MAS70 antibodies (Pre) or antibodies directed against ISP42 or MAS70. Immunoprecipitates were analyzed by SDS-PAGE and fluorography (lanes 3–8). x, cross-link of MAS70.
Biogenesis of the Mitochondrial MOM Complex

Fig. 8. Interaction of MOM72 in N. crassa mitochondria with MOM22. A, MOM72 precursor was imported into N. crassa mitochondria. Then resolated mitochondria were incubated without or with EDC (0.5 mM) for 30 min at 0°C. As control reticulocyte lysate was also incubated without or with the addition of EDC. Samples were analyzed as in Fig. 6A. B, import of MOM72 precursor into mitochondria and cross-linking with EDC was performed as in A. After resolation, the mitochondria were lysed in Triton X-100 buffer. Immunoprecipitation was performed using preimmune antibodies (Pre) or antibodies directed against MOM19, MOM22, or MOM72. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. I, cross-link of MOM72 to MOM22; II, SDS stable dimer of MOM72, which is also observed with endogenous MOM72 (6).

cross-linked to N. crassa MOM22 after their import into mitochondria of the heterologous organism. Under the conditions described for Fig. 6, no cross-linked proteins of these molecular masses were observed (not shown), which indicates that assembly of MOM72 and of MAS70 in heterologous mitochondria may not follow the correct pathway.

DISCUSSION

In this report we investigated the biogenesis and assembly of MOM72 and MAS70 into the MOM complex. The efficient insertion of these proteins into mitochondrial membranes is stimulated by the addition of ATP. Such a stimulatory effect of ATP on the membrane insertion has been reported for most outer membrane proteins studied (23–25). Components that are responsible for this ATP effect could be located either in the cytosol or at the mitochondrial surface (26). Cytosolic factors known to stimulate the mitochondrial protein import in an ATP-dependent manner are cytosolic HSP70, Ydj1p, and the mitochondrial import stimulating factor (27–29). We show that cytosolic HSP70 interacts in an ATP-dependent manner with the precursor form of MAS70. As the hydrophilic 60-kDa domain of MAS70 and MOM72 exists as a tightly folded domain, cytosolic HSP70 could bind to the N-terminal amino acid residues of the precursor form comprising the signal anchor domain. This interaction with HSP70 might help to prevent aggregation of the cytosolic precursor due to the presence of the hydrophobic segments of the signal anchor domain (30–33) and in addition help to expose the signal anchor domain such that it can be recognized by the mitochondrial outer membrane.

Because the cytosolic domain of MOM72 and MAS70 is tightly folded in the cytosol, it is likely to be in its functional conformation (20). Therefore folding of MOM72 in the cytosol is different from precursors of other outer membrane proteins, for example monoamine oxidase A. This latter protein changes its conformation from an inactive to an active conformation upon binding to the outer membrane as shown by inhibitor interaction (34).

What are the mitochondrial components that control the insertion of outer membrane proteins into the lipid phase? For some outer membrane proteins it was shown that import receptors are involved in the insertion process. Two components of the MOM complex, MOM22 and MOM38, use both the receptors MOM19 and MOM72 for their binding to and insertion into the outer membrane (9, 10). On the other hand, import of MOM19 is independent on surface receptors, such as pre-existing MOM19 or MOM72, but depends on MOM38 (8).

Experiments presented here suggest that protease-sensitive surface components are not essential for insertion of MOM22/MAS70 into the outer membrane. MOM22 does not influence the insertion of MOM72, because in a mutant, in which MOM22 is missing, import of MOM72 is not impaired (35). Furthermore, the drastically reduced levels of MOM19 in this mutant indicate that MOM19 is not needed for the insertion of MOM72 into the outer mitochondrial membrane. The observation that anti-MOM19 IgG interferes with the import of MOM72 at low temperatures (3) might be related to a kinetic effect triggered by binding of the antibody to the MOM complex. Yet it could be that MOM38/ISP42 is involved in MOM72/MAS70 integration, although membrane insertion of MOM72/MAS70 was not impaired in protease-treated mitochondria in which MOM38/ISP42 was clipped by added protease. These cleavage products might retain insertion activity as was shown for the SecY protein in Escherichia coli (36). Interestingly, a fusion protein between the MAS70 signal anchor and dihydrolate reductase was not able to interfere with the import of matrix proteins (32). This observation would indicate that for its membrane insertion MAS70 does not use the same entry site into the MOM complex as precursors destined for other subcompartments.

MAS70 assembles in the outer membrane into the MOM complex (3, 7, 8, 22). The assembly step of MAS70 occurs at a stage that can be differentiated from the insertion step because pretreatment of mitochondria with trypsin did not influence insertion but abolished assembly into the MOM complex. Thus, proteinaceous components are needed for the assembly step to occur. This raises the question of which components are associated with MAS70 and MOM72 in the outer membrane. Using a cross-linking approach, we found MAS70 in the neighborhood of a protease-sensitive 37-kDa protein in the outer membrane of yeast mitochondria. In yeast the 37-kDa protein appears to be needed for the assembly of MAS70 by anchoring it to the MOM complex. The 37-kDa protein is so far uncharacterized. It could represent either a novel component or be identical to the protein MAS37, which is located in the mitochondrial outer membrane and was suggested to function in combination with MAS70 (37, 38). Another component that could correspond to the 37-kDa protein is MSP1 (apparent molecular mass of 40 kDa), an outer membrane ATPase that upon over-expression was reported to mislocalize a MAS70 fusion protein to the inner membrane (39). We are currently investigating whether the 37-kDa protein is one of these proteins or a new outer membrane component.

In N. crassa mitochondria MOM72 was found in contact with MOM22. Recently, MOM19 was also reported to physically interact with MOM22 in N. crassa mitochondria (40–42). Therefore, receptor and translocation sites for precursors appear to be in close proximity.

It is interesting to note that MOM72 and MAS70 do not interact in yeast and Neurospora in the same manner with their heterologous components as with their partners in the
homologous mitochondria. This observation would explain why MOM72 is not able to complement the import and growth defect of a JMAS70 mutant. On the other hand, the N. crassa proteins MOM19 and MOM38 assemble properly into the yeast MOM complex in vitro, and MOM19 does so in vivo (8, 10, 22). So far there is no data to support that these proteins become functional in the yeast MOM complex. However, because MAS22 of yeast could be cloned by complementation with Neurospora MOM22 (43), such a functional exchangeability might be essential for the assembly of MAS70.

From the experiments presented here we suggest the following model for the biogenesis of MAS70. Most likely insertion of MAS70 is mediated by interaction of its signal anchor domain with the general insertion pore, followed by insertion of the protein into the outer membrane. For assembly into the MOM complex, MAS70 then needs to interact with the cytosolic exposed part of a 37-kDa protein, which is located in the yeast mitochondrial outer membrane.

In summary, we showed that assembly into the MOM complex but not membrane insertion of MAS70 is dependent on trypsin-sensitive mitochondrial surface components. In the MOM complex, MAS70 and MOM72 are in contact with specific components that are a 37-kDa protein in yeast and the protein MOM22 in N. crassa. The interaction with the 37-kDa protein likely anchors MAS70 to the MOM complex and therefore might be essential for the assembly of MAS70.

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