Metaxin Is a Component of a Preprotein Import Complex in the Outer Membrane of the Mammalian Mitochondrion*

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Metaxin, a novel gene located between the glucocerebrosidase and thrombospondin 3 genes in the mouse, is essential for survival of the postimplantation mouse embryo. In this study, the subcellular location, domain structure, and biochemical function of metaxin were investigated. Anti-recombinant metaxin antibodies recognized 35- and 70-kDa proteins in mitochondria from various tissues; the 35-kDa protein is consistent in size with the predicted translation product of metaxin cDNA. When metaxin cDNA was transfected into COS cells, immunofluorescence staining demonstrated that the protein is located in mitochondria. Metaxin contains a putative mitochondrial outer membrane signal anchor domain at its C terminus, and a truncated form of metaxin lacking this signal anchor domain had a reduced association with mitochondria. In addition, metaxin was highly susceptible to proteases in intact mitochondria. We therefore conclude that metaxin is a mitochondrial protein that extends into the cytosol while anchored into the outer membrane at its C terminus. In its N-terminal region, metaxin shows significant sequence identity to Tom37, a component of the outer membrane portion of the mitochondrial preprotein translocation apparatus in Saccharomyces cerevisiae, but important structural differences, including apparently different mechanisms of targeting to membranes, also exist between the two proteins. Given the similar subcellular locations of metaxin and Tom37, the possible role of metaxin in mitochondrial preprotein import was investigated. Antibodies against metaxin, when preincubated with mitochondria, partially inhibited the uptake of radiolabeled preadrenodoxin into mitochondria. Metaxin is therefore the second mammalian component of the protein translocation apparatus of the mitochondrial outer membrane to be characterized at the molecular level and the first for which an inherited mutation has been described. The early embryonic lethal phenotype of mice lacking metaxin demonstrates that efficient import of proteins into mitochondria is crucial for cellular survival. The characterization of metaxin provides an opportunity to elucidate similarities and possible differences in the mechanisms of protein import between fungi and mammals and in the phenotypes of fungi and mammals lacking mitochondrial import receptors.

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During studies of thrombospondin 3 (Thbs3), which encodes an extracellular matrix protein (2–4), and glucocerebrosidase (Gba), which encodes a lysosomal enzyme (5), a novel gene, termed metaxin (Mtx), was found to span the interval between Thbs3 and Gba on chromosome 3ES-F1 in the mouse (6). Mtx and Thbs3 are situated in a head-to-head orientation, with the translation start sites 1.4 kilobase pairs apart. Gba and Mtx lie in a tail-to-tail orientation, with the polyadenylation sites spaced 431 base pairs apart. The Mtx promoter resembles the promoters for housekeeping genes in that it is TATA-less, GC-rich, and regulated by tandem Sp1 elements within 300 base pairs of the transcription start site (7). Although the close apposition and head-to-head orientation of Mtx and Thbs3 raise the possibility that the two genes share a bidirectional promoter, several lines of evidence suggest that these genes are regulated independently. First, Mtx mRNA is ubiquitously distributed in the mouse, whereas Thbs3 mRNA is restricted to lung, cartilage, the gastrointestinal tract, and hippocampus (2, 8, 9). Secondly, the basal elements of the Mtx and Thbs3 promoters can largely be dissociated, which indicates that the Mtx and Thbs3 genes are not regulated by a true bidirectional promoter (7).

In the process of producing a subtle mutation in Gba, a neomycin cassette was inadvertently placed in exon 8 of Mtx (6). Mice homozygous for this disruption have an embryonic lethal phenotype, indicating that metaxin is necessary for the early development of the mouse embryo. This severe phenotype is in contrast to the phenotype of mice lacking glucocerebrosidase, which die shortly after birth with Gaucher disease-like symptoms. Because Mtx mRNA is ubiquitously expressed in the adult mouse, it is likely that metaxin has a fundamental role in an as-yet-undefined cellular process. To gain insight into its function and to examine the possible metabolic relationship between metaxin and glucocerebrosidase and/or thrombospondin 3, we have investigated the subcellular location of metaxin and have found that it is a mitochondrial outer membrane protein. Metaxin has significant sequence identity to a component of the yeast mitochondrial protein import machinery, Tom37 (10). We demonstrate here that metaxin also functions in the import of mitochondrial preproteins into mammalian mitochondria, although its mode of function may differ in important respects from that of Tom37.

EXPERIMENTAL PROCEDURES

Antibodies to Metaxin—A full-length mouse metaxin cDNA was placed in frame with the GST gene in pGEX2T (Pharmacia Biotech Inc.) or with the polyhistidine gene of pTrcHis (Invitrogen). The resulting GST-metaxin fusion protein, synthesized in Escherichia coli, was insoluble and was partially purified by fractionation of inclusion bodies. The polyhistidine/metaxin fusion protein was also insoluble and was...
purified from guanidinium-solubilized inclusion bodies on a ProBond nickel affinity column under denaturing conditions, as described by the manufacturer (Invitrogen). Inclusion bodies containing GST-metaxin were cleaved with thrombin, and the metaxin portion was purified by electrophoresis and electroelution. The electroeluted metaxin was injected into New Zealand White rabbits. Subcellular fractions of mouse liver were obtained as described (11). Purity of the fractions was assessed by assays for DNA, citrate synthase (12), acid phosphatase (13), lactate dehydrogenase (14), Na⁺/K⁺ ATPase (15), and glucose 6-phosphatase (16). Western blot analysis was performed with anti-metaxin antibodies at a 1:500 dilution and with a secondary anti-guinea pig IgG–alkaline phosphatase conjugate. Blots were developed with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

Preparation of Tissue Lysates and Liver Subcellular Fractions—Tissue lysates were prepared by homogenization with a Dounce homogenizer in 100 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS, 1 mM leupeptin. Subcellular fractions of mouse liver were obtained as described (11). Purity of the fractions was assessed by assays for DNA, citrate synthase (12), acid phosphatase (13), lactate dehydrogenase (14), Na⁺/K⁺ ATPase (15), and glucose 6-phosphatase (16). Western blot analysis was performed with anti-metaxin antibodies at a 1:500 dilution and with a secondary anti-guinea pig IgG–alkaline phosphatase conjugate. Blots were developed with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

Immunofluorescence Analysis of COS Cells Expressing Mouse Metaxin cDNA—Metaxin was placed under control of the cytomegalovirus promoter in pcDNA3 (Invitrogen) and transfected into COS cells by electroporation. Electroporations were performed in a BRL Cell-4

Submitochondrial Distribution of Metaxin—Mitochondria were pre-

servers

Mitochondrial Import Assays—Mitochondrial import of preadreno-

corticochrome C, spans amino acids 1–303 and was

Western blots were performed with mouse liver mitochondrial (100 μg) in 100 μl of the “import buffer” (10 mM HEPES-KOH buffer, pH 7.4 containing 1 mM EDTA, 1 mM diethyleth	

other tissues, mouse heart, kidney, liver, and lung were separated into a mitochondrial fraction and a post mitochondrial supernatant, which contains microsomes and cytosol. In subcellular fractions of mouse heart, kidney, and lung, no cytosolic band was detected, whereas the mitochondrial 35- and 70-kDa bands were present (Fig. 1B). Mitochondrial metaxin has a tissue distribution like that of metaxin mRNA and therefore likely represents the authentic metaxin protein. It is unclear whether the 34-kDa liver cytosolic band is related to metaxin or is a cross-reactive protein. The relationship of the 70-kDa protein to metaxin is also unclear, but the fact that the two proteins are found together in mitochondria and in the same tissues suggests that the 70-kDa form could represent a denaturation-resistant homo- or heterodimer containing the 35-kDa protein.

The subcellular distribution of metaxin was also determined in cultured cells by immunofluorescence. Several mouse cell lines were stained for metaxin, but the endogenous concentrations of metaxin were too low to permit detection by immunofluorescence. Therefore, COS cells were transiently transfected with a full-length metaxin cDNA, and after 48 h, indirect immunofluorescence was performed with anti-metaxin antibody. The overexpressed metaxin protein had a punctate distribution within the cytoplasm with heavier staining in the perinuclear region that was consistent with a mitochondrial localization (Fig. 2a). In addition, weaker diffuse staining throughout the cytosol was observed. Mitochondria were detected by incubation of the cells with MitoTracker® dye prior to fixation and staining for metaxin (Fig. 2b). A merged image of the staining for metaxin and mitochondrial (Fig. 2b) revealed a partial colocalization (indicated by yellow color). In this experiment, metaxin does have a wider distribution than mitochondria, which can be due to the possibility that overexpressed metaxin has saturated chaperones and/or receptors responsible for its mitochondrial localization. A similar staining pattern

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Mitochondrial Metaxin Protein—Mitochondrial metaxin was assayed as described previously (18) with a minor modification. Briefly, 125I-labeled preadrenodoxin was incubated with 150–200 μg of rabbit reticulocyte lysate in 50 μl of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM potassium acetate and 2 mM magnesium acetate at 30 °C for 30 min. The substrate mixture was incubated with mouse liver mitochondria (100 μg) in 100 μl of the “import buffer” (10 mM HEPES-KOH buffer, pH 7.4 containing 1 mM EDTA, 1 mM dithiothreitol, 0.22 mM mannitol, and 0.07 mM sucrose) containing 5 mM NADH, 20 mM sodium succinate, 0.5 mM ATP, 0.1 mM GTP, 1.5 mM creatine phosphate, and 15 μg/ml creatine phosphokinase at 30 °C for 50 min. Mitochondria were then separated by centrifugation and resolved by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis gels were analyzed with a Fuji Bioimage Analyzer BAS2000.

RESULTS

Subcellular Distribution of Metaxin—Antibodies against recombinant metaxin recognize a 35- and a 70-kDa protein in lysates of all mouse tissues that were examined (data not shown). The predicted translation product of metaxin cDNA is 317 amino acids in length, which corresponds closely to the 35-kDa band that we observe. The ubiquity of metaxin protein is in keeping with the wide distribution of metaxin mRNA (6). Mouse liver was fractionated into its constituent subcellular organelles, and these fractions were assayed for the presence of metaxin by Western blotting (Fig. 1A) and for marker enzymes (not shown). Antibodies against metaxin recognized 35- and 34-kDa bands in mitochondria and in the cytosol, respectively. The 70-kDa band was found solely in mitochondria. To determine whether this subcellular distribution was characteristic of metaxin in other tissues, mouse heart, kidney, liver, and lung were separated into a mitochondrial fraction and a post mitochondrial supernatant, which contains microsomes and cytosol. In subcellular fractions of mouse heart, kidney, and lung, no cytosolic band was detected, whereas the mitochondrial 35- and 70-kDa bands were present (Fig. 1B). Mitochondrial metaxin has a tissue distribution like that of metaxin mRNA and therefore likely represents the authentic metaxin protein. It is unclear whether the 34-kDa liver cytosolic band is related to metaxin or is a cross-reactive protein. The relationship of the 70-kDa protein to metaxin is also unclear, but the fact that the two proteins are found together in mitochondria and in the same tissues suggests that the 70-kDa form could represent a denaturation-resistant homo- or heterodimer containing the 35-kDa protein.

The subcellular distribution of metaxin was also determined in cultured cells by immunofluorescence. Several mouse cell lines were stained for metaxin, but the endogenous concentrations of metaxin were too low to permit detection by immunofluorescence. Therefore, COS cells were transiently transfected with a full-length metaxin cDNA, and after 48 h, indirect immunofluorescence was performed with anti-metaxin antibody. The overexpressed metaxin protein had a punctate distribution within the cytoplasm with heavier staining in the perinuclear region that was consistent with a mitochondrial localization (Fig. 2a). In addition, weaker diffuse staining throughout the cytosol was observed. Mitochondria were detected by incubation of the cells with MitoTracker® dye prior to fixation and staining for metaxin (Fig. 2b). A merged image of the staining for metaxin and mitochondrial (Fig. 2b) revealed a partial colocalization (indicated by yellow color). In this experiment, metaxin does have a wider distribution than mitochondria, which can be due to the possibility that overexpressed metaxin has saturated chaperones and/or receptors responsible for its mitochondrial localization. A similar staining pattern
was observed in cell lines stably expressing metaxin cDNA (data not shown). In sum, examination of its distribution by immunofluorescence and by subcellular fractionation indicates that metaxin has a mitochondrial location within the cell.

**Subcellular Location of Metaxin**—The majority of proteins that are to be directed to the interior of mitochondria contain a cleavable, N-terminal, amphiphilic, positively charged sequence of 12–70 amino acids that acts as a signal peptide (19). Metaxin contains no positively charged residues within its first 20 amino acids, and a helical wheel projection of the N-terminal 20 amino acids of metaxin did not reveal a predilection toward amphiphilicity (data not shown). Therefore, it is unlikely that metaxin resides in the interior of mitochondria. Near its C terminus, metaxin contains a 23-amino acid stretch of hydrophobic and hydroxy amino acids that is predicted from a transmembrane sequence by the PHDhtm transmembrane topology prediction computer program (20). This transmembrane sequence is flanked at its N terminus by three consecutive arginine residues and is followed by a sequence of 7 amino acids that contains 2 arginines and no negatively charged amino acids. This sequence motif resembles the C-terminal mitochondrial outer membrane targeting domains of Bcl-2 (21) and monoamine oxidase A (22) (Fig. 3A). Yeast Tom70 contains a motif of similar character but at its N terminus (23).

The hypothesis that metaxin is an outer membrane protein with a C-terminal signal anchor sequence and a majority of the protein in the cytosol was first tested by determining its protease susceptibility in intact mitochondria. Mitochondria were incubated with increasing concentrations of trypsin, and the presence of metaxin was examined by Western blotting. Metaxin was found to be susceptible to very low concentrations of trypsin (Fig. 3B). At lower concentrations of trypsin, a 30-kDa proteolytic fragment of metaxin was evident, whereas at higher concentrations, complete disappearance of metaxin was observed. In mitochondria incubated with 1% trypsin (w/v mitochondria), a 30-kDa band also appears in the supernatant (not shown); presumably, the supernatant form is a metaxin fragment lacking a membrane anchor. Under these same conditions, cytochrome c oxidase subunit IV, an inner membrane protein that protrudes into the inner membrane space and is partially cleaved by trypsin in mitoplasts (24), was not digested by trypsin, demonstrating that general degradation of proteins within the interior of the mitochondria did not occur (Fig. 3B, COX IV). However, when the outer membrane of mitochondria was removed from the inner membrane/matrix fraction (mitoplasts), metaxin remained in association with the mitoplast fraction (data not shown). Certain outer membrane proteins, such as Bcl-2, exhibit this behavior, presumably as a result of their association with contact sites between the outer and the inner membrane (21). Interestingly, the 70-kDa cross-reactive species was resistant to trypsin in intact mitochondria but was susceptible in mitoplasts (data not shown).

**Metaxin Contains a C-terminal Outer Membrane Targeting Sequence**—The requirement for the putative transmembrane sequence in the mitochondrial localization of metaxin was tested by making truncations of the metaxin C-terminal region. The first deletion construct, pcDNA3metΔC, encodes a metaxin that lacks the C-terminal 15 amino acids, which are not predicted to contain targeting information (Fig. 3A). As judged by indirect immunofluorescence of COS cells transfected with pcDNA3metΔC, metaxin lacking its C-terminal 15 amino acids retained its mitochondrial location (Fig. 4A, a–c), and furthermore the COS cells appeared to have a lower background of cytosolic staining than did cells transfected with the intact metaxin cDNA. The second deletion construct, pcDNA3metΔTM/C, encodes a metaxin that lacks the 48 C-terminal amino acids, a region that includes the transmembrane domain (Fig. 3A). The metaxin lacking this transmembrane domain was distributed diffusely throughout the cell (Fig. 4A, d–f), indicating that the transmembrane domain is required for proper mitochondrial distribution of metaxin.

Stable cell populations expressing full-length metaxin and the two C-terminal deletion constructs were prepared by selection with G418. Mitochondrial and post-mitochondrial supernatant fractions were prepared by differential centrifugation and analyzed for metaxin by Western blotting (Fig. 4B). The percentage of total cellular overexpressed metaxin that was associated with mitochondria was calculated by densitometric analysis of Western blots from three separate experiments (Fig. 4C). Small amounts of metaxin were detectable in the mitochondrial fraction of COS cells transfected with vector alone. COS/metaxin cells accumulated metaxin to a high concentration in the mitochondrial fraction and contained a lower concentration of metaxin in the post-mitochondrial supernatant fraction (Fig. 4B). The percentage of total cellular metaxin associated with mitochondria (28.1 ± 1.2% (mean ± S.D.)) was lower than that in the cytosol (Fig. 4C), because the protein...
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Metaxin Functions in Mitochondrial Preprotein Import—
Several proteins with distant sequence identity to metaxin were detected by searching the protein data bases with the BLAST function. Metaxin has 25% sequence identity over 143 amino acids to Tom37, a yeast protein that is located in the mitochondrial outer membrane and participates in the import of mitochondrial preproteins into the mitochondria (10). The highest degree of identity occurs between the N-terminal regions of the proteins, from amino acids 35 to 165 of metaxin and amino acids 43 and 185 of Tom37; no significant sequence identity occurs within the C-terminal halves of the proteins (data not shown). Another apparent difference exists in the mitochondrial outer membrane targeting domains of the two proteins, because Tom37 has an N-terminal targeting sequence, and metaxin has a C-terminal targeting sequence.

We have attempted to answer the question of whether metaxin is a functional mammalian homologue of Tom37 by assessment of the ability of metaxin cDNA to restore a wild type phenotype to YSG1®, a tom37 strain of Saccharomyces cerevisiae (kindly provided by Sabine Rospert and Gottfried Schatz, Biozentrum, Basel). The plasmid pRS316OXmet, which contains the metaxin cDNA, did not restore the ability of the mutant strain to grow at 37°C on dextrose-containing medium, whereas pEL204, which contains the Tom37 gene, did complement the mutation. Although metaxin mRNA was present, the protein was not detectable in yeast transfected with metaxin cDNA; this failure to complement the tom37 mutation may be due to instability of metaxin in yeast, which may in turn be a consequence of an inability of the protein to bind productively to yeast mitochondria.

Data Base Searches Reveal Several Additional Homologous Proteins—Metaxin shares sequence identity with a Caenorhabditis elegans hypothetical protein (GenBank® accession number P34599); a 29% identity over 222 amino acids was determined by the FASTA algorithm. The function of the protein encoded by this gene is unknown, and it lacks any sequences resembling the C-terminal transmembrane domain of metaxin. Metaxin also has 29% identity over 206 amino acids with fuxed axon connections protein (FAX), the product of a Drosophila gene that in its heterozygous state exacerbates the phenotype of abl tyrosine kinase null mutants (25). FAX is thought to function as a participant in the abl kinase pathway, but its precise biochemical function is unknown. Metaxin re-
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A

yeast Tom70

+ + +

NH2-KKSFTTRNN-KAILATVAATGTAIGAYYVQD-...

Bcl-2

+ + +

...KTLSTALVGCAITLGVLYGH-COOH

monoamine oxidase A

+ + +

...KIQGSTVTAQGFLVRYKGRLPS-COOH

B

% trypsin (w/w mito)

0 0.2 1 4 10

kDa

66-45-31-21-

metaxin

14-

-COX IV

FIG. 3. Mitochondrial outer membrane localization of metaxin. A. mitochondrial outer membrane proteins contain a continuous stretch of approximately 20 hydrophobic and uncharged amino acids (underlined) near the C or the N terminus, flanked by several positively charged amino acids (denoted by +). Metaxin contains such a sequence near its C terminus. Sequences at the C terminus of metaxin, which lacks the N-terminal amino acids, and metaxinMTM/C, which lacks the transmembrane domain and C-terminal residues, are indicated. B, intact mitochondria were incubated with varying concentrations of trypsin and were then centrifuged. The pellet and the supernatant fraction were analyzed by Western blotting for metaxin and for cytochrome c oxidase subunit IV (COX IV), an inner membrane protein that protrudes into the intermembrane space.

Each has weak sequence similarity to numerous glutathione S-transferases. The GST superfamily is large and highly variable, but a consensus sequence for GSTs has been determined (27). Metaxin and its relatives partially conform to this consensus and contain the most sequence identity to one another in the regions that match the GST consensus. In particular, those residues determined by x-ray crystallography to be involved in conferring structural stability to GSTs (28) are almost completely conserved among GSTs and between metaxin and its relatives, whereas residues involved in binding to glutathione are not (data not shown). These findings suggest that metaxin and its relatives probably do not function as GSTs.

DISCUSSION

The metaxin gene was discovered during investigations of its two flanking genes, which encode glucocerebrosidase and thrombospondin 3. We describe here the characterization of metaxin and conclude that it is a mitochondrial outer membrane protein that functions as an import receptor for mitochondrial preproteins. Metaxin resembles the yeast mitochondrial preprotein import subunit, Tom37, in sequence, in outer membrane location, and in participation in mitochondrial import. Although many components of the fungal mitochondrial protein import apparatus have been characterized, only two mammalian import proteins, human Tom20 and human Tim17, have heretofore been described at the molecular level (29–32). Mammalian Tom 20 and Tim 17 are similar to the yeast proteins at the amino acid sequence level, and the finding of a third mammalian protein that resembles a yeast mitochondrial protein import component suggests a general conservation between fungi and mammals in their mechanisms of uptake of proteins into mitochondria.

Is metaxin a functional mammalian homologue of S. cerevisiae Tom37? Our results indicate that, although metaxin and Tom37 both participate in preprotein import, they differ in some important aspects. First, the amino acid sequence identity between metaxin and Tom37 is significant only within the N-terminal regions of the proteins. The presence of divergent C termini within metaxin and Tom37 suggests that some functional characteristics of the two proteins differ. Second, the mechanisms by which metaxin and Tom37 associate with the outer membrane are apparently different. Tom37 contains at its N terminus an amphiphilic, positively charged sequence followed by a predicted transmembrane sequence; these sequences are thought to function like the N-terminal signal anchor domain of Tom70 (10). Metaxin contains neither of these sequences at its N terminus. These differences in targeting domains may indicate differences in the mechanism of insertion into the outer membrane and/or in the arrangement of the proteins in the outer membrane. Third, metaxin cannot carry out the biochemical functions of Tom37, because metaxin cDNA fails to complement the tom37 mutation of S. cerevisiae. The inability of the yeast import apparatus to recognize the C-terminal targeting sequence of metaxin or the failure of metaxin to oligomerize with the partners of Tom37 in yeast may preclude metaxin from reaching the yeast mitochondrial outer membrane and may result in the rapid degradation of metaxin. Metaxin therefore appears to bear a general structural and functional resemblance to Tom37 but has diverged evolutionarily from Tom37 in several key details.

With the characterization of metaxin as a mitochondrial import protein, the metaxin-null mutation (6) represents the first reported mammalian inherited deficiency in a mitochondrial import protein. Mutations in fungal mitochondrial import proteins, in contrast, have been well studied, and phenotypes of such mutants range from temperature-sensitive deficiency in...
Effect of C-terminal deletions on mitochondrial targeting of metaxin.

A. COS cells transiently transfected with pcDNA3metΔC (a–c) and metΔTM/C (d–f) were stained for metaxin (a and d) and mitochondria (b and e) and analyzed by confocal laser scanning microscopy to produce merged images (c and f) as described in Fig. 2. Metaxin ΔC shows a strong association with mitochondria, whereas metaxin ΔTM/C shows little association.

B. COS cells stably transfected with pcDNA3 containing no insert (vector), full-length metaxin, metaxin ΔC, or metaxin ΔTM/C were obtained by selection with G418. Cells were fractionated into mitochondrial (mito) and post-mitochondrial supernatant (S/N) fractions, and analyzed by Western blotting for metaxin. Equal amounts of protein (20 μg) were loaded onto each lane. Metaxin was detected by Western blotting with affinity-purified anti-metaxin IgG and horseradish peroxidase-based chemiluminescence. The blot was overexposed to make visible the metaxin bands of lesser intensity. C, bands from Western blots from three separate experiments, as in B, were quantified by scanning densitometry. Total metaxin associated with mitochondria and post-mitochondrial supernatant was calculated; data are expressed as the percentage of metaxin associating with mitochondria (mean ± S.D.).
respiration to lethality. Yeast lacking Tom37 are unable to grow on any carbon source at 37 °C but grow on both fermentable and nonfermentable carbon sources at 23 °C (10). Mouse metaxin-null cells appear to have limited replicative potential in vivo, because metaxin-null mutant embryos survive to the blastocyst stage and implant, but by day 6.5 the embryos die and become resorbed.2 Precise evaluation of the consequences of a deficiency of metaxin is complicated by the possibility that metaxin and/or metaxin mRNA contributed by the fertilized egg persists long enough to enable the blastocyst to develop and implant. The capacity of a zygote entirely lacking metaxin to divide and differentiate may therefore be even more limited than that observed in metaxin-null mutant mice.

Several possibilities exist for the disparity between the severe phenotype of metaxin-null mutant mice and the conditional phenotype of Tom37 mutant yeast. Tom37 facilitates import of selected mitochondrial proteins (10); presumably yeast lacking Tom37 import proteins into mitochondria by means of other outer membrane receptors, such as Tom 22/20. Metaxin may import a broader subset of mitochondrial proteins than does Tom37, and other receptors may be less able to compensate for the lack of metaxin than for Tom37. In this case, all embryonic cell types would be compromised by a lack of metaxin. Alternatively, embryos lacking metaxin may be defective in the

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2 E. I. Ginns, unpublished data.
function of a certain cell type, which may result from the action of metaxin in stimulating import of a subset of proteins that are strictly required for survival of that cell type in the early mouse embryo. Examination of metaxin-null blastocysts during outgrowth in vitro might permit identification and phenotypic characterization of cell types affected by metaxin deficiency.

In yeast, Tom37 forms a heterodimer with Tom70, and they act together as a receptor for a subset of mitochondrial preproteins (10). A chaperone, MSF, purified from rat liver cytosol, complexes specifically with mitochondrial precursor proteins and mediates their association with Tom37-Tom70 (33). Although a yeast counterpart of MSF has not yet been described, the productive interaction between MSF and yeast Tom37-Tom70 suggests that such a homologue exists. After the MSF-preprotein complex docks at the Tom37-Tom70 site on the outer membrane, ATP stimulates the dissociation of MSF from the preprotein and the Tom37-Tom70 complex, and the preprotein is then transferred to another outer membrane receptor complex, Tom20-Tom22, to begin traversal of the outer membrane pore (33). Thus far, no other protein besides metaxin has been found to have significant sequence identity to Tom37, but Tom70 belongs to the tetratricopeptide family of proteins. Tom37 also contains a tetratricopeptide motif that is partially conserved in metaxin. Tetratricopeptide sequences are involved in the association of Tom20 and Tom70 with one another (34), and these motifs may also mediate associations between Tom37, and perhaps metaxin, and other subunits of the import machinery.

In mammalian mitochondria, docking of the preprotein at the outer membrane and subsequent passage of the preprotein to the outer membrane pore appear to occur in stages similar to those observed in yeast (35), but the components of the outer membrane that facilitate this passage are only beginning to be characterized. A component of the outer membrane receptor for the MSF-preprotein complex, OM37, has been characterized with a specific antisera but has not yet been characterized at the molecular level (35). OM37 and metaxin appear to be distinct entities, because antibodies against each protein fail to recognize the other.3 An important next step in the characterization of the function of metaxin and its relation to that of Tom37 is the evaluation of its participation in the recruitment of MSF-preprotein complexes to the mitochondrial outer membrane.

The sequence directing metaxin to the mitochondrial outer membrane resembles the C-terminal signal anchor sequences of monoamine oxidase and Bcl-2, although metaxin contains 15 amino acids C-terminal to the signal anchor that are not present in the other two proteins. The positively charged character of these outer membrane targeting sequences probably allows them to utilize the same chaperones and receptors that classical mitochondrial matrix signals do, whereas the hydrophobic stretch serves as a transmembrane domain. Interestingly, the C terminus of metaxin contains six consecutive acidic residues; clusters of acidic residues in Tom22 are thought to be important in the binding of mitochondrial presequences to the trans side of the mitochondrial outer membrane during import (36), although this finding is controversial (37, 38).

We observed that exogenous metaxin was not associated completely with mitochondria, whereas endogenous metaxin was found exclusively in mitochondria. Incomplete targeting is presumed to occur as a result of saturation of specific chaperones and receptors involved in correctly targeting the protein. We also observed that metaxin lacking its 15 C-terminal amino acids was more efficiently targeted to mitochondria than was the intact protein. The six consecutive acidic residues mentioned above may inhibit integration of overexpressed metaxin into the mitochondrial outer membrane, because an overall positive charge is generally a requirement for import. However, we cannot rule out the possibility that a concentration threshold exists for efficient targeting of metaxin to mitochondria and that full-length metaxin has exceeded that threshold as a result of its relatively high expression level. On the other hand, metaxin missing these C-terminal amino acids and the putative transmembrane region was severely compromised in its association with mitochondria, thereby demonstrating that metaxin associates with mitochondria largely by means of a C-terminal signal anchor. A residual amount of metaxin lacking its signal anchor does continue to associate with mitochondria, perhaps by specifically dimerizing with another protein of the mitochondrial outer membrane. Such putative interactions could also explain why the C-terminal signal anchor domain alone acts rather weakly in directing EGFP to mitochondria.

The relationships among metaxin, FAX, and the C. elegans hypothetical proteins are unclear. None of these relatives of metaxin contains identifiable N- or C-terminal mitochondrial targeting signals, and they are therefore likely to reside in other compartments of the cell. Perhaps these metaxin-like sequences have a common molecular function that can be employed in various cellular locations. The distant similarity of metaxin and its relatives to the GSTs indicates that the former class of proteins may be members of the ancient GST superfamily, although further information about the tertiary structure of metaxin will be needed to properly address this question. The conservation in metaxin of residues that in GSTs are responsible for binding glutathione suggest that metaxin adopts a GST-like conformation but is not an enzymatically active GST.

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REFERENCES

1. Armstrong, L. C., Vos, H., Bergman, B., and Bornstein, P. (1995) Mol. Biol. Cell 6, 434 (abstr.)
2. Vos, H. L., Devarayalu, S., De Vries, Y., and Bornstein, P. (1992) J. Biol. Chem. 267, 12192–12196
3. Bornstein, P., and Sage, E. H. (1994) Methods Enzymol. 245, 62–85
4. Bornstein, P., Devarayalu, S., Edelhoss, S., and Disteche, C. M. (1993) Genomics 15, 607–615
5. Beutler, E. (1995) Adv. Genet. 32, 17–49
6. Bornstein, P., McKinney, C. E., LaMarca, M. E., Winfield, S., Shingu, T., Devarayalu, S., Vos, H. L., and Ginns, E. I. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 4547–4551
7. Collins, M., and Bornstein, P. (1996) Nucleic Acids Res. 24, 3661–3669
8. Iruela-Ario, M. L., Liska, D. J., Sage, E. H., and Bornstein, P. (1995) Dev.

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Table:

| IgG (μg/ml) | α Porin | α Metaxin |
|------------|---------|-----------|
| 500 | 1000 | 0.75 | 1.5 | 3.0 | 7.5 | 15 |

**Fig. 6. Antibodies against metaxin inhibit import of preadrenodoxin.** Mouse liver mitochondria (100 μg) were incubated with the indicated amounts of the antibodies in 50 μl of the import buffer at 0°C for 60 min, washed once with the import buffer, and then subjected to import at 30°C for 50 min. pAd, preadrenodoxin; mAd, mature form of adrenodoxin.

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3. L. C. Armstrong, T. Komiya, B. E. Bergman, K. Mihara, and P. Bornstein, unpublished observations.
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Dyn. 197, 40–56
9. Qabar, A. N., Lin, Z., Wolf, P. W., O’Shea, K. S., Lawler, J., and Dixit, V. (1994) J. Biol. Chem. 269, 1262–1269
10. Gratzer, S., Lithgow, T., Bauer, R. E., Lamping, E., Paltauf, F., Kohlwein, S. D., Haucke, V., Junne, T., Schatz, G., and Horst, M. (1995) J. Cell Biol. 129, 25–34
11. Fleischer, S., and Kervina, M. (1974) Methods Enzymol. 31, 6–40
12. Srere, P. A. (1969) Methods Enzymol. 13, 3–5
13. Trouet, A. (1974) Methods Enzymol. 31, 323–329
14. Stolzenbach, F. (1966) Methods Enzymol. 9, 278–288
15. Schimmel, S. D., Kent, C., Bischoff, R., and Vagelos, P. R. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3195–3199
16. Swanson, M. A. (1955) Methods Enzymol. 2, 541–543
17. Schnaitmann, C., and Greenawalt, J. W. (1968) J. Cell Biol. 38, 158–175
18. Kamiya, T., Sakaguchi, M., and Mihara, K. (1996) EMBO J. 15, 399–407
19. Roise, D., and Schatz, G. (1988) J. Biol. Chem. 263, 4509–4511
20. Rost, B., Casadio, R., Fariselli, P., and Sander, C. (1995) Protein Sci. 4, 521–533
21. Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993) J. Biol. Chem. 268, 25265–25268
22. Mitoma, T., and Ito, A. (1992) J. Biochem. 111, 20–24
23. Hase, T., Muller, U., Riezman, H., and Schatz, G. (1984) EMBO J. 3, 3157–3164
24. Jarausch, J., and Kadenbach, B. (1985) Eur. J. Biochem. 146, 219–225
25. Hill, K. K., Bedian, V., Juang, J.-L., and Hoffmann, F. M. (1995) Genetics 141, 595–606
26. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
27. Koonin, E. V., Mushegian, A. R., Tatusov, R. L., Altschul, S. F., Bryant, S. H., Bork, P., and Valencia, A. (1994) Protein Sci. 3, 2045–2054
28. D irr, H., Reinemer, P., and Huber, R. (1994) Eur. J. Biochem. 220, 645–661
29. Hansen, B., Nuttall, S., and Hoogenraad, N. (1996) Eur. J. Biochem. 235, 750–753
30. Geping, I. S., Millar, D. G., and Shore, G. C. (1995) FEBS Lett. 373, 45–50
31. Seki, N., Moczko, M., Nagase, T., Zufall, N., Ehmann, B., Dietmeier, K., Schäfer, E., Nomura, N., and Pfanner, N. (1995) FEBS Lett. 375, 307–310
32. Bomer, U., Rassow, J., Zufall, N., Pfanner, N., Meijer, M., and Maarse, A. C. (1996) J. Mol. Biol. 262, 389–395
33. Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G., and Lithgow, T. (1995) Nature 376, 705–708
34. Haucke, V., Horst, M., Schatz, G., and Lithgow, T. (1996) EMBO J. 15, 1231–1237
35. Kamiya, T., and Mihara, K. (1996) J. Biol. Chem. 271, 22105–22110
36. Bollinger, L., Junne, T., Schatz, G., and Lithgow, T. (1995) EMBO J. 14, 6318–6326
37. Court, D. A., Kleene, R., Neupert, W., and Lill, R. (1996) FEBS Lett. 390, 73–77
38. Nakai, M., Kinoshita, K., and Endo, T. (1995) J. Biol. Chem. 270, 30571–30575