Import of ADP/ATP Carrier into Mitochondria: Two Receptors Act in Parallel

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Abstract. We have identified the yeast homologue of *Neurospora crassa* MOM72, the mitochondrial import receptor for the ADP/ATP carrier (AAC), by functional studies and by cDNA sequencing. Mitochondria of a yeast mutant in which the gene for MOM72 was disrupted were impaired in specific binding and import of AAC. Unexpectedly, we found a residual, yet significant import of AAC into mitochondria lacking MOM72 that occurred via the receptor MOM19. We conclude that both MOM72 and MOM19 can direct AAC into mitochondria, albeit with different efficiency. Moreover, the precursor of MOM72 apparently does not require a positively charged sequence at the extreme amino terminus for targeting to mitochondria.

An essential step in the biogenesis of cell organelles is the specific import of cytosolically synthesized precursor proteins (Wickner and Lodish, 1985; Douglas et al., 1986; Walter and Lingappa, 1986; Attardi and Schatz, 1988; Hartl et al., 1989; Pfanner and Neupert, 1990). Targeting signals in the precursor proteins are decoded by complementary structures (receptors) on the organelle surfaces, thus ensuring selective transport of precursor proteins into the correct compartments. With respect to mitochondrial protein import, two import receptors were identified on mitochondria from *Neurospora crassa (N. crassa)*. MOM19, a mitochondrial outer membrane protein with an apparent molecular mass of 19 kD, seems to function as import receptor for most precursor proteins studied, including all that carried an amino-terminal presequence (Söllner et al., 1989). MOM72, an outer membrane protein of 72 kD, acts as import receptor for the ADP/ATP carrier (AAC)[1] that is synthesized without a cleavable extension sequence (Söllner et al., 1990). The presence of at least two mitochondrial import receptors raises the problem of selectivity of the receptors and in particular the question as to whether the "master receptor" MOM19 may also be able to interact with the precursor of AAC. Moreover, with mitochondria of the yeast *Saccharomyces cerevisiae*, a 42-kD protein of the outer membrane was identified that is involved in protein import (Vestweber et al., 1989). In the assumption that this "import site protein 42" (ISP 42) may act as surface receptor, the problem arises whether yeast mitochondria possess equivalents of MOM19 and MOM72 or whether *N. crassa* has developed mechanisms for import of precursor proteins that are different from the mechanisms in yeast.

For this report, we analyzed the import pathway of the most abundant mitochondrial protein, the inner membrane protein AAC, in yeast and *N. crassa*. Through a combined biochemical and molecular approach we made the following observations. (*a*) The yeast protein MOM72 was identified as import receptor that appears to fulfill the same functions as *N. crassa* MOM72. We discuss evidence that suggests that ISP 42 acts at a later stage of the import pathway, probably at the level of membrane insertion of precursor proteins (Pfaller et al., 1988). (*b*) Mitochondria from a yeast mutant lacking MOM72 are impaired in binding and import of AAC. However, they are still able to perform a residual, but significant import of AAC. We found that MOM19 can substitute for MOM72 in importing AAC (although with lower efficiency) and that MOM72 and MOM19 apparently act in parallel in the transport of AAC. (*c*) A comparison of the amino-terminal sequences of MOM72 of *N. crassa* and yeast suggests that a positively charged sequence at the extreme amino terminus is not essential for targeting of precursor proteins to the mitochondrial outer membrane.

Materials and Methods

Biochemical Procedures

Published procedures were used for the following steps: isolation of mitochondria from *N. crassa* (Schleyer et al., 1982; Pfanner and Neupert, 1985) and *Saccharomyces cerevisiae* (growth on ethanol- and glycerol-containing medium at 30°C) (Daum et al., 1982; Hartl et al., 1987); production of antisera in rabbits and purification of IgGs by protein A-Sepharose chromatography (Söllner et al., 1989); synthesis of mitochondrial precursor pro-

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1. *Abbreviations used in this paper*: AAC, ADP/ATP carrier; GIP, general insertion protein.

teins in rabbit reticulocyte lysates and labeling with [^{35}S]methionine (Pfaller et al., 1988); preincubation of mitochondria with IgGs (Söllner et al., 1989); binding to and import of precursor proteins into mitochondria in a buffer containing 250 mM sucrose, 3% (wt/vol) BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH (pH 7.2) (Pfanner and Neupert, 1987; Pfanner et al., 1987*b*; Söllner et al., 1989, 1990); treatment of mitochondria with trypsin, proteinase K, or elastase (Pfanner and Neupert, 1987; Pfaller et al., 1988; Söllner et al., 1989); SDS-PAGE, transfer of proteins to nitrocellulose paper, and immunodecoration (Laemmli, 1970; Schleyer et al., 1982; Söllner et al., 1989).

For isolation of outer membranes of yeast mitochondria, mitochondria (4 mg/ml) were incubated in 5 mM KPi (pH 7.2) and 1 mM PMSF for 5 min on ice. The outer membranes were sheared off by 15 strokes in a Potter homogenizer. Sucrose was added to a final concentration of 45% (wt/wt). The sample was overlayered with a solution containing 30% (wt/wt) sucrose, 1 mM EDTA, 1 mM PMSF, and 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.2), followed by a similar solution containing 8% (wt/wt) sucrose. After centrifugation for 5 h at 240,000 *g* in a SW41 rotor (Beckman Instruments, Fullerton, CA) the outer membrane was recovered from the 8/30% interphase. The purity of the outer membrane was analyzed by immunodecoration with antibodies directed against proteins of various mitochondrial subcompartments, including porin (outer membrane), AAC (inner membrane), and hsp60 (matrix). The outer membrane fraction only reacted with antiserum directed against porin.

DNA Manipulations

A full-length cDNA-clone coding for *N. crassa* MOM72 was isolated from a λgt11 library of *N. crassa* cDNA (Schneider et al., 1990) by means of antibody-screening and subcloned into pGEM4 (Promega Biotec; Melton et al., 1984; Söllner et al., 1990). In vitro transcription/translation resulted in authentic MOM72 precursor protein (Söllner et al., 1990). The cDNA insert was sequenced according to the Exonuclease III method (Henikoff, 1984), by using internal restriction sites and by using MOM72-specific oligonucleotide primers. Both strands were sequenced overlapping at least three times.

The gene coding for yeast 70-kD protein/MOM72 was isolated from a yeast genomic library by screening with specific oligonucleotides (two 44-mers corresponding to nucleotide positions 70–113 and 1,688–1,731 of the published sequence [Hase et al., 1983]). DNA-sequencing using both specific primers revealed that correct clones were isolated. The gene was disrupted essentially as described (Riezman et al., 1983*b*; Rothstein, 1983): the *URA3* gene (which served as a selectable marker) was inserted into an internal BstE II site. The haploid yeast strain S150 (*leu 2-3, leu 2-112, his 3-Δ1, trp 1-289, ura 3-52*; gift of Dr. A. Haid, Universität München) was used. Southern-blot analysis revealed that the *URA3* gene was correctly inserted thereby disrupting the MOM72 gene.

Results

Identification of the Yeast Homologue of MOM72

For our attempts to identify the protein import receptor MOM72 of yeast mitochondria, we assumed the following characteristics of yeast MOM72: the protein should be exposed on the mitochondrial surface and possess a similar size as *N. crassa* MOM72; antibodies prepared against the protein should specifically inhibit the binding of AAC to the mitochondria. We produced a collection of polyclonal antisera each of them specifically directed against a mitochondrial outer membrane protein of the yeast *Saccharomyces cerevisiae*. Immunoglobulins G (IgGs) were prepared from the antisera and tested for a possible inhibitory effect on specific binding of AAC.

To prepare large amounts of antigens, we developed a rapid and efficient method for the isolation of mitochondrial outer membranes of *Saccharomyces cerevisiae*. In brief, isolated mitochondria were swollen as described (Riezman et al., 1983*a*); the outer membranes were sheared off in a potter homogenizer and separated from the residual mitochondrial membranes by flotation on a discontinuous sucrose gradient. The protein pattern of yeast mitochondrial outer membrane obtained by separation on SDS-polyacrylamide gels is shown in lane *1* of Fig. 1. The proteins were transferred to nitrocellulose, distinct bands were excised and used as antigens for production of rabbit antisera. IgGs prepared from the various antisera that were monospecific for outer membrane proteins were tested for an inhibitory effect on specific binding of AAC to isolated yeast mitochondria. We thereby found that IgGs against an outer membrane protein of ~70 kD selectively inhibited binding of AAC. As described below, this protein apparently represents the equivalent of *N. crassa* MOM72 and was consequently termed MOM72. Lane *2* of Fig. 1 shows that the antibodies against MOM72 are monospecific as they recognize a single band of yeast mitochondrial outer membranes.

For analysis of specific binding of AAC to the mitochondrial surface, the precursor of AAC was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and

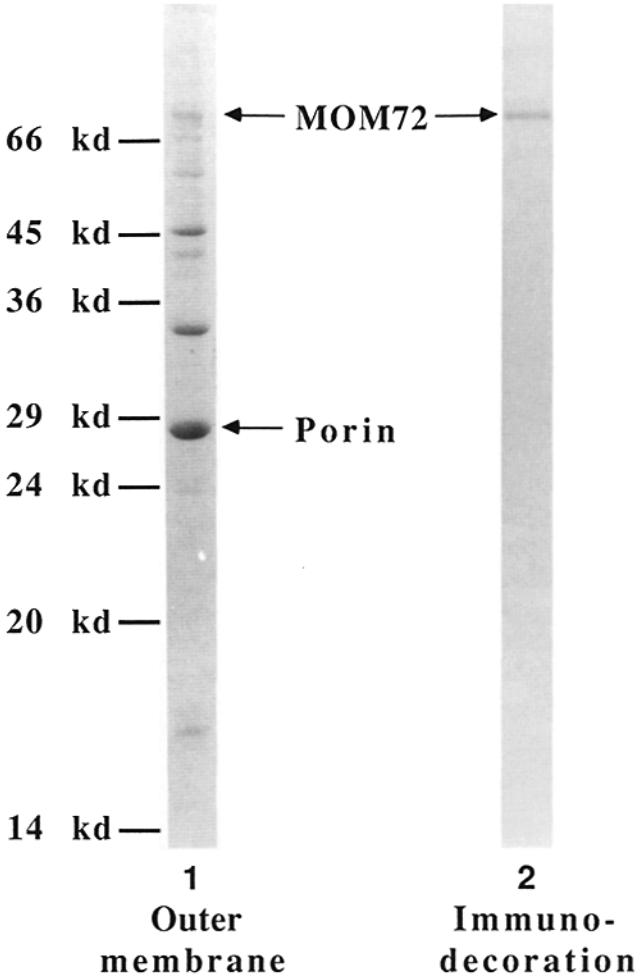

Figure 1. Identification of MOM72 in yeast mitochondrial outer membranes. Mitochondrial outer membranes were prepared as described in Materials and Methods. Lane *1* shows the protein pattern of the outer membrane (30 μg) after separation by SDS-PAGE and staining with Coomassie blue R-250. For lane *2*, 30 μg of outer membrane proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose paper, and immunodecorated with antibodies against yeast MOM72.

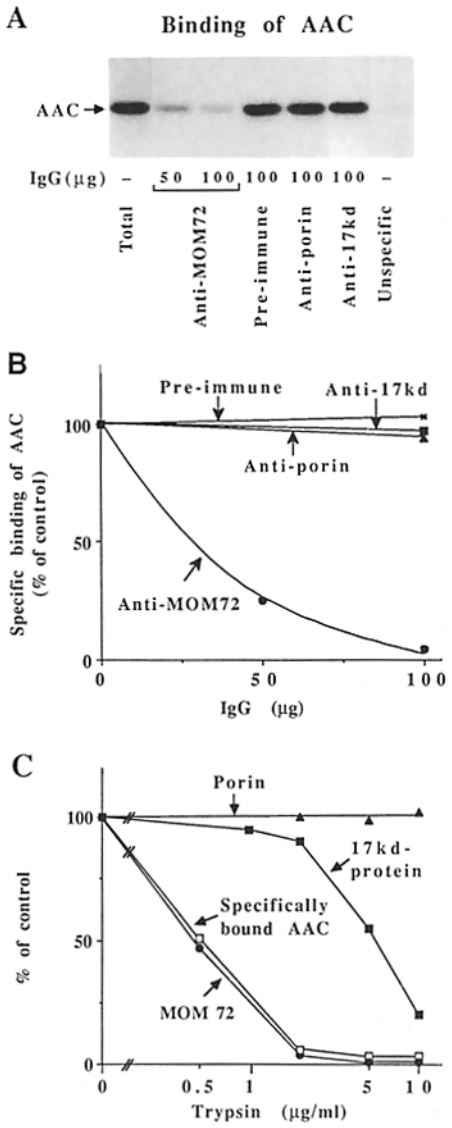

Figure 2. MOM72 is responsible for specific binding of the ADP/ATP carrier to yeast mitochondria. (*A* and *B*) Antibodies against yeast MOM72 inhibit specific binding of AAC. Isolated yeast mitochondria (10 μg of protein) were pre-incubated with IgGs prepared from preimmune serum or from antisera directed against yeast MOM72, porin, or a 17-kD outer membrane protein for 30 min at 0°C (Söllner et al., 1989). In a parallel sample, mitochondria were pre-treated with trypsin (20 μg/ml) as described (Pfanner and Neupert, 1987). The precursor of AAC was synthesized in rabbit reticulocyte lysate by coupled transcription/translation in the presence of [^{35}S]methionine. The reticulocyte lysate was depleted of endogenous ATP by preincubation with apyrase (5 U/ml) for 25 min at 25°C (Pfanner and Neupert, 1986). Mitochondria and reticulocyte lysate were incubated in the presence of 20 μM oligomycin (to inhibit the mitochondrial F_oF_1-ATPase) and 1 μM valinomycin (to dissipate the mitochondrial membrane potential) for 15 min at 25°C as described in Materials and Methods. The mitochondria were reisolated, and the samples were analyzed by SDS-PAGE, fluorography, and laser densitometry. To determine the amount of specific binding of AAC, the amount of nonspecific binding of AAC (mitochondria pretreated with 20 μg/ml of trypsin [Pfaller et al., 1988; Söllner et at., 1990]) was subtracted from the values obtained. In a control sample, we determined that >95% of the AAC molecules were sensitive to proteinase K (10 μg/ml), i.e., were located on the mitochondrial surface (Pfanner and Neupert, 1987; Pfanner et al., 1987*b*). (*C*) The presence of MOM72 correlates with the presence of binding sites for AAC. Mitochondria were pretreated with trypsin as indicated and incubated with reticulocyte lysate as described for *A* and *B*. The amount of specifically bound AAC was determined as described for *B*. The amounts of MOM72, porin, and 17-kD protein were determined by immunodecoration as described (Söllner et al., 1989, 1990).

incubated with isolated mitochondria at low levels of ATP and in the absence of a membrane potential $\Delta\Psi$ across the inner mitochondrial membrane (Fig. 2, *A* and *B*) (Pfanner et al., 1987*b*; Pfaller et al., 1988; Söllner et al., 1988, 1990). When IgGs directed against MOM72 were bound to the mitochondria, the binding of AAC was strongly inhibited (Fig. 2, *A* and *B*). The residual binding of 10–20% corresponds to the unspecific (nonproductive) binding of AAC that is also observed after degradation of the surface receptors by a treatment of mitochondria with trypsin (Fig. 2, *A*, *last lane*) (Pfaller et al., 1988; Söllner et al., 1990). Control antibodies did not inhibit binding of AAC, including IgGs directed against the major outer membrane protein porin, IgGs directed against a 17-kD protein that is exposed on the outer membrane surface, and IgGs prepared from preimmune sera (Fig. 2, *A* and *B*). To obtain independent evidence that MOM72 mediates the specific binding of AAC, mitochondria were treated with various low concentrations of trypsin under conditions that preserve the intactness of the outer membrane barrier (Hartl et al., 1989). The degradation of MOM72 correlated well with the degradation of the specific binding sites for AAC (Fig. 2 *C*).

Primary Structure of MOM72

When comparing the protein pattern of our preparation of yeast mitochondrial outer membranes (Fig. 1, lane *I*) with that of Riezman et al. (1983*a*), MOM72 appeared to be very similar in size and abundance to a 70-kD protein that had been used as a tool to study the biogenesis of mitochondrial outer membrane proteins (Riezman et al., 1983*b*; Hase et al., 1984; Hurt et al., 1985*a*; Nakai et al., 1989). The primary structure of this protein had been determined (Hase et al., 1983). If this 70-kD protein was the equivalent of *N. crassa* MOM72, one would expect sequence homology between these proteins.

We determined the nucleotide sequence of a full-length cDNA clone encoding *N. crassa* MOM72 (Söllner et al., 1990). The derived amino acid sequence (Fig. 3) predicts a protein of 619 amino acid residues and a molecular weight of 68,839 in good agreement with the apparent molecular weight of ~72,000 (Söllner et al., 1990). MOM72 is an essentially hydrophilic protein, but contains a putative membrane-spanning sequence in the amino-terminal portion (Fig. 3). MOM72 most probably exposes a large hydrophilic domain to the cytosol that can be cleaved off by protease treatment of intact mitochondria (Söllner et al., 1990; T. Söllner, N. Pfanner, and W. Neupert, unpublished results). *N. crassa* MOM72 shares 33.0% amino acid identity with yeast 70-kD protein/MOM72. Including isofunctional amino acid exchanges, the proteins exhibit 45.9% similarity (Fig. 4).

Studies of Hurt et al. (1985*a*) had suggested that the extreme amino-terminus of yeast MOM72, in particular the presence of positively charged amino acid residues, served

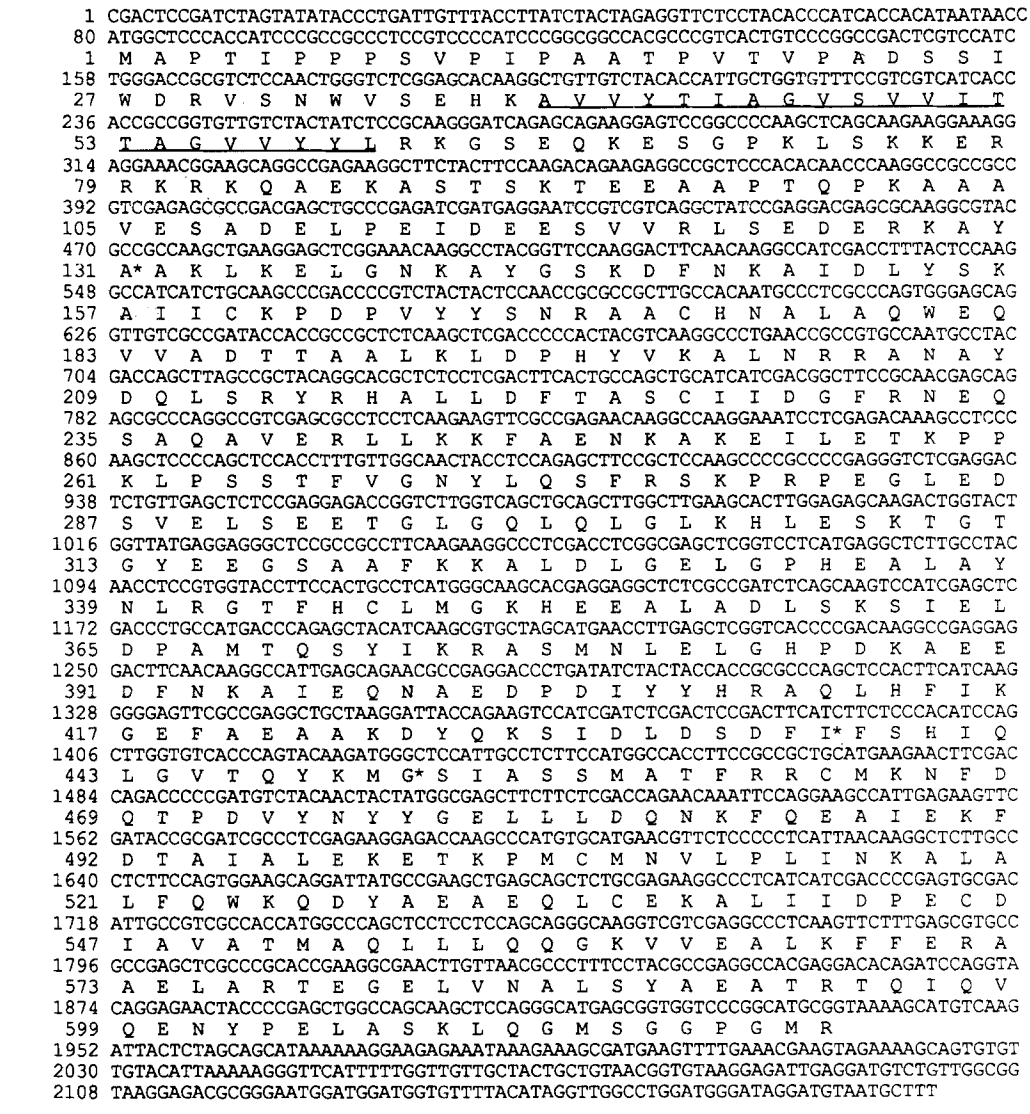

Figure 3. Nucleotide sequence of the cDNA coding for *N. crassa* MOM72 and deduced amino acid sequence. Amino acids are given in the one-letter code. A protein of 619 amino acid residues and a molecular weight of 68,839 is predicted. A putative membrane spanning segment (amino acid residues 39–60) is underlined. Stars indicate the first amino acid residue of sequences with a putative homology to the tetratricopeptide (TPR) motif (Sikorski et al., 1990). The nucleotide sequence data are available from EMBL GenBank/DDBJ under accession number X53735.

as mitochondrial targeting signal (Hurt and van Loon, 1986). We have previously shown that the precursor of *N. crassa* MOM72 used the import receptor MOM19 as do the precursor proteins carrying a positively charged presequence (Söllner et al., 1989, 1990). However, the positively charged amino acid residues in the extreme amino terminus of yeast MOM72 are not conserved in *N. crassa* MOM72 (Fig. 4). The implication on the role of positive charges in mitochondrial protein import will be discussed below.

MOM72 of *N. crassa* (Fig. 3) and yeast (not shown) contain a repeating 34-amino acid motif, termed the tetratricopeptide (TPR) motif, that was recently discovered by Sikorski et al. (1990). The TPR motif is characterized by a loosely defined consensus sequence (AEAWFG*L*G*H*IYEK LGDLEKALDAFQKALLLDPNN; underlined amino acid residues are found in at least 40% of the TPR sequences) and a predicted predominantly helical conformation. The TPR motif was found in proteins that are encoded by genes required for mitosis or RNA synthesis. The TPR gene family includes *CDC23*, *CDC16*, *nuc2*$^{+}$, *bimA*, *SSN6*, and *SKI3* (Sikorski et al., 1990). So far, it can only be speculated on the relation between these proteins. In addition to a possible evolutionary relationship, the presence of the TPR motif might for example indicate a putative role of MOM72 in interaction with the cytoskeleton (Söllner et al., 1990).

Mitochondria Lacking MOM72 Are Impaired in Import of AAC

In attempting to identify the function of the yeast 70-kD protein/MOM72, Riezman et al. (1983*b*) had constructed a yeast mutant lacking the protein. The mutant cells grew slowly on nonfermentable carbon sources at 23°C and not at all at 37°C, but grew nearly like wild-type cells on glucose-containing rich medium (we now found, however, that at elevated temperature [35–37°C] yeast cells lacking MOM72 are impaired also in growth on glucose-containing medium [see legend to Fig. 5]). No function could be assigned to this outer membrane protein so far.

As we now know that MOM72 functions as import receptor for AAC, we wondered why cells lacking MOM72 were still viable and we constructed a yeast mutant lacking MOM72 (Fig. 5 *A*) to characterize the role of MOM72 in protein im-

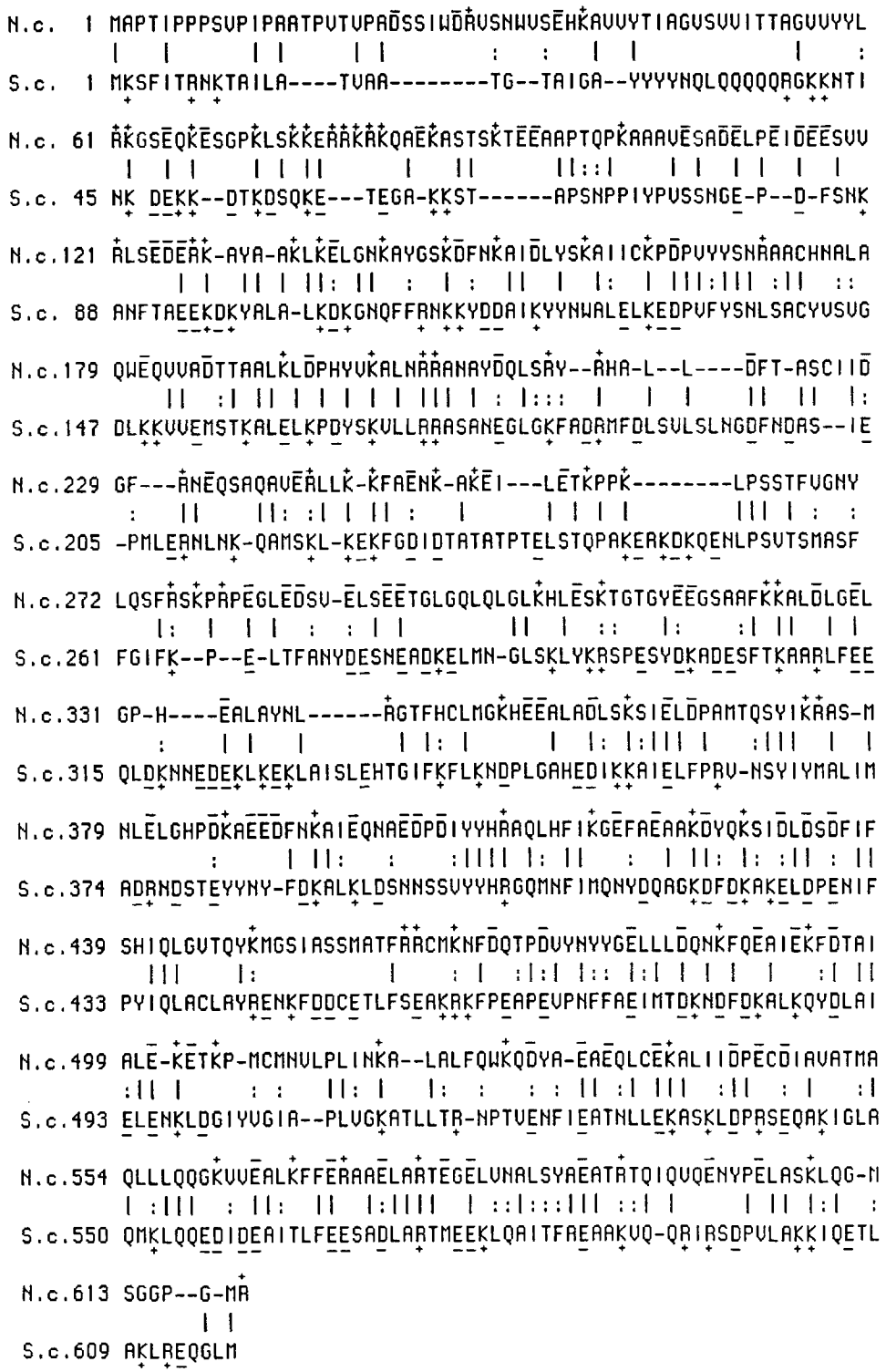

Figure 4. Comparison of MOM72 from *N. crassa* (*N.c.*) and yeast (*S.c.*). The predicted amino acid sequence of *N. crassa* MOM72 was compared with that of *Saccharomyces cerevisiae* MOM72/70-kD protein (Hase et al., 1983). Vertical lines indicate identical amino acid residues, and dots indicate isofunctional amino acid residues.

port in more detail. Fig. 5 *B* demonstrates that a deletion of MOM72 leads to the identical phenotype as the blocking of MOM72 with specific antibodies (Fig. 2; Söllner et al., 1990): AAC did not bind in a productive manner to mitochondria isolated from the mutant cells. To analyze the specificity of MOM72 we studied the import of various precursor proteins into the mutant mitochondria under conditions where the amount of imported protein depended on the time of import in a linear fashion. Precursor proteins synthesized in reticulocyte lysates were incubated with the mitochondria for 10 min at 25°C and the amount of imported proteins was assessed by monitoring their proteolytic processing and/or their protection against externally added proteinase K (Pfanner and Neupert, 1987; Pfaller et al., 1988; Söllner et al., 1989, 1990). As expected, the import of AAC into the mutant mitochondria was inhibited. Surprisingly, however, a re-

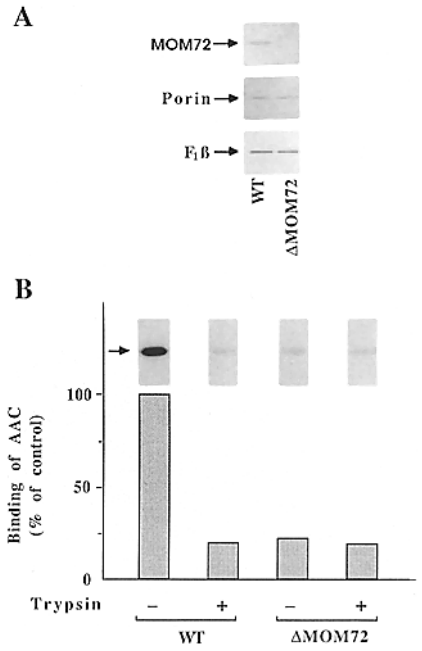

Figure 5. Mitochondria of a yeast mutant lacking MOM72 are defective in specific binding of the ADP/ATP carrier. (*A*) A yeast mutant lacking MOM72. Mitochondria (30 μg of protein) of wild-type yeast (WT) and of the yeast mutant (ΔMOM72) in which the gene for MOM72 was disrupted (see Materials and Methods; growth of the yeast cells on ethanol- and glycerol-containing medium) were resolved by SDS-PAGE. After transfer to nitrocellulose paper, immunodecoration with antisera against MOM72, porin, and F_1-ATPase subunit β ($F_1β$) was performed. Anti-MOM72 antiserum did not react with a protein band of the mutant mitochondria. The mutant yeast cells were impaired in growth on non-fermentable glycerol-containing medium with a doubling time of 5 h at 30°C as compared with 2.5 h of wild-type cells. The mutant cells did not grow at all on non-fermentable medium at 35°C. On fermentable glucose-containing rich-medium the growth rates of the mutant cells were close to wild-type cells at 25°C and at 30°C; at 35°C the growth rates were strongly decreased. (*B*) Specific binding of AAC. The experiment was performed as described in the legend of Fig. 2 C. Mitochondria from wild-type (*WT*) and mutant (*ΔMOM72*) yeast were pre-treated with trypsin (20 μg/ml) or left untreated, and precursor of AAC was bound to the mitochondria. The means of five experiments are given (with a standard error of the mean <3% in each case). The arrow marks AAC on fluorographs.

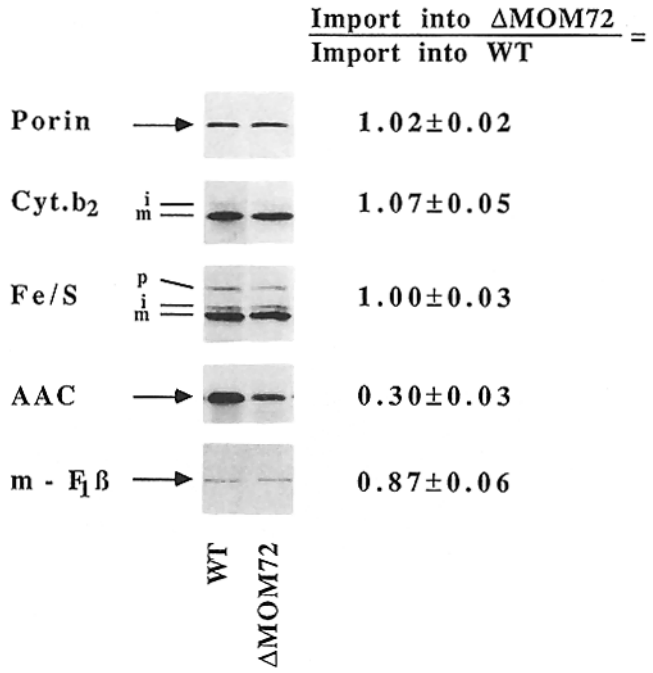

Figure 6. Import of various precursor proteins into mitochondria from MOM72-deficient yeast. Mitochondria (10 μg of protein) from wild-type (*WT*) and mutant (*ΔMOM72*) yeast were incubated with reticulocyte lysate containing radiolabeled precursor proteins in the presence of 1 mM ATP and 2 mM NADH for 10 min at 25°C as described in Materials and Methods. The samples were treated with proteinase K as described (Pfanner and Neupert, 1987; Pfaller et al., 1988). The mitochondria were reisolated. Analysis was performed by SDS-PAGE, fluorography, and laser densitometry. For quantitation, the means of five experiments were used (± SEM). In control samples, the import of AAC into wild-type mitochondria pretreated with trypsin (20 μg/ml) was <10% of the import into untreated mitochondria. *Cyt. b_2*, cytochrome b_2; *Fe/S*, Fe/S protein of the bc_1-complex; *$F_1β$*, F_1-ATPase subunit β; *p-*, *i-*, *m-*, precursor-, intermediate-, and matured-sized forms, respectively.

sidual import of AAC was still possible in these mitochondria (Fig. 6), a finding that apparently explains the viability of the mutant cells. Import of porin, cytochrome b_2, Fe/S protein of the bc_1-complex, and of F_1-ATPase subunit β ($F_1β$) occurred at rates that were close to the import rates observed with wild-type mitochondria (Fig. 6). It remains possible that a (minor) fraction of the import of precursor proteins such as $F_1β$ depends on the presence of MOM72, although it is evident that the bulk of $F_1β$-import does not require MOM72 (Fig. 6; Söllner, T., H. F. Steger, N. Pfanner and W. Neupert, unpublished data). By pulse–chase experiments in intact yeast cells we found a two- to threefold decrease in the import rates of several precursor proteins, including AAC, $F_1β$, and cytochrome b_2, in vivo (data not shown). In a first view, this result may indicate that the import of various mitochondrial precursor proteins strongly depends on the presence of MOM72, in contrast to the results obtained in vitro. It has to be emphasized, however, that the conclusions which can be drawn from an in vivo analysis of mitochondrial import mutants are limited due to possible indirect effects on mitochondrial functions which virtually cannot be controlled in intact cells (summarized in Pfanner et al., 1988*b*; Hartl et al., 1989; Pfanner, N., and W. Neupert, unpublished). The in vitro studies described above clearly suggest that the major fraction of the import of presequence-carrying precursor proteins does not require MOM72.

MOM19 Can Mediate Import of AAC

A pretreatment of yeast mitochondria with low concentrations of trypsin led to a stronger inhibition (>90%) of AAC import than the deletion of MOM72 (Fig. 6). It is generally assumed that by this mild trypsin treatment the mitochondrial import receptors are degraded (Attardi and Schatz, 1988; Hartl et al., 1989). It has to be emphasized that the 42-kD protein (ISP 42 [Vestweber et al., 1989]) is obviously

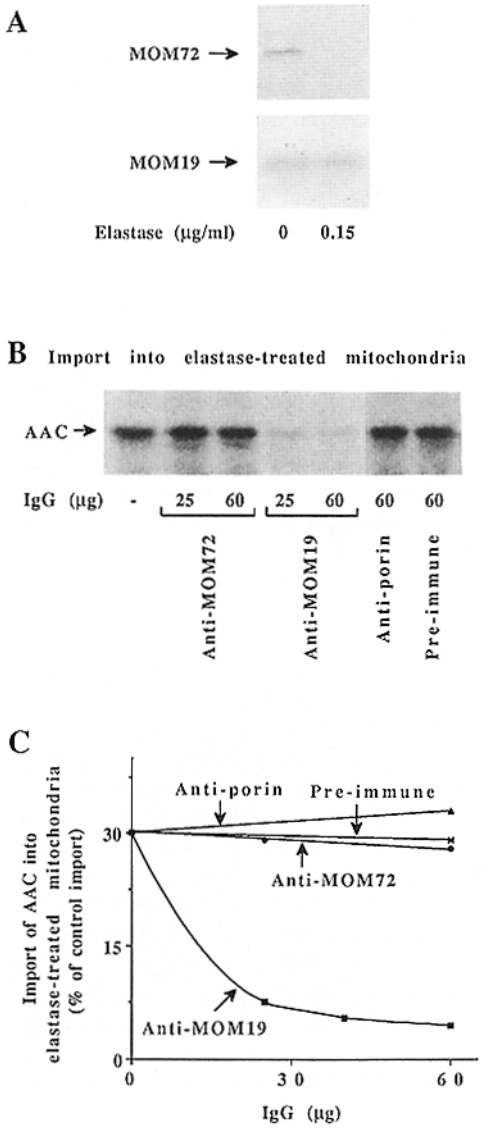

Figure 7. *N. crassa* mitochondria lacking MOM72 are able to import the ADP/ATP carrier via MOM19. (*A*) Selective degradation of MOM72 by elastase. *N. crassa* mitochondria (50 μg of protein) were treated with elastase (0.15 μg/ml [Pfaller et al., 1988; Söllner et al., 1989]) or left untreated. Mitochondrial proteins were separated by SDS-PAGE, transferred to nitrocellulose paper, and immunodecorated with antisera directed against *N. crassa* MOM72 and MOM19. (*B* and *C*) Antibodies against MOM19 block import of ADP/ATP carrier into MOM72-deficient mitochondria. Mitochondria (10 μg of protein) were pretreated with elastase (0.15 μg/ml) and incubated with IgGs as indicated (Söllner et al., 1989, 1990). Mitochondria were isolated and incubated with reticulocyte lysate containing radiolabeled precursor of AAC in the presence of 8 mM potassium ascorbate and 0.2 mM *N*, *N*, *N'*, *N'*-tetramethylphenylenediamine (TMPD) for 7 min at 25°C as described in Materials and Methods. The samples were treated with proteinase K as described (Pfanner and Neupert, 1987; Pfaller et al., 1988). Mitochondria were reisolated and analyzed by SDS-PAGE, fluorography, and laser densitometry.

not degraded under the conditions applied (Ohba and Schatz, 1987). These results suggest that an unknown surface component of yeast mitochondria is able to mediate import of AAC when MOM72 is missing. An experimental analysis, however, is difficult as no other import receptor of yeast mitochondria has been identified, and in particular the equivalent to the receptor MOM19 of *N. crassa* mitochondria (Söllner et al., 1989) is not known.

To overcome this problem, we applied biochemical means to selectively degrade MOM72 in *N. crassa* mitochondria without affecting MOM19. A pretreatment of *N. crassa* mitochondria with very low concentrations of elastase led to degradation of MOM72, whereas MOM19 remained intact (Fig. 7 *A*). Other outer membrane proteins analyzed were also not affected by this treatment with elastase (data not shown). The reduction of import of AAC by the treatment of *N. crassa* mitochondria with elastase (Fig. 7, *B* and *C*) was comparable to that observed after disruption of the gene for MOM72 in yeast (Fig. 6). This residual import of AAC was strongly inhibited by a prebinding of antibodies against MOM19 to the mitochondria whilst control antibodies, including those directed against MOM72, had no effect (Fig. 7, *B* and *C*). Thus, MOM19 can function as import receptor for AAC when MOM72 is missing.

What is the function of MOM19 in AAC import when MOM72 is present? We found that antibodies against MOM72 did not completely block the import of AAC into intact mitochondria (Fig. 8 *A*; Söllner et al., 1990). In fact, the three methods for inactivation of MOM72, i.e., binding of antibodies, disruption of the gene (Fig. 6), and treatment of mitochondria with elastase (Fig. 7), led to practically the same inhibition of AAC import (70–75% inhibition). On the other hand, antibodies against MOM19 reproducibly exhibited a weak inhibitory effect on AAC import (Fig. 8 *A*) of previously unclear significance (Söllner et al., 1989, 1990). This inhibition of ~25% correlates well with the amount of residual import into mitochondria lacking MOM72. In view of the results reported above, the inhibition of AAC import into intact mitochondria by anti-MOM19 antibodies indicates that the rate of AAC import via MOM19 does not depend on the presence or absence of MOM72. Moreover, we previously demonstrated that the rate of AAC import via MOM72 is not influenced by antibodies against MOM19: both the high-affinity binding of AAC molecules to the mitochondrial surface (i.e., binding to MOM72) and the completion of AAC import from MOM72 into the inner membrane, are not inhibited by anti-MOM19 antibodies (Söllner et al., 1990). As the inactivation of either receptor does not inhibit the import of AAC via the other receptor, MOM72 and MOM19 obviously do not act sequentially, but in parallel to mediate import of AAC into mitochondria. We reasoned that a combination of antibodies against MOM72 and MOM19 should lead to a practically complete block of AAC import. Fig. 8 *A* shows that this is indeed the case. Various combinations of control antibodies with antibodies against MOM72 or MOM19 did not reveal any effect (Fig. 8 *A*; and data not shown).

To confirm that both MOM72 and MOM19 can direct AAC onto the correct import pathway, we made use of a translocation intermediate of AAC, namely precursor inserted into the GIP (general insertion protein) site in the outer membrane (Pfanner and Neupert, 1987; Pfanner et al., 1987*b*, 1988*a*; Pfaller et al., 1988; Söllner et al., 1988). Generation of the GIP intermediate of AAC is performed by

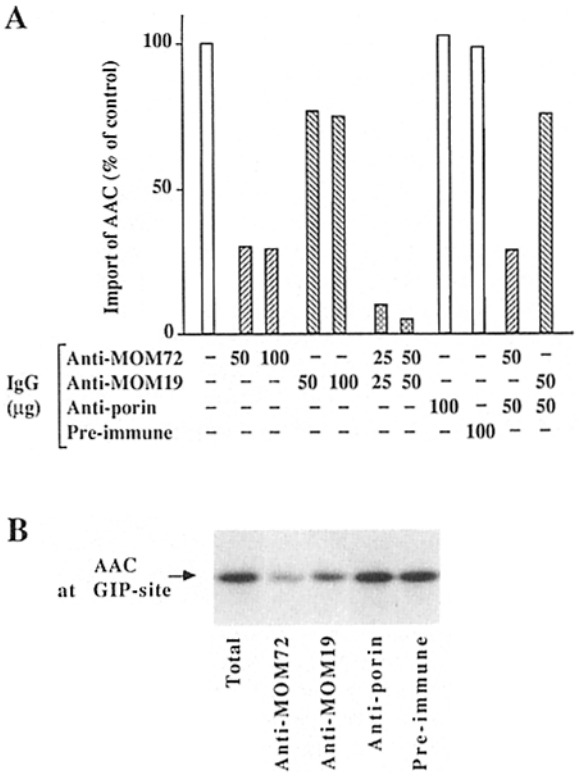

Figure 8. Role of MOM19 in import of the ADP/ATP carrier into intact *N. crassa* mitochondria. (*A*) Mitochondria loaded with antibodies against MOM72 and MOM19 are completely blocked in import of AAC. The experiment was performed as described in the legend of Fig. 7, *B* and *C*, except that IgGs were prebound to untreated mitochondria. The values given are the means of five or more experiments (with a standard error of the means <3%). (*B*) Both MOM72 and MOM19 direct AAC to the GIP site. Mitochondria (10 μg of protein) were preincubated with IgGs (60 μg) as indicated (Söllner et al., 1990). Mitochondria were reisolated and incubated with reticulocyte lysate containing radiolabeled precursor of AAC in the presence of 1 μM valinomycin for 20 min at 25°C as described in Materials and Methods and treated with proteinase K (10 μg/ml) (Pfanner and Neupert, 1987; Pfanner et al., 1987*b*).

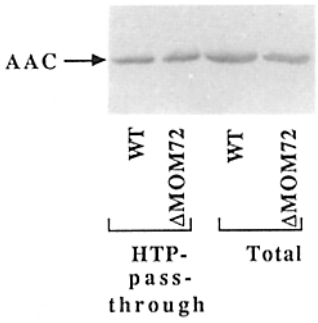

Figure 9. Levels of ADP/ATP carrier in MOM72-deficient yeast cells *in vivo*. For analysis of total AAC levels, an experiment was performed as described in the legend of Fig. 5 *A* and immunodecoration was carried out with antiserum directed against AAC. For analysis of assembled AAC, binding of carboxyatractyloside and passage of the lysed mitochondria over hydroxylapatite were performed as described (Klingenberg et al., 1979; Schleyer and Neupert, 1984; Knirsch et al., 1989). The pass-through of the hydroxylapatite (HTP) columns (containing correctly assembled AAC) was subjected to SDS-PAGE, transferred to nitrocellulose paper and immunodecorated with antiserum against AAC. The levels of AAC in the mutant compared to wild-type yeast did not change when the cells were grown on glucose-containing rich medium instead of nonfermentable glycerol-containing medium.

incubation of precursor with mitochondria in the presence of ATP, but in the absence of the membrane potential across the inner membrane (by a subsequent treatment with proteinase K, AAC molecules still located on the surface of the outer membrane are removed). Both antibodies against MOM72 and antibodies against MOM19 inhibited the insertion of AAC into the outer mitochondrial membrane (Fig. 8 *B*) to about the same extent as they inhibited the import of AAC. This fits well to the recent observation made with other precursor proteins using MOM19, namely that the precursors are transferred from MOM19 to the GIP site in the outer membrane (Söllner et al., 1989). Moreover, our finding that the transport of AAC from MOM72 via GIP into the inner membrane is not affected by anti-MOM19 antibodies (Söllner et al., 1990) suggests that MOM72 and MOM19 also act in parallel when the transfer of AAC into the GIP site is analyzed.

In summary, by use of three different methods, we conclude that MOM72 functions as the major receptor for the precursor of AAC, responsible for ~70–75% of the total import of AAC into mitochondria. MOM19 seems to be responsible for ~25% of AAC import. Both receptors apparently function independently of each other in importing AAC. The two import pathways of AAC converge at the common membrane insertion site (GIP) in the outer mitochondrial membrane. As many examples have shown that relatively low import rates of precursor proteins can lead to nearly normal mitochondrial levels of the respective mature proteins in vivo (Nelson and Schatz, 1979; Hurt et al., 1985*b*, 1986; Pfanner et al., 1988*b*; Thompson and McAlister-Henn, 1989), one would expect that yeast cells lacking MOM72 should accumulate nearly wild-type levels of AAC in their mitochondria. In Fig. 9 we compared the total amounts of immunoreactive AAC in wild-type and mutant mitochondria. Moreover, we used a specific assay for assembled dimeric AAC, namely binding of the inhibitor carboxyatractyloside and passage over hydroxylapatite (Klingenberg et al., 1979; Schleyer and Neupert, 1984; Knirsch et al., 1989). We found that the mutant mitochondria contained ~70–90% of the wild-type levels of correctly assembled AAC. We suggest that the import of AAC into the mutant yeast mitochondria occurs via the putative yeast equivalent of MOM19 in vivo and in vitro.

Discussion

Import Receptors of Yeast Mitochondria

We have identified an import receptor for the ADP/ATP carrier (AAC) in mitochondrial outer membranes of the yeast *Saccharomyces cerevisiae* with comparable function and a homologous primary structure to the receptor MOM72 of *N. crassa* mitochondria. While the import receptor of *N. crassa* mitochondria was characterized essentially through the inhibitory effects of specific antibodies (Söllner et al., 1990), the function of yeast MOM72 could be analyzed by both genetic and biochemical procedures. Besides confirming the results obtained with antibodies, the genetic approach led us to a second import receptor for AAC (see below). The

identification of MOM72 in yeast should now allow a detailed mutational analysis of structure and function of the receptor molecule. MOM72 contains the repeating tetratricopeptide (TPR) motif that was found in a number of proteins required for mitosis or RNA synthesis (Sikorski et al., 1990). This homology suggests a possible evolutionary relationship among these proteins (Sikorski et al., 1990) and might give hints for additional functions of MOM72. For example, several proteins encoded by cell division cycle genes may interact with the cytoskeleton and it is thus conceivable that MOM72 might play a role in the interaction of mitochondria with the cytoskeleton in agreement with our previous speculation (Söllner et al., 1990).

As yeast mitochondria possess the import receptor MOM72, the question arises what the function of ISP 42, the 42-kD outer membrane protein (Vestweber et al., 1989), may be. We suggest that ISP 42 does not act as a surface receptor, but that it may be related to the GIP (Pfaller et al., 1988; Pfanner et al., 1988*a*). First, yeast mitochondria could be pretreated with relatively high concentrations of trypsin and ISP 42 was not degraded: antibodies could still bind to ISP 42 and inhibit protein import (Ohba and Schatz, 1987). In contrast, the surface receptors were digested at low concentrations of trypsin (Riezman et al., 1983*c*). Second, a fraction of ISP 42 could be cross-linked to a precursor protein that was trapped in mitochondrial contact sites (Vestweber et al., 1989), i.e., was already partially inserted into the mitochondrial membranes. These properties would fit very well to that of the proposed general site for insertion of precursor proteins into the outer mitochondrial membrane (GIP) (Pfanner and Neupert, 1987; Pfaller et al., 1988).

Two Mitochondrial Import Receptors for the ADP/ATP Carrier

We obtained the unexpected result that MOM19, the receptor for most mitochondrial precursor proteins (Söllner et al., 1989), is also able to import AAC into mitochondria. With all likelihood, MOM19 not only functions as ACC-receptor when MOM72 is missing, but also in parallel to MOM72 in intact mitochondria. Both MOM72 and MOM19 appear to act independently of each other in recognizing the precursor of AAC and transferring it to the GIP in the outer membrane.

On the basis of the endosymbiont hypothesis of mitochondrial origin, we previously suggested that MOM19 served as import receptor for the proteins that were originally synthesized in the organelle and are now expressed in the cytosol (Söllner et al., 1989, 1990). AAC that was probably introduced by the eukaryotic cell (Klingenberg, 1985) uses MOM72 as main receptor. MOM72 allows a more efficient specific binding of the precursor to the mitochondrial surface than does MOM19 for all the precursor proteins studied (Pfanner et al., 1987*a*; Hartl et al., 1989; Söllner et al., 1989, 1990), consistent with the finding that the high-affinity binding of AAC to the mitochondrial surface is largely due to the interaction with MOM72 (Figs. 2 and 5; Söllner et al., 1990). We now extend our model as the precursor of AAC can also interact with MOM19 albeit with clearly lower efficiency as with MOM72. AAC might have originally used MOM19 as its only receptor and then the specific and very efficient interaction with MOM72 might have been developed. Additionally (or alternatively) the presence of MOM19 may serve as a safety system to ensure import of AAC when MOM72 is defective. This is evidenced by the quite considerable levels of AAC in mitochondria of yeast cells lacking MOM72. Furthermore, while it is evident that MOM19 functions as main receptor for the import of presequence-carrying precursor proteins (Söllner et al., 1989), it is possible that a (small) fraction of the import of precursors such as $F_1\beta$ may depend on MOM72 (Fig. 6; Söllner, T., H. F. Steger, N. Pfanner, and W. Neupert, unpublished data). Both MOM72 and MOM19 might thus exhibit an overlapping specificity for some precursor proteins, allowing each receptor to function as back up receptor for its counterpart.

Several laboratories have performed an extensive search for yeast mutants that are defective in mitochondrial protein import (Yaffe and Schatz, 1984; Yaffe et al., 1985; Pollock et al., 1988; Yang et al., 1988; Jensen and Yaffe, 1988; Cheng et al., 1989). So far, however, only soluble components of the mitochondrial matrix were identified by these screening procedures. The phenotype of the yeast mutant lacking MOM72, i.e., import of AAC via a second receptor, suggests that it is virtually impossible to select mutants for an AAC receptor by the usual screening procedures. Riezman et al. (1983*b*) had in fact constructed a yeast mutant lacking MOM72 without obtaining evidence for its function in import of AAC. The existence of parallel pathways as in the case of import of AAC underscores the necessity of strictly linear assay systems (that are often only possible in vitro) and the importance of defined translocation intermediates for the analysis of protein sorting.

Targeting Signals of Mitochondrial Precursor Proteins

A comparison of the primary structure of MOM72 in *N. crassa* and yeast showed that both proteins possess a putative membrane-spanning sequence at a similar position, in the amino-terminal portion, suggesting that both proteins expose a large hydrophilic domain containing the carboxy terminus to the cytosol. There is, however, an important difference between the two homologous proteins. The positively charged sequence that is present in the extreme amino terminus of yeast MOM72 (Hase et al., 1983) is not found in *N. crassa* MOM72. These positively charged amino acid residues were implicated to play an essential role in the targeting of the precursor of MOM72 to mitochondria (Hurt et al., 1985*a*; Hurt and van Loon, 1986; Nakai et al., 1989). Since the precursor of MOM72 uses MOM19 as import receptor (Söllner et al., 1990) like the numerous precursor proteins with a positively charged presequence, the possibility arises that a positively charged sequence (at the extreme amino terminus) is not essential for interaction of precursors with MOM19. This assumption is supported by the observation that porin, another outer membrane protein using MOM19 (Söllner et al., 1989), does also not contain a typical positively charged sequence (Mihara and Sato, 1985; Kleene et al., 1987). Moreover, nonmitochondrial targeting signals that bypass mitochondrial surface receptors on their (inefficient) way to the inner membrane are characterized by the presence of positive charges (discussed in Pfanner et al., 1988*b*). We speculate that positively charged amino acid residues in targeting signals are of particular importance for responding to the membrane potential $\Delta\Psi$ across the inner membrane (Pfanner and Neupert, 1985), while their role in the actual targeting process remains to be further analyzed.

We thank Drs. G. Arnold, R. Mertz, and A. Oswald (MPI, Martinsreid) for providing oligonucleotides and Dr. A. Haid for providing the yeast strain S150. The presence of the TPR motif in MOM72 was discovered by Dr. M. S. Boguski; we are grateful to Drs. V. Hines and G. Schatz for drawing our attention to this. We thank Ulrike Hanemann for expert technical assistance.

This work was supported by the Sonderforschungsbereich 184 (project B1), the Genzentrum München and the Fonds der Chemischen Industrie.

Received for publication 22 June 1990 and in revised form 25 July 1990.

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