THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 16, Issue of April 22, pp. 11893–11901, 1994
Printed in U.S.A.

Specific Recognition of Mitochondrial Preproteins by the Cytosolic Domain of the Import Receptor MOM72*

(Received for publication, December 30, 1993, and in revised form, February 4, 1994)

Jens Schlossmann, Klaus Dietmeier, Nikolaus Pfanner‡, and Walter Neupert§

From the Institut für Physiologische Chemie, Universität München, Goethestraße 33, D-80336 München, Germany

The import receptor MOM72 constitutes part of the protein translocation machinery of the outer mitochondrial membrane, the receptor-general insertion pore complex. The protein contains a membrane anchor at the NH_2 terminus and a large cytosolic domain. In yeast and *Neurospora crassa* the cytosolic domain comprises about 570–580 amino acid residues. The cytosolic domain of yeast MOM72 was purified after expression in *Escherichia coli* as a homogeneous monomeric protein. It can recognize precursor proteins as demonstrated by its ability to compete for binding and import into the mitochondria and to physically interact with preproteins. A subset of preproteins including the ADP/ATP carrier and the phosphate carrier interact with very high affinity, precursors that are known to be targeted via MOM72. Thus, the cytosolic domain of MOM72 plays a critical function in the recognition of preproteins by directly binding to precursor proteins and thereby facilitating their targeting to mitochondria.

Translocation of nuclear-encoded proteins from the cytosol into mitochondria is a process that can be divided into a number of consecutive steps. In an initial reaction preproteins, present in the cytosol in association with chaperones and other factors to maintain their translocation competence, are recognized by surface components of the outer mitochondrial membrane (1–6). They are then translocated across or inserted into the outer membrane using a set of proteins that form a complex, termed general insertion pore. Practically all preproteins irrespective of their final location within the mitochondria use this complex (7–10). Proteins sorted to the matrix and inner membrane then interact with a translocation machinery in the inner membrane which mediates, in cooperation with heat shock proteins, the translocation of the presequences and of the mature parts of the preproteins (11–18).

During recent years it has become apparent that the specificity of transport is due to interaction of signals in the preproteins with mitochondrial surface components termed receptors (19). The signals in mitochondrial preproteins are, in most cases, contained in amino-terminal presequences of about 20–70 amino acid residues which are cleaved after entering the matrix space by the mitochondrial processing peptidase (20). Several preproteins, however, are not made with amino-terminal extensions and have targeting signals within the sequence of the mature protein itself (21–24).

What are the components that identify the targeting signals? Two proteins of the outer membrane have been described which qualify for this function. The first candidate is the surface-exposed integral membrane protein MOM19 (5) which was found to play a general role in import of preproteins; antibodies against MOM19 inhibit import of most preproteins tested. Consistent with the antibody studies, mutants deficient in MOM19 exhibit severe defects in import of preproteins (25, 26). The second candidate is the outer membrane protein MOM72 (also termed MAS70 in yeast) which is required for import of a limited number of preproteins, in particular of the most abundant inner membrane protein the ADP/ATP carrier (AAC)[1] and of the phosphate carrier (P_iC) (2, 3, 24). Deletion of MOM72 in yeast has no particular phenotype, but mitochondria isolated from the null mutant had a strongly reduced ability to bind the precursor of the AAC to the outer mitochondrial membrane (4). Related *in vitro* experiments with mitochondria from the null mutant led to the view that yeast MOM72 can act as receptor for precursor proteins in addition to AAC and P_iC (27).

We have studied the properties of MOM72 in order to determine if this protein acts as a true receptor in the sense of a specific and reversible binding of preproteins. So far a direct interaction between MOM surface proteins and preproteins had been implied but not directly demonstrated. As described here, MOM72 has a well defined topology with a tightly folded 65-kDa COOH-terminal domain exposed to the cytosol and is anchored in the outer membrane by a single hydrophobic transmembrane segment close to the NH_2 terminus. Treatment of *Neurospora crassa* mitochondria with various proteases led to the release of the cytosolic domain as a protease-resistant fragment. This fragment could compete for binding and import of AAC to mitochondria. We have also expressed in *Escherichia coli* the cytosolic domain of yeast MOM72 and have purified this monomeric protein to homogeneity. Like the *N. crassa* fragment, the purified domain of yeast MOM72 was able to compete for specific binding and import of precursor proteins into mitochondria. The most strongly affected precursors were those of AAC and phosphate carrier, but most preproteins analyzed were not impaired in their import. The precursor of cytochrome c_1 was also affected but only to a low extent. Furthermore, direct interaction of the precursor of AAC with the 65-kDa domain was demonstrated. Thus, MOM72 acts as a high affinity import receptor for a subset of preproteins and possibly as a receptor for other precursors with low affinity.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmid Encoding the 65-kDa Fragment of Yeast MOM72

To generate a vector to overexpress the 65-kDa fragment of yeast MOM72, comprising amino acids 38–617, a combination of polymerase

* The work was supported by the Sonderforschungsbereich 184 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Biochemisches Institut, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: AAC, ADP/ATP carrier; P_iC, phosphate carrier; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; PAS, protein A-Sepharose; PAGE, polyacrylamide gel electrophoresis.

chain reaction (28, 29) and standard cloning techniques were used. This was necessary because no appropriate restriction sites were available. First the 65-kDa fragment was cloned by polymerase chain reaction using the sense primer 5′-GGGAATTCGCATGCGAGGAAAAAAGAACACGAT-3′ with a *Eco*RI and a *Sph*I-site containing the start ATG for the 65-kDa fragment and the antisense primer 5′-GGGAATTCGGATCCTACATTAAACCCTGTTCGCG-3′ with a *Eco*RI and a *Bam*HI site. The amplified DNA fragment was cleaved with *Sph*I and *Bam*HI and ligated into the *Sph*I-*Bam*HI linearized vector pJLA504 (a gift of Dr. McCarthy, Braunschweig, Germany). To avoid polymerase chain reaction errors in the 65-kDa fragment, most of the coding region was exchanged by a genomic fragment isolated from a YEp13-MOM72 clone (nucleotides 317–1912). This genomic clone was cleaved with *Acc*I, blunted with Klenow enzyme, and cleaved with *Bst*EII. The fragment was isolated and ligated in the pJLA504/65K vector cleaved with *Bam*HI, treated with Klenow fragment, and cleaved with *Bst*EII.

Purification of the Recombinant 65-kDa Fragment

Expression of the 65-kDa Fragment in E. coli—E. coli strain DH1 transformed with the plasmid pJLA504/65K was grown overnight in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 86 mM NaCl containing 100 mg/liter ampicillin) at 30 °C. This culture (40 ml) was diluted 50-fold into LB medium, grown up to an OD of ~0.2. Then 2 liters of media at 56 °C was added (final temperature 42 °C), and the culture was incubated at 42 °C for 2 h. The OD of the culture was about 1.0. Cells were collected by centrifugation and resuspended in 2% of the original volume in buffer A (250 mM sucrose, 50 mM Tris/HCl, pH 8.0, 1 mM PMSF, 20 mM dithiothreitol). Lysozyme was added to yield 0.5 mg/ml, and the cells were incubated under gentle shaking at room temperature for 10 min. Cells were diluted to 8% of the original volume with buffer A, and EDTA (15 mM final concentration) and Triton X-100 (0.5% (w/v) final concentration) were added. After freezing-thawing, the suspension was sonified in a Branson sonifier at 40% duty and setting 8 with a microtip by 6 × 15 pulses to break the cells. After centrifugation at 20,000 × *g* in a Beckman centrifuge, the supernatant (extract) was taken for chromatographic steps.

DEAE-cellulose Chromatography—The supernatant was loaded onto a DE52 column (Whatman) (2.5 × 35 cm) equilibrated with buffer B (30 mM Tris/HCl, pH 8.0, 0.6 mM EDTA, 0.1% Triton X-100, 5% glycerol). After washing with 700 ml of buffer B, proteins were eluted with 550 ml of a salt gradient from 0 to 100 mM NaCl in buffer B (flow rate: 2 ml/min), followed by a salt step of 100 mM NaCl in buffer B (550 ml). The amounts of 65-kDa fragment in the different fractions were determined by Coomassie Blue staining. The fractions were also analyzed by immunodecoration with antibodies directed against yeast MOM72. Fractions containing the 65-kDa fragment (eluting at ~100 mM NaCl) were pooled.

Hydroxylapatite Chromatography—Hydroxylapatite (Bio-Rad) (1.5 × 10 cm) was equilibrated with buffer C (30 mM MOPS, 5 mM NaP_i, pH 7.0, 2% glycerol). The DE52 pool was applied to the HTP column. The column was first washed with 200 ml of buffer C, then with 200 ml of buffer C containing 100 mM NaP_i, pH 7.0, and subsequently eluted with a gradient from 100 to 170 mM NaP_i, pH 7.0 (60 ml, flow rate: 1 ml/min), followed by a passage of 60 ml of 170 mM NaP_i, pH 7.0. Fractions were assayed with Coomassie Blue staining and immunodecoration. The fractions containing pure 65-kDa fragment were pooled and stored at −20 °C.

Protein Import into Isolated Mitochondria

N. crassa (wild type 74A) was grown, and mitochondria were isolated as described (30, 31). The mitochondria were washed in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2). Standard import assays contained import buffer (250 mM sucrose, 3% (w/v) bovine serum albumin (BSA), 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH, pH 7.2), 2–10% reticulocyte lysate containing *in vitro* synthesized ^{35}S-labeled mitochondrial precursor proteins, unlabeled methionine (5 mM), and mitochondria (10 μg of protein). For assaying import of preproteins, 8 mM potassium ascorbate and 0.2 mM *N,N,N′,N′*-tetramethylphenylenediamine was added (to support a membrane potential). Incubation was performed for 3–15 min at 25 °C, then mitochondria were treated with proteinase K (50 μg/ml; 200 μg/ml after import of phosphate carrier) for 15 min at 0 °C. For analyzing binding of the AAC to the mitochondria, pretreatment with apyrase (5 units/ml) was performed separately of the reticulocyte lysate for 15 min at 25 °C and of mitochondria for 25 min at 4 °C (32). Binding was assayed in the presence of antimycin A (8 μM), oligomycin (20 μM), and valinomycin (0.5 μM) (22, 33) at the conditions described above (the assay contained 120 mM KCl). Thereafter the mitochondria were reisolated and washed with SEM. Unspecific binding to mitochondria was analyzed by treating mitochondria with trypsin (20 μg/ml) for 15 min at 4 °C (3).

To determine the effects of antibodies against receptors on import or binding of precursor proteins to mitochondria, IgGs (100 μg) were first incubated with mitochondria for 35 min at 4 °C in the presence of α_2-macroglobulin (130 μg/ml).

For competing, import or binding to mitochondria with the cytosolic domain of yeast MOM72 radiolabeled precursors were incubated with the 65-kDa fragment (1–25 μg) in the presence of 85 mM KP_i before the import reaction, in the presence of 20 mM KP_i and 100 mM KCl before the binding reaction for 5 min at 4 °C. Then import or binding was performed and analyzed as described above.

Coimmunoprecipitation of Radiolabeled Precursor Proteins with the 65-kDa Fragment

Protein A-Sepharose (PAS) (2.5 mg of protein A) was incubated with antibodies against yeast MOM72 and preimmmune antibodies (25 μl of serum) for 30 min. After one washing step with buffer D (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% (w/v) Triton X-100, or 0.5% (w/v) digitonin), the 65-kDa fragment (1–10 μg) was added to the PAS-antibody complex and incubated in buffer D with 3% (w/v) BSA for 45 min. The complex was washed once with buffer D and incubated with radiolabeled precursor proteins in buffer D with 3% (w/v) BSA and 5 mM methionine for 45 min. Then the PAS complex was washed three times with buffer D and once with 10 mM Tris/HCl, pH 7.5.

The interaction of the 65-kDa fragment with precursor proteins translated from *N. crassa* poly(A)$^+$ RNA was assayed by coimmunoprecipitation. For this purpose, poly(A)$^+$ RNA was translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The radiolabeled lysate (10%) was incubated with 5 μg of the 65-kDa fragment in buffer D with 3% (w/v) BSA and 5 mM methionine for 15 min at 4 °C followed by centrifugation for 15 min at 20,000 × *g*. 25 μl of antiserum against MOM72 was added to the supernatant, and the mixture was incubated for 1 h, then incubation with protein A-Sepharose (2.5 mg of protein A) was performed and the PAS complex was washed as described above. For immunoprecipitation of coimmunoprecipitated AAC with AAC antibodies the PAS complex was dissolved in SDS-containing buffer (2% SDS, 50 mM Tris/HCl, pH 7.5) for 5 min at 95 °C. After 40-fold dilution with buffer D, immunoprecipitation was performed as described above (8). The PAS complex was analyzed by SDS-PAGE and fluorography.

Processing Peptidase Assay

Radiolabeled precursor of cytochrome c_1 (1 μl) was incubated with isolated mitochondrial processing peptidase in 15 μl of processing buffer (30 mM Tris/HCl, pH 7.7, 0.5 mM $MnCl_2$, 1% Triton X-100, 1 mM PMSF) for 40 min at 25 °C (20). In a parallel reaction, 1 mM EDTA was added to achieve partial processing. The processing reaction was stopped by addition of 5 mM EDTA in buffer D (150 μl).

Purification of AAC after Overexpression in E. coli

The gene of AAC was introduced into the expression vector pJLA 503 (34) and the plasmid transformed in *E. coli* (strain DH1). Cells were grown overnight in LB medium, diluted 100-fold, and grown for an additional 3 h up to an OD of about 0.9. For overexpression of the protein, cells were then exposed to 42 °C for 2 h. The cells were harvested, treated with lysozyme, subjected to freezing-thawing and sonication (see above). Inclusion bodies were pelleted by centrifugation (15 min, 20,000 × *g*) and washed (including sonication) in the following buffers: buffer E (20 mM Tris/HCl, pH 7.5, 1 mM PMSF, 0.1% 2-mercaptoethanol) containing 1% (w/v) Triton X-100; buffer E containing 1 M urea; buffer E containing 3 M urea. The pellet was then solubilized in buffer E containing 7 M urea by sonication, centrifuged (15 min at 20,000 × *g*), and the supernatant was stored at −20 °C.

Miscellaneous Procedures

The following procedures were carried out as described previously: determination of protein content (35); alkaline treatment of mitochondria (32); coupling of peptides to keyhole limpet hemocyanin (36); raising of antisera and preparation of IgGs (3, 37); translation of precursor proteins in rabbit reticulocyte lysate and labeling with [^{35}S]methionine (38); isolation of mitochondria from yeast *Saccharomyces cerevisiae* (39); SDS-PAGE (40); transfer of proteins to nitrocellulose by semidry blotting, followed by immunodecoration and detection by ECL Western blotting system (Amersham) (3, 37, 41); and quantitation of fluorographs by laser densitometry (42).

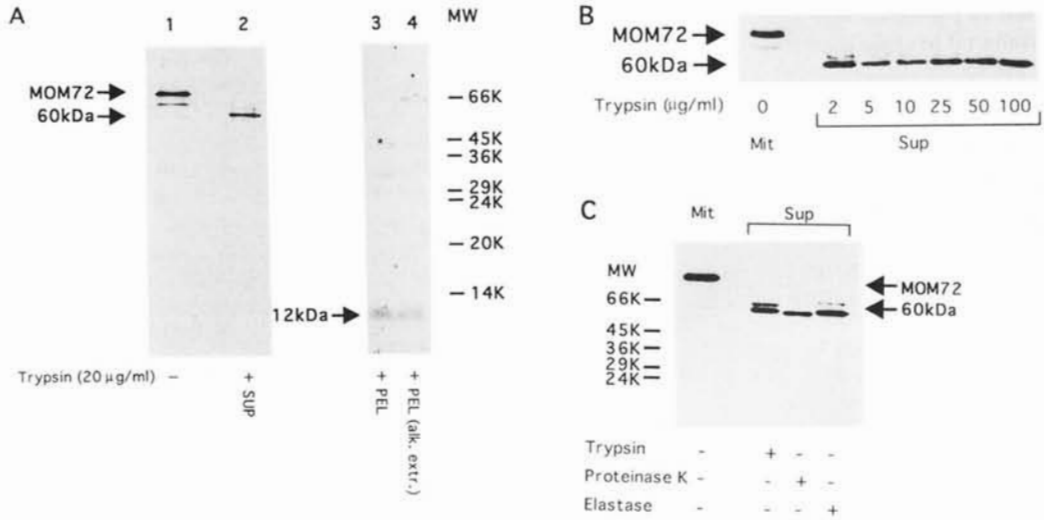

FIG. 1. **Topology of MOM72 in the mitochondrial outer membrane.** *A*, trypsin treatment of *N. crassa* mitochondria releases a 60-kDa fragment into the supernatant, whereas a 12-kDa fragment is membrane-associated. Mitochondria (2 mg of protein/ml of SEM) were incubated with and without trypsin for 15 min at 0 °C. Trypsin was then inactivated by incubation with a 20-fold excess of soybean trypsin inhibitor for 5 min at 0 °C. Mitochondria and supernatant were separated by centrifugation (12 min at 12,000 × *g*). One-half of the mitochondria not treated with trypsin (*lane 1*), or mitochondria treated with trypsin (*lane 3*) as well as one-half of the supernatant from mitochondria treated with trypsin (*lane 2*), was subjected to SDS-PAGE. The other half of the mitochondria treated with trypsin was subjected to alkaline treatment with Na_2CO_3 (53), and the pellet obtained after centrifugation was also subjected to SDS-PAGE (*lane 4*). Immunoblotting was performed with antibodies against MOM72 raised against the protein purified from outer membranes. *SUP*, supernatant; *PEL*, pellet. *B*, the 60-kDa fragment of MOM72 is highly protease-resistant. Mitochondria (2 mg of protein/ml) were treated with increasing concentrations of trypsin for 15 min at 0 °C and then incubated with soybean trypsin inhibitor. After centrifugation for 12 min at 12,000 × *g*, supernatants (equivalent to 40 μg of protein) and mitochondria not treated with trypsin were subjected to SDS-PAGE, followed by immunoblotting with anti-MOM72-antibodies. *Mit*, mitochondria; *Sup*, supernatant. *C*, the 60-kDa fragment is produced by various proteases and reacts with antibodies against a COOH-terminal peptide of MOM72. Mitochondria (2 mg of protein/ml) were treated with trypsin (10 μg/ml), proteinase K (5 μg/ml), or elastase (10 μg/ml) and then incubated with soybean trypsin inhibitor (trypsin) or 1 mM PMSF for 5 min at 0 °C (elastase or proteinase K). Mitochondria were reisolated, and both the mitochondria and supernatants were analyzed by SDS-PAGE and immunoblotting with antibodies directed against a COOH-terminal peptide of MOM72 (comprising 28 amino acid residues). *Mit*, mitochondria; *Sup*, supernatant.

RESULTS

Topology of MOM72 in the Outer Mitochondrial Membrane—MOM72 was identified in intact isolated mitochondria from *N. crassa* by immunoblotting with an antibody against the purified protein. A major band of ~72 kDa was seen and occasionally one or two minor bands that represent degradation products generated during the isolation of mitochondria (Fig. 1*A*). When mitochondria were treated with trypsin, the supernatant obtained after sedimentation of the mitochondria contained a 60-kDa fragment that strongly reacted with the antibody. In the trypsinized mitochondria the antibody decorated a 12-kDa band. The sequence of MOM72 predicts a single transmembrane domain close to the NH_2 terminus. The 12-kDa fragment apparently represents the NH_2-terminal part of MOM72, including the membrane anchor and a short sequence of ~30 residues protected from protease acting on the outside of the mitochondria. The latter fragment was not extracted by carbonate treatment of the mitochondria and therefore is most probably firmly integrated into the outer membrane (Fig. 1*A*).

Rather low concentrations of trypsin were sufficient for release of the 60-kDa fragment; on the other hand even high concentrations of trypsin did not cause a degradation of the fragment (Fig. 1*B*). Not only trypsin, but also proteinase K and elastase in rather low concentrations, were able to cleave MOM72 in intact *N. crassa* mitochondria, yielding fragments of slightly different sizes. Immunoblotting with antibodies generated against a peptide corresponding to the COOH-terminal 28 amino acid residues showed that these fragments contained the COOH terminus of the protein (Fig. 1*C*). This result confirmed the prediction that the COOH terminus is exposed to the cytosol.

In summary, MOM72 possesses a tightly folded hydrophilic domain protruding into the cytosol compartment and is linked to its membrane anchor and the very amino-terminal sequence through a region that is readily accessible to proteases.

The Cytosolic 60-kDa Domain of N. crassa MOM72 Is Involved in Transport of Preproteins—As described previously, MOM72 antibodies inhibit the binding and import of precursors, suggesting that the surface available regions of MOM72 are involved in the binding and import of the precursors. We therefore investigated the role of the hydrophilic cytosolic domain in precursor binding and import. Antibodies against the COOH-terminal 28 amino acid residues of *N. crassa* MOM72 were also used for assaying their effect on binding and import of the precursor of the AAC. These antibodies had virtually the same inhibitory effect as polyclonal antibodies raised against total MOM72 both on specific binding to the mitochondrial surface and on transfer into the inner membrane (Fig. 2, *A* and *B*). As reported previously, the MOM72 polyclonal antibodies reduce the efficiencies of both reactions by about 75%, whereas antibodies against MOM19 inhibit import only by 25–40% and binding only by a marginal degree (Fig. 2, *A* and *B*). MOM19, although being able to facilitate import of AAC, does not bind tightly to the precursor.

Isolated mitochondria were treated with elastase to release the cytosolic domain into the supernatant. Afterwards elastase was inactivated by addition of PMSF. Supernatant prepared in this manner contained the 60-kDa domain as major component as visualized by protein staining and by immunoblotting (Fig. 3*A*). To test for the ability of the 60-kDa domain to interact with precursors, increasing amounts of supernatant were added to assay for import and receptor binding of AAC. The import of AAC was reduced by about 50%, whereas import of pre-F1β was not affected (Fig. 3*B*). This is in good agreement with the previous observation that selective inactivation of MOM72 either by protease treatment or by deletion of the gene leads to

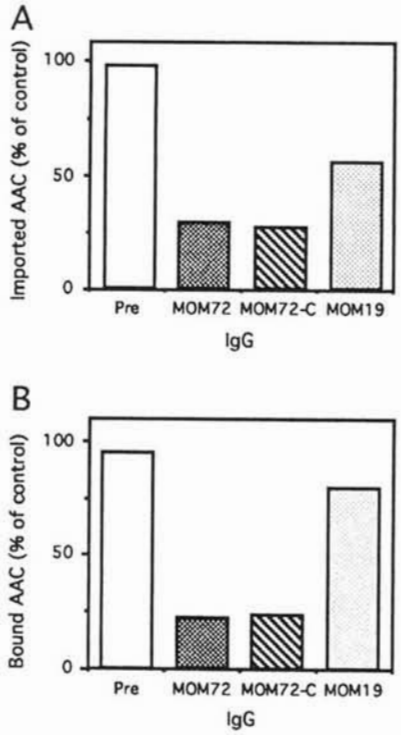

FIG. 2. **Antibodies directed against the COOH terminus of MOM72 inhibit the binding and import of preproteins.** *A*, IgGs against the COOH terminus of MOM72 inhibit import of the AAC. Mitochondria (10 μg of protein) were incubated with IgGs (100 μg) prepared from either preimmune sera or antisera directed against intact MOM72, MOM72-C (peptide comprising 28 amino acid residues of MOM72) or MOM19 for 35 min at 0 °C. Then [^{35}S]methionine-labeled AAC was added and import performed under standard import conditions. Mitochondria were then treated with proteinase K, reisolated, and analyzed by SDS-PAGE and fluorography. *B*, IgGs against the COOH terminus of MOM72 inhibit specific binding of AAC. IgGs were prebound to mitochondria (10 μg of protein) that had been preincubated with apyrase to reduce ATP levels. AAC-containing reticulocyte lysate, which was also treated with apyrase, was then added to mitochondria. The binding assay was performed as described under "Experimental Procedures."

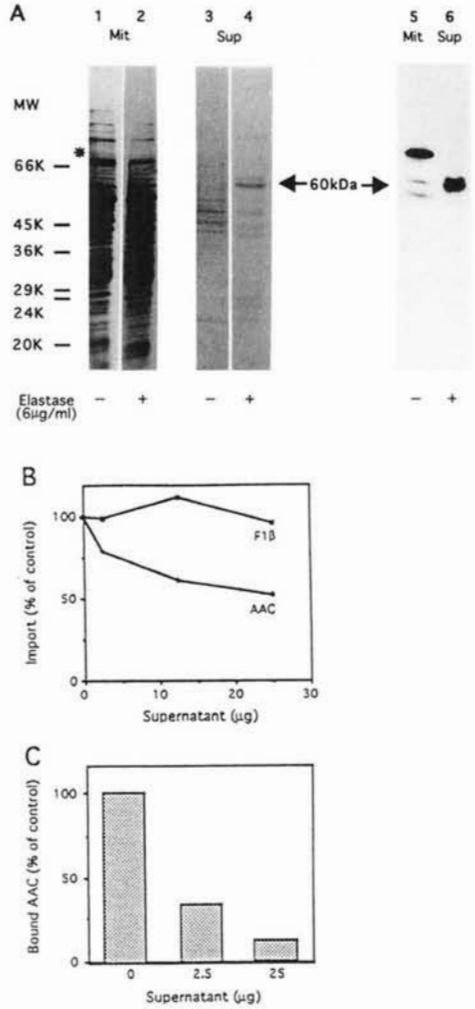

FIG. 3. **Polypeptides released from mitochondria by elastase treatment selectively inhibit the import of MOM72-dependent preproteins.** *A*, pattern of proteins released into supernatant. Mitochondria (6 mg of protein/ml) were treated with elastase (6 μg/ml) (*lanes 2, 4,* and *6*) or without elastase (*lanes 1, 3,* and *5*) for 12 min at 25 °C. Elastase was then inactivated with 2 mM PMSF. Mitochondria were separated from supernatant by centrifugation. Then mitochondria (40 μg of protein) (*lanes 1, 2,* and *5*) and supernatants (equivalent of 200 μg of protein) (*lanes 3, 4,* and *6*) were subjected to SDS-PAGE, Coomassie staining (*lanes 1–4*), and immunodecoration for MOM72 (*lanes 5* and *6*). The *asterisk* indicates the band corresponding to MOM72 in intact mitochondria. *B*, import of AAC is inhibited by the elastase supernatant. Reticulocyte lysate containing precursor proteins of AAC and F1β (β-subunit of ATP-synthase) were mixed with different amounts of supernatant of elastase-treated mitochondria (supernatant of 1 mg of elastase-treated mitochondria contained 25 μg of protein). Mitochondria (10 μg) were added, and import was followed under standard import conditions. *C*, binding of AAC is inhibited by the elastase supernatant. Precursor of AAC in reticulocyte lysate was incubated with elastase supernatant, and specific binding to mitochondria was assayed as described under "Experimental Procedures."

moderate reduction of import of AAC (as MOM19 acts as a back-up receptor) but does not interfere with pre-F1β import, which predominantly uses MOM19 (1, 4). Binding of AAC precursor to mitochondria, however, was strongly impaired, again in agreement with the observed low affinity of MOM19 for the AAC precursor (Fig. 3*C*). Supernatants obtained from mock-treated mitochondria had no effect on the binding of the precursor to mitochondria (data not shown).

These data suggest that blocking with antibodies of the cytosolic domain of MOM72 impairs import of precursors and that the released domain alone or in cooperation with other protease-released components can reduce import by competing for binding of precursors.

The Cytosolic Domain of Yeast MOM72 Can Be Expressed in E. coli and Prepared in Purified Form—A segment of the MOM72 gene of yeast was cloned into the heat-inducible plasmid pJLA that extended from codon 38 (*i.e.* directly after the sequence specifying the membrane anchor) to the end of the coding sequence. The resulting construct encoded a protein of 581 amino acid residues, corresponding to a molecular mass of ~65 kDa. *E. coli* cells harboring the plasmid pJLA504/65K synthesized a protein of 65 kDa when kept at 42 °C, but not at 30 °C, as seen upon SDS-PAGE of whole cell extracts and staining with Coomassie Blue (Fig. 4*A*). The 65-kDa fragment was present in inclusion bodies and in the soluble fraction of *E. coli* cell extracts. It was purified from the soluble fraction in two steps, chromatography on DEAE-cellulose and on hydroxylapatite, to homogeneity (Table I and Fig. 4*B*). Both protein staining and immunoblotting with an antibody against MOM72 showed a single band after SDS-PAGE of the final preparation.

The purified protein, when subjected to chromatography on a Superose 12 sizing column, displayed an apparent molecular mass of around 60 kDa (not shown). Thus, the purified cytosolic domain of MOM72 represents a monomeric protein.

The Purified Cytosolic Domain of MOM72 Competes with Outer Membrane-integrated MOM72 for Binding of a Subset of Precursor Proteins—The effect of adding increasing amounts of the 65 kDa-fragment of yeast MOM72 on the import of various

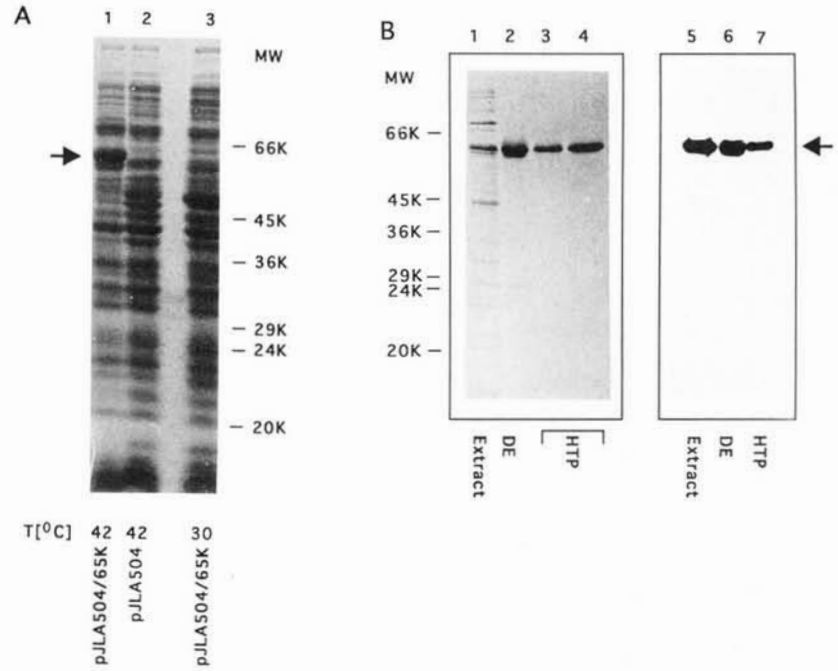

FIG. 4. **Expression of the 65-kDa fragment of yeast MOM72 in *E. coli* and purification.** *A*, expression. The DNA encoding the 65-kDa fragment was cloned into the heat shock-inducible vector pJLA504. Expression of the protein was analyzed at 42 °C (*lane 1*) and at 30 °C (*lane 3*) as well as after transforming *E. coli* with pJLA504 not containing DNA for the fragment (*lane 2*). The *arrow* indicates the position of the 65-kDa fragment. *B*, purification. After expression of the protein, cells were lysed by freezing-thawing and sonication and then centrifuged at 20,000 × *g* for 30 min. Most of the protein remained in the soluble fraction (*lanes 1* and *5*) and was purified by DEAE-cellulose chromatography (*lanes 2* and *6*) and hydroxylapatite chromatography (*lanes 3, 4,* and *7*). Fractions were analyzed by SDS-PAGE and Coomassie staining (*lanes 1–4*) and by immunodecoration with yeast MOM72 antibodies (*lanes 5–7*). In *lanes 3* and *4*, 2 and 4 μg, respectively, of protein were loaded.

TABLE I
Purification of the 65-kDa fragment of yeast MOM72 after expression in E. coli

The 65-kDa fragment of yeast MOM72 was overexpressed in *E. coli* (4-liter culture) and then isolated by two chromatographic steps as indicated (see also Fig. 4). The amounts of 65-kDa fragment in fractions was measured by scanning of Coomassie-stained gel bands in comparison with a BSA standard curve.

	Volume	Protein	65-kDa fragment/ total protein	Purification factor	Yield
	ml	*mg*			*%*
E. coli extract	70	1050	0.069	1	100
DEAE-cellulose	105	63	0.41	5.9	36
Hydroxylapatite	15	10	1	14.3	14

precursor proteins into isolated mitochondria was tested (Fig. 5*A*). A strong inhibitory effect was observed with the precursors to AAC and the phosphate carrier. A roughly 200–500-fold excess of the 65-kDa fragment resulted in 50% inhibition of import. On the other hand, no inhibition of import was observed with other precursors, such as porin, pre-F1β, and MOM38 (Fig. 5*A*), as well a precytochrome b_2 (not shown). A moderate inhibition was seen with precytochrome c_1. This suggests that certain precursors such as AAC and phosphate carrier bind strongly to the cytosolic domain of MOM72 and therefore are efficiently sequestered, whereas other precursors, in fact the majority of those tested, do not bind or bind only weakly to MOM72.

In the presence of the 65-kDa fragment the rate of import of the AAC precursor was reduced, suggesting competition between the intact receptor and its soluble cytosolic domain for binding of the precursor (Fig. 5*B*).

To demonstrate directly that the initial binding reaction of the AAC precursor was affected by the 65-kDa domain, competition for binding to the mitochondrial surface was tested. As has been shown previously, the AAC precursor binds to the mitochondria in a specific and productive manner in the absence of ΔΨ and under strongly reduced ATP levels in the incubation medium (33). Specific binding was efficiently impaired, and a 50% reduction was observed at a ~50–100-fold molar excess of the fragment over the endogenous MOM72 (Fig. 5*C*). When binding of the AAC precursor was first performed in the absence of the 65-kDa fragment and then an incubation in the presence of the fragment was performed, binding was not reversed (Fig. 5*C*), suggesting that the off rate of the reaction is slow or, perhaps more likely, that binding to MOM72 is immediately followed by another reaction that shifts the equilibrium of the binding reaction. The requirement of higher concentrations of the 65-kDa fragment needed for competing import into the inner membrane as compared with binding is similarly explained by the continuous removal of precursor from the binding equilibrium by the import system. Higher levels of the fragment then are needed to keep precursor from being transferred into the mitochondria.

Interaction of Preproteins with the Isolated 65-kDa Domain of Yeast MOM72—The data described so far suggested binding of precursors to the 65-kDa domain, but it was necessary to prove a physical interaction between these components. Antibodies against MOM72 were bound to protein A-Sepharose and then the 65-kDa fragment was bound to the antibodies. This immunocomplex was then used as a tool to probe the interaction of the 65-kDa fragment with the precursor of the AAC. Under those experimental conditions a significant portion of total AAC precursor present in a reticulocyte lysate and labeled with [^{35}S]methionine could be recovered with the immunocomplex (~10%, Fig. 6*A*). Unspecific binding was ruled out by employing preimmune IgGs in the formation of the immunocomplex. Only a minor binding was observed in this control reaction, which could be virtually completely suppressed by increasing the salt concentration in the reaction mixture (Fig. 6*A*). In addition, AAC binding was dependent on the amount of specific immunocomplex employed in the assay (Fig. 6*B*).

When different preproteins were tested in this assay, distinct specificity was found. The precursors of AAC and of the mitochondrial phosphate carrier were bound with highest affinity. The precursor of cytochrome c_1 was also bound; however, binding was less efficient. Other preproteins showed no significant binding, as only 1% or less of input was recovered in the immunocomplex, a value which was similar to that of non-mitochondrial control proteins such as yeast invertase, prepro-α-factor, and dihydrofolate reductase (Fig. 6*C*).

To further analyze precursor binding to the 65-kDa fragment, a soluble form of AAC was purified after expression in *E. coli*. Then AAC was isolated from inclusion bodies and solubi-

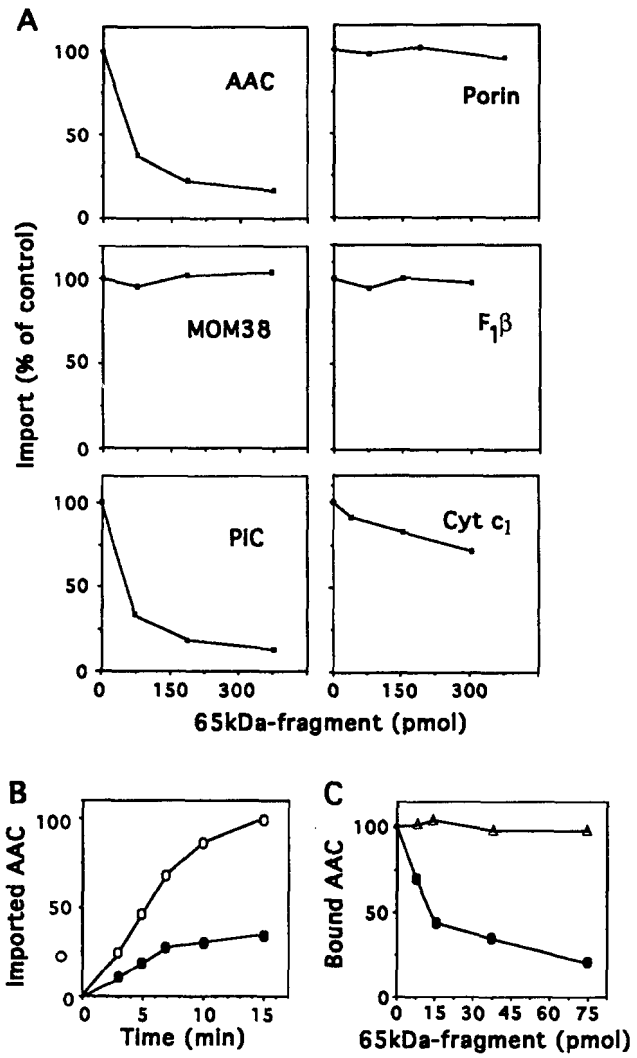

FIG. 5. **Selective inhibition of import of mitochondrial preproteins by the 65-kDa fragment.** *A*, the 65-kDa fragment inhibits import of a subset of precursor proteins. Radiolabeled precursor proteins were imported into *N. crassa* mitochondria (10 μg) for 10 min at 25 °C in the presence of various amounts of the 65-kDa fragment. The total volume of the reaction was 100 μl. After treatment with proteinase K, mitochondria were analyzed by SDS-PAGE and fluorography, and the amounts of imported proteins were quantified. *B*, the rate of AAC import is reduced in the presence of the 65-kDa fragment. Import of AAC into yeast mitochondria (10 μg) was performed as in *A* in the presence (●) or absence (○) of the 65-kDa fragment (150 pmol). *C*, binding of AAC to mitochondria is inhibited by the 65-kDa fragment. AAC, synthesized in reticulocyte lysate, was incubated with the indicated amounts of the 65-kDa fragment and then binding to *N. crassa* mitochondria was determined (●) (see "Experimental Procedures"). In another reaction, AAC precursor was first bound to mitochondria for 3 min at 25 °C, then the 65-kDa fragment was added, incubated with the import mixture for further 7 min at 25 °C, then mitochondria were reisolated and analyzed (△).

lized in 7 M urea. After 50-fold dilution out of urea and centrifugation, about 10% of the protein remained in the supernatant. This soluble AAC could be quantitatively coimmunoprecipitated with MOM72 antibodies when the 65-kDa fragment was present. Preimmune antibodies did not immunoprecipitate such a complex (Fig. 6*D*). If the binding reaction was performed in the presence of reticulocyte lysate, the same result was obtained, suggesting that unspecific interaction with lysate proteins or an inhibitory effect of chaperones contained in the lysate did not affect the binding reaction. These data provide strong evidence that MOM72 directly interacts with the precursor of AAC.

Next, we analyzed whether the interaction of precytochrome c_1 with the 65-kDa fragment represented a specific binding reaction for the precursor form in comparison with the processed form. Precytochrome c_1 was synthesized in a reticulocyte lysate which was then incubated with mitochondrial processing peptidase under conditions where processing of only half of the precursor occurred (Fig. 7, *lane 2*). An immunocomplex was then formed, as described above, with antibodies against MOM72 and the 65-kDa fragment. This immunocomplex reacted preferentially with the unprocessed precursor (Fig. 7, *lane 4*), whereas control immunocomplex with preimmune IgGs showed a rather low unspecific interaction with both forms (not shown). These observations support the view that the presequence in precytochrome c_1 is important for specific binding to MOM72. If there was an unspecific hydrophobic interaction, the overall more hydrophobic processed form might have exhibited stronger binding.

Finally, if the AAC and P_iC precursors are indeed high affinity binding partners of MOM72, it was to be predicted that the 65-kDa fragment should be able to bind these precursors out of a mixture of newly synthesized proteins. Poly(A)$^+$ RNA was prepared from *N. crassa* and translated in reticulocyte lysate. Then incubation with the immunocomplex containing anti-MOM72 and the 65-kDa fragment was performed, and the complex was recovered, washed, and analyzed by SDS-PAGE and autoradiography. Several proteins made from poly(A)$^+$ RNA were associated with the complex; the most prominent one had the mobility of the AAC (Fig. 8, *lane 2*). That this band indeed represented AAC was verified by dissolving the complex with SDS and immunoprecipitating with antibodies against the AAC (Fig. 8, *lane 4*). A substantial part of the total AAC present in the poly(A)$^+$-translation mixture could be collected with the 65-kDa fragment as is apparent from a comparison with a direct immunoprecipitation of the AAC from the lysate (Fig. 8, *lane 3*). We conclude that MOM72 interacts with the AAC precursor with such high affinity and specificity that it can bind it with high selectivity from a mixture of newly synthesized cellular proteins.

In summary, the cytosolic domain of MOM72 has the ability to directly interact with precursor proteins with high affinity and with high selectivity. In the experiments described here, the 65-kDa fragment had a distinct preference for the AAC and P_iC precursors, whereas most other precursors were not bound. The cytochrome c_1 precursor interacted with the fragment with lower affinity, but still in a specific fashion.

DISCUSSION

Mitochondrial import receptors MOM72 (MAS70 in yeast) and MOM19 have been identified by showing that specific antibodies against these surface-exposed protease-sensitive components of the outer membrane inhibit binding and import of precursor proteins (1, 3, 4). The structures of these receptors have been deduced from their cDNA or genomic DNA sequences (4, 5, 43), but further analysis has so far been hampered by the low abundance of these components preventing preparation from mitochondria in larger amounts and by the lack of expression of these membrane-integrated components in heterologous systems such as *E. coli*.

In the present study we show that the MOM72 receptor consists of a tightly folded carboxyl-terminal domain exposed to the cytosol with a molecular mass of about 60 kDa that is linked to the membrane by the hydrophobic amino-terminal membrane spanning segment. The piece between the membrane anchor and 60-kDa domain is readily accessible to various proteolytic enzymes, suggesting that this may be a flexible region perhaps serving as a hinge-like structure. Interestingly, this region contains abundant proline residues in both the

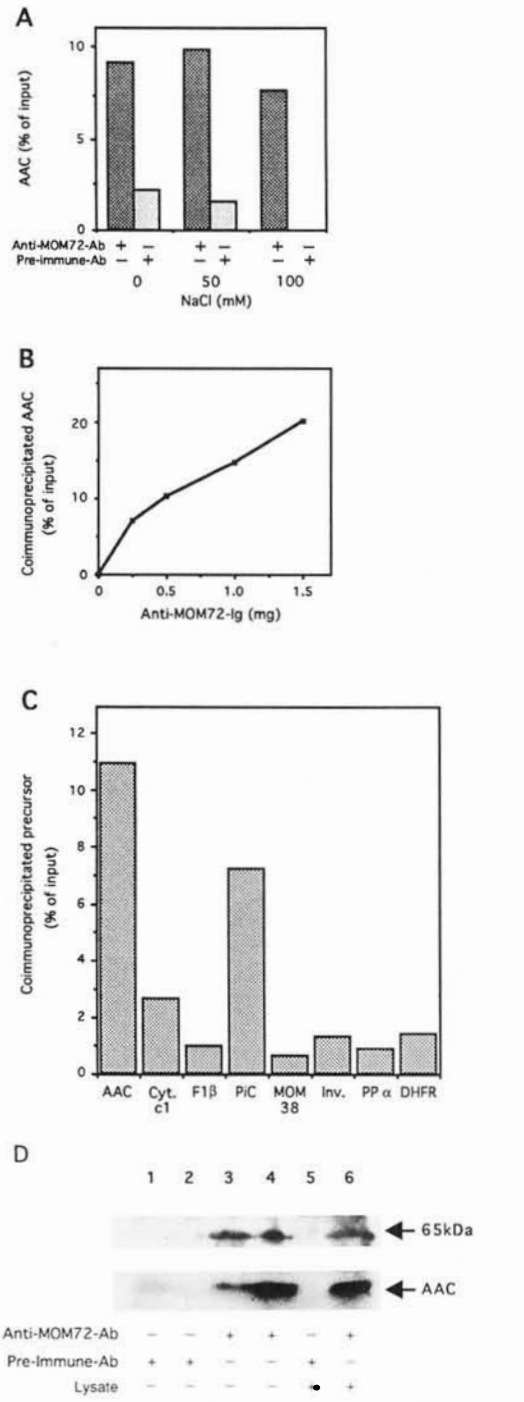

FIG. 6. **Specific binding of preproteins to the 65-kDa fragment.** *A*, AAC and the 65-kDa fragment are coimmunoprecipitated with anti-MOM72 antibodies. Anti-MOM72 or preimmune antibodies were first bound to protein A-Sepharose beads, then the 65-kDa fragment (3 μg) was added. In a further step, incubation with radiolabeled AAC synthesized in reticulocyte lysate was performed at different concentrations of added salt. Bound AAC was determined by SDS-PAGE, fluorography, and laser densitometry. *B*, coimmunoprecipitation of AAC is limited by the amount of immunoadsorbed 65-kDa fragment. Anti-MOM72 antibodies were bound to PAS. Half of the beads were then incubated with the 65-kDa fragment, mixed in different proportions with beads lacking fragment, and incubated with radiolabeled AAC. In the absence of the 65-kDa fragment, 1% of input AAC was found associated with the PAS anti-MOM72 pellet, and this background was subtracted from all values. *C*, a subset of mitochondrial precursor proteins is bound by the 65-kDa fragment. Binding of various mitochondrial and non-mitochondrial precursor proteins to the 65-kDa fragment was determined by coimmunoprecipitation as described in *A* and under "Experimental Procedures." *Inv.*, yeast invertase; *PPa*, yeast prepro-α-factor; *DHFR*, mouse dihydrofolate reductase. *D*, purified AAC is bound by the 65-kDa fragment. AAC was overexpressed in *E. coli*, purified as inclusion bodies, and solubilized in 7 M urea (34). AAC (0.3 μg) (*lanes 1* and *3*) or AAC (3 μg) (*lanes 2* and *4*) were diluted 50-fold in buffer D with 3% (w/v) BSA (150 μl) and incubated with the 65-kDa fragment (3 μg) in the absence or presence of reticulocyte lysate (20 μl) for 30 min at 0 °C. After centrifugation (15 min at 20,000 × *g*), the supernatant was incubated with anti-MOM72 antibodies or preimmune antibodies for 1 h at 4 °C, then protein A-Sepharose was added; after 45 min, the immunocomplex was sedimented and analyzed by SDS-PAGE, blotting to nitrocellulose, and immunodecoration with the Luminol system using antibodies against MOM72 and AAC.

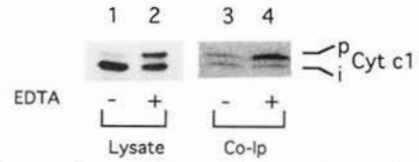

FIG. 7. **The matrix-targeting sequence of precytochrome c_1 is required for binding to the 65-kDa fragment.** Precursor of cytochrome c_1 synthesized in reticulocyte lysate was incubated with purified mitochondrial processing peptidase in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of 1 mM EDTA for 40 min at 25 °C. Aliquots corresponding to 2% of the total reactions were then directly analyzed by SDS-PAGE and fluorography (*lanes 1* and *2*). To the remaining portions of the two reactions, the 65-kDa fragment (3 μg) was added and coimmunoprecipitation with anti-MOM72 antibodies was performed (see "Experimental Procedures"). Bound cytochrome c_1 was then analyzed by SDS-PAGE and fluorography (*lanes 3* and *4*). *p*, unprocessed precursor form of cytochrome c_1; *i*, intermediate sized form of cytochrome c_1.

yeast and *N. crassa* proteins. Inhibition of binding and import of precursors by antibodies against the cytosolic domain suggested that at least part of the receptor function is localized in this part of the molecule. Several lines of evidence indicate that the cytosolic domain indeed interacts with precursors. Addition of the purified domain to import assays reduced binding and import of precursors, apparently by competing with the endogenous MOM72. Furthermore, binding of precursors to the cytosolic MOM72 domain was observed. These findings establish the role of MOM72 as a receptor on the surface of the mitochondria. They are in full agreement with previous results obtained with intact mitochondria.

The relatively high molar excess of the MOM72 fragment over precursor required for competing binding and import and the incomplete coimmunoprecipitation of precursor with excess MOM72 fragment probably reflect a complex situation in the *in vitro* assay. There are several reasons why precursors synthesized in a cell-free system may show incomplete binding and apparently reduced affinity. Only a part of the precursor may be free to bind rapidly to the MOM72 fragment. In fact, only a portion of the precursor made in reticulocyte lysate can be imported into isolated mitochondria. In intact mitochondria, interaction with additional components may influence the apparent affinity of the precursor. The reported dimerization of intact MOM72 in the outer membrane may alter the binding equilibrium (10, 44). These uncertainties do not, however, interfere with the conclusion that MOM72 interacts specifically and with high affinity with a limited set of precursors. Detailed studies with purified precursor proteins may help to answer these open questions.

In addition these results shed further light on the functional relationship of the two identified receptors MOM72 and MOM19. The cytosolic domain of MOM72 was able to specifically bind with high affinity the precursors of the two major inner membrane proteins AAC and P_iC. This is in very good agreement with the observed inhibition of import of these precursors by antibodies against MOM72. In fact, MOM72 was initially identified as a major receptor for the AAC (3). Of the various precursors tested, besides AAC and P_iC, only the pre-

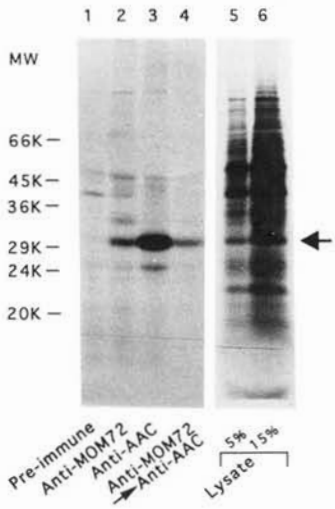

FIG. 8. **Selective binding of the 65-kDa fragment to preproteins translated from *N. crassa* poly(A)$^+$ RNA.** Proteins were synthesized in reticulocyte lysate programmed with *N. crassa* poly(A)$^+$ RNA in the presence of [^{35}S]methionine. To four aliquots of the lysate (reactions 1–4) the 65-kDa fragment was added, and immunoprecipitation with the following antibodies was performed. Reaction 1, preimmune antibodies; reactions 2 and 4, anti-MOM72 antibodies; reaction 3, anti-AAC antibodies. Immunocomplexes were harvested by adding PAS, and in case of the reactions 1–3, the immunoadsorbed material was analyzed by SDS-PAGE. The immunocomplex obtained in reaction 4 was dissociated by addition of SDS, the sample was diluted by adding Triton X-100-containing buffer, and a second immunoprecipitation with antibodies against AAC was performed. Then also this immunoprecipitate was analyzed by SDS-PAGE. Reactions 1–4 correspond to *lanes 1–4*. For comparison, aliquots of the poly(A)$^+$ RNA-programmed reticulocyte lysate were analyzed directly by SDS-PAGE. *Lane 5* corresponds to 5% and *lane 6* to 15% of the input in each of the four reactions analyzed in *lanes 1–4*. The *arrow* marks the position of AAC on the fluorographs.

cursor of cytochrome c_1 showed a significant binding to the MOM72 cytosolic domain. This does, however, not exclude that other precursors are also recognized by MOM72, although binding may occur with much lower affinity. On the other hand, deletion of MOM72 in yeast had no effect on growth and mitochondrial protein import at 25 °C, and only a minor deficiency was observed at 37 °C (2, 4). Targeting via MOM72 is not essential, and other components such as MOM19 appear to mediate recognition and initial import of AAC and P_iC. In agreement with this notion, genetic inactivation of MOM19 in *N. crassa* leads to cessation of cellular growth, strong alterations of mitochondrial structure, and composition and impairment of import of many precursor proteins (25, 45).

The exceptional high affinity binding of the AAC and the P_iC to the cytosolic domain of MOM72 may be related to the particular structure of these precursors. They do not have a cleavable signal, but instead multiple internal signals are present (46, 47). In principal it seems possible that the interaction of the AAC and P_iC precursors with MOM72 does not occur directly, but that a chaperone-like component binds to the precursors in the cell-free system, and it is this component that is recognized by MOM72. However, the observed binding of purified AAC precursor and the reported cross-linking of the AAC precursor when bound to MOM72 (10) argue strongly for a direct interaction.

Quite interestingly, MOM72/MAS70 has six or seven so-called tetratricopeptide motifs which are also found in a number of transcription factors and mitotic genes (48–51), in MOM19, and in a putative peroxisomal import receptor, Pas8p of *Pichia pastoris* (52). One might speculate that the tetratricopeptide motif has a function in precursor recognition; however, other roles of these structural elements, such as interaction with further components of the receptor complex or with cytosolic factors, are equally well possible.

In summary, our data show that the hydrophilic cytosolic domain of mitochondrial import receptor MOM72 interacts in a specific manner with precursor proteins. Binding of precursors to the receptors may stabilize a certain conformation, *e.g.* an amphiphilic helix structure, which then makes the precursor competent for further steps in the import pathway, in particular for insertion into the general insertion pore. In the light of our results, it appears possible that also recognition of precursors by MOM19 occurs by binding to the cytosolic domain of this other important mitochondrial import receptor.

Acknowledgments—We thank Dr. M. Arretz for providing the mitochondrial processing peptidase, Dr. K. Mahlke for preparing the AAC expression clone, and Dr. McCarthy for the gift of the vector pJLA504 and for analyzing the secondary structure of the MOM72-mRNA. We are grateful to Dr. D. Cyr for his advice.

REFERENCES

1. Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N. & Neupert, W. (1989) *Cell* **59,** 1061–1070
2. Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brütsch, H. & Schatz, G. (1990) *EMBO J.* **9,** 3191–3200
3. Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. & Neupert, W. (1990) *Cell* **62,** 107–115
4. Steger, H. F., Söllner, T., Kiebler, M., Dietmeier, K. A., Pfaller, R., Trülzsch, K. S., Tropschug, M., Neupert, W. & Pfanner, N. (1990) *J. Cell Biol.* **111,** 2353–2363
5. Schneider, H., Söllner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trülzsch, B., Neupert, W. & Pfanner, N. (1991) *Science* **254,** 1659–1662
6. Moczko, M., Gärtner, F. & Pfanner, N. (1993) *FEBS Lett.* **326,** 251–254
7. Baker, K. P., Schaniel, A., Vestweber, D. & Schatz, G. (1990) *Nature* **348,** 605–609
8. Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N. & Neupert, W. (1990) *Nature* **348,** 610–616
9. Moczko, M., Dietmeier, K., Söllner, T., Segui, B., Steger, H., Neupert, W. & Pfanner, K. (1992) *FEBS Lett.* **310,** 265–268
10. Söllner, T., Rassow, J., Wiedmann, M., Schloßmann, J., Keil, P., Neupert, W. & Pfanner, N. (1992) *Nature* **355,** 84–87
11. Pfanner, N., Rassow, J., van der Klei, I. J. & Neupert, W. (1992) *Cell* **68,** 999–1002
12. Maarse, A. C., Blom, J., Grivell, A. & Meijer, M. (1992) *EMBO J.* **11,** 3619–3628
13. Dekker, P. J. T., Keil, P., Rassow, J., Maarse, A. C., Pfanner, N. & Meijer, M. (1993) *FEBS Lett.* **330,** 66–70
14. Emtage, J. L. T. & Jensen, R. E. (1993) *J. Cell Biol.* **122,** 1003–1012
15. Horst, M., Jenö, P., Kronidou, N. G., Bolliger, L., Oppliger, W., Scherer, P., Manning-Krieg, U., Jascur, T. & Schatz, G. (1993) *EMBO J.* **12,** 3035–3041
16. Neupert, W. & Pfanner, N. (1993) *Phil. Trans. R. Soc. Lond. B* **339,** 355–362
17. Stuart, R. A., Gruhler, A., van der Klei, I., Guiard, B., Koll, H. & Neupert, W. (1994) *Eur. J. Biochem.*, **220,** 9–18
18. Voos, W., Gambill, B. D., Guiard, B., Pfanner, N. & Craig, E. A. (1993) *J. Cell Biol.* **123,** 119–126
19. Pfanner, N., Söllner, T. & Neupert, W. (1991) *Trends Biochem. Sci.* **16,** 63–67
20. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. & Neupert, W. (1988) *Cell* **53,** 795–806
21. Zimmermann, R. & Neupert, W. (1980) *Eur. J. Biochem.* **109,** 217–229
22. Pfaller, R., Steger, H. F., Rassow, J., Pfanner, N. & Neupert, W. (1988) *J. Cell Biol.* **107,** 2483–2490
23. Lill, R., Stuart, R. A., Drygas, M. E., Nargang, F. E. & Neupert, W. (1992) *EMBO J.* **11,** 449–456
24. Dietmeier, K., Zara, V., Palmisano, A., Palmieri, F., Voos, W., Schloßmann, J., Moczko, M., Kispal, G. & Pfanner, N. (1993) *J. Biol. Chem.* **268,** 25958–25964
25. Harkness, T. A. A., Nargang, F. E., van der Klei, I., Neupert, W. & Lill, R. (1994) *J. Cell Biol.* **124,** in press
26. Ramage, L., Junne, T., Hahne, K., Lithgow, T. & Schatz, G. (1993) *EMBO J.* **12,** 4115–4123
27. Hines, V. & Schatz, G. (1993) *J. Biol. Chem.* **268,** 449–454
28. Inis, M. A., Myambo, K. B., Gelfand, D. H. & Brow, M. A. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8432–8436
29. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491
30. Pfanner, N. & Neupert, W. (1985) *EMBO J.* **4,** 2819–2825
31. Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. & Neupert, W. (1986) *Cell* **51,** 1027–1037
32. Pfanner, N., Tropschug, M. & Neupert, W. (1987a) *Cell* **49,** 815–823
33. Pfanner, N. & Neupert, W. (1987) *J. Biol. Chem.* **262,** 7528–7536
34. Mahlke, K. (1991) *Biogenese Mitochondrialer Innenmembranproteine: Sortierung und Assemblierung der F_oF_1-ATPase-Untereinheit 9 und des ADP/ATP-Translokators,* Thesis, University of Munich
35. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
36. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Kiebler, M., Keil, P., Schneider, H., van der Klei, I., Pfanner, N. & Neupert, W. (1993) *Cell* **74,** 483–492
38. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67,** 247–256

39. Daum, G., Böhni, P. C. & Schatz, G. (1990) *Nature* **348,** 605–609
40. Laemmli, U. K. (1970) *Nature* **227,** 680–685
41. Kyshe-Andersen, J. (1984) *J. Biochem. Biophys. Methods* **10,** 203–207
42. Söllner, T., Rassow, J. & Pfanner, N. (1991) *Methods Cell Biol.* **34,** 345–358
43. Hase, T., Riezman, H., Suda, K. & Schatz, G. (1983) *EMBO J.* **2,** 2169–2172
44. Millar, D. G. & Shore, G. C. (1993) *J. Biol. Chem.* **268,** 18403–18406
45. Harkness, T. A. A., Metzenberg, R. L., Schneider, H., Lill, R., Neupert, W. & Nargang, F. (1994) *Genetics* **136,** 107–118
46. Pfanner, N., Hoeben, P., Tropschug, M. & Neupert, W. (1987b) *J. Biol. Chem.* **262,** 14851–14854
47. Smagula, C. S. & Douglas, M. G. (1988) *J. Cell. Biochem.* **36,** 323–328
48. Schultz, J. & Carlson, M. (1987) *Mol. Cell. Biol.* **7,** 3637–3645
49. Hirano, T., Hiraoka, Y. & Yanagida, M. (1988) *J. Cell Biol.* **106,** 1171–1183
50. Sikorski, R. S., Boguski, M. S., Goebl, M. & Hieter, P. (1990) *Cell* **60,** 307–317
51. Williams, F. E., Varanasi, U. & Trumbly, R. J. (1991) *Mol. Cell. Biol.* **11,** 3307–3316
52. McCollum, D., Monosov, E. & Subramani, S. (1993) *J. Cell Biol.* **121,** 761–774
53. Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. (1982) *J. Cell Biol.* **93,** 97–102