Mutational analysis of action of mitochondrial fusion factor mitofusin-2

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Summary

Mitofusin-2 (Mfn2) is an essential component of mitochondrial fusion machinery, but its molecular mechanism of action is not clear. We found that a Mfn2 deletion mutant lacking two transmembrane spans (Mfn\(^{\Delta TM}\)) acts as a dominant-negative mutant and blocks mitochondrial fusion. Furthermore, detailed analysis of various mutants of Mfn\(^{\Delta TM}\) revealed that GTPase activity and four regions highly conserved from nematodes to mammals are necessary for the dominant-negative effect. Immunoprecipitation studies of the N- and C-terminal cytosolic tails of Mfn2 showed that in addition to the coiled-coil domains previously identified, a highly conserved domain in the most N-terminal region and GTPase activity are necessary for the interaction between the N- and C-terminal tails, which is in turn required for the dominant-negative effect. In addition, we found unexpectedly that overexpression of the deletion mutant composed of one short region each in the N- and C-terminal tails of Mfn2 resulted in loss of mitochondrial membrane potential, suggesting that Mfn2 might also be connected to maintenance of mitochondrial membrane potential.

Key words: mitochondria, mitofusin, mitochondrial morphology, mitochondrial membrane potential

Introduction

The mitochondrion is a highly compartmentalized organelle consisting of inner and outer membranes separated by an intermembrane space and it plays a key role in energy metabolism, thermogenesis, calcium signaling and apoptosis (Green and Reed, 1998; Saraste, 1999; Brenner and Kroemer, 2000). It forms an interacting network called a mitochondrial reticulum the dynamic structure of which is maintained by an equilibrium between fusion and fission. Proteins that are involved in the control of mitochondrial dynamics (i.e. shape, number and subcellular distribution) are being identified, including the fusion protein mitofusin (Mfn) and the division factor Drp, both belonging to the family of large GTP-binding proteins (for recent reviews, see Yaffe, 1999; Griparic and van der Bliek, 2001; Danino and Hinshaw, 2001; Westermann, 2002; Mozdy and Shaw, 2003; Westermann, 2003; Osteryoung and Nunnari, 2003; Chen and Chan, 2004; Rube and van der Blik, 2004). According to the prevailing model for mitochondrial division in yeast (Shaw and Nunnari, 2002; Osteryoung and Nunnari, 2003), fission is initiated by the recruitment of the dynamin-related large GTPase, Dnm1p, to the mitochondrial outer membrane and its assembly with the adaptor Mdv1p and the integral membrane protein Fis1p (Bleazard et al., 1999; Sesaki and Jensen, 1999; Mozdy et al., 2000; Tieu et al., 2002). Fusion processes of yeast mitochondria are mediated by Fzo1p (Hermann et al., 1998; Rapaport et al., 1998), a large GTPase, which interacts with the adaptor Ugo1p in the outer membrane (Sesaki and Jensen, 2001) and associates with the inner membrane dynamin-related protein Mgm1p (Wong et al., 2000; Sesaki et al., 2003; Wong et al., 2003). Mammalian orthologues of Dnm1p and Fis1p, termed Drp1 and hFis1, respectively, have been identified and shown to participate in mitochondrial fission (Labrousse et al., 1999; Smirnova et al., 2001; James et al., 2003). Mammalian counterparts of the yeast fusion factors Fzo1p and Mgm1p are mitofusins and the inner membrane dynamin-related protein OPA1, respectively.

Mitofusins, Mfn1 and Mfn2, were identified as homologues to the Drosophila fuzzy onions protein (Fzo), which is required for mitochondrial fusion during spermatogenesis of the fruit fly (Hales and Fuller, 1997). They are mitochondrial outer membrane proteins with large N-terminal and relatively short C-terminal domains exposed towards the cytosol (Hermann et al., 1998; Rapaport et al., 1998; Fritz et al., 2001; Rojo et al., 2002). Their domain structure is schematically shown in Fig. 1B, and consists of a GTPase domain near the N terminus, a coiled-coil domain, two transmembrane spans and a coiled-coil domain in the C-terminal tail facing the cytoplasm. Loss of the mitofusin function causes the mitochondrial network to rapidly fragment and no fusion of mitochondria occurs when this protein is defective or absent. Site-directed mutagenesis revealed that mitochondrial fusion machinery of fzo/mitofusin is regulated in a GTPase-dependent manner in fruit fly, yeast and mammals (Hales and Fuller, 1997; Hermann et al., 1998; Santel and Fuller, 2001). In yeast Fzo1p, the short loop facing the intermembrane space has been demonstrated to associate with the contact sites between inner and outer membranes and to be essential for mitochondrial four-bilayer fusion (Fritz et al., 2001). Rojo et al. (Rojo et al., 2002) showed that a construct covering the double transmembrane spans and C-terminal tail of Mfn2 is targeted to mitochondria and the N- and C-terminal tails of Mfn2 are able to interact through their coiled-coil
domains. Mitofusin has been suggested to be a component of multieprotein fusion machinery (Rapaport et al., 1998; Fritz et al., 2001; Santel et al., 2003). Mice deficient in either Mfn1 or Mfn2 die in midgestation and the embryonic fibroblasts established from the knockout mice displayed fragmented mitochondria (Chen et al., 2003). In mammals, two isoforms, Mfn1 and Mfn2, have been reported to cooperate to fuse mitochondria (Chen et al., 2003; Santel et al., 2003; Eura et al., 2003). Functional differences between Mfn1 and Mfn2 are being revealed. For example, it has been shown that embryonic fibroblasts established from knockout mice lacking either Mfn1 or Mfn2, display distinct types of fragmented mitochondria (Chen et al., 2003), that OPA1 requires Mfn1 but not Mfn2 to induce mitochondrial fusion (Cipolat et al., 2004), and that Mfn1 tethers mitochondrial membranes with a higher efficiency than Mfn2 (Ishihara et al., 2004). However, the mechanisms of mitochondrial fusion and the role of Mfn/Fzo family remain essentially unknown.

To gain insight into the mechanism governing mitochondrial dynamics, we prepared a series of deletion mutants of mouse Mfn2 and succeeded in generating a soluble dominant-negative mutant that causes a marked shift in the balance between fusion and fission towards fission. Detailed analysis of its mode of action revealed that GTPase-dependent interaction between the N-terminal and C-terminal tails of Mfn2 through their coiled-coil domains and a highly conserved domain in the most N-terminal region is essential for the dominant-negative effect. In addition to the GTPase domain, four other regions (two each in the N- and C-terminal tails including the coiled-coil domain of the C-terminal tail) were found to be essential for the dominant-negative activity. Furthermore, through the analysis of soluble deletion mutants, we unexpectedly found two regions (one each in the N- and C-terminal tails) that can induce, if present in combination, loss of mitochondrial membrane potential and hence fragmentation of mitochondria. Interestingly, these regions are highly conserved among mitofusins of various species. The reason we chose Mfn2 first is that it has higher amino acid sequence similarity than Mfn1 to those of Drosophila Fzo and yeast Fzo1p, the latter being the only known members of the Fzo/Mfn family in the corresponding species.

Materials and Methods

Cloning of mouse Mfn2 has been described previously (Honda and Hirose, 2003). The Mfn2614-647 deletion construct was derived by using PCR to amplify the defined region of a Mfn2-pBlueScript construct. The primers used were: 5′-GAGCGGCTGACCTGGAGCC (forward primer) and 5′-CCTAGACGTCAAGAGGCC (reverse primer). All other mutants diagrammed in Fig. 2 were generated in a similar manner using appropriate primers. The Mfn2T130A point mutation was generated using the method of Ito et al. (Ito et al., 1991) with Drp-pBlueScript as a template and the primer 5′-AGCAATTGCGCTGATCCAGACC (forward primer) and 5′-CCATCGACCTGCAGAGGCC (reverse primer). Other mutants diagrammed in Fig. 2 were generated in a similar manner using appropriate primers. The Mfn21635 was generated by PCR using the forward primer 5′-GGAATTCATGTCCCTGCTTCATCTCAG and the reverse primer 5′-CCAGGTACCGACCTCGAGAGGCCAGGC and Mfn2648-757 was with the forward primer 5′-GAAGATCTGACGTCGATACCGGACCA and the reverse primer 5′-GGAATTCATGTCCCTGCTTCATCTCAG.

Cell culture and transfections

COS7 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Transfections were done using Lipofect-AMINE Plus and Lipofect-AMINE 2000 (Invitrogen) according to the supplier’s instructions.

Antibodies

Anti-Mfn2C was produced as described previously (Honda and Hirose, 2003). Anti-Hsp60 (SPA-807) was purchased from StressGen; anti-FLAG M2 (F3165) was from Sigma; and anti-mouse IgG Alexa Fluor 350 and anti-mouse IgG Alexa Fluor 488 were from Molecular Probes.

Immunofluorescence staining of transfected COS7 cells

COS7 cells were transfected with expression constructs for Mfn2 deletion mutants. After 16 hours, living cells were incubated with 500 nM MitoTracker Red CMXRs (Molecular Probes) at 37°C for 30 minutes, washed twice with PBS, fixed with 4% formaldehyde/PBS for 15 minutes, washed three times with PBS. Cells were permeabilized for 15 minutes in PBS with 0.2% Triton X-100 and blocked for 30 minutes with PBS containing 5% FBS. For double and triple staining, cells were first incubated with mouse anti-FLAG antibody for 3 hours at room temperature, washed three times with PBS, subsequently incubated with anti-mouse IgG Alexa Fluor 488 for 1 hour at room temperature, and washed three times with PBS. Cells were then incubated with rabbit anti-Mfn2C antibody overnight at room temperature, washed three times with PBS, subsequently incubated with anti-rabbit IgG Alexa Fluor 350 for 3 hours at room temperature, and washed three times with PBS. Coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) on glass slides. Cells were examined with an Olympus fluorescence microscope, model IX70, equipped with a 100× oil-immersion objective and filters optimized for triple-label experiments. Pictures were taken using a Princeton Instruments cooled CCD camera (MicroMAX5 MHz; Rooper Scientific) and analyzed using the MetaMorph software (Universal Imaging).
Results

Mitofusins domains are highly conserved

A sequence analysis revealed that mouse Mfn2 shares the domain structure, characterized by the presence of the GTPase, two transmembrane (TM) domains and two coiled-coil domains (Fig. 1B), with those of the fruit fly (Hales and Fuller, 1997), yeast (Hermann et al., 1998; Rapaport et al., 1998), and human (Santel and Fuller, 2001). The GTPase domain is highly conserved among these species; for example, mouse and fruit fly Fzo/Mfn are more than 80% similar in this region. Other regions, however, exhibit only 40-60% similarities. Although the GTPase domain, two coiled-coil domains, double transmembrane spans and an intermembrane region of the Mfn/Fzo family members are reported to be essential for their activity, the function of the rest, which amounts to two thirds of the full length, is still unknown. To obtain structural and functional clues to these regions, we first performed a ClustalW analysis using mammalian, fruit fly and nematode Mfn/Fzo sequences (Fig. 1A) and found seven highly conserved regions R1-R7 (Fig. 1B) in addition to the GTPase domain and transmembrane spans. The conserved region R4 partially overlaps with the N-terminal coiled-coil domain (N-CC); R7 overlaps extensively with the C-terminal coiled-coil domain (C-CC; Fig. 1). R4 and N-CC (R4/N-CC) and R7 and C-CC (R7/C-CC) are therefore treated as single domains in the following construction of deletion mutants, namely ΔR4 and ΔR7 represent ΔR4/N-CC and ΔR7/C-CC, respectively.

Deletion mutant lacking transmembrane spans acts as a dominant-negative mutant

Overexpression of Mfn in cultured cells by transient transfection is reported to induce perinuclear aggregation of mitochondria, independent of the mitochondrial fusion activity of Mfn (Santel and Fuller, 2001), as also observed in transient overexpression of the mitochondrial outer membrane.
protein OMP25 (Nemoto and De Camilli, 1999). To avoid this artifactual effect, we made a deletion mutant of mouse Mfn2 that lacks double transmembrane spans (Mfn2ΔTM; Fig. 2A). When expressed in COS7 cells, Mfn2ΔTM protein caused mitochondria to fragment into small pieces (Fig. 2B,b). This result indicates that the balance between mitochondrial fusion and fission was shifted toward fission by the mutant protein. Such a shift is consistent with a block of the fusion by a dominant-negative mutant of a fusion factor such as Mfn2. Noteworthy is the fact that the dominant-negative effect was exerted by a mutant, Mfn2ΔTM, that became soluble because of deletion of the transmembrane spans (Fig. 2B,a), suggesting depletion by Mfn2ΔTM of soluble factors necessary for mitochondrial fusion.

Next, to determine the relationship between the dominant-negative effect and GTPase activity, we introduced a mutation in the active site of the GTPase domain (Mfn2T130A, ∆TM; Fig. 2A), which corresponds to a loss-of-function mutation in the G2 motif of the GTPase domain of yeast Fzo1p (Fzo1T221A), a mutation that gives a similar phenotype as in G1 motif mutants.

Fig. 2. Segments R1, R3, R6 and R7, and GTPase activity of Mfn2ΔTM are necessary for mitochondrial fragmentation in COS7 cells. (A) Schematic diagram of the Mfn2 deletion and point mutants used to analyze mitochondrial fragmentation, and percentage of transfected cells with fragmented mitochondria. All constructs are epitope-tagged with the 3×FLAG sequence present at the N terminus. Boxed C, predicted coiled-coil domain; gray, GTPase domain; black, predicted transmembrane domains. The G2 Mfn2-T130A mutation is equivalent to a mutation in the Ras GTPase that abolishes effector interactions. (B) COS7 cells were transiently transfected with expression vector for FLAG-Mfn2ΔTM (a,b) and FLAG-Mfn2ΔR4/R5/ΔTM (c,d). After 16 hours, the cells were labeled with MitoTracker to visualize mitochondria (b,d), fixed, and stained with anti-FLAG antibody followed by Alexa Fluor 488-conjugated secondary antibody (a,c). The star indicates untransfected cell with normal reticular mitochondria. Bar, 20 µm.
such as Fzo1K200A (Hermann et al., 1998), Mfn2K109T (Santel and Fuller, 2001) and Mfn2K109A (Santel et al., 2003). In the case of human Mfn1, overexpression of the G2-T109A mutant has been reported to result in fragmentation of mitochondria while introduction of point mutation in the G1 motif (G1-K88T mutant) only reduces mitochondrial networks (Santel et al., 2003), suggesting that the G2 motif mutant exerts stronger inhibitory effects on mitochondrial fusion. We therefore decided to use the G2 mutant of mouse Mfn2 (i.e. Mfn2T130A, TM). Mfn2T130A,TM caused a loss of the dominant-negative activity, yielding normal mitochondrial morphology with an elongated tubular network (Fig. 2A; micrographs not shown). This normal mitochondrial morphology in the presence of Mfn2T130A,TM is noteworthy since it has been shown, as mentioned above, that even a loss-of-function mutant of Mfn2 causes perinuclear clustering of mitochondria if it has the transmembrane spans (Santel and Fuller, 2001). We next tried to determine which part of Mfn2T is responsible for the inhibitory effect, by expressing a series of deletion mutants of Mfn2T (Mfn2AR1/TM, Mfn2AR2/TM, Mfn2AR3/TM, Mfn2AR4/R5/TM, Mfn2AR5/R6/TM, Mfn2AR6/TM). As summarized in Fig. 3A, deletion mutants of Mfn2TVM lacking the R1 and R5 regions in the presence of either the R1-R6 or the GTPase/TM caused a loss of the dominant-negative phenotype (Mfn2AR1/TM, Mfn2AR6/TM; Fig. 2A). Furthermore, Mfn2TVM lacking R3, R4 and R5 regions (Mfn2AR3-R5/TM) also resulted in loss of the dominant-negative phenotype but this did not occur with Mfn2TVM lacking the R4 and R5 regions (Mfn2AR4/R5/TM; Fig. 2A,B,d). These results indicated that four highly conserved regions (R1, R3, R6 and R7) are necessary for the dominant-negative activity.

The N- and C-terminal tails of Mfn2 appear to interact through their highly conserved regions including coiled-coil domains in a GTPase-dependent manner.

We next determined the effects of the N-terminal and C-terminal tails of Mfn2 on mitochondrial morphology when they were expressed as separated molecules (Fig. 3A). As expected, neither the N-terminal tail alone (Mfn2N-FLAG; Fig. 3B,f; asterisk) nor the C-terminal tail alone (Myc-Mfn2C; Fig. 3B,f; arrowhead) affected the mitochondrial morphology. Coexpression of Mfn2N-FLAG and Myc-Mfn2C (Fig. 3A) resulted in dominant-negative phenotypes (Fig. 3B,c; arrow) in a GTPase-dependent manner (Mfn2NTR30a; Fig. 3A,B). The result described above suggests an interaction between the N-terminal and C-terminal tails. To confirm the interaction, we performed immunoprecipitation analysis. When the C-terminal tail (Myc-Mfn2C) was immunoprecipitated with anti-C-terminal antibody (anti-Mfn2C), the N-terminal tail was found to coprecipitate (data not shown). Similarly, the C-terminal fragment (Myc-Mfn2C) was coimmunoprecipitated with the N-terminal fragment (Mfn2N-FLAG) (Fig. 3C).

To determine the regions involved in the interaction, we prepared deletion mutants of the large N-terminal tail as depicted in Fig. 3A, and examined their effects on mitochondrial morphology by coexpressing them with the C-terminal fragment Mfn2C. As summarized in Fig. 3A, deletion of either the R2-R4 regions (Mfn2NAR2-R4) or the GTPase domain including its N-terminal flanking region (Mfn2NAR1 and Mfn2NAR2/GTPase) resulted in complete loss of the dominant-negative activity that leads to fission of the mitochondrial reticulum (Fig. 3A). This dominant-negative activity correlated well with their ability to interact with the C-terminal fragment Mfn2C, as demonstrated by immunoprecipitation (Fig. 3C). An interesting finding was that interaction between Mfn2NAR1 and Mfn2C is relatively weak despite the fact that both constructs contain intact coiled-coil domains (i.e. R4-N-CC and R7-C-CC), indicating that the coiled-coil domains alone are not sufficient for tight association and the R1 region is also involved in the interaction between the N- and C-terminal tails (Fig. 3C). Furthermore, as mentioned above, deletion of R4 and R5, had no effect on the dominant-negative phenotype (Mfn2AR4/R5/TM; Fig. 2A,B,d). These results strongly suggest that the N- and C-terminal interaction leading to the dominant-negative effect occurs mainly through the R1-GTPase-R2-R3 regions. The fact that the conserved R regions alone (R1, R2, R3 and R4) are not sufficient for the interaction indicates that the GTPase contributes to maintain the conformation of the N-terminal R1-R4 region so as to fit the complementary structure of the C-terminal R6-R7.

GTPase domain- and transmembrane span-lacking soluble mutants of Mfn2 disrupt mitochondrial membrane potential

When a deletion mutant lacking the GTPase and transmembrane domains (Mfn2GTPaseTM; Fig. 4A) was expressed in COS7 cells, it caused loss of mitochondrial membrane potential and mitochondria became insensitive to MitoTracker, a membrane potential-sensitive dye (Fig. 4B,a,b). Further deletion of the two coiled-coil regions (Mfn2R2/R6; Fig. 4A) did not affect the membrane potential-disrupting activity (Fig. 4B,c,d), leaving two short regions as the sites responsible for the activity. Deletion of either of them (Mfn2AR2/GTPase/TM, Mfn2AR2/GTPase/TM/R6; Fig. 4A) resulted in loss of the disrupting activity, yielding normal potential and morphology (data not shown). Interestingly, these regions (R2 and R6) are highly conserved among Mfn1 and Mfn2 of various species (Fig. 4A).

Loss of mitochondrial membrane potential is known to lead to fragmentation of mitochondria (Legros et al., 2002; Ishiihara et al., 2003), we therefore determined whether Mfn2GTPaseTM induces mitochondrial fragmentation by staining mitochondria with anti-Hsp60, a mitochondrial protein present in the intermembrane space. Mfn2GTPaseTM caused fragmentation of mitochondria (Fig. 4C) without causing loss of mitochondrial DNA (Fig. 4D). Concerning the mechanisms of Mfn2GTPaseTM-induced loss of mitochondrial inner membrane potential, direct activation of the uncoupling protein (UCP) appears unlikely since UCP is an inner membrane protein and Mfn2GTPaseTM is present in the cytosol and their direct interaction is physically impossible. Mfn2GTPaseTM may interact with certain component(s) of the apoptotic signaling system in the cytosol and trigger the signal that leads to loss of mitochondrial membrane potential and fragmentation. However, the possibility remains that a minor part of the overexpressed molecules goes to mitochondria and exerts such a deteriorating effect.
Fig. 3. GTPase-dependent interaction of the Mfn2 amino and carboxyl termini exposed to cytoplasm is necessary for mitochondrial fragmentation in COS7 cells. (A) Schematic diagram of the Mfn2 deletion and point mutants used to analyze mitochondrial fragmentation, and percentage of transfected cells with fragmented mitochondria. All constructs of Mfn2 with the N terminus exposed to the cytoplasm were epitope-tagged with the 3×FLAG sequence present at the C terminus. Mfn2C construct is epitope-tagged with the Myc sequence present at the N terminus. Boxed C, predicted coiled-coil domain; gray, GTPase domain; black, predicted transmembrane domains. The G2 Mfn2-T130A mutation is equivalent to a mutation in the Ras GTPase that abolishes effector interactions. (B) COS7 cells were transiently cotransfected with expression vectors for Myc-Mfn2C and Mfn2N-FLAG (a-f) or Mfn2NTR2-R4-FLAG (g-i). After 16 hours, the cells were labeled with MitoTracker to visualize mitochondria (c,f,i), fixed, and stained with anti-FLAG antibody and anti-Mfn2C antibody followed by Alexa Fluor 488-conjugated secondary antibody (a,d,g) and Alexa Fluor 350-conjugated secondary antibody (b,e,h), respectively. Mitochondrial fragmentation was induced in COS7 cells expressing both Mfn2N and Mfn2C (arrow) but not Mfn2N alone (star; reticular structure is normal) or Mfn2C alone (arrowhead). Faint mitochondrial staining, seen in b, e and h, is not due to staining of endogenous Mfn2 on mitochondria by anti-Mfn2C since it disappeared when Alexa Fluor 350-labeled secondary antibody (blue) was replaced with Alexa Fluor 488-labeled one (green). The staining is probably an artifact caused by autofluorescence: on blue laser irradiation, mitochondria become weakly autofluorescent (blue). Fluorescence of Alexa Fluor 350 is relatively weak compared to that of Alexa Fluor 488 and requires longer exposure for imaging. Under such conditions, mitochondrial autofluorescence becomes inevitable. (C) HEK293T cells were transiently cotransfected with expression vectors for Myc-Mfn2C and Mfn2N-FLAG, Mfn2NTR2-R4-FLAG, Mfn2NAR1/GTPase-FLAG, Mfn2NAR1-FLAG, or Mfn2NAR1/GTPase-FLAG. After 16 hours, cell lysates were immunoprecipitated with anti-FLAG affinity agarose, separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with rabbit anti-Mfn2C antiserum or anti-FLAG antibody. Bar, 20 µm.
Fig. 4. Loss of mitochondrial membrane potential induced by overexpression of the fusion protein lacking the GTPase domain and transmembrane domain of Mfn2, in COS7 cells. (A) Schematic diagram of the Mfn2 deletion mutants used and percentage of transfected cells with loss of mitochondrial membrane potential. All constructs are epitope-tagged with the 3×FLAG sequence. Yellow, predicted coiled-coil domains; gray, GTPase domain; black, predicted transmembrane domains. (B) COS7 cells transiently transfected with expression vector for FLAG-Mfn2GTPase/TM (a,b) and FLAG-Mfn2R2/R6 (c,d). After 16 hours of transfection, the cells were stained by the membrane potential-sensitive dye MitoTracker Red (b,d), fixed, and then immunostained with anti-FLAG antibody followed by Alexa Fluor 488-conjugated secondary antibody (a,c). An arrow and an arrowhead indicate transfected cells expressing FLAG-Mfn2GTPase/TM and FLAG-Mfn2R2/R6, respectively; stars indicates normal mitochondria with a reticular network. (C) COS7 cells transiently transfected with an expression vector for FLAG-Mfn2GTPase/TM (a-d). After 16 hours of transfection, the cells were stained with MitoTracker as the membrane potential-sensitive dye (b), fixed, and stained with anti-Mfn2-C antibody (a) and anti-Hsp60 antibody as mitochondrial marker (c) followed by Alexa Fluor 350-conjugated secondary antibody and Alexa Fluor 488-conjugated secondary antibody, respectively. A merged image is shown in panel d. (D) Mfn2GTPase/TM-induced loss of mitochondrial membrane potential without loss of mitochondrial DNA. COS7 cells expressing Mfn2GTPase/TM were stained with MitoTracker, with Hoechst 33342 as DNA dye (b), and anti-Hsp60 antibody as mitochondrial marker (k) followed by Alexa Fluor 488-conjugated secondary antibody. Merged images are shown in a and d; (b-d) higher magnifications of the boxed area in a. Bar, 20 µm.
Discussion

Mitochondrial fusion is essential for various biological functions in eukaryotic cells. The *Drosophila* protein Fzo is required for formation of the giant mitochondrial derivative during spermatogenesis (Hales and Fuller, 1997). The *S. cerevisiae* homologue fzo1p mediates mitochondrial fusion events during mitotic growth and mating and is required for long-term maintenance of mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998). Knockout mice for the mammalian homologue, Mfn1 or Mfn2, die in midgestation, indicating that these proteins are essential for embryo development (Chen et al., 2003). The molecular mechanisms controlling mitochondrial fusion, however, are not well understood. In the present study, we demonstrated an interaction between the N- and C-terminal fragments of mouse Mfn2 by activity assay and immunoprecipitation. Such interaction has been suggested by Rojo et al. (Rojo et al., 2002) who expressed truncated forms of Mfn2 [i.e. the N-terminal soluble fragment (Mfn2-N) and the rest of the molecule consisting of the transmembrane spans and the C-terminal tail (Mfn2-TM-C)], and found that (i) Mfn2-TM-C can be targeted normally to mitochondria and (ii) Mfn2-N can also be targeted to mitochondria if coexpressed with Mfn2-TM-C whereas it remains in the cytosol when expressed alone. They further showed that deletion of the coiled-coil domain from Mfn2-N eliminates its accumulation in the Mfn2-TM-C-expressing mitochondria. Our contribution is not merely a direct confirmation of the previously suggested coiled-coil-mediated interaction between the N- and C-terminal domains of mitofusins, but demonstrates (i) its dependency on the GTPase activity, (ii) functional significance of the interaction as shown by a marked, dominant-negative effect of the resulting soluble complex (Mfn2N-Mfn2C), and (iii) identification of the novel regions that are necessary for their interaction.

Among the highly conserved regions of Mfn2, the N-terminal R1-GTPase-R2-R3-R4/CC segment was shown, by immunoprecipitation analysis, to interact with the C-terminal tail through not only the coiled-coil domains but also the R1 region in a GTPase-dependent fashion. Interestingly, the N-terminal flanking region of the Fzo/Mfn family members from yeast, fly and nematode, but not from mammals, has an additional third coiled-coil domain (Hermann et al., 1998; Mozdy and Shaw, 2003), which might also be involved in the interaction between the N- and C-terminal tails, suggesting the functional importance of the N-terminal region throughout eukaryotic species. The N- and C-terminal interaction may create new binding site(s) for other fusion factor(s) and initiate the fusion reaction. In yeast, the following three proteins have been shown to interact directly in the mitochondrial outer membrane (Sesaki et al., 2003; Wong et al., 2003): (i) Fzo1p, a GTPase identified as a homologue of *Drosophila* fuzzy onions, which is required for mitochondrial fusion during fly spermatogenesis (Sesaki and Jensen, 1999) and corresponds to mammalian mitofusins; (ii) Ugo1p, an integral membrane protein anchored in the mitochondrial outer membrane (Sesaki and Jensen, 2001); (iii) Mgm1p, a dynamin-related GTPase located, as a peripheral membrane protein, in the intermembrane space (Sesaki et al., 2003; Wong et al., 2003). Gel filtration studies of detergent-solubilized mitochondria showed that Fzo1p is found in an ~800-kDa complex (Rapaport et al., 1998; Fritz et al., 2001) and human Mfn1 in an ~350-kDa complex (Santel et al., 2003). Therefore similar multicomponent fusion machinery such as Fzo1p, Mgm1p and Ugo1p may also be functioning in the mammalian system. The inhibitory effect caused by such a soluble Mfn2N-Mfn2C complex(es) may be due to depletion of protein components essential for making an active fusion machinery around mitofusins on the mitochondrial surface, and the multiple regions of Mfn2 identified here as indispensable segments (R1, R2, R3, R6 and R7) may provide binding sites for the assembly.

Recently, Koshiba et al. (Koshiba et al., 2004) have reported that a heptad repeat region of the Mfn1 C-terminal tail (residues 660-735) forms a dimeric, antiparallel coiled coil and proposed that this interaction mediates tethering between adjacent mitochondria before fusion. The dominant-negative effect observed here is, however, not due to the direct interaction of Mfn2C with the heptad repeat region of endogenous mitofusins expressed on the surface of mitochondria, since addition of Mfn2C alone had no effect on mitochondrial morphology.

In addition, we unexpectedly found deletion mutants of Mfn2 (Mfn2∆GTPase/TM and Mfn2R2/R6; Fig. 4A) that can induce loss of mitochondrial membrane potential. Two groups have reported that mitochondrial fusion is completely inhibited by treatment with protonophores that dissipate mitochondrial membrane potential (Legros et al., 2002; Ishihara et al., 2003). Furthermore, loss or reduction of mitochondrial membrane potential has been observed in a significant population of embryonic fibroblasts established from Mfn1- or Mfn2-knockout mice (Chen et al., 2003), and in cultured cells in which Mfn2 was depleted using an adenovirus vector expressing Mfn2-specific antisense cDNA (Bach et al., 2003). These data indicate that the activity of Mfn is regulated directly or indirectly by mitochondrial membrane potential, and lowering of the membrane potential triggers the fusion reaction, leading to rescue of abnormal mitochondrial phenotypes by complementation with components of the fusion counterparts (normal mitochondria). R2 and R6 segments of Mfn2 identified here may be a key regulator of mitochondrial membrane potential or bind such regulator molecules; the soluble constructs Mfn2∆GTPase/TM and Mfn2R2/R6 may be useful for identifying such factors. More analysis of the segments may shed new light on the relationship between mitochondrial fusion and mitochondrial membrane potential.

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