Cell, Vol. 59, 1061–1070, December 22, 1989, Copyright © 1989 by Cell Press

MOM19, an Import Receptor for Mitochondrial Precursor Proteins

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Summary

We have identified a 19 kd protein of the mitochondrial outer membrane (MOM19). Monospecific IgG and Fab fragments directed against MOM19 inhibit import of precursor proteins destined for the various mitochondrial subcompartments, including porin, cytochrome c_1, Fe/S protein, F_0 ATPase subunit 9, and F_1 ATPase subunit β. Inhibition occurs at the level of high affinity binding of precursors to mitochondria. Consistent with previous functional studies that suggested the existence of distinct import sites for ADP/ATP carrier and cytochrome c, we find that import of those precursors is not inhibited. We conclude that MOM19 is identical to, or closely associated with, a specific mitochondrial import receptor.

Introduction

Most proteins of cell organelles are synthesized on cytosolic polysomes and imported into the organelles. One important aspect of this process is the specificity of targeting to the various organelles. It is generally assumed that precursor proteins carry signals that direct them to the correct cellular compartment and that organellar surfaces contain receptor-like structures able to decode the information contained in the targeting sequences (Wickner and Lodish, 1985). Considerable functional data have accumulated to support this concept for mitochondria, chloroplasts, and endoplasmic reticulum (ER).

Studies with Neurospora crassa mitochondria have provided a great deal of information about the functional properties of proteinaceous receptor sites (summarized in Pfanner et al., 1988a; Hartl et al., 1989). Precursor proteins can be reversibly arrested at the level of binding to the mitochondrial surface. They can bind with high affinity to saturable binding sites. These sites are exposed on the mitochondrial surface, since they are accessible to proteases added to mitochondria. Not all precursors use the same sites, suggesting the existence of distinct specific receptor sites.

Despite the bulk of functional evidence supporting the existence of mitochondrial protein import receptors, none of the putative receptor sites could be identified thus far. This lack of identification of an import receptor even led to speculations that receptor proteins may not be involved or may not be important for mitochondrial protein import (Roise et al., 1986; Hurt and van Loon, 1986; Jordi et al., 1989). On the other hand, receptor-like protein components have been reported for other organelles. The existence of a signal sequence receptor in the ER has been proposed on the basis of cross-linking experiments (Wiedmann et al., 1987; Walter, 1987). This receptor is believed to bind signal sequences, following their release from the signal recognition particle (SRP) and docking protein (SRP receptor) system (Walter and Blobel, 1982; Meyer et al., 1982; Gilmore et al., 1982). With anti-idiotypic antibodies (mimicking signal sequences), a protein of the chloroplast envelope was identified that participates in protein import (Pain et al., 1988). Functional characterization of the properties of receptor sites, including high affinity binding of precursors, will be required to determine whether the identified components are identical to an ER signal sequence receptor or a chloroplast import receptor, or whether they represent other components of the translocation machineries. Antibodies prepared against 45 kd proteins of yeast mitochondrial outer membranes were found to reduce import of precursor proteins (Ohba and Schatz, 1987); however, it appears that this was not due to blocking of receptors, because protease pretreatment of mitochondria was required to allow binding of the antibodies.

For this paper, we have prepared antibodies and Fab fragments against a number of outer membrane proteins of N. crassa mitochondria. Fab fragments against a 19 kd protein (MOM19) inhibit import of a subset of precursor proteins at the level of high affinity binding to the mitochondrial surface. MOM19 appears to exhibit all of the properties expected of a specific mitochondrial import receptor.

Results

MOM19 Is an Outer Membrane Protein Exposed on the Mitochondrial Surface

Our attempts to identify a mitochondrial import receptor were based on the following observations. Functional studies suggested that receptor sites are exposed on the mitochondrial surface, i.e., they are accessible to proteases and probably also to antibodies added to intact mitochondria (summarized in Pfaller et al., 1988; Söllner et al., 1988). Because the outer membrane contains only a limited number of proteins (see below), it was possible to prepare monospecific antibodies against the various proteins and to test their effect on the import of precursor proteins.

Mitochondrial outer membrane of N. crassa was isolated using a procedure previously described (Freitag et al., 1982). In brief, mitochondria were swollen, the outer membrane was sheared off in a potter homogenizer, and the membranes were separated on sucrose gradients (Figure 1A). The distribution of outer membrane, inner

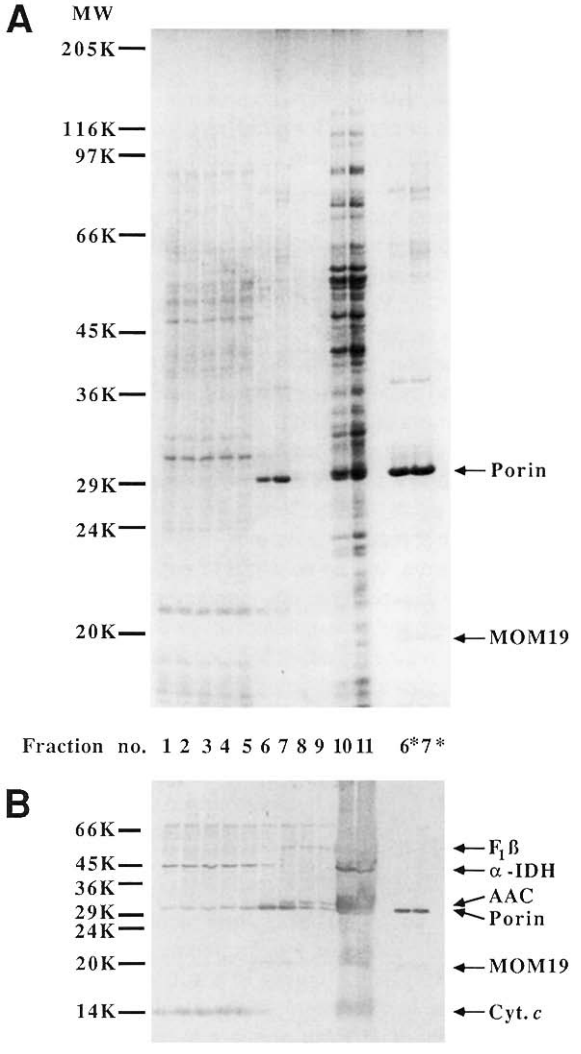

Figure 1. Purification of Outer Membrane from N. crassa Mitochondria

Mitochondria were swollen and ruptured in a potter homogenizer to remove the outer membrane as described in Experimental Procedures. The resulting membrane vesicles were layered on the top of a sucrose step gradient and centrifuged. Fractions (1 ml) were collected from the gradient.

(A) Protein pattern. Aliquots (200 μl) of fractions 1–9 and 40 μl of fractions 10 and 11 were precipitated with trichloroacetic acid (TCA), separated on an SDS–polyacrylamide gel, and stained with Coomassie blue R-250. Lane 1 corresponds to the top of the gradient, and lane 11 corresponds to the bottom. Aliquots (600 μl) of fractions 6 and 7 were then flotated by centrifugation as described in Experimental Procedures. The fractions enriched in porin (lanes 6* and 7*) were precipitated with TCA and resolved by SDS–PAGE, and proteins were stained with Coomassie blue R-250.

(B) Marker proteins. Aliquots (200 μl) of each fraction were precipitated with TCA, separated on SDS–polyacrylamide gels, and transferred to nitrocellulose paper. Immunolabeling was performed with antisera directed against marker proteins of different mitochondrial compartments: porin (outer membrane), cytochrome c (Cyt. c, intermembrane space), ATP/ADP carrier (AAC, inner membrane), subunit β of ATPase ($F_1\beta$, matrix side of inner membrane), and α subunit of isocitrate-dehydrogenase (α-IDH, matrix space). Bound antibodies were visualized by decoration with ^{14}C-labeled protein A and autoradiography.

membrane, and soluble fractions was followed by the use of marker proteins (Figure 1B). Fraction 6 contained the outer membrane marker porin, whereas marker proteins for inner membrane, intermembrane space, and matrix were almost undetectable. Flotation of fraction 6 led to a further purification, resulting in essentially pure outer membrane free of contamination from other mitochondrial fractions (Figure 1, lane 6*). Separation on SDS–polyacrylamide gels revealed approximately 25 distinct polypeptides (Figure 1A). After transfer to nitrocellulose, distinct protein bands were excised, and rabbit antisera against the proteins were generated. In pilot studies, immunoglobulins G (IgGs), prepared from the various antisera that specifically recognized only one polypeptide of N. crassa mitochondria, were screened for their ability to inhibit import of in vitro–synthesized precursor proteins into isolated N. crassa mitochondria. Whereas most IgG preparations had no significant effect on import of precursor proteins, IgG against a 19 kd protein (MOM19) selectively inhibited import of a subset of precursor proteins. We thus characterized the properties of MOM19 in detail (see below).

IgG prepared against MOM19 recognized only MOM19, both upon immunoprecipitation from ^{35}S-labeled mitochondria lysed in SDS-containing buffer and upon immunodecoration of mitochondrial proteins transferred to nitrocellulose (Figure 2A, lanes 1 and 2). MOM19 could be purified to homogeneity by the use of an affinity matrix carrying covalently coupled IgG (Figure 2A, lane 3). MOM19 cofractionated with the outer membrane of mitochondria (Figure 1B) and remained membrane associated even after sonication of mitochondria at various concentrations of salt (Figure 2B). MOM19 is exposed on the mitochondrial surface as shown by the accessibility to low concentrations of proteases and to antibodies added to intact mitochondria (Figure 2C). MOM19 is present at about 2–4 pmol/mg mitochondrial protein as calculated by the abundance of the Coomassie blue–stained protein band. For comparison, porin, the major protein (and the only one characterized thus far) of the mitochondrial outer membrane of N. crassa, is present at about 100 pmol/mg mitochondrial protein (Freitag et al., 1982).

Mitochondria were preloaded with anti-MOM19 antibodies and labeled with protein A–gold particles before fixation and processing for electron microscopy. Figures 3A and 3B demonstrate that MOM19 was accessible to antibodies added to intact mitochondria. In addition, cryosections of mitochondria were prelabeled with anti-MOM19 antibodies and decorated by protein A–gold particles (Figures 3C and 3D). MOM19 was found solely in the outer mitochondrial membrane. Contact sites between mitochondrial outer and inner membranes are the major site for translocation of precursor proteins across the mitochondrial membranes (Schleyer and Neupert, 1985; Schwaiger et al., 1987; Pfanner et al., 1987a; Rassow et al., 1989). We therefore investigated the distribution of MOM19 across the outer membrane in respect to contact site regions (marked by arrowheads in Figure 3). MOM19 appears to be distributed over the entire mitochondrial surface with only slight, if any, enrichment in contact sites

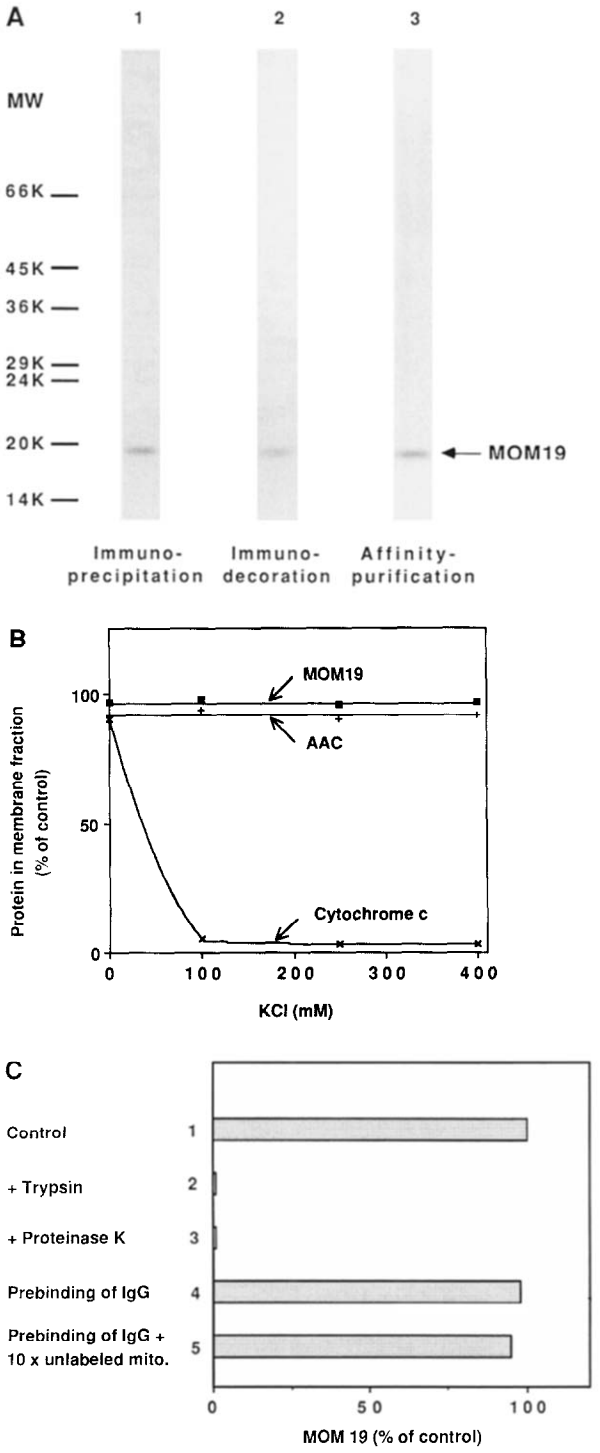

Figure 2. MOM19 Is an Outer Membrane Protein Exposed on the Mitochondrial Surface

(A) Antibodies against MOM19 recognize only MOM19. Mitochondria were isolated from N. crassa cells grown in presence of [^{35}S]sulfate, solubilized in SDS-containing buffer (Schleyer and Neupert, 1985), and diluted 20-fold with Triton X-100 buffer (10 mM Tris–HCl [pH 7.5], 1% Triton X-100, 300 mM NaCl, 1 mM PMSF). MOM19 was immunoprecipitated from extracts corresponding to 50 μg of mitochondrial protein and analyzed by SDS–PAGE and fluorography (lane 1). Mitochondrial proteins (60 μg) were separated on an SDS–polyacrylamide gel and electrotransferred to nitrocellulose. Immunolabeling was performed with antisera directed against MOM19, and bound antibodies were visualized by decoration with ^{14}C–protein A and autoradiography (lane 2). Isolated mitochondria were lysed in Triton X-100 buffer and incubated with anti-MOM19 IgGs covalently coupled to Reacti-Gel (Pierce). After washing, MOM19 was eluted with 0.1 M glycine–HCl (pH 2.5), neutralized with 2 M Tris base, and precipitated with TCA. The pellet was analyzed by gel electrophoresis and staining with Coomassie blue R-250 (lane 3).

(B) MOM19 is not released from the mitochondrial membrane by salt and sonication. Mitochondria (200 μg protein/ml SEM; see Experimental Procedures) were sonified with a Branson Sonifier with tapered microtip (setting 3, 40% duty, 3 × 60 s with an interval of 60 s). PMSF was added to a final concentration of 1 mM. Membranes were pelleted by centrifugation for 60 min at 166,000 × g and analyzed by immunoblotting for ADP/ATP carrier, cytochrome c, and MOM19. The autoradiogram was quantified by laser densitometry.

(C) MOM19 is accessible to antibodies and proteases in intact mitochondria. Mitochondria were isolated from Neurospora cells grown in the presence of [^{35}S]sulfate and divided into aliquots. The control sample (reaction 1) was left on ice for 20 min. Mitochondria were treated for 20 min at 0°C either with trypsin at a final concentration of 15 μg/ml (reaction 2) or with proteinase K at a final concentration of 10 μg/ml (reaction 3). Mitochondria (reactions 1–3) were then solubilized in Triton X-100 buffer, and MOM19 was immunoprecipitated. In a second aliquot IgGs directed against MOM19 were prebound to mitochondria (reactions 4 and 5). The mitochondria were reisolated and washed once with SEM buffer. Mitochondria containing prebound MOM19 IgG were lysed either directly in Triton X-100 buffer (reaction 4) or in the presence of a 10-fold excess of nonradiolabeled mitochondria (reaction 5). MOM19 was then precipitated from both samples with protein A Sepharose.

(Table 1). We suggest that receptor sites collect precursor proteins from all over the mitochondrial surface and eventually transfer them to contact sites.

MOM19 Is Involved in Import of a Subset of Mitochondrial Precursor Proteins

IgG and Fab fragments directed against MOM19 (anti-MOM19) were analyzed for their effect on the import of different mitochondrial precursor proteins. Mitochondria were preincubated with anti-MOM19. Parallel samples were preincubated with anti-porin or with IgG or Fab fragments prepared from "preimmune" serum obtained from rabbits before the injection of N. crassa antigens. After reisolation, mitochondria were incubated with radiolabeled mitochondrial precursor proteins at 25°C. For each precursor protein, conditions were chosen in order to test the import of the precursor in the linear range (Pfanner et al., 1987c; Pfaller et al., 1988). Translocation of precursors into mitochondria was determined by monitoring proteolytic processing (removal of the presequence) and/or protection of the imported precursor against high concentrations of proteinase K (for further details, see Pfanner et al., 1987d; Pfaller et al., 1988). Anti-MOM19 inhibited the import of porin, cytochrome c_1, Fe/S protein of the bc_1 complex, F_0 ATPase subunit 9 ($F_0$9), and F_1 ATPase subunit β (F_1β) (Figures 4A–4E), whereas anti-porin and preimmune IgG or Fab fragments showed no significant effect on import of these precursors. The import of ADP/ATP carrier (AAC), however, was practically not inhibited (Figure 4F). It might be argued that AAC binds with a much higher affinity to MOM19 than other precursor proteins, and therefore its import is not inhibited by anti-MOM19. However, this does not appear to be the case for several rea-

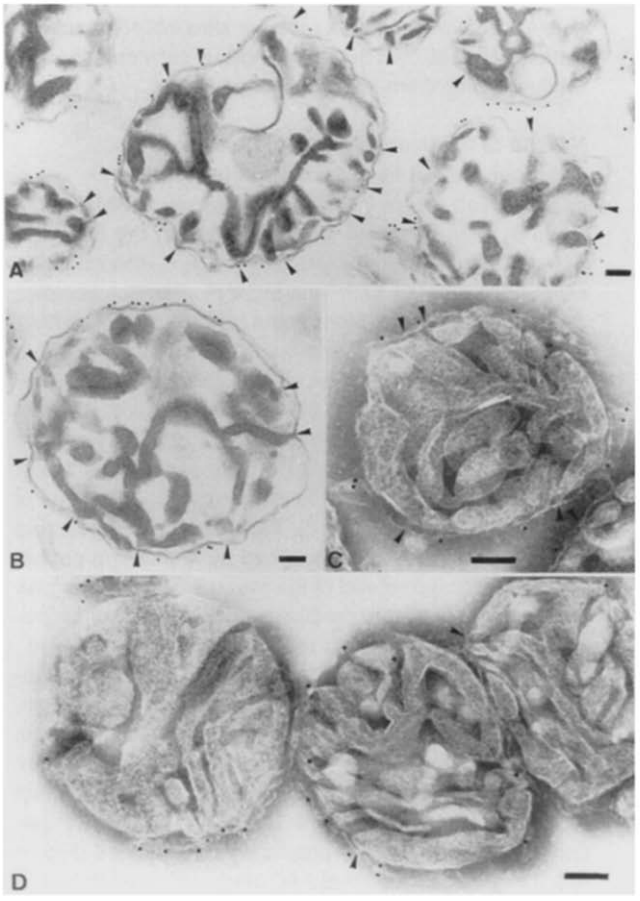

Figure 3. MOM19 Is Located in the Mitochondrial Outer Membrane

(A) and (B) show plastic sections of mitochondria that were prelabeled with anti-MOM19 IgG and decorated by protein A gold before fixation. The gold particles are visible on the periphery of the outer membrane. The arrowheads indicate the position of the contact sites.

(C) and (D) show cryosections of aldehyde-fixed mitochondria that were labeled with anti-MOM19 IgG followed by protein A–gold. The sections were negatively stained with a mixture of methyl cellulose and ammonium molybdate. Almost all of the gold particles are either on or close to the outer membrane. The contact sites are again shown by arrowheads. A: ×38,720; B: ×43,520; C: ×70,400; D: ×70,400. Bars, 100 nM.

sons: We previously reported that the affinity for binding of AAC and porin to mitochondria was similar and that AAC and porin appeared to use different import receptors (Pfaller et al., 1988; Pfaller and Neupert, 1987). Moreover, IgG and Fab fragments directed against another outer membrane protein inhibit binding and import of AAC but not of MOM19-dependent precursor proteins (T. Söllner, R. Pfaller, G. Griffiths, N. Pfanner, and W. Neupert, unpublished data). Import of cytochrome c, as assayed by the covalent addition of heme to apocytochrome c (Nicholson et al., 1987), was not affected either (Figure 5). The amounts of IgG required for inhibition of import were the same as the amounts required for immunoprecipitation of MOM19 after preincubation of mitochondria with IgG (data not shown).

The Inhibitory Effect of IgG and Fab Fragments on Import Is a Result of Specific Interaction with MOM19

The import of an unfolded form of the porin precursor (Pfaller et al., 1985) was inhibited by anti-MOM19 to the same extent as import of the native porin precursor (see Figures 4A and 8). Because this unfolded precursor form does not require cytosolic cofactors and ATP for its import (Pfaller and Neupert, 1987; Pfanner et al., 1988b), the inhibitory effect of anti-MOM19 is apparently not due to interference with cytosolic cofactors, the ATP-dependent import step, or the unfolding of precursors (Pfanner et al., 1987d). None of the precursor proteins themselves is recognized by anti-MOM19, either in their native conformation or after denaturation by heating in SDS-containing

Table 1. Distribution of MOM19 over the Mitochondrial Outer Membrane

	Experiment 1	Experiment 2	Average
Ratio of the length of contact sites to the length of outer membrane (%)	12.40 ± 0.80	9.40 ± 0.40	11.25 ± 0.50
Concentration of gold particles in contact sites (particles/μm)	3.52 ± 0.59	3.60 ± 0.34	3.57 ± 0.50
Concentration of gold particles on the outer membrane (minus contact sites) (particles/μm)	3.01 ± 0.33	2.63 ± 0.38	2.86 ± 0.22

The experiments were performed as described in the legend to Figure 3. Quantitation was performed as described in Experimental Procedures.

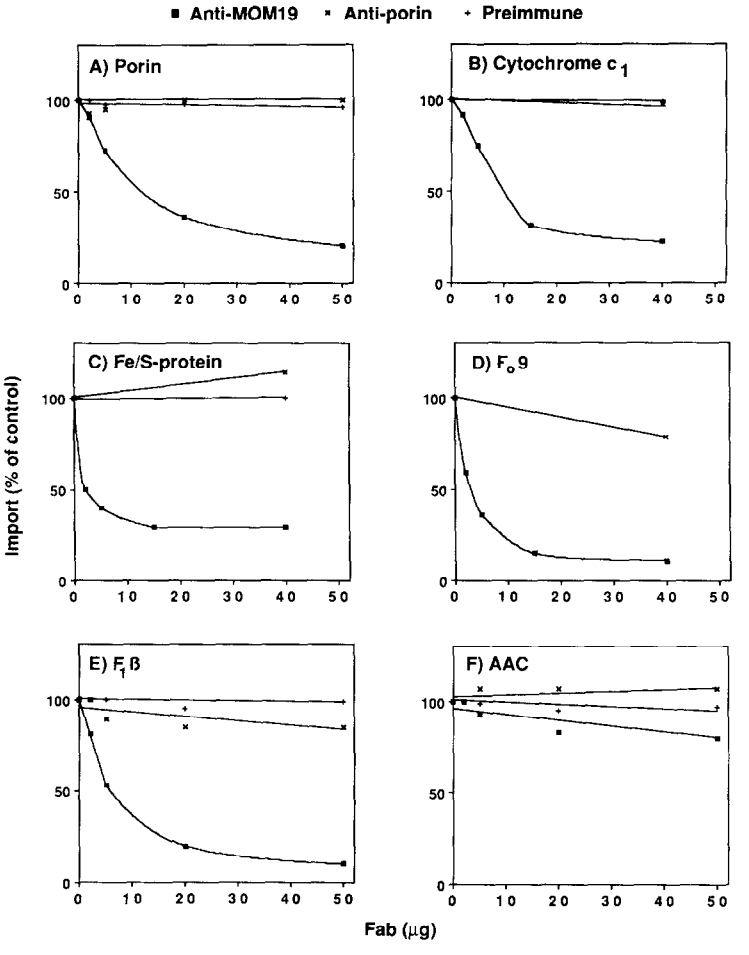

Figure 4. Antibodies against MOM19 Inhibit Import of a Subset of Mitochondrial Precursor Proteins

Fab fragments were prepared either from preimmune serum (+) or from antiserum directed against porin (x) or MOM19 (■) and incubated with mitochondria (10 μg of protein) for 35 min at 0°C in BSA buffer as described in Experimental Procedures. Following reisolation by centrifugation, mitochondria were incubated for 7 min at 25°C with various reticulocyte lysates containing [^{35}S]methionine-labeled precursor proteins in the presence of K-ascorbate (4 mM) and TMPD (0.2 mM) in BSA buffer. Import of porin, $F_1\beta$, and AAC was analyzed by resistance to treatment with 100 μg/ml proteinase for 20 min at 0°C, while import of Fe/S and cytochrome c_1 was controlled by resistance to 15 μg/ml proteinase K. Import of F_0 ATPase subunit 9 (F_09) was monitored by the processing of F_09 to its mature size. Mitochondria were reisolated, lysed in SDS-containing sample buffer, and resolved by SDS–PAGE. Imported porin (A), cytochrome c_1 (B), Fe/S protein (C), F_09 (D), $F_1\beta$ (E), and AAC (F) were quantified by laser densitometry of fluorographs of the resulting gels (Pfanner et al., 1987c).

buffer (data not shown). This excludes the trivial possibility that anti-MOM19 might bind to and consequently inactivate the cytosolic precursors proteins.

Is the inhibitory effect of anti-MOM19 caused by selective blocking of a mitochondrial surface component? We showed previously that precursor proteins can bypass protease-accessible surface components, thus resulting in a residual import (bypass import). Bypass import exhibits several criteria of mitochondrial protein import, including transport through contact sites between both mitochondrial membranes and dependence on both ATP and a membrane potential ($\Delta\psi$) across the inner membrane (Pfaller et al., 1989). If anti-MOM19 specifically inactivates a surface component, bypass import should not be inhibited. This is indeed the case (Figure 6). Unspecific effects of anti-MOM19, furthermore, can be excluded by the observation that the import of AAC, which, like the other precursor proteins, requires ATP, the general insertion protein (GIP) in the outer membrane, contact sites, and the membrane potential (Pfanner et al., 1987a, 1987d; Pfaller et al., 1988), is not inhibited (Figure 4F).

MOM19 Is Required for Specific Binding of Precursor Proteins to Mitochondria

The import pathway of $F_1\beta$ experimentally can be divided into two steps: insertion into contact sites between the two mitochondrial membranes and completion of translocation into the mitochondrial matrix (Schleyer and Neupert, 1985; Schwaiger et al., 1987; Pfanner et al., 1987a). In the case that MOM19 acts as a surface receptor, only the first step, transport into contact sites, should be inhibited by anti-MOM19. Figure 7A shows that preincubation of mitochondria with anti-MOM19 inhibited insertion of $F_1\beta$ into translocation contact sites, whereas the completion of transport from contact sites into the mitochondrial matrix was unaffected (Figure 7B).

Is MOM19 required for high affinity binding of precursor proteins to mitochondria? Binding of precursor proteins to mitochondria can be divided into specific high affinity binding that involves a protease-accessible receptor site and unspecific (nonproductive) low affinity binding (Pfaller and Neupert, 1987). Precursor proteins can be accumulated at the level of binding to mitochondria, e.g., by incubation with isolated mitochondria at 0°C (in case of porin) or in the absence of a membrane potential across the inner membrane (in case of F_09) (Pfaller and Neupert, 1987; Pfanner et al., 1987c). Scatchard analysis revealed that anti-MOM19 completely inhibited the high affinity binding of the precursor of porin to mitochondria (Figure 8), strongly suggesting that MOM19 is a specific mitochondrial sur-

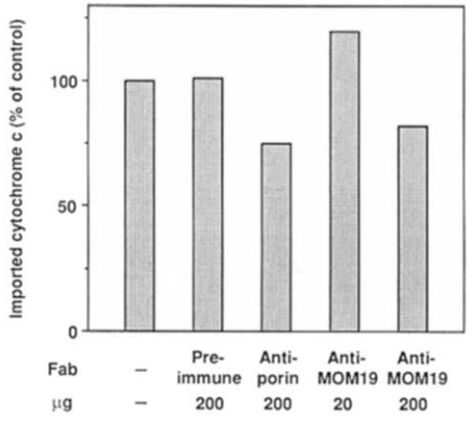

Figure 5. Antibodies against MOM19 Do Not Inhibit Import of Cytochrome c

Prebinding of Fab fragments to mitochondria (50 μg of protein) was performed exactly as described in Figure 4. Following reisolation, mitochondria were incubated with 30 μl of reticulocyte lysate containing [^{35}S]cysteine-labeled apocytochrome c in SEM buffer containing hemin (3 μM) and sodium dithionite (1 mg/ml) for 10 min at 25°C. Levels of holocytochrome c formed were determined following immunoprecipitation, digestion of immunocomplexes with trypsin, and subjection to reverse-phase HPLC (Nicholson et al., 1987).

face receptor. In further support of this, we found that anti-MOM19 inhibited the specific binding of the precursor of $F_0$9 to the mitochondrial surface (Figure 9).

A 17 kd Fragment of MOM19 Is Able to Mediate Import of $F_1\beta$, but Not of Other Precursor Proteins

We reported previously that pretreatment of mitochondria with relatively low concentrations of elastase strongly inhibited subsequent import of precursors like porin, Fe/S protein, and $F_0$9; however, import of $F_1\beta$ was practically

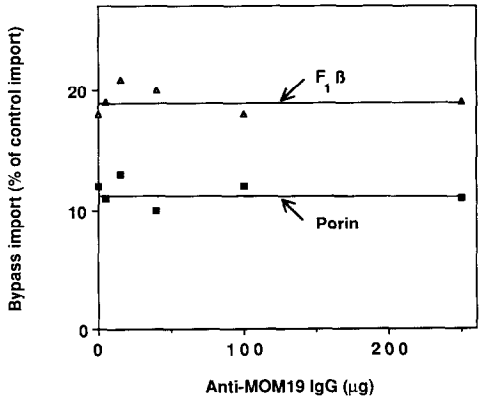

Figure 6. Antibodies against MOM19 Do Not Inhibit Bypass Import

Isolated mitochondria were pretreated with trypsin (15 μg/ml) as described (Pfanner and Neupert, 1987a). IgGs were preincubated with the trypsin-treated mitochondria as described in the legend of Figure 4. Mitochondria were reisolated and tested for their ability to import [^{35}S]methionine-labeled porin and $F_1\beta$ precursor proteins. For comparison, import of both porin and $F_1\beta$ into non-trypsin-treated mitochondria was inhibited by ~75% by prebinding 40 μg of IgG against MOM19 (data not shown).

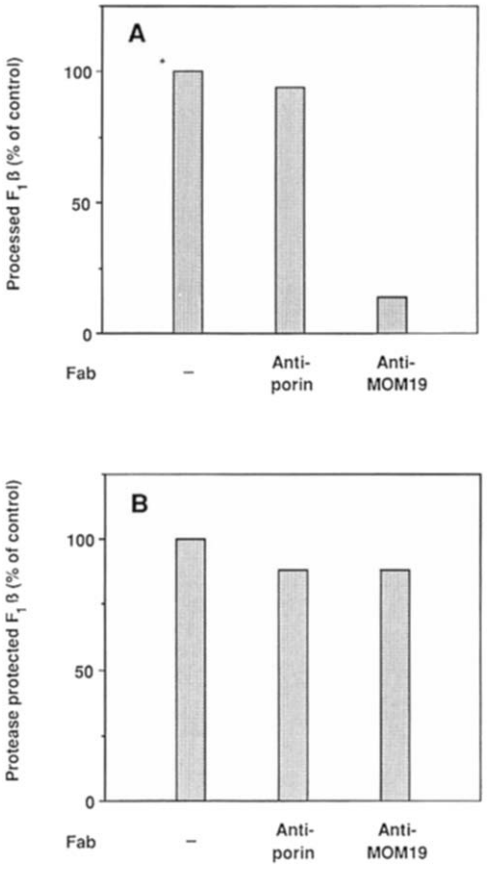

Figure 7. Antibodies against MOM19 Inhibit Translocation of $F_1\beta$ into Contact Sites

(A) Inhibition of formation of contact site intermediate. Reticulocyte lysates containing [^{35}S]methionine-labeled $F_1\beta$ precursor ($pF_1\beta$) and mitochondria (1 mg/ml protein in SEM buffer) were treated (separately) with 0.7 U/ml apyrase as described (Pfanner et al., 1987d). Aliquots of mitochondria were then preincubated either with SEM or with 40 μg of Fab fragments against porin or MOM19 as described in the legend to Figure 4. Mitochondria were reisolated and combined with the apyrase-treated reticulocyte lysate in BSA buffer in the presence of 6 mM ADP, 4 μM antimycin A, 10 μM oligomycin, 4 mM K-ascorbate, and 0.2 mM TMPD. Following incubation for 25 min at 25°C, mitochondria were reisolated and resolved by SDS–PAGE. $F_1\beta$ of mature size was quantified by laser densitometry of the fluorograph. Eighty-eight percent of the processed $F_1\beta$ was accessible to externally added protease (data not shown), i.e., it was trapped in contact sites (Schleyer and Neupert, 1985).

(B) Completion of translocation into the matrix is not inhibited. Apyrase treatment of isolated mitochondria and reticulocyte lysate containing [^{35}S]methionine-labeled $pF_1\beta$ was performed as described for (A). Mitochondria (10 μg of protein) were then incubated with the reticulocyte lysate for 25 min at 25°C as described for (A) to accumulate $F_1\beta$ in contact sites (Pfanner et al., 1987d). Following reisolation, mitochondria were resuspended in BSA buffer containing 1 μM valinomycin, and 40 μg of Fab fragments against either porin or MOM19 was added. After incubation for 30 min at 0°C, GTP to a final concentration of 6 mM was added, and the reaction was continued for 15 min further at 25°C to complete translocation into the matrix (Pfanner et al., 1987d). Samples were treated with 100 μg of proteinase K/ml.

unaffected at these elastase concentrations (Zwizinski et al., 1984; Pfaller et al., 1988). We had concluded, therefore, that the receptor sites for $F_1\beta$ and the other precursors are either two (or more) distinct proteins, or that one

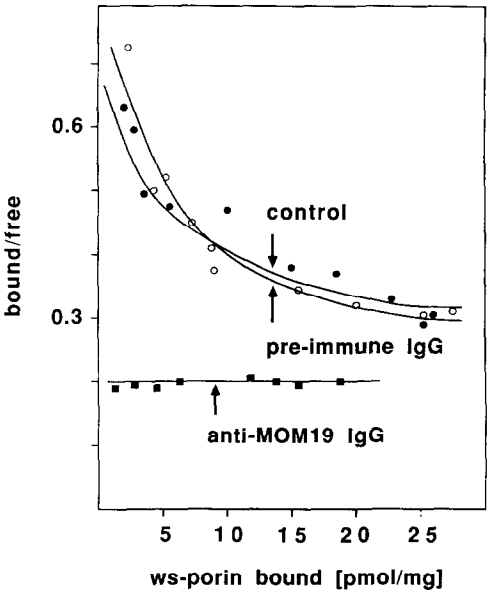

Figure 8. Antibodies against MOM19 Inhibit High Affinity Binding of Porin to Mitochondria

Aliquots of mitochondria (40 μg of protein) were preincubated with either no IgG or 100 μg of preimmune IgG, or IgG against MOM19 as described in the legend to Figure 4. Binding of ^{14}C-labeled water-soluble porin to the reisolated mitochondria was analyzed as described in Experimental Procedures.

protein possesses distinct functional sites. Figures 10A and 10B show that treatment of mitochondria with elastase generated an 18 kd fragment of MOM19, followed by formation of a 17 kd fragment. Import of porin and other precursors correlated with the presence of MOM19 and the 18 kd fragment, whereas import of $F_1\beta$ still occurred when only the 17 kd fragment remained (Figure 10C). This provides further strong evidence for the role of MOM19 as a specific import receptor and suggests that distinct parts

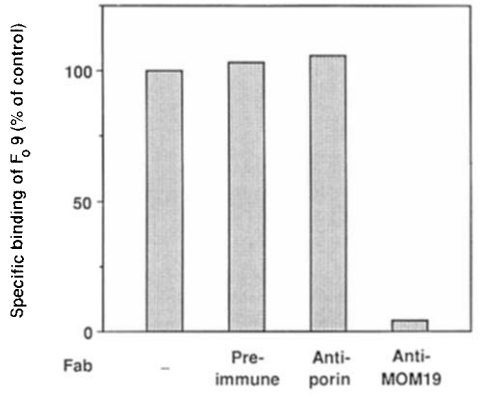

Figure 9. Antibodies against MOM19 Inhibit Specific Binding of F_o9 to the Mitochondrial Surface

Aliquots of mitochondria (10 μg of protein) were preincubated with Fab fragments (40 μg) prepared from antiserum against porin or MOM19 as described in Figure 4. Specific binding of F_o9 to the reisolated mitochondria was analyzed as described in Experimental Procedures.

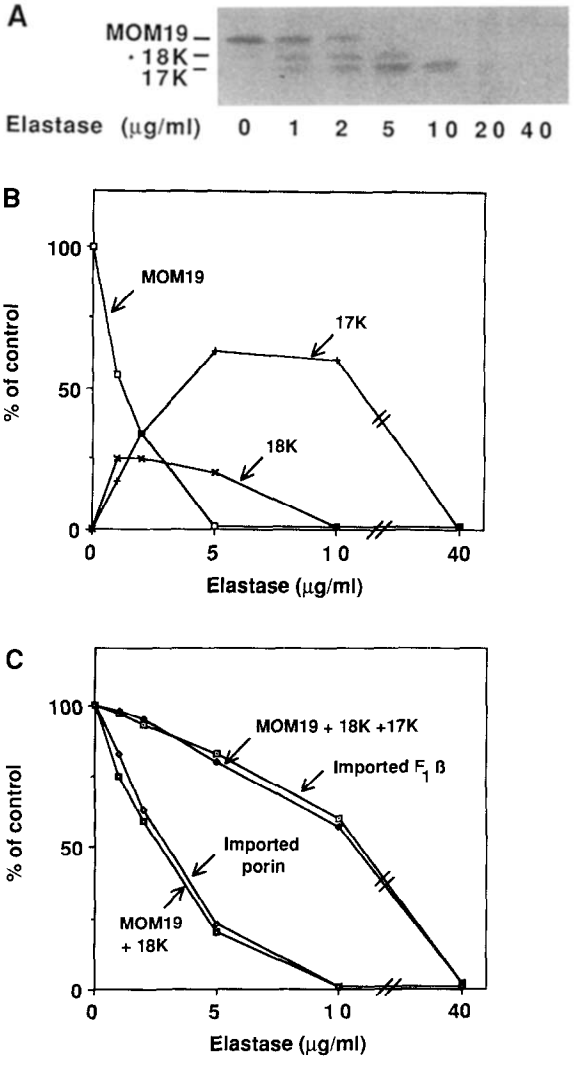

Figure 10. A 17 kd Fragment of MOM19 Can Mediate Import of $F_1\beta$

Isolated mitochondria were pretreated with various concentrations of elastase (Pfaller et al., 1988).

(A) Western blotting with antibodies against MOM19. Mitochondrial protein were separated by SDS–PAGE and immunoblotted with antibodies against MOM19.

(B) Degradation of MOM19. Bands in (A) were quantified by laser densitometry. The amount of MOM19, 18 kd, and 17 kd are expressed as percent of MOM19 in the control.

(C) Correlation of degradation of MOM19 with import of precursors. Precursors to $F_1\beta$ and porin were incubated with mitochondria pretreated with various concentrations of elastase, and import was determined. For comparison, the amounts of MOM19 plus 18 kd and 17 kd are plotted, as well as the amounts of MOM19 plus 18 kd fragment (see A and B). One hundred percent control represents mitochondria not pretreated with elastase.

of MOM19 may be involved in the interaction with various precursor proteins.

Discussion

We have identified an organellar membrane component involved in high affinity binding of cytosolic precursor proteins, the mitochondrial outer membrane protein MOM19.

MOM19 is required for import of precursor proteins (porin, cytochrome c_1, Fe/S protein, $F_0$9, and $F_1\beta$) into the different mitochondrial subcompartments.

The role of MOM19 in high affinity binding of precursor proteins, in conjunction with the following observations, provides strong evidence that MOM19 acts as a specific import receptor. First, consistent with the previous finding that porin and AAC use different receptor sites (Pfaller et al., 1988), we find that import of AAC does not involve MOM19. Second, a mild elastase treatment of mitochondria initially generates an 18 kd fragment and subsequently a 17 kd fragment of MOM19. Import of porin and other precursors depends upon the presence of MOM19 (or the 18 kd fragment), whereas the import of $F_1\beta$ remains unaffected as long as the 17 kd fragment is present. This clarifies the observation from previous studies that suggested the existence of distinct receptors for porin and $F_1\beta$, because of a differential sensitivity towards treatment with elastase (Zwizinski et al., 1984; Pfaller et al., 1988). MOM19 may thus carry functionally distinct segments for different precursors. Next, MOM19 cannot be the common membrane insertion site (GIP) in the outer membrane for several reasons. Porin and AAC use the same GIP sites (Pfaller et al., 1988). GIP, in contrast to MOM19, is apparently not accessible to low concentrations of proteases added to intact mitochondria, such as trypsin or proteinase K (Pfaller et al., 1988; Pfanner and Neupert, 1987a). After protease treatment of mitochondria, the residual import, that bypasses receptor sites, still involves GIP sites (Pfaller et al., 1989) but is not inhibited by anti-MOM19 antibodies or Fab fragments. Finally, anti-MOM19 antibodies do not interfere with the precursor proteins or cofactors in the cytosol, and anti-MOM19 antibodies do not inhibit the ATP-dependent import step, the unfolding of precursor proteins, or translocation of precursors from the outer membrane into and across the inner membrane. MOM19 thus fulfills all of the various criteria established for a mitochondrial import receptor. We conclude that MOM19 is (or is closely associated with) a specific receptor protein.

How many different receptors exist to direct precursor proteins into mitochondria? Our results suggest that at least two distinct surface receptors exist, MOM19 and a further receptor for ADP/ATP carrier (AAC) and similar precursors. In fact, IgG and Fab fragments directed against another outer membrane protein inhibit import and high affinity binding of AAC, but not of porin, $F_1\beta$, and other precursors (Söllner et al., unpublished data). AAC and probably similar proteins, such as the uncoupling protein, are unique in that they do not carry a signal sequence at the amino terminus of the precursor. They display a tripartite structure and are assumed to contain a signal sequence in the carboxy-terminal half of each of the three domains (Pfanner et al., 1987b; Smagula and Douglas, 1988). Moreover, these proteins were most likely not present in the prokaryotic ancestors of mitochondria (Klingenberg, 1985) but probably introduced after the endocytosis event had taken place. They may have acquired particular targeting sequences and their own specific surface receptor (Pfanner and Neupert, 1987b). In contrast, proteins like cytochrome c_1, Fe/S protein, $F_0$9, and $F_1\beta$, which use MOM19, were already present in the prokaryotic cell, and after transfer of their genes to the nucleus, they acquired amino-terminal signal sequences that direct them to their surface receptor. Neither the evolutionary origin nor the targeting sequence(s) of porin, a protein that also uses MOM19, are known.

The import pathway of cytochrome c is quite distinct from the pathways of all other mitochondrial precursor proteins studied thus far. The precursor protein apocytochrome c apparently does not use a protease-sensitive surface receptor (Nicholson et al., 1988) but may spontaneously insert into the outer membrane (Rietveld et al., 1985). Translocation into the intermembrane space is tightly coupled to covalent addition of heme by the intermembrane space enzyme cytochrome c heme lyase (Nicholson et al., 1988; Nargang et al., 1988). Consistently, anti-MOM19 antibodies do not inhibit import of cytochrome c.

In summary, import of mitochondrial precursor proteins involves at least three distinct targeting pathways: recognition of precursor proteins by MOM19; recognition of AAC and related precursors by a different surface receptor; and import of cytochrome c that is independent of protease-accessible surface components. Our current data suggest that the pathway involving MOM19 is the major one used by many different precursor proteins. The first two of these three import pathways converge at the common membrane insertion site in the outer membrane (GIP) (Pfaller et al., 1988).

Experimental Procedures

Isolation of Mitochondria

N. crassa wild-type 74 A was grown and harvested as described (Schleyer et al., 1982). For labeling with [^{35}S]sulfate, the concentration of unlabeled sulfate in the growth medium was reduced to 0.08 mM, and 4 mCi of [^{35}S]sulfate (Amersham) was added per liter of culture. Mitochondria were isolated either by differential centrifugation (Pfanner and Neupert, 1985) or by Percoll density-gradient centrifugation (Hartl et al., 1986) as described, except that the hyphae were ground for 30–60 s. After washing in SEM (250 mM sucrose, 1 mM EDTA, 10 mM 3-[N-morpholino]propanesulfonic acid [MOPS], adjusted to pH 7.2 with KOH), mitochondria were resuspended in SEM at a protein concentration of 1–6 mg/ml.

Preparation of Outer Membrane

Mitochondria were resuspended in swelling buffer (5 mM KPi [pH 7.2], 1 mM PMSF [phenylmethylsulfonyl fluoride], 300 μg/ml cytosolic protease inhibitor fraction from N. crassa [Schmidt et al., 1984]) at a final concentration of 5–6 mg/ml. After 25 min at 0°C the same volume of swelling buffer was added, and the mitochondria were ruptured by 15 strokes in a potter homogenizer. The homogenate was layered on a discontinuous sucrose gradient (1 ml of 60% sucrose, 4 ml of 32% sucrose, 1.5 ml of 15% sucrose [w/v], each containing 1 mM EDTA, 1 mM PMSF and buffered with 10 mM MOPS/KOH [pH 7.2]) and centrifuged at 134,000 × g for 60 min at 2°C in a SW41 rotor (Beckman Instruments, Inc.). The outer membrane fraction was recovered from the 32%/15% interface, and a 70% sucrose solution was added to achieve a final sucrose concentration of 50%. The outer membrane was further purified by flotation on a second discontinuous sucrose gradient (5 ml of 50% sucrose containing the outer membrane, 5 ml of 32% sucrose [w/v], 1.5 ml of gradient buffer [10 mM MOPS/KOH (pH 7.2), 1 mM EDTA, 1 mM PMSF]) by centrifugation in a SW41 rotor at 240,000 × g for 5 hr. The outer membrane fraction from the 0%/32% interface was spun at 166,000 × g for 60 min or precipitated with trichloroacetic acid (TCA).

Raising of Antisera and Preparation of IgG and Fab Fragments

Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 12% acrylamide, 0.24% N,N'-methylene-bis-acrylamide) and transferred to nitrocellulose overnight at 4°C at 6 V/cm in 20 mM Tris, 150 mM glycine, 0.02% SDS, 20% methanol. After staining with Ponceau S, distinct bands were excised, solubilized with dimethyl sulfoxide, and used as immunogens in rabbits as described (Knudsen, 1985; Hawlitschek et al., 1988). Antisera were prepared, the complement was heat inactivated (20 min at 56°C), and IgGs were prepared by using protein A Sepharose column (Pharmacia) chromatography. Bound IgGs were eluted with 0.1 M citrate (pH 3), neutralized with 2 M Tris base, dialyzed against distilled water, and concentrated by lyophilization.

IgGs were digested with papain according to Mage (1981). Fab fragments were separated from intact IgGs and Fc fragments by using a protein A Sepharose column. Fab fragments were dialyzed against water, lyophilized, and dissolved in SEM buffer.

Binding and Import of Precursor Proteins in Vitro

Precursor proteins were synthesized in rabbit reticulocyte lysates (Pelham and Jackson, 1976) that were programmed with specific RNA transcribed by SP6-RNA-polymerase (Melton et al., 1984) from pGEM3 plasmids (Promega) and labeled with [^{35}S]methionine (500 Ci/mmol, Amersham, Buchler). Postribosomal supernatants were prepared and supplemented as described (Zimmermann and Neupert, 1980).

IgG or Fab fragments were incubated with mitochondria (10 μg of protein) in BSA buffer (250 mM sucrose, 3% [w/v] BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH [pH 7.2]) containing α_2–macroglobulin (130 μg/ml), N. crassa protease inhibitor fraction (100 μg/ml; Schmidt et al., 1984) in a final volume of 150 μl. Import mixtures contained 5%–30% reticulocyte lysate in BSA buffer and 10 μg of mitochondria in a final volume of 200 μl. Addition of antimycin A, oligomycin, valinomycin, potassium ascorbate, N,N,N',N'-tetramethylphenylenediamine (TMPD) and treatment of mitochondria with trypsin or proteinase K were performed as published (Pfanner and Neupert, 1987a). Immunoprecipitations and SDS–PAGE were performed according to Laemmli (1970), Schleyer et al. (1982), and Pfanner and Neupert (1985). All reactions were made chemically identical by adding the same volume of reagent-free solvent to the control sample.

Analysis of High Affinity Binding of Porin to Mitochondria

^{14}C-labeled water-soluble porin was incubated with mitochondria in 0.5 ml of a buffer containing 2.4% (w/v) BSA, 0.2 M KCl, 20 mM NaPi, 0.2 mM PMSF, 8 mM MOPS/KOH (pH 7.2) for 20 min at 0°C. Mitochondria were reisolated by centrifugation, and aliquots of the supernatant were removed to determine free radioactivity. Pellets were resuspended in KMBP buffer (3% [w/v] BSA, 180 mM KCl, 0.2 mM PMSF, 10 mM MOPS/KOH [pH 7.2]) and transferred to new tubes, and mitochondria were pelleted once again. Radioactivity associated with mitochondria was determined by liquid scintillation counting (Pfaller and Neupert, 1987).

Binding of the Precursor of F_o9 to the Mitochondrial Surface

Reticulocyte lysate containing [^{35}S]methionine-labeled precursor of F_o ATPase subunit 9 (pF_o9) was incubated with isolated mitochondria in the presence of antimycin A, oligomycin, and BSA buffer. Samples were incubated for 20 min at 25°C. Mitochondria were reisolated, washed once in SEM buffer, and lysed in SDS-containing sample buffer. Samples were analyzed by electrophoresis and quantified by densitometry of the resulting fluorograph. Levels of specifically bound pF_o9 were calculated by subtracting the amount of pF_o9 unspecifically bound to mitochondria. Unspecific binding of pF_o9 was measured by quantifying the levels of pF_o9 bound to mitochondria that had been pretreated with 15 μg/ml trypsin (Pfanner et al., 1987c).

Electron Microscopy

Small pieces of the pellets of fixed mitochondria were mounted on copper stubs and prepared for cryosectioning and labeling as described previously (Griffiths et al., 1983, 1984). The prelabeled mitochondria were centrifuged and fixed in glutaraldehyde, and the pellets were treated with 1% OsO_4 in 0.1 M cacodylate buffer for 30 min followed by 0.5% uranyl acetate in water for 30 min. Following ethanol/propylene oxide dehydration, the pellets were embedded in Epon.

For quantitation of the prelabeled mitochondria, 24 random micrographs were taken of the Epon sections at a primary magnification of ×24,000. These were enlarged ×2.5 and printed. The position of the contact sites were marked with a pen, and a double square lattice grid (Weibel, 1979) was positioned over the image. Intersections of the outer membrane with the coarse lines of the lattice grid were counted as well as intersections of the contact site with the fine mesh lines. Each coarse line was equivalent to five fine lines. The ratio of intersections with the fine lines over the contact sites in relation to the total intersections with the outer membrane produced the ratio of the surface area of contact sites to the total surface of outer membrane. The gold particles associated with the contact sites as well as the total gold particles were counted. The number of gold particles per length of contact sites and total membrane were calculated as described in Griffiths and Hoppeler (1986).

Acknowledgments

We thank Heinz Horstmann for preparing electron micrographs and Christine Forster and Brigitte Stelzle for expert technical assistance. We are grateful to Dr. Rosemary Stuart for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 184, project B1) and the Fonds der Chemischen Industrie.

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Received July 31, 1989; revised September 20, 1989.

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