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# Cloning and Characterization of a 35-kDa Mouse Mitochondrial Outer Membrane Protein MOM35 with High Homology to Tom40[1]

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We have cloned a 35-kDa protein from a mouse cDNA library with a 25% overall amino acid identity to yTom40 and 27% identity to nTom40. This homolog to Tom40 was named MOM35. It contains two possible start codons 36 amino acids apart from each other. Both the long and the short version of MOM35 can be imported *in vitro* into mouse mitochondria. The identified protein is imported into the outer mitochondrial membrane and comprises a trypsin-resistance pattern similar to that of nTom40. Tom40 of *N. crassa*, *S. cerevisiae*, and the protein identified herein contains a highly conserved region with possible physiological importance. Subsequent investigation has revealed that this region interacts specifically *in vitro* with preproteins proposed to be imported by a Tom40-dependent pathway.

**KEY WORDS:** Mitochondria; protein import; Tom40; mammalian Tom complex.

## INTRODUCTION

Most of the proteins that reside in the mitochondria are nuclear gene products. These proteins are translated in the cytoplasmic compartment and subsequently transported to the organelle. The mitochondrial import machinery has been very well characterized for lower eukaryotes. An import machinery comprised of receptor proteins and the general insertion pore in the outer membrane and two distinct translocation apparatuses in the inner membrane were identified (Neupert, 1997; Pfanner, 1998; Schatz, 1996; Schleiff, 1999). The molecular mechanisms involved in mitochondrial targeting are found to be partly conserved through evolution, although not much is known about the translocation mechanism in higher animals so far. To date, just a few proteins have been described and these are mostly involved in the outer mitochondrial membrane translocation (Mori and Terada, 1998).

After synthesis the precursor is transferred to the receptor complex on the mitochondrial surface. The initial recognition process of the preproteins by a receptor complex is followed by the transfer of these proteins to the general insertion pore (GIP) (Neupert, 1997; Pfanner, 1998; Schatz, 1996; Schleiff, 2000). The mitochondrial outer membrane contains at least four different proteins that recognize precursor proteins; Tom70, Tom37, Tom20, and Tom22 [translocase of the outer membrane followed by their approximate molecular weight in kDa (Pfanner *et al.*, 1996)]. Tom70, Tom37, and Tom20 are only involved in recognition of preproteins, whereas Tom22 is also involved in formation and regulation of the GIP (Dekker *et al.*, 1998). The GIP is the protein import channel in the

[1] *Key to abbreviations:* DHFR, dihydrofolate reductase; GIP, general insertion pore; GST, glutathione-*S*-transferase; ISL, inner side loop; MDH, malate dehydrogenase; OSL, outer side loop; PCR, polymerase chain reaction; pOT, pre-ornityl-carbamyl-trans-ferase; pO-peptide, residues 1–29 of pOCT; SDS—PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UCP, uncoupling protein; VDAC, voltage-dependent anion channel.

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outer mitochondrial membrane composed of at least five proteins: Tom40, Tom22, Tom7, Tom6, and Tom5. Tom5 represents the final preprotein recognition site on the *cis* side of the outer membrane, Tom6 and Tom7 are involved in the regulation of the dynamics of the GIP, and Tom22 comprises the first binding site in the intermembrane space (Pfanner, 1998). Tom40 constitutes the core subunit of this pore (Vestweber *et al.*, 1989) and is essential for protein import and viability.

This outer membrane protein appears to be deeply embedded in the membrane and is in contact with preproteins as they traverse the outer membrane (Rapaport *et al.*, 1997). To date, only the Tom40 of *Neurospora crassa* and *Saccharomyces cerevisiae* could be identified (Neupert, 1997; Schatz, 1996). However, a potato protein of similar size was found to cross-react with anti-Tom40 antibodies from yeast and *N. crassa* and was shown to be involved in import (Perryman *et al.*, 1995), suggesting a relation to the Tom40 protein family.

Tom40 was found to be an integral membrane protein and its core is protease resistant in intact mitochondria. It has been suggested that this protein forms a β-strand structure embedded in the membrane. This model suggests that both the N- and the C-termini of the *N. crassa* protein face the intermembrane space (Kiebler *et al.*, 1993). The channel activity was first manifested by Thieffry *et al.* (1988) who described a peptide-sensitive channel, which was subsequently identified as Tom40 (Juin *et al.*, 1997).

Here we describe the cloning and characterization of a novel mouse protein (denoted MOM35) with a 25% overall amino acid identity to yTom40 and 27% identity to nTom40. Comparison of sequence and hydrophobicity suggests a strong relation to the Tom40 family but not to porins. This protein is imported *in vitro* into mitochondria and is localized in the outer membrane. Its trypsin digestion pattern is similar to that observed for nTom40. Furthermore, all proteins of the Tom40 family contain a highly conserved region, which can interact with preproteins *in vitro*.

## MATERIALS AND METHODS

### Cloning of MOM35

A DNA probe was constructed using the region with highest homology after aligning the amino acid sequence of yTom40 and nTom40 (Genebank, accession numbers X56885 and X556883, respectively). This alignment was made using the program LALIGN (WWW; Eerie Nimes, France). It resulted in a high homology region (see results), which was used for a Blast search (WWW, National Center for Biotechnology Information). The resulting mouse est (expressed sequence tag) was used to design primers that would generate the probe used for screening. Two primers were designed: on the coding strand 5′-GGTGACATGGACAATAGTG-3′, and on the minus strand 5′-CCGGTGGTAGACGAGCTC-3′. These were used to amplify a 263bp fragment by PCR. A mouse embryonic cDNA library (11.5 days) was used as a template. The fragment was labeled with $^{32}P$ by the random primer method (Pharmacia) and used as a probe to screen a cDNA library.

We used two cDNA libraries: a mouse kidney library cloned into λZap and a pre B-cell mouse cDNA library, cloned into λgt10. The kidney library was prepared by random primers, while the pre-B-cell library was poly-dT-generated.

The libraries were prepared and screened as detailed previously (Sambrook *et al.*, 1989). Positive plaques were picked and purified by dilutional secondary and tertiary screenings. In the case of the kidney library, phagemids were excised as described by Stratagene.

The phage DNAs from the B-cell library were digested with *Eco*RI (New England Biolab, Inc.) and agarose gel purified. The released fragments were cloned into pBluescript plasmid KS (+) (Stratagene) using XL1-Blue *E. coli* (Stratagene).

DNA sequencing of double-stranded DNA was accomplished using the dideoxy chain termination method with Sequenase ver. 2.0 (Amersham) according to the manufacturer's specifications and using synthetic oligonucleotides as primers. The nucleic acid and deduced amino acid sequence were analyzed using the DNA Strider package, BCM Search Launcher Multiple Sequence Alignments (WWW, Baylor College of Medicine) and SeqVu 1.0 (Galvan Institute of Medical Research).

### Introduction of a Kozac Sequence

In order to increase translation efficiency, we introduced a Kozac sequence, which is an eukaryotic counterpart of the bacterial Shine-Dalgarno sequence (Lewin, 1995). The coding region of the gene was amplified by 30 cycles of PCR annealing at 52°C using:
GCGAATTCACCATGATGAAGTCCGGGAACT
and the T7 primer. The generated fragment was agarose

gel-purified and digested with *Eco*RI and *Stu*I (New England Biolab, Inc.). This digestion resulted in a 400-bp fragment, which replaced the 5′ end of the original clone.

### *In vitro* Import of MOM35

Isolation of mitochondria from rat heart and *in vitro* import assays were performed as described in previous articles (Argan *et al.*, 1983; Li and Shore, 1992).

### Trypsin Assays of *in Vitro* Imported Proteins

After import, tubes were incubated with 0.5 μg of trypsin for 30 min, or for 5, 15, 30, and 60 min in kinetic experiments. Digestion and arrest of the reaction were performed at 4°C. Digestion was stopped by the addition of 5 μg (i.e., 10-fold excess) of soybean trypsin inhibitor for 30 min at 4°C. After protease treatment, tubes were treated as described in (Argan *et al.*, 1983). For the osmotic shock experiments, after standard import reaction pelleted mitochondria were resuspended in 20 μl 10 mM HEPES pH 7.5. The suspension was incubated on ice for 15 min and sonicated for 45 sec at 4°C in a sonication bath (Heating systems-Ultrasonic Inc, Plainsview NY). These tubes were treated with 0.2 μg of protease for 5, 15, 30 and 60 min at 4°C and the digestions stopped with tenfold soybean trypsin inhibitor, incubated for 30 min on ice, and transferred to Eppendorf tubes with 7 μl of SDS sample buffer.

### *In vitro* Binding Assays

A GST fusion protein was constructed by fusing in frame amino acids 173 to 201 of MOM35 to glutathione-*S*-transferase using a pGEX vector (Invitrogen). The resulting fusion protein was overexpressed in *E. coli* Topp2 cells (Stratagene). The purification and binding of the fusion protein to glutathione-sepharose 4B beads (Pharmacia) was performed as described for GST-$\Delta_{30}$hTom20 (Schleiff *et al.*, 1997b). Binding of this construct to preproteins was studied using an assay previously developed by Schleiff *et al.* (1997a).

### Protein Topology Model

The model was predicted using prediction programs available on the Internet (DAS, Toppred 2; WWW.Stockholm University, Protein Prediction Server) and the rules described in Sternberg (Sternberg 1996). The side hydrophobicity was calculated using

$$H(i) = 1/4[h(i - 2) + h(i) + h(i + 2) + h(i + 4)]$$

with the hydrophobicity scale from Eisenberg (Steinberg 1996). When residue $i - 2$ or $i + 4$ was found to be aromatic, the hydrophobicity was set to 1.6. Subsequently, the prediction was manually altered using aromatic amino acids as capping amino acids and the circumstance that regions with high homology as well as regions were gaps had to be introduced into the alignment are usually found in surface-exposed structures.

## RESULTS

### Cloning of MOM35

A DNA probe was designed by aligning the yeast and the *N. crassa* homolog of Tom40 (yTom40 and nTom40, respectively). A high homology region was identified to run from amino acid 129 to amino acid 219 in yTom40 and from amino acid 109 to residue 197 in nTom40. This region was used for a BLAST search that detected a mouse est with accession number of AA018026. This is a sequence of 446bp, of which the region from bp36 to bp300 codes for a high-homology region.

This sequence was amplified by PCR and labeled with $^{32}$P (as described in Materials and Methods). This fragment was used as a probe for screening several cDNA libraries. Using a mouse kidney library, we isolated one clone that showed homology at the amino acid level with the previously described TOM40 genes. Although this clone contained a methionine that could initiate translation, it did not appear to be the initiation site for translation, since the open reading frame did not start at this ATG. This clone is subsequently mentioned as met36. A mouse preB-cell library, poly-dT generated, yielded two identical clones. These clones were determined to be identical by restriction mapping and were named Clone 41b1. Clone met36 starts at bp 279 corresponding to amino acid 32 in the open reading frame of 41b1. The nucleotide sequence and the predicted amino acid sequence of the later clone

are shown in Fig. 1. The nucleotide sequence has been deposited on the GenBank (accession number AF109918). The clone has a length of 1363 bp and an open reading frame of 963 bp encoding a 321 amino acid protein with a predicted molecular mass of 35.31 kDa. This protein has been termed MOM35 (Mitochondrial Outer Membrane protein of 35 kDa).

The deduced amino acid sequence of MOM35 was used for a BLAST search. This protein has 25.5% identity with yeast Tom40, 27.9% with *N. crassa* Tom40, and 43.6% with a *C. elegans* protein with Gene bank accession number Z70034. The alignments are shown in Fig. 2A. Furthermore, MOM35 aligns to peptide 3 and 4 of Tom40 found in the plant complex (Jansch *et al.*, 1998). The relatively low identity of MOM35 with the yeast and *N. crassa* homologs is expected for two reasons, the identity between the two evolutionary closely related proteins nTom40 and yTom40 is only 36% and a low identity was also observed for other classes of channel proteins [using LALIGN we observed only 30% identity between nVDAC (voltage-dependent anion channel) and humanVDAC(1)]. Using MOM35 in a blast search

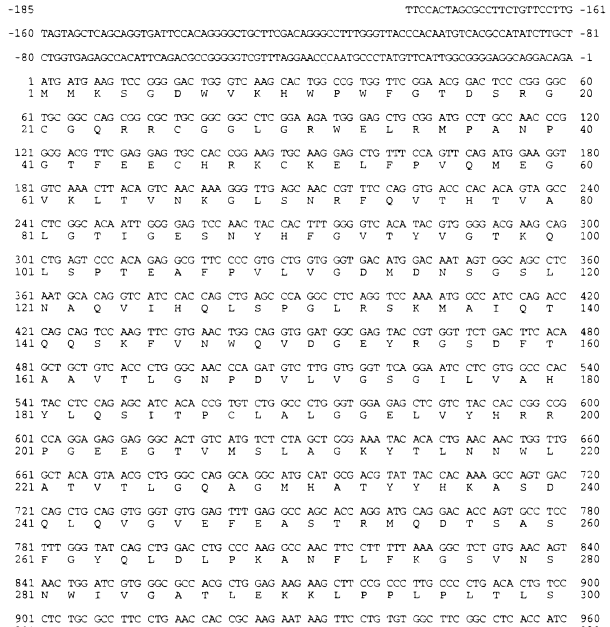


**Fig. 1.** Nucleotide sequence of mTom40. The 1363bp cDNA sequence of mouse MOM35 is shown. The predicted amino acid sequence is indicated by single-letter codes for amino acids below the nucleotide sequence. It is indicated from the first probable ATG according to high-homology regions with yTom40 and nTom40.

(WWW, BLAST, NCBI), nTom40 and yTom40 were the only proteins with significant homology. The identity of the third ranked protein (CGMP-dependent 3′, 5′-cyclic phosphodiesterase from rat) was found to be 11.8%. Therefore, the alignments suggest that the predicted 35-kDa protein represents the mouse homolog of Tom40. Interestingly, the degree of homology varies throughout the protein, being the highest from amino acid 173 to 201 (of MOM35) and might be that this region is essential for either the structural organization of the protein or its function.

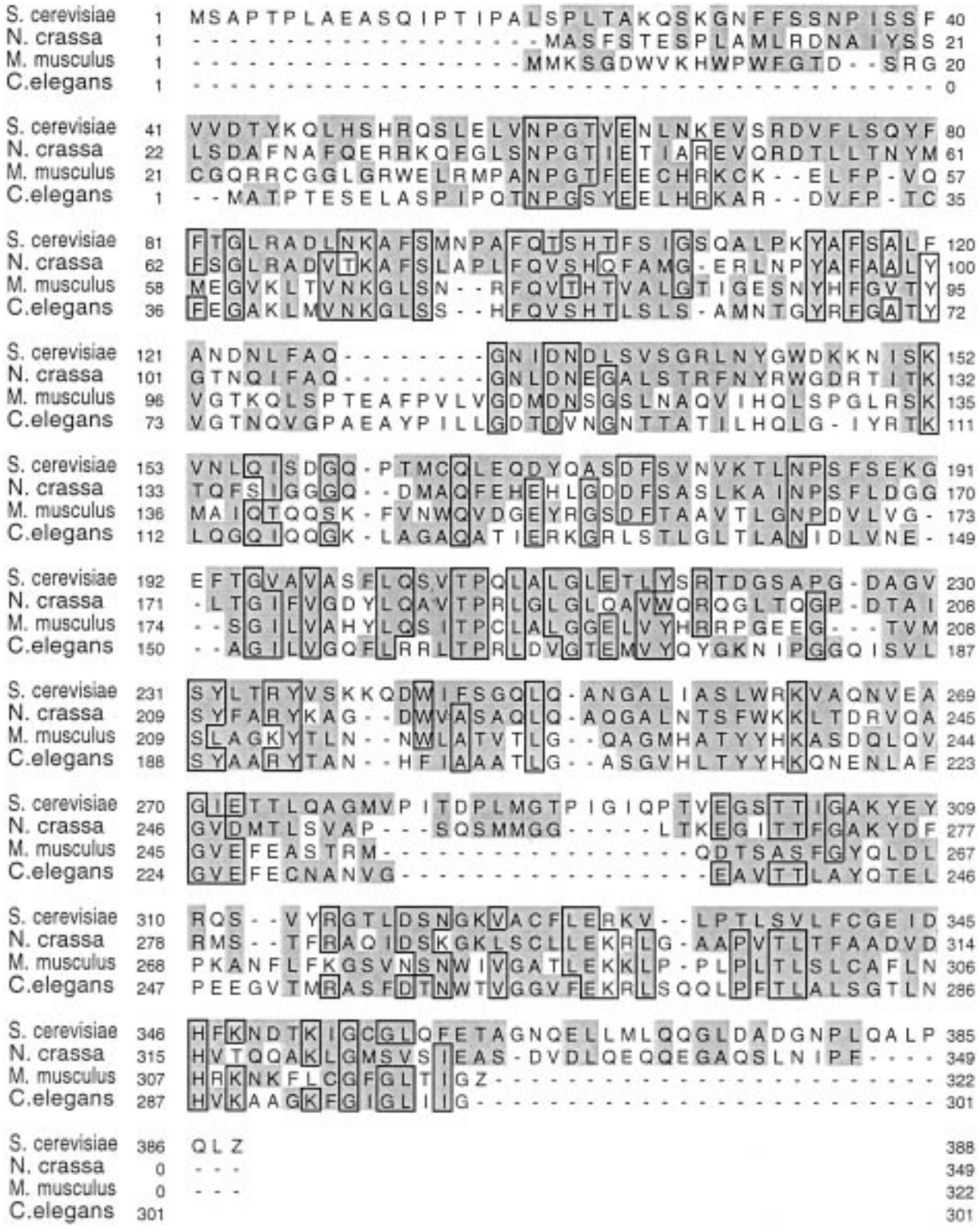
The protein encoded by the *C. elegans* gene is proposed to have 301 amino acids and a molecular weight of 32.38 kDa. The *C. elegans* protein was found to have 25% identity with yeast Tom40, similar to the identity found for MOM35. We speculate that the *C. elegans* sequence represents the nematode homolog of Tom40.

The *N. crassa* homolog of Tom40 is thought to form a β-barrel structure with 14 amphipathic transmembrane regions (Court *et al.*, 1995). The only other mitochondrial protein with a similar structure is the mitochondrial porin VDAC (Mannella *et al.*, 1996). Kyte–Doolittle hydropathy plots of nTom40, MOM35, and human VDAC(1) are compared in Fig. 2B. All are characterized by a series of alternating hydrophobic and hydrophilic domains along the proteins. However, overall patterns are comparable between *N. crassa* and the mouse protein, in contrast to the comparison with human VDAC(1). Furthermore, alignment of MOM35 with the consensus sequence for VDAC (Ha *et al.*, 1993) reveals less than 10% identity (data not shown).

## *In vitro* Import of MOM35

Radioactively labeled MOM35 was synthesized using the reticulocyte lysate system. *In vitro* translation of MOM35 from the first ATG results in a protein of approximately 35 kDa. However, translation initiation was also observed at Met36 as well as at Met58 as seen in Fig. 3. In order to examine whether the N-terminal region of MOM35 has an effect on insertion of the protein, we assayed the translation and import of clone met36. It generates a translation product of approximately 30 kDa.

Both MOM35 constructs were imported along with other mitochondrial proteins in order to determine its submitochondrial localization. VDAC and UCP (uncoupling protein) were used as controls since these proteins are both integral membrane proteins. VDAC is an outer membrane protein and UCP is located in the inner membrane of the organelle. Neither of these


S. cerevisiae 1 MSAPTPLAEASQIPTIPALSPLTAKQSKGNFFSSNPISSF 40
N. crassa 1 ----------MASFSTESPLAMLRDNAIYSS 21
M. musculus 1 ----------MMKSGDWVKHWPWFGTD--SRG 20
C.elegans 1 ---------- 0
S. cerevisiae 41 VVDTYKQLHSHRQSLELVNPGTVENLNKEVSRDVFLSQYF 80
N. crassa 22 LSDAFNAFQERRKQFGLSNPGTIETIAREVQRDTLLTNYM 61
M. musculus 21 CGQRRCGGLGRWELRMPANPGTFEECHRKCK--ELFP-VQ 57
C.elegans 1 --MATPTESELASPIPQTNPGSYEELHRKAR--DVFP-TC 35
S. cerevisiae 81 FTGLRADLNKAFSMNPAFQTSHTFSIGSQALPKYAFSALF 120
N. crassa 62 FSGLRADVTKAFSLAPLFQVSHQFAMG-ERLNPYAFAALY 100
M. musculus 58 MEGVKLTVNKGLSN--RFQVTHTVALGTIGESNYHFGVTY 95
C.elegans 36 FEGAKLMVNKGLSS--HFQVSHTLSLS-AMNTGYRFGATY 72
S. cerevisiae 121 ANDNLFAQ--------GNIDNDLSVSGRLNYGWDKKNISK 152
N. crassa 101 GTNQIFAQ--------GNLDNEGALSTRFNYRWGDRTITK 132
M. musculus 96 VGTKQLSPTEAFPVLVGDMDNSGSLNAQVIHQLSPGLRSK 135
C.elegans 73 VGTNQVGPAEAYPILLGDTDVNGNTTATILHQLG-IYRTK 111
S. cerevisiae 153 VNLQISDGQ-PTMCQLEQDYQASDFSVNVKTLNPSFSEKG 191
N. crassa 133 TQFSIGGGQ--DMAQFEHEHLGDDFSASLKAINPSFLDGG 170
M. musculus 136 MAIQTQQSK-FVNWQVDGEYRGSDFTAAVTLGNPDVLVG- 173
C.elegans 112 LQGQIQQGK-LAGAQATIERKGRLSTLGLTLANIDLVNE- 149
S. cerevisiae 192 EFTGVAVASFLQSVTPQLALGLETLYSRTDGSAPG-DAGV 230
N. crassa 171 -LTGIFVGDYLQAVTPRLGLGLQAVWQRQGLTQGP-DTAI 208
M. musculus 174 --SGILVAHYLQSITPCLALGGELVYHRRPGEEG---TVM 208
C.elegans 150 --AGILVGQFLRRLTPRLDVGTEMVYQYGKNIPGGQISVL 187
S. cerevisiae 231 SYLTRYVSKKQDWIFSGQLQ-ANGALIASLWRKVAQNVEA 269
N. crassa 209 SYFARYKAG--DWVASAQLQ-AQGALNTSFWKKLTDRVQA 245
M. musculus 209 SLAGKYTLN--NWLATVTLG--QAGMHATYYHKASDQLQV 244
C.elegans 188 SYAARYTAN--HFIAAATLG--ASGVHLTYYHKQNENLAF 223
S. cerevisiae 270 GIETTLQAGMVPITDPLMGTPIGIQPTVEGSTTIGAKYEY 309
N. crassa 246 GVDMTLSVAP---SQSMMGG-----LTKEGITTFGAKYDF 277
M. musculus 245 GVEFEASTRM----------------QDTSASFGYQLDL 267
C.elegans 224 GVEFECNANVG----------------EAVTTLAYQTEL 246
S. cerevisiae 310 RQS--VYRGTLDSNGKVACFLERKV--LPTLSVLFCGEID 345
N. crassa 278 RMS--TFRAQIDSKGKLSCLLEKRLG-AAPVTLTFAADVD 314
M. musculus 268 PKANFLFKGSVNSNWIVGATLEKKLP-PLPLTLSLCAFLN 306
C.elegans 247 PEEGVTMRASFDTNWTVGGVFEKRLSQQLPFTLALSGTLN 286
S. cerevisiae 346 HFKNDTKIGCGLQFETAGNQELLMLQQGLDADGNPLQALP 385
N. crassa 315 HVTQQAKLGMSVSIEAS-DVDLQEQQEGAQSLNIPF---- 349
M. musculus 307 HRKNKFLCGFGLTIGZ---------------------- 322
C.elegans 287 HVKAAGKFGIGLIIG---------------------- 301
S. cerevisiae 386 QLZ 388
N. crassa 0 --- 349
M. musculus 0 --- 322
C.elegans 301 301

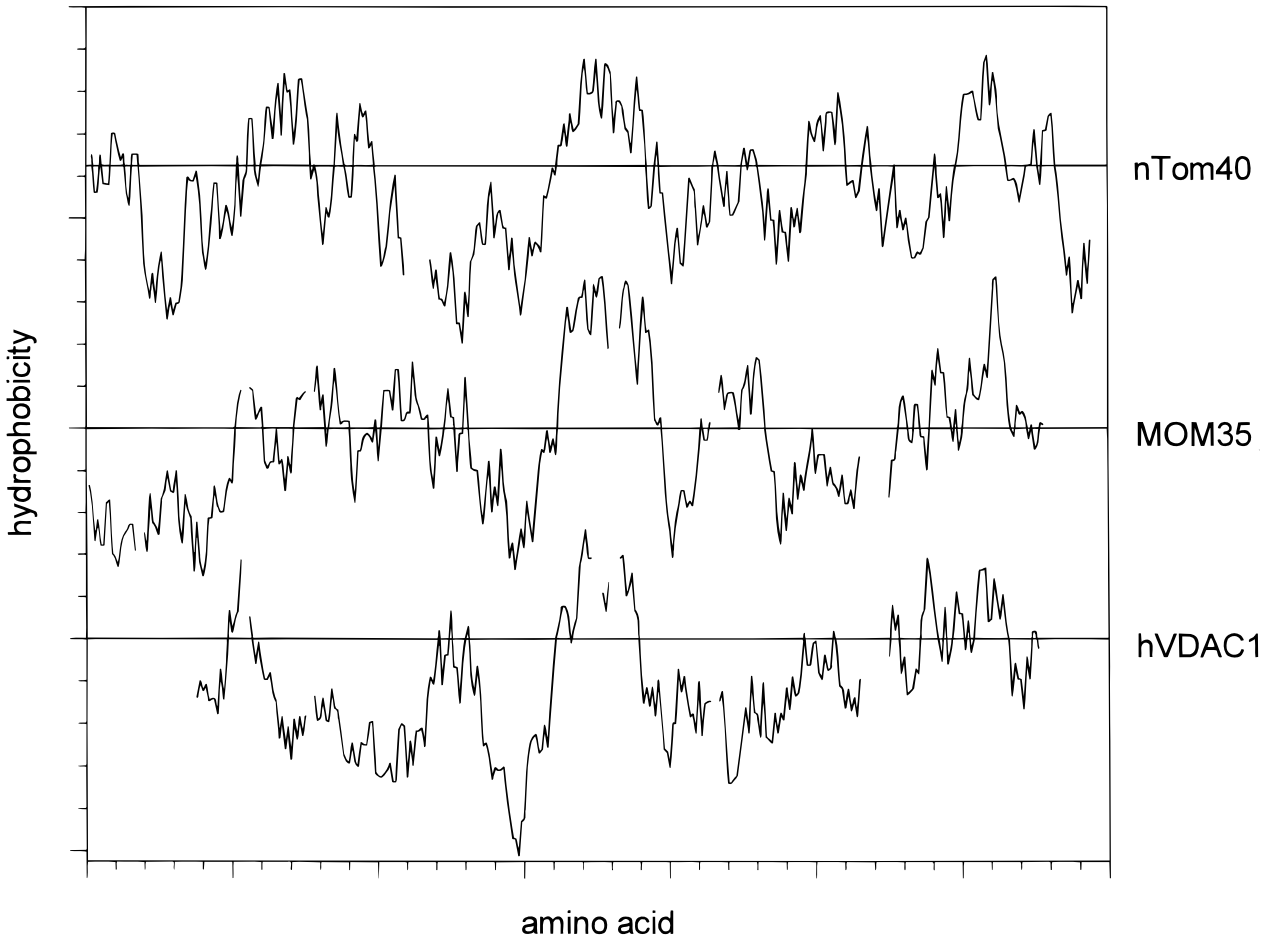



**Fig. 2.** Sequence alignment of MOM35. (A) Amino acid sequence alignment of MOM35, the *C. elegans* gene product, and Tom40 sequences from *S. cerevisiae* and *N. crassa*. Sequences are shown according to the degree of homology, yTom40 and nTom40 being the ones showing the highest homology, followed by the mouse sequence and the *C. elegans* sequence. Identical amino acids are shown in boxes, while homology is indicated by shading. Alignment was produced using the ClustalW (ver. 1.7) program (WWW, BCM Launcher, Bayler College of Medicine). (B) Hydropathy plot alignment of nTom40, MOM35, and hVDAC1. The predicted protein sequence of MOM35 was compared to the protein sequence of nTom40 and human hVDAC1, using the DNA Strider program according to the paradigms of Kyte and Doolittle (1982). Gaps were introduced according to the best-observed alignment of MOM35 to either nTom40 or hVDAC1.

proteins contains an N-terminal cleavable sequence. Bcl-2 is an outer membrane protein, but unlike VDAC, it exposes a flexible domain, which can be degraded by protease treatment. pODHFR is a chimerical protein that contains the matrix targeting signal of preornityl carbamyltransferase attached to the cytosolic protein dihydrofolate reductase. The signal will be processed after insertion of the protein into the mitochondrial matrix (Sheffield *et al.*, 1990).

Figure 3 shows that both forms of MOM35 are imported into mitochondria (lane 3). The translation of both proteins resulted in two further products starting atmethionine 58 (Fig. 3, M58) and methionine 113 (not labeled). However, these products where not always observed (Fig. 4), but can be imported into mitochondria (Fig. 3, lane 3). No cleavage of either construct after import was observed indicating that MOM35 does not contain a cleavable presequence. Furthermore, both proteins and the translation product of the internal methionine 58 are alkali resistant (Fig. 3, lane 5), which means that they are embedded in the membrane and this insertion is not dependent on the first 58 amino acids. However, the protein starting at amino acid 113 was not alkali-resistant suggesting that this protein was not inserted into the membrane. Moreover, depletion of the membrane potential, $\Delta\psi$, by pretreatment of the mitochondria with CCCP (carbonyl-cyanide *m*-chlorophenylhydrazone) does not affect the import nor the localization of either MOM35, VDAC, or Bcl-2 in the membrane fraction (not shown). In contrast,

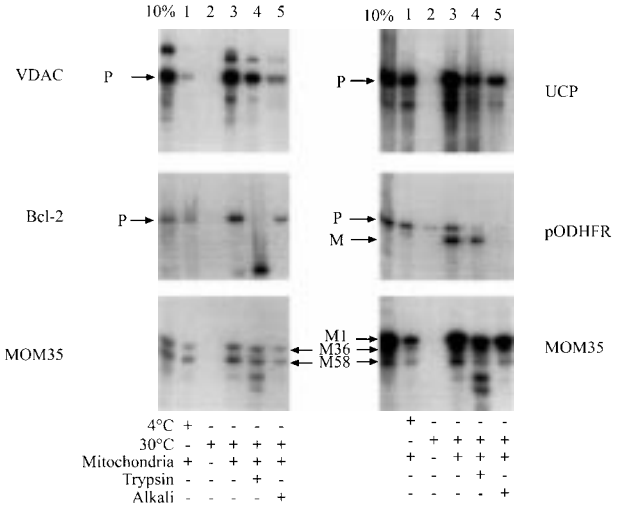



**Fig. 3.** *In vitro* import of mTOM40. Import of the [$^{35}$S] methionine labeled preproteins MOM35 starting from both methionine 1 (MOM 35, right panel) and methionine 36 (MOM35, left panel), VDAC, UCP, pODHFR, and BCL-2 was performed as described (Materials and Methods). Translated product were (5μl) incubated with 25 μg of purified mitochondria for 30 min at the indicated temperature and pelleted in order to determine inserted protein. Translated product (5 μl) is shown as 10% input control. Lane 1 represents incubation of translation product and mitochondria at 4°C, whereas, in lane 2 no mitochondria were present. Lane 3 is the import reaction at 30°C. After import, mitochondria were treated with 2.5 μg/μl trypsin (lane 4) or 0.1 *M* sodium carbonate (lane 5) for 30 min. M1 indicates translation started at methionine 1, M36 at methionine 36, and M58 at methionine 58. P, indicates the nonprocessed signal containing protein; M, mature form of the preprotein after processing.

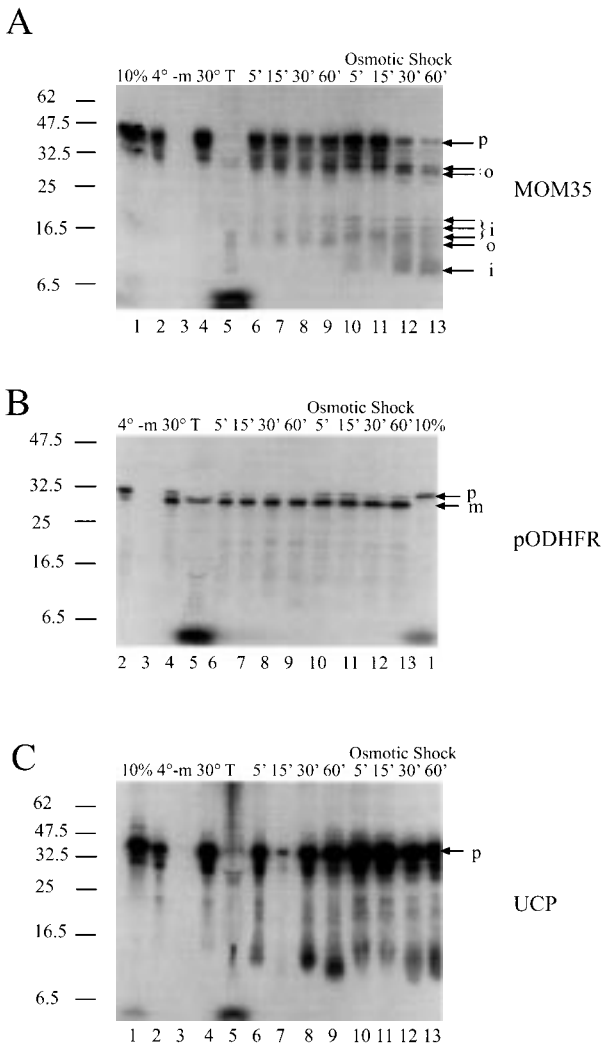



**Fig. 4.** Protease sensitivity of imported MOM35. Import of pODHFR, UCP, and MOM35 was carried out as described (Materials and Methods). Ten percent of *in vitro* translation product, which was used in every experiment, is shown before (lane 1) and after trypsin treatment (lane 5, T). Import was performed at 4°C (lane 2) or 30°C (lanes 3, 4, 6–13) with 25 μg mitochondria present (lanes 2, 4, 6–13). After preprotein import for 30 min, the outer membrane of the mitochondria was disrupted by osmotic shock (lanes 10–13), and incubated with trypsin for 5 (lanes 6 and 10), 15 (lanes 7 and 11), 30 (lanes 8 and 12), and 60 min (lanes 9 and 13). Digestion was stopped by addition of soybean trypsin inhibitor for 30 min at 4°C. Lane 3 represents experimental background. o represents digest on the outer and i on the inner side of the membrane; p, indicates precursor; m, mature preprotein.

import of UCP and pODHFR was inhibited by depletion of the membrane potential (not shown), since $\Delta\psi$ is needed for the import of inner membrane and matrix proteins. These results suggest that MOM35 is localized in the outer membrane after *in vitro* import into the mitochondria.

As expected, trypsin treatment of the organelle after import does not result in cleavage of VDAC, UCP, or pODHFR. In contrast, the outer membrane protein Bcl-2, which contains large surface exposed regions, is largely degraded by trypsin after import. Incubation with trypsin after insertion of MOM35 generates two main fragments of approximately 28 and 27 kDa, although one other band of approximately 12 kDa also appears (Fig. 4). Further, the fragment size is independent of the length of the N-terminus since the same bands are observed with met36 and can be explained in two ways. On one hand, it is possible that MOM35 is deeply embedded in the membrane (as found for VDAC) and exposes only a small fragment to the outside. When mitochondria are treated with a protease, this fragment is cleaved leaving intact just those parts of the protein that were protected by lipids. It has been reported that treatment of outer membrane vesicles of *N. crassa* with proteinase K resulted in the formation of three fragments of about 36, 26, and 12

kDa (Kunkele *et al.*, 1998). On the other hand, it may be that MOM35 traverses the membrane and a large domain is exposed to the inner mitochondrial space.

In order to distinguish between both possibilities and to address the question of the orientation of the protein, the protease resistance of MOM35 was studied in more detail. After import was performed mitochondria were treated with trypsin for 5, 15, 30, and 60 min. (Fig. 4, lanes 6–9). The digest was stopped by the addition of tenfold molar excess of soybean trypsin inhibitor. It can be observed that prolonged exposure of the mitochondrial surface to the protease does not affect the Tom40 digestion pattern described above. When the outer mitochondrial membrane was disrupted by osmotic shock prior to treatment with trypsin, MOM35 still remained largely trypsin resistant. After 60 min, smaller fragments were detectable (see lanes 10–13). MOM35 not inserted in any membrane surface was protease sensitive (lane 5), suggesting that after import MOM35 was not surface exposed but rather integrated in the outer membrane.

## The Highly Conserved Region of MOM35 Is Able to Interact with Preproteins

MOM35 was cloned by using a high-homology region within yTom40 and nTom40. As mentioned earlier, this region accounts for a high-homology region present in MOM35 (aa 173–201) and *C. elegans*. Since this domain is the most conserved one of the protein, we assumed that it is of particular importance for either the protein's physiology or its structural organization. The models of nTom40 (Court *et al.*, 1995) and MOM35 suggest that the major part of this region is exposed to the intermembrane space. Furthermore, it has been suggested that Tom40 plays an important role in the formation of the *trans* site (Rapaport *et al.*, 1997) and the contribution of Tom40 to the *trans* site is found to be exposed to the intermembrane space (Rapaport *et al.*, 1997). In a study involving the peptide-sensitive channel (PSC), it was demonstrated that after reconstituting the channel into lipid bilayers, the transfer to a trypsin bath induced the loss of gating properties (Thieffry *et al.*, 1988). In the light of these results, we decided to probe the highly conserved region for its ability to bind to preproteins. Recently, a technique has been developed to assess the interaction between preproteins and import receptors. This method was successfully used to determine the ability of Tom20 to bind preproteins *in vitro* (Schleiff *et al.*, 1997b). Therefore, amino acids 173 to 201 of MOM35 were fused to GST. This fusion protein was bound to glutathione–sepharose and incubated with various radioactively labeled precursor proteins. Afterward, specific interactions were determined by eluting the proteins from the beads with reduced glutathione, and separating them by SDS–PAGE followed by autoradiography. We tested various matrix-targeted proteins and an outer membrane protein for binding (Fig. 5). All proteins tested have a similar ability to bind the cytosolic portion of hTom20 and the highly conserved region of Tom40 (see lanes 3 and 4), whereas no interaction between the preprotein and GST alone was observed (lane 2). This interaction between Tom20 and matrix-targeting signal-containing proteins was found to be salt sensitive and enhanced by detergent (Schleiff *et al.*, 1997b). However, the binding of these signals to MOM35 (173–201) was not found to be salt sensitive and was only slightly decreased in the presence of Triton X-100 (lane 5 and 6). The specificity of this interaction could be demonstrated by competition using 5 μM of a pO peptide (lane 7) representing the N-terminal targeting signal of pre-ornityl carbamyltransferase (pOCT), which directs OTC toward the matrix. These results agree with those that have been proposed for the interactions of preproteins with the

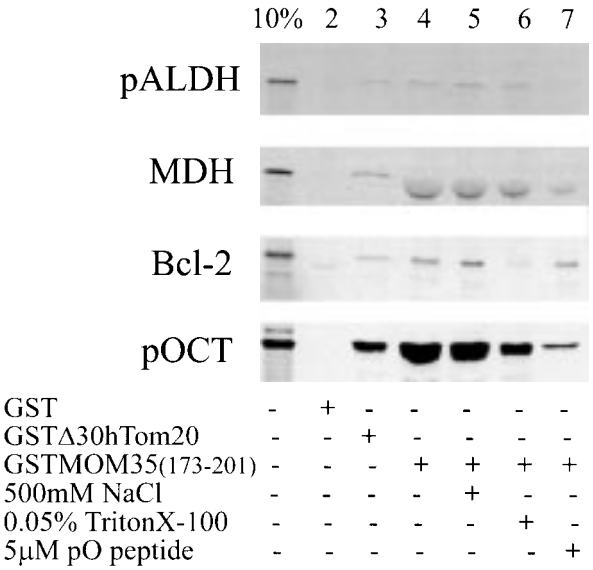



**Fig. 5.** *In vitro* binding of MOM35 amino acids 173–201 to preproteins. [$^{35}$S] methionine labeled pALDH, MDH, Bcl-2, and pOCT were incubated with GST (lane 2), GST-$\Delta_{30}$hTom20 (lane 3) or GST-MOM35(173–201) (lanes 4–7) prebound to glutathione–sepharose as described (Material and Methods). Binding was performed in the presence of 500 mM NaCl (lane 5), 0.05% Triton X-100 (lane 6), or 5 μM pO peptide (lane 7). Lane 1 shows 10% of the radiolabeled preprotein incubated with the beads.

*trans* site (Rapaport *et al.*, 1998). *Trans*-site interactions of matrix-targeted preproteins, in contrast to *cis*-site interactions are independent of ionic strength. However, the characteristics of this binding are not the same for all the proteins tested. Here Bcl-2 did interact with MOM35(173–201) in a detergent-sensitive manner and this interaction could not be competed with the matrix-targeting signal peptide even at concentrations up to 20 μM (not shown). This might suggest that Bcl-2 will not be recognized by this binding site and, therefore, not by the *trans* site of Tom40. The interaction between Bcl-2 and Tom20 was found to have the same characteristics as the interaction of the internal targeting signal-containing protein VDAC; the binding was salt insensitive and decreased by detergent (Schleiff, 1999). Moreover, luciferase, a protein not imported into the mitochondria (Schleiff *et al.*, 1997a), and the transmembrane domain of hTom20 fused to DHFR (Goping *et al.*, 1995) were recognized neither by Tom20 nor by MOM35(173–201) (not shown).

## DISCUSSION

We have cloned a 35 kDa protein from a mouse library using a probe generated using the region of highest homology in yeast and *N. crassa* Tom40. This protein was named MOM35. The predicted weight correlates with the one observed on transcription-translation assays. MOM35 possesses a considerable degree of homology with the previously described Tom40 proteins of *N. crassa* and *S. cerevisiae*. While aligning MOM35 a gene of *C. elegans* with high identity to the Tom40 family was identified and it might be proposed that the gene product represents the Tom40 protein of *C. elegans*.

yTom40 does not contain an amino-terminal targeting sequence and the internal targeting information has not been identified thus far. Interestingly, none of the proteins forming the outer mitochondrial import complex have typical targeting signals, so their import pathway into mitochondria is an active field of study (Schleiff, 2000). We could demonstrate that MOM35 will be inserted into the outer mitochondrial membrane, independent of the presence of the first 36 amino acids. This documents that the insertion is mediated by an internal targeting signal. Interestingly, the translation product starting at Met58 was also imported and, therefore, a dependence of the first 58 amino acids can be excluded.

The insertion of MOM35 into the outer mitochondrial membrane is consistent with a relation to the Tom40 family, since Tom40 is proposed to form an β-barrel channel in the outer mitochondrial membrane (Neupert, 1997; Schleiff, 1999). The transmembrane segments of the barrel structure are proposed to be amphipathic regions (Court *et al.*, 1995) and it has been proposed that the charges in putative membrane-spanning segments are compensated internally or by interaction of the transmembrane regions with other members of the complex. The model of Tom40 further suggests that both termini of the protein face the intermembrane space (Kiebler *et al.*, 1993). When imported into mitochondria, MOM35 exhibits a relative resistance to trypsin digestion, suggesting that it is deeply embedded in the membrane.

It is remarkable that even after 60 min of trypsin digest, three bands of 28, 27, and 12 kDa are visible and the majority of the protein remains uncleaved. However, when the outer membrane is disrupted by osmotic shock, the protein becomes more accessible to the protease. The resulting fragments are in the range of 17 kDa, still suggesting that at least one part of the protein is buried in the membrane. These results are consistent with the interpretation that lipids protect the main core of the protein, yet there is a part of the protein that is exposed to the intermembrane space. Using structure prediction routines (DAS, TopPred 2; Stockholm University, Protein Prediction Server; www.biokemi.su.se/~erikw), MOM35 was predicted to be a β-barrel rather then a helix spanning protein. The predicted transmembrane domains [shown in Fig. 6; modeling was performed according to Sternberg (1996) and references within] are somewhat consistent with the prediction by Court et al., (1995) (63% overlap based on alignment). Helical extensions at both termini of the protein face the intermembrane space. The core of the protein is composed of fourteen membrane spanning β-strands connected by short surface-exposed loops. α-Helical regions, which might be involved in the regulation of translocation, are predicted in loops 3 and 5 on the outer membrane surface and loop 4 on the inner side of the membrane. This idea is consistent with the finding that helix 4 (residues 179–190) is located in the most conserved region of Tom40 and is involved in recognition of preproteins. Furthermore, the model is consistent with the trypsin cleavage pattern. The fragmentation of MOM35 is consistent with the fragment sizes observed for trypsin diges-

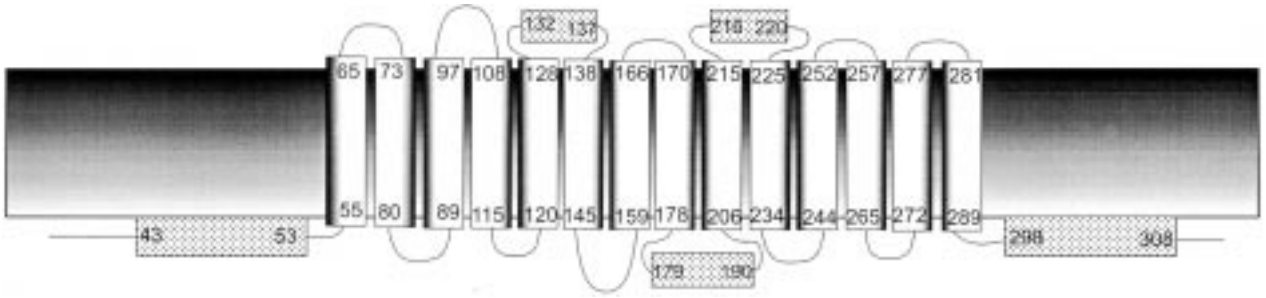



**Fig. 6.** Transmembrane model of MOM35. Presented is the transmembrane model of MOM35. Numbers indicate the capping amino acids of each beta strand. Stippled rectangles represent α-helices and open rectangles represent β-strands. Modeling procedure is outlined (Materials and Methods).

tion of nTom40 (Kunkele *et al.*, 1998). Therefore, MOM35 shows topology similar to Tom40 from *N. crassa*.

Translocation through the outer membrane of mitochondria is driven by the specific and reversible interaction of the presequence with two binding sites. The *cis* site is formed by a multicomponent receptor complex at the membrane surface, whereas the *trans* site is located on the inner face of the outer membrane and is exposed to the intermembrane space. Tom40 has been demonstrated to play a major role on the preprotein binding on the *trans* site and subsequently it has been suggested that the binding site of Tom40 for preproteins is surface exposed (Rapaport *et al.*, 1997). Moreover, the sensitivity of the peptide-sensitive channel (identified as Tom40-; Juin *et al.*, 1997) to trypsin treatment (Thieffry *et al.*, 1988) suggests that Tom40 possesses a domain, which is capable of binding presequences, and that maybe is exposed to the intermembrane space. Our data shows that, as found for Tom40, MOM35 is mainly protected by the membrane. Analysis of the sequences of yTOM40, nTom40, MOM35, and a *C. elegans* homolog, show that there is a highly conserved region in all four proteins. This region was proposed to be surface exposed by Court *et al.* (1995) and in our model of MOM35. We demonstrated that this segment (that in MOM35 corresponds to amino acids 173–201) is able to bind preproteins *in vitro* in a specific manner. Competition of the preproteins can not occur by increasing the ionic strength or hydrophobicity but by a matrix-targeting signal peptide. This is consistent with what is found for the behavior of the *trans*-binding site (Rapaport *et al.*, 1998), suggesting a role for MOM35 in the translocation of preproteins across the mitochondrial membrane.

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