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Metaxin 1 Interacts With Metaxin 2, a Novel Related Protein Associated With the Mammalian Mitochondrial Outer Membrane

Lucas C. Armstrong, Adam J. Saenz, and Paul Bornstein*

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Abstract A recently described protein, metaxin 1, serves as a component of a preprotein import complex in the outer membrane of the mammalian mitochondrion. A yeast two-hybrid screen with metaxin 1 as bait has now identified a novel protein, which we have termed metaxin 2, as a metaxin 1-binding protein. Metaxin 2 shares 29% identity with metaxin 1 at the amino acid level, but metaxin 2, unlike metaxin 1, lacks a C-terminal mitochondrial outer membrane signal-anchor domain. Two *C. elegans* hypothetical proteins, CelZC97.1 and CelF39B2.i, share high sequence similarity with metaxin 2 and metaxin 1, respectively, and likely represent the *C. elegans* orthologs. Affinity-purified antibodies against metaxin 2 were prepared against the recombinant protein produced in *E. coli* and were used to analyze the subcellular distribution of metaxin 2. In subcellular fractions of mouse liver, a 29 kD immunoreactive protein, consistent in size with the predicted translation product of metaxin 2 cDNA, was found solely in mitochondria. Alkali extraction of mitochondria indicated that metaxin 2 is peripherally associated with mitochondrial membranes. Metaxin 2 in intact mitochondria was susceptible to digestion with proteinase K, indicating that metaxin 2 is located on the cytosolic face of the mitochondrial outer membrane. Finally, baculoviruses encoding a His_6-tagged metaxin 2 and an untagged metaxin 1 lacking its C-terminal transmembrane domain were produced and used separately or in combination to infect Sf21 insect cells. Metaxin 1 bound to a Ni^{2+}-chelate affinity column only in the presence of metaxin 2, indicating that metaxin 1 and metaxin 2 interact when overexpressed in insect cells. These results suggest that metaxin 2 is bound to the cytosolic face of the mitochondrial outer membrane by means of its interaction with membrane-bound metaxin 1, and that this complex may play a role in protein import into mammalian mitochondria. J. Cell. Biochem. 74:11–22, 1999.

Key words: metaxin; metaxin 2; mitochondria; import; outer membrane

Although mitochondria contain DNA that encodes several resident proteins, the majority of mitochondrial proteins are encoded in the nucleus and are imported posttranslationally. Most nuclear-encoded mitochondrial proteins contain, at their N-termini, cleavable presequences that are positively charged and amphiphilic in character [Roise and Schatz, 1988]. A complex set of proteins in the cytosol and mitochondrial outer membrane, inner membrane, and matrix selectively discern mitochondrial signal sequences and actively transport mitochondrial preproteins into the interior of the organelle [for review, see Kübrich et al., 1995; Schatz, 1996; Ryan and Jensen, 1995; Neupert, 1997].

After translation is completed, subsets of mitochondrial preproteins are targeted to the outer membrane in either an ATP-dependent or -independent fashion [Wachter et al., 1994]. The variable ATP requirement reflects the association of the preprotein with distinct chaperones. Preproteins that do not require cytosolic ATP associate with cytosolic hsp70, whereas preproteins whose import requires ATP associate with MSF, a mammalian 14-3-3 protein with ATPase activity that maintains the preprotein in a disaggregated, import-competent form [Mihara and Omura, 1996; Hachiya et al., 1993]. A recently described cytosolic chaperone from yeast,

Abbreviations used: COX IV, cytochrome c oxidase subunit IV; GST, glutathione S-transferase; MSF, mitochondrial import stimulatory factor; FAX, failed axon connections protein; GAL4AD, GAL4 activation domain; GAL4BD, GAL4 DNA-binding domain; PMSF, phenylmethylsulfonyl fluoride.

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*Correspondence to: Paul Bornstein, Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195-7350. E-mail: bornsten@u.washington.edu

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Mft52, also binds specifically to mitochondrial preproteins [Cartwright et al., 1997].

The mitochondrial preprotein then binds to, and is translocated across, the outer membrane by a multiple subunit apparatus that is best characterized in fungi. Differential association of the preprotein with hsp70 or MSF dictates the mode of association of the precursor protein with the outer membrane. Hsp70-bound preproteins have their positively charged presequences exposed, and directly contact the "acid bristle" domains of the Tom20-Tom22 dimer, which is positioned on the mitochondrial outer membrane [Lithgow et al., 1995; Haucke et al., 1995]. Tom refers to a translocase of the outer membrane [Pfanner et al., 1996]. However, MSF binds to and masks the presequence, and the MSF/preprotein complex binds to a distinct, heterodimeric outer membrane receptor, Tom37-Tom70 [Mihara and Omura, 1996; Hachiya et al., 1995]. Tom70 is anchored in the mitochondrial outer membrane by its N-terminus, while its C-terminus contains seven tetratricopeptide repeats that are presumed to mediate protein/protein interactions [Hase et al., 1984; Hines et al., 1990; Steger et al., 1990]. Tom37, which has been described in yeast but not in *Neurospora* [Gratzer et al., 1995], contains a region that is homologous to metaxin, a mitochondrial outer membrane protein that participates in protein import into mouse mitochondria [Bornstein et al., 1995; Armstrong et al., 1997]. On the mammalian mitochondrial outer membrane, a protein termed OM37 that is uncharacterized at the molecular level functions as a docking protein [Komiya and Mihara, 1996]. ATP hydrolysis by MSF releases MSF from the preprotein and Tom37-Tom70 or OM37, and the preprotein is transferred from Tom37-Tom70 to Tom20-Tom22 [Mihara and Omura, 1996; Komiya and Mihara, 1996]. From Tom20-Tom22, the preprotein traverses a pore through the outer membrane, the primary component of which is Tom40 [Kiebler et al., 1990; Vestweber et al., 1989].

Recent information about the import process in mammalian mitochondria supports the idea that the mechanisms and components of the mitochondrial import machinery are grossly conserved between fungi and mammals, but structural differences and potential mechanistic variations in the components exist. An overall conservation of the import process is suggested by the observation that preproteins from fungi can usually be imported into mammalian mitochondria, and vice versa. Furthermore, import of a precursor protein into rat mitochondria can be resolved into stages that correspond to those of import into fungal mitochondria [Komiya and Mihara, 1996]. Several of the components of the mammalian mitochondrial import apparatus that have been characterized at the molecular level also point to a functional equivalence between fungal and mammalian mitochondrial preprotein import mechanisms. First, MSF, which was purified from rabbit reticulocyte lysates, stimulates import of preproteins into yeast mitochondria, thereby demonstrating a degree of conservation of chaperone/outer membrane interactions [Hachiya et al., 1995]. Second, a human homologue of Tom20 can complement the *tom20* mutation in yeast, demonstrating overall functional equivalence between the human and yeast proteins [Goping et al., 1995]. However, the presence of mammalian mitochondrial outer membrane protein hTom34 [Nuttall et al., 1997], which participates in mitochondrial protein import but appears not to have a close fungal homolog, provides evidence of dissimilarity between mammalian and yeast TOMs.

Metaxin, which was discovered as a gene closely interposed between the glucocerebrosidase and the thrombospondin 3 genes in the mouse, shares a modest sequence similarity with Tom37 [Bornstein et al., 1995; Armstrong et al., 1997; Gratzer et al., 1995]. However, metaxin has a C-terminal, and Tom37 has an N-terminal mitochondrial targeting domain, and the two proteins have dissimilar C-terminal domains. To determine whether metaxin interacts with mouse mitochondrial outer membrane proteins that might be similar to or distinct from previously characterized yeast proteins, we undertook a yeast two-hybrid screen with the cytosolic domain of metaxin as bait. Interestingly, we found that metaxin binds to a related protein, which we have termed metaxin 2, that is not present in yeast. Although metaxin 2 does not contain a signal-anchor sequence, it is associated with the cytosolic face of the mitochondrial outer membrane, presumably by means of its interaction with metaxin. We propose that metaxin now be designated metaxin 1, and that the designations metaxin 1 and metaxin 2 be replaced with standardized names for mitochondrial protein translocases [Pfanner et al., 1996] when the relationship between metaxin 1 and

yTom37, and the role of metaxin 2 in protein import into mitochondria, become clarified.

MATERIALS AND METHODS

Reagents, Plasmids, Yeast Strain, and Cell Lines

Plasmids pGBT9.C [Bartel et al., 1996] and pLAM-CYH [Bartel et al., 1993], and the yeast strain PJ696 (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*), a derivative of PJ69-4A [James et al., 1996], were generously provided by Dan Lockshon and Stanley Fields (Dept. of Molecular Biotechnology, UW). Plasmids pBacPAK9 and pBacPAK-His1, mouse liver MATCHMAKER cDNA library in pGAD10, monoclonal antibody against GAL4BD, *Bsu*36 I-digested baculovirus DNA and *S. frugiperda* cell line Sf21 were obtained from Clontech. Plasmid pGEX4T-1 was obtained from Pharmacia (Gaithersburg, MD). Plasmid pTrcHisB and ProBond resin were obtained from Invitrogen (La Jolla, CA). Monoclonal antibody 20E8-C12 against COX IV was obtained from Molecular Probes (Eugene, OR).

Yeast Two-Hybrid Screen

The metaxin 1 ΔTM/C cDNA (encoding amino acids 1-269) [Bornstein et al., 1995; Armstrong et al., 1997] was subcloned downstream from and in frame with the GAL4 DNA-binding domain cDNA in pGBT9.C to create pGBT9.C-met1ΔTM/C. Yeast strain PJ696 was cotransformed by the LiAc method with pGBT9.C-met1ΔTM/C and pGAD10 containing a mouse liver cDNA library cloned downstream from the GAL4 activation domain. The cells were plated on synthetic medium lacking leucine, tryptophan, histidine and adenine, and grown at 30°C for 5 days. pGAD10 and pGBT9.C contain the LEU2 and TRP1 genes, respectively, as selection markers. For two-hybrid selection, PJ696 contains the following genes: 1) the HIS3 gene under control of the GAL1 promoter, 2) the ADE2 gene under control of the GAL2 promoter, and 3) the lacZ gene under control of the GAL7 promoter. As each of these GAL promoters contains a GAL4 binding site, productive interaction between the GAL4BD-metaxin1ΔTM/C and the GAL4AD-metaxin 1-interacting protein allows for transcription of the marker genes (i.e., a $His^+/Ade^+/LacZ^+$ phenotype). β-galactosidase activity was determined by a liquid phase assay, and $LacZ^+$ clones were studied further. Daughter colonies that had lost pGBT9.C-met1ΔTM/C were selected on the basis of their Leu^+/Trp^- phenotypes, and were found to be His^-/Ade^-, which indicates that the GAL4AD-candidate fusion proteins were not autonomously activating the markers. Plasmids were purified from the Leu^+ daughter colonies and introduced into *E. coli* strain HB101 for propagation. Authentic positives were sequenced by automated fluorescent dideoxy sequencing.

Antibodies Against Metaxin 2

The full-length mouse metaxin 2 cDNA was subcloned into pGEX4T-1 in frame with the GST cDNA. The GST-metaxin 2 fusion protein was purified from inclusion bodies as previously described for metaxin [Armstrong et al., 1997]. Rabbits were injected with 250 μg fusion protein mixed with complete Freund's adjuvant, and were boosted twice with the same dose of fusion protein mixed with incomplete Freund's adjuvant. To produce a different fusion protein for affinity purification of anti-metaxin 2 antibodies, the full-length metaxin 2 cDNA was subcloned into pTrcHisB in frame with the cDNA sequence encoding a hexahistidine tag. Polyhistidine metaxin 2 was purified from inclusion bodies under denaturing conditions, as described by the manufacturer. The inclusion bodies were subjected to SDS-PAGE, and transferred to nitrocellulose. The fusion protein band was excised and incubated with crude anti-metaxin 2 serum. Antibodies were eluted with 0.1 M glycine, pH 2.5, and neutralized with 0.1 volume of 1 M Tris, pH 8.0.

Subcellular Fractionation and Western Blotting

Organellar fractions of mouse liver were prepared and analyzed as described [Armstrong et al., 1997]. Western blots were incubated with affinity-purified anti-metaxin 1 [Armstrong et al., 1997] at a dilution of 1:500, or affinity-purified anti-metaxin 2 at a dilution of 1:250, followed by anti-guinea pig (for anti-metaxin 1) or anti-rabbit (for anti-metaxin 2) IgG-horseradish peroxidase conjugate. Proteins were visualized by an enhanced chemiluminescence method (ECL, Amersham, Arlington Heights, IL). For protease sensitivity experiments, purified mouse liver mitochondria (200 μg) were incubated in 2 mM HEPES, pH 7.4, 220 mM mannitol, 70 mM sucrose (Buffer A) and varying concentrations of proteinase K in a final volume of

100 µl for 90 min at room temperature. The reaction was stopped by the addition of PMSF to 1 mM and soybean trypsin inhibitor to 2.5 mg/ml. Mitochondria were pelleted and washed once in Buffer A containing protease inhibitors as above. The pellet was resuspended in SDS-PAGE sample buffer containing 7% SDS and 1 mM PMSF, and subjected to SDS-PAGE and Western blotting analysis with anti-metaxin 2 and anti-COX IV antibodies.

Production of Metaxin 1 and Metaxin 2 in Insect Cells Using A Baculovirus Vector

Metaxin 1 ΔTM/C cDNA was cloned downstream from the polyhedrin promoter in pBacPAK9, and metaxin 2 cDNA was cloned downstream from and in frame with the hexahistidine-coding sequence in pBacPAK-His 1. Plasmids were combined with *Bsu*36 I-digested baculovirus DNA and Lipofectin, and incubated with Sf21 cells according to the manufacturer's instructions. Viral clones were plaque-purified and assessed for the presence of recombinant metaxin 1 and metaxin 2 by Western blotting analysis.

For metaxin 1/metaxin 2 association experiments, 1×10^6 Sf21 cells in 35 mm dishes were infected with either metaxin 1 or His_6-metaxin 2 baculovirus, or with a mixture of the two viruses, and incubated for 3 days. Cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 1 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 µg/ml pepstatin). Supernatant (100 µl) was incubated with 50 µl packed Pro-Bond resin for 30 min, and the resin was washed four times with lysis buffer. Bound proteins were eluted in 0.1 M EDTA and subjected to Western blotting analysis.

RESULTS

Yeast Two-Hybrid Screen

To identify proteins that interact directly with metaxin 1, we employed the yeast two-hybrid system [Bartel and Fields, 1995] with metaxin 1 as bait. A truncated metaxin 1 cDNA (metaxin 1 ΔTM/C, described in Armstrong et al., 1997) was chosen because of the likelihood that the presence of the C-terminal transmembrane domain would cause misfolding or mislocalization of the fusion protein in yeast. Metaxin 1 ΔTM/C cDNA was placed downstream from the GAL4BD cDNA in pGBT9.C to create pGBT-met1ΔTM/C. *S. cerevisiae* strain PJ696, derived from progenitor strain PJ69-4A [James et al., 1996], was transformed with pGBT9.C or pGBT9.C-met1ΔTM/C, and lysates were prepared from the cells. Western blotting analysis of the lysates with antibodies against metaxin and GAL4BD revealed an immunoreactive protein of 50 kD, corresponding to GAL4BD-metaxin 1 ΔTM/C fusion protein, in the cells that were transformed with pGBT-met1ΔTM/C (data not shown).

A mouse liver library, cloned downstream from the GAL4AD cDNA in pGAD10, was selected for the two-hybrid screen. We cotransformed PJ696 with pGBT9.C-met1ΔTM/C and the library, and plated the cells on synthetic medium lacking leucine, tryptophan, histidine, and adenine. Growth in the absence of leucine and tryptophan indicates that successful transformation with each plasmid has occurred, and growth in the absence of histidine and adenine occurs as a result of the GAL4BD-metaxin fusion protein and the GAL4AD-candidate fusion protein interacting, binding GAL4 elements in the promoters of the *HIS3* and *ADE2* genes, and activating transcription of the reporter genes. An initial screen of an estimated 10^7 clones yielded 17 $Leu^+/Trp^+/Ade^+/His^+$ colonies, of which eight were $lacZ^+$. As the library contained approximately 3×10^6 independent clones, the screen is most likely saturating. Plasmids isolated from these positive colonies were characterized by restriction mapping and sequencing, and one clone was found to be identical to a novel protein, which we have termed metaxin 2, that is related in sequence to metaxin 1 (see below). PJ696 was cotransformed with pGAD10-metaxin 2 and either pGBT9.C, pGBT9.C-met1ΔTM/C, or pLAM-CYH, which encodes a GAL4BD-lamin fusion protein prone to producing false positive interactions [Bartel et al., 1993], and plated on SD lacking leucine, tryptophan, histidine, and adenine. Transformants with pGAD10-metaxin 2 were $Leu^+/Trp^+/Ade^+/His^+$ in combination with pGBT9.C-met1ΔTM/C, and $Leu^+/Trp^+/Ade^-/His^-$ in combination with pGBT9.C and pCYH-LAM; phenotypes of cotransformations are shown in Figure 1. Transformants were also analyzed by liquid phase β-galactosidase assay, and only PJ696 transformed with both pGBT9.C-met1ΔTM/C and pGAD10-metaxin 2 was *LacZ*-positive (data not shown).

Homologs of Metaxin 2

The sequence for metaxin 2 matched that of several human and mouse ESTs. To confirm the

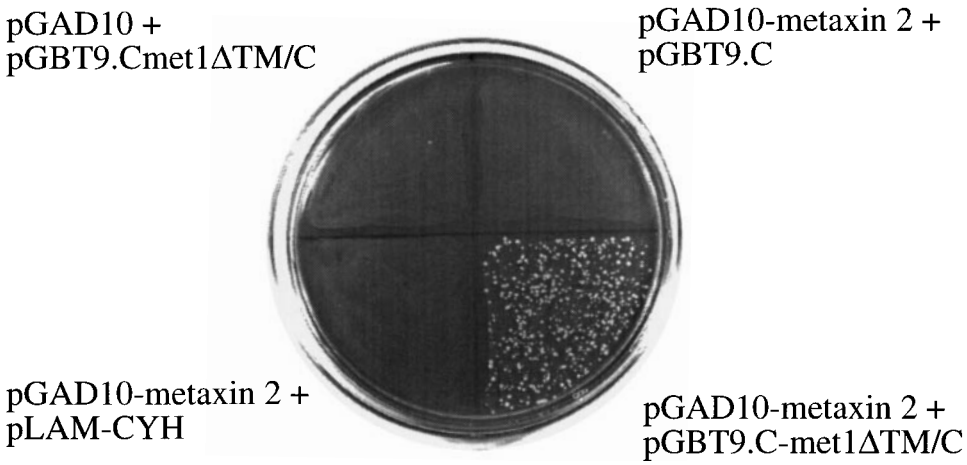

Fig. 1. Yeast two-hybrid interaction between metaxin 1 and metaxin 2. *Saccharomyces cerevesiae* strain PJ696 was transformed with the GAL4 DNA-binding domain vector, pGBT9.C (either empty or containing a cDNA encoding metaxin 1 lacking its C-terminal transmembrane domain, metaxin 1 ΔTM/C), and with the GAL4 activation domain vector, pGAD10 (either empty or containing a cDNA encoding metaxin 2). Plasmid pLAM-CYH is a GAL4 activation domain vector containing a cDNA encoding lamin C. Transformed yeast were plated onto SD agar lacking leucine, tryptophan, histidine, and adenine, and grown for 5 days at 30°C.

sequence of the mouse metaxin 2 cDNA clone obtained in the yeast two-hybrid screen and to determine the human metaxin 2 cDNA sequence, the clones from which the ESTs were derived were obtained and sequenced in their entireties. The mouse metaxin 2 clones obtained from the yeast two-hybrid screen and from the EST database were identical. The human and mouse metaxin 2 cDNAs (GenBank accession #AF053551 and #AF053550, respectively) are 1270 and 1223 base pairs in length, respectively, and both encode proteins of 263 amino acids that are 97% identical to one another (data not shown) and 29% identical to metaxin 1 (Fig. 2). A search of the nonredundant protein database with the translated mouse metaxin 2 cDNA identified the most similar protein (34% identity) as CelZC97.1, a *C. elegans* hypothetical protein (#P34599) which was identified during sequencing of *C. elegans* chromosome III, cosmid ZC97 [Wilson et al., 1994]. Other proteins sharing significant sequence similarity with metaxin 2 were *C. elegans* hypothetical proteins F39B2.i (#Z92834), C25H3.7 (#U29535), and K01D12.13 (#Z75543), and *Drosophila* failed axon connections protein (FAX) [Hill et al., 1995]. In addition, metaxin 2 conforms to a glutathione S-transferase consensus sequence that is also found in metaxin 1 and FAX [Koonin et al., 1994; Armstrong et al., 1997]. A search of the SCOP database [Murzin et al., 1995] with the metaxin 2 sequence and its secondary structure predicted by the PHDsec algorithm [Rost and Sander, 1993] reveals GST isoenzyme 3-3 as the best match ($P = 0.08$).

Comparison of the metaxin 1 and metaxin 2 sequences revealed that metaxin 2 lacks the transmembrane domain of metaxin 1. We have previously demonstrated that this transmembrane domain acts as a mitochondrial outer membrane signal-anchor domain [Armstrong et al., 1997]; therefore, metaxin 2 is not predicted to be an integral protein of the mitochondrial outer membrane. A search for putative subcellular targeting sequences with PSORT [Nakai and Kanehisa, 1992] failed to reveal any such targeting sequences, including N-terminal mitochondrial targeting domains. Therefore, metaxin 2 is predicted to be a cytosolic protein. Interestingly, CelF39B2.i, like metaxin 1, contains a C-terminal transmembrane sequence, whereas CelZC97.1, like metaxin 2, lacks such a domain (Fig. 2). This observation, in combination with the observation of higher degrees of sequence similarity between CelF39B2.i and metaxin 1, and CelZC97.1 and metaxin 2, suggests that CelF39B2.i is the *C. elegans* ortholog of metaxin 1, whereas CelZC97.1 is the *C. elegans* ortholog of metaxin 2.

Cellular and Subcellular Distribution of Metaxin 2

Multiple tissue Northern blotting analysis with the mouse metaxin 2 cDNA as a probe

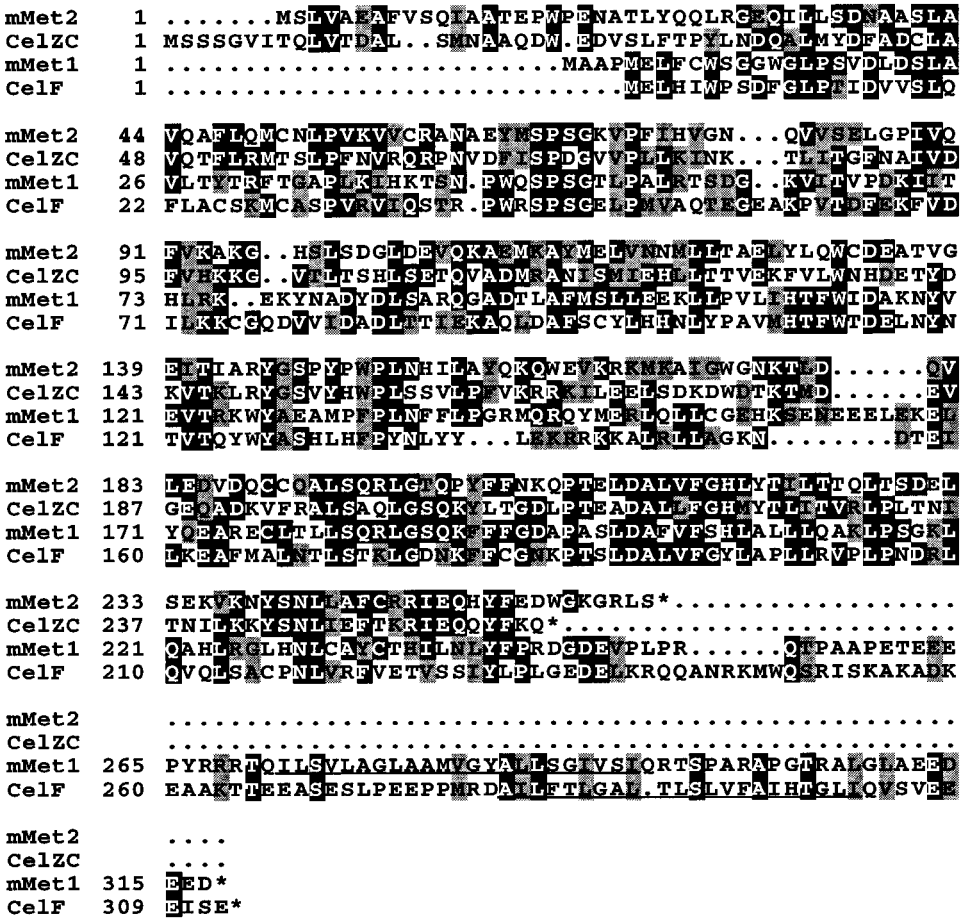

Fig. 2. Protein sequence alignment of metaxin 1, metaxin 2, and two *C. elegans* hypothetical proteins. Murine metaxin 2 (mMet2, GenBank accession #AF053550), CelZC97.1 (CelZC, #P34599), murine metaxin 1 (mMet1, #L36962), and CelF39B2 (CelF, #Z92834) protein sequences were aligned by the PileUp function of GCG. Sequence identities and similarities (I/M/L/V, R/K, E/D, Q/N, W/F/Y, G/A, S/T, D/N, Q/E) were highlighted by BOXSHADE. Identical residues are indicated by a black background, and similar residues are indicated by grey shading. Gaps, indicated by dots, are inserted to optimize global alignment. Asterisks indicate translational stop sites. Predicted transmembrane sequences are underlined.

revealed a ubiquitously expressed 1.4 kb mRNA (data not shown). With the length of the polyA tail taken into account, this size is consistent with that of a full-length metaxin 2 cDNA. Western blotting analysis of a range of mouse tissues with affinity-purified anti-metaxin 2 antibodies also revealed that a 29 kD band, consistent in size with the predicted translation product of metaxin 2 cDNA, was ubiquitously expressed (data not shown).

To determine the subcellular distribution of metaxin 2, subcellular fractions of mouse liver were prepared by differential centrifugation and analyzed by Western blotting with anti-metaxin 2 antibodies. Metaxin 2 was found solely in mitochondria (Fig. 3A), and was present in mitochondria from mouse kidney, lung, and heart (Fig. 3B). Therefore, metaxin 2 appears to be a widely expressed component of mitochondria. Metaxin 2 appears to be tightly bound to mitochondria, as no metaxin 2 could be detected in cytosolic fractions from the same tissues (data not shown). In mitochondria from lung, and to a lesser extent in heart, an 85 kD immunoreactive band, which could represent either a complex containing metaxin 2 or an unrelated cross-reactive protein, was observed. This band is distinct from the 70 kD band observed in mitochondrial lysates probed with anti-metaxin 1 antibodies [Armstrong et al., 1997].

To determine the nature of the interaction between metaxin 2 and mitochondrial membranes, mitochondria were subjected to extraction with alkali or nondenaturing detergent, and the fractions were analyzed by Western

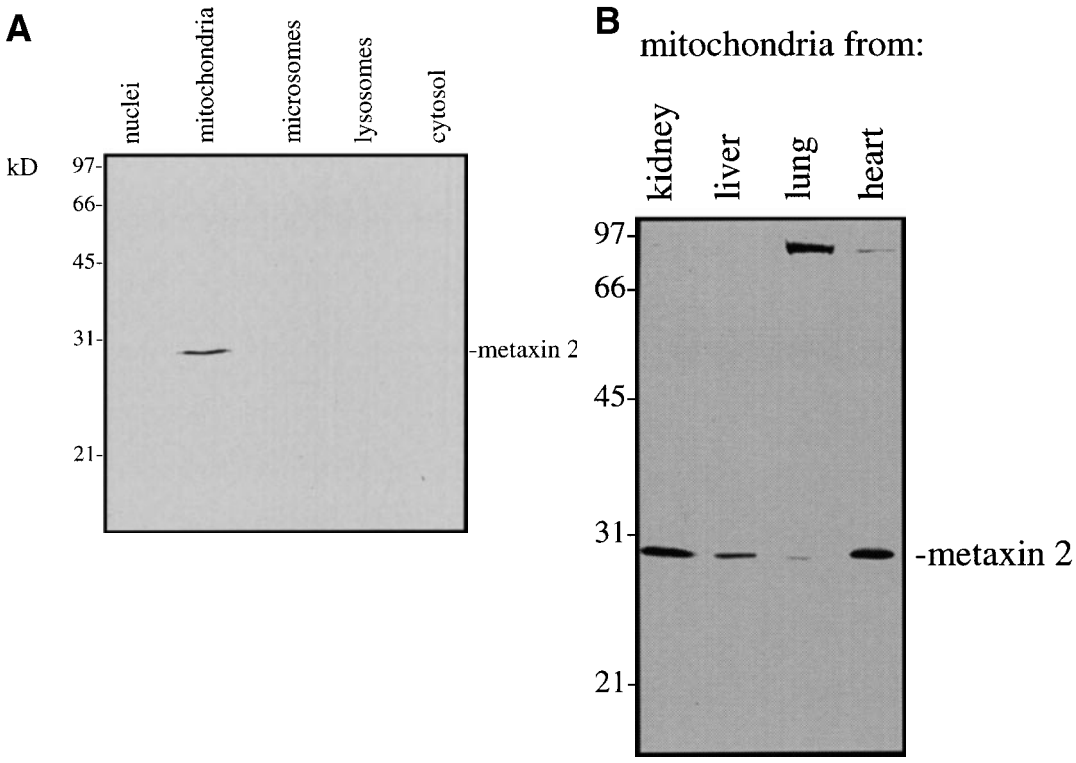

Fig. 3. Subcellular distribution of metaxin 2 in mouse tissues. **A:** Mouse liver organelles were fractionated by sucrose density centrifugation and subjected to Western blotting analysis with affinity-purified anti-metaxin 2 antibodies. Metaxin 2 is present solely in mitochondria. **B:** Mitochondria were isolated from mouse kidney, liver, lung, and heart and subjected to Western blotting analysis as in A. Metaxin 2 is present in mitochondria from all tissues examined.

blotting. Although metaxin 1 was found to be insoluble in alkali, which is consistent with its previously described outer membrane location, metaxin 2 was found to be in the alkali-soluble fraction (Fig. 4A). This observation suggests that metaxin 2 is peripherally associated with mitochondrial membranes.

Given the lack of obvious mitochondrial targeting sequences in metaxin 2, and the finding that metaxin 2 interacts with the cytosolic domain of metaxin 1 in a yeast two-hybrid assay, we predicted that metaxin 2 would be located on the cytosolic face of the mitochondrial outer membrane. To test this hypothesis, intact mitochondria were incubated with proteinase K. Under these conditions, proteins on the cytosolic face of the outer membrane are degraded by protease, while proteins within the interior of mitochondria are protected from protease digestion by the outer membrane. Digestion was halted by addition of protease inhibitors, and the mitochondria were pelleted and analyzed by Western blotting analysis with antibodies against metaxin 2 and COX IV. Metaxin 2 was digested by proteinase K treatment (Fig. 4B, top panel), while COX IV was unaffected by proteinase K (Fig. 4B, bottom panel). The slightly higher mobility of metaxin 2 in proteinase K-treated mitochondria most likely results from cleavage at a highly sensitive site near one of the termini. Chymotrypsin digestion also resulted in degradation of metaxin 2 in the absence of degradation of COX IV (data not shown). In mitochondria in which the outer membrane was removed by treatment with digitonin, COX IV was susceptible to protease treatment (data not shown), indicating that COX IV was protected from degradation in intact mitochondria by the presence of an intact outer membrane and not by an intrinsic resistance to proteolytic digestion. The susceptibility of metaxin 2 to digestion by proteinase K indicates that metaxin 2 is exposed to the cytosol while bound to the outer membrane.

Interaction Between Metaxin 1 and Metaxin 2

To evaluate the biochemical interaction between metaxin 1 and metaxin 2, baculovirus vectors containing metaxin 1 and metaxin 2 cDNAs were constructed. The metaxin 2 baculo-

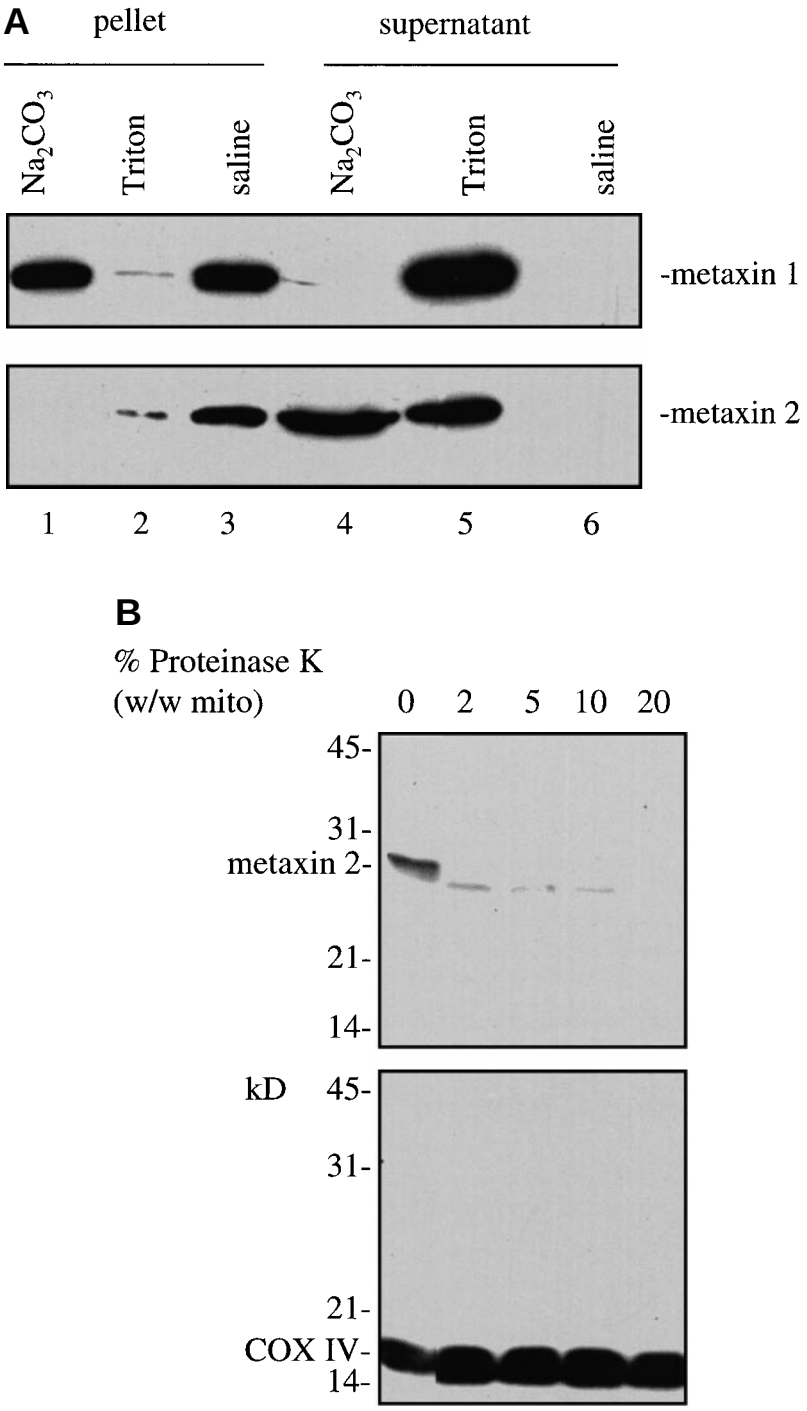

Fig. 4. Solubility and protease accessibility of metaxin 2 in mouse mitochondria. **A:** Mouse liver mitochondria (20 μg) were resuspended in either 0.1 M Na_2CO_3, pH 11.5 (**lanes 1** and **4**), 1% Triton X-100 (**lanes 2** and **5**), or Tris-buffered saline (**lanes 3** and **6**). Samples were centrifuged at 10,000 *g*, and the pellet (lanes 1–3) and supernatant (lanes 4–6) fractions were subjected to Western blotting analysis with affinity-purified antibodies against metaxin 1 (top panel) or metaxin 2 (bottom panel). Insolubility of metaxin 1 (top panel, lane 1) and solubility of metaxin 2 (bottom panel, lane 4) in alkali indicate that metaxin 1 is an integral membrane protein, whereas metaxin 2 is a peripheral membrane protein. **B:** Mouse liver mitochondria were incubated with the indicated amounts of proteinase K (w/w mitochondrial protein) and centrifuged. The pellet was washed with a solution of protease inhibitors and subjected to Western blotting analysis with anti-metaxin 2 antibodies (top panel). The blot was stripped and analyzed with a monoclonal antibody against COX IV (bottom panel), an inner membrane space protein that extends into the intermembrane space. Susceptibility of metaxin 2 to proteinase K suggests that metaxin 2 is present on the cytosolic face of the mitochondrial outer membrane. Lack of degradation of COX IV indicates that proteins in the mitochondrial interior are protected from protease under the conditions of the experiment.

virus contained the metaxin 2 coding sequence in frame with an N-terminal hexahistidine-coding sequence, whereas the metaxin 1 baculovirus contained an untagged version of metaxin 1 lacking its C-terminal transmembrane sequence. Sf21 cells were infected with each baculovirus alone, or with the two together, and were lysed after several days. Western blotting analysis of the lysates revealed that metaxin 1 and His_6-metaxin 2 were expressed at high levels in the appropriate cells (Fig. 5, lanes 1–4). Lysates were incubated with a Ni^{2+}-chelate affinity resin and bound proteins were eluted with EDTA. His_6-metaxin 2 in lysates from all cells infected with metaxin 2 baculovirus was found to be bound to the resin (Fig. 5, lanes 7 and 8). However, metaxin 1 from cells infected with metaxin 1 baculovirus alone failed to bind the resin (Fig. 5, lane 6), whereas metaxin 1 from cells infected with both metaxin 1 and His_6-metaxin 2 baculovirus was associated with the resin (Fig. 5, lane 8). Therefore, metaxin 1 associates with metaxin 2 when both are expressed at high levels in insect cells.

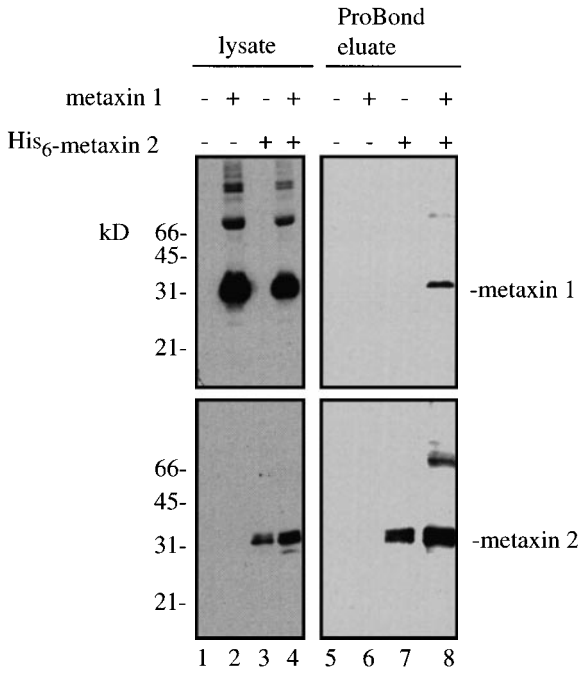

Fig. 5. Recombinant metaxin 1 and metaxin 2 associate when coexpressed in insect cells infected with baculoviruses. Sf21 insect cells were infected with recombinant baculovirus containing metaxin 1 ΔTM/C cDNA (**lanes 2** and **6**), or with metaxin 2 cDNA in frame with a hexahistidine tag (**lanes 3** and **7**), or with both baculoviruses (**lanes 4** and **8**). **Lanes 1** and **5** represent an uninfected control. After 3 days, cells were lysed with NP40-containing buffer. A fraction of each lysate was incubated with Ni^{2+}-chelate affinity resin (ProBond, Invitrogen), washed with lysis buffer and eluted with 0.1 M EDTA. Crude lysates (lanes 1–4) and eluates from ProBond resin (lanes 5–8) were subjected to Western blotting analysis with affinity-purified antibodies against metaxin 1 (top panels) or metaxin 2 (bottom panels).

DISCUSSION

Several lines of evidence indicate that metaxin 2, a novel protein related in sequence to metaxin 1, physically interacts with metaxin 1: 1) metaxin 2 interacts with metaxin 1 as indicated by the results of a yeast two-hybrid screen, 2) metaxin 2 associates with metaxin 1, as judged by binding to a Ni^{2+}-chelate affinity matrix, when both are overexpressed in insect cells by means of a baculovirus vector, 3) metaxin 2 is located on the cytosolic face of the mitochondrial outer membrane, as is metaxin 1. However, metaxin 2 differs from metaxin 1 in its lack of a C-terminal mitochondrial outer membrane signal-anchor domain and consequent peripheral association with mitochondrial membranes. The most likely explanation for the association of metaxin 2 with mitochondria is that metaxin 1 tethers metaxin 2 to the mitochondrial outer membrane. It is unclear what fraction of metaxin 2 is bound to metaxin 1, as quantitative coimmunoprecipitation experiments from detergent-solubilized mitochondria were prevented by the insolubility of metaxin 1 and metaxin 2.

We have previously demonstrated that metaxin 1 is a component of the mammalian mitochondrial preprotein translocase of the outer membrane (TOM) complex [Armstrong et al., 1997]. Therefore, it is likely that metaxin 2 plays a role in protein translocation into mitochondria. However, antibodies against metaxin 2 failed to inhibit translocation of preadrenodoxin into isolated mouse mitochondria (L.C. Armstrong, T. Komiya, K. Mihara, unpublished observations). This result does not exclude a role for metaxin 2 in protein import into mitochondria, since our antibodies against metaxin 2 may not be neutralizing antibodies, or may be sterically blocked, by the presence of an adjacent protein, from binding to metaxin 2 on the mitochondrial surface. Supporting this possibility is the observation that metaxin 2 in detergent-solubilized mitochondria remains in a particulate fraction that is sedimented at 100,000*g* (data not shown). Alternatively, metaxin 2 may not play a constitutive and obligatory role in protein import into mitochondria, but may serve instead to modulate import by interacting with

cytosolic structural or regulatory molecules. Nevertheless, metaxin 2 appears to be tightly associated with mitochondria, as no metaxin 2 could be detected in cytosolic fractions from various mouse tissues, and is therefore unlikely to function as a cytosolic chaperone.

Metaxin 1 shares significant sequence similarity with yTom37, a yeast mitochondrial outer membrane protein that binds to yTom70 and facilitates the binding of certain mitochondrial proteins to the outer membrane [Gratzer et al., 1995]. However, metaxin 2 appears to share no significant sequence similarity with any characterized or hypothetical yeast proteins, including yTom37, as judged by a search with metaxin 2 against the entire yeast genome translated in six reading frames. One possible explanation for the relationships between yTom37, metaxin 1, and metaxin 2 is that after the common ancestor of yeast and mammals diverged, the single, ancestral Tom37 gene underwent a duplication event, and the second copy subsequently diverged considerably in sequence and lost its transmembrane domain. It is likely that this duplication occurred prior to or early in the evolution of metazoans, as *C. elegans* contains two hypothetical proteins, CelF39B2.i and CelZC97.1, that are strikingly similar in sequence to metaxin 1 and metaxin 2, respectively.

At present, our lack of information regarding the degree of relatedness between metaxin 1 and yTom37 prevents us from renaming metaxin 1 to conform with standardized nomenclature for translocases of the outer membrane [Pfanner et al., 1996]. The facts that metaxin 1 and yTom37 share sequence similarity only in their N-terminal halves and have mitochondrial outer membrane signal-anchor sequences at opposite ends of the molecules raise the possibility that the proteins may have significant functional differences. Further functional information is therefore necessary to determine whether metaxin 1 deserves to be renamed as the mammalian counterpart to yTom37 or be given a unique Tom designation. Similarly, further studies clarifying the role of metaxin 2 in protein import into mitochondria are necessary before giving it a Tom designation.

Each member of the metaxin protein family, including metaxin 2, appears to conform to a cytosolic GST consensus sequence [Koonin et al., 1994]. As with metaxin 1, metaxin 2 appears to have diverged at residues important for catalysis, so it would seem that metaxin 2 is not an enzymatically active GST. Cytosolic GSTs exist either as homo- or heterodimers, and heterodimers have only been observed to form between members of the same class [Wilce and Parker, 1994]. In this respect, the observation that metaxin 1 and metaxin 2 interact is also consistent with the predicted relationship of these proteins with GSTs.

The interaction between metaxin 1 and metaxin 2 has implications for the phenotypic consequences of loss of metaxin 1 in mice. A targeted disruption of the metaxin 1 gene in the mouse was serendipitously produced during experiments designed to produce a subtle mutation in the adjacent *Gba* gene; mice homozygous for this metaxin 1-null allele display an early embryonic lethal phenotype [Bornstein et al., 1995]. Given that metaxin 1 participates in anchoring metaxin 2 to the mitochondrial outer membrane, the absence of metaxin 1 most likely results in mislocalization and loss of function of metaxin 2. The extent to which the severe phenotype of the metaxin 1-null mouse is dependent upon a loss of function of metaxin 2 could be determined by the construction and analysis of a metaxin 2-null mouse.

The interaction between metaxin 2 and metaxin 1, a component of the mammalian TOM complex, supports the concept that the components of the TOM complex that act early in the process of import have diverged between yeast and mammals. Tom37 in yeast appears to be replaced by metaxin 1 and metaxin 2 in mammals, although further experiments are required to assess the functional similarities between the molecules. Thus far, no direct mammalian counterpart to yTom70 has been described. A novel mammalian outer membrane translocase, termed hTom34, appears to contain one tetratricopeptide motif characteristic of yTom70, but otherwise bears no similarity to any other yeast proteins [Nuttall et al., 1997]. However, Tom20, which acts at a later stage in the import process and imports proteins unaccompanied by a chaperone or bound to hsp70, is structurally and functionally similar between yeast and mammals [Iwahashi et al., 1997; Seki et al., 1995; Goping et al., 1995]. One possible explanation for the divergence of early stage TOMs and conservation of later stage TOMs is that the primary function of early stage TOMs is to recognize preproteins bound to specific chaperones, and that the mitochon-

drial preprotein-specific chaperones of yeast and mammals may have also diverged. At the present time, the relationship between yeast and mammalian cytosolic chaperones specific for mitochondrial preproteins is unclear. Although MSF can promote protein import into isolated yeast mitochondria by way of Tom37-Tom70 [Hachiya et al., 1995], a yeast counterpart to MSF has not been described. Conversely, Mft52 has been characterized in yeast, but has not been described in mammals [Cartwright et al., 1997].

It is important to note that the precise functions of neither yTom37 nor metaxin 1 in the mechanism of import have been characterized. Although antibodies against yTom37 inhibit MSF-dependent protein import into yeast mitochondria, recombinant yTom70 appears to be sufficient for binding an MSF/preprotein complex [Hachiya et al., 1995; Komiya et al., 1997]. Perhaps yTom37 in yeast and the metaxin 1/metaxin 2 complex in mammals modify the affinity of yTom70 and its mammalian counterpart, respectively, for precursors and/or chaperones, and the apparent redundancy of the metaxin 1/metaxin 2 component in mammals adds an extra layer of discrimination to the process.

It is also possible that the early stage TOMs have diverged to facilitate the mitochondrial import process in mammalian cells with complex and varied architectures. As mammalian mitochondria are frequently distant from the site of protein synthesis, added complexity in import mechanisms may be required for efficient protein import. For example, in skeletal muscles, distinct subpopulations of mitochondria that reside in the subsarcolemmal space and adjacent to myofibrils differ in rates of protein import, which suggests that subcellular heterogeneity of mitochondria can be generated by variations in protein import [Hood et al., 1994; Takahashi and Hood, 1996]. In neuronal cells, mitochondria appear to import preproteins within the cell body, and subsequently are transported a great distance to distal dendrites and axon terminals [Liu and Wong-Riley, 1994]. The mechanisms by which mitochondrial protein import and subcellular positioning of mitochondria are coordinated are unknown, but the added complexity of the mammalian early stage TOMs may facilitate such processes.

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