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Insertion of MOM22 into the mitochondrial outer membrane strictly depends on surface receptors

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Targeting of preproteins to mitochondria and their translocation across the outer membrane are mediated by the mitochondrial receptor complex. This protein complex contains the import receptors MOM19 and MOM72 and the general insertion pore GIP. All seven components of the receptor complex are synthesized in the cytosol and thus have to be targeted to the mitochondria themselves. Here we investigated the import pathway of the precursor of MOM22 into the outer membrane. In contrast to other mitochondrial preproteins studied so far, the import of MOM22 absolutely depended on the presence of surface receptors. In fact, both receptors MOM19 and MOM72 were involved in its import pathway. The targeting of MOM22 to mitochondria is thus highly specific and controlled.

Mitochondrion; Outer membrane; Protein translocation; Import receptor

1. INTRODUCTION

The mitochondrial outer membrane contains a machinery for the recognition and translocation of cytosolically synthesized preproteins [1,2]. This machinery seems to be present in a high molecular weight complex that consists of (at least) seven proteins. The complex contains the two preprotein receptors MOM19 and MOM72 (mitochondrial outer membrane proteins of 19 kDa and 72 kDa, respectively), and five other proteins, MOM38, MOM30, MOM22, MOM8 and MOM7 [3,4]. MOM19 and MOM72 expose large domains to the cytosol that are easily degraded by proteases added to isolated mitochondria. MOM38, MOM30, MOM8 and MOM7 are quite well protected aginst added proteases and are in close proximity to the general insertion pore GIP that mediates the translocation of preproteins across the outer membrane. The seventh component, MOM22, is partially exposed on the outer membrane surface [5] and is involved in the transfer of preproteins from the receptors to GIP (M. Kiebler et al., in preparation).

All components of the receptor complex are encoded by nuclear genes and are synthesized on cytosolic polysomes. This raises the question as to how these components are targeted to mitochondria themselves. The precursor of MOM72 was found to use MOM19 as its import receptor [6], emphasizing the role of MOM19 as mitochondrial master receptor. The precursor of MOM9, however, did not require a protease-sensitive surface receptor for its import into the mitochondrial receptor complex; the specificity of import of MOM19 appeared to be controlled by its assembly with MOM38 [7].

For this report, we analysed the import of MOM22 and found a third pathway for targeting a preprotein into the mitochondrial receptor complex. Both receptors, MOM19 and MOM72, were involved in the import of MOM22. Moreover, the import of MOM22 was completely blocked by a removal of the surface receptors. This is in contrast to the import of all other preproteins that had been analysed so far; these preproteins could be imported in the absence of surface receptors via the so-called bypass route, albeit with a usually low efficiency [8]. On the bypass route, preproteins probably directly enter the GIP (in some cases such as the import of MOM19 or the subunit Va of yeast cytochrome *c* oxidase, the 'bypass pathway' is even the major route [7,9]). MOM22 is thus the first preprotein that is unable to use the bypass route.

2. MATERIALS AND METHODS

The following procedures were performed as described previously [6,10–12]: in vitro transcription and translation of a preprotein in rabbit reticulocyte lysates in the presence of [^{35}S]methionine; isolation of mitochondria from *Neurospora crassa* wild-type 74A; prebinding of antibodies to mitochondria; in vitro import of a preprotein into mitochondria; treatment of mitochondria with trypsin or proteinase K; co-immunoprecipitation of the mitochondrial receptor complex after lysis of mitochondria with digitonin [3–5]; SDS-polyacrylamide gel electrophoresis, fluorography and laser densitometry.

A typical import assay contained 15 μl reticulocyte lysate, isolated mitochondria (25 μg protein), and BSA-buffer (3% [w/v] bovine serum

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Abbreviations: GIP, general insertion protein/pore; MOM*y*, mitochondrial outer membrane protein of *y* kDa.

albumin, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.2) in a final volume of 100–200 μl. Import was performed for 7 min at 25°C, followed by a treatment with trypsin for 15 min at 0°C. Prebinding of antibodies to mitochondria was performed in SEM-buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) for 35 min at 4°C.

3. RESULTS AND DISCUSSION

A full-length cDNA of *N. crassa* MOM22 inserted into pGEM4 (M. Kiebler et al., in preparation) was used to synthesize the precursor of MOM22 in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. As expected for mitochondrial outer membrane proteins (that are not proteolytically cleaved during import [1,2]), the preprotein had the same apparent size as the mature protein. When incubated with isolated *N. crassa* mitochondria, the preprotein efficiently associated with the mitochondria. A treatment of the mitochondria with trypsin led to the formation of fragments of MOM22, in particular a 12 kDa-fragment (Fig. 1A), that were similarly found with the endogenous MOM22 (not shown). When the mitochondria were lysed with Triton X-100 before the protease treatment, MOM22 and the 12 kDa-fragment were digested (Fig. 1B, columns 3 and 4). The amount of MOM22 and 12 kDa-fragment resistant to 80 μg/ml trypsin (Fig. 1B, columns 1 and 2) was taken as measure for the import of MOM22 into mitochondria. The import of MOM22 was not inhibited by dissipation of the membrane potential across the inner membrane (Fig. 1C), but depended on the presence of ATP (not shown). Its import thus fulfils the typical properties of import of outer membrane proteins [1,2,13].

Is the MOM22 imported in vitro correctly inserted into the mitochondrial receptor complex? We lysed mitochondria with the detergent digitonin after the import reaction and co-immunoprecipitated the mitochondrial receptor complex with antibodies directed against MOM19 [3–5] or MOM38. The imported MOM22 was indeed coprecipitated (Fig. 2), indicating that the MOM22 was not only inserted into the outer membrane, but also assembled into its functional location in the receptor complex.

We then tested if the import of MOM22 depended on mitochondrial surface receptors. We used the standard procedure, a mild pre-treatment with trypsin [12], that led to a degradation of MOM19 and MOM72, but did not damage the outer membrane barrier. Surprisingly, the import of MOM22 was thereby completely blocked (Fig. 3, column 2). When the mitochondria were preloaded with antibodies or Fab fragments directed against MOM19, the import of MOM22 was strongly inhibited (Fig. 3, columns 4–6), indicating that the master receptor MOM19 was required for the import of MOM22. Moreover, antibodies against MOM72 also showed a clear inhibitory effect on the import of MOM22 (Fig. 3, columns 7 and 8). This result was unexpected as all other preproteins which were previously found to use MOM19 as main receptor were practically not inhibited by antibodies against MOM72 [6,14]. Control antibodies against the major outer membrane protein porin or from preimmune sera did not show any inhibitory effect (Fig. 3, columns 3 and 9).

Could the inhibitory effect of antibodies against MOM19 or MOM72 on the import of MOM22 be just explained by indirect effects and not be due to an inactivation of the import receptor function? This seems to be excluded for several reasons. (i) The antibodies are

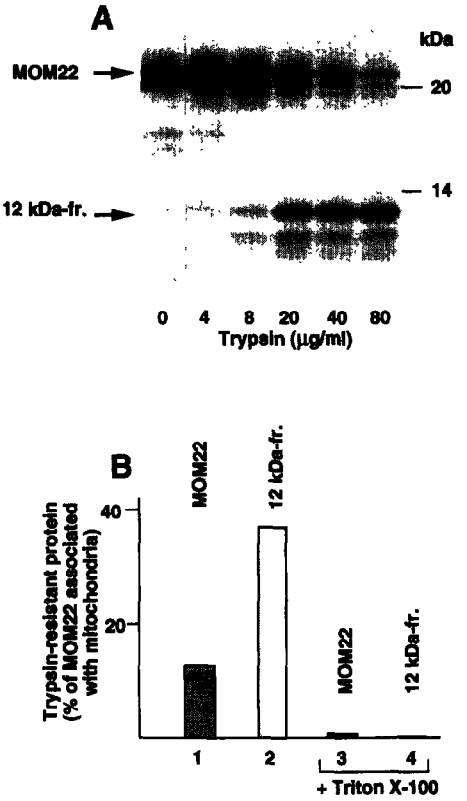

Fig. 1. Characteristics of import of MOM22 into isolated mitochondria. (A) Formation of tryptic fragments of imported MOM22. Reticulocyte lysate with ^{35}S-labeled precursor of MOM22 was incubated with isolated *N. crassa* mitochondria, followed by a treatment with trypsin as described in section 2. The mitochondria were re-isolated and analysed by SDS-PAGE and fluorography. Similar fragments were found after a treatment of mitochondria with proteinase K. 12 kDa-fr., 12-kDa fragment of MOM22. (B) After import of MOM22, the mitochondria were re-isolated, and samples 3 and 4 received 1% (w/v) Triton X-100. Then all samples were treated with trypsin (80 μg/ml). Triton X-100 was now added to samples 1 and 2. The proteins were precipitated by trichloroacetic acid and analysed by SDS-PAGE, fluorography and laser densitometry, including correction for the different number of methionines in MOM22 and the 12 kDa-fragment [12]. The total amount of ^{35}S-labeled MOM22 associated with the mitochondria (without treatment with trypsin) was set to 100%. (C) Import of MOM22 does not require a membrane potential $\Delta\Psi$ across the inner membrane. The import of MOM22 into isolated mitochondria (column 1) was not inhibited by dissipation of $\Delta\Psi$ (column 2: addition of 0.2 μM valinomycin, 8 μM antimycin A and 20 μM oligomycin [10]). As control, the import of F_1-ATPase subunit β (column 3) was inhibited by dissipation of $\Delta\Psi$ (column 4). The mitochondria were treated with trypsin (80 μg/ml) after the import reaction.

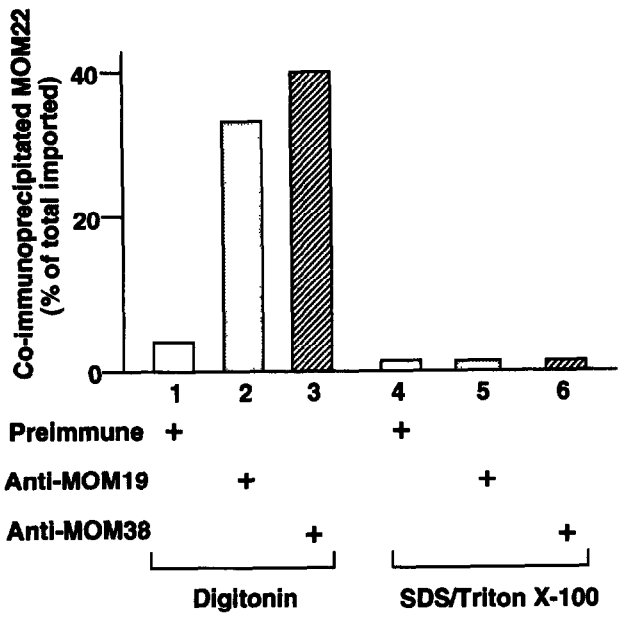

Fig. 2. In vitro imported MOM22 is assembled into the mitochondrial receptor complex. MOM22 was imported into isolated mitochondria as described in section 2. The mitochondria were re-isolated and lysed in buffer with 0.5% digitonin (samples 1–3) [3–5]. Immunoprecipitation with preimmune serum, antiserum directed against MOM19 or antiserum directed against MOM38 [5,11] was performed. The amount of precipitated ^{35}S-labeled MOM22 was compared to the total amount of MOM22 imported into the mitochondria (as quantified in Fig. 1, set to 100%). As control (samples 4–6), the re-isolated mitochondria were boiled in SDS-containing buffer and immunoprecipitations were performed in Triton X-100 containing buffer; since the mitochondrial receptor complex was thereby dissociated [3–5], no co-immunoprecipitations were observed.

prebound to intact mitochondria that contain plenty of endogenous MOM22 in the receptor complexes. Therefore, antibodies that would pose a steric hindrance on the final position of MOM22 in the receptor complex should not bind to these mitochondria. (ii) The mild pretreatment with trypsin completely blocks the import of MOM22. In this case a steric hindrance is of course excluded, and we should keep in mind that under the identical conditions the import of MOM19 was not inhibited at all [7], demonstrating that the mitochondria were not unspecifically damaged.

We conclude that the import of MOM22 into the mitochondrial outer membrane is controlled by the two surface receptors MOM19 and MOM72. A dependence on both MOM19 and MOM72 had also been found for the import pathway of the ADP/ATP carrier [3,14]. In the case of the ADP/ATP carrier, three parallel import pathways exist, the major pathway via MOM72 (about 70% of import), a second one via MOM19 (about 25%) and a third one via the bypass route without surface receptors (5–10%). For the import pathway of MOM22, the situation is quite different as no bypass import is possible and the inhibitory effects of anti-MOM19 and anti-MOM72 antibodies sum up to much more than 100%, suggesting that the receptors do not act on parallel and independent import routes, but are required on the same unique pathway.

The import of MOM22 also provides additional evidence for the mechanism of the bypass import. As its import is fully blocked by a trypsin-pretreatment, it can be excluded that the bypass import, which is measured after a trypsinization of mitochondria [6,8], is due to a small amount of remaining MOM19 or MOM72 that may not have been degraded. Bypass import has been proposed to occur by a direct insertion of preproteins into the GIP [1,2,8] and the results reported here are

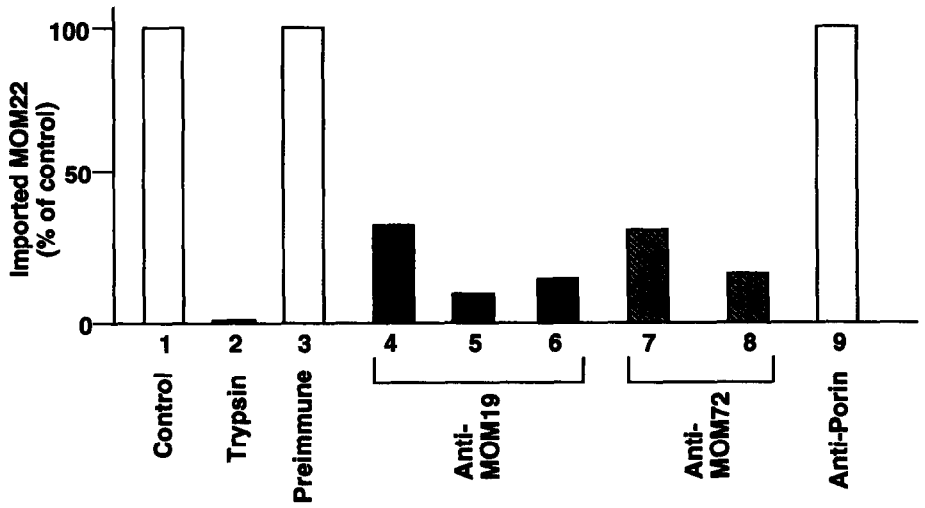

Fig. 3. MOM19 and MOM72 are involved in the import pathway of MOM22. Isolated mitochondria were pretreated with trypsin (20 μg/ml) and re-isolated (sample 2), or preincubated with immunoglobulins G from preimmune serum (150 μg, sample 3) or directed against MOM19 (40 μg, sample 4; 80 μg, sample 5; 60 μg Fab fragments, sample 6), MOM72 (70 μg, sample 7; 150 μg, sample 8) or porin (150 μg, sample 9). Then MOM22 was imported. Further analysis was performed as described in section 2 and the legend of Fig. 1.

consistent with this model. The assembly route of MOM22 into the mitochondrial receptor complex obviously deviates from the general import pathway before the bypass entry site (i.e. GIP) is reached, explaining the inability of MOM22 to use the bypass pathway.

In summary, the targeting of MOM22 to mitochondria and its assembly into the mitochondrial receptor complex occur by a new and strictly receptor-controlled pathway.

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