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MOM22 is a receptor for mitochondrial targeting sequences and cooperates with MOM19

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Recognition of targeting signals is a crucial step in protein sorting within the cell. So far, only a few components capable of deciphering targeting signals have been identified, and insights into the chemical nature of the interaction between the signals and their receptors are scarce. Using highly purified mitochondrial outer membrane vesicles, we demonstrate that MOM22 and MOM19, components of the protein import complex of the outer membrane, bind preproteins at the mitochondrial surface in a reversible fashion. Interaction specifically and directly occurs with the N-terminal presequence and is abolished after inactivation of either MOM22 or MOM19. Binding is salt sensitive, suggesting that recognition involves electrostatic forces between the positive charges of the presequence and the acidic cytosolic domain of MOM22. MOM19 and MOM22 can be cross-linked with high efficiency. We propose that the two proteins form a complex which functions as the presequence receptor at the mitochondrial surface and facilitates the movement of preproteins into the translocation pore.
Keywords: mitochondria/MOM22/MOM19/presequence receptor/protein import

Introduction

Transport of newly synthesized proteins to their sites of function within the cell involves the specific recognition of targeting signals. The identification of the components deciphering and discriminating the various targeting signals is fundamental for our understanding of the chemical principles underlying protein sorting. Moreover, knowledge of the components serving as the preprotein receptors is also a prerequisite for describing the steps of preprotein movement across membranes at the molecular level. Only a few components functioning as receptors for targeting signals have been identified so far. For instance, the eukaryotic signal recognition particle, SRP, binds to signal sequences as they emerge from the ribosome (Walter *et al.*, 1981). Bacterial SecA protein has been shown to recognize both the signal and mature domains of preproteins (Lill *et al.*, 1990).

In the case of protein targeting to mitochondria, initial recognition of the targeting signals also may occur in the cytosol by mitochondrial import stimulation factor, MSF (Hachiya *et al.*, 1994), even though this step may not be obligatory for all preproteins and can be bypassed (Becker *et al.*, 1992; Hajek and Bedwell, 1994). At the mitochondrial surface, protease-sensitive components (Pfaller and Neupert, 1987), together with membrane-embedded components (Söllner *et al.*, 1992), facilitate the translocation of preproteins across the mitochondrial outer membrane (Mayer *et al.*, 1995b). Despite the fact that the involvement of the protease-sensitive components has long been documented, only one component of the protein import complex of the outer membrane, MOM72/Mas70p, has been demonstrated directly to function as a 'preprotein receptor' (Söllner *et al.*, 1990; Schlossmann *et al.*, 1994). It may act in cooperation with Mas37p, which so far has been identified in yeast only (Gratzer *et al.*, 1995). Another likely candidate to perform such a function in preprotein recognition at the mitochondrial surface is MOM19/Mas20p (Söllner *et al.*, 1989; Moczko *et al.*, 1993; Ramage *et al.*, 1993; Harkness *et al.*, 1994b; Moczko *et al.*, 1994). This protein is important for normal growth of cells and is involved in the transport of many mitochondrial precursor proteins. An interaction of MOM19/Mas20p with preproteins at the mitochondrial surface has been suggested (Söllner *et al.*, 1989; Haucke *et al.*, 1995), but has never been proven in direct binding experiments. MOM22/Mas22p, a component which is exposed at the mitochondrial surface and is essential for cell viability (Lithgow *et al.*, 1994; Nakai and Endo, 1995; Nargang *et al.*, 1995), has been reported to play a role in the transfer of the ADP/ATP carrier (AAC) from the MOM72-bound state into the translocation pore (Kiebler *et al.*, 1993b). From these data, the functional role of MOM22 appeared to be downstream of the receptor-bound state. However, it remained unclear from this investigation whether MOM22 plays a similar role for preproteins which enter the mitochondria in a MOM19-dependent fashion (Kiebler *et al.*, 1993a).

Here, we investigated the binding of preproteins to the mitochondrial surface in order to identify components deciphering the N-terminal mitochondrial targeting sequence (presequence). Previously, two main obstacles prohibited the study of preprotein binding to the mitochondrial surface and therefore precluded the identification of the preprotein receptors. First, preparations of mitochondria are contaminated with other cellular membranes and exhibit a high degree of unspecific binding of the mostly hydrophobic preproteins (Mayer *et al.*, 1995a). Second, it was impossible to arrest preproteins at the surface and prevent them from entry into the translocation machinery (cf. Mayer *et al.*, 1995b). To circumvent these problems, we took advantage of the low unspecific binding of preproteins observed with highly purified outer membrane vesicles (OMV) from *Neurospora crassa*, and we employed fusion proteins between mitochondrial pre-

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sequences and dihydrofolate reductase (DHFR). These preproteins can be prevented from translocation across the outer membrane by stabilizing their DHFR moiety against unfolding with methotrexate (MTX) and NADPH (Eilers and Schatz, 1986; Mayer *et al.*, 1995b). Thereby, a surface-bound translocation intermediate can be created.

The binding studies presented here assign a novel role to MOM22 in that it acts as a preprotein receptor at the mitochondrial surface. In this function, it cooperates with MOM19, since no significant interaction with preproteins is observed when either of these two components of the protein import complex is inactivated, e.g. by blocking them with specific IgG or by using OMV purified from MOM22 and MOM19 disruption mutants. The interaction specifically occurs with the presequence and is labile, especially at higher ionic strength. This suggests that electrostatic forces between the positively charged presequence and the negatively charged cytosolic domain of MOM22 are an important feature of the recognition process.

Results

For our studies of surface binding, we used fusion proteins of the presequences of subunit 9 of the F_0-ATPase (pSu9-DHFR; Pfanner *et al.*, 1987b) or of cytochrome c_1 (pCytc1-DHFR; Stuart *et al.*, 1994) and DHFR. Binding of the preproteins to isolated OMV was performed in the presence of MTX and NADPH to prevent unfolding of the DHFR domain. This precludes the insertion of the preprotein into the translocation machinery (see below). A substantial fraction, 50 and 25%, respectively, of the added preproteins became associated with the OMV (Figure 1A). The interaction of the preprotein specifically occurred with components exposed at the mitochondrial surface, since trypsin pretreatment of OMV strongly reduced the amount of bound material. Binding was highly sensitive to increased ionic strength. Hardly any specific surface binding to OMV was observed when the salt concentration was increased above 50 mM. Obviously, at higher ionic strength, the interaction between the preproteins and the receptor components becomes too labile to be detectable by our sedimentation binding assay. However, interaction between the preprotein and the receptors must occur in a transient fashion, since the overall protein import into intact mitochondria is barely affected by KCl concentrations up to 130 mM (Figure 1B). Under all ionic conditions tested, import was strongly dependent on the function of the protease-sensitive receptor components. This indicates that surface receptors are involved in the import reaction even at higher ionic strength although, under these conditions, their interaction with preproteins is too labile to be detectable by our binding assay. The lability of preprotein binding to the mitochondrial surface, especially under more physiological conditions, may be an important prerequisite for the rapid movement of the preprotein into the translocation pore.

To determine which membrane components facilitate preprotein recognition, individual surface proteins were blocked with specific IgG. Surface binding of pSu9-DHFR and pCytc1-DHFR was strongly reduced by IgG against either MOM22 or MOM19, but not by IgG against MOM72 or IgG purified from preimmune serum (Figure 2).

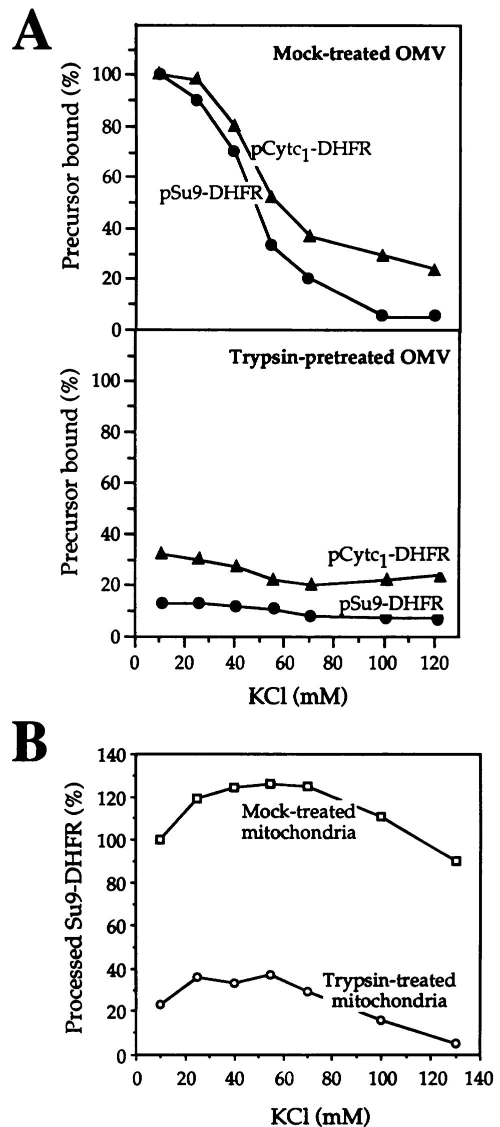

Fig. 1. Binding of preproteins to the surface of the mitochondrial outer membrane. (**A**) ^{35}S-labelled preproteins were incubated in import buffer (containing 10 mM KCl) with methotrexate (MTX; 1 μM) and NADPH (1 mM) for 5 min at 25°C. After addition of mock-treated or trypsin-pretreated OMV (5 μg/sample), incubation was continued for 5 min at 25°C. Samples were chilled to 0°C, diluted 3-fold with 10 mM MOPS-KOH pH 7.2 and adjusted to different KCl concentrations. OMV were reisolated, dissolved in sample buffer and analysed for bound preprotein by SDS-PAGE, fluorography and densitometry. The amount of preprotein bound to mock-treated OMV at 10 mM KCl was set to 100%. (**B**) Import of preSu9-DHFR into trypsin-treated or mock-treated isolated mitochondria was as described (Lill *et al.*, 1992; Mayer *et al.*, 1993) in buffer M (220 mM sucrose, 30 mg/ml fatty acid-free BSA, 5 mM $MgCl_2$ and 10 mM MOPS-KOH, pH 7.2) containing the indicated concentrations of KCl. Data are given relative to the amount of mature Su9-DHFR, which had been imported into mock-treated mitochondria at 10 mM KCl.

In contrast, surface binding of translocator AAC (Pfanner and Neupert, 1987) was affected only by IgG against MOM72. Interaction of AAC with the mitochondrial surface is known to be mediated by MOM72, and not by MOM22 or MOM19 (Söllner *et al.*, 1990; Schlossmann *et al.*, 1994; Nargang *et al.*, 1995).

In an independent approach, we performed binding experiments with OMV isolated from disruption mutants of MOM22 and MOM19. Mitochondria isolated from

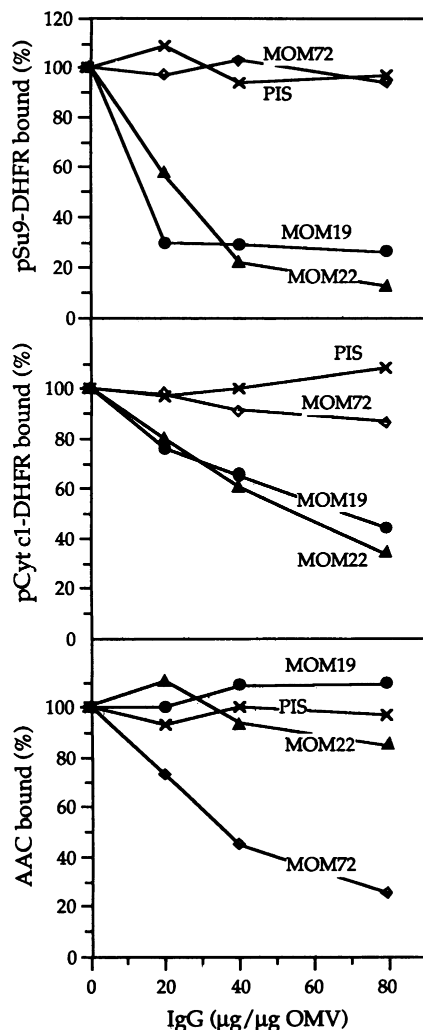

Fig. 2. Preprotein binding to OMV is inhibited by antibodies against MOM22 and MOM19. OMV (2.5 μg/sample) were incubated with IgG against the indicated proteins or IgG purified from preimmune serum (PIS) in import buffer. After ATP depletion of samples, preproteins were incubated with MTX–NADPH, bound to these OMV, and binding (at 20 mM KCl) was analysed as in Figure 1A.

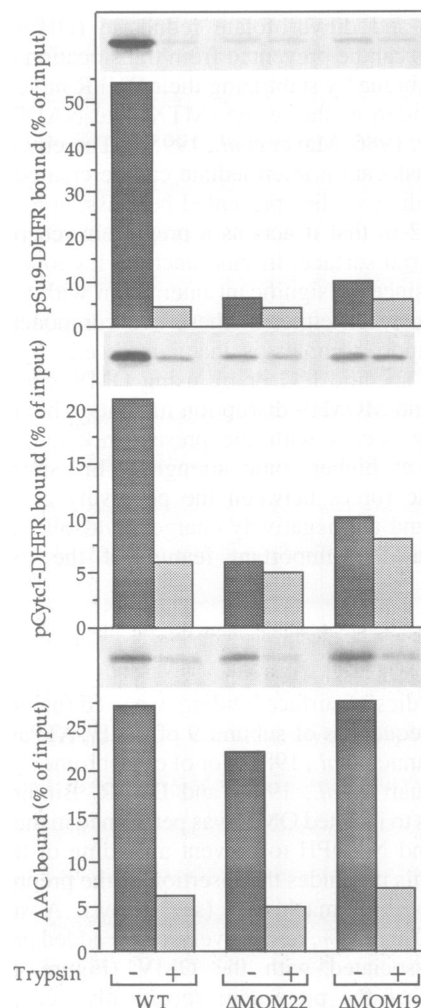

Fig. 3. OMV isolated from MOM22 and MOM19 disruption mutants are impaired in preprotein binding. OMV prepared from wild-type or MOM22 (ΔMOM22; Nargang *et al.*, 1995) or MOM19 (ΔMOM19; Harkness *et al.*, 1994b) mutant strains. The vesicles were used for binding reactions under conditions as in Figure 2. Analysis and data evaluation were as in Figure 1A.

these mutants are depleted of these components and display a strong reduction in the import of preproteins (Harkness *et al.*, 1994b; Nargang *et al.*, 1995). Binding of pSu9-DHFR and pCytc1-DHFR to the two mutant OMV was strongly impaired in comparison with wild-type OMV (Figure 3). Residual binding was insensitive to protease pretreatment (Figure 3) and could not be reduced further by prebinding of IgG against MOM19 or MOM22 (not shown). On the contrary, binding of AAC was unaffected in both mutant OMV which contain MOM72 at virtually unchanged levels (not shown; Harkness *et al.*, 1994b; Nargang *et al.*, 1995). AAC binding remained sensitive to trypsin pretreatment, confirming that the interaction occurred with MOM72 and was independent of MOM22 and MOM19. In summary, efficient interaction of presequence-bearing preproteins with the outer membrane requires the cooperative function of MOM22 and MOM19.

We also asked whether bound precursor protein was still interacting with MOM22 and MOM19, or whether it had left them and had already been passed on to other components acting in a subsequent stage of the membrane transport pathway. In the latter case, it should not be possible to displace bound preprotein by IgG against MOM22 or MOM19. However, both antibodies were able to cause dissociation of prebound pSu9-DHFR from the OMV, whereas no displacement was observed upon the addition of IgG against MOM72 or IgG purified from preimmune serum (Figure 4). The dependence on the IgG concentration in the displacement assay was similar to that observed in the binding experiments using IgG-pretreated OMV (cf. Figure 2). We therefore conclude that the surface-bound preprotein was interacting with both MOM22 and MOM19, and that it had not been forwarded to components acting downstream of the receptor stage.

Does the interaction between the preprotein and MOM22/MOM19 occur in a direct fashion or is it mediated by soluble factors present in the cytosol or the reticulocyte lysate? To address this problem, we analysed binding using a purified preprotein (preSu9-DHFR carrying a C-terminal hexahistidinyl tag) which had been radiolabelled *in vivo*. A significant fraction of the added preprotein

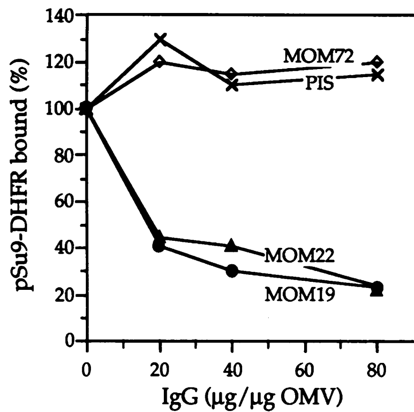

Fig. 4. Displacement of prebound preprotein by antibodies against MOM22 and MOM19. pSu9-DHFR was prebound (10 min, 25°C) to OMV in import buffer (20 mM KCl) as described in Figure 1A. After reisolation of OMV and resuspension in the same buffer, increasing amounts of IgG purified from preimmune serum (PIS) or antisera against MOM19, MOM22 and MOM72 were added. After 10 min at 25°C, OMV were reisolated and binding was analysed as in Figure 1A.

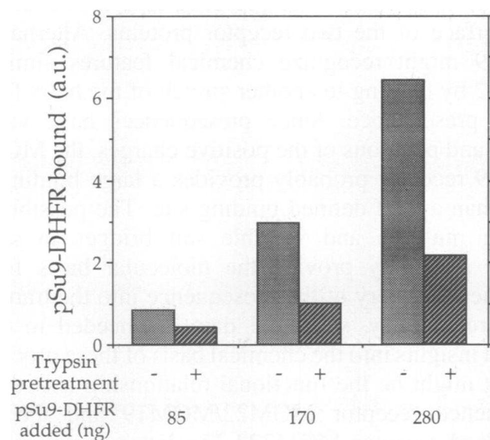

Fig. 5. PreSu9-DHFR directly interacts with MOM22 and MOM19. Purified radiolabelled preSu9-DHFR carrying a hexahistidinyl tag in 7 M urea was diluted 40-fold into import buffer containing 3 mg/ml BSA, 1 μM MTX and 1 mM NADPH. After incubation for 10 min at 25°C, samples were chilled on ice and centrifuged for 15 min at 17 000 *g*. Increasing amounts of preprotein were added to mock-treated or trypsin-pretreated OMV (10 μg/sample) in the same buffer. After 5 min on ice, binding was analysed as described in Figure 1A. a.u., arbitrary units.

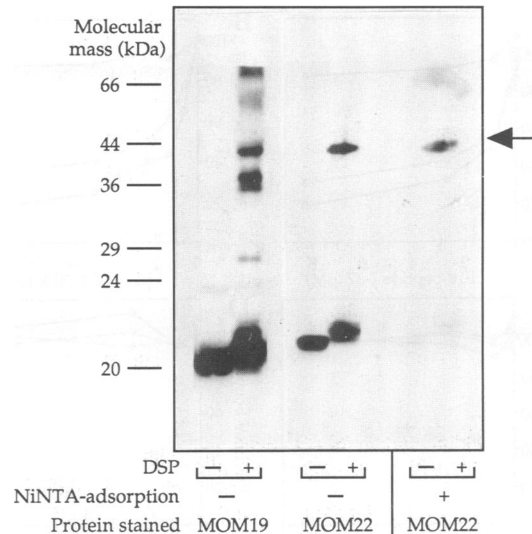

Fig. 6. Cross-link between MOM22 and MOM19. OMV (10 μg/lane) isolated from *Neurospora* strain 2-2 expressing MOM19 with a hexahistidinyl tag were incubated in import buffer with or without 0.5 mM dithio*bis*(succinimidylpropionate) (DSP) for 30 min at 0°C. Cross-linking was terminated by adding 0.2 M Tris–HCl, pH 7.2 (5 min, 25°C). After TCA precipitation, two aliquots were solubilized in sample buffer. A third aliquot was solubilized in buffer N (6 M urea, 0.1% Triton X-100, 50 mM MOPS-KOH, pH 7.5, 0.1 M KCl, 10 mM imidazole). Hexahistidinyl-tagged MOM19 was adsorbed to and eluted from Ni-NTA beads as described in Materials and methods. All samples were analysed by SDS–PAGE and immunostaining of MOM22 and MOM19 as indicated. The cross-linked product is marked with an arrow. Note that MOM19 antiserum recognizes DSP-modified MOM19 only poorly, probably due to partial masking of epitopes.

(between 10 and 30% in different experiments) became bound to the OMV. Binding was decreased significantly after removal of the receptors by trypsin pretreatment (Figure 5). Addition of cytosol did not further increase the binding (not shown). Thus, the interaction of preproteins with MOM22 and MOM19 does not require accessory soluble components.

As shown above, the simultaneous interaction with MOM22 and MOM19 is crucial for retaining precursor proteins at the mitochondrial surface. To investigate whether this requirement was reflected by a close physical contact of the two proteins, we performed cross-linking experiments. A major cross-linking product of 43 kDa was detected by immunostaining with antisera against either MOM22 or MOM19, suggesting that it is a heterodimer composed of these two proteins (Figure 6). To confirm this, we made use of OMV isolated from a *Neurospora* strain carrying a hexahistidinyl-tagged *mom-19* gene instead of the wild-type copy. When the cross-linking reaction was performed with these OMV, the 43 kDa band could be isolated by Ni-NTA affinity chromatography, and was immunostained by antibodies against MOM22 (Figure 6). In contrast, non-cross-linked MOM22 (Figure 6) and other outer membrane proteins (not shown) were not retained on the affinity resin. Thus, the close functional cooperation of MOM22 and MOM19 in recruiting preproteins to the mitochondrial surface is reflected by their physical neighbourhood.

Which part of the preprotein is recognized by MOM22 and MOM19? We addressed this question by using a displacement assay. Both a presequence peptide derived from the β-subunit of the F_1-ATPase (F1β; Cyr and Douglas, 1991) and a purified preprotein (pCoxIV-DHFR; Eilers and Schatz, 1986) dislodged prebound pSu9-DHFR and pCytc1-DHFR efficiently, with half-maximal effects at a concentration of 1 μM (Figure 7A and B). In contrast, AAC as a control was not displaced. Importantly, a truncated presequence peptide of F1β, which did not inhibit protein import into mitochondria efficiently (Cyr and Douglas, 1991), failed to compete for prebound precursor proteins (Figure 7C). Likewise, neither a peptide lacking typical features of a presequence nor the DHFR domain alone were able to displace the three precursors (Figure 7D and E). Thus, MOM22 and MOM19 specifically recognize N-terminal presequences. Interestingly, the interaction between MOM72 and AAC, which carries an internal targeting signal (Pfanner *et al.*, 1987a), seems to

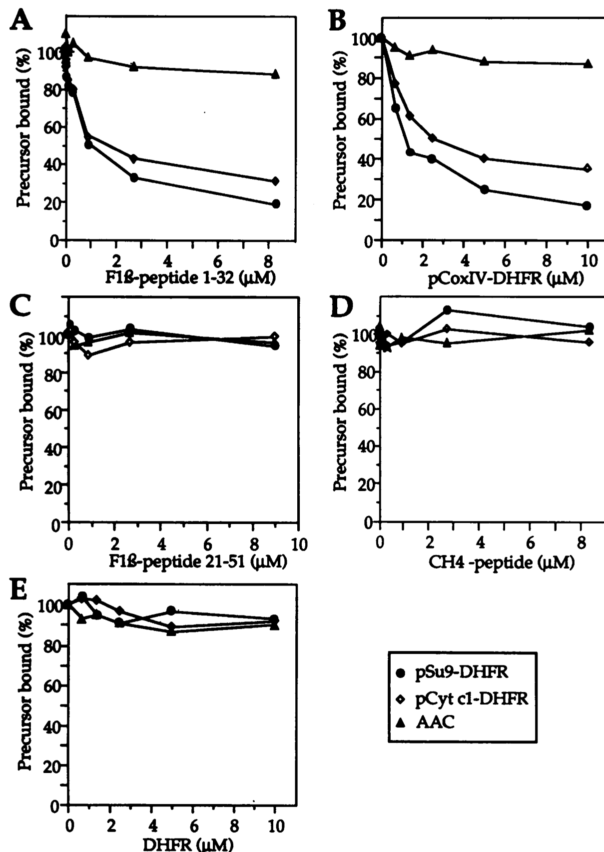

Fig. 7. Binding to MOM22/19 is specific for the presequence. Displacement of prebound preproteins was performed as in Figure 4 using the following purified peptides or preproteins as competitors. (**A**) A presequence peptide corresponding to amino acids 1–32 of the precursor of the β-subunit from yeast F_1-ATPase (Cyr and Douglas, 1991), (**B**) a fusion protein between the presequence of subunit IV of cytochrome oxidase and DHFR (pCoxIV-DHFR; Eilers and Schatz, 1986), (**C**) a peptide corresponding to amino acids 21–51 of the precursor of the β-subunit from yeast F_1-ATPase (Cyr and Douglas, 1991), (**D**) a control peptide (CH4) corresponding to amino acids 1–25 of *N.crassa* cytochrome *c* heme lyase (CCHL; Drygas *et al.*, 1989), and (**E**) DHFR. The N-terminus of CCHL does not contain targeting information (R.Lill, unpublished).

be of a different nature, since AAC remained stably bound to the OMV under all conditions tested.

Discussion

Our data establish a novel function for MOM22 in the recognition of preproteins. Together with MOM19, it binds mitochondrial presequences at the surface of the outer membrane. From the protease sensitivity and the importance for the overall translocation reaction, such a function has been suggested to be performed by MOM19 (Söllner *et al.*, 1989; Moczko *et al.*, 1994; Haucke *et al.*, 1995). Until now, however, direct experimental support for this proposal has been lacking. This was due to the fact that no assay for the direct study of preprotein binding to the mitochondrial surface was available. The development of such an assay allowed us to characterize MOM22 and MOM19 as the key components for the initial binding of preproteins to mitochondria. Both components are needed simultaneously to allow efficient interaction with the mitochondrial targeting sequence. Based on our results, we propose that MOM22 and MOM19 form a complex which operates as the presequence receptor at the mitochondrial surface. In this way, the complex is the physical basis for the so-called *cis* site, which was defined recently in a mechanistic study of preprotein translocation across the mitochondrial outer membrane (Mayer *et al.*, 1995b). MOM22 and MOM19 are the first components of mitochondria known to recognize specifically presequences.

The salt sensitivity of presequence binding to MOM22/19 suggests that it is mediated largely by electrostatic forces. Most probably, the acidic cytosolic domain of MOM22 (Kiebler *et al.*, 1993b; Lithgow *et al.*, 1994) interacts with the positively charged face of the amphipathic helical structure which mitochondrial presequences can adopt (von Heijne, 1986; Roise and Schatz, 1988). This could occur via salt bridges formed between α-helical structures of the presequence and of MOM22. What role may then be envisaged for MOM19? Its net charge is close to zero (Schneider *et al.*, 1991; Ramage *et al.*, 1993; Moczko *et al.*, 1994). Thus, it could decode the uncharged side of the amphipathic α-helix. In this view, the interaction with presequences would occur at the interface of the two receptor proteins. Alternatively, MOM19 might recognize chemical features similar to MOM22 by binding to another stretch of the helix formed by the presequence. Since presequences have variable lengths and positions of the positive charges, the MOM22/MOM19 receptor probably provides a large binding area rather than a well defined binding site. The possibility of forming multiple and variable salt bridges in such a binding area may provide the molecular basis for the subsequent delivery of the presequence into the translocation pore. Clearly, structural data are needed to obtain detailed insights into the chemical basis of these processes.

What might be the functional relationship between the presequence receptor MOM22/MOM19 and the more specialized receptor MOM72? The latter component has been demonstrated to act as the surface receptor for AAC and related proteins (Söllner *et al.*, 1990; Dietmeier *et al.*, 1993; Schlossmann *et al.*, 1994). Neither MOM22 nor MOM19 is involved in high affinity binding of AAC (cf. Figures 2 and 3), yet the transfer of MOM72-bound AAC across the outer membrane requires the function of MOM22 (Kiebler *et al.*, 1993b). MOM19, on the other hand, plays a minor role in this reaction (Söllner *et al.*, 1990; Moczko *et al.*, 1994; Schlossmann *et al.*, 1994), and is even dispensable (Harkness *et al.*, 1994b). Based on these data, a model can be proposed in which MOM72 is presenting AAC in a conformation which permits faster or more efficient recognition of its internal targeting signal by MOM22 (Figure 8). This interaction may then serve to feed AAC into the general import route. The model provides an explanation for the observation that the MOM72-bound stage is not an obligatory step in AAC transport (Steger *et al.*, 1990). Inactivation of MOM72 by biochemical or genetic methods results in a decrease in the import efficiency of AAC, but import remains dependent on MOM22/MOM19, suggesting that in this situation AAC enters the mitochondria directly via these proteins.

The labile character of the interaction between presequences and MOM22/19 at the mitochondrial surface may be important for facilitating efficient transfer of preproteins into the translocation channel or for rapid

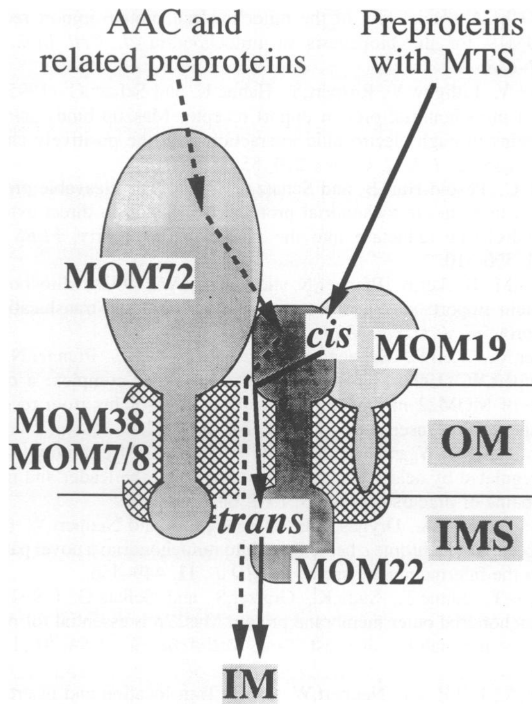

Fig. 8. Working model for preprotein recognition at the mitochondrial outer membrane. The majority of mitochondrial preproteins, in particular those carrying an N-terminal matrix targeting signal (MTS; presequence), are recognized by MOM22/MOM19. From this so-called *cis* site (Mayer *et al.*, 1995b) the presequence is reversibly transferred into the putative translocation pore and eventually reaches the *trans* site, which is located on the intermembrane space side of the outer membrane (Mayer *et al.*, 1995b). The *trans* site may, in part, be provided by the C-terminal portion of MOM22. A subset of preproteins including the ADP/ATP carrier (AAC) and related preproteins are first bound to the specialized receptor MOM72 from which they are transferred to MOM22 (and possibly MOM19) before being passed across the membrane. For further explanations see text. Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

removal of non-cognate proteins from the receptor. Accuracy of presequence recognition may be enhanced by a second, presequence-specific interaction with the so-called *trans* site (Mayer *et al.*, 1995b; see Figure 8). This interaction occurs at the inner face of the outer membrane and drives translocation of the presequence. Since in OMV the *trans* site reacts with presequences in a largely salt-resistant fashion (A.Mayer, unpublished), it appears to recognize different properties of the presequence. The sequential decoding may provide a 'double-check' system for mitochondrial presequences which could improve the accuracy of the overall translocation reaction. The molecular nature of the *trans* site is still enigmatic. The most likely candidate for forming at least part of the *trans* site is the intermembrane space domain of MOM22 which is also acidic (Kiebler *et al.*, 1993b; Lithgow *et al.*, 1994; Nakai and Endo, 1995). Thus, MOM22, an essential component of the translocation machinery (Nargang *et al.*, 1995), may well serve a dual function in preprotein transport. Its cytosolic domain is involved in surface binding of presequences, whereas its intermembrane space segment may participate in stably binding the presequences after their reversible movement through the translocation pore of the outer membrane (Mayer *et al.*, 1995b).

Materials and methods

General biochemical procedures

The following published procedures were used: growth of *N.crassa* wild-type strain 74*A* and the mutant strains of *mom-19* (28.17; Harkness *et al.*, 1994b) and *mom-22* genes (ND-113-1; Nargang *et al.*, 1995). To avoid a concomitant decrease in MOM22 and MOM19, mutant cells were cultivated for the minimal times required to achieve depletion of MOM19 and MOM22, respectively. Residual levels of MOM22 and MOM19 were 50–60% as compared with wild-type strains. Purification of mitochondrial OMV (Mayer *et al.*, 1993); raising antisera and purification of immunoglobulin G (IgG; Söllner *et al.*, 1989), with the modification that IgGs were concentrated by ultrafiltration in Centriprep tubes (Amicon); transcription and translation reactions in reticulocyte lysate using [^{35}S]methionine (ICN Radiochemicals) as radioactive label (Söllner *et al.*, 1991); SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography of the resulting gels (Nicholson *et al.*, 1987); blotting of proteins onto nitrocellulose and immunostaining of blotted proteins using the ECL chemiluminescence detection system (Amersham; Mayer *et al.*, 1993). The resulting films and those from fluorography of radioactive proteins were scanned on an Image Master densitometer (Pharmacia). Protein concentrations were determined by the Coomassie dye binding assay using IgG as a standard (Bio-Rad).

Manipulation of reticulocyte lysates

The following precursor proteins were synthesized in reticulocyte lysates: the fusion proteins preSu9-DHFR (Pfanner *et al.*, 1987b) and preCytc1-DHFR (Stuart *et al.*, 1994), containing the presequences of subunit 9 of the mitochondrial F_0-ATPase or of precytochrome c_1, respectively, in front of mouse DHFR; and the AAC (Pfanner and Neupert, 1987)

To stabilize the DHFR domain of the fusion proteins against unfolding, the proteins were preincubated in import buffer containing 1 mM NADPH and 1 μM MTX for 5 min at 25°C. Then, they were added to the binding reactions. For depletion of ATP and in binding studies using AAC precursor, reticulocyte lysates and OMV were incubated separately in import buffer with 10 U/ml apyrase for 10 min at 25°C before mixing.

Assay for surface binding of precursor proteins

All vials were coated with fatty acid-free bovine serum albumin (BSA; 1 mg/ml, 15 min) before use in order to reduce unspecific interaction of precursor proteins with cup walls. OMV (5 μg) were suspended in 200 μl of import buffer (0.2 mg/ml BSA, 20 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS–KOH, pH 7.2) with 1 mM NADPH and 1 μM MTX. OMV were incubated with reticulocyte lysate (1–5 μl) containing the radiolabelled precursor protein for 10 min at 25°C. Samples were diluted 3-fold with ice-cold buffer A (10 mM MOPS–KOH, pH 7.2, 10 mM KCl), and OMV were reisolated (20 min, 125 000 *g*). The resulting pellets were spun again for 5 min at 30 000 *g* and residual buffer was removed. Pellets were then resuspended in 20 μl of SEM buffer (220 mM sucrose, 1 mM EDTA and 10 mM MOPS, pH 7.2), and transferred to new cups, where they were finally dissolved in sample buffer. Proteins were analysed by SDS–PAGE, fluorography and densitometry of the resulting bands.

Displacement of surface-bound precursor proteins

Precursor proteins were bound to OMV (2.5 μg/sample) as outlined above, under conditions of ATP depletion. After reisolation (20 min, 75 000 *g*), the OMV were resuspended in import buffer and split into aliquots. Increasing amounts of peptides, precursor proteins or IgG were added as competitors. After 10 min at 25°C, OMV were reisolated (20 min, 125 000 *g*) and analysed for bound precursor protein as above.

Purification of chemical amounts of preproteins and DHFR

Genes encoding pCoxIV-DHFR (Hurt *et al.*, 1984) and DHFR, both carrying a C-terminal hexahistidinyl tag, were cloned into the vector pUHE 73-1 and overexpressed in *Escherichia coli*. Soluble proteins were prepared according to Arretz *et al.* (1994) in buffer B (30 mM Tris–HCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethyl-sulphonyl fluoride) containing 2 mM $MgCl_2$, 10 μg/ml *N.crassa* protease inhibitor (Söllner *et al.*, 1989) and 0.2% Triton X-100. The sample was loaded on a Ni-NTA column (2 ml resin; 0.4 ml/min), washed with 30 ml of buffer B (0.2 ml/min) and eluted with 40 ml of an imidazole gradient (0–0.5 M in buffer B plus 200 mM KCl; 0.1 ml/min flow rate). Fractions of 1 ml were collected and assayed by SDS–PAGE for their protein composition. Fractions containing pure proteins were pooled and dialysed against buffer A. The protein was concentrated by ultrafiltration to 2 mg/ml, frozen in aliquots in liquid nitrogen and stored at –80°C.

Aliquots were thawed only once. Purified radiolabelled preSu9-DHFR carrying a C-terminal hexahistidinyl tag was kindly provided by I.Wagner.

Construction of a Neurospora strain expressing a hexahistidinyl-tagged MOM19

Plasmid pM19H6 containing the coding sequence of *mom-19* and an additional six histidine codons at the C-terminus was co-transformed with a plasmid conferring resistance to bleomycin (Austin *et al.*, 1990) into *Neurospora* strain 28.17 (Harkness *et al.*, 1994a). One of the two nuclei in this heterokaryotic strain carries a null allele of *mom-19*, a requirement for uridine and a resistance marker for *p*-fluorophenylalanine (fpa). To select for transformation of this nucleus, transformants were plated onto medium containing bleomycin, fpa and uridine. Transformants were picked to slants containing fpa and uridine, purified once by streaking to single colonies on the same medium, and then tested for uridine requirement. Strains requiring uridine should be homokaryotic for the nucleus containing that marker, while restoration of rapid growth indicates complementation of the defective gene by the hexahistidinyl-tagged *mom-19*. Mitochondria from such a strain (termed 2-2; grown as the wild-type strain 74A in the presence of uridine) were examined by immunostaining for hexahistidinyl-tagged MOM19. The modified protein was distinguishable from wild-type MOM19 by its slightly higher molecular mass.

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