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Molecular characterization of mitofilin (HMP), a mitochondria-associated protein with predicted coiled coil and intermembrane space targeting domains

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SUMMARY

We have identified and characterized a human protein of the mitochondria which we call mitofilin. Using monoclonal and polyclonal antibodies, we have isolated cDNA clones and characterized mitofilin biochemically. It appears as a 90 and 91 kDa doublet in western blots and is translated from a single 2.7 kb mRNA. Antibodies raised against cellular and bacterially-expressed protein give identical cytoplasmic immunofluorescence and immunoblot results. Mitofilin co-localizes with mitochondria in immunofluorescence experiments and co-purifies with mitochondria. Double label studies show co-localization only with mitochondria and not with Golgi or endoplasmic reticulum. Co-localization with mitochondria is retained when actin or tubulin are de-polymerized, and mitofilin is expressed in all human cell types tested. The cDNA encodes a polypeptide with a central α-helical region with predicted coiled coil domains flanked by globular amino and carboxy termini. Unlike coiled coil motor proteins, mitofilin is resistant to detergent extraction. The presence of mitochondrial targeting and stop-transfer sequences, along with the accessibility of mitofilin to limited proteolysis suggests that it resides predominantly in the intermembrane space, consistent with immuno-electron micrographs which show mitofilin mainly at the mitochondrial periphery. The cDNA sequence of mitofilin is identical to that recently reported by Icho et al. (1994; *Gene* 144, 301-306) for a mRNA preferentially expressed in heart muscle (HMP), consistent with the high levels of mitochondria in cardiac myocytes.

Key words: Mitochondria, Inter-membrane space, Coiled coil protein, Immunofluorescence, Monoclonal antibody, Matrix targeting sequence, HMP

INTRODUCTION

Mitochondria are dynamic organelles which replicate semi-autonomously, bifurcate and re-fuse, and are actively transported through the cytoplasmic volume (reviewed by Attardi and Schatz, 1988; Bereiter-Hahn and Voth, 1994). Differing cellular energy requirements result in variations in the number of mitochondria present, and mitochondrial morphologies also vary greatly with cell type. These range from the familiar submicron structures seen in hepatocytes to highly complex, anastomosing, filamentous networks many microns long in growing fibroblasts (Johnson et al., 1980; Amchenkova et al., 1988).

Most proteins required for the respiratory chain, for mitochondrial structure and motility, and for import and export are translated in the cytoplasm from nuclear-transcribed RNAs, and must then be delivered to the organelle and translocated to their appropriate compartments. This transport is directed largely by short, amphiphilic amino acid sequences which target proteins to their appropriate destinations (van Loon et al., 1986; von Heijne, 1986; reviewed by Hartl et al., 1989; Attardi and Schatz, 1988). The electrochemical gradients required for ATP synthesis are maintained through complex topological arrangements of enzymes and transport proteins with specific orientations relative to the mitochondrial double membrane system. These gradients also provide the means to selectively label living mitochondria with fluorescent dyes.

Because of stringent topological requirements for the assembly and coupling of holoenzymes across the membrane system, the mechanisms for protein import and processing are subject to complex regulatory steps (Attardi and Schatz, 1988; Hartl et al., 1989). A number of protein components of the translocation machinery have been characterized biochemically and at the sequence level (reviewed by Hannavy et al., 1993; Schwarz and Neupert, 1994; van der Klei et al., 1994). Ultrastructural and immunocytochemical analyses suggest that import may occur primarily at sites where the two membranes come into close apposition (Schleyer and Neupert, 1985; Pfanner et al., 1990). These ‘contact sites’ persist after ultrasonic disruption of mitochondria and appear to resist digitonin extraction of most of the outer membrane (Hackenbrock and Miller, 1975).

Although much is known at the molecular level about mitochondrial metabolism and electron transport, there is still much

to be learned about mitochondrial movement, morphogenesis, import/export, and cytoskeletal interactions. Studies of yeast mutants have identified proteins which regulate mitochondrial morphology, possibly through interactions with the cytoskeleton, but the mechanisms underlying the effects of the mutations are not understood (Sogo and Yaffe, 1994; Burgess et al., 1994). While mitochondria have been observed to co-localize with other cytoskeletal elements, several lines of evidence implicate microtubules as the cytoskeletal element along which mitochondria are translocated in a variety of cell types from higher organisms. These include ultrastructural analyses by immunofluorescence (Heggeness et al., 1978) and quick-freeze EM imaging (Hirokawa, 1982) as well as in vitro studies of reconstituted systems (Brady, 1985; Martz et al., 1984). An unusual monomeric, kinesin-like protein called KIF1B was recently described which functions as a mitochondria-specific motor protein in microtubule transport assays (Nangaku et al., 1994). Like other motor proteins, KIF1B is detergent-extractable; however, unlike other motors, it appears to act as a monomer. How KIF1B recognizes mitochondria is currently unknown.

Here, we report the molecular characterization of a previously unknown constituent of mitochondria which resists detergent extraction. As part of an ongoing study of nuclear matrix proteins, monoclonal antibodies were raised using total nuclear matrix proteins as antigen. One resulting antibody, however, showed a distinctive cytoplasmic distribution, and we have used that antibody to clone and characterize the protein it recognizes. Because of its predicted α-helical coiled coil content, its filamentous appearance in fluorescently labeled fibroblasts, and its mitochondrial localization, we have named the protein mitofilin. The cDNA sequence of mitofilin is identical to that recently published by Icho et al. (1994) for a mRNA preferentially transcribed in heart muscle, HMP (heart muscle protein). We present evidence which argues against those authors' suggestion that the protein product of the mRNA is a kinesin-like motor protein.

MATERIALS AND METHODS

Nuclear matrix protein antibody production

Total nuclear matrix proteins were isolated by the method of Fey and Penman (1988) from the human cervical tumor cell line Caski and were used for the production of monoclonal antibodies. Antibodies were prepared by Matritech, Inc. (Newton, MA). Balb/c by J mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with purified Caski NM protein every 2 weeks for a total of 16 weeks. Spleen cells were fused with the SP2/O-Ag14 mouse myeloma line using the method of Koehler and Milstein (1975). One clone, 302.47, was selected which reacted with a 90 and 91 kDa protein on immunoblots. A second generation monoclonal, clone 2-8, was raised using purified recombinant protein (see below) following the same procedures. One New Zealand White rabbit was also immunized over a three-month period with a total of 40 μg of recombinant protein to produce a polyclonal antiserum (3584).

Library screening

cDNA clones were obtained from a λgt11 human carcinoma cDNA expression library (Stratagene, La Jolla CA.). Library screening followed manufacturer's instructions. One clone contained a 2.25 kb insert whose expressed protein was recognized by antibody 302.47. The cDNA was subcloned into pBluescript II plasmid (Stratagene). The resulting plasmid, pGT1, was sequenced directly and further subcloned to produce fusion protein (see below). Four independent clones were obtained by further library screening using cDNA probes derived from the pGT1 sequences.

Sequencing and sequence analysis

pGT1 cDNA sequences were obtained using the dideoxy method of Sanger et al. (1977). Double-stranded sequencing was done utilizing the T3 and T7 primers and internal primers as needed. Some of the independent cDNA clones were sequenced by automated cycle sequencing (Applied Biosystems, Foster City, CA) for several hundred bases to confirm their identities. The predicted amino acid sequence was analyzed by protein motif algorithms using GeneWorks software (Intelligenetics, Inc., Mountain View, CA), and also by a DOS-compatible translation (Odgren et al., 1996) of the Coils 2.1 coiled coil prediction program of Lupas et al. (1991). Mitochondrial targeting signals were analyzed by the PSORT program (Nakai and Kanehisa, 1992).

Preparation of recombinant protein

A fusion protein was obtained using the insert from pGT1 by subcloning into the pMAL-C vector (New England Biolabs Inc., Beverly MA), expressing in *Escherichia coli* strain TB1, and purifying by amylose affinity chromatography. Experimental details of the pMAL system were carried out according to the manufacturer's instructions. The ligation was such that the first Met codon in the pGT1 cDNA was inserted in-frame (nucleotide position 437 of D21094; Fig. 1A), following the Factor Xa protease cleavage site encoded by the vector. After cleavage, the released maltose-binding protein was removed by a second amylose affinity chromatography step. Recombinant protein, free of low molecular mass fragments, was purified to homogeneity by preparative SDS-PAGE using a model 491 system (Bio-Rad, Hercules, CA) with an 8% gel column. Fractions were assayed by SDS-PAGE, immunoblot and Coomassie staining, and appropriate fractions were pooled.

Immunofluorescence microscopy

Cell extraction and fixation

Human diploid fibroblasts WI38 and IMR-90, SV40-transformed IMR-90, and ME-180 human cervical carcinoma cells were grown in basal medium Eagle (IMR-90) or Dulbecco's modified Eagle's medium (WI38 and ME-180) supplemented with 10% fetal bovine serum on glass coverslips and washed twice with PBS. Non-extracted cells were fixed by three different methods: (1) some cells were fixed at room temperature for 12 minutes in PBS containing 3.7% formaldehyde. After fixation and washing away residual fixative, the cells were permeabilized with PBS containing 0.1% Triton X-100 for three minutes. (2) In some experiments cells were fixed for 10 minutes in −20°C acetone, air dried, and re-hydrated in PBS. (3) Some cells were fixed in −20°C absolute methanol for 10 minutes, after which the methanol was replaced in steps by PBS.

Cytoskeleton preparations, which contain actin and intermediate filaments but not microtubules, phospholipids, or other soluble components, were made by extracting three times for 1 minute with ice-cold cytoskeleton buffer (CSK: 300 mM sucrose, 100 mM NaCl, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5 % Triton X-100, pH 6.8; Fey et al., 1984) and fixing for 15 minutes on ice with 3.7% formaldehyde in CSK. Permeabilized cells containing intact microtubules were prepared either by fixation in −20°C methanol as above (Doxsey et al., 1994) or by extraction at room temperature with a microtubule stabilization buffer (MSB), a modification of that described by Mitchison and Kirschner (1984): 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100, pH 6.8. Following a single 30- to 60-second MSB extraction, cells were fixed at room temperature for 15 minutes in MSB containing 1% glutaraldehyde. After rinsing twice with MSB, glutaraldehyde active sites were quenched

by three incubations of five minutes each in MSB containing 1% sodium borohydride.

Antibodies and fluorescence labeling of cells

Monoclonal antibodies to mitofilin, clones 302.47 and 2-8, were from Matritech, Inc. (Newton, MA). Monoclonal antibody (clone 58k-9) to Golgi-associated 58k protein (Bloom and Brashear, 1989), and β-tubulin (clone TUB2.1) were from Sigma Chemical (St Louis, MO). Monoclonal antibody to IP90 (calnexin), an endoplasmic reticulum protein (Hochstenbach et al., 1992), was kindly provided by Dr Michael Brenner (Harvard University). Primary antibodies were detected with fluorochrome-labeled goat (Sigma) or donkey (Jackson Immunological Laboratories, Minneapolis, MN) anti-mouse or anti-rabbit IgG. MitoTracker FM, phalloidin-rhodamine conjugate, and monoclonal antibody to human cytochrome oxidase subunit II (clone A-6404) were from Molecular Probes (Eugene, OR). Mitochondria were labeled by incubation at 37°C for 40 minutes in MitoTracker FM at a final concentration of 1 μM in medium, after which cells were fixed in formaldehyde and permeabilized as above. Following fixation, cells were washed with PBS and PBS with 0.5% bovine serum albumin (PBSA). Working dilutions of antibodies and optimal incubation times were determined empirically. Antibodies were diluted in PBSA, added to coverslips in multi-well trays, and incubated at 37°C. After removal of primary antibody followed by three 5 minute washes in PBSA, secondary antibody was added and incubated as above. In some double label experiments, primary antibodies were combined in a single incubation, as were secondary antibodies labeled with different fluorochromes. Following the final antibody incubations, coverslips were washed once in PBSA containing 0.1% Triton X-100 and the DNA counterstain DAPI (14 μM), once with PBSA, twice with PBS, and then mounted with 90% glycerol in PBS containing phenylenediamine.

Cytoskeleton disruption studies

SV40 transformed IMR-90 cells growing on coverslips were first exposed to MitoTracker FM (Molecular Probes) at a concentration of 1 μM in medium for 30 minutes at 37°C to label mitochondria. Actin filaments or microtubules were then de-polymerized for 30 minutes by adding cytochalasin D (final concentration 5 μg/ml; Taneja et al., 1992), or nocodazole (final concentration 10 μg/ml; Doxsey et al., 1994), respectively. De-polymerization was confirmed by staining with anti-β-tubulin or by fluorescent phalloidin. Duplicate coverslips were labeled with anti-mitofilin antibodies and detected with Texas red- or rhodamine-conjugated secondary antibodies to assess co-localization with the green-fluorescing mitochondria.

Photomicrography

Fluorescence was visualized using a Zeiss Axiofot microscope equipped for epifluorescence with a ×100 objective and single and multiple bandpass filter sets (Johnson et al., 1991). Images were photographed on Ektachrome film (400 ASA, Kodak, Rochester, NY), with exposure times between 2 and 60 seconds.

Immunoelectron microscopy

Steps were carried out at room temperature unless noted. WI-38 human diploid fibroblasts grown in plastic tissue culture dishes were washed twice with PBS and fixed for 20 minutes in PBS with 2% paraformaldehyde (freshly made) and 0.1% glutaraldehyde, after which they were scraped up and transferred to microfuge tubes. Glutaraldehyde active sites were quenched with 0.5 M ammonium chloride for 90 minutes, the pellets washed 3 times with PBS, and transferred to BEEM capsules. The pelleted cells were dehydrated, infiltrated, and embedded in Lowicryl K4M (Polysciences, Warrington, PA) according to the manufacturer's instructions. Sections (60-90 nm) were collected on 200-mesh nickel grids. The buffer for blocking, immuno-staining, and washes was 0.5 M NaCl, 20 mM Tris-HCl, 0.02% sodium azide, pH 7.5 (TBS). After a 15 minute block in 8% BSA, grids were rinsed in TBS and inverted onto drops of antibody 3584 diluted 1:100 in 4% BSA. After 30 minutes at 37°C, incubation was continued overnight at 4°C. Grids were then thoroughly rinsed with TBS and inverted onto secondary antibody (goat anti-rabbit IgG, 6 nm gold conjugate, Jackson) diluted 1:10 in 4% BSA and incubated at 37°C for 30 minutes. After rinses in TBS with 0.1% Triton X-100 and TBS, grids were post-fixed with 2% glutaraldehyde for 2 minutes, rinsed thoroughly with water, wicked dry, and then air-dried. Sections were stained with lead citrate/uranyl acetate and photographed on a Philips 400 transmission electron microscope operated at 60 kV.

Sequential extraction of cellular proteins

In this procedure, cells were chemically dissected into five protein fractions which were analyzed in comparison with total cellular proteins. All extraction solutions were supplemented with the following protease inhibitors (Boehringer Mannheim) at the concentrations indicated: bestatin, 40 μg/ml; pepstatin, 0.7 μg/ml; phenylmethylsulfonyl fluoride, 50 μg/ml; aprotinin, 2 μg/ml; leupeptin, 2 μg/ml; E64, 1 μg/ml. ME-180 cells were grown to approximately 80% confluence. Total cell proteins were solubilized in UNM as described below. The other cell protein fractions were obtained from duplicate dishes by the method of Fey et al. (1984). Briefly, the 'soluble' fraction was first obtained by extraction in ice-cold CSK buffer (see above), and 'cytoskeleton' proteins by a subsequent extraction with CSK containing 250 mM ammonium sulfate, which solubilizes the actin cytoskeleton and other salt-extractable proteins. Next, following DNase and RNase digestion (each at 100 μg/ml for 20 minutes at room temperature), the 'chromatin' protein fraction was obtained by another ammonium sulfate extraction. The remaining NM-IF pellet was solubilized in urea-containing disassembly buffer. Intermediate filaments were collected by ultracentrifugation following dialysis against urea-free assembly buffer (Zackroff et al., 1982). The supernatant is enriched in nuclear matrix proteins. The intermediate filament pellet was then re-solubilized in UNM. The soluble, cytoskeleton, and chromatin fractions were dialyzed against cold PBS. These, along with the nuclear matrix fraction, were precipitated by cold ethanol and dried under argon, after which they were re-dissolved in UNM. Fractions were aliquotted and stored at −70°C until use.

Isolation of mitochondria

Mitochondria from HeLa cells grown in suspension were isolated according to standard differential centrifugation methods, where $\boldsymbol{g}_{av}$ equals centrifugal force at the midpoint of the centrifuge tube (O'Brien and Kalf, 1967; Carvalho Guerra, 1974). Cells were washed with PBS and a sample was taken to solubilize total cell proteins. This was done by brief homogenization in 8 M urea, 2% Non-Idet P40, and 2% β-mercaptoethanol (UNM; Klose and Zeindl, 1984), followed by 5 minute, 10,000 radial centrifugal force (rcf) microfuge centrifugation at room temperature. Aliquots of the supernatant were snap frozen. All steps below were carried out at 0-2°C. Cells were suspended in hypo-osmotic buffer (0.15 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After a 5 minute incubation, cells were Dounce homogenized for 10 strokes with a glass pestle, diluted with an equal volume of hyper-osmotic buffer (same, but with 0.45 M sucrose) and centrifuged at 1,000 $\boldsymbol{g}_{av}$ for 10 minutes. The mitochondria-containing supernatant was harvested, and the remaining first pellet was washed in PBS, a sample of which was solubilized in UNM for the 'nuclear' fraction. The supernatant from the 1,000 $\boldsymbol{g}_{av}$ spin was re-centrifuged at 9,000 $\boldsymbol{g}_{av}$ for 10 minutes, and the post-mitochondrial supernatant was harvested. The mitochondrial pellet was resuspended in iso-osmotic buffer (IB: 0.3 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and washed twice in IB. A sample of the post-mitochondrial supernatant was precipitated by incubation on ice in 10% trichloroacetic acid, pelleted at 10,000 rcf for 10 minutes, the pellet washed once with 70% ethanol, and dried. Proteins from the low-speed pellet, the post mitochondrial supernatant, and the mitochon-

dria were solubilized with UNM. Fractions were either analyzed immediately by SDS-PAGE and immunoblotting, or frozen, as above. Protein concentrations were measured by the Coomassie Plus microassay (Pierce Chemical, Rockford, IL) standardized with bovine serum albumin.

Protease treatment of mitochondrial compartments

All steps were carried out on ice. Samples of mitochondria were resuspended to 180-200 μg protein per ml either in IB or in osmotic shock buffer (OS: 1 volume of IB plus five volumes of 20 mM Tris-HCl, 1 mM EDTA, pH 7.5; Daum et al., 1982). Mitochondrial suspensions were rocked gently for 30 minutes prior to trypsin incubations. For detergent-permeabilized mitochondria, Triton X-100 was added to IB-suspended samples from 20% (v/v) stock to a final concentration of 2% five minutes before trypsin incubations began. Samples of intact, OS, and Triton-permeabilized mitochondria were then treated with trypsin (type XIII, TPCK-treated; Sigma) at concentrations of 0, 5 or 15 μg/ml for thirty minutes on ice. Reactions were stopped by adding Pefabloc SC (Boehringer) to a concentration of 12.5 μM (100-fold and 33-fold molar excess for the 5 and 15 μg/ml trypsin doses, respectively). After a 5 minute incubation on ice, SDS-PAGE sample buffer was added, the samples placed in a boiling water bath for 10 minutes and loaded on gels for analysis.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed according to the method of Laemmli (1970) using 6.5 cm × 9.5 cm minigels. Coomassie staining of gels was done using colloidal Coomassie stain (Sigma). For protein transfer, gels were blotted onto Immobilon P PVDF membranes (Millipore, Bedford MA) using the semi-dry method of Kyhse-Andersen (1984). Some transferred proteins were stained with Coomassie by the method of LeGendre and Matsudaira (1989). For immunoblots, whether developed by chemiluminescent (Immun-Lite AMPPD, Bio-Rad) or by chromogenic (NBT/BCIP) substrates for alkaline phosphatase-conjugated secondary antibodies, the blocking, washing, and antibody dilution buffers were as instructed for the Immun-Lite chemiluminescent system.

Northern blot analysis

Total RNA was prepared from ME-180 cells by the guanidinium isothiocyanate procedure (Chirgwin et al., 1979). Poly(A)$^+$ selection by oligo(dT) cellulose chromatography was performed as described by Aviv and Leder (1972). Samples of total and poly(A) selected RNA (30 μg each) were solubilized in formaldehyde-urea-vanadate buffer, subjected to agarose-formaldehyde gel electrophoresis as described by Melloni et al. (1992), and blotted onto Zeta-Probe (Boehringer) nylon membrane. The probe was a random-primed [α-^{32}P]dCTP labeled (High Prime, Boehringer), 728 base pair PCR product encompassing bases 528-1,256 of the cDNA. Hybridization was carried out at 42°C overnight under standard conditions (Selden, 1990).

RESULTS

Cloning and sequence analysis of mitofilin cDNA

A series of immunizations was carried out in which total nuclear matrix proteins, prepared by the method of Fey and Penman (1988), were used as the immunogen. While many of the resulting hybridomas recognized nuclear proteins, one clone, 302.47, recognized a protein doublet of 90 and 91 kDa by immunoblot which was present at extremely low levels in the nuclear matrix protein fraction. This antibody showed a distinctive cytoplasmic distribution in immunofluorescence studies.

Results of cDNA library screening and sequence analysis are shown schematically in Fig. 1A. Expression library screening with 302.47 antibody yielded a cDNA clone (Fig. 1A, pGT1) with a 2.25 kb insert. Sequence analysis showed a single open reading frame with 3′ untranslated region, poly-adenylation signal, and a poly(A) tail. Further screening yielded several additional clones. One of these, MF.4, while shorter, had an additional 60 nucleotides and 20 amino acids of open reading frame at the 5′ end. The others (MF.1-.3) started further 3′ than the original clone. Sequencing confirmed that all clones derived

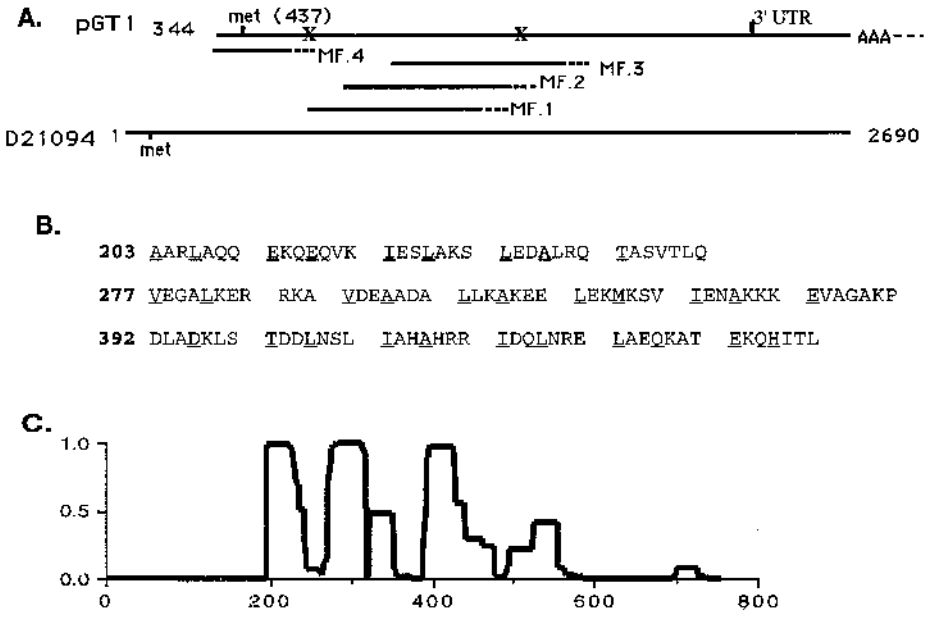


Fig. 1. (A) Mitofilin cDNA clones aligned with HMP (Icho et al., 1994; GenBank accession number D21094). Clones pGT1 and MF.4 were obtained by library screening with monoclonal antibody 302.47. Clones MF.1-.3 were obtained by hybridization screening with a PCR-generated probe encompassing the region between the two Xs on pGT1 (also used for northern blots). The pGT1 insert was completely sequenced, and the other 4 clones were partially sequenced. pGT1 begins at nucleotide 344 of D21904. 'met (437)' indicates the position of the first codon of the mitofilin moiety of a pMAL fusion construct used to produce recombinant protein. (B) Coiled coil domains predicted by the Coils program (Lupas et al., 1991). Regions with > 0.98 probability of coiled coil formation are shown, with the number of the first residue of each line at the left. Amino acids occupying heptad positions *a* and *d* are shown underlined. Three stretches 5 to 6 heptads in length were identified. (C) Plot of coiled coil probability, with amino acid number on the *x*-axis, and probability on the *y*-axis.

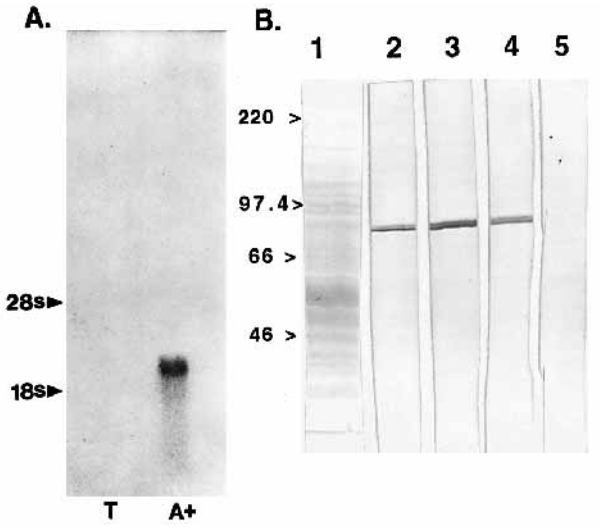


Fig. 2. RNA (A) and protein (B) blot analyses of mitofilin. (A) Total RNA (lane T) and poly(A)$^+$ RNA (lane A+) from ME-180 cells (30 μg/lane) were probed. Positions of the 28 S and 18 S ribosomal RNA bands are indicated by arrows. A single mRNA band of approximately 2.7 kb is seen in lane A+ that was not detected in lane T. (B) Immunoblots of mitofilin. ME-180 total proteins were separated on an 8% gel and blotted, with molecular mass marker positions shown at left. Lane 1 is Coomassie-stained. Lane 2 was probed with monoclonal antibody 302.47. Lanes 3 and 4 were probed with antibodies raised against recombinant mitofilin: monoclonal (clone 2-8) in lane 3 and rabbit polyclonal (3584) in lane 4. Lane 5 was probed with pre-immune serum from the same rabbit. Mitofilin appears as a closely-spaced doublet at 90 and 91 kDa and is detected specifically by all three antibodies.

from various regions of the same cDNA. During the course of the preparation of this manuscript, a match of over 99% was found in the GenBank database to sequence D21094, reported by Icho et al. (1994) for a mRNA preferentially transcribed in growing heart muscle, which the authors called HMP (heart muscle protein). We conclude that our clones represent the same mRNA; however, we have found the protein product of this mRNA to be present in all human cells tested, and further, that it is a constituent of mitochondria (see below).

Schematics of cDNA clones we obtained shown in Fig. 1A are aligned with the D21094 sequence, and nucleotide positions are numbered accordingly. Our application of predictive computer algorithms to analyze the amino acid sequence of mitofilin confirms those reported (Icho et al., 1994). This includes an extended, α-helical region through the central portion of the sequence which contains several domains of heptad repeats. We used the coiled coil prediction program of Lupas et al. (1991) and found three regions of extremely high (>98%) coiled coil forming probability (Fig. 1B,C). For reasons discussed below, however, we feel that putative kinesin-like motor protein qualities attributed to HMP are incorrect. Because of its exclusive association with mitochondria, its interrupted coiled coil domains along an extended α-helical region reminiscent of intermediate filament proteins, and the filamentous appearance of the mitochondrial network labeled with this antibody in certain cell types, we have called this protein 'mitofilin'.

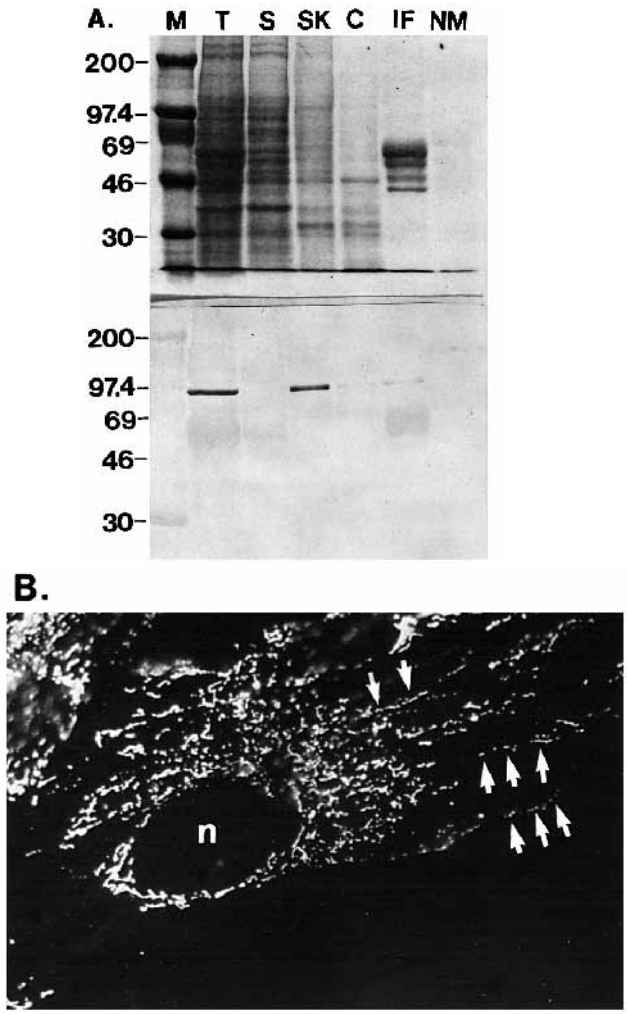


Fig. 3. Partitioning of mitofilin in sequentially extracted cells (see Materials and Methods), and the effect of non-ionic detergent on mitofilin distribution. (A) ME-180 cell protein extracts analyzed by duplicate SDS-PAGE gels stained by Coomassie (upper) or blotted and probed with antibody 302.47 (lower). Lanes were loaded with samples representing equal cell numbers. Lane T is total proteins; lane S is soluble protein released by Triton-containing CSK buffer; lane SK is the cytoskeleton fraction released by CSK plus ammonium sulfate; lane C is the chromatin fraction; lane IF is the intermediate filament fraction; lane NM is the nuclear matrix-enriched fraction. CSK buffer alone extracted no detectable mitofilin (lane S), after which ammonium sulfate released nearly all of it (lane SK). The chromatin (lane C) and intermediate filament (lane IF) fractions contained small amounts of mitofilin. The nuclear matrix fraction contained no detectable mitofilin at this loading (lane NM). Lane M, molecular mass markers (sizes shown in kDa).
(B) Immunofluorescence of WI38 cells extracted with cold CSK buffer, then fixed in CSK-formaldehyde. Despite the quantitative retention of mitofilin by cells in this treatment (see A, lane S), its distribution is altered. Instead of the filamentous network usually seen, the protein is present in a series of punctate bodies, some of which lie along filamentous paths (arrows). n, nucleus.

Production and purification of recombinant mitofilin

The insert from pGT1 was cloned into the pMalC vector (New England Biolabs) to produce a MalE:mitofilin fusion protein. After proteolytic cleavage of the peptide linking the maltose

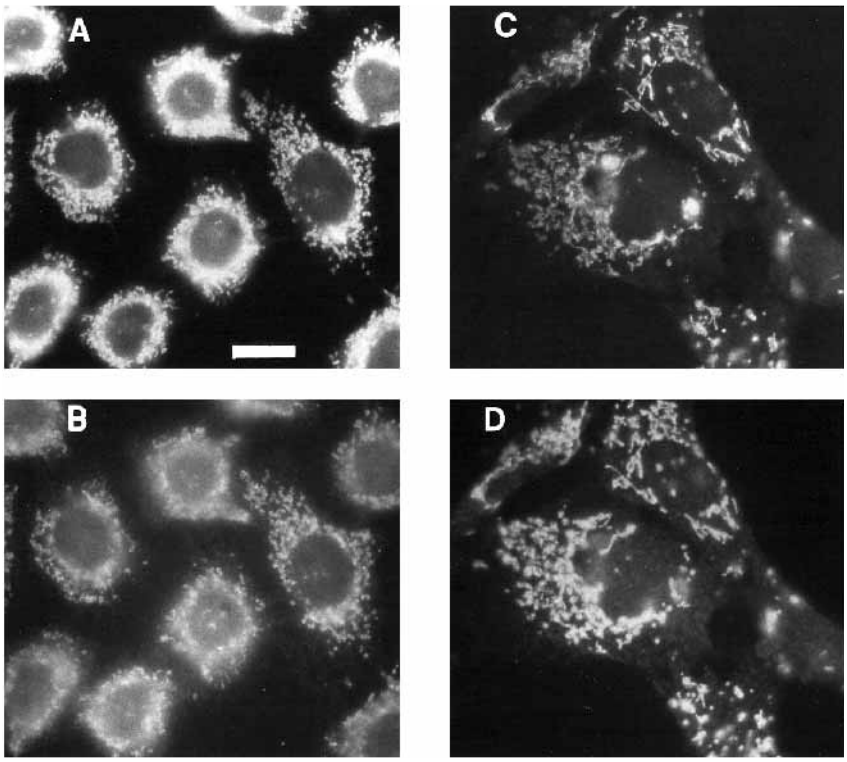


Fig. 4. Co-localization of mitofilin with mitochondria. Live cells were labeled with fluorescent MitoTracker dye, fixed with formaldehyde, permeabilized with Triton, and incubated with anti-mitofilin antibodies followed by Texas red-conjugated secondary antibodies. (A and B) ME-180 cervical epithelial cells. (C and D) SV40-transformed IMR90 human fibroblasts. In both cell types (as in all human cell types examined), the fluorescent mitochondria signal (A and C) co-localizes with anti-mitofilin antibodies. (B) Monoclonal antibody 302.47; (D) Rabbit polyclonal 3584. Bar, 12 μm.

binding protein to the mitofilin moiety, the recombinant molecule extends for 640 residues, from amino acid 117 (Met 437; Fig. 1A) of the cellular protein through to the carboxy terminus. In addition to this full-length product, many lower molecular mass bands were seen in immunoblots of induced bacteria. The full-sized recombinant mitofilin was purified to homogeneity by preparative SDS-PAGE and used as antigen to produce second-generation monoclonal and polyclonal antibodies.

Mitofilin is transcribed from a single mRNA

Northern blot analysis of ME-180 RNA is shown in Fig. 2A. No signal was visible when total RNA was probed (lane T), but when poly(A) enriched RNA was tested (lane A+), a single band of approximately 2.7 kb was seen. The fact that 30 μg of poly(A) enriched RNA, along with a 24 hour exposure, was required to detect the band indicated that the mitofilin mRNA is of relatively low abundance in these cervical epithelial cells.

Clone confirmation by antibodies to recombinant mitofilin

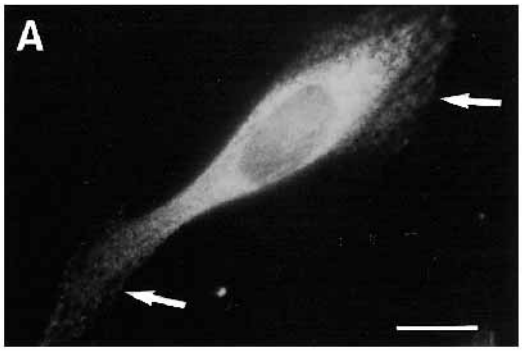
The identity of the cDNA clones was confirmed, and the correspondence of immunoblot and fluorescence results with the original 302.47 antibody was verified, by the antibodies raised against recombinant protein. All three antibodies recognize the same 90 and 91 kDa doublet in cell extracts. Fig. 2B shows immunoblot analysis of ME180 total protein extracts. The two monoclonals recognize distinct epitopes, as determined by immunoblot analyses both of the multiple bands seen in the bacterial expression product described above and of *N*-chlorosuccinimide-cleaved recombinant protein (not shown). Double label immunofluorescence experiments with the antibodies used in combinations all gave identical cytoplasmic co-localization, although the 2-8 antibody also gave non-specific nuclear staining at high concentrations (not shown). The 90 and 91 kDa mitofilin bands were seen in protein extracts from all human tissue and cell types examined except the red blood cell.

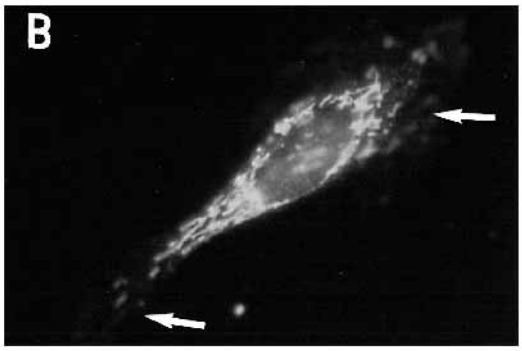
Resistance to non-ionic detergents

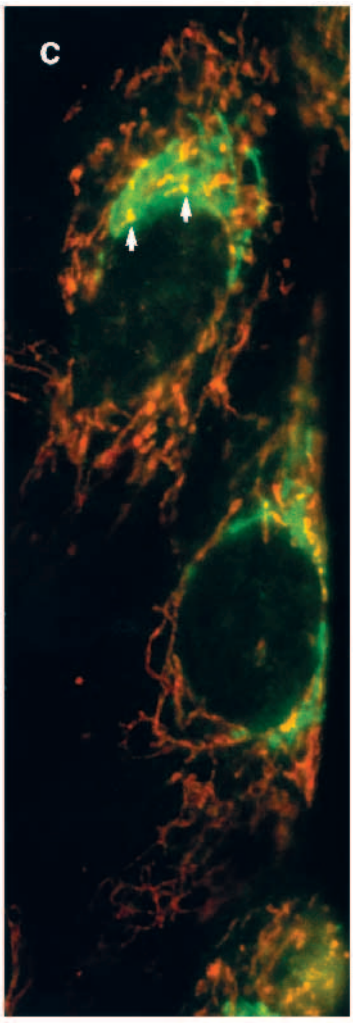
Analysis of protein fractions obtained by sequential cell extraction (Fey et al., 1984) revealed that mitofilin is quantitatively retained after Triton X-100 treatment, but that subsequent salt extraction removes nearly all of the protein, with trace amounts recovered in the remaining fractions (Fig. 3A). CSK buffer extracted no detectable mitofilin. The protein obtained by this extraction, which removes phospholipids, certain membrane proteins, soluble cytoplasmic proteins, microtubules, and microtubule-associated proteins virtually quantitatively, is called the soluble fraction (lane S). Subsequent treatment of cells with CSK buffer supplemented with ammonium sulfate, which solubilizes the actin-based cytoskeleton and certain other proteinaceous components, yields the cytoskeleton protein fraction (Fey et al., 1984). This treatment released nearly all the mitofilin protein from the remaining structures (lane SK). The chromatin and intermediate filament fractions each contained a small amount of mitofilin (lanes C and IF). The nuclear matrix fraction, which is greatly enriched in lamin B, NuMA, certain nuclear porins, and other nuclear matrix proteins, contained no detectable mitofilin when loaded at equal cell number (lane NM); however, when 10-fold greater cell equivalents were tested, the 90 and 91 kDa mitofilin doublet was faintly detected (not shown). The presence of small amounts of mitofilin in the nuclear matrix fraction accounts for the original monoclonal antibody 302.47 being produced by immunizations with this material.

Effect of fixation and non-ionic detergent

The biochemical fractionation results above would seem to suggest that mitofilin is stable during extraction with detergent;







however, immunofluorescence (Fig. 3B) shows that, at the structural level, cold CSK extraction has a marked effect. Despite all the mitofilin remaining in the cell after this treatment, the protein is re-organized into an array of large punctate spots and short strands which are often arranged along filamentous trajectories. We tested whether the structural disruption was mainly due to the effects of Triton or to the depolymerization of microtubules, which also occurs in this treatment, by examining cells fixed via preparative methods which retain microtubules intact. Extraction at room temperature with a Triton-containing microtubule stabilization buffer which is very similar to CSK (see Materials and Methods) preserves microtubule ultrastructure (Mitchison and Kirschner, 1984). This affected mitofilin in a manner indistinguishable from cold CSK (not shown), indicating that the disruption of the mitofilin organization by Triton occurs whether or not microtubules are also removed. We also examined non-extracted cells which had been fixed at –20°C in either methanol or acetone. The effect of these fixatives on the appearance of mitofilin in fibroblasts was intermediate between PBS-formaldehyde (Figs 4, 5) and Triton (Fig. 3B). That is, some filamentous structures remained in these cells, but some of the mitofilin was present as punctate bodies (not shown).

Immunofluorescence co-localization with mitochondria

Mitofilin-specific antibodies co-localize with fluorescently-labeled mitochondria in all cell types examined. Fig. 4 shows epithelial cells and fibroblasts double-labeled with MitoTracker FM dye (Molecular Probes) and anti-mitofilin antibodies. The morphology of mitochondria in the two cell types is quite distinct. In the fibroblasts (Fig. 4C,D), mitochondria appear predominantly as an anastomosing, filamentous network, with some rod-like and vesicular forms, similar to those seen in the original publications by Chen and co-workers of mitochondria-specific fluorescence labeling with rhodamine 123 (see for example Johnson et al., 1980, 1981). In the cuboidal epithelial ME180 cells (Fig. 4A,B) the mitochondria are smaller and less interconnected. In both cell types, the mitofilin and the MitoTracker signals coincide. Because the cytoplasm of fibroblasts extends as a thin layer, the morphology of mitochondria and other cell constituents is more easily seen in photographic reproductions; therefore the remaining images presented in this report are of fibroblasts, although

Fig. 5. Mitofilin is associated only with mitochondria, and not with endoplasmic reticulum or Golgi. IMR90 cells were fixed in formaldehyde and permeabilized with 0.1% Triton X-100 before labeling. (A and B) Cells were double-labeled with monoclonal anti-IP90 (calnexin), and anti-mitofilin antibody 3584, respectively. The distribution of the two antigens is quite different in appearance and extent. Mitochondria are distinctly more filamentous and rod-like and occupy a smaller area of the cytoplasm. The arrows denote peripheral, thinner regions of cytoplasm where mitochondria are virtually absent, but which contain a lacy network of ER. (C) IMR90 cells double-labeled with monoclonal antibody to the Golgi-associated 58k protein (green) and mitofilin 3584 (red), photographed with a double bandpass filter. While the two organelle systems appear closely interwoven, there is little of the yellow signal (arrows) produced by coincident sources of red and green. The small amount of yellow signal is likely due to vertical stacking of mitochondria and Golgi along the optical axis.

similar results have been obtained with epithelial and other cell types.

Because other organelle systems can appear similar to mitochondria in some cell types, we performed double label experiments with antibodies to Golgi and endoplasmic reticulum (ER; Fig. 5) proteins. These results confirmed that mitofilin labeling was confined to the mitochondria. ER (Fig. 5A) was labeled with a monoclonal antibody to calnexin, originally called IP90 (Hochstenbach et al., 1992; kindly provided by Dr M. B. Brenner). Calnexin distribution was clearly distinguished from the mitofilin pattern in both its extent and morphological appearance (Fig. 5B). Similar results were obtained with antibodies to protein disulfide isomerase and oligosaccharyl transferase as well as with the fluorescent dye DiOC6 (not shown). The separation of Golgi, labeled with antibody to the 58k Golgi-associated protein (Bloom and Brashear, 1989), from mitofilin signal is most easily seen in color images (Fig. 5C). Despite the apparent intertwining of the structures labeled by the antibodies, the red (mitofilin) and green (Golgi) signals are actually quite well separated, showing very little of the yellow signal which results from coincident red and green sources.

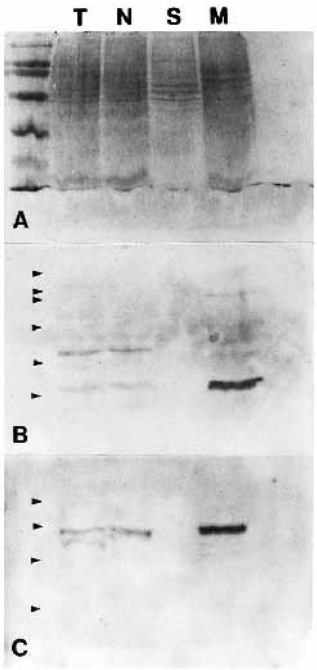


Fig. 6. Co-purification of mitofilin with mitochondria, analyzed by SDS-PAGE. HeLa cell mitochondria were purified as described in Materials and Methods. (A and B) Duplicate 12% SDS-PAGE gels of the fractions obtained. A is stained with Coomassie, B is an immunoblot probed with anti-cytochrome oxidase subunit II; C is an immunoblot of an 8% gel probed with mitofilin antibody 302.47. Masses of markers are indicated in A, and their positions are indicated by arrowheads in B and C, with the 46 kDa marker the lowest resolved in C. T represents total cell proteins; N represents the nucleus-enriched low-speed pellet; S represents the post-mitochondrial supernatant; lane M represents the 9,000 g_{av} mitochondrial pellet. Lanes T, N, and S were loaded at equal cell numbers, and lane M at equal protein to lane T. In B, the genuine 26 kDa band of cytochrome oxidase II is barely detectable in lanes T and N, while faint, non-specific bands are seen at other positions. As expected, lane M is greatly enriched in this enzyme. In C, some mitofilin protein is seen in lanes T and N, none is detected in the post-mitochondrial supernatant, while in the mitochondrial pellet mitofilin is greatly enriched.

Mitofilin co-purifies with mitochondria

SDS-PAGE and immunoblot analyses of HeLa cell mitochondria isolated by differential centrifugation (O'Brien and Kalf, 1967; Carvalho Guerra, 1974) are shown in Fig. 6. In Fig. 6C, mitofilin is detected weakly in the total (lane T) and low-speed (1,000 g_{av}) nuclear pellet fractions (lane N). The low-speed pellet contains mostly nuclei, cytoskeletal elements, and dense nuclear-associated ER. After centrifugation at 9,000 g_{av}, the post-mitochondrial supernatant (lane S) contained no detectable mitofilin. The twice-washed mitochondrial pellet (lane M) shows great enrichment of mitofilin. In Fig. 6B, antibody to cytochrome oxidase II (COxII) confirms the enrichment of mitochondria in the washed pellet. COxII is encoded by the mitochondrial genome, is translated in the mitochondria, and is inserted into the inner mitochondrial membrane with both its amino- and carboxy termini exposed to the inter-membrane space (reviewed by Capaldi, 1990). It is thus well-suited as a mitochondria-specific marker protein. At the loading used in Fig. 6, the level of COxII in the total and nuclear pellet lanes was barely detectable even by high-sensitivity chemiluminescent immunoblot. In the mitochondrial pellet, however, the 26 kDa COxII band is greatly enriched. The co-purification of mitofilin with COxII in the mitochondrial pellet, together with its specific co-localization with mitochondria in immunofluorescence experiments, establish that mitofilin is a previously unknown mitochondria-associated protein.

Mitofilin association with mitochondria is independent of intact actin filaments or microtubules

We examined whether the association of mitofilin with mitochondria was disrupted by the de-polymerization of actin and tubulin for several reasons. First, it has been shown for a number of cell types that mitochondria bind to and move along microtubules, and Nangaku et al. (1994) recently identified KIF1B, a mitochondria-specific microtubule-associated motor protein which has three short coiled coil domains reminiscent of the mitofilin sequence (Fig. 1). We therefore wished to examine whether the association of mitofilin with mitochondria would persist in the absence of microtubules. And, as noted above (Fig. 4), although Triton extraction leaves mitofilin behind quantitatively, its distribution is altered (Fig. 3B); moreover, subsequent treatment with 250 mM ammonium sulfate, which removes the actin cytoskeleton, also extracted mitofilin nearly quantitatively, suggesting a possible interaction between mitofilin and actin.

In immunofluorescence experiments in which either actin filaments (Fig. 7A,C) or microtubules (Fig. 7B,D) were disrupted, the co-localization of mitofilin with MitoTracker was unaffected. Duplicate coverslips included in the experiment and stained with anti-β-tubulin antibody or with phalloidin-rhodamine conjugate confirmed that de-polymerization had occurred (not shown). In the nocodazole-treated cells (Fig. 7A,C) there was no marked effect on either cellular or mitochondrial morphology by the 30 minute time point at which

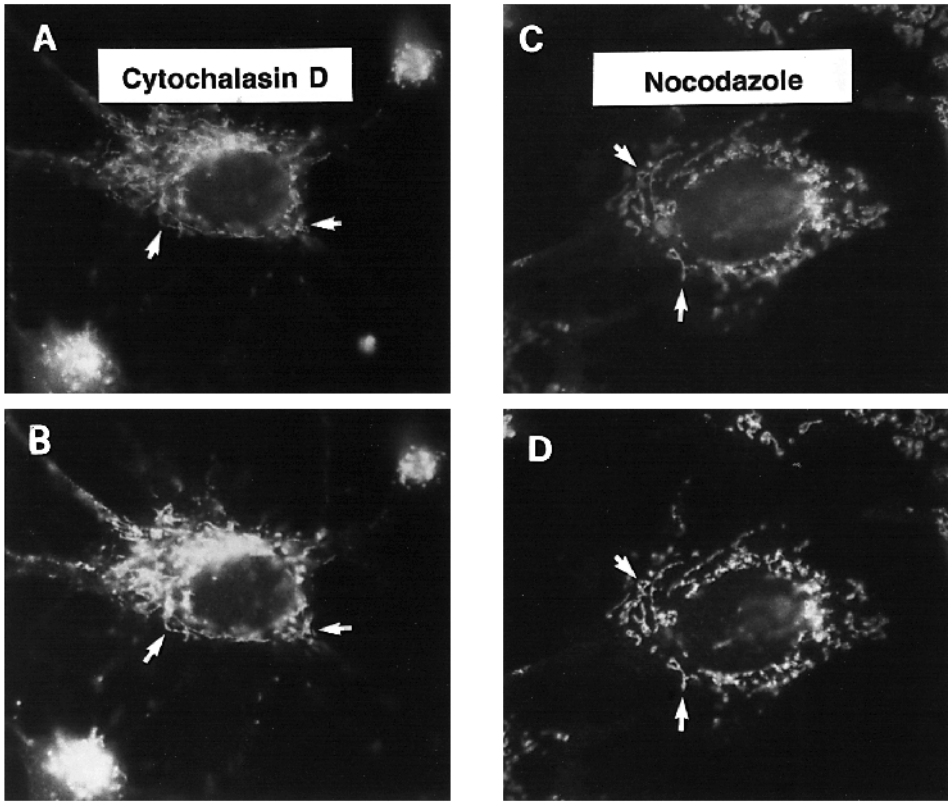


Fig. 7. Double label fluorescence shows mitochondrial association of mitofilin is independent of actin filaments or microtubules. SV40 transformed IMR90 cells were labeled with MitoTracker dye, after which either cytochalasin D, to depolymerize actin (A and B), or nocodazole, to depolymerize microtubules (C and D), were added. A and C show MitoTracker fluorescence; B and D show anti-mitofilin antibody 3584 signal. Neither treatment disturbed the localization of mitofilin protein with mitochondria. Some conspicuous structures are denoted by arrows for comparison.

cells were fixed, nor was the mitochondrial localization of mitofilin affected. Similarly, although actin de-polymerization had a marked effect on the morphology of the fibroblasts, most notably rounding up and shrinking back of the cytoplasm toward the nucleus, the mitofilin signal remained coincident with mitochondria (Fig. 7B,D).

Matrix targeting and stop-transfer signals

In their description of the HMP cDNA sequence, Icho et al. (1994) noted coiled coil regions reminiscent of kinesin. In addition, they identified a region with some similarities to known nucleotide triphosphate-binding domains (residues 45-61: KIAGAGLLFVGGGTGGT). Together, these observations led them to conclude that the protein was a heart muscle-specific motor protein. The domain thought to be a potential NTP-binding motif, however, lacks the invariant lysine residue which is required for binding to occur (i.e. the penultimate G should be a K; Walker et al., 1982; Traut, 1994; Rapiejko and Gilmore, 1994). The mitochondrial localization of the protein led us to search for mitochondrial targeting motifs. The results are summarized in Fig. 8. The putative NTP-binding domain in fact lies within a region encompassing both a mitochondrial matrix targeting signal and a stop-transfer sequence from residues 21-65. The PSORT sequence analysis program (Nakai and Kanehisha, 1992) calculated a probability of 82% that mitofilin lies in the mitochondrial inter-membrane space.

Accessibility to limited proteolysis

The predicted topology of mitofilin within mitochondria was addressed experimentally by limited trypsinization of isolated mitochondria after three different treatments (Fig. 9): intact mitochondria (lanes I); osmotic shock (lanes OS; Daum et al., 1982) to disrupt selectively the outer membrane; and Triton-solubilized (lanes T) to solubilize both membranes and allow protease access to all compartments. Samples were mock-

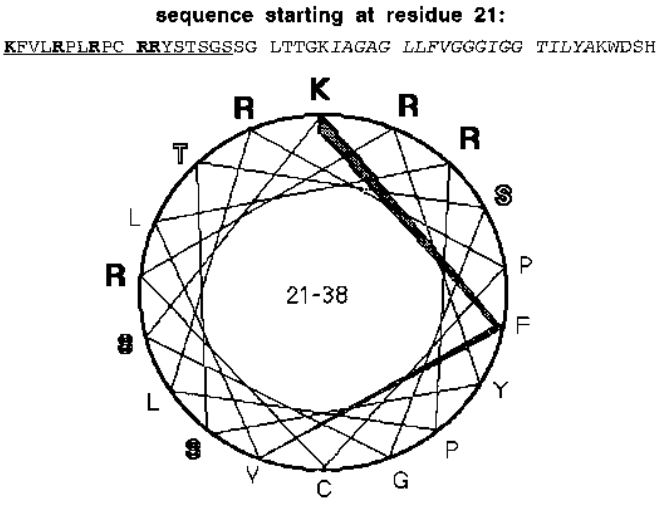


Fig. 8. Mitochondrial matrix targeting signal and stop-transfer sequence of mitofilin. Above is the amino acid sequence of mitofilin from residue 21-70. The region underlined has characteristics of a mitochondrial matrix targeting signal: positively charged amino acids (boldface type) spaced roughly four residues apart, with the remainder being either non-polar or serines/threonines. Following this, in italics, is a non-polar stretch of sufficient length to span the lipid bilayer. Below, the matrix targeting sequence is shown in a helical wheel diagram. The basic residues lie along one face of the helix, a hallmark of matrix targeting signals (van Loon et al., 1986; Glick et al., 1992a,b).

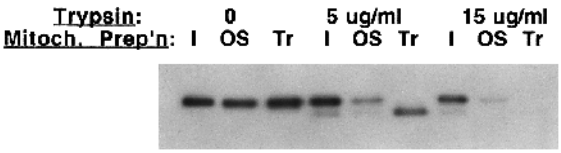


Fig. 9. Mitofilin sensitivity to trypsin in selectively permeabilized mitochondria. Mitochondria were intact (lanes I), osmotically shocked (lanes OS), or permeabilized with 2% Triton (lanes Tr) and exposed to trypsin as indicated. Western blot was probed with antibody 302.47. In intact mitochondria mitofilin was protected, whereas osmotic shock, which selectively disrupts the outer mitochondrial membrane, permitted digestion. Triton allowed complete degradation of mitofilin by the high trypsin dose (right-most lane), while the lower dose (middle lane), produced mainly a partial digestion product by cleavage at a hyper-sensitive site. Its accumulation in the middle lane is likely due to competition for limited enzyme by the released matrix components, since a three-fold increase in trypsin concentration accomplished complete degradation.

digested or digested with trypsin at the concentrations indicated. In the intact mitochondria (lanes I), mitofilin was protected from proteolysis. In the osmotically shocked mitochondria (lanes OS), trypsin degraded mitofilin quite effectively. In the Triton-treated samples (lanes Tr), the highest dose of trypsin (15 μg/ml) degraded mitofilin below detectable levels. The 5 μg/ml trypsin treatment of these solubilized mitochondria yielded mainly a partial digestion product which ran approximately 9.6 kDa smaller than the native protein. This same band can be seen faintly in other lanes, and represents cleavage at a hyper-sensitive site. Its preferential accumulation in the 5 μg/ml trypsin digest is very likely due to increased competition for limited enzyme by the Triton-released contents of the mitochondrial matrix, since a three-fold increase in trypsin was sufficient to digest the protein completely (right-most lane). The accessibility of mitofilin protein to proteolytic cleavage in osmotically shocked mitochondria is consistent with the matrix targeting and stop-transfer signals, and indicates that the protein resides predominantly in the mitochondrial inter-membrane space.

Immunoelectron microscopic localization of mitofilin

To confirm further the localization of mitofilin within mitochondria, immunogold electron microscopy was performed using anti-mitofilin antibody to probe Lowicryl-embedded sections. Fig. 10 shows a field containing a typical fibroblast mitochondrion labeled with antibody 3584 and gold-conjugated secondary antibody. As denoted by arrowheads, the antibody preferentially and specifically decorated the mitochondrial periphery. Some beads are also seen on other parts of the mitochondrion (arrows). This is perhaps not surprising, since not only do the 60-90 nm-thick sections contain much of the thickness of the organelle (about 150 nm in this case), but it is also possible that mitofilin occupies cristae surfaces. Of the 38 beads bound to the mitochondrion in Fig. 10, 25 (66%) were localized within 20 nm of the apparent boundary of the organelle. Controls using just secondary antibody showed no non-specific binding of gold beads to mitochondria (not shown). Although embedment conditions which preserve antigen (see Materials and Methods) make the inter-membrane space difficult to resolve, these results converge well with the sequence and proteolysis results (above), and together they are consistent with mitofilin being localized primarily in the inter-membrane space.

DISCUSSION

The purification of mitofilin protein in the mitochondrial pellet, along with its co-localization with mitochondria by immunofluorescence, establishes it as a previously unknown mitochondria-associated protein. The presence of a small amount of mitofilin in the nuclear matrix preparations used in the original immunizations accounts for the generation of the mitofilin-specific hybridoma 302.47, especially when one considers that the recombinant protein is highly immunogenic (see Fig. 2). The identical reactivities of both a monoclonal and a polyclonal antibody raised against highly purified recombinant protein verify the identity of the cDNA clone obtained with the original monoclonal antibody.

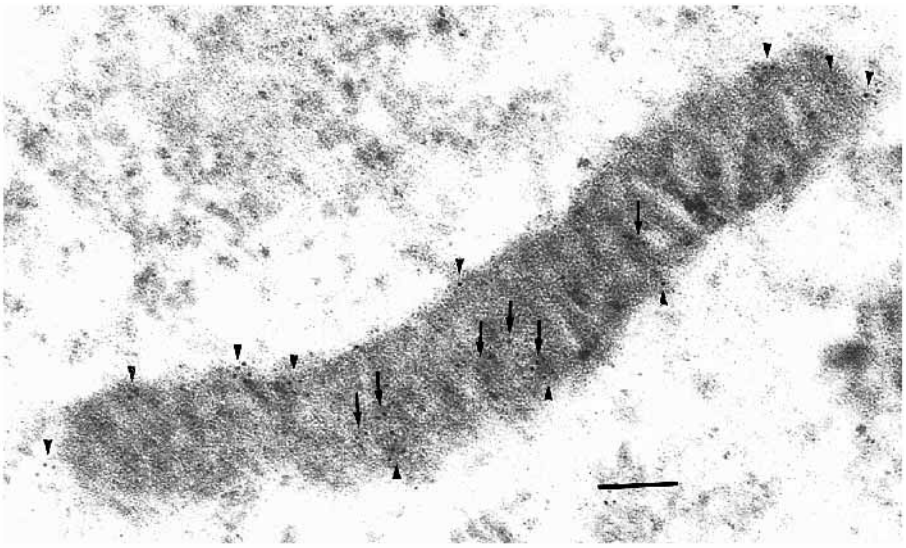


Fig. 10. Immunoelectron microscopic localization of mitofilin (×133,000). Sections of Lowicryl-embedded WI-38 fibroblasts were probed with antibody 3584 and visualized with 6 nm gold-conjugated secondary antibody. The periphery of the mitochondria is preferentially labeled (arrowheads denote some of these beads and bead clusters), with some beads also seen across the mitochondrial profile (arrows), while the rest of the cytoplasm shows little non-specific binding. Bar, 100 nm.

Because of the reorganization of mitofilin fluorescence signal induced by extraction with cold Triton X-100 (see Fig. 3), we investigated whether its mitochondrial association was affected by the removal of microtubules or actin filaments, and whether the re-organization was due primarily to the detergent. The drug-induced depolymerization of either filament system had no discernible effect on the disposition of mitofilin along the entire length of the mitochondria. When cells were Triton-treated under conditions which preserve microtubules, the same re-organization of the protein signal seen in cold CSK treatments occurred. The resistance to extraction implies that mitofilin is attached to other cellular structures. The fact that it is retained quantitatively through three Triton extraction steps, with none detected in the supernatant, argues strongly against artifactual binding produced de novo by detergent exposure. The predicted coiled coil domains of mitofilin (Fig. 1) very likely provide sites for its stable interaction with itself and/or with other proteins (Cohen and Parry, 1990). Such domains are most commonly found in microtubule-associated motor proteins, in structural proteins of the nuclear matrix-intermediate filament scaffold, or in subunits of certain holoenzymes (Odgren et al., 1996). Their occurrence within a region of mitofilin identified by other algorithms to form an extended α-helix, along with the extremely high probability values (i.e. >0.98), adds strength to the coiled coil prediction. It also suggests that cross-linking experiments may help to identify dimerization partners.

Icho et al. (1994) identified potential ATP-binding motifs in the amino-terminal region of the mitofilin/HMP sequence, which, together with the coiled coil domains, led them to speculate that the protein is a kinesin-like motor. Unlike all known motor proteins, however, mitofilin is not extracted in cold Triton-containing buffers, nor is it present in supernatants of cell homogenates after the 100,000 rcf spins commonly used to isolate microtubule-associated motor proteins (see Fig. 5). Only one mitochondria-specific motor protein has to date been identified, KIF1B (Nangaku et al., 1994), and its biochemical purification is similar to that of other motor proteins. Moreover, the proposed ATP-binding site of mitofilin/HMP both lacks the lysine residue required for triphosphate binding and falls within a region with a high probability of acting as a matrix targeting and stop-transfer signal (see Results and Fig. 8). Together, these findings make mitofilin unlikely to function as a motor.

The fact that the mRNA encodes a mitochondria-specific protein explains its abundance in heart muscle (Icho et al., 1994), given the extremely high numbers of mitochondria in cardiac myocytes. Utilizing antibodies specific for the protein product of this mRNA, however, we have found it is readily detected both by fluorescence microscopy and by immunoblot in every human cell and tissue type examined with the exception of erythrocytes (which lack mitochondria), and have consistently found the same 90 and 91 kDa doublet and mitochondrial localization. We conclude that mitofilin is a constituent of all human mitochondria. Antibodies 302.47 and 3584, but not 2-8, also recognize 90 kDa mouse and rat homologues and label mitochondria, implying that the protein is conserved, at least among mammals (not shown). Whether there is wider phylogenetic conservation of mitofilin remains to be investigated.

It is interesting that mitofilin remains attached, be it directly or indirectly, to the structures remaining after cold Triton extraction (see Fig. 3). This implies that certain mitochondrial constituents are stably linked to non-microtubule cytoskeletal elements. Examination of the biochemical behavior of other mitochondrial proteins in situ may provide further insight into the nature of this linkage. Differences in the disposition of protein import contact sites, for example, have been observed in comparing isolated mitochondria with mitochondria stained in situ (van der Klei et al., 1994), and could reflect disruption of these associations during the isolation. In isolated mitochondria, the contact sites were highly localized to small, stable regions of the mitochondrial surface, whereas in situ they were arrayed dynamically along the entire mitochondrial surface. Mitofilin may thus be a candidate for participation in the association of contact sites with cytoskeletal elements; however, the precise role of this ubiquitous, conserved protein in the life of the mitochondrion awaits further functional and structural analysis.

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