Primary Structure of a Dynamin-related Mouse Mitochondrial GTPase and Its Distribution in Brain, Subcellular Localization, and Effect on Mitochondrial Morphology*

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A new member of the dynamin GTPase family (OPA1) was recently identified in humans and shown to be mutated in patients with dominant optic atrophy. To understand better the function of mammalian OPA1, we isolated a mouse ortholog (mOPA1) from brain and raised a specific antibody against its C terminus. The subcellular distribution of mOPA1, own expressed in COS-7 cells largely overlapped that of endogenous cytochrome c, a well known mitochondrial marker, and dramatically affected mitochondrial morphology, altering it from tubular to vesicular. Mitochondrial targeting was mediated by the N-terminal region of mOPA1 as follows: deletion of the 124 N-terminal amino acids eliminated mitochondrial targeting, although fusion of the N-terminal 60 or 90 amino acids of mOPA1 with green fluorescent protein resulted in its mitochondrial targeting. mOPA1 was expressed widely in the mouse brain, especially in neurons of olfactory bulb, cerebral cortex, piriform cortex, hypothalamus, hippocampus, red nucleus, cochlear nucleus, motor trigeminal nucleus, facial nucleus, cerebellar nucleus, and Purkinje cells. Within dissociated cerebellar cells, mOPA1 protein was clearly observed in the dendrites and somas of neuronal cells, as well as in astrocytes and meningeal cells. In each case, it was distributed in the vesicular pattern seen in other cell types.

GTP-binding proteins (G proteins)¹ are known to play key roles in a variety of cellular processes. In addition to the two major families, the trimeric and low molecular weight G proteins, there is a third family composed of relatively large proteins having molecular masses ranging from 70 to 120 kDa. Members of this family are diverse in terms of their overall primary structures and their functions (1, 2), but all share highly homologous GTPase domains located near their N termini. Among this group of proteins are dynamin, known to be involved in coated vesicle formation and endocytosis (3, 4); Vps1, which is required for the protein sorting in the trans-Golgi network of yeast (5); Mx, the expression of which is induced in vertebrates by interferon (6); and Drp1/Dnm1 (7, 8) and Mgm1 (9). Drp1/Dnm1 are cytoplasmic proteins known to regulate mitochondrial fission, although they are not localized to mitochondria (7, 10). Instead, they assemble into punctate structures on the outer mitochondrial membrane at sites associated with membrane fission (7, 8, 10–13). On the other hand, Mgm1, which contains a mitochondrial targeting signal (14) at its N terminus, is localized to mitochondria and is reportedly involved in determining normal mitochondrial morphology and division (15, 16).

We previously isolated a cDNA encoding a novel, large (971 amino acids) G protein from salmon brain (17). This protein had a clear GTP-binding motif but showed only limited homology with the other members of this family known at that time (for example 25% amino acid identity with dynamin). The transcript was expressed in the brain and ovary, and in situ hybridization analysis of brain sections revealed the mRNA to be most intensely expressed in the large motor neurons of the brain stem. The specific function of this protein remains unknown, however.

In the present work, we first isolated a mouse ortholog of the salmon cDNA clone in order to investigate the function of this large G protein in mammals. During the course of this study, it was reported that human OPA1 (hOPA1), which is highly homologous with our salmon clone (76.4%) and is described as the only known large G protein in vertebrates that has an N-terminal mitochondrial targeting sequence, is mutated in patients with autosomal dominant optic atrophy (18, 19). Delettre et al. (18) demonstrated that hemagglutinin-tagged hOPA1 localized to mitochondria in transfected HeLa cells, but the subcellular distribution of the untagged hOPA1 protein is not yet known due to the lack of a specific antibody. That mutation of hOPA1 caused optic atrophy suggests it has a biologically important role, presumably affecting mitochondrial function. However, as with the salmon clone, the functional significance of hOPA1 and its regulation by GTP binding have not yet been studied in detail. Here we describe our use of a specific antibody raised against a mouse OPA1 to analyze the subcellular...
Distribution of OPA1, as well as the structural basis for that localization, its GTP-dependent regulation, and its posttranscriptional distribution within brain.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—We initially did a TBLASTN search of an EST database using the amino acid sequence of the salmons that provided the sequence information. We then used the sequence information to design a primer corresponding to amino acids 96–104 (salmon clone number 5′-GGAGCCAACTTTTAAAACTTCATATGAT-3′) and an antisense primer corresponding to amino acids 152–159 (5′-GGAGGGCCCTTATATT- TTTCC-3′). By using this primer pair and PCR, we screened subdivided pools of a mouse brain cDNA plasmid library (Takara, Japan). One positive pool was then repeatedly subcloned until a single clone was obtained, after which the entire nucleotide sequence was determined using a BigDye Terminator Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (Applied Biosystems type 377 and type 310).

A Sculptor in situ mutagenesis system (Amersham Biosciences) and QuickChange site-directed mutagenesis kit (Stratagene) were used with mutated oligonucleotide DNA primers as instructed by the manufacturer to construct a group of mOPA1 point mutants: Met125 to Ala (M125A), Glu297 to Val (Q297V), Glu297 to Gly (Q297G), Glu297 to Leu (Q297L), Glu297 to Phe (Q297F), Glu297 to Trp (Q297W), Lys96 to Ala (K301A), and Thr96 to Asn (T302N). mOPA1 FLAG-tagged at the N terminus was constructed using PCR protocol that inserted DYKD-DDEK between Met1 and Trp2. An N-terminal deletion mutant in which the N-terminal region upstream of Met5 (ΔN-mOPA1) was also constructed by PCR. mOPA1 Myc tagged at the C terminus (960Myc) was constructed using PCR that added EQKLISEEDL after Lys96. A C terminus deletion mutant (355MCy), in which the C-terminal region downstream of Asp595 was deleted and Myc tag was added at the C terminus, was constructed by inserting the SalI- BglII fragment of mOPA1 cDNA into the Myc-tagged vector. Fusion proteins composed of N-terminal fragments of mOPA1 fused to enhanced green fluorescent protein (EGFP) were constructed by inserting the appropriate PCR product into the EGFP-N1 vector (CLONTECH). DNA sequencing of the primer and surrounding regions confirmed all of the mutations. Wild type (WT) mOPA1 and its mutants were also subcloned into pCXS, a plasmid vector for efficient expression in mammalian cells (20).

**Northern Hybridization**—Poly(A)+ RNA samples were isolated from BALB/c mice. After determining the concentrations of the samples by absorbance at 260 nm, a 2-μg aliquot of the poly(A)+ RNA was fractionated on a 0.7% agarose formaldehyde gel and transferred to a nylon membrane. Thereafter, the entire mOPA1 cDNA was labeled with 32P through random priming and hybridization. Finally, the membranes were washed with 0.1× SSC, 0.1% SDS at 65 °C for 15 min, and exposed to an X-ray film for 1 day. The integrity of the loaded RNA was confirmed by staining the RNA gel with ethidium bromide and the nylon membrane with methylene blue. In some cases, a mouse Multiple Tissue Northern blot filter (CLONTECH) was simultaneously used for hybridization.

**Cell Culture**—COS-7, HEK 293, and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 μM glutamine. For immunocytochemistry, cells were seeded onto poly-L-lysine-coated glass coverslips, where they were transiently transfected with the appropriate expression plasmids using the LipofectAMINE PLUS (Invitrogen) according to the manufacturer's instructions. In addition, primary cultures of rat cerebral cells were prepared as described previously (21).

**Preparation of Anti-mOPA1 Antibody**—A peptide corresponding to amino acids 938–960 of mOPA1 (CDLKKVRERQEKLDFAFELHKEK) was synthesized with an artificial Cys placed at the N-terminal end for conjugation with keyhole limpet hemocyanin. Three rabbits were then immunized by subcutaneous injection with the keyhole limpet hemocyanin-conjugated peptide every 4 weeks. A specific antibody against mOPA1 was purified from the collected antiserum as follows. Epoxy-activated Sepharose 6B (Amersham Biosciences) was coupled for 16 h at 37 °C with synthetic peptide in 0.2 M sodium carbonate buffer (pH 9.5). The Sepharose was then blocked with 1 M ethanolamine (pH 8.0) for 4 h at 50 °C and washed with 10 column volumes of 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (TBS). Antiserum was then loaded onto the column and incubated for 2 h at 4 °C, after which the column was washed with 10 volumes of TBS. The antibody was then eluted with 0.1 M glycine hydrochloride (pH 2.5) and immediately neutralized with Tris base. The specificities of the antiserum and the affinity-purified antibody were confirmed by comparing Western blot and immunocytochemical data obtained from mOPA1-transfected and untransfected HEK 293 cells.

**Other Antibodies**—Mitochondria were labeled with mouse anti-cytochrome c mAb (clone 6H2.B4, BD Pharmingen); neurons with mouse anti-MAP-2 mAb (clone AP20, Roche Molecular Biochemicals); astrocytes and Bergman glial cells with mouse anti-GFAP mAb (clone G-A-5, Roche Molecular Biochemicals); FLAG tag with mouse anti-FLAG mAb (M2, Sigma); rabbit anti-c-Myc antibody (A-14, Santa Cruz Biotechnology). Alexa-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes), Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch), biotinylated anti-rabbit IgG (Vector Laboratories), and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) served as secondary antibodies.

**Western Blot**—Brains from adult BALB/c mice were homogenized in a glass-Teflon Potter homogenizer in ice-cold PBS supplemented with a mixture of proteinase inhibitors (Complete, Roche Molecular Biochemicals) and then centrifuged at 500 × g for 10 min at 4 °C to remove unbroken tissues. The supernatant was mixed with Laemmli SDS-PAGE sample buffer and boiled for 10 min. Alternatively, HEK 293 cells transfected with mOPA1 were washed three times with ice-cold PBS (10 mM NaCl, 0.5% BSA, 0.5% sodium pyruvate, and 1 mM glutamine). The cells were then collected by centrifugation, suspended in Laemmli SDS-PAGE sample buffer, neutralized with Tris base, and boiled for 10 min. In either case, protein samples were resolved on 7.5% SDS-polyacrylamide gels and electrophoresed to polyvinylidene difluoride membranes (Millipore). The membranes were then blocked for 30 min at room temperature with blocking solution (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20 and 5% skim milk) and probed for 1 h at 37 °C with affinity-purified anti-mOPA1 antibody diluted in blocking solution. After washing, membranes were treated first with horseradish peroxidase-conjugated secondary antibody and then with ECL Plus reagent (Amersham Biosciences).

**Immunocytochemistry**—Cells plated on coverslips were transfected with wild type mOPA1 or its mutants. After incubation (ordinarily 24 h), they were fixed for 15 min at room temperature in 4% paraformaldehyde in PBS and then washed with PBS and blocked for 1 h at 37 °C in PBS containing 2% skim milk and 0.1% Triton X-100. The coverslips were then incubated for 1 h at 37 °C with the appropriate primary antibodies in PBS + 2% skim milk, washed three times with PBS, and incubated with fluorescence-conjugated secondary antibodies in PBS + 2% skim milk. After aspiration of the secondary antibody, the coverslips three times with PBS, they were incubated for 5 min with PBS containing 0.5 μg/ml DAPI and washed again with PBS. Finally, the coverslips were mounted on microscope slide glass using PermaFluor mounting medium (Shandon). Fluorescent images were acquired using a Zeiss AxioCam camera, and pseudocolors were added to the digitized images. For DAB staining, a biotinylated secondary antibody was used as described below.

**Immunohistochemistry**—Sections from adult mice were prepared as described above. Free-floating sections were blocked for 1 h at room temperature in PBS + 1% skim milk and then incubated overnight at 4 °C with anti-mOPA1 antibody in the blocking solution. The sections were then rinsed with PBS and incubated with biotinylated anti-rabbit IgG (Vector Laboratories), rinsed again, and reacted with avidin-biotin

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peroxidase complex (Elite ABC kit, Vector Laboratories). Finally, the sections were reacted with 0.02% DAB and 0.002% hydrogen peroxide in 0.1M sodium phosphate buffer.

RESULTS

cDNA Cloning and the Primary Structure of mOPA1

To isolate a mouse ortholog of the large G protein previously cloned from salmon brain (17), we carried out a TBLASTN search of a mouse EST database using the amino acid sequence of the salmon clone as a probe. We then prepared a set of PCR primers based on the registered sequence of one of the clones obtained, screened a mouse brain cDNA library, and through repeated subdivision of a positive pool, eventually identified a single cDNA clone. It was 6 kbp in length and encoded 960 amino acids. The deduced amino acid sequence (Fig. 1A) showed 76% identity with the salmon clone (17) and 96% identity with the recently isolated hOPA1 (18, 19). We there-

![Fig. 1. Primary structure of the mOPA1.](image)

**Fig. 1. Primary structure of the mOPA1.** A, deduced amino acid sequence of mOPA1 and alignment with human and salmon clones; * indicates the conserved residues. Three GTP-binding motifs are boxed. The two double dashed regions are predicted coiled-coil structures. The GenBank accession numbers are as follows: mouse, AB044138; human, AB011139; salmon, AB012720. B, sequence of the N-terminal 280 amino acids of mOPA1. Positively charged amino acids (R and K) in the N-terminal 100 amino acids indicative of mitochondrial targeting are underlined. Conserved mitochondrial endopeptidase cleavage motifs are boxed.

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fore designated the isolated mouse clone mOPA1.

Like hOPA1 and the salmon clone, the N-terminal region of mOPA1 contained an obvious GTP-binding motif (Fig. 1A, boxes), a putative mitochondrial targeting signal with scattered positively charged amino acid residues (14), and three mitochondrial endopeptidase cleavage motifs (RXF/L/IxxG/S/T) (XX) (22) (Fig. 1B). There were also two predicted coiled-coil structure motifs (Fig. 1A, amino acids 208–251 and 922–951) (23).

**Tissue-specific Distribution of mOPA1**—Although hOPA1 was found to be expressed in almost every tissue investigated (18, 19), the salmon clone was most abundantly expressed in brain and ovary (17). When we analyzed tissue-specific expression of mOPA1 using Northern hybridization (Fig. 2), we observed that an mRNA of ~6 kb, comparable in size to the isolated cDNA, was expressed in the forebrain, cerebellum, and brain stem, as well as in the heart. A faint 6-kb band was also detected in liver, kidney, ovary, and skeletal muscle; however, a stronger band was detected at ~3.5 kb. Although the identity of the lower molecular weight product is presently unknown, it was also clearly detectable on a commercially available RNA blot sheet (CLONTECH; data not shown), and its presence was also reported in conjunction with hOPA1 (18, 19).

**Processing of mOPA1 Protein in Mouse Brain and in Transfected HEK 293 Cells**—As the mOPA1 amino acid sequence contains three cleavage motifs (Fig. 1B), we used Western blot analyses to examine the extent to which this protein is processed in cells. When mouse brain was probed using a specific anti-mOPA1 antibody, a major band at ~90 kDa and double bands at 80 kDa were detected (Fig. 3, lane 1). In the HEK 293 cells transfected with WT mOPA1, bands were detected at 100 and 90 kDa, and a major band appeared at 80 kDa (Fig. 3, lane 2). No protein was detected in HEK 293 cells transfected with empty vector, confirming the specificity of the antibody (Fig. 3, lane 6).

To exclude the possibility that the presence of various sized mOPA1 proteins reflects multiple translation initiation sites, we constructed a deletion mutant in which the N-terminal region upstream of the 2nd Met (Met125) was deleted (ΔN-mOPA1). In that case, only a single major band was detected (Fig. 3, lane 4). In addition, analysis of an M125A point mutant yielded three bands like those obtained with the wild type protein (Fig. 3, lane 5). It thus appears that all three bands obtained with WT mOPA1 isolated from HEK cells were derived from a single protein translated from the 1st Met (Met1) and that the lower molecular weight bands resulted from processing at the N-terminal end of that protein.

We also evaluated a mOPA1 construct having a FLAG tag attached at its N terminus. This construct yielded only one band at 100 kDa plus FLAG and was detectable using antibodies against either the C-terminal end of mOPA1 (Fig. 3, lane 3) or FLAG (data not shown), which confirms that the 100-kDa protein is the unprocessed form of mOPA1 and indicates that the presence of a FLAG tag at the N-terminal end inhibits processing of the protein.

**Subcellular Localization of mOPA1 and Its Effect on Mitochondrial Morphology in Transfected Cells**—Immunofluorescence microscopy (Fig. 4A) and DAB staining (Fig. 4B and B’) revealed mOPA1 to be distributed in a pattern of small vesicles throughout the cytoplasm of transfected COS-7 cells. The same vesicular pattern was observed in mOPA1-transfected HeLa and HEK 293 cells (data not shown). In addition, some staining presumed a ring-like pattern (arrows in Fig. 4B’), suggesting mOPA1 might be situated on the membrane of an organelle in close proximity to mitochondria.
Note that EGFPs fused with 60- and 90-amino acid fragments almost completely co-localized with cytochrome \( c \). As expected, the N-terminal deletion mutant truncated just upstream of Met 125 (\( \Delta N\)-mOPA1), after which they were labeled with anti-mOPA1 (red in A) or anti-cytochrome \( c \) (green in A’). Their overlap is seen in the merged image (A’). The typical tubular mitochondrial morphology is revealed by cytochrome \( c \) labeling (A’). B–E, COS-7 cells were transfected with EGFP (B) or a 60-, 90-, or 90-amino acid fragment of the N-terminal of mOPA1 fused to the N-terminal end of EGFP (C–E, respectively). The merged images show the distributions of EGFP (green) and anti-cytochrome \( c \) (red). Note that EGFPs fused with 60- and 90-amino acid fragments almost completely co-localized with cytochrome \( c \) (D and E). Scale bars, 10 \( \mu \)m.

The functional significance of the putative mitochondrial targeting signal of mOPA1—The functional significance of the putative mitochondrial targeting signal at the N terminus of hOPA1 has not been demonstrated experimentally. To clarify this point with respect to mOPA1, we first examined the subcellular distribution of \( \Delta N\)-mOPA1, which started at Met 125 and thus lacked the mitochondrial targeting signal. As expected, the intracellular distribution of the truncated protein was diffuse, although some aggregates were seen (Fig. 5A). The morphology of mitochondria revealed by anti-cytochrome \( c \) showed the standard tubular shape (Fig. 5A’), and the distributions of the two proteins did not overlap (Fig. 5A”).

We next created a group of fusion proteins composed of 30-, 60-, or 90-amino acid fragments from the N terminus of mOPA1 fused with EGFP at its N-terminal end and examined their subcellular distributions. Comparison of the distributions of cytochrome \( c \) (red) and the EGFP fusion proteins (green) revealed that the 60- and 90-amino acid fragments were sufficient for mitochondrial targeting, but the 30-amino acid fragment was not (Fig. 5, C–E). The mitochondria retained their standard tubular morphology in these experiments, indicating that the N-terminal sequence of mOPA1 is necessary and sufficient for mitochondrial targeting but that expression in mitochondria, itself, is not sufficient to alter mitochondrial morphology.

Effects on Mitochondrial Morphology of mOPA1 Mutants in GTP-binding Motif or C Terminus—Although mOPA1, hOPA1, and the salmon clone all contain an obvious GTP-binding motif, little is known about the regulation of their function by GTP. To gain some idea, we transfected COS-7 and HeLa cells with either WT mOPA1 or one of the constructs containing a point mutation (Q297V, K301A, or T302N) in the GTP-binding motif and then monitored the morphology of the mitochondria in the transfecteds (Fig. 6, A–F). Glu\(^n\) of mOPA1 corresponds to the residue at which Gly to Val mutation was shown to make Rho protein constitutively active by depriving GTP hydrolyzing activity (25). The actual biochemical nature of Q297V mutant of mOPA1, however, is not characterized. K301A and T302N of mOPA1 correspond to the mutants that were shown to be constitutively negative, the former lacked nucleotide binding activity (26), although the latter stabilized the GDP-bound form (25, 27). In COS-7 cells, all three mutants co-localized with cytochrome \( c \) and changed tubular mitochondrial morphology (Fig. 6, B–D). In the case of the Q297V mutant, small vesicular mitochondria were observed. In approximately half of COS-7 cells expressing the Q297V mutant (46%, \( n = 170 \)), the fragmented vesicular mitochondria no longer distributed throughout the cytoplasm but accumulated in one region adjacent to the nucleus (Fig. 6B). The accumulation of fragmented mitochondria was also observed in part of COS-7 cells transfected with Q297G (24%, \( n = 311 \)), Q297F (17%, \( n = 535 \)), and Q297A (16%, \( n = 100 \)).
Q297L (13%, n = 295), or Q297W (8%, n = 324) (data not shown). This phenotype was never observed in COS-7 or HeLa cells transfected with WT mOPA1 but in Q297V-transfected HeLa cells (Fig. 6F). In the case of K301A and T302N mutants, they changed the mitochondrial morphology of transfected COS-7 cells to a short tubular or a large vesicular pattern (Fig. 6, C and D), which is distinctly different from the small vesicular pattern observed in the cells transfected with WT mOPA1 (Figs. 4D’ and 6A). Taken together, mutations in GTP-binding motif of mOPA1 do not change the localization of the mOPA1 in mitochondria, but actually affect the function of mOPA1 to alter the morphology and distribution of mitochondria.

To confirm further that the morphological change of mitochondria is not artifacts due to simple overexpression, we made a deletion mutant of the C terminus of mOPA1 up to Asp324 with a Myc tag at the C-terminal end (355Myc), and we analyzed its effect on mitochondrial morphology in transfected COS-7 cells. A full-length mOPA1 with a Myc tag (960Myc), which was prepared as a control, localized to mitochondria and changed mitochondrial morphology to the vesicular pattern (Fig. 6, G and G’) as WT mOPA1 did (Fig. 6, A and A’). On the other hand, 355Myc could hardly change the standard tubular shape of mitochondria in transfected cells (Fig. 6H’), although the mutant protein actually localized to mitochondria and showed a vesicular pattern in itself (Fig. 6H). These results demonstrate that the expression of incomplete mOPA1 protein and its localization in mitochondria are not sufficient to alter mitochondrial morphology to a vesicular pattern.

Distribution of mOPA1 mRNA in the Mouse Brain—Northern hybridization showed levels of mOPA1 mRNA to be highest in the brain (Fig. 2). By in situ hybridization using a digoxigenin-labeled cRNA probe, we found that unlike the salmon clone, which was expressed mostly in the large motor neurons of the brain stem (17), the mOPA1 transcript was also detected in numerous other sites and cell types throughout the gray matter of the brain (Fig. 7 and Table I). That expression was not detected in the white matter suggests mOPA1 is not expressed in oligodendrocytes.

Distribution of mOPA1 Protein in the Brain—When expression of the mOPA1 protein was studied immunohistochemically...
using anti-mOPA1 antiserum as a probe (Fig. 8), we observed sufficient overlap between the distributions of the mRNA and the mOPA1 protein in layer V cells in the cerebral cortex; in pyramidal, dentate and polymorphic cells in the hippocampus; in Purkinje and cerebellar nucleus cells; and in inferior olive cells (Figs. 7 and 8). In the Purkinje cells, mOPA1 was apparent in both the somas and dendrites (Fig. 8C, arrow in right panel). Dendritic signals were also detected in cerebral cortical neurons and in neurons in the CA1 region of the hippocampus (Fig. 8, A and B, arrows). mOPA1 protein is thus widely expressed in a variety of neurons and is localized in both the somas and the dendrites. No labeling was detected when the antiserum was preincubated with the peptide used for the immunization, confirming its specificity and validity for immunohistochemical analysis (data not shown).

To better visualize the subcellular localization of mOPA1, a primary culture of dissociated rat cerebellar cells was prepared. mOPA1 labeling, distributed in a vesicular pattern, was detected in the somas of MAP-2-positive neurons (Fig. 9A), and a weaker signal was detected in the dendrites (Fig. 9B). mOPA1 was also expressed in flat cells, some of which were identified as astrocytes by co-labeling them with anti-GFAP (Fig. 9C). There were also GFAP-negative cells among the mOPA1-positive flat cells; they were presumed to be meningeal cells (Fig. 9D). Interestingly, the mOPA1 signal did not necessarily overlap that of cytochrome c in these cells (Fig. 9, E and F), suggesting that the distribution of endogenous mOPA1 in the brain might not be confined to mitochondria.

**DISCUSSION**

We have isolated a mouse ortholog of the cDNA encoding a novel large GTP-binding protein previously cloned from salmon brain (17) and identified as OPA1 in humans (18, 19); we designated the mouse clone mOPA1. Although mutation of hOPA1 has been identified as a cause of dominant optic atrophy (18, 19), little or nothing was known about the cellular function of OPA1; the presence of a mitochondrial targeting signal and several cleavage motifs have been noted, but their significance remained unknown. We therefore raised a specific antibody against the C-terminal end of mOPA1 and used it to study the processing of this protein, its subcellular distribution, its macroscopic distribution in the brain, and its effect on mitochondrial morphology.
N-terminal Processing and Intracellular Localization of mOPA1—By using Western blot analysis to probe samples from mOPA1-transfected HEK 293 cells, we detected proteins with molecular masses of about 100, 90, and 80 kDa (Fig. 3, lane 2). Based on results obtained using a FLAG-tagged construct (Fig. 3, lane 3), we concluded that the 100-kDa protein is the unprocessed mOPA1 translated from the 1st Met, although its size is slightly smaller than that estimated from the amino acid sequence (111 kDa; 960 amino acid residues). Furthermore, the 90- and 80-kDa proteins are not translated from the 2nd Met (Met125); rather they are formed through processing of the 100-kDa protein.

Whether isolated from mouse, human, or salmon, the N terminus of this protein contains a putative mitochondrial targeting signal, enriched in positively charged amino acid residues (14), and a mitochondrial peptidase cleavage motif (22) immediately downstream (Arg103) (Fig. 1B). If mOPA1 is processed here, the resultant protein would be ~10 kDa smaller than the 100-kDa protein (the 90-kDa form). Downstream of the cleavage motif is a highly hydrophobic region recognized to be a signal sequence for targeting to the intermembrane space (28), and downstream of that is another putative cleavage motif (Arg256) (Fig. 1B). The protein processed at this site is estimated to be 80 kDa and is thought to be the final and major product expressed in HEK 293 cells. The presence of the 100-kDa protein in transfected HEK 293 cells most likely reflects incomplete processing; indeed the 100-kDa form was not clearly detected in brain samples (Fig. 3, lane 1).

We observed a high degree of overlap between the distributions of mOPA1 and cytochrome c in transfected COS-7 cells, which confirmed that mOPA1 is, in fact, localized to mitochondria (Fig. 4). Precisely where within the mitochondria OPA1 is situated is not yet clear. But when COS-7 cells were co-transfected with mOPA1 and DsRed1-Mito (CLONTech), the distribution of mOPA1 was somewhat different from that of DsRed1-Mito, which contains the matrix-targeting signal from subunit VIII of cytochrome c oxidase (29) (data not shown). This suggests that at least in transfected COS-7 cells, mOPA1 is situated in the intermembrane space rather than in the matrix, an interpretation supported by the observation that DAB staining often showed mOPA1 to be distributed in a ring-like pattern (Fig. 4, B and B’). Like mOPA1, the yeast proteins Mgm1 (9) and Msp1 (30) also contain an N-terminal, mitochondrial targeting signal; their localization within mitochondria differs from each other, however (15, 16, 31). Consequently, the precise localization of OPA1 and other dynamin family members that contain an N-terminal mitochondrial targeting signal remains controversial.

Influence of mOPA1 and Its Mutants on Mitochondrial Morphology of Transfected COS-7 Cells—The most striking immunocytochemical finding was that overexpression of mOPA1 dramatically altered the morphology of mitochondria from tubular to vesicular (Fig. 4, C–D”). In that regard, some large G proteins expressed in yeast are known to be involved in the fusion and fission of mitochondria (2, 32). Dnm1, for instance, is present in punctate structures at the constriction sites on mitochondrial tubules, and dnm1 mutants possess single, net-like mitochondria (8, 11–13). Drp1, a cytoplasmic G protein that lacks a mitochondrial targeting signal, nevertheless associates with the cytoplasmic surface of the outer mitochondrial membrane (7, 10), and expression of a dominant negative Drp1 mutant causes mitochondria to collapse into a clump near the nucleus (7). Finally, Mgm1 contains a clear mitochondrial targeting signal, is localized at mitochondria, and is known to be involved in mitochondrial morphogenesis (15, 16). These large G proteins all appear to have key functions related to mitochondrial fission. As overexpression of mOPA1 in COS-7 cells resulted in the fragmentation of mitochondria (Fig. 4), it seems likely that mOPA1 might affect the mitochondrial morphology in mammalian cells, although its function at a physiological expression level is not yet clear.

We introduced mutations in the GTP-binding motif of mOPA1 which are analogous to the mutants known to be constitutively active or negative in other GTP-binding proteins (25–27). As it is informative to determine biochemically if the GTP binding activity is actually changed, we tried purification of the His-tagged recombinant mOPA1 protein of the mutants expressed in Escherichia coli. However, the recombinant mOPA1 protein could not be solubilized unless using strong detergents, and we could not carry out the biochemical analyses concerning GTP bindings and GTPase activities (data not shown).
In transfected COS-7 cells, the expressed mutant proteins in the GTP-binding motif were localized at the mitochondria, where they had a marked effect on their distribution and morphology (Fig. 6). In particular, the Gln\textsuperscript{297} mutants (Q297V, -G, -F, -L, or -W) caused to various extents an accumulation of fragmented vesicular mitochondria near the nucleus (Fig. 6, B and F). Although the biochemical nature of these mutations are uncharacterized, it is fair to conclude that the Gln\textsuperscript{297} mutation affecting the GTPase activity somehow causes the mitochondrial accumulation in the cells. It is well known that many proteins, including microtubules, modulate the distribution of mitochondria within cells (32). We therefore hypothesized that, as with mitochondria, mOPA1 mutants might disrupt the structures of microtubules, and \alpha-tubulin labeling revealed microtubules to be unaffected by expression of mOPA1 or its mutants (data not shown). Therefore, the true cause of mitochondrial accumulation in cells expressing the Gln\textsuperscript{297} mutants remains to be determined.

Although all our data concerning the effect of mOPA1 on mitochondrial morphology were obtained using the overexpressing transfected cells, there are some evidences supporting that the phenomenon is not simply due to overexpression of mOPA1. 1) mOPA1 protein localized at mitochondria in a small vesicular pattern as early as 9 h after transfection, when the protein just started to be detected and when the expression level was not in excess (data not shown). 2) The small vesicular pattern of mitochondrial characteristic of cells expressing WT mOPA1 could not be observed in transfected cells with K301A or T302N mutants that are expected to be negative mutants, although the mutant proteins localized at mitochondria (Fig. 5, C and D). 3) The mitochondria retained their normal tubular morphology in cells transfected with cDNA encoding a fusion protein of EGFP with 60- or 90-amino acid fragments from the N terminus of mOPA1, although it is overexpressed in mitochondria (Fig. 5, D and E). 4) The C-terminal deletion mutant of mOPA1 did not change mitochondrial morphology from a tubular to a vesicular pattern (Fig. 6G), although it localized in mitochondria (Fig. 6H).

It remains, however, to be determined what the function of mOPA1 is in cells expressing mOPA1 at a physiological level. **Comparison of the Distributions of mOPA1 in Brain Sections and in Primary Cell Culture—**In brain sections, mOPA1 appeared to be expressed in both the somas and dendrites of neuronal cells (Fig. 8). In primary cultures of dissociated cerebellar cells, however, the dendritic labeling was quite faint (Fig. 9, A and B), perhaps indicating that environmental factors affect the expression of mOPA1 in these cells. We also observed intense mOPA1 labeling in GFAP-positive astrocytes (Fig. 9C), and from their spine shape, some of the GFAP-positive cells were to be Bergmann glia. The intense expression seen in cultured astrocytes was not detected by in situ hybridization or immunohistochemical analysis (Figs. 7 and 8), suggesting that expression of mOPA1 was up-regulated in glial cells under our culture conditions.

When mOPA1-positive cells were also labeled with anti-cytochrome c, the expression patterns sometimes did not overlap very well (Fig. 9, E and F): although mOPA1 was clearly distributed in a vesicular pattern, cytochrome c labeling some-times exhibited the tubular pattern characteristic of normal mitochondrial morphology. In some instances, mOPA1 could be observed at the tip of a cytochrome c-positive tubular structure, which is consistent with a role in mitochondrial fission and/or fusion. The presence of vesicular mOPA1 labeling that is cytochrome c-negative could indicate that mOPA1 also exists in other organelles or that the fragmented mitochondria contain little cytochrome c. Additional studies comparing the distributions of mOPA1 and those of mitochondrial markers other than cytochrome c should clarify this point.

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Primary Structure of a Dynamin-related Mouse Mitochondrial GTPase and Its Distribution in Brain, Subcellular Localization, and Effect on Mitochondrial Morphology

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