Molecular Cloning and Characterization of MT-ACT48, a Novel Mitochondrial Acyl-CoA Thioesterase*

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While characterizing Eps15 partners, we identified a 48-kDa polypeptide (p48) which was precipitated by Eps15-derived glutathione S-transferase fusion proteins. A search in a murine expressed sequence tag data base with N-terminal microsequences of p48 led to the identification of two complete cDNA clones encoding two isoforms of a 439-amino acid protein sharing 95% nucleic and amino acid identity. Northern blot and immuno blotting studies showed that p48 was ubiquitously expressed. A significant homology (19% identity and 40% similarity) between p48 and rat brain cytosolic acyl-CoA thioesterase was observed in an 80-amino acid C-terminal domain, retrieved from proteins from human, nematode, and plants. The thioesterase function of p48 was further demonstrated against long chain acyl-CoAs in a spectrophotometric assay. Furthermore, data obtained from sequence analysis showed that p48 contained a mitochondrial targeting signal, cleaved in mature protein as assessed by microsequencing. The mitochondrial localization of both endogenous and transfected p48 was confirmed by confocal microscopy. These results indicate that p48, called MT-ACT48 (mitochondrial acyl-CoA thioesterase of 48 kDa), defines a novel family of mitochondrial long chain acyl-CoA thioesterases.

Acyl-CoA hydrolases (EC 3.1.2.2) are enzymes that cleave thioester bonds of fatty acyl-CoA and liberate free fatty acids and CoASH. Acyl-CoA thioesterase activity is widely distributed among organisms from prokaryotes to eukaryotes (1). In eukaryotes, acyl-CoA thioesterase activities are detected in various subcellular organelles (2–4) including lysosomes (5) and peroxisomes and mitochondria (6, 7) as well as in cytosol (8).

The physiological functions of most acyl-CoA thioesterases are not clearly understood. The ability of thioesterases to regulate acyl-CoA concentration in the cell may provide a mechanism for the control of lipid metabolism. So far, the termination of chain elongation in fatty acid synthesis has been attributed to an acyl-CoA hydrolase/thioesterase in the mammary glands of certain species as well as the thioesterase domain of fatty acid synthase (9, 10). Thioesterases also control the acylation state of some proteins, such as signal transduction proteins, and their intracellular localization (11). Thus, a recently cloned thioesterase, the acyl-protein thioesterase 1, deacylates a subunit of heterotrimeric G proteins and Ha-Ras in vivo (12) and thereby regulates the membrane association of these proteins and their activation. Acyl-CoA and acyl-CoA thioesterases were also involved in intracellular traffic. The formation of transport vesicles from the cis to the medial Golgi was stimulated by long chain fatty acyl-CoA, and blocked by a nonhydrolyzable analog of palmitoyl-CoA, suggesting a role of acyl-CoA thioesterase(s) in vesicular budding (13, 14). Finally, thioesterases may play a role in endocytosis. Recent data have shown that association of human immunodeficiency virus-1 Nef protein with an acyl-CoA thioesterase, called human thioesterase, is required for the Nef-induced down-regulation of CD4 (15, 16).

The Eps15 protein is a recently identified component of plasma membrane clathrin-coated pits required for receptor-mediated endocytosis (17–19). Eps15 does not contain a characterized enzymatic site, and its function seems to rely on its ability to drive the formation of multimolecular complexes. Its C-terminal domain (DIII) contains binding sites for the AP-2 clathrin adaptor complex (20, 21). Its central coiled-coil domain (DIID) is involved in homodimerization (22, 23). Its N-terminal (DI) contains three Eps15 homology domains, which bind to several NPF (asparagine-proline-phenylalanine)-containing proteins (24) including synaptotagmin-1 (25), a phosphatidylinositol 4,5-bisphosphate phosphatase, and epsin (26), a protein involved in endocytosis. To characterize new Eps15-associated proteins, 35S-labeled cell lysates were precipitated with a glutathione S-transferase (GST)-Eps15 fusion protein. This approach led to the identification of a 48-kDa protein that specifically bound to fusion proteins derived from the central domain (DII) of Eps15. However, the association between Eps15 and p48 may be fortuitous, since it could not be confirmed in vivo. Characterization of p48 indicates that this protein belongs to a new family of acyl-CoA thioesterases. Tissue expression and intracellular distribution were analyzed.

* This work was supported by grants from the Association pour la Recherche contre le Cancer and from the Ligue Nationale contre le Cancer. The confocal microscope was purchased with a donation from Marcel and Liliane Pollack. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank§§ and EBI Data Bank with accession number(s) AJ238893 and AJ238894.

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¶ Supported by Ligue Nationale contre le Cancer.

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1 The abbreviations used are: AP-2, plasma membrane clathrin-associated protein complex; ACT, acyl-CoA thioesterase; EST, expressed sequence tag; GFM, MitoTracker® green fluorescent mitochondrial probe; GST, glutathione S-transferase; MT-ACT48, mitochondrial acyl-CoA thioesterase of 48 kDa; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; VSV, vesicular stomatitis virus; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
MATERIALS AND METHODS

Cells, Antibodies, and Probes—Human B cell line transformed by Epstein-Barr virus (a gift of P. De Leit, Hôpital Necker, Paris, France), human 293T (a gift of J. P. De Villaray, Hôpital Necker, Paris, France), and murine NIH3T3 and human HeLa cell lines (ATCC, Manassas, VA) were grown in RPMI 1640 or in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamicin (Life Technologies, Inc., Cergy Pontoise, France).

The production and characterization of the rabbit polyclonal antibody AC139 against mouse p48 is described below and under “Results.” Mouse monoclonal antibody P5D4 (IgG1) against the YTDIEMNRLGK epitope of the G protein of vesicular stomatitis virus (VSV) was a kind gift of Dr. T. Kreis (27). Texas Red-conjugated goat anti-rabbit IgG antibody was obtained from Molecular Probes, Inc. (Eugene, OR). Green fluorescence protein (GFP) microscope slide MitoTracker Green FM (GFP) was purchased from Molecular Probes.

Plasmids—The different EpS15-derived GST fusion proteins encoding the full-length protein (GST-Eps15), the N-terminal domain (GST-DI), the central domain (GST-DII), and the C-terminal domain (GST-DIII) were described previously (20). The cDNA of human eps15 subcloned in pBluescript II KS (Stratagene, La Jolla, CA) (28) was used as a template to generate the different cDNA fragments encoding parts of the central domain of EpS15 as described previously (20). The cDNA fragments were subcloned between the BamHI and the Xhol site of the PGEX5.1 vector (Amersham Pharmacia Biotech, Orsay, France) in frame with the GST moiety.

The cDNA of the two murine p48 isoforms corresponded to EST I.M.A.G.E. clone ID 514415 (GenBank™ accession number AA061148) and clone ID 602142 (GenBank™ accession number AA145599) (29) cloned in pBluescript SK and were obtained from the UK HGMP Resource Center (Hinxton, Cambridge, United Kingdom). The EST clones were sequenced using the T3 and T7 primers and internal 18-20-mer primers. Sequencing reactions were performed using the double-stranded sequencing reaction termination method (Dye Terminator cycle sequencing Ready Reaction kit, Applied Biosystems Inc., Foster City, CA) and an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). Exploitation of the obtained sequences was performed using the ABI PRISM program, and their alignment was performed using the GenoKyoo II program.

The cDNA of murine clone 514415 was used as a template for polymerase chain reaction to generate the p48-derived constructs. For GST fusion proteins, EcoRI and XhoI sites were introduced into the upper and the lower primers, respectively, allowing subcloning of the polymerase chain reaction products in the PGEX5.1 vector. The VSVG-tagged form of p48 was generated by introducing a Smal site in the lower primer, replacing the stop codon of p48 and allowing the in-frame introduction of the VSVG epitope beginning at the C terminus of p48. The VSVG-tagged p48 construct was subcloned in the eukaryotic expression vector pCDNA3 (Invitrogen BV, Leek, The Netherlands) and transfected into HeLa cells for transient expression. For transient expression, the CalPhos Maximizer Transfection Kit (CLONTECH, Palo Alto, CA).

Northern Blotting—Murine multiple tissue Northern blot (CLONTECH) containing 2 μg/glane of poly(A) RNA was first incubated with the hybridization solution (ExpressHyb, CLONTECH) at 68 °C for 30 min. A 1.5-kilobase pair probe, corresponding to the entire sequence of p48 (cDNA clone 514415), was randomly primed with [α-32P]dCTP using Megaprime™ DNA labeling system (Amersham Pharmacia Biotech) and added to fresh hybridization solution at a final concentration of 2 × 106 cpm/ml. The blot was further incubated with probe-containing hybridization solution with gentle shaking at 68 °C for 1 h and then washed four times, for a total of 40 min, with 2× sodium chloride/ sodium citrate, 0.05% sodium dodecyl sulfate at room temperature and twice, for a total of 20 min, with 0.1× sodium chloride/sodium citrate containing 0.1% sodium dodecyl sulfate at 50 °C. The blot was used for autoradiography at 70 °C for 60 h.

Production of GST Fusion Proteins—GST fusion proteins derived from EpS15 were produced as described previously (20, 28). The protocol for production of GST fusion proteins derived from p48 was modified, since these fusion proteins proved to be insoluble, remaining in the bacterial pellet. For an injection, a solubilization assay was attempted, shaking sonicated bacterial pellets in a 6 μl urea, 50 mM Tris, pH 8, 1 mM EDTA, 25 mM 1,4-dithiothreitol solution for 1 h at 4 °C. Solubilized proteins were recovered after a 15,000 × g centrifugation at 4 °C for 50 min and diluted 1:10 (v/v) to a final concentration of 0.6 μM urea in PBS, 1 mM EDTA, 25 mM 1,4-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Sigma). The second way to extract insoluble fusion proteins from the bacterial pellet consisted in growing bacteria at room temperature and increasing the number of sonication cycles.

Immunization of the Rabbits—Before immunization, the rabbits were bled in order to obtain preimmune sera. For immunization, 200 μg of GST-p48 fusion proteins resolubilized in 0.6 M urea solution were injected intradermically with complete Freund’s adjuvant (Sigma, Saint Quentin Fallavier, France) in the back of the rabbit. Five consecutive injections were performed on day 15 (200 μg of fusion proteins in incomplete Freund’s adjuvant), day 30 (same conditions), and day 31 (100 μg of fusion proteins injected intravenously). Rabbids were bled from the ear artery on day 45 to test the immune response by Western blot and then bled once a week for 4 weeks. Finally, rabbits were rechallenged with 100 μg of fusion protein, completely bled 2 weeks later. Partially purified IgG fractions were prepared from nonimmune and immune sera by (NH4)2SO4 precipitation.

Biochemical Procedures—Cells were lysed in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 (Sigma), containing a mix of protease inhibitors (4 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, pepstatin, 50 μg/ml trypsin inhibitor (Sigma)).

For precipitation, cell lysates were cleared with GST coupled to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). After removal of the beads, lysates were incubated 1 h with Eps15- or p48-derived GST fusion proteins (25 μg of fusion proteins/μl of glutathione-Sepharose 4B beads/107 cells). Precipitated proteins were separated by SDS-PAGE under reducing conditions.

For Western blotting with exogenous 35S-labeled amino acids (Amerham Pharmacia Biotech), the precipitates were washed six times with 100 mM borate buffer, pH 8.3 containing 1.3 M NaCl and 0.5% Triton X-100, twice with Hepes buffer, pH 8, containing 10% glycerol, twice with Hepes buffer, pH 8, containing 1 M urea, and twice with Hepes buffer, pH 8.

For Western blotting, acrylamide gels were transferred onto nitrocellulose membranes (Amerham Pharmacia Biotech) in 10 mM Tris, 0.2 mM glycine, and 30% methanol. Nonspecific binding sites were blocked by incubation in Tris-HCl, pH 7.6, containing 5% bovine serum albumin and 0.1% Tween. Blots were incubated for 1 h with rabbit anti-p48 polyclonal antibody AC139 (1:1000) revealed using peroxidase-labeled pig anti-rabbit immunoglobulin antiserum (1:20,000), and ECL (Amerham Pharmacia Biotech).

Purification and Microsequencing of the p48 Protein—Purification of the p48 protein was performed according to a modification of a published procedure (28). Briefly, Triton X-100 lysates of 1 × 109 NIH3T3 or 4 × 109 HeLa cells were cleared successively with GST and GST-DII EpS15-derived fusion protein deprived of the p48 interaction site (GST-DIII308/471) and then precipitated for 2 h using a GST-DII EpS15-derived fusion protein containing the p48 interaction site (GST-DIII1/471/538) (50 μg of fusion protein/20 μl of beads/1 × 107 cells). After extensive washes of beads, the precipitated proteins were resuspended in electrophoresis sample buffer containing 20 mM 1,4-dithiothreitol, subjected to SDS-PAGE, and transferred on a polyvinylidene difluoride membrane (Priba, Applied Biosystems) in CAPS buffer (Sigma) containing 10% methanol. Transferred proteins were revealed with Coo massie Blue R (Bio-Rad). The bands to be analyzed were excised and directly processed for amino acid sequence determination in a sequencer (model 477A, Applied Biosystems) equipped with an on-line phenylthiohydantoic analyzer (model 1201, Applied Biosystems).

Thioesterase Activity Assay—Acyl-CoA thioesterase activity was measured at 22 °C using a spectrophotometric assay (30). NIH3T3 cell lysates obtained from 12 × 107 cells were successively precipitated with 50 μg of GST fusion proteins. After extensive washes (six times with 100 mM borate buffer, pH 8.3, containing 1.3 M NaCl and 0.5% Triton X-100; twice with Hepes buffer, pH 8, containing 10% glycerol; and twice with Hepes buffer, pH 8), beads were resuspended in 1 ml of 0.05 mM potassium phosphate buffer, pH 8, containing 0.1 mM 5,5'-dithiobis-(2-nitrobenzoate), 20 μM/ml bovine serum albumin, and 10 μM of acyl-CoA of different lengths (C8-CoA, C10-CoA, C14-CoA, and C18-CoA from Sigma) as substrates. After a 7-min incubation, the reaction was stopped by a brief centrifugation to eliminate beads. Supernatants were immediately monitored spectrophotometrically by recording absorbance at 412 nm.

Mitochondrial Labeling and Intracellular Immunofluorescence—For mitochondrial labeling, NIH3T3 or transfected HeLa cells were grown on coverslips. After a 1-day culture, cells were washed in 37 °C prewarmed serum-free Dulbecco’s modified Eagle’s medium and incubated for 30 min at 37 °C with a green-labeled mitochondrial probe (GFM, Molecular Probes) at 200 μM in Dulbecco’s modified Eagle’s medium. After removing the medium containing nonincorporated probe, cells were incubated for 1 h at 37 °C in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum and then washed five times in PBS.

A Novel Mitochondrial Thioesterase
Precipitates were run on SDS-PAGE under reducing conditions or with GST fusion proteins encoding each of the three domains of the 48-kDa polypeptide precipitated by the central domain of Eps15. An additional band was observed that corresponded specifically to the 48-kDa polypeptide of murine NIH3T3 cells (Fig. 1B, lane 2) or 4 × 10^6 human HeLa cells (not shown) for microsequencing by Edman degradation. Microsequences of the N termini of murine and human p48 were obtained in one run and confirmed by three other runs (Fig. 1C and data not shown). The amino acids at positions 14 and 16 of murine microsequence could not be determined and are represented by X in Fig. 1C.

For intracellular immunofluorescence, cells grown on coverslips were fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 1 at 4°C and then for 30 min at room temperature. After quenching for 10 min in 50 mM NH_4Cl-PBS, cells were washed in PBS and permeabilized for 30 min at room temperature in PBS containing 0.1% Triton X-100 and 1 mg/ml bovine serum albumin (permeabilization buffer). Subsequent steps were performed at room temperature in permeabilizing buffer. Cells were incubated with primary antibodies for 45 min. After three washes, primary antibodies were revealed by incubating the cells for 45 min with Texas Red-coupled secondary antibodies. After three washes in permeabilizing buffer and one wash in PBS, cells were mounted on microscope slides in 100 mg/ml Mowiol (Calbiochem), 25 mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma), 25% glycerol (v/v), 100 mM Tris-HCl, pH 8.5. Samples were examined under an epifluorescence microscope (Axiophot, Carl Zeiss, Oberkochen, Germany) attached to a cooled CCD camera (Photometrics, Tucson, AZ) or to a confocal microscope (Carl Zeiss). No immunofluorescence staining was observed when secondary antibodies were used without the first antibody or with an irrelevant first antibody.

RESULTS

Identification and Microsequencing of a Novel 48-kDa Polypeptide Precipitated with the Central Domain of Eps15—For microsequencing by Edman degradation. Microsequences of the N termini of murine and human p48 were obtained in one run and confirmed by three other runs (Fig. 1C and data not shown). The amino acids at positions 14 and 16 of murine microsequence could not be determined and are represented by X in Fig. 1C.

cDNA Cloning and Sequence Analysis of Murine p48—N terminus sequences of both human and murine p48 were submitted to a set of protein data bases using the BLAST program. No homology with any known protein were found, indicating that p48 is a new protein. The sequences were then compared with GenBank™ EST data bases.

In the human EST data base, one clone (EST 98062, GenBank™ accession number AA884507) was found identical to the sequenced N terminus of human p48 but was incomplete. This clone shows high sequence similarity to murine p48 protein, indicating that the p48 polypeptides precipitated from both murine and human cells correspond to the same protein (not shown). However, since it was not possible to obtain a complete cDNA for the human p48 (see “Discussion”), our efforts were concentrated on murine p48.

In murine EST data bases, two EST clones (I.M.A.G.E. Consortium clone ID 602142 and 514415) showed, respectively, 100% and 90% sequence identity with the sequenced N terminus of murine p48 (Fig. 1C and data not shown). Undetermined amino acids indicated by an X at positions 14 and 16 corresponded in both clones to a histidine residue. These two clones derived from a Stratagene mouse testis library had been obtained by unidirectional cloning using an oligo(dT) primer. Complete sequencing of the two clones revealed that they contained the entire coding region and encoded two isoforms sharing 95% identity at the nucleotide level. The nucleotide and predicted amino acid sequences for EST clones 514415 and 602142 are shown in Fig. 2. The cDNA clone 514415 contains a 5'-untranslated region of 97 nucleotides with a Kozak box upstream from the ATG (position 98) coding for the putative initial methionine, a coding region of 1317 nucleotides, and a 3'-untranslated region of 143 nucleotides, for a total of 1557 nucleotides. The 3'-untranslated sequence contains several polyadenylation consensus sequences, AATTAA (positions 1470, 1497, 1504, 1508, and 1512), followed by a short poly(A)⁺ tail starting at position 1536. The cDNA clone 602142 contains a 5'-untranslated region of 110 nucleotides, a coding region of 1317 nucleotides, and a 3'-untranslated region of 85 nucleotides including a polyadenylation sequence at position 1482 and a poly(A)⁺ tail starting at position 1503, for a total of 1512 nucleotides.

The amino acid sequences deduced from both murine cDNA clones contain 439 amino acids, with a calculated molecular mass of 50.6 kDa. These two isoforms share 96% identity and 98% similarity. The amino acid and nucleotide differences between clones 602142 and 514415 are indicated in Fig. 2. In both isoforms, the putative initial methionine is located 21 amino acids upstream of the beginning of the sequence obtained by microsequencing of the N terminus (Fig. 2, boldface residues). It is interesting to notice that a cleavage site is predicted between amino acids Gly²¹ and Leu²², using the Signal PV1.1 consensus sequences, AATAAA (positions 1470, 1497, 1504, 1508, and 1512), followed by a short poly(A)⁺ tail starting at position 1536. The cDNA clone 602142 contains a 5'-untranslated region of 110 nucleotides, a coding region of 1317 nucleotides, and a 3'-untranslated region of 85 nucleotides including a polyadenylation sequence at position 1482 and a poly(A)⁺ tail starting at position 1503, for a total of 1512 nucleotides.

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cleavage site analysis program. Therefore, the initial 21 amino acids potentially constitute a leader peptide (see below), and the 48-kDa apparent size of the band observed on SDS-PAGE is in agreement with the size predicted for the expressed proteins after removal of the potential leader sequence (48.3 kDa).

p48 Is a Ubiquitously Expressed Protein—Northern blot analysis using the EST clone 514415 cDNA as a probe revealed in all mouse tissues a single mRNA transcript of approximately 2 kilobase pairs (Fig. 3A), a result in keeping with the fact that EST clones corresponding to p48 were obtained from cDNA libraries of various cell types and organs (not shown).

In order to study the tissue distribution of p48 at the protein level, a polyclonal antibody against murine p48 was produced. GST fusion proteins encoding the full length of the EST clone 514415 coding sequence or the same sequence deleted of the putative peptide leader sequence were constructed and used as immunogens. A partially purified IgG fraction derived from serum 139 was further used for biochemical studies. The AC139 antibody recognized a 48-kDa band in murine cell lysates (Fig. 3B, lane 1) and in cell lysates precipitated with GST-DII471/538 (Fig. 3B, lane 3). This 48-kDa band was observed neither when cell lysates were precipitated with GST-DII308/471 (Fig. 3B, lane 2) nor when the preimmune serum was used instead of AC139 (data not shown). The AC139 antibody stained a 48-kDa band in GST-DII471/538 precipitates obtained from homogenates of all tested murine organs (Fig. 3C and data not shown), confirming the ubiquitous expression of p48.

p48 Is a Conserved Protein Showing Homology to Acyl-CoA Thioesterase—Homology searches in the GenBank™ human EST data base using the murine coding sequence (EST clone 514415) led to the identification of a series of human EST clones in cDNA libraries derived from various tissues including thyroid, ovary, fetal liver, spleen, placenta, testis, and aorta. Nucleic acid alignment of all available EST clones led to a coding sequence starting at the same level as protein sequence obtained by microsequencing (not shown). This sequence was further confirmed by sequencing polymerase chain reaction products encompassing the alignment. The deduced protein sequence encompassed 418 amino acids (out of 439 for the complete murine sequence) and showed 85% amino acid iden-
Acyl-CoA Thioesterase Activity and Substrate Specificity of p48—Homology studies showing that p48 is homologous to a cytosolic acyl-CoA thioesterase suggested a possible thioesterase function for p48. Potential p48 thioesterase activity was tested in a spectrophotometric assay using a series of acyl-CoAs as substrates.

In a first set of experiments, thioesterase activity was assessed using recombinant GST fusion proteins encoding either p48 or human thioesterase, the Nef-associated acyl-CoA thioesterase (15), as positive control. No activity could be demonstrated for GST-p48, whereas GST-human thioesterase produced in the same conditions actively hydrolyzed C10 and C14 acyl-CoAs (not shown), as described (15). Since almost all produced GST-p48 was insoluble, the small amount of soluble p48 produced in bacteria and tested for thioesterase activity might be unfolded and thus inactive. The thioesterase activity of p48 was therefore measured on endogenous p48 precipitated from NIH3T3 cell lysates using the GST-DII-derived fusion protein. GST-DII471/538 precipitates showed a strong thioesterase activity, whereas precipitates obtained with GST alone or with GST-DII308/471 lacking the p48 interaction site were nonreactive. The p48 interaction site was therefore mapped to a region in the C-terminal domain (Fig. 5). A maximal activity was observed when myristoyl-CoA (C14.0) was used as a substrate, whereas precipitates obtained with GST alone or with GST-DII308/471 lacking the p48 interaction site showed no thioesterase activity (Fig. 5). When myristoyl-CoA (C14.0) was used as a substrate, whereas precipitates obtained with GST alone or with GST-DII308/471 lacking the p48 interaction site showed no thioesterase activity (Fig. 5).

Mitochondrial Localization of p48—Data obtained from both protein microsequencing and sequence analysis of the deduced primary sequence suggested that p48 possessed a cleaved N-terminal leader peptide. Various N-terminal signal peptides have been described to be involved in targeting of proteins into subcellular compartments including endoplasmic reticulum and mitochondria.

Proteins bearing endoplasmic reticulum leader peptides follow the secretory pathway and are glycosylated in the endoplasmic reticulum and in the Golgi apparatus. Four potential N-linked glycosylation sites are found at amino acid residues 133, 137, 175, and 236 of murine p48. However, incubation of precipitates of p48 with endoglycosidase F to release high mannos and oligosaccharides from the protein did not modify the migration of the protein on SDS-PAGE (data not shown), showing that p48 is not N-glycosylated and does not enter the secretory pathway.

Mitochondrial nucleus-encoded proteins are synthesized in...
The intracellular targeting of endogenous p48 was also investigated using immunofluorescence. Staining of p48 using the AC139 antibody showed a specific bright, comma-like pattern when observed with the AC139 antibody and colocalized with GFM, a specific mitochondrial probe (Fig. 6, compare c and d). The mitochondrial targeting of p48 was confirmed by confocal microscopy, showing a complete colocalization of endogenous p48 stained by AC139 with the mitochondrial probe GFM (Fig. 7).

**DISCUSSION**

The current study describes molecular cloning, expression pattern, and intracellular localization of a new protein of 48 kDa that is the prototype of a novel family of highly conserved mitochondrial proteins. Its acyl-CoA thioesterase function was suggested by its homology to rat cytochrome C oxidase and demonstrated by an *in vitro* assay that showed optimal activity with C14 myristoyl-CoA. The mitochondrial targeting of the p48 polypeptide was suggested by the presence of a cleaved N-terminal signal sequence rich in basic amino acids and was demonstrated by fluorescence microscopy for both endogenous and transfected VSV-tagged proteins. This 48-kDa protein has therefore been named MT-ACT48 (for mitochondrial acyl-CoA thioesterase of 48 kDa).

The initial aim of our work was to identify new binding partners for Eps15 by precipitation using Eps15-derived GST fusion proteins. This approach led to the identification of a 48-kDa polypeptide that specifically binds to the central domain of Eps15. Further deletion studies mapped the precise site of interaction to a small region at the end of the coiled-coil domain of Eps15 (data not shown). Sequence analysis and functional studies identified the 48-kDa polypeptide as a novel acyl-CoA thioesterase. Recent work has implicated acyl-CoA and/or acyl-CoA thioesterases in vesicular transport (see Introduction), suggesting that the association of Eps15 with a thioesterase may be relevant to its function in endocytosis. However, several lines of evidence argued against a functional link between Eps15 and MT-ACT-48. First, the mitochondrial localization of MT-ACT48 is not easy to reconcile with a putative function in plasma membrane clathrin-coated pits. However, the cytosol contains large amounts of a 48-kDa polypeptide reactive with AC139, the antiserum anti-mouse p48 (data not shown). For at least two mitochondrial thioesterases, cytosolic isoenzymes differing by the lack of N-terminal mitochondrial targeting sequence have been described (7, 33). The existence of an as yet unidentified cytosolic isoform of MT-ACT48, which could be available for recruitment by Eps15, is thus not excluded. In addition, it must be stressed that the thioesterase that binds to the human immunodeficiency virus-1 Nef protein and is required for Nef-induced down-regulation of CD4 is a peroxisomal protein (15). Second, *no in vivo* interaction between Eps15 and MT-ACT48 could be demonstrated. Unfortunately, it was not possible to use the anti-MT-ACT48 polyclonal

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2 S. Benichou and R. Benarous, personal communication.
antibody AC139 for coimmunoprecipitation studies, since it was not an immunoprecipitating antibody (data not shown). To bypass the use of a precipitating anti-MT-ACT48 antibody, a VSV-tagged form of MT-ACT48 was generated. The anti-VSV tag antibody P5D4 immunoprecipitated VSV-tagged MT-ACT48 but did not immunoprecipitate endogenous Eps15 (data not shown). Furthermore, AC139 did not detect any 48-kDa protein in immunoprecipitates obtained with the anti-Eps15 antibody 6G4 (data not shown). Altogether, these results suggest that Eps15 and p48 are not associated in vivo. However, it is possible that the Eps15/p48 association is labile in experimental conditions, and/or regulated and transient.

MT-ACT48 is conserved from mouse to human. The coding sequence of human MT-ACT48 could not be fully determined. All the various approaches used to clone the 5′ missing part, including reverse transcription-polymerase chain reaction with 5′ primers derived from the murine sequence and anchored polymerase chain reaction, remained unsuccessful. Nevertheless, the human protein sequence deduced from the determined cDNA sequence encompassed 418 amino acids and showed 85% identity and 92% similarity with murine MT-ACT48. Furthermore, homology searches led to the identification of two sets of proteins homologous to MT-ACT48. The first set included ACT and cytosolic long chain acyl-CoA thioesterase II, two conserved mammalian cytosolic acyl-CoA thioesterases further referred to as ACT (31, 34). The second set of proteins shows high sequence similarity to MT-ACT48, including four proteins from C. elegans. The one showing the highest sequence similarity to MT-ACT48 was further named C.E.1 (AF016682); the protein of A. thaliana was further called A.T. (AC002340). Both C.E.1 and A.T. are good candidates as functional homologs of MT-ACT48. They share a comparable length with 440 and 438 amino acids, respectively, versus 439 amino acids for MT-ACT48 (Fig. 4D). They exhibit a similar structural organization with a comparable hydrophobic profile (not shown), a basic rich leader peptide predicting their mitochondrial or chloroplastic localization, respectively (Fig. 4C), and a conserved region of 80 amino acids in the C terminus sharing 65 and 69% amino acid similarity with the same domain in both murine and human MT-ACT48 (Fig. 4, B and D).

In conclusion, homology searches suggest that MT-ACT48 defines a novel family of acyl-CoA thioesterases characterized by a highly conserved C-terminal 80-amino acid domain (Fig. 4B). This conserved domain is likely to play an important role in the function of the protein. Indeed, MT-ACT48, as the other homologous proteins from A. thaliana and C. elegans, does not contain any of the consensus sequences present in many acyl-CoA thioesterases such as the serine active site GXSXG or the carboxy-terminal histidine active site GXH. Mutational analysis of the most conserved amino acids in this 80-amino acid domain should help to define its involvement in esterase function and the consensus sequence for this putative new active site.

Acknowledgments—We thank R. Benarous, S. Benichou, and L. Liu for the kind gift of the GST-human thioesterase construct and helpful advice; P. Rustin for helpful advice in the mitochondrial field; V. Collin for expert help with rabbit immunization; R. Hello for expert assistance with confocal microscopy; and D. Ojcius for careful reading of the manuscript.

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