Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo

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Summary

Two human Fzo-homologs, mitofusins Mfn1 and Mfn2, are shown by RT-PCR and western blot to be ubiquitous mitochondrial proteins. Protease digestion experiments reveal that Mfn2 is an outer membrane protein with N-terminal and C-terminal domains exposed towards the cytosol. The transmembrane and C-terminal domains of Mfn2 (Mfn2-TMCT) are targeted to mitochondria and deletion of these domains leads to the cytosolic localization of truncated Mfn2 (Mfn2-NT). Mfn2 is targeted to the endoplasmic reticulum or to mitochondria when the C-terminal domain is replaced by short stretches of neutral/hydrophobic (Mfn2-IYFFT) or polar/basic (Mfn2-RRD) amino acids. The coiled-coil domains of Mfn2, upstream and downstream of the transmembrane domain, are also important for mitochondrial targeting: Mfn2-mutants deleted of any of its coiled-coil domains are only partially targeted to mitochondria and significant protein amounts remain cytosolic. We show that these coiled-coil domains interact with each other: mistargeted Mfn2-NT or Mfn2-IYFFT localize to mitochondria if co-expressed with Mfn2-TMCT. This relocalization is abolished when the coiled-coil domain is deleted in any of the co-transfected molecules. We also found that Mfn2 can cluster active mitochondria in the perinuclear region independently of the cytoskeleton, bring mitochondrial membranes into close contact and modify mitochondrial structure, without disturbing the integrity of the inner and outer membrane.

Key words: Transmembrane protein, Protein targeting, GTPase, Mitochondria, Membrane fusion

Introduction

Mitochondria represent a tubular and branched membrane system whose morphology and intracellular distribution vary significantly between tissues and cell types and are further influenced by metabolic conditions and developmental stage (Bereiter-Hahn and Voth, 1994; Smiley et al., 1991; Tzagoloff, 1982). The observation that mitochondria are dynamic and undergo fusion and fission reactions (Nunnari et al., 1997; Rizzuto et al., 1998) has led to the notion that the mitochondrial network represents a single cellular organelle. This should enable complementation between mitochondrial DNA molecules carrying different deleterious mutations, but the available studies are contradictory concerning the frequency and/or efficiency of intermitochondrial complementation (Enriquez et al., 2000; Nakada et al., 2001; Ono et al., 2001). For this and for other reasons, the extent and frequency of mitochondrial fusion in mammals remains a matter of debate.

In contrast to other intracellular membranes, little is known about the factors governing mitochondrial membrane dynamics. In the budding yeast, mitochondrial fission is mediated by a dynamin-related protein (Dnm1p) together with other recently discovered proteins (reviewed by Yoon and McNiven, 2001). Another dynamin-related protein, Mgm1p, is required for the maintenance of fusion competent mitochondria (Wong et al., 2000). The fission activity of Dnm1p is antagonized by that of a mitochondrial transmembrane protein, the predicted GTPase Fzo (Bleazard et al., 1999; Sesaki and Jensen, 1999). Yeast Fzo is constitutively expressed, localizes to the mitochondrial outer membrane and is essential for mitochondrial fusion and biogenesis (Hermann et al., 1998; Rapaport et al., 1998). The mammalian homologs of the dynamin-related proteins Dnm1p (Drp1) and Mgm1p (OPA1) are ubiquitously expressed and contribute to mitochondrial distribution and function (Delettre et al., 2000; Smirnova et al., 1998). The expression of the *Drosophila* homolog of Fzo is restricted to spermatids during the short time period where mitochondrial fusion occurs and its absence leads to male sterility (Hales and Fuller, 1997). Two human Fzo homologs, called mitofusins Mfn1 and Mfn2, have been recently identified. Their expression pattern remains unknown. They appear to be involved in fusion, as they can antagonize the fission activity of human Drp1, like Fzo does in yeast (Santel and Fuller, 2001).

Sequence analysis of the relatively large Fzo/Mfn proteins (90-100 kDa) reveals a multidomain structure containing a GTP-binding and a transmembrane domain, as well as two predicted coiled-coil regions upstream and downstream of the transmembrane domain. The transmembrane domain of human Mfn2 is necessary for mitochondrial targeting (Santel and Fuller, 2001) and yeast Fzo is imported into the outer membrane in a receptor-dependent manner (Rapaport et al., 1998). However, the mechanisms and motifs responsible for

mitochondrial targeting and membrane insertion of Fzo/Mfn remain unknown. Mutations of conserved GTP-binding motifs abolish the capacity of yeast Fzo to mediate mitochondrial fusion (Hermann et al., 1998) and alteration of a small domain exposed to the intermembrane space interfere with its capacity to mediate fusion (Fritz et al., 2001). However, the precise function of Fzo in the fusion process remains unknown.

In this study we show that mitofusins Mfn1 and Mfn2 are ubiquitous mitochondrial proteins. We determine the membrane topology of Mfn2 and the role of its C-terminal domain in membrane targeting. Furthermore, we show that the coiled-coil domains of Mfn2 interact with each other, and that these interactions are important for mitochondrial targeting. Finally, we show that Mfn2 can modify mitochondrial morphology and bring mitochondrial membranes into close contact.

Materials and Methods

Sequence analysis

Transmembrane domains, coiled-coil regions and mitochondrial presequences were predicted with TMPRED (Hofmann and Stoffel, 1993), COILS (Lupas et al., 1991) and MitoProt (Claros and Vincens, 1996), respectively. Protein alignments and dendograms were performed with CLUSTAL W (Thompson et al., 1994) and Multalin (Corpet, 1988).

Cloning and mutagenesis

Total RNAs were reverse transcribed with Superscript II (Gibco/BRL) using oligo dT as a primer, and the chosen cDNA-fragments were amplified by PCR. For semi-quantitative analysis of RT-PCR, gels were stained with ethidium bromide, photographed with a CCD camera and stain-intensity of the bands was quantified with NIH-Image. The open reading frame of human Mfn1 (deduced from overlapping cDNA sequences AK000700 and U95822) was amplified by RT-PCR from total RNA of human skin fibroblasts using Expand High Fidelity polymerase (Roche Molecular Biochemicals). The cDNA encoding human Mfn2 [KIAA protein 0214 (Nagase et al., 1996)] was kindly provided by Kazusa DNA Research Institute. Cloning was performed according to standard procedures and all PCR products were verified by sequencing. The cDNAs encoding two fragments of Mfn2, NG (residues 1-405) and CT (residues 648-757), were amplified with primers appended with restriction sites and inserted between the *Xho*I and *Bam*HI sites of the pET15b vector (Novagen). A mammalian expression vector coding for a myc-epitope downstream of a Kozak sequence (pCB6-MYC) was generated by ligation of annealed primers encoding a Kozak sequence and a myc-epitope. Mfn1, Mfn2 and truncated Mfn2 molecules (MF2-NT, MF2-TMCT, MF2-ΔC2, MF2-IYFFT) were amplified with primers appended with restriction sites and the PCR products were inserted in pCB6 (Rojo et al., 2000) or pCB6-MYC. Individual residues of the GTP binding motifs of Mfn2 were mutagenized (K109A, S110N and R259L) with Quikchange (Stratagene). Mfn2 molecules devoid of the GTP-binding domain (MF2-ΔGB: residues 97-264 deleted) or of the first coiled-coil domain (MF2ΔC1: residues 390-439 deleted) were generated by separate amplification of upstream and downstream regions with primer couples containing a sequence overlap. These PCR products were linked by fusion PCR (Ho et al., 1989), and the final PCR products were cloned into PCB6-MYC. A GFP molecule targeted to the mitochondrial matrix (mtGFP) was constructed as described (Rizzuto et al., 1998) and cloned into pCB6.

Cell culture and microscopy

HeLa cells were maintained and transfected as described (Rojo et al., 2000). A permissive clone of the mouse myogenic cell line C2 [C27C4 (Pinset et al., 1988)] was maintained in DMEM (4.5 g Glc/l) supplemented with 20% fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were processed for fluorescence microscopy as described (Rojo et al., 1997). Cells were viewed using a Zeiss Axiophot microscope and images were recorded with a CCD camera or on Ilford HP5 Plus film. Micrographs were processed with Metaview and Adobe Photoshop. Confocal laser scanning microscopy and electron microscopy were performed as described (Bakker et al., 2000).

Reagents, proteins and antibodies

JC-1 was obtained from Molecular Probes, nocodazole from Calbiochem, and FITC-Phalloidin, cytochalasin B and carbonyl cyanide m-chlorophenyl hydrazone (cccp) were from Sigma. The stock solution of JC-1 (5 mg/ml in water) was kept at 4°C; all other stock solutions (10 mM nocodazole in DMSO, 0.2 mg/ml FITC-phalloidin in DMSO, 1 mg/ml cytochalasin B in ethanol, 20 mM cccp in DMSO) were kept at –20°C. His-tagged Mfn2-fragments NG or CT were expressed in *E. coli* strain C41 (DE3) (Miroux and Walker, 1996). Inclusion bodies containing NG and CT were solubilized in buffers containing 6 M urea and purified with Ni-NTA agarose (Qiagen). Antisera against a mixture of NG and CT (1:1 by mass) were generated in rabbits. For affinity purification of antibodies, NG and CT were refolded on Ni-NTA columns with a gradient of decreasing urea concentration. Refolded NG and CT were coupled separately to Affi-Gel 10 (Bio-Rad) and antibodies were purified as described (Rojo et al., 1997). Protein analysis (protein determination, SDS-PAGE and western blot) was performed as described (Rojo et al., 1997). For western blot analysis, equal amounts of protein (20 μg) were loaded in each lane, and equal loading was confirmed by Ponceau protein staining of membranes. Antibodies specific for the following antigens and/or compartments were used: ATP/ADP carrier (Rojo and Wallimann, 1994), subunits of cytochrome c oxidase [COX2 and COX4 (Bakker et al., 2000)], calnexin (StressGen Biotechnologies Corporation), unknown Golgi-specific antigen [CTR433 (Jasmin et al., 1989)], α-tubulin (Amersham Pharmacia Biotech), Hsp60 (Sigma), cytochrome c (BD PharMingen) and myc-epitope [9E10 (Evan et al., 1985)].

Subcellular fractionation and protease digestion

Mitochondria from rat tissues were enriched as described (Rojo and Wallimann, 1994) except that the Percoll gradient step was omitted. Adherent culture cells were homogenized in Hepes-Sucrose-medium (HS) as described (Rojo et al., 1997). For sedimentation of nuclei, homogenates were centrifuged twice for 5 minutes at 200 ***g*** (Sigma tabletop centrifuge). To sediment mitochondria, the post-nuclear supernatant was centrifuged for 10 minutes at 5000 ***g*** (Sigma tabletop centrifuge). For separation of microsomes and cytosol, the post-mitochondrial supernatant was centrifuged for 1 hour at 100,000 ***g*** (Beckman TLA-45 rotor). Freshly isolated mitochondria (2 mg/ml in HS) were incubated with Proteinase K (10 μg/ml) at 0°C in the presence or absence of 0.5% Triton X-100. Digestions were stopped by the addition of an excess of HS containing 2 mM PMSF.

Results

Expression and localization of human mitofusins

The search for mammalian Fzo-homologs with BLAST (Altschul et al., 1997) led to the identification of sequences predicting the existence of two mammalian homologs (unigene clusters Hs.197877-Mm.27914 and Hs.3363-Mm.24578-Rn.8570). These Fzo-homologs were identical to the recently described mitofusins Mfn1 and Mfn2 (Santel and Fuller, 2001).

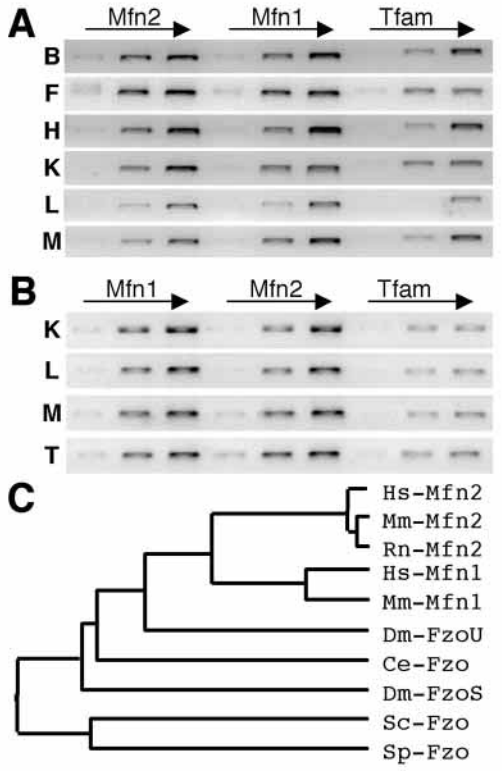

Fig. 1. Mammalian mitofusins are ubiquitously expressed. (A,B) RT-PCR of mitofusins (Mfn1, Mfn2) and of mitochondrial transcription factor A (Tfam) from human (A) and mouse (B) RNA. Shown are equal aliquots of the amplification reactions after 25, 30 and 35 cycles. Both mitofusins are ubiquitously expressed at similar levels. Tissues: B, brain; F, fibroblasts; H, heart; K, kidney; L, liver; M, muscle; T, Testis. (C) Dendogram of the Fzo-family. Mammalian homologs are called mitofusins (Mfn). Species: Ce, *C. elegans*; Dm, *D. melanogaster*; Hs, *H. sapiens*; Mm, *M. musculus*; Rn, *R. norvegicus*; Sc, *S. cerevisiae*; Sp, *S. pombe*. Accession numbers (p, protein; n, nucleotide): Ce-Fzo, p-AAC71095; Dm-FzoS, n-U95821; Dm-FzoU, p-AAF46162; Hs-Mfn1, n-AF329637; Hs-Mfn2, n-D86987; Mm-Mfn1, n-AK018181; Mm-Mfn2, n-AF384100; Rn-Mfn2, n-U41803; Sc-Fzo, n-Z36048; Sp-Fzo, p-CAA19004.

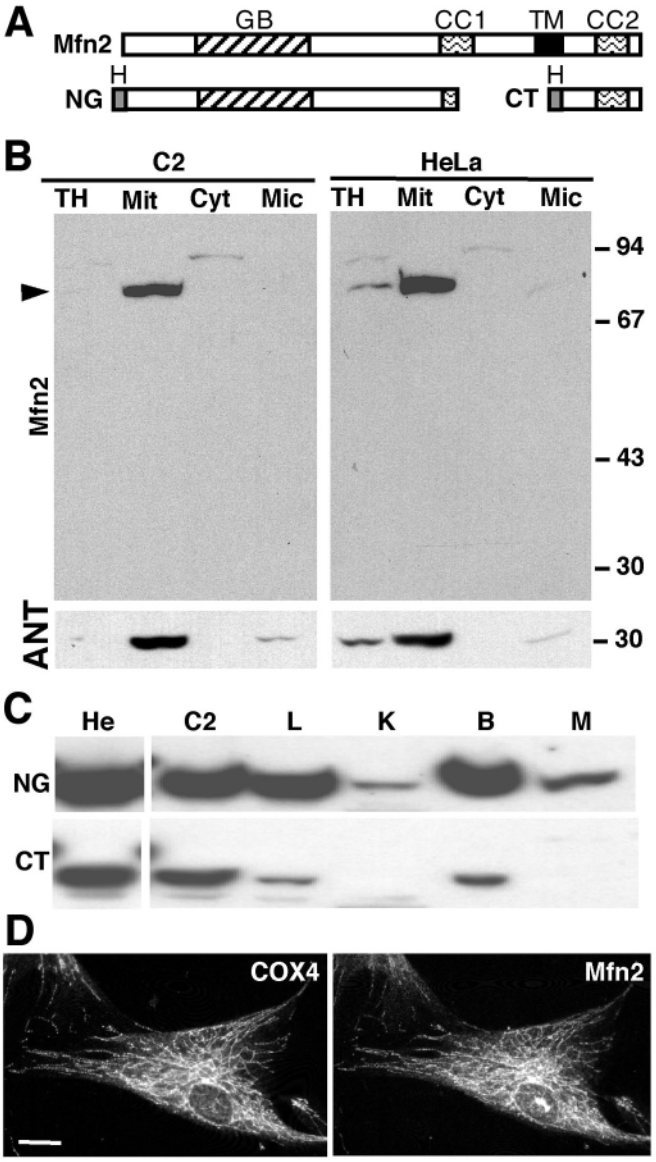

Fig. 2. Mitofusin 2 is a ubiquitous mitochondrial protein. (A) Domain organization of mitofusin 2 (Mfn2) and of Mfn2-fragments (NG, CT) used for antibody generation. GB, GTP-binding; CC1, CC2, coiled-coil; TM, transmembrane domain; H, 6His-tag. (B) Western blots of subcellular fractions from mouse C2 and human Hela cells: TH, total homogenate; Mit, mitochondria; Cyt, cytosol; Mic, microsomes. The Mfn2-specific antiserum decorates a polypeptide (arrowhead) that is highly enriched in mitochondria, and whose apparent molecular mass is similar to that predicted for Hs-Mfn2 (86.4 kDa). The distribution of the adenine nucleotide translocator (ANT) confirms mitochondrial enrichment. The position and size (in kDa) of molecular mass markers is indicated. (C) Western blot analysis of mitochondrial fractions from human HeLa (He) and mouse C2 cells (C2) and from rat liver (L), kidney (K), brain (B) and muscle (M) with affinity purified antibodies specific for Mfn2-fragments NG or CT. (D) Double immunofluorescence on human skin fibroblasts with antibodies against the mitochondrial marker COX4 and against an N-terminal fragment (NG) of Mfn2. Mfn2 and COX4 colocalize in mitochondria. Bar, 15 μm.

The open reading frame of Mfn1 was amplified from human RNA by RT-PCR. The cDNA encoding Mfn2 had been cloned previously (KIAA protein 0214, D86987). We investigated the expression of mammalian mitofusins by RT-PCR. As a control, we amplified the transcript of mitochondrial transcription factor A (Tfam), a ubiquitous protein that is essential for mitochondrial function (Parisi and Clayton, 1991). As shown in Fig. 1, both mitofusins are transcribed at similar levels in a variety of mammalian tissues, including testis. Semi-quantitative analysis of the PCR reactions reveal that Mfn1 and Mfn2 are expressed at similar levels (ratio Mfn2/Mfn1=0.91±0.22; n=24) and that their transcripts are more abundant than that of Tfam (ratio Mfn1/Tfam=1.97±0,45; n=21).

The ubiquitous co-expression of the two mammalian mitofusins differs from the restricted expression of *Drosophila* Fzo (Hales and Fuller, 1997). This discrepancy prompted us to search for other Fzo-homologs in DNA databases. We identified Fzo-homologs in the genomes of *C. elegans* and *S. pombe* (Fig. 1C, Ce-Fzo, Sp-Fzo) and a new Fzo-homolog of in the genome of *D. melanogaster* (Fig. 1C, Dm-FzoU). In addition, we found expressed sequence tags (ESTs) originating

from *C. elegans* and *D. melanogaster*. In *C. elegans*, all ESTs (*n*=18) were identical to the predicted protein Ce-Fzo. In *Drosophila*, all ESTs (*n*=10) were identical to the new Fzo homolog, Dm-FzoU, and originated from embryo, head and ovary libraries. This predicts the existence of two Fzo homologs in *Drosophila*: Dm-FzoS, whose expression is restricted to developing spermatids (Hales and Fuller, 1997) and Dm-FzoU, whose ESTs are found at different developmental stages and in various tissues. Interestingly, mammalian mitofusins are more similar to the putatively ubiquitous Dm-FzoU than to the spermatid-specific Dm-FzoS (Fig. 1C).

Mitofusins display, like all Fzo-homologs, a multi-domain structure that includes a GTP-binding domain, two coiled-coil regions and a predicted transmembrane domain (Fig. 2A, Mfn2). To investigate the localization of Mfn2, we generated a rabbit antiserum against Mfn2-fragments encoding N-terminal and C-terminal fragments of the protein (Fig. 2A, NG, CT). In western blots, this antiserum decorated a band with the expected apparent molecular mass of Mfn2 (86.4 kDa) that was highly enriched in mitochondrial fractions of mouse C2 and human HeLa cells (Fig. 2B, arrowhead). Affinity purified antibodies against each fragment identified a polypeptide of the same apparent molecular mass in mitochondrial fractions of human HeLa cells, mouse C2 cells, and different rat tissues (Fig. 2C), demonstrating the presence of Mfn2 in mitochondria of several tissues. Immunohistochemical analysis of human skin fibroblasts (Fig. 2D), human HeLa cells and mouse C2 cells (not shown) with affinity purified NG-antibodies confirmed the mitochondrial localization of Mfn2. It is important to note that endogenous Mfn2 distributes throughout the entire mitochondrial network (Fig. 2D). Affinity purified CT-antibodies were too weak to reveal endogenous levels of Mfn2 by immunofluorescence, but recognized the overexpressed protein in transfected cells (not shown).

The N-terminal and C-terminal domains of the transmembrane protein Mfn2 are oriented towards the cytoplasm

The membrane topology of Mfn2 was investigated by protease treatment of isolated mitochondria followed by western-blot analysis with domain-specific antibodies. To confirm the specificity of affinity purified antibodies, we analyzed extracts of cells expressing myc-tagged versions of Mfn1, Mfn2 and Mfn2-mutants devoid of the C-terminal or N-terminal domain (Fig. 3A). The blots shown in Fig. 3B visualize the highly abundant overexpressed proteins under conditions where endogenous Mfn2 is hardly detected. Myc-specific antibodies

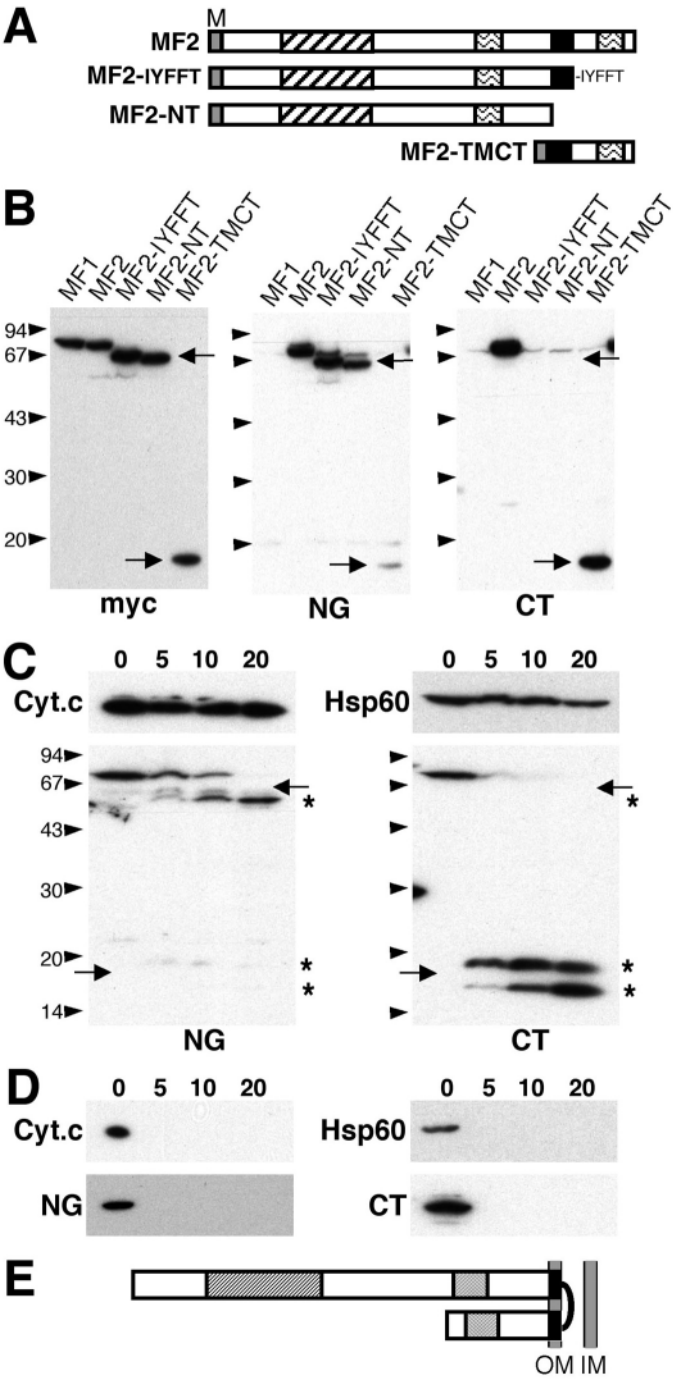

Fig. 3. Membrane topology of mitofusin 2. **(**A) Structure of myc-tagged (M) Mfn2 (MF2) and Mfn2-mutants (MF2-IYFFT, MF2-NT, MF2-TMCT). (B) Antibody specificity. Cells were transfected with myc-tagged mitofusins (MF1, MF2) and Mfn2-fragments (MF2-IYFFT, MF2-NT, MF2-TMCT) and analyzed by western-blot with the indicated antibodies (myc, NG, CT). The images visualize overexpressed proteins at exposures where endogenous Mfn2 is hardly visible (NG, CT). Myc-specific antibodies decorate both mitofusins and the Mfn2-constructs (arrows). NG and CT do not decorate Mfn1 and are specific for their respective Mfn2-domains (arrows). (C,D) Western blot analysis of isolated mitochondria treated with Proteinase K for the indicated times (minutes) in the absence (C) or presence (D) of Triton X-100. Proteins of the intermembrane space (Cyt.c) and of the mitochondrial matrix (Hsp60) are protected in intact mitochondria (C) and are degraded after membrane solubilization (D). In the absence of detergent (C), Mfn2 is gradually degraded into fragments (asterisks) that are differentially recognized by domain-specific antibodies (NG, CT). These fragments are smaller than MF2-NT/MF2IYFFT (upper arrow) and MF2-TMCT (lower arrow), respectively. This reveals the cleavage of N-terminal and C-terminal domains and their exposure towards the protease-containing solution. The position and size (in kDa) of molecular mass markers is indicated by arrowheads. (E) Deduced membrane topology of Mfn2. OM and IM, outer and inner mitochondrial membranes.

(Fig. 3B, myc) decorated Mfn1, Mfn2 and the different Mfn2-fragments (arrows). Affinity purified antibodies against N-terminal and C-terminal Mfn2-fragments (NG and CT, Fig. 2A) were isoform and domain-specific: (1) neither antibody decorated Mfn1; (2) NG decorated MF2-IYFFT and MF2-NT but hardly recognized MF2-TMCT; and (3) CT revealed MF2-TMCT, but did not decorate MF2-IYFFT and MF2-NT (Fig. 3B). The weak recognition of TMCT by NG-antibodies suggests that, despite affinity purification, NG-antibodies were still contaminated with a minor amount of CT-antibodies.

Next we treated freshly isolated mitochondria with proteinase K in the absence or presence of detergent. Proteins of the intermembrane space (Cyt.c) and of the matrix space (Hsp60) were protected in intact mitochondria (Fig. 3C) and were digested after membrane solubilization with detergent (Fig. 3D). By contrast, various Mfn2-fragments were generated during protease treatment of intact mitochondria (Fig. 3C, asterisks). Some of these fragments, which were labelled differentially by domain-specific antibodies (Fig. 3C, NG, CT), were smaller than MF2-NT (Fig. 3C, upper arrow) or MF2-TMCT (Fig. 3C, lower arrow), respectively. These results reveal the cleavage of the N-terminal and the C-terminal domains and demonstrate their exposure towards the protease-containing outside medium. The membrane topology revealed by these experiments is shown in Fig. 3E: Mfn2 is anchored in the mitochondrial outer membrane via its transmembrane domain and exposes N-terminal and C-terminal domains to the cytosol.

The C-terminal domain of Mfn2 specifies mitochondrial targeting

The transmembrane domain of Mfn2 has been shown to be required for mitochondrial targeting (Santel and Fuller, 2001). To better understand the targeting mechanism of Mfn2, we analyzed the subcellular localization of Mfn2-mutants truncated before the transmembrane domain (Fig. 4A, MF2-NT) and of Mfn2-molecules devoid of their N-terminal domain (Fig. 4A, MF2-TMCT). The cytoplasmic distribution of MF2-NT (not shown) confirmed that the transmembrane domain of Mfn2 is necessary for mitochondrial targeting. The colocalization of the MF2-TMCT molecule with mitochondrial GFP (mtGFP, Fig. 4B) demonstrated that the transmembrane and C-terminal domains of Mfn2 are sufficient for mitochondrial targeting. The targeting properties of Mfn2 and Mfn2-fragments are similar to those of tail-anchored proteins that expose their N-terminal domain towards the cytosol (Borgese et al., 2001). Although their targeting mechanism is still poorly understood, the available data indicate that their subcellular localization is specified by the flanking region downstream of the transmembrane domain: proteins are inserted in the mitochondrial outer membrane when this flanking region contains basic residues and in the endoplasmic reticulum (ER) when it contains neutral or hydrophobic amino acids (Borgese et al., 2001; Isenmann et al., 1998; Kuroda et al., 1998). Similar motifs also function in proteins that display the opposite membrane orientation (Kanaji et al., 2000).

We noticed that all Fzo-homologs contain numerous basic amino acids scattered along their C-terminal domains. To investigate whether they specify insertion of Mfn2 in the mitochondrial outer membrane, we replaced the C-terminal

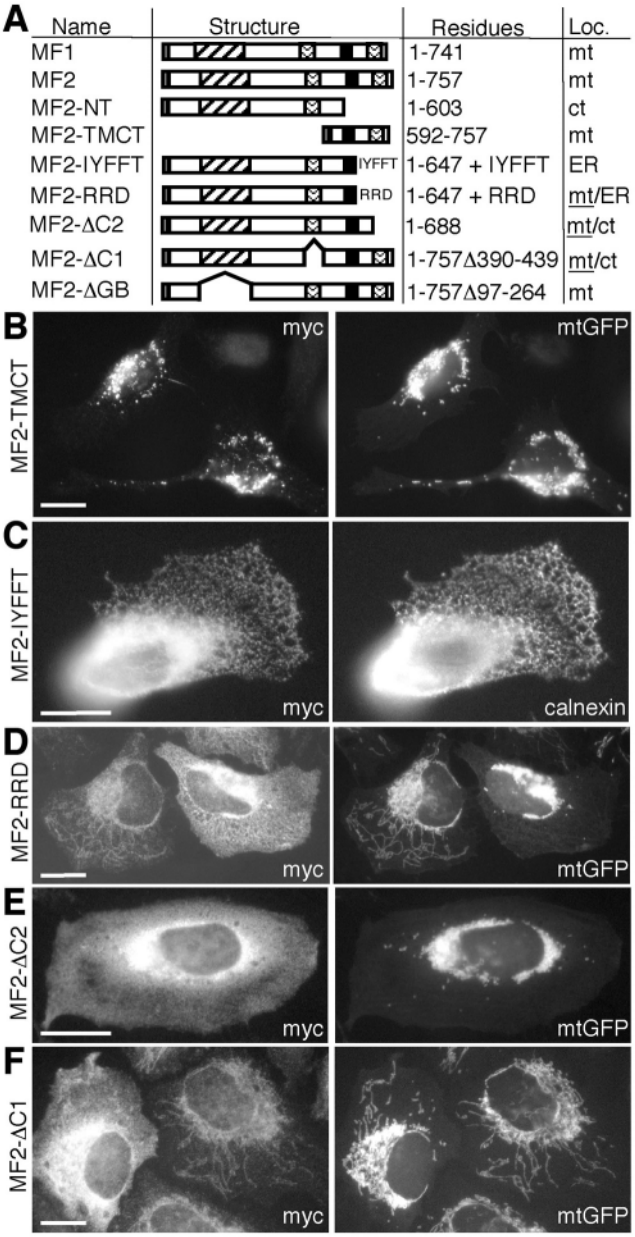

Fig. 4. Involvement of the C-terminal and of the coiled-coil domains in mitochondrial targeting. (A) Structure and localization (Loc.) of myc-tagged mitofusins and Mfn2-constructs. mt, mitochondria; ct, cytoplasma; ER, endoplasmic reticulum. (B-F) Cells were transfected with mitochondrial GFP (mtGFP) and/or with the indicated Mfn2-constructs. Myc-tagged Mfn2-mutants (myc) and the ER-marker calnexin were visualized with specific antibodies. (B) MF2-TMCT colocalizes with mtGFP in mitochondria. (C) MF2-IYFFT colocalizes with calnexin in the ER. (D-F) MF2-RRD, MF2-ΔC1 and MF2-ΔC2 are targeted to mitochondria, albeit inefficiently. A fraction of these molecules is mistargeted, especially at high expression levels. Bars, 15 μm.

domain of Mfn2 by the stretch of hydrophobic amino acids (IYFFT) that targets an isoform of the SNARE protein VAMP-1 (VAMP-1A) to the ER (Isenmann et al., 1998). MF2-IYFFT (Fig. 4A) was targeted to the ER as demonstrated by its colocalization with the ER-marker calnexin (Fig. 4C). The C-

terminal domain of Mfn2 was then replaced by the three polar residues (RRD) that target the other VAMP-1 isoform (VAMP-1B) to mitochondria (Isenmann et al., 1998). MF2-RRD (Fig. 4A) was targeted mainly to mitochondria, as revealed by colocalization with mtGFP (Fig. 4D). A minor fraction of MF2-RRD was targeted to the ER-membranes, especially at high expression levels (not shown). Together, these results show that mitochondrial targeting of Mfn2 is specified by the C-terminal domain via motifs and mechanisms resembling, at least partially, those of tail-anchored proteins. The behaviour of MF2-RRD suggests the presence of further targeting determinants in the C-terminal domain of Mfn2.

Coiled-coil interactions are required for efficient mitochondrial targeting

Even though coiled-coil domains (Fig. 2A, CC1, CC2) are conserved in all members of the Fzo-family (Hales and Fuller, 1997; Santel and Fuller, 2001), their function remains unknown. To investigate their function, we analyzed the localization of Mfn2-molecules devoid of either coiled-coil domain (Fig. 4A, MF2-ΔC2, MF2-ΔC1). Although both molecules were targeted to mitochondria, significant protein amounts remained cytosolic (Fig. 4E,F).

The reduced targeting efficiency of MF2-ΔC2 may be due to the removal of C-terminal targeting determinants. In contrast, it is more difficult to understand the reduced targeting efficiency of MF2-ΔC1, which has the same C-terminal domain as full length Mfn2 and MF2-TMCT. The fact that both deletion mutants display similar targeting properties suggest that, beside the motifs of the C-terminal domain, coiled-coil interactions are required for efficient mitochondrial targeting of full-length Mfn2. These interactions could be established between the coiled-coil domains of Mfn2 or with the coiled-coil domains of other proteins. To investigate these possibilities, MF2-NT and a version of Mfn2-TMCT devoid of a myc-tag (F2-TMCT) were expressed simultaneously by cotransfection. Co-expressed molecules were visualized with antibodies against the myc-tag (MF2-NT) and with CT-antibodies (F2-TMCT). Affinity purified CT-antibodies are too weak to reveal endogenous levels of Mfn2, but recognized the CT epitope of F2-TMCT (Fig. 5). We observed that MF2-NT colocalized with F2-TMCT in mitochondria (Fig. 5A). In contrast, MF2-NT molecules devoid of their coiled-coil domain (MF2-NTΔC1) remained cytosolic despite the mitochondrial localization of co-expressed F2-TMCT (Fig. 5B). Similarly, MF2-NT remained cytosolic when co-expressed with a version of F2-TMCT devoid of its coiled-coil domain (F2-TMCTΔC2, not shown). Interestingly, MF2-IYFFT was also targeted to mitochondria when cotransfected with F2-TMCT (Fig. 5C,D). MF2-IYFFT localized to the ER when the coiled-coil domain was deleted in any of the co-transfected molecules (not shown). It is interesting to note that the properties of co-transfected Mfn2-fragments resemble those of full length Mfn2: they can cluster mitochondria in the perinuclear region at high expression levels (Fig. 5D). Quantitative analysis of these experiments revealed that mitochondrial localization of MF2-NT or MF2-IYFFT by F2-TMCT was highly efficient and was abolished upon deletion of the coiled-coil domain in any of the co-transfected molecules (Fig. 5E). These results, which demonstrate specific and strong interactions between both Mfn2-fragments, further imply that the coiled-coil

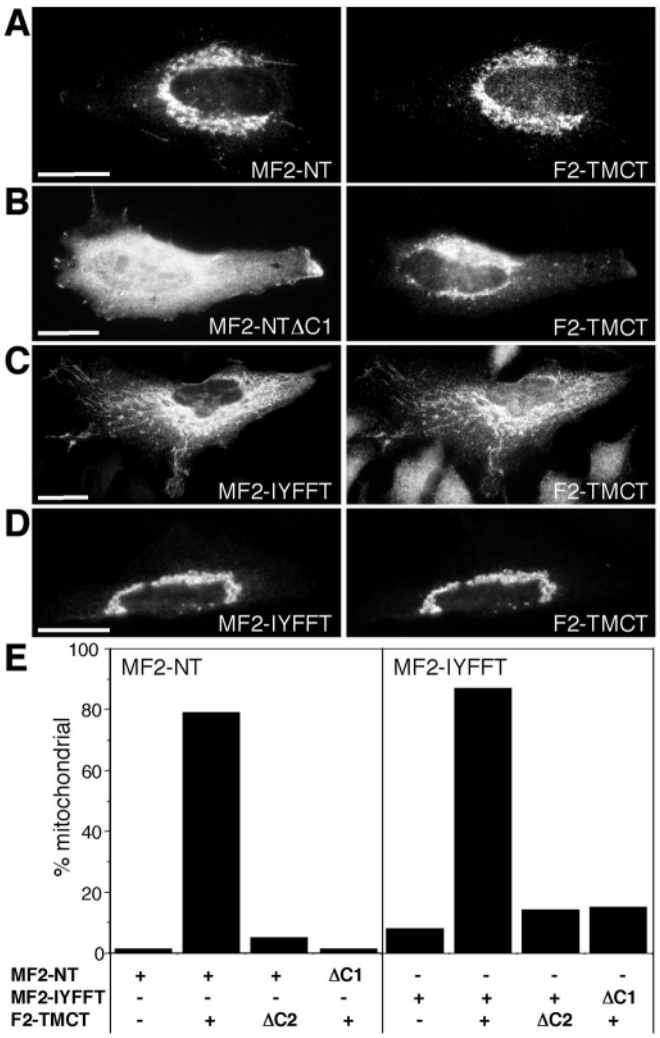

Fig. 5. The coiled-coil domains of Mfn2 interact with each other and mediate mitochondrial localization of mistargeted Mfn2-fragments. (A-D) HeLa cells were co-transfected with plasmids encoding Myc-tagged Mfn2-fragments (MF2-NT, MF2-IYFFT) and with a plasmid encoding the transmembrane and C-terminal domain of Mfn2 (F2-TMCT). Molecules were visualized with antibodies against myc and CT, respectively. (A) Soluble Mfn2-fragments truncated before the transmembrane domain (MF2-NT) localize to mitochondria upon co-expression of the transmembrane and C-terminal domains of Mfn2 (F2-TMCT). (B) MF2-NT molecules devoid of their coiled-coil domain (MF2-NTΔC1) remain cytosolic despite the co-expression of F2-TMCT. (C,D) Mfn2-mutants normally targeted to the endoplasmic reticulum (MF2-IYFFT) localize to mitochondria upon co-expression of F2-TMCT. (D) Co-expressed Mfn2-fragments can redistribute and cluster mitochondria in the perinuclear region. (E) Cells transfected with the indicated plasmids were processed for immunofluorescence and the localization of MF2-NT or MF2-IYFFT was determined. Where indicated (ΔC1, ΔC2), molecules were devoid of their coiled-coil domains. The results are expressed as percentage of transfected cells in which MF2-NT or MF2-IYFFT localize to mitochondria ($n \geq 200$ cells for each category). The mitochondrial relocalization of MF2-NT and MF2-IYFFT by F2-TMCT depends on the presence of coiled-coil domains on both Mfn2-fragments. Bars, 15 μm.

domains of truncated F2-TMCT and MF2-IYFFT are oriented towards the cytoplasma. Although it is probable that the coiled-coil domains of Mfn2-fragments interact directly with each other, it cannot be excluded that unknown bridging molecules mediate these interactions. The interactions between domains upstream and downstream of the transmembrane domain confirm the membrane topology established previously by protease protection experiments.

Microtubules and actin filaments are dispensable for Mfn2-mediated mitochondrial redistribution

Overexpression of human mitofusins leads to massive mitochondrial clustering (Santel and Fuller, 2001). Also in this work, the overexpression of Mfn1 (not shown) and Mfn2 (Fig. 6C,D) led to perinuclear clustering of mitochondria in cells expressing high protein levels (routinely in 35-45% of transfected cells). Immunofluorescence microscopy with organelle-specific antibodies showed that Mfn2 overexpression did not affect morphology or intracellular distribution of the endoplasmic reticulum and the Golgi apparatus (not shown), demonstrating that Mfn2 acts specifically on mitochondria. Mutations in key residues of their predicted G1 (K109A, S110N) and G4 motifs (R249L) did not modify the capacity of excess Mfn to cluster mitochondria (not shown), confirming that a functional GTP-binding domain is not required for mitochondrial clustering (Santel and Fuller, 2001). A mutant devoid of the entire GTP-binding domain (Fig. 4A, MF2-ΔGB), which was efficiently targeted to mitochondria, clustered mitochondria similarly to wild-type Mfn2 (not shown). This definitely excludes the involvement of the GTP-binding domain in the protein-protein interactions leading to mitochondrial redistribution and clustering. Mfn2-molecules devoid of the entire C-terminal domain (MF2-RRD) or of either coiled-coil domain (MF2-ΔC1, MF2-ΔC2) retained the capacity to redistribute and cluster mitochondria (Fig. 4D-F), albeit in a lower proportion of transfected cells (15-25%). It is important to note that expression of Mfn2-fragments containing the GTP-binding domain, but mistargeted to the cytosol (MF2-NT) or to the ER (MF2-IYFFT) did not appear to influence mitochondrial morphology or distribution (see below; Fig. 7C).

Mitochondrial clustering by excess Mfn2 was paralleled by the redistribution of mitochondria to the perinuclear region (Fig. 6C,D). Since microtubules and actin filaments modulate the intracellular distribution of mitochondria (Bereiter-Hahn and Voth, 1994; Krendel et al., 1998; Morris and Hollenbeck, 1995), we investigated whether excess Mfn2 required a functional cytoskeleton for mitochondrial redistribution. To this end, we used drugs (nocodazole, cytochalasin B) that severely interfere with the dynamics of the tubulin or actin cytoskeleton. In untransfected cells, the distribution of

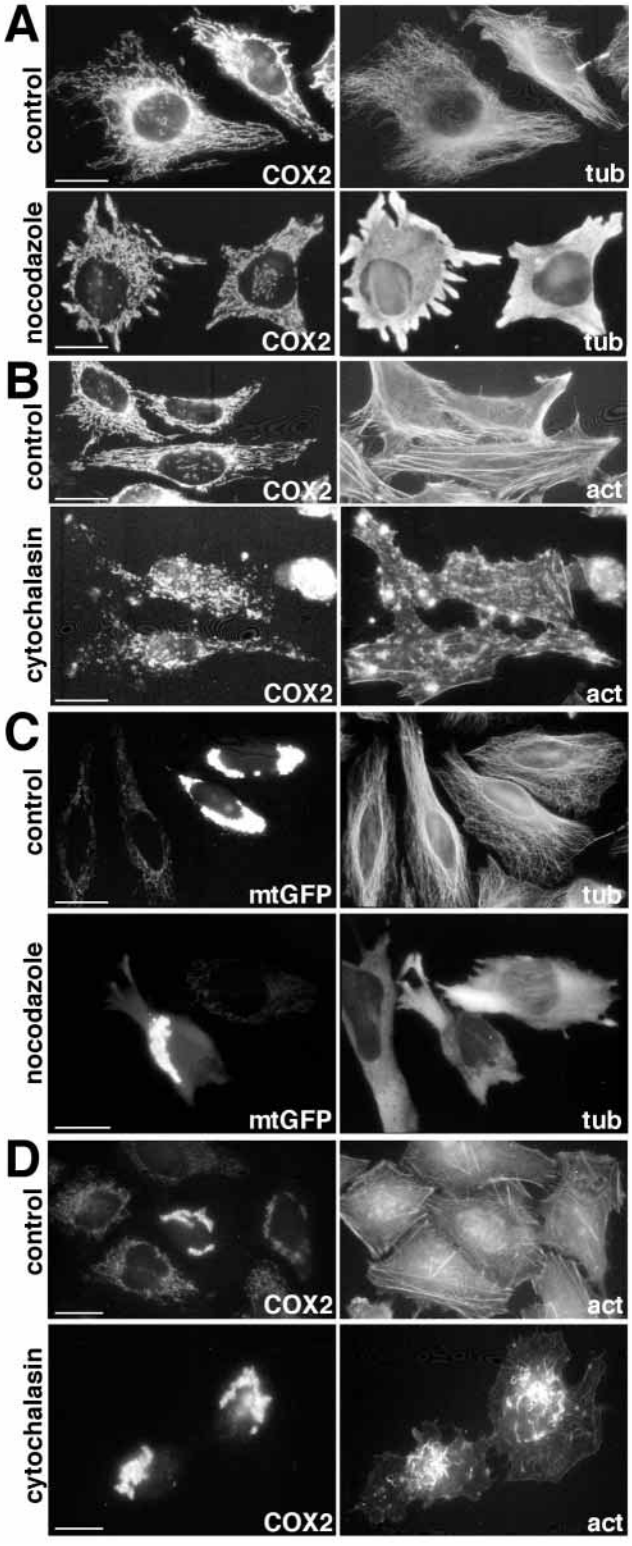

Fig. 6. Excess Mfn2 redistributes and clusters mitochondria in a process independent of the cytoskeleton. Immunofluorescence analysis of untransfected HeLa cells (A,B) and of Hela cells expressing mtGFP and/or Mfn2 (C,D). (A) The distribution and morphology of elongated mitochondria (COX2) is mildly perturbed upon deplymerazation of microtubules (tub) with nocodazole. (B) Addition of cytochalasin B (cytochalasin) leads to the dissappearance of elongated stress fibers (act) and to the appearance of punctated mitochondria scattered throughout the cell (COX2). Both drug treatments lasted 2 hours. (C,D) Mfn2-overexpression leads to mitochondrial redistribution and clustering (mtGFP, COX2) in the presence (control) and absence (nocodazole, cytochalasin) of a tubulin or actin cytoskeleton. Drug treatments started 9 hours after transfection, before significant expression of exogenous Mfn2. Cells were processed for immunofluorescence after a further 15 hours. Bars, 15 μm.

elongated mitochondria (Fig. 6A, control) was mildly perturbed upon depolymerization of microtubules (Fig. 6A, nocodazole). In contrast, the depolymerization of actin filaments led to the appearance of punctate mitochondria distributing throughout the cell (Fig. 6B). In cells transfected with plasmids encoding Mfn2 alone, or Mfn2 and mtGFP, the drug treatment started 9 hours after transfection. At this early time point, only a minority of cells (≤2%) expresses any transgene. Fifteen hours later (24 hours after transfection), when more than 30% of the cells expressed visible amounts of mtGFP, cells were processed for immunofluorescence microscopy. The formation of mitochondrial clusters and their redistribution to the perinuclear region also occurred in the absence of a functional tubulin (Fig. 6C) or actin cytoskeleton (Fig. 6D). When drugs were added after cluster formation (24 hours after transfection), they did not affect the size or intracellular distribution of pre-existing mitochondrial clusters (not shown). These results exclude the involvement of the tubulin and actin cytoskeleton in Mfn2-mediated mitochondrial redistribution. They further suggest that Mfn2 mediates intermitochondrial adhesion and that this leads to the retraction of mitochondria from the cell periphery and to their accumulation in the perinuclear region.

Excess Mfn2 modifies mitochondrial morphology and brings mitochondrial membranes into close contact without damaging membrane integrity

Next we analyzed the structure of clustered mitochondria by confocal microscopy. In control cells expressing mtGFP alone, most mitochondria appeared as tubules of several microns in length, and 400 to 450 nm diameter. The inner membrane marker COX2 and the matrix marker mtGFP colocalized extensively, and their intramitochondrial distributions displayed only minimal differences (Fig. 7A, control). In cells co-expressing Mfn2 and mtGFP, clustered mitochondria appear as discrete spherical or ovoid structures whose diameters (860±160 nm, n=34) were significantly larger than that of mitochondrial tubules in control cells (Fig. 7A, +Mfn2). Interestingly, the inner membrane appeared to surround the matrix space in enlarged spherical mitochondria (Fig. 7A, +Mfn2). This emphasizes the significant increase in size and suggests partial segregation of the inner membrane and the matrix. In cells expressing high levels of MF2-IYFFT, MF2-IYFFT molecules segregated within the ER and became enriched in discrete membrane domains (Fig. 7B). The spherical structure of these ER-subdomains is reminiscent of that of clustered mitochondria.

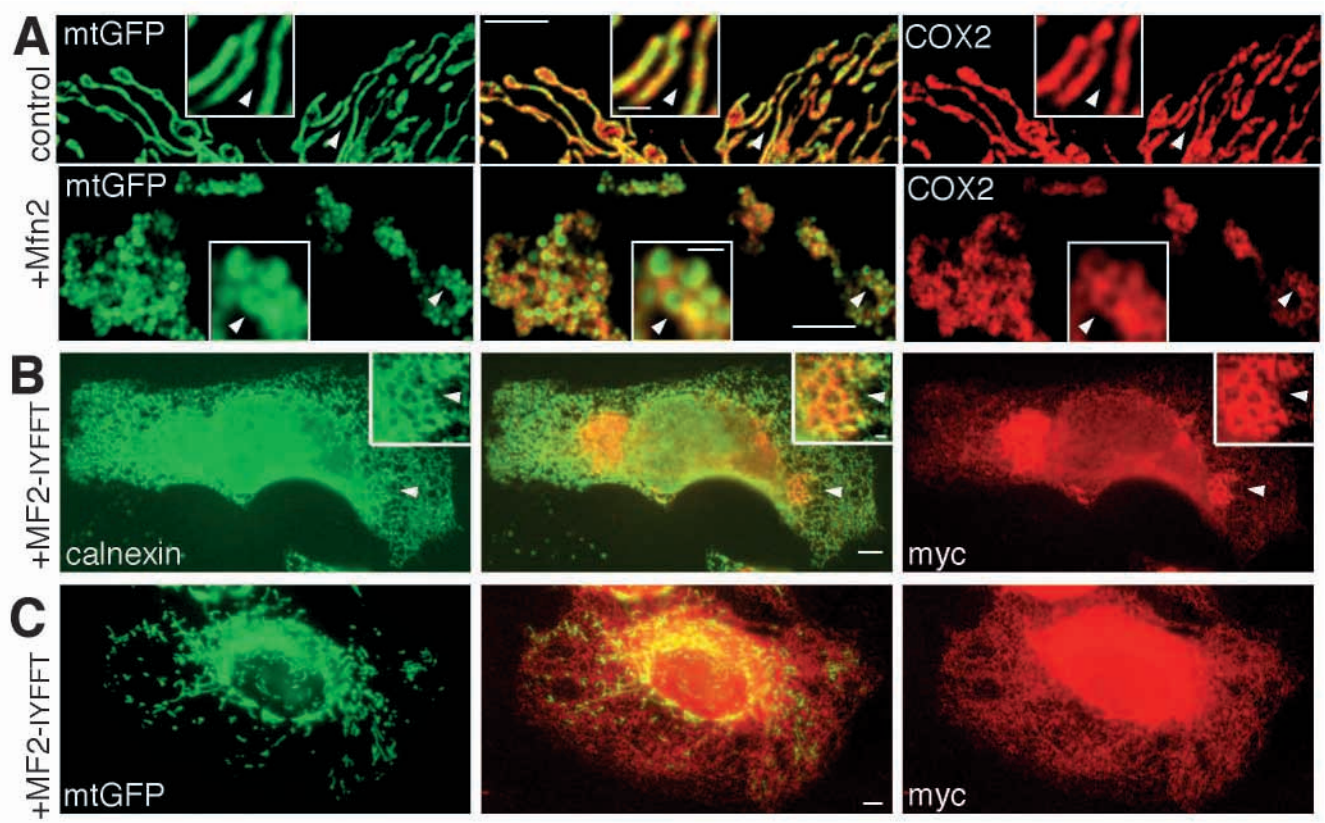

Fig. 7. Excess Mfn2 modifies mitochondrial morphology. HeLa cells were transfected with expression vectors encoding mtGFP (A, control), mtGFP and Mfn2 (A, +Mfn2) or MF2-IYFFT (B,C). Cells were fixed and decorated with specific antibodies directed against an inner membrane marker (COX2), calnexin or the myc-epitope (myc). Cells were visualized by confocal (A) or conventional microscopy (B,C). The images depict selected cell regions (A) or entire cells (B,C). The insets show enlargements of the areas marked with arrowheads. (A) In control cells, mtGFP and COX2 colocalize in mitochondrial tubules of several μm length and 400-450 nm diameter. In Mfn2 overexpressing cells, mitochondria are spherical (mean diameter, 860±160 nm, n=34) and clustered. In enlarged spherical mitochondria, markers of the inner membrane (COX2) appear to surround the matrix space (mtGFP). (B) At high expression levels, MF2-IYFFT (myc) segregates within the ER (calnexin) and forms membrane networks with modified morphology. (C) MF2-IYFFT expression (myc) does not affect mitochondrial morphology and distribution (mtGFP). Bars, 5 μm (main images) and 1 μm (insets).

We then investigated the integrity of mitochondrial membranes in clusters induced by Mfn2 overexpression. Cytochrome c, a protein that is released from the mitochondrial intermembrane space when the outer membrane is damaged and/or permeabilized (for a review, see Desagher and Martinou, 2000), remained in mitochondrial clusters induced by Mfn2 expression (not shown). The vital dye JC-1, which accumulates in mitochondria and forms red-fluorescent aggregates in the presence of a high transmembrane potential (Smiley et al., 1991), labelled normal and clustered mitochondria in a similar fashion (not shown). Together, these experiments show that mitochondrial clustering by excess Mfn2 does not affect the integrity of mitochondrial inner and outer membranes.

The ultrastructure of HeLa cells transfected with an empty plasmid or with a plasmid encoding Mfn2 was analysed by electron microscopy. In control cells, mitochondria displayed a standard morphology: spherical or tubular mitochondrial profiles (minimum diameter 250-350 nm) delimited by a double membrane with an electron dense matrix space and closely apposed cristae membranes (Fig. 8A). Cells with mitochondrial clusters in the perinuclear region were found only in dishes transfected with Mfn2 (Fig. 8B,C). The size and localization of these mitochondrial clusters was in accordance with those observed by confocal microscopy. All mitochondrial profiles were spherical/ovoid, and their diameters were significantly increased, reaching up to 1 μm (Fig. 8B). The internal structure of clustered mitochondria was also affected: whereas the cristae membranes appeared normal in smaller mitochondria, they were swollen in mitochondria of intermediate size and were difficult to identify in the largest mitochondria (Fig. 8B,C). The increase in size and the change from tubular to globular morphology may lead to the relocalization of the inner cristae membranes (which are probably continuous with the inner membrane) to the periphery of enlarged mitochondria. This would explain why, in confocal microscopy, the inner membrane of enlarged mitochondria appears to surround the matrix (Fig. 7A). The higher magnification in Fig. 8C shows that the structure of the limiting inner and outer

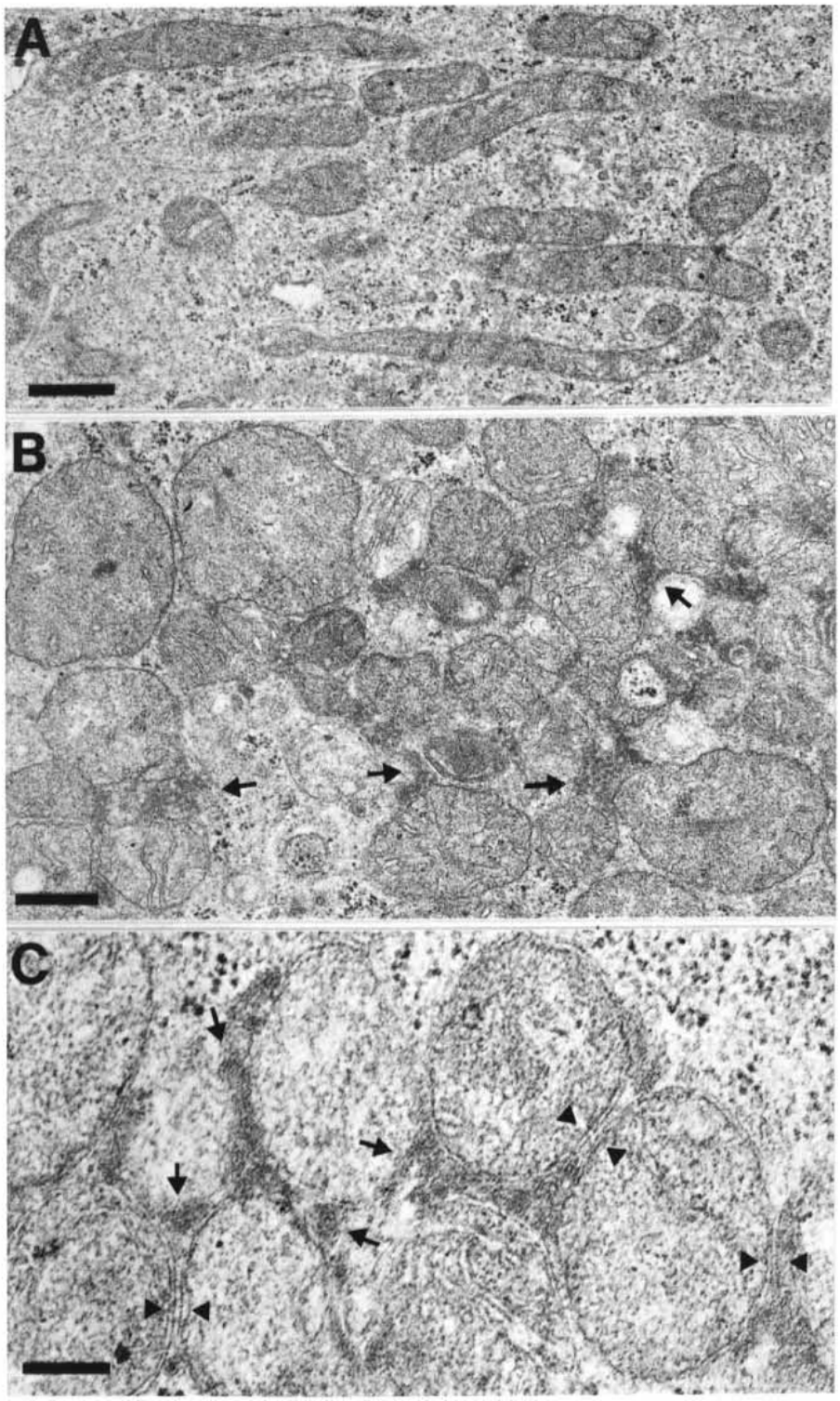

Fig. 8. The ultrastructure of clustered mitochondria is modified and their enveloping double membranes are closely apposed. Hela cells were transfected with an empty vector (A) or with a vector encoding Mfn2 (B,C) and processed for electron microscopy. (A) In control cells, sections depict tubular mitochondria of 250-350 nm diameter. (B) Mitochondrial distribution and morphology is modified in Mfn2- transfected cells. Mitochondria are clustered, and their diameters can surpass 1 μm (upper left region). In enlarged mitochondria, cristae membranes are swollen and/or difficult to identify. (C) The double membranes of clustered mitochondria are in direct contact (arrowheads), but do not merge. Arrows in B and C point to electron dense material that is absent from control cells, and accumulates between mitochondria. Bars, 500 nm (A,B); 200 nm (C).

mitochondrial membranes was well preserved, and that the double membranes of apposed mitochondria could be in close contact without merging (Fig. 8C, arrowheads), confirming the capacity of excess Mfn2 to mediate intermitochondrial adhesion.

Discussion

Expression of mitofusins

The existence of Fzo-homologs (mitofusins) in mammals implies strong similarities between the mitochondrial fusion machineries of all eukaryotes. The involvement of Fzo-proteins in fusion (Hales and Fuller, 1997; Hermann et al., 1998), together with the ubiquitous expression of mitofusins, indicate that mitochondrial fusion is not restricted to certain tissues and/or developmental stages in mammals. This work, together with a previous study (Santel and Fuller, 2001), reveals similarities between both mammalian mitofusins. It is not known whether, in vivo, endogenous mitofusins display different or similar activities and whether they act in an independent and/or synergistic manner. In contrast to yeast and nematodes, the fly *D. melanogaster* appears to have two Fzo-homologs: Dm-FzoS and Dm-FzoU. Whereas Dm-FzoU may be ubiquitously expressed, Dm-FzoS is expressed only during spermatid development (Hales and Fuller, 1997). In mammals, as in *Drosophila*, spermatid mitochondria fuse extensively in the late stages of spermatogenesis (reviewed by Tzagoloff, 1982). Therefore, and despite the expression of ubiquitous Mfn1 and Mfn2 in the testis, it is possible that a spermatid-specific Fzo-homolog also exists in mammals.

Targeting and membrane topology

The membrane topology of Mfn2, with N-terminal and C-terminal domains exposed towards the cytosol, implies that the hydrophobic domain spans the outer membrane twice, as seen in yeast Fzo (Fritz et al., 2001). This topology was confirmed by the interactions between N-terminal and C-terminal domains of Mfn2. Given the fact that the domain organization is conserved between all Fzo-homologs (Hales and Fuller, 1997; Santel and Fuller, 2001), it is probable that Fzo-proteins also share their membrane topology.

It was found that the transmembrane and C-terminal domains of Mfn2 specify its targeting via motifs resembling those of other transmembrane proteins of the outer mitochondrial membrane (Borgese et al., 2001; Isenmann et al., 1998; Kanaji et al., 2000; Kuroda et al., 1998). These targeting motifs are sufficient for localization of Mfn2-fragments to the ER (MF2-IYFFT) or to mitochondria (MF2-TMCT), but not for mitochondrial targeting of full-length Mfn2. Co-transfection experiments revealed that the coiled-coil domains of Mfn2 mediate direct or indirect interactions between different Mfn2-fragments and that these interactions are necessary for efficient mitochondrial targeting of full-length Mfn2. These experiments further showed that the mitochondrial targeting of interacting Mfn2-fragments is dominant over the ER-targeting of MF2-IYFFT. Although protein transport to the mitochondrial outer membrane is still poorly understood, it has been recently shown that mitochondria and ER recruit tail-anchored proteins directly from the cytosol and can compete for the same polypeptide (Borgese et al., 2001). Co-expressed Mfn2-fragments could interact with each other in the cytosol, mask the ER-targeting signal of MF2-IYFFT and the resulting protein complex would then be translocated to mitochondria. Alternatively, F2-TMCT could be first targeted to mitochondria, from where it would recruit mistargeted MF2-NT and MF2-IYFFT. It is reasonable to assume that MF2-IYFFT would be recruited by F2-TMCT before its insertion in the ER-membrane. The fact that Mfn2-molecules devoid of either coiled-coil domain remain partially cytosolic suggests that the mitochondrial transport machinery can discriminate between proteins with and without assembled coiled-coils. This would argue for the first hypothesis, that is, for the assembly of coiled-coil domains in the cytosol, prior to mitochondrial import.

Modulation of mitochondrial distribution and morphology by Mfn2

Overexpressed mitofusins induced perinuclear clustering of mitochondria without affecting membrane integrity. To our knowledge, the capacity to cluster mitochondria upon overexpression has been reported only for the mitochondrial outer membrane protein OMP25, and requires its PDZ domain (Nemoto and De Camilli, 1999). Numerous outer membrane proteins, including porin/VDAC isoforms (Yu et al., 1995), a SNARE protein (Isenmann et al., 1998), a cytochrome b isoform (Kuroda et al., 1998) and Tom20 (Kanaji et al., 2000), do not induce mitochondrial clustering when overexpressed in mammalian cells. Although we cannot completely exclude that mitochondrial clustering is provoked non-specifically, the ability of Mfn2 to redistribute mitochondria in the absence of a functional cytoskeleton suggests that Mfn2 has the capacity to mediate intermitochondrial adhesion. It is possible that the coiled-coil domains of Mfn2-molecules mediate intermitochondrial adhesion/docking by the formation of a four helix bundle, as seen in SNARE proteins (Sutton et al., 1998). However, it is important to note that molecules devoid of a coiled-coil domain also mediated mitochondrial clustering, albeit with lower efficiency. The diminished clustering capacity of the coiled-coil mutants could reflect a direct involvement of coiled-coil domains in adhesion, or result from the lower efficiency of mitochondrial targeting. Therefore, it is possible that Mfn2 mediates intermitochondrial adhesion by a mechanism other than the formation of a (SNARE-like) four helix bundle and/or that it participates in other membrane rearrangements leading to membrane merge and mitochondrial fusion.

Since a functional GTP-binding domain is necessary for Mfn2-function (Hales and Fuller, 1997; Hermann et al., 1998; Santel and Fuller, 2001), it is tempting to speculate that this domain either modulates the activity of Mfn2 or catalyzes further downstream reactions. Thus, mitochondrial clustering by excess Mfn2 could be either provoked by Mfn2 molecules that overrun modulation or result from a combination of increased intermitochondrial adhesion and lack of downstream fusion events. Like other GTP-binding proteins Mfn/Fzo could require modulator and/or effector molecules for proper function. The fact that expression of mistargeted Mfn2-constructs (MF2-NT, MF2-IYFFT) did not affect mitochondrial distribution and morphology, showed that hypothetical factors were not titrated out. This may indicate

that they are highly abundant, membrane-bound (and cannot interact with mistargeted molecules), or that they do not exist. Further characterization of Mfn/Fzo and its GTP-binding domain, as well as identification and characterization of further proteins (including hypothetical modulators of Mfn/Fzo) will improve our understanding of Fzo-function and will help to unravel the mechanisms governing mitochondrial fusion.

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