Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins

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The mitochondrial import receptors MOM19 and MOM72 form a complex with two other proteins of the mitochondrial outer membrane, MOM38 and MOM22. This receptor complex is involved in recognition, membrane insertion and translocation of precursor proteins with MOM38 constituting (at least part of) the general insertion site GIP.

TRANSLOCATION of precursor proteins into and across organelle membranes is an essential step in the biogenesis of a number of cell organelles, such as the endoplasmic reticulum, mitochondria, chloroplasts and peroxisomes[1]. Intriguing questions are how the cytosolically synthesized precursor proteins are targeted to the correct organelle and how the precursor polypeptides are transferred across the membrane barriers.

By analysing translocation intermediates, the import of precursor proteins into mitochondria has been dissected into a series of steps[2-4], including the specific binding of precursors to receptors on the mitochondrial surface, the insertion of precursors into the outer mitochondrial membrane at a general insertion site, GIP, and the further translocation of precursors through contact sites between both mitochondrial membranes. Several of the components mediating the transfer of precursor proteins have been identified. Cytosolic hsp70 heat-shock proteins are involved in preventing misfolding of the polypeptide chains[5-8]. The import of most precursor proteins occurs by way of the general import receptor MOM19, a mitochondrial outer membrane protein of relative molecular mass 19,000 (M_r 19K) in *Neurospora crassa*[9,10]. MOM72, a second surface receptor identified in *N. crassa* and in yeast mitochondria, is important for the import of ADP/ATP carrier, the most abundant mitochondrial membrane protein[10,11]. A 42K protein of the yeast mitochondrial outer membrane seems to interact with precursor proteins, although its exact function is unknown[12]. Moreover, several proteins of the mitochondrial matrix have been identified as components of the protein import machinery, including the heat-shock proteins mitochondrial hsp70 (ref. 13) and hsp60 (refs 14, 15), the mitochondrial processing peptidase MPP and the processing enhancing protein PEP[16,17].

Little is known about the components mediating insertion and membrane translocation of mitochondrial precursor proteins. Both receptors, MOM19 and MOM72, seem to transfer the precursor proteins to a common insertion site in the outer membrane, termed the general insertion protein (GIP)[18,19]. An isolated mitochondrion contains roughly 200–400 GIP sites[18] (for comparison, one mitochondrion contains a similar number of receptor sites, yet roughly 10 times the number of translocation contact sites[9,11,20,21]). How are the precursors transferred from the receptors to GIP and what is the structural equivalent of GIP? In this report, we identify a high-molecular-weight complex in the mitochondrial outer member that contains both receptors and two additional components. One of them, MOM38, apparently represents GIP or at least a part of it. Thus, the receptors directly interact with GIP and thereby donate the precursors to this membrane insertion site. Furthermore, we present evidence that this 'receptor complex' is also involved in the translocation of precursor proteins through mitochondrial contact sites.

Identification of the receptor complex

To investigate possible interactions of the mitochondrial import receptors with other outer membrane proteins, isolated *N. crassa* mitochondria were lysed with the mild detergent digitonin and analysed by gel filtration. MOM19 fractionated in a range expected for molecules of about 400–600 K (Fig. 1*a*). A considerable amount of MOM72 also appeared in those fractions. Although an exact sizing is evidently impossible as a result of the presence of detergent, this finding implied that the receptors may not be present as monomers, but rather in oligomeric forms.

To purify putative protein complexes, we made use of specific antibodies directed again MOM19 (ref. 9). *N. crassa* cells were grown in the presence of [^{35}S]sulphate and isolated mitochondria lysed with digitonin. Immunoprecipitation was then performed with anti-MOM19 antibodies prebound to protein A-Sepharose and the precipitates were analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Three protein bands of apparent M_rs 22K, 38K and 72K were coprecipitated with MOM19 (Fig. 1*b*, lane 1). The 38K protein and part of the 72K and 22K proteins also cofractionated with MOM19 on gel filtration (Fig. 1*a*). Controls show the specificity of the coprecipitation. None of the four components was precipitated with pre-immune serum obtained from the rabbit before immunization with MOM19 (Fig. 1*b*, lane 3). After degradation of MOM19 by pretreating intact mitochondria with low concentrations of trypsin (leading to degradation of a few mitochondrial surface proteins[9,18,22]), none of the proteins was coprecipitated (Fig. 1*b*, lane 2). As MOM38 is not degraded by this treatment with trypsin (see Fig. 6*a*), coprecipitation of MOM38 apparently results from its association with a mitochondrial surface component and not from unspecific precipitation. Lanes 9–12 of Fig. 1*b* show the total amount of mitochondrial proteins (for each lane, one tenth of the amount of mitochondria used for one immunoprecipitation was taken), demonstrating that the immunoprecipitated proteins represent a selective and very minor fraction of mitochondrial proteins, in agreement with the low abundance of these four outer membrane proteins (see below). In view of the large number of mitochondrial proteins that are 10- to 100-fold more abundant, an unspecific coprecipitation of these four outer membrane proteins is extremely unlikely. In the presence of Triton X-100 and 300 mM NaCl (standard conditions for immunoprecipitation) only MOM19 was brought down by anti-MOM19 antibodies (Fig. 1*b*, lane 5). Similarly, after dissolving mitochondria in SDS-containing

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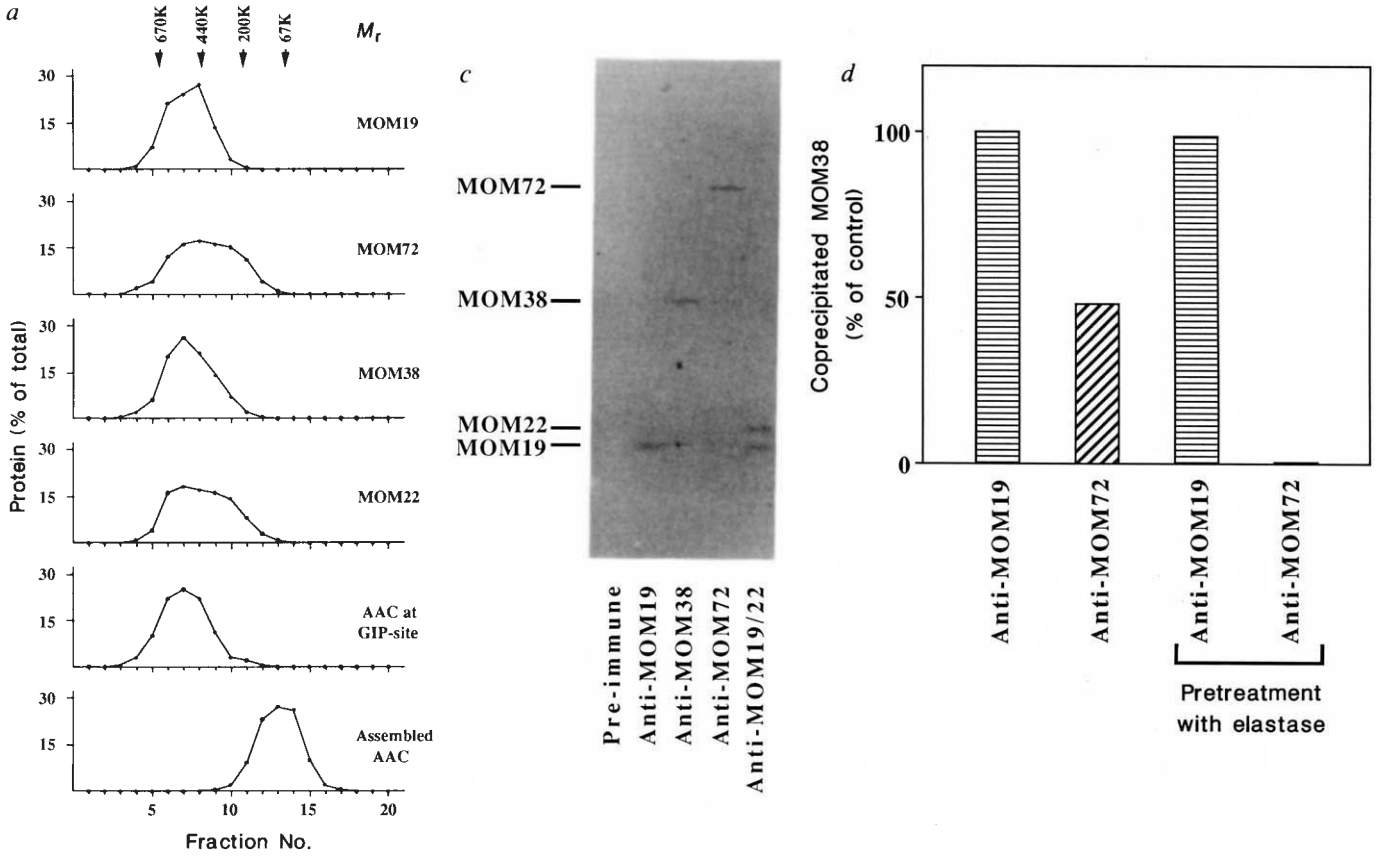


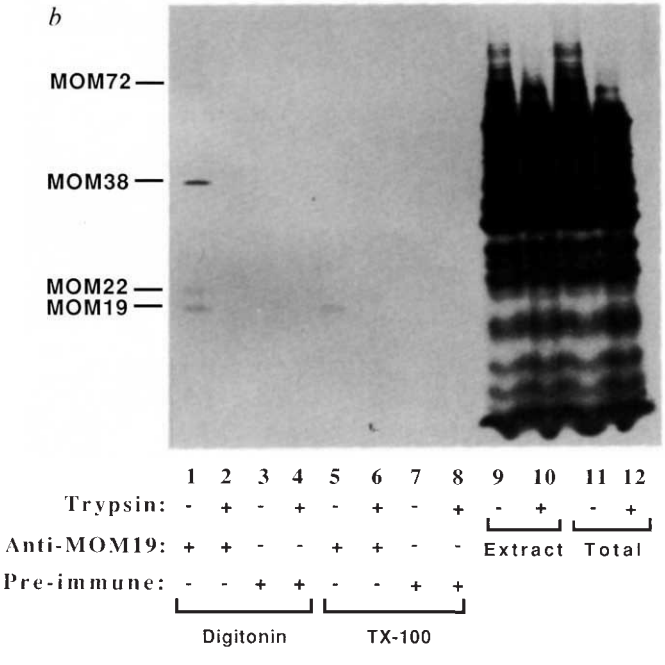


FIG. 1 Identification of a receptor complex in the outer membrane of *N. crassa* mitochondria. *a*, Analysis of outer membrane proteins and ADP/ATP carrier by gel filtration. AAC, ADP/ATP carrier; assembled AAC, mature dimeric ADP/ATP carrier in the inner membrane. Arrows indicate M_r standards. *b*, Precipitation of a protein complex with anti-MOM19 antibodies. Trypsin, mitochondria pretreated with trypsin; TX-100, Triton X-100; total, labelled mitochondrial proteins; extract, mitochondrial proteins extracted with digitonin. *c*, Identification of mitochondrial outer membrane proteins in the protein complex. *d*, Coprecipitation of MOM38 with anti-MOM72 antibodies. Elastase, mitochondria pretreated with elastase.

METHODS. Mitochondria (1 mg protein ml^{-1}) were lysed in digitonin buffer (0.5% (w/v) digitonin, 3% (w/v) BSA, 250 mM sucrose, 1 mM EDTA, 10 mM 3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.2, 1 mM phenylmethylsulphonyl fluoride) as described[11], followed by a centrifugation for 15 min at 25,000*g*. For immunoprecipitation, the mitochondrial extracts were incubated with protein A-Sepharose carrying specific antibodies in digitonin buffer (including 100–200 mM NaCl). The immunoprecipitates were washed in the digitonin–NaCl buffer (without BSA)[11]. Accumulation of the precursor of ADP/ATP carrier at the GIP site was performed by incubation of rabbit reticulocyte lysate containing ^{35}S-labelled precursor with isolated mitochondria in the presence of BSA-buffer (3% (w/v)) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.2) and 0.1 μM valinomycin, 8 μM antimycin A and 20 μM oligomycin (to dissipate a mitochondrial membrane potential) for 20 min at 25 °C as described[11,18,23,24]. Analysis of samples (reisolated mitochondria, immunoprecipitates, or TCA-precipitated fractions) was performed by SDS-PAGE[38], fluorography or immunodecoration and autoradiography, followed by laser densitometry as described[9,11,18,39]. *a*, Isolated mitochondria carrying the GIP intermediate of radiolabelled ADP/ATP carrier were lysed in digitonin buffer (without BSA) containing 0.05% (w/v) Lubrol PX. The extract was analysed by Superose 6 chromatography (Pharmacia-LKB) using the buffer described above. The protein-containing fractions are shown. Immunodecoration was performed with antibodies specific for MOM19, MOM38, MOM72 and an antibody preparation recognizing MOM19 and MOM22. ADP/ATP carrier at the GIP site was analysed by fluorography. Radiolabelled ADP/ATP carrier imported into the inner membrane was found in the same fractions as shown for endogenous (assembled) ADP/ATP carrier. *b*, ^{35}S-labelled mitochondria[9,11] were lysed in digitonin buffer and an immunoprecipitation with anti-MOM19 antibodies or with pre-immune antibodies was performed. Where indicated, the mitochondria were pretreated with trypsin as described[18,23]. For lanes 5–8, the immunoprecipitates were washed in a buffer containing 1% (w/v) Triton X-100 and 300 mM NaCl as described[40]. Lanes 1–8 are derived from samples containing 50 μg mitochondrial protein, lanes 9–12 correspond to 5 μg mitochondrial protein. *c*, The protein complex was precipitated with anti-MOM19 antibodies as described for (*b*) and dissolved in SDS-containing sample buffer. After a 40-fold dilution with buffer containing Triton X-100, a second immunoprecipitation with the indicated antibodies was performed under standard conditions[40]. *d*, The protein complex was precipitated with anti-MOM19 or anti-MOM72 antibodies, and MOM38 was analysed as described above. Where indicated, the mitochondria were pretreated with elastase (1 μg ml^{-1}) as described[10,18]. The amount of MOM38 precipitated with anti-MOM19 antibodies was set to 100%.

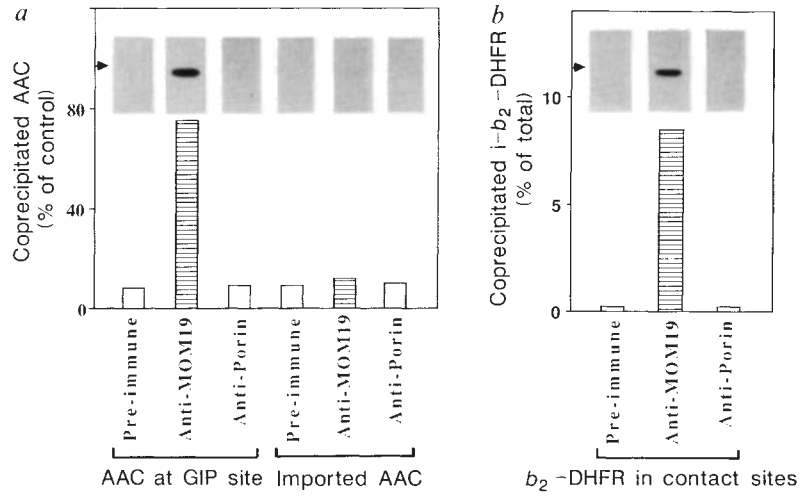
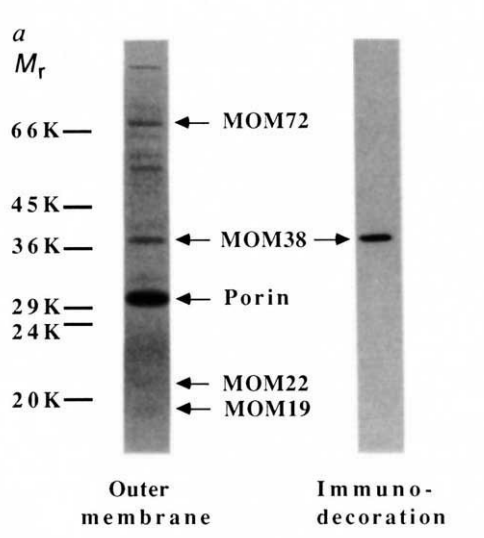


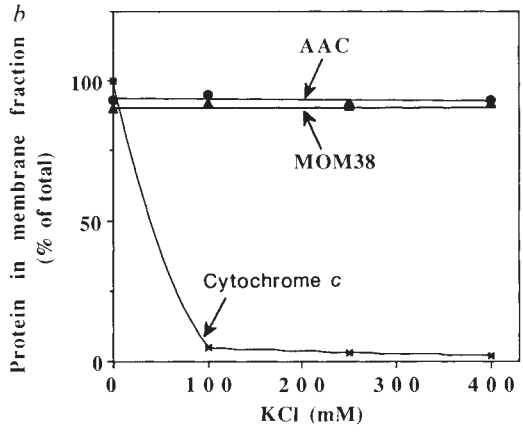
FIG. 2 Copurification of translocation intermediates with the receptor complex. *a*, Copurification of the GIP intermediate of ADP/ATP carrier. The arrowhead indicates copurified ADP/ATP carrier (AAC). *b*, Copurification of the contact site intermediate of b_2-DHFR. The arrowhead indicates copurified intermediate-sized i-b_2-DHFR.
METHODS. *a*, ADP/ATP carrier precursor was accumulated at the GIP site of isolated mitochondria as described in the legend to Fig. 1. For import of ADP/ATP carrier into the inner membrane, 8 mM potassium ascorbate and 0.2 mM N,N,N',N'-tetramethylphenylenediamine were added (to generate a mitochondrial membrane potential) and valinomycin, antimycin and oligomycin were omitted[23]. The reisolated mitochondria were lysed in digitonin buffer and immunoprecipitations were performed as described in the legend to Fig. 1. The amount of ADP/ATP carrier at the GIP site or of imported ADP/ATP carrier (determined by immunoprecipitation with anti-AAC antibodies[23]) was set to 100%. *b*, Reticulocyte lysate containing ^{35}S-labelled precursor of cytochrome b_1(1-167)-DHFR[21] was preincubated with 1 μM methotrexate for 5 min at 0 °C and then added to isolated mitochondria in the presence of a membrane potential as described above[21]. Lysis of the reisolated mitochondria with digitonin buffer and further treatment were performed as described above. The total amount of i-b_2-DHFR was set to 100%.

buffer, only MOM19 was precipitated, emphasizing the monospecificity of the anti-MOM19 antibodies[9].

The coprecipitated components were identified using a collection of antisera prepared against mitochondrial outer membrane proteins from N. crassa[9,11]. We found that the 72K protein was indeed MOM72 (Fig. 1*c*). The 38K protein was recognized by antibodies prepared against an outer membrane protein of this size and consequently termed MOM38. Finally, the 22K protein was immunoprecipitated by an antiserum that recognizes both MOM19 and a 22K outer membrane protein, now termed MOM22 (Fig. 1*c*). Each of the four components of the complex represents roughly 0.01–0.04% of total mitochondrial protein as assessed by the abundance of the Coomassie blue-stained protein bands.

The molar ratios of MOM19:MOM22:MOM38:MOM72 after coprecipitation with anti-MOM19 antibodies are about 1:0.6 (±0.2):1.0 (±0.1):0.5 (±0.15), determined as the average of 10 experiments. Assuming that the receptors MOM19 and





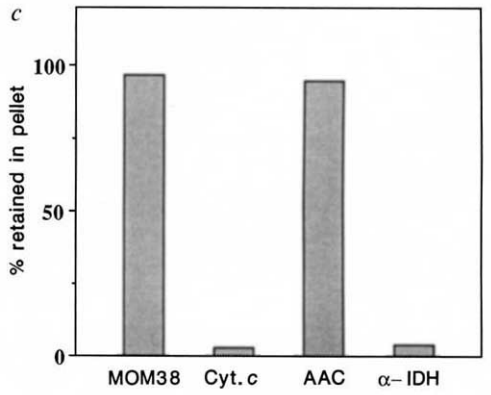


FIG. 3 MOM38 is an integral protein of the mitochondrial outer membrane. *a*, Protein pattern of the outer membrane of *N. crassa* mitochondria and immunodecoration of MOM38. *b*, MOM38 is not released from the membranes by salt and sonication. *c*, MOM38 is membrane-associated at pH 11.5. Cyt. *c*, cytochrome *c* (intermembrane space); AAC, ADP/ATP carrier (inner membrane); α-IDH, α subunit of isocitrate dehydrogenase (matrix).
METHODS. *a*, Mitochondrial outer membranes of *N. crassa* were isolated as described[9] and resolved by SDS-PAGE. Proteins were stained with Coomassie blue R-250. Mitochondrial proteins were transferred to nitrocellulose and immunodecorated with anti-MOM38 antibodies[9,11]. A similar result was obtained when purified outer membrane proteins were immunodecorated with anti-MOM38 antibodies. *b*, Mitochondria (0.2 mg protein ml^{-1}) were sonicated at various concentrations of KCl[9,11]. Membranes and supernatants were separated by centrifugation for 60 min at 166,000*g*. *c*, Mitochondria (0.1 mg protein ml^{-1}) were incubated in 100 mM Na_2CO_3 (pH 11.5) for 30 min at 0 °C. Separation of pellets and supernatants was performed as described[41].

MOM72 are present in roughly equimolar amounts in mitochondria[9,11], this implies that only about 50% of the MOM72 molecules are coprecipitated with anti-MOM19 antibodies (a similar conclusions applies to MOM22). The extent of coprecipitation of MOM38 with anti-MOM19 antibodies compared with that with anti-MOM72 antibodies is shown in Fig. 1*d* (we previously showed that the anti-MOM19 antibodies quantitatively precipitated MOM19 and similarly the anti-MOM72 antibodies quantitatively precipitated MOM72 (refs 9, 11)). About half of the MOM38 molecules that were coprecipitated with anti-MOM19 antibodies were coprecipitated with anti-MOM72 antibodies, suggesting that (at least) two types of MOM19/MOM38 complexes exist: those containing MOM72 and those not containing MOM72. The finding that only a fraction of the MOM72 molecules is associated with the MOM19/MOM38 complex agrees well with the previous observation of a partially different distribution of MOM19 and MOM72 over the outer membrane. In particular, MOM72 is more enriched in contact sites areas than MOM19 (refs 9, 11).

MOM72 is selectively degraded by treatment of mitochondria with low concentrations of elastase[10,11], whereas other outer membrane proteins, including the three other components of the complex, are not affected. After such pretreatment, anti-MOM72 antibodies did not coprecipitate the other complex components as shown for MOM38 in Fig. 1*d*. This represents an additional independent control for the specificity of the coprecipitation.

Functions of the receptor complex

Does this mitochondrial receptor complex have further functions in addition to being involved in recognition of precursors? Mitochondrial precursor proteins can be arrested at different stages of their translocation pathway (translocation intermediates), including as intermediates inserted into the outer membrane at the GIP site[18,23,24] and intermediates spanning both mitochondrial membranes at contact sites[20,21,25–27]. We asked whether precursors trapped at these intermediate stages are associated with the receptor complex: that is, can they be co-purified with the complex.

The GIP intermediate of the precursor of ADP/ATP carrier was formed by incubating precursor synthesized *in vitro* with isolated mitochondria in the presence of ATP, but in the absence of an electrical potential across the inner membrane[18,23,24]. Mitochondria were then lysed with digitonin and immunoprecipitation performed with anti-MOM19 antibodies. Figure 2*a* shows that the GIP intermediate of the ADP/ATP carrier was efficiently coprecipitated by anti-MOM19 antibodies, whereas antibodies from pre-immune serum or antibodies directed against the major outer membrane protein porin did not precipitate the precursor. In contrast, we previously reported that ADP/ATP carrier bound to its high-affinity receptor MOM72 on the mitochondrial surface was virtually not coprecipitated with anti-MOM19 antibodies[11], indicating that at this stage the precursor is not associated with the complex. As expected, both intermediates of the ADP/ATP carrier, the surface-bound form and the GIP-associated form, were coprecipitated with antibodies to MOM72 (ref. 11 and data not shown). On the other hand, ADP/ATP carrier fully imported into the inner membrane was neither coprecipitated with anti-MOM19 nor with anti-MOM72 antibodies (Fig. 2*a*, and ref. 11). ADP/ATP carrier at the GIP site, but not fully imported dimeric ADP/ATP carrier, also cofractionated with MOM19 in a gel filtration (Fig. 1*a*).

Together with our previous findings that the precursor of ADP/ATP carrier first binds to its receptor MOM72, then inserts into the GIP site and eventually is translocated into the inner membrane[11,18,23,24], these results suggest the following import pathway for the ADP/ATP carrier. The precursor predominantly binds to the MOM72 molecules that are not present in the complex. On assembly of MOM72 into the complex, the precursor is delivered to the general insertion site, GIP, that seems to be formed by one or more components of the receptor complex (as discussed below, MOM72 itself does not constitute the GIP site). From the GIP site, the precursor is then transported into the inner membrane and assembled into the dimeric form[28].

In order to arrest a precursor stably in mitochondrial contact sites, a fusion protein betwen the 167 N-terminal amino-acid residues of the precursor of cytochrome b_2 and entire dihydrofolate reductase (b_2-DHFR) was preincubated with methotrexate[21]. The tertiary structure of the DHFR-domain was thereby stabilized and, on addition to isolated mitochondria in the presence of ATP and a membrane potential, the cytochrome b_2-part of the fusion protein was inserted into the mitochondrial membranes and the presequence cleaved off in the matrix. The folded DHFR remained on the cytosolic side and the precursor therefore spanned both mitochondrial membranes[21]. The mitochondria were lysed with digitonin and a co-precipitation with anti-MOM19 antibodies was performed. About 8–10% of the contact site intermediates were coprecipitated, whereas control antibodies did not show an effect (Fig. 2*b*). The efficiency of coprecipitation agrees with the relative abundances of translocation sites and receptor complexes (about 10-fold more

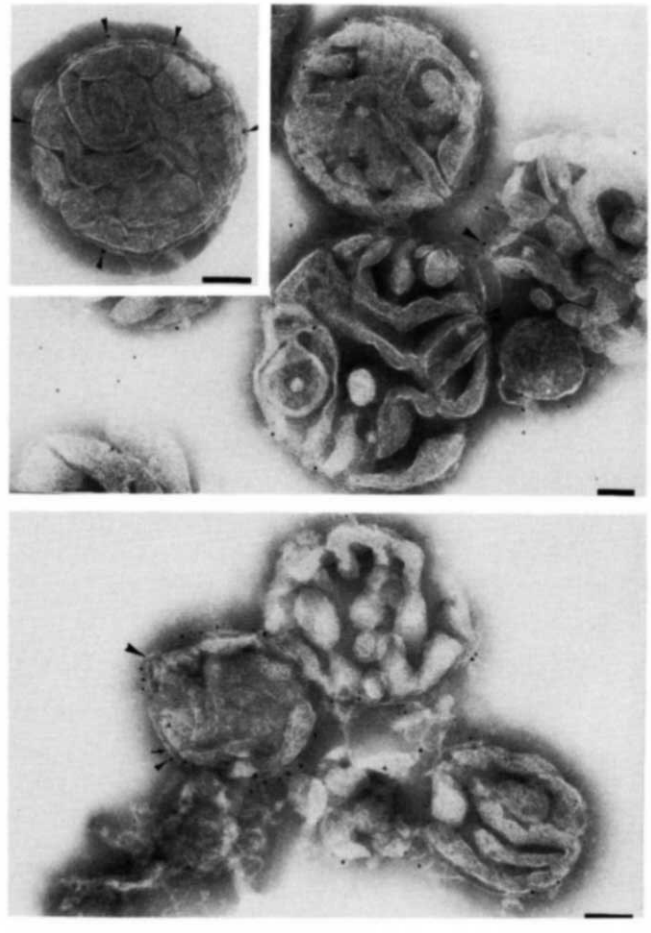


FIG. 4 Immunocytochemical localization of MOM38 in the mitochondrial outer membrane. Upper half, cryosections of mitochondria labelled with anti-MOM38 antibodies and protein A-gold particles (large arrowhead); small arrowheads, outer membrane. Insert, cryosections of mitochondria labelled with anti-MOM38 antibodies and protein A-gold particles (arrowheads). Lower half, mitochondria pre-labelled with anti-porin antibodies and 9-nm protein A-gold particles (small arrowhead) were cryosectioned and labelled with anti-MOM38 antibodies and 6-nm protein A-gold particles (arrow); large arrowhead, outer membrane. Bars represent 100 nm.
METHODS. Cryosections of *N. crassa* mitochondria were prepared and labelled as described[9,11,42,43].

translocation sites). Moreover, MOM38 can be crosslinked to a precursor protein arrested in contact sites (J. Rassow, M. Wiedmann, N. P. and W. N., unpublished results). It is concluded that the receptor complex is in contact with a fraction of the contact site intermediates, suggesting that the complex is also involved in the translocation of precursors through contact sites. Because of its low abundance compared with translocation contact sites, the receptor complex is probably not a permanent structural part of such sites. We propose that the receptor complex may transiently interact with contact sites and partake in the membrane insertion and translocation of precursor proteins, while (more abundant) proteins that have not been identified so far would form the structural basis of the contact sites.

MOM38 and the GIP

We analysed the properties of MOM38, the major complex component besides MOM19, in order to determine its possible function. Figure 3*a* shows the protein band corresponding to MOM38 on a Coomassie blue-stained SDS-polyacrylamide gel of purified outer membranes. MOM38 is not released from the membranes by sonication of mitochondria at various salt concentrations (Fig. 3*b*) or by treatment at alkaline pH (Fig. 3*c*), indicating that MOM38 is an integral membrane protein. Its localization in the outer mitochondrial membrane was confirmed by immunocytochemistry. Cryosections of *N. crassa* mitochondria were labelled with antibodies directed against MOM38 followed by protein A-gold particles. Figure 4 (upper part including insert) shows that the vast majority of gold particles were close to the outer membrane. As a further control for the outer membrane localization of MOM38, its colocalization with porin is shown (Fig. 4, lower part).

A full-length complementary DNA clone of MOM38 was isolated from a λgt11 library of *N. crassa* cDNA[29]. The nucleotide sequence and the deduced amino-acid sequance are shown in Fig. 5*a*. A protein with 349 amino-acid residues and M_r 38,108, containing at least one possible membrane spanning sequence, is predicted. Two stretches with similarity to the A and B regions, respectively, of an ATP-binding consensus sequence[30] are present in the C-terminal half of MOM38 (Fig. 5*a*). This finding may be related to the previously observed dependence on ATP for the transfer of precursor proteins from the receptor sites to the GIP site[24]. Two regions with a predicted α-helical conformation (amino acid residues 142–154 and 329–340) are characterized by a high content of negatively charged amino-acid residues and the complete absence of positively charged residues. In both cases, the negative charges are located on one side of the helix. These regions are thus putative candidates for sequences interacting with the positively charged mitochondrial presequences[4,31,32]. It is also notable that MOM38 contains an unusally high proportion of phenylalanine residues (7% of all residues).

MOM38 contains a sequence of about 70 amino-acid residues

a

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-  28                                                    GTCCCTGTCTACTGCGACCGTTACAATC
+   1 ATGGCTTCGTTTTCCACCGAGTCGCCTTTGGCGATGCTGCGCGACAATGCCATTTATTCG
    1  M  A  S  F  S  T  E  S  P  L  A  M  L  R  D  N  A  I  Y  S
+  61 AGCCTTTCCGATGCCTTCAACGCCTTTCAGGAAAGGAGCAAACAGTTCGGTCTTTCCAAC
   21  S  L  S  D  A  F  N  A  F  Q  E  R  R  K  Q  F  G  L  S  N
+ 121 CCCGGCACGATCGAGACCATCGCCCGCGAGGTCCAACGCGATACCCTCCTCACCAACTAC
   41  P  G  T  I  E  T  I  A  R  E  V  Q  R  D  T  L  L  T  N  Y
+ 181 ATGTTCTCTGGCCTCCGCGCCGACGTCACCAAGGCTTTCAGCCTCGCCCCTCTCTTCCAA
   61  M  F  S  G  L  R  A  D  V  T  K  A  F  S  L  A  P  L  F  Q
+ 241 GTTTCCCACCAGTTTGCCATGGGCGAGAGGTTGAACCCTTATGCCTTTGCTGCTCTCTAC
   81  V  S  H  Q  F  A  M  G  E  R  L  N  P  Y  A  F  A  A  L  Y
+ 301 GGAACCAACCAGATCTTCGCTCAGGGTAACCTGGACAACGAGGGCGCTCTCTCGACAAGA
  101  G  T  N  Q  I  F  A  Q  G  N  L  D  N  E  G  A  L  S  T  R
+ 361 TTCAACTACAGATGGGGTGACAGGACCATCACCAAGACGCAGTTCTCGATTGGTGGCGGC
  121  F  N  Y  R  W  G  D  R  T  I  T  K  T  Q  F  S  I  G  G  G
+ 421 CAGGATATGGCCCAGTTTGAGCATGAACACCTTGGCGACGACTTCAGTGCCTCCCTCAAG
  141  Q  D  M  A  Q  F  E  H  E  H  L  G  D  D  F  S  A  S  L  K
+ 481 GCCATCAACCCCTCTTTCCTTGACGGCGGTCTCACCGGTATCTTTGTCGGCGACTACCTC
  161  A  I  N  P  S  F  L  D  G  G  L  T  G  I  F  V  G  D  Y  L
+ 541 CAGGCCGTCACTCCCAGACTCGGCCTCGGTCTCCAGGCCGTCTGGCAACGTCAGGGTCTC
  181  Q  A  V  T  P  R  L  G  L  G  L  Q  A  V  W  Q  R  Q  G  L
+ 601 ACTCAGGGCCCCGACACCGCTATCTCCTACTTTGCCCGCTACAAGGCCGGTGACTGGGTT
  201  T  Q  G  P  D  T  A  I  S  Y  F  A  R  Y  K  A  G  D  W  V
+ 661 GCTAGCGCTCAGCTCCAGGCTCAGGGTCGCTCTCAACACTTCCTTCTGGAAGAAGCTGACG
  221  A  S  A  Q  L  Q  A  Q  G  A  L  N  T  S  F  W  K  K  L  T
+ 721 GATAGGGTGCAGGCTGGTGTTGATATGACGCTATCTGTTGCTCCCTCTCAGAGCATGATG
  241  D  R  V  Q  A  G  V  D  M  T  L  S  V  A  P  S  Q  S  M  M
+ 781 GGTGGCCTTACCAAGGAAGGCATCACCACCTTTGGTGCCAAGTACGACTTCAGAATGTCC
  261  G  G  L  T  K  E  G  I  T  T  F  G  A  K  Y  D  F  R  M  S
+ 841 ACCTTCAGGGCTCAGATCGACTCCAAGGGCAAGCTCAGCTGCTTGCTCGAGAAGCGTCTT
  281  T  F  R  A  Q  I  D  S  K  G  K  L  S  C  L  L  E  K  R  L
+ 901 GGTGCCGCCCCCGTCACTCTGACCTTCGCTGCTGATGTTGACCACGTCACTCAACAAGCC
  301  G  A  A  P  V  T  L  T  F  A  A  D  V  D  H  V  T  Q  Q  A
+ 961 AAGCTCGGCATGTCCGTCTCCATTGAGGCGTCTGATGTCGATCTCCAGGAGCAGCAAGAG
  321  K  L  G  M  S  V  S  I  E  A  S  D  V  D  L  Q  E  Q  Q  E
+1021 GGTGCCCAGTCCCTCAACATCCCCTTTTAAACTGGAATATGTATATTGGGGATGAAGAAC
  341  G  A  Q  S  L  N  I  P  F  *
+1081 ACTGGTCGATTGGGCCAGCATTTGGTTTCCGCTCCACCCTTGCACTCTACTCATTGTGTT
+1141 GACAACGTAATCTAATCACACAGATGCCCACCTTGTACCAAGGAACAGAAAGGGTGAGG
+1201 GTCATACCTTTTCACCGTCCCTCGCTACCAGCGCCATTTCTGTTTTGACCACATTCTCAT
+1261 CACATCATCTAGTACAAGCTGGTCACGGCGAGTTGCCATGGGAAAAATCGAAAGTCGGAC
+1321 GTTGCGAACCTCCCCATCGCAATGGTCCCGCATGGCTTGGGTCTTTGCATACAAGTTCCA
+1381 CGGAACGATAGACATCTCTCACCACATTTTCCACCTTAAAAAAAG
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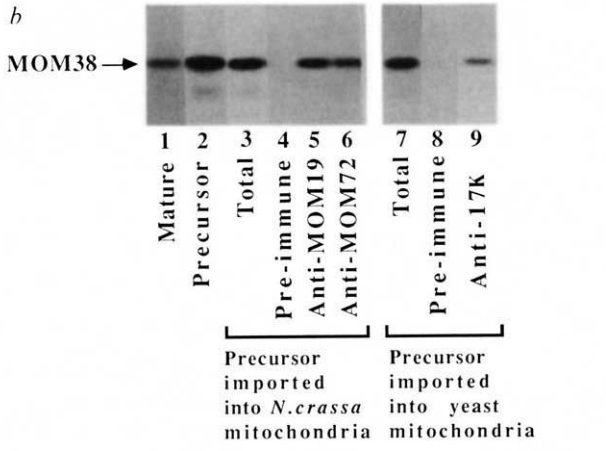
FIG. 5 Primary sequence of MOM38 and assembly of the precursor *in vitro*. *a*, Nucleotide sequence of the cDNA coding for *N. crassa* MOM38 and derived amino-acid sequence (single letter code). A full-length cDNA close coding for MOM38 was isolated from a λgt11 library of *N. crassa* cDNA[29] by



antibody screening and subcloned into pGEM4 (Promega Biotec)[44]. The cDNA insert was sequenced using internal restriction sites and MOM38-specific oligonucleotide primers. Both strands were sequenced overlapping at least three times. A possible membrane-spanning sequence is underlined. Additional uncharged segments are found at amino-acid positions 72–88, 219–236 and 249–264. Boxes indicate the *A* and *B* regions of a putative ATP-binding domain[30]. *b*, The precursor of *N. crassa* MOM38 is imported and assembled in isolated *N. crassa* and yeast mitochondria. Lane 1 shows MOM38 immunoprecipitated from ^{35}S-labelled *N. crassa* mitochondria. The MOM38 precursor was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine by coupled transcription/translation[18] (lane 2) (the precursor was efficiently recognized by anti-MOM38 antibodies). The reticulocyte lysate was then incubated with isolated mitochondria from *N. crassa* (lanes 3–6) or *Saccharomyces cerevisiae*[45] (lanes 7–9) in BSA buffer (see legend to Fig. 1) for 20 min at 25 °C. Samples 3 and 7 were treated with proteinase K (20 μg ml^{-1}) for 20 min at 0 °C and the mitochondria reisolated. The mitochondria of samples 4–6, 8 and 9 were reisolated, lysed in digitonin buffer (see legend to Fig. 1) and immunoprecipitated with the indicated antibodies. Samples were analysed by SDS-PAGE and fluorography. The 17K protein is an outer membrane protein of yeast mitochondria[10] (the putative homologue to *N. crassa* MOM19). The precursor of MOM38 in reticulocyte lysate was completely digested by low concentrations of trypsin (20 μg ml^{-1}) and proteinase K (10 μg ml^{-1}), in contrast to the protein imported into mitochondria.

(residues 146–218) that exhibits 68% similarity (including 25% identity) to a sequence (residues 66–136) of the bovine mitochondrial phosphate carrier protein, an inner membrane protein[33]. Homology is also found to the similar region of the yeast mitochondrial phosphate carrier protein[34]. Furthermore, a sequence of about 60 amino-acid residues (residues 288–346 of MOM38) shows 78% similarity (including 35% identity) to a sequence (residues 522–584) of the *czcA* gene product of the divalent cation efflux system from the bacterium *Alcaligenes eutrophus*[35]. As these sequences are present in membrane proteins with transport function, a role for structure and/or function of membrane carrier proteins might be possible.

The precursor of MOM38 was synthesized in rabbit reticulocyte lysate by coupled transcription/translation. As expected for an outer membrane protein, the precursor exhibited the same apparent molecular size as the mature mitochondrial protein (Fig. 5*b*, lanes 1 and 2), indicating the absence of a cleavable targeting sequence. The precursor was efficiently imported into isolated *N. crassa* mitochondria (Fig. 5*b*, lane 3) and correctly assembled into the receptor complex as shown by the specific coprecipitation with anti-MOM19 and anti-MOM72 antibodies (Fig. 5*b*, lanes 5 and 6). Moreover, the precursor of *N. crassa* MOM38 was imported into isolated yeast mitochondria (Fig. 5*b*, lane 7) and could be coprecipitated with antibodies directed against a yeast mitochondrial outer membrane protein of 17K[10] (Fig. 5*b*, lane 9), the putative homologue of MOM19 (H. F. Steger, T. S., N. P. and W. N., manuscript in preparation). This result suggests that a structurally and functionally equivalent receptor complex may exist in yeast mitochondria.

What is the function of MOM38 in protein import? We previously reported a characteristic feature of the GIP site, namely its resistance to very high concentrations of trypsin whereas it is degraded at intermediate concentrations of proteinase K[3,18,23,24,36]. In the following experiment (Fig. 6) we determined whether a component of the receptor complex showed this property. MOM19, MOM22 and MOM72 were digested by treatment of isolated mitochondria with trypsin. MOM38 was completely resistant to treatment with trypsin as was the precursor of ADP/ATP carrier accumulated at the GIP site (Fig. 6*a*). The precursor of ADP/ATP carrier itself was very sensitive to digestion by trypsin, both in reticulocyte lysate and on lysis of mitochondria with detergent[23], whereas MOM38 was also resistant to trypsin after lysis of mitochondria. Moreover, MOM38 in the outer membrane was not accessible to antibodies prepared against denatured MOM38 (data not shown). As the precursor of MOM38 in reticulocyte lysate is digested by trypsin and recognized by antibodies (see legend to Fig. 5), it is concluded that the imported MOM38 acquires a conformation in the outer membrane that does not allow access for typsin or antibodies.

In summary, the GIP site copurifies with the receptor complex (Fig. 2*a*). Three of the complex components are digested at protease concentrations that do not degrade the GIP site, excluding them as possible candidates for GIP (under these conditions, the precursor of ADP/ATP carrier at the GIP-site remains protected and is fully competent for completion of import[18,23,24]). With all likelihood, MOM38 is responsible for the protection of precursors accumulated at the GIP site and thus forms an essential part of GIP[18,19]. Furthermore, when mitochondria were treated with proteinase K the sensitivity of MOM38 again correlated well with that of the GIP site (Fig. 6*b*). The function of MOM22 is unknown; its presence in the receptor complex and its exposure on the mitochondrial surface

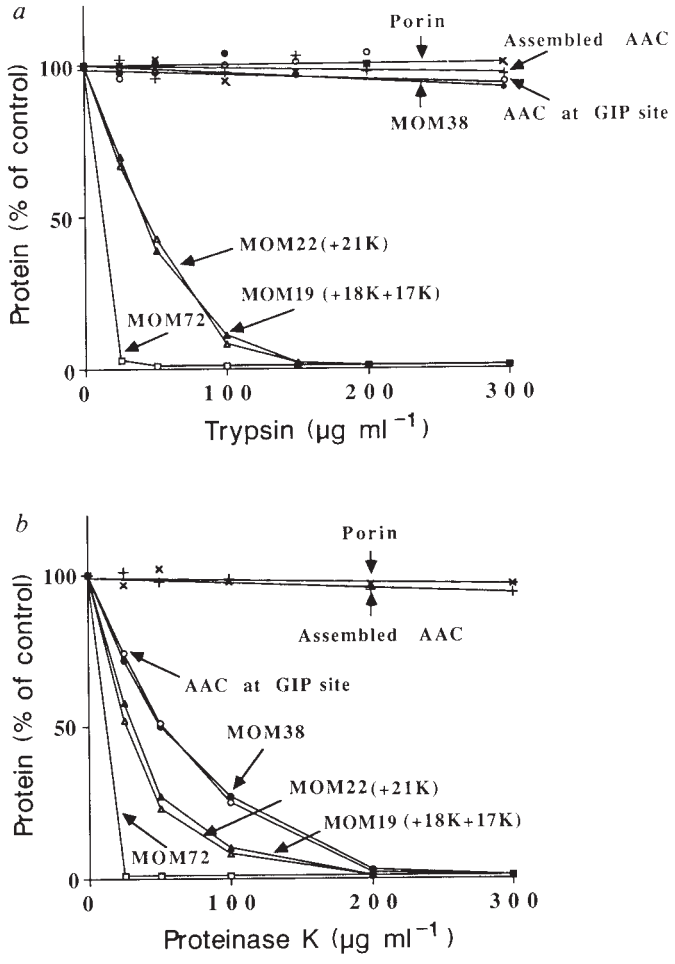


FIG. 6 The protease sensitivities of GIP site and MOM38, but of no other component of the complex, show close correlation. Isolated *N. crassa* mitochondria (0.25 mg protein ml^{-1}) carrying the GIP intermediate of the ADP/ATP carrier (AAC) were treated with tryspin (*a*) or proteinase K (*b*) in BSA buffer (see legend to Fig. 1) as described[23]. The mitochondria were reisolated and analysed for GIP-associated ADP/ATP carrier by SDS-PAGE and fluorography and for MOM38, MOM19 (and 18K and 17K fragments derived from MOM19 (ref. 9)), MOM22 (and a 21K fragment derived from MOM22), MOM72, assembled AAC, and porin by immunodecoration.

like the receptors MOM19 and MOM72 (Fig. 6) may suggest an involvement in initial steps of protein import.

Discussion

We report here that both mitochondrial protein import receptors, the master receptor MOM19 and the receptor for the ADP/ATP carrier (MOM72)[9-11], are present in a protein complex in the outer membrane of *N. crassa* mitochondria. Analysis of the constituents of the complex led to the identification of two new components, the outer membrane proteins MOM38 and MOM22. MOM38, an integral membrane protein, apparently represents the general insertion site GIP (or a part of it) which is responsible for the accumulation of precursor proteins in the outer membrane before their transfer into the inner membrane[18,19]. This implies that the transfer of precursor proteins from the bound state at the receptors to the membrane-inserted GIP state occurs by way of direct interaction of the receptors with MOM38. MOM22, the fourth component of this receptor complex, is exposed on the outer membrane surface, yet its function is currently unknown.

Our results suggest the following working hypothesis. The association between MOM19 and MOM38 forms the core of the receptor complex. Precursor proteins that are recognized by MOM19 can thus be immediately donated to the GIP site, that is MOM38, and thereby be inserted into the outer membrane. MOM72 may assemble with and disassemble from this complex. The precursor of ADP/ATP carrier first binds to those MOM72 molecules that are not in the complex and thereby remains on the mitochondrial surface[11]. Upon assembly of MOM72 into the complex, the precursor is donated to MOM38 and inserted into the outer membrane. The further transport of precursors occurs through contact sites between both mitochondrial membranes[2,3,20,21,25-27]. As the constituents of the complex are not exclusively located in contact sites[9,11], lateral mobility of these constituents and/or contact sites in the outer membrane is implied. The association of the receptor complex with a fraction of precursors in transit through contact sites also suggests a role in membrane translocation.

This model agrees well with results obtained with yeast mitochondria. The import receptors MOM72 (ref. 10) and MOM19 (H. F. Steger, T. S., N. P. and W. N., manuscript in preparation) have been identified in yeast. These components seem to be present in a complex similar to that of *N. crassa* as indicated by the observed ability of *N. crassa* MOM38 to assemble with the yeast components after import into isolated mitochondria. Vestweber *et al.*[12] have identified a protein of 42K in the yeast mitochondrial outer membrane by cross-linking to a precursor arrested in contact sites. The properties of this 42K protein, that is apparent size, association with a fraction of precursors trapped in contact sites[12], high resistance to trypsin[37], and distribution over the entire outer membrane[12], correlate with the expected properties of the yeast equivalent to MOM38. In fact, this 42K protein seems to be present in a receptor complex of yeast mitochondria (N. P., T. S., K. Dietmeier, H. F. Steger and W. N., unpublished results). We propose that the yeast 42K protein is the equivalent of MOM38 and thus represents the GIP of yeast mitochondria. The hypothesis of cycles of assembly and disassembly of components of the protein transport apparatus and of lateral mobility of the components in the membrane is thus supported by findings both with *N. crassa* and yeast. We speculate that similar dynamic behaviour may also be important for other membrane systems where recognition, insertion and translocation of precursor proteins takes place.

Note added in proof: *N. crassa* MOM38 shows 40% amino-acid sequence identity (plus 37% isofunctional amino-acid exchanges) with the 42K protein identified in yeast mitochondria (see accompanying article[46]). The similarity extends over the entire lengths of the proteins (with the exception of 21 additional amino-acid residues at the N-terminus of the yeast protein). An identity of 40% is in the usual range observed with equivalent proteins of *N. crassa* and *S. cerevisiae* (for example, refs 10 and 29). This supports our proposal that the 42K protein represents the yeast mitochondrial general insertion site GIP or part of it. □

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