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The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space

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Abstract **Mutations in the *OPA1* gene are associated with autosomal dominant optic atrophy. *OPA1* encodes a dynamin-related protein orthologous to Msp1 of *Schizosaccharomyces pombe* and Mgm1p of *Saccharomyces cerevisiae*, both involved in mitochondrial morphology and genome maintenance. We present immuno-fluorescence and biochemical evidences showing that OPA1 resides in the mitochondria where it is imported through its highly basic amino-terminal extension. Proteolysis experiments indicate that OPA1 is present in the inter-membrane space and electron microscopy further localizes it close to the cristae. The strong association of OPA1 with membranes suggests its anchoring to the inner membrane.**

Key words: Mitochondrion; Dynamin; Morphology; Membrane; Optic atrophy

1. Introduction

Conventional dynamins are large GTPases involved in vesicular traffic and endocytosis [1]. Different subfamilies of dynamin-like proteins share a highly conserved GTPase domain and take part in a variety of cellular processes including antiviral activity, plant cell plate formation and chloroplast biogenesis or mitochondrial morphology [1].

Two subfamilies of dynamin-like proteins have recently been linked to mitochondrial morphology and distribution. One comprises Dnm1p in *Saccharomyces cerevisiae* and its orthologues Drp1 in *Caenorhabditis elegans* and DLP1/DRP1 in mammals. These proteins punctuate constriction sites of the mitochondrial network where they promote fission of the outer membrane (OM) [2–5]. The morphology of this membrane is regulated by fission forces driven by Dnm1p and antagonistic fusion events controlled by Fzo1p or mitofusin, a large GTPase unrelated to dynamins [6–10]. A second subfamily of mitochondrial dynamin-like proteins includes Mgm1p in *S. cerevisiae* [11–14] and Msp1 in *Schizosaccharomyces pombe* [15,16] which were shown to be involved in the maintenance of the mitochondrial genome and network, possibly by modulating inner membrane (IM) dynamics.

We have recently identified OPA1 [17,18], the human orthologue of Msp1/Mgm1p, and found that mutations in the *OPA1* gene were associated with the most frequent form of autosomal dominant optic atrophy (ADOA, MIM165500), which features a progressive loss of retinal ganglion cells that often lead to legal blindness [19,20]. By analogy with Msp1/Mgm1p functions and since the mitochondrial network of monocytes from ADOA patients appears abnormally condensed [17], this may represent the first case of a mitochondrial disease caused by alteration of the mitochondrial network. A controversy persists on the localization of these proteins. Msp1 has been reported to be anchored to the matrix side of the IM [16], whereas Mgm1p has been shown to be anchored to the cytoplasmic face of the OM [13], or peripheral to the IM exposed to the inter-membrane space (IMS) [14]. To gain insights into the pathophysiology of ADOA, we address here the issue of OPA1 localization.

2. Materials and methods

2.1. Recombinant DNA technologies

For expression as green fluorescent protein (GFP) fusion proteins, OPA1 fragments corresponding to full length protein (1–960) or to deleted forms (1–123 and 125–960), were cloned into pEGFP-N3 (Clontech) yielding OPA1-GFP, mtsOPA1-GFP and ΔmtsOPA1-GFP, respectively. For in vitro import, OPA1 (1–123) was fused at the NH_2-terminus of dihydrofolate reductase (DHFR) into pSP65 (Promega) yielding mtsOPA1-DHFR.

2.2. Antibodies

OPA1 (261–904) produced from the pET15-b (Novagen) *Escherichia coli* expression vector was extracted from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to immunize rabbits (Eurogentec). Anti-OPA1 antibodies were affinity-purified from crude serum by incubation on strips of nitrocellulose containing the OPA1 (261–904). Antisera to human Tim13 and to rat UCP1 were provided respectively by C. Koehler (UCLA, Los Angeles, CA, USA) and L. Casteilla (UMR5018, Toulouse, France). Commercial antibodies were from the sources indicated: anti-HSP60 (LK2, Sigma), anti-FGF (SC79, Santa Cruz Biotechnology), anti-cytochrome *c* (7H8.2C12, Zymed Laboratories), anti-Bcl-2 (clone 124, Roche Diagnostics), anti-cytochrome *b* and Alexa-594 anti-rabbit IgG and Alexa-488 anti-mouse IgG (Molecular Probes), anti-rabbit IgG-HRP and anti-

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Abbreviations: mts, mitochondrial targeting signal; ADOA, autosomal dominant optic atrophy type 1; DHFR, dihydrofolate reductase; IM, inner membrane; OM, outer membrane; IMS, inter-membrane space; GFP, green fluorescent protein

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mouse IgG-HRP (New England Biolabs) and gold-conjugated goat anti-rabbit IgG (British BioCell).

2.3. Culture, transfection and fluorescence microscopy

HeLa and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), and transfected with ExGen-500 (Euromedex). For fluorescence microscopy, cells were fixed (30 min, 4°C) in phosphate-buffered saline (PBS), 3.7% paraformaldehyde and permeabilized in 100% methanol (10 min, −20°C) and in PBS, 0.25% Triton X-100 (5 min, room temperature). Immunolabeling was in PBS, 1% bovine serum albumin (BSA), using antibodies as indicated in the figure legends, and Alexa-594 anti-rabbit IgG and Alexa-488 anti-mouse IgG (1/500).

2.4. Cell fractionation

Cells were suspended in mitochondrial buffer (MB, 210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES pH 7.5) supplemented with a cocktail of protease inhibitors (Boehringer) and broken by 15–20 strokes of a tight-fitting Dounce homogenizer. The suspension was centrifuged (800×*g*, 4°C, 10 min) and the supernatant spun (15 000×*g*, 4°C, 15 min) to separate the mitochondrial pellet from the cytosol *plus* light membrane fraction.

Tissues were collected, minced and trimmed for contaminating tissues in ice-cold MB. They were blended at maximal speed in a glass-Teflon Potter–Elvenjehm homogenizer. The homogenates were decanted for 5 min and mitochondrial fractions were obtained as above.

To assess membrane association, mitochondria in hypo-osmotic buffer (MB with 1/10 concentrations of mannitol and sucrose) were fragilized by three freeze-thaw cycles and sonicated three times for 15 s on ice. Residual mitochondria were removed (12 000×*g*, 4°C, 10 min) and the supernatant made either 1 M NaCl or 0.1 M Na_2CO_3, pH 11 and incubated for 30 min on ice with frequent vortexing. The solution was centrifuged (150 000×*g*, 4°C, 45 min) to separate the supernatant from the membrane pellet.

To determine the sub-compartmentalization, mitochondria were suspended in MB containing proteinase K and the indicated concentrations of digitonin or Triton X-100. After 30 min on ice, proteolysis was terminated by adding 5 mM PMSF and Laemli's sample buffer, and heating at 90°C for 10 min.

Samples (100–200 μg of mitochondrial proteins) were analyzed by SDS–PAGE and Western blotting using the antibodies indicated and anti-rabbit IgG-HRP or anti-mouse IgG-HRP (1/5000).

2.5. In vitro import of OPA1 into isolated mitochondria

The mtsOPA1-DHFR and mtsATP synthase su9-DHFR (gift from G. Schatz, Basel, Switzerland, [21]) were translated in vitro (TNT® SP6 Quick Coupled Transcription/Translation kit, Promega) in the presence of [^{35}S]methionine (Amersham). Import of the translated products into isolated mitochondria (400 μg of protein), inhibition by valinomycin (1 μg/ml) and sensitivity of the imported peptides to trypsin digestion (1 mg/ml) were performed as described elsewhere [16].

2.6. Immuno-electron microscopy

Rat brown adipose tissue was fixed for 2 h with 4% paraformaldehyde in PBS, pH 7.2, incubated in 1% NH_4Cl for 1 h and dehydrated in increasing concentrations of ethanol. Ethanol was progressively exchanged with LRWhite (Euromedex) and polymerization lasted for 2 days at 46°C. Thin sections adsorbed on to nickel grids were labeled in PBS, 0.2% Tween-20, 2% BSA using affinity-purified anti-OPA1 antibodies (1/5), or anti-UCP1 serum (1/20), and 10 nm gold particles conjugated to anti-rabbit IgG antibodies (1/100). Sections were stained with 1% uranyl acetate and 0.3% lead citrate, and imaged in a JEOL-1200 EX electron microscope at 80 kV.

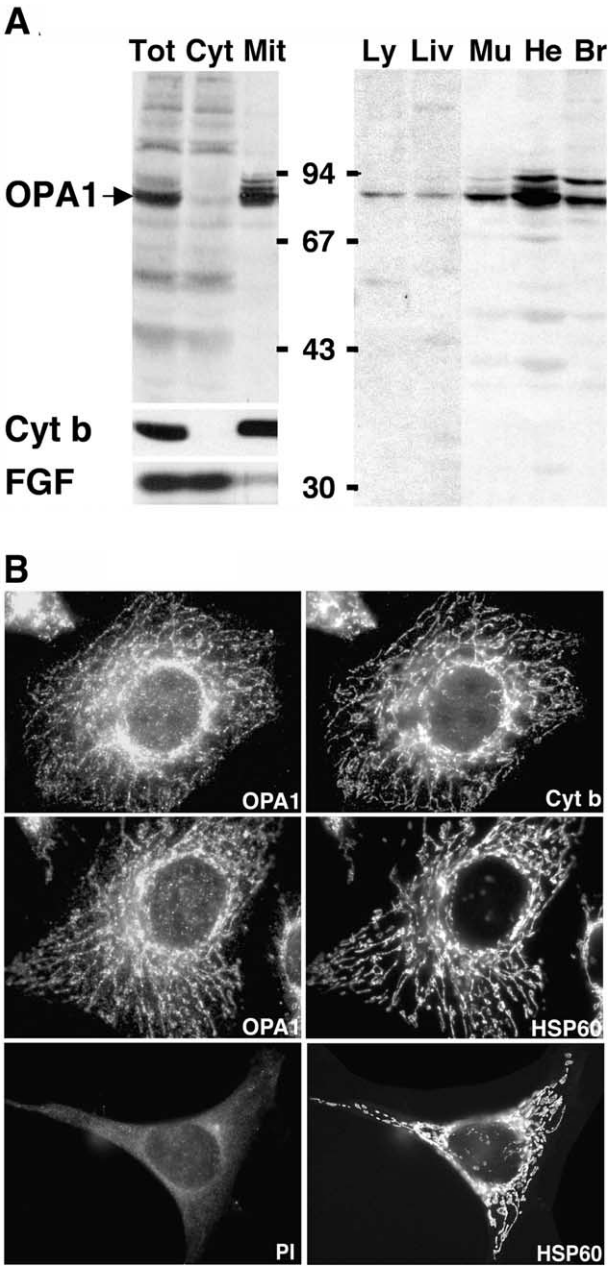


Fig. 1. OPA1 is localized in the mitochondria. A: Identical cell-equivalent amounts of HeLa cells post-nuclear supernatant (Tot, 400 μg of protein) separated into cytosol *plus* light membranes (Cyt) and mitochondrial (Mit) fractions, and mitochondria (100 μg of protein) from human lymphocytes (Ly) and rat liver (Liv), skeletal muscle (Mu), heart (He) and brain (Br) were analyzed by Western blots using antibodies against OPA1 (1/300), mitochondrial cytochrome *b* (Cyt *b*, 1/400) and cytosolic fibroblast growth factor (FGF, 1/1000). Positions of molecular weight markers (kDa) are shown between the two panels. B: Fluorescence micrographs of fixed HeLa cells treated with antibodies against OPA1 (1/150), or the corresponding pre-immune serum (PI, 1/150), or against mitochondrial cytochrome *b* (Cyt *b*, 1/150) or HSP60 (1/150).

3. Results

3.1. OPA1 is localized in the mitochondrial network

The cellular localization of OPA1 was studied by raising antibodies against recombinant OPA1 which were used to characterize fractions of HeLa cells by Western blots (Fig. 1A). Cell fractionation was controlled using antibodies against the cytosolic fibroblast growth factor and the mitochondrial cytochrome *b*. OPA1 was present in equal amounts in the total and mitochondrial fractions but absent from the fraction containing the cytosol *plus* light membranes. The estimated molecular weight of OPA1 (86 kDa) was somewhat lower than the 102 kDa predicted for the mature OPA1 product (see below). Similar results (not shown) were obtained using

other cell lines (NIH-3T3 and MCF7) and/or another immune serum against an OPA1 peptide (571–585). The 86 kDa form also predominated in human lymphocytes, and in rat liver, muscle and heart, but a 92 kDa form was almost equally represented in brain (Fig. 1A).

In immuno-fluorescence experiments on HeLa cells (Fig. 1B), filamentous elongated structures distributed throughout the cytoplasm were similarly decorated by antibodies against OPA1 or the mitochondrial protein HSP60 or cytochrome *b*, showing colocalization of these proteins. No specific fluorescence was observed using the pre-immune serum.

3.2. The basic-rich N-terminal part of OPA1 is a bona fide mitochondrial targeting signal (mts)

The highly basic amino-terminus of OPA1 presumably constitutes an mts. This was checked by studying the cellular distribution of GFP chimeric proteins comprising various OPA1 fragments, after transient expression in NIH-3T3 cells (Fig. 2A). Whereas fluorescence associated with pEGFP was concentrated in the nucleus and fainter in the cytoplasm, OPA1-GFP labeled a network of cytoplasmic tubules superimposed to that visualized using antibodies to HSP60. The basic-rich 120 amino-terminal residues of OPA1 were sufficient to address mtsOPA1-GFP to the mitochondrial network which appeared in this case as dots (the different morphology of the mitochondrial network between these two situations is addressed in Section 4). In contrast, the OPA1Δmts-GFP chimera, which lacked this amino-terminal extension, displayed diffuse cytoplasmic fluorescence.

The mts property of the OPA1 NH_2-terminal region was further established by fusing it with DHFR, a cytoplasmic enzyme, and assaying the localization of the chimera upon import into isolated mitochondria (Fig. 2B, top). The in vitro translated precursor of mtsOPA1-DHFR (p_1) was associated with mitochondria, and yielded two maturation products (m_1 and m_1') only when the membrane potential was not disrupted by valinomycin. OPA1 was addressed inside mitochondria since the imported peptides were resistant to trypsin digestion. This assay was validated using the mts of IM ATP synthase subunit 9 fused to DHFR. In the presence of an intact membrane potential, the precursor (p_2) yielded a maturation product (m_2) which was also resistant to proteolysis. In the amino-terminal part of OPA1 (Fig. 2B, bottom), the cleavage region predicted from the apparent molecular weights of the maturation peptides indeed displayed a canonical sequence for a two-step cleavage site by matrix processing peptidases [22].

3.3. OPA1 resides in the IMS tightly attached to the membrane

The submitochondrial localization of endogenous OPA1 was analyzed by electron microscopy using antibodies to OPA1 and rat brown adipocytes that showed large mitochondria with well-defined and highly structured cristae. Most gold grains were found within the mitochondria ($89 \pm 5\%$, $n = 736$), close to the cristae and no labeling occurred on the external border delimited by the OM. Labeling of the cristae by anti-OPA1 was confirmed by the similar pattern of gold grain distribution obtained using antibodies to UCP1 (Fig. 3A), a cristae marker [23].

Proteolysis experiments on intact mitochondria or after progressive solubilization of the OM by digitonin (Fig. 3B), indicated that OPA1 was digested together with the IMS marker Tim13 whereas Bcl-2, an OM protein facing the cytosol, was readily digested on intact mitochondria, and cytochrome *b*, a matricial IM marker, required solubilization of the IM by Triton X-100. A similar result was obtained by

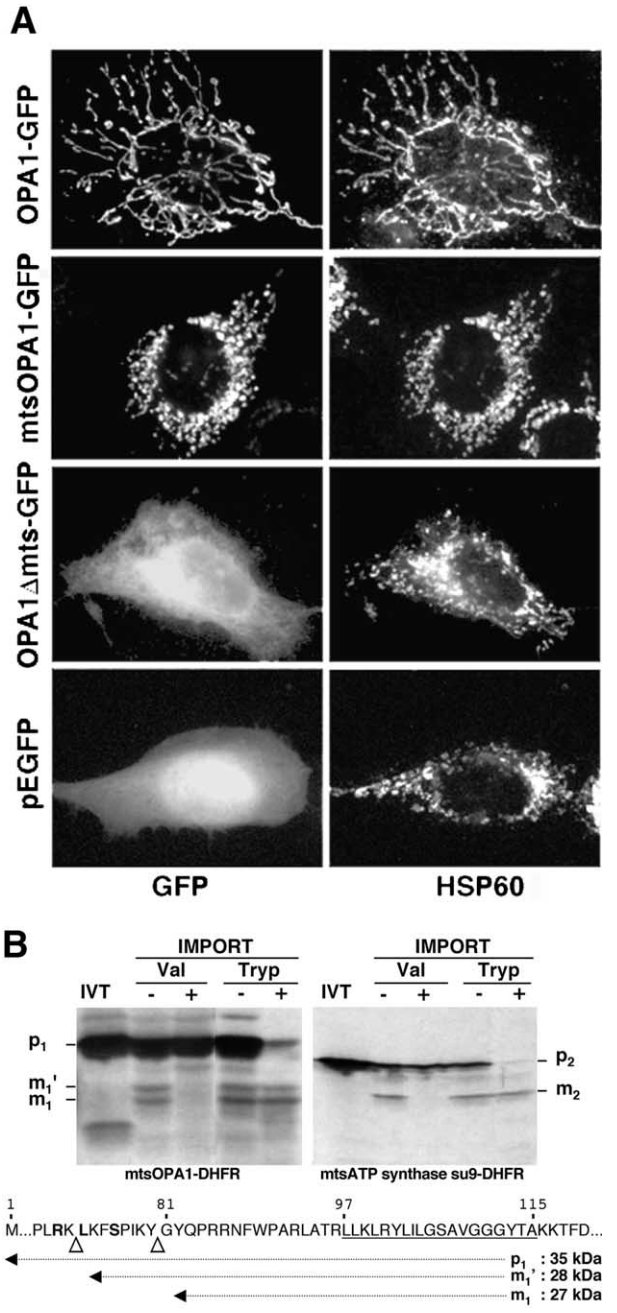
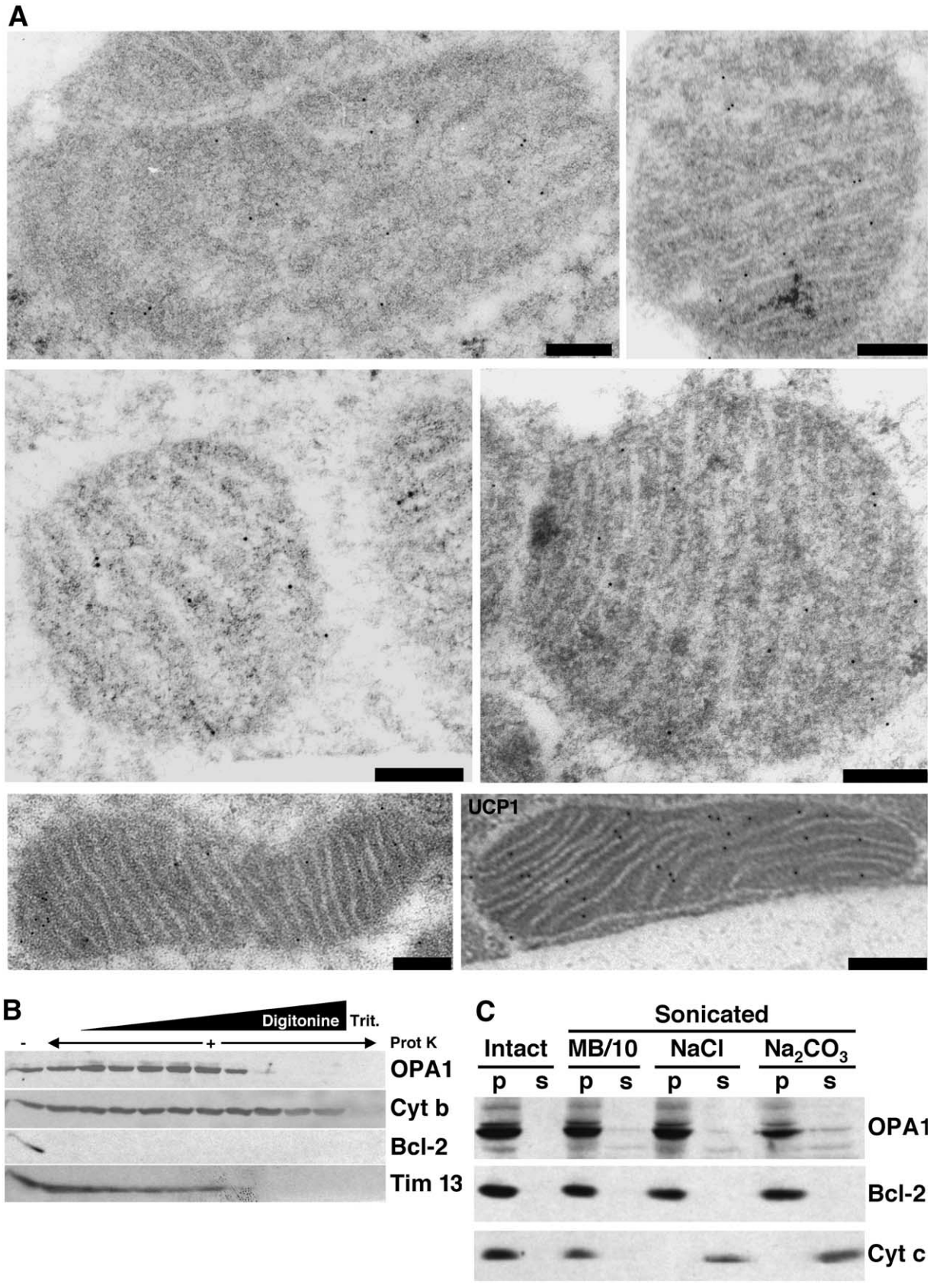


Fig. 2. The basic-rich 120 amino-terminal residues of OPA1 constitute a bona fide mts. A: NIH-3T3 cells transiently expressing the plasmids indicated on the left side were visualized by direct GFP fluorescence (left panels) and by staining with anti-HSP60 (1/150) (right panels). B: In vitro translation products (IVT) of plasmids mtsOPA1-DHFR and mtsATP synthase su9-DHFR were imported into mitochondria in the absence (−) or presence (+) of valinomycin (Val) and washed mitochondria were digested (+) or not (−) with trypsin (Tryp). The NH_2-terminal sequence of OPA1 is displayed at the bottom, showing the consensus motif **RXΦXXS/T** (**Φ** is any of the hydrophobic residues F, L, V or I) for two-step cleavage sites (triangles) of mitochondrial precursors and the putative transmembrane domain (underlined). The migration and estimated molecular weights of the OPA1-DHFR precursor (p_1) and intermediate (m_1') and mature (m_1) import products are indicated.



osmotic swelling of mitochondria: OPA1 was digested to the same extent as Tim13 (respectively 78 and 76%), while most of the matrix protein Hsp60 (78%) was protected (data not shown).

The association of OPA1 to membranes was studied on isolated mitochondria after incubation with either 1 M NaCl or 0.1 M Na_2CO_3, pH 11 (Fig. 3C). Whatever the treatment used, OPA1 remained in the membrane pellets as did the

Fig. 3. OPA1 is a membrane-associated protein of the IMS. A: Electron microscope micrographs of thin sections from rat brown adipocytes treated with anti-OPA1 (all except bottom right) or anti-UCP1, and gold-conjugated secondary antibodies. Bars, 200 nm. B: Mitochondria were incubated in the absence (−) or presence (+) of proteinase K (50 μg/ml) without (lanes 1 and 2) or with increasing concentrations of digitonin (0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0, 3.0 and 4.0 mg/ml) or with 2% Triton X-100 (Trit.). C: Mitochondria (intact or sonicated) were incubated in hypo-osmotic MB (MB/10) alone or supplemented with 1 M NaCl, or 0.1 M Na_2CO_3, pH 11, and centrifuged to separate the membrane pellets (p) from soluble protein supernatants (s). B,C: Samples were analyzed by Western blotting using anti-OPA1 (1/300), anti-Bcl-2 (1/100), anti-Cyt *c* (1/200), anti-Cyt *b* (1/400) and anti-Tim13 (1/1000) antibodies.
←

integral membrane protein Bcl-2 [24], whereas cytochrome *c*, peripherally associated to the IM [25], was readily extracted by either treatment.

4. Discussion

OPA1 is the first dynamin-related protein so far identified as responsible for a human disease. As an approach to its function and given the controversy on the presence of orthologous yeast proteins in the different mitochondrial compartments, it was of interest to clarify its submitochondrial localization. We have shown here by cellular fractionation and immuno-fluorescence studies that OPA1 is localized in the mitochondrial network. As expected from the distribution of its mRNA [17,18], OPA1 was present in each mammalian tissue and cell line examined. The mitochondrial nature of OPA1 correlated with structural features of its N-terminal region [26,27], which was shown to contain a bona fide mts within the first 120 residues. Electron microscopy further localized OPA1 within the mitochondria with no labeling on the periphery, exactly as observed for the IM-associated cristae marker UCP1 [23]. This suggested that OPA1 is associated to cristae, facing either the matrix or the IMS volume contained within cristae cisternae [28]. Since OPA1 is accessible to protease, as is the IMS marker Tim13, and strongly associated with membranes, we propose that it is anchored to the IM through its N-terminal transmembrane segment, the bulk of the protein facing the intracristae IMS. This contrasts with data from the literature suggesting that Mgm1p is cytoplasmic, anchored to the OM [13], and that Msp1 is attached to the IM, facing the matrix [16]. In these reports however, protease access to IMS markers, either before [13] or after [16] osmotic rupture of the OM, was not assessed. Nevertheless, in a recent study [14], the Mgm1p localization indeed agrees with that of OPA1, in the IM facing the IMS.

OPA1 was detected as multiple molecular weight forms among which the 86 kDa one predominated. This molecular weight is significantly lower than the 102 kDa anticipated from the use of the two-step cleavage site proposed in the NH_2-terminus of the precursor. N-terminal maturation of OPA1 to yield the 86 kDa form would have necessitated the cleavage of about 220 amino acid residues, in a region devoid of recognition sites for mitochondrial processing peptidases, eliminating the apparently essential membrane anchor. C-terminal maturation is also unlikely since deletion of few residues from this region has been associated with ADOA [29]. Nor can alternative splicing explain this seemingly low molecular weight, since the smallest form identified would have a predicted molecular weight of 98 kDa [29]. The apparent 86 kDa molecular weight of OPA1 may rather result from post-translational modifications and/or from an aberrant 'conformational' migration.

Modifications of the mitochondrial morphology were observed in cells transfected by different OPA1 constructs. A punctuate structure, as seen in untransfected cells, was obtained with mtsOPA1-GFP while OPA1-GFP induced a filamentous network. As for Mgm1p in *S. cerevisiae* [14], ectopic expression of OPA1 may thus modify the fusion/fission equilibrium of the mitochondrial network. Given its likely localization in the intracristae IMS, the dynamin-related OPA1 could modulate the cristae dynamics. It may for example participate in cristae formation during increased respiration and/or energy production. OPA1 may also play a major role in mobilization of the highly sequestered content of cristae cisternae [28], as may be the case during apoptosis-induced cytochrome *c* release [30]. Extensive dysfunction of OPA1 on IM dynamics would ultimately lead to the more drastic alteration of the mitochondrial network as observed here.

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References

[1] Hinshaw, J.E. (2000) Annu. Rev. Cell Dev. Biol. 16, 483–519.
[2] Bleazard, W., McCaffery, J.M., King, E.J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J. and Shaw, J.M. (1999) Nat. Cell Biol. 1, 298–304.
[3] Labrousse, A.M., Zappaterra, M.D. and Rube, D.A. (1999) Mol. Cell 4, 815–826.
[4] Smirnova, E., Griparic, L., Shurland, D.L. and van Der Bliek, A.M. (2001) Mol. Biol. Cell 12, 2245–2256.
[5] Yoon, Y., Pitts, K.R. and McNiven, M.A. (2001) Mol. Biol. Cell 12, 2894–2905.
[6] Hales, K.G. and Fuller, M.T. (1997) Cell 90, 121–129.
[7] Rapaport, D., Brunner, M., Neupert, W. and Westermann, B. (1998) J. Biol. Chem. 273, 20150–20155.
[8] Sesaki, H. and Jensen, R.E. (1999) J. Cell Biol. 147, 699–706.
[9] Fritz, S., Rapaport, D., Klanner, E., Neupert, W. and Westermann, B. (2001) J. Cell Biol. 152, 683–692.
[10] Santel, A. and Fuller, M.T. (2001) J. Cell Sci. 114, 867–874.
[11] Guan, K., Farh, L., Marshall, T.K. and Deschenes, R.J. (1993) Curr. Genet. 24, 141–148.
[12] Jones, B.A. and Fangman, W.L. (1992) Genes Dev. 6, 380–389.
[13] Shepard, K.A. and Yaffe, M.P. (1999) J. Cell Biol. 144, 711–720.
[14] Wong, E.D., Wagner, J.A., Gorsich, S.W., McCaffery, J.M., Shaw, J.M. and Nunnari, J. (2000) J. Cell Biol. 151, 341–352.
[15] Pelloquin, L., Belenguer, P., Menon, Y. and Ducommun, B. (1998) Biochem. Biophys. Res. Commun. 251, 720–726.
[16] Pelloquin, L., Belenguer, P., Menon, Y., Gas, N. and Ducommun, B. (1999) J. Cell Sci. 112, 4151–4161.
[17] Delettre, C. et al. (2000) Nat. Genet. 26, 207–210.
[18] Alexander, C. et al. (2000) Nat. Genet. 26, 211–215.
[19] Johnston, R.L., Seller, M.J., Behnam, J.T., Burdon, M.A. and Spalton, D.J. (1999) Ophthalmology 106, 123–128.
[20] Votruba, M., Moore, A.T. and Bhattacharya, S.S. (1998) J. Med. Genet. 35, 793–800.

[21] Pfanner, N., Muller, H.K., Harmey, M.A. and Neupert, W. (1987) EMBO J. 6, 3449–3454.
[22] Hendrick, J.H.P. and Rosemberg, L. (1989) Proc. Natl. Acad. Sci. USA 86, 4056–4060.
[23] Cinti, S., Cancello, R., Zingaretti, M.C., Ceresi, E., De Matteis, R., Giordano, A., Himms-Hagen, J. and Ricquier, D. (2002) J. Histochem. Cytochem. 50, 21–31.
[24] Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J. and Shore, G.C. (1993) J. Biol. Chem. 268, 25265–25268.
[25] Subramanian, M., Jutila, A. and Kinnunen, P.K. (1998) Biochemistry 37, 1394–1402.
[26] Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917.
[27] Pfanner, N. (2000) Curr. Biol. 10, R412–415.
[28] Frey, T.G. and Mannella, C.A. (2000) Trends Biochem. Sci. 25, 319–324.
[29] Delettre, C. et al. (2001) Hum. Genet. 109, 584–591.
[30] Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S.A., Mannella, C.A. and Korsmeyer, S.J. (2002) Dev. Cell 2, 55–67.