Regulation of mitochondrial fission and apoptosis by the mitochondrial outer membrane protein hFis1

Tianzheng Yu[1], Randall J. Fox[1], Lindsay S. Burwell[2] and Yisang Yoon[1,*]

[1]Department of Anesthesiology and of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA
[2]Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA
*Author for correspondence (e-mail: yisang_yoon@urmc.rochester.edu)

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Summary

Mitochondrial fission is a highly regulated process mediated by a defined set of protein factors and is involved in the early stage of apoptosis. In mammals, at least two proteins, the dynamin-like protein DLP1/Drp1 and the mitochondrial outer membrane protein hFis1, participate in mitochondrial fission. The cytosolic domain of hFis1 contains six α-helices that form two tetratricopeptide repeat (TPR) motifs. Overexpression of hFis1 induces DLP1-mediated fragmentation of mitochondria, suggesting that hFis1 is a limiting factor in mitochondrial fission by recruiting cytosolic DLP1. In the present study, we identified two regions of hFis1 that are necessary for correct fission of mitochondria. We found that the TPR region of hFis1 participates in the interaction with DLP1 or DLP1-containing complex and that the first helix (α1) of hFis1 is required for mitochondrial fission presumably by regulating DLP1-hFis1 interaction. Misregulated interaction between DLP1 and hFis1 by α1 deletion induced mitochondrial swelling, in part by the mitochondrial permeability transition, but significantly delayed cell death. Our data suggest that hFis1 is a main regulator of mitochondrial fission, controlling the recruitment and assembly of DLP1 during both normal and apoptotic fission processes.

Key words: Mitochondria, Fission, Dynamin-like protein, hFis1, Permeability transition, Apoptosis

Introduction

Mitochondria in mammalian cells frequently change their shapes by fission and fusion (Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994). While the precise reason for the shape changes is not fully understood, perturbations of these processes affect cell viability and the evolutionarily conserved proteins mediate these processes (Karbowski and Youle, 2003), suggesting that mitochondrial fission and fusion are important for cell survival. A set of proteins that participate in mitochondrial fission has been identified in yeast and mammals (Cerveny et al., 2001; James et al., 2003; Karbowski et al., 2004; Mozdy et al., 2000; Otsuga et al., 1998; Pitts et al., 1999; Smirnova et al., 1998; Tieu and Nunnari, 2000; Yoon et al., 2003). One of these proteins, DLP1/Drp1 in mammals and Dnm1p in yeast, is a member of the dynamin family of large GTPases and mediates the scission of mitochondrial membranes through GTP hydrolysis. The mitochondrial outer membrane protein Fis1p/hFis1 is a small 17 kDa protein with a single transmembrane domain at the C-terminal end such that the bulk of the molecule is exposed to the cytosol (Mozdy et al., 2000). It has been suggested that hFis1 recruits DLP1 from the cytosol to mitochondria for the fission reaction (Mozdy et al., 2000; Shaw and Nunnari, 2002; Yoon et al., 2003). Structural analyses of hFis1 revealed that the N-terminal cytosolic domain of hFis1 forms six α-helices (α1-α6) (Dohm et al., 2004; Suzuki et al., 2003). Among those, α2-α3 and α4-α5 were identified to adopt tetratricopeptide repeat (TPR) folds that are known to mediate protein-protein interactions (Dohm et al., 2004; Suzuki et al., 2003).

Recent studies indicate that mitochondrial fission contributes to mitochondria-mediated apoptosis (Frank et al., 2001; James et al., 2003; Lee et al., 2004). Upon apoptotic stimuli, mitochondria often display dramatic shape change, namely a thread-grain transition, which is mediated by mitochondrial fission proteins, before releasing apoptotic factors (Skulachev et al., 2004). It has been found that overexpression of hFis1 induces mitochondrial fragmentation and apoptosis (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003) whereas inhibition or knockdown of hFis1 or DLP1 blocks cytochrome *c* release and apoptotic progression (Frank et al., 2001; Lee et al., 2004). While it is clear that mitochondrial fission proteins are necessary for cytochrome *c* release and apoptotic progression (Frank et al., 2001; Lee et al., 2004), the molecular mechanisms by which fission proteins participate in the early stage of apoptosis are not fully understood.

To define the role of hFis1 in mitochondrial fission, in this study, we generated multiple truncated and mutated hFis1 proteins and assessed mitochondrial morphology and DLP1-binding properties. We have identified two domains of hFis1 important for mitochondrial fission. We found that the TPR of hFis1 is involved in the DLP1 binding and the N-terminal α1-helix of hFis1 in regulating the interaction between DLP1 and

hFis1. Our results indicate that aberrant interaction between DLP1 and hFis1 induces mitochondrial swelling, and delays downstream apoptotic progression. These data provide mechanistic insight as to how mitochondrial fission proteins interact and how this interaction is regulated during normal and apoptotic fission processes.

Materials and Methods

Cell culture, plasmid construction and transfection

The cell lines Clone 9 (ATCC CRL-1439) and BHK-21 (ATCC CCL-10) were used for all experiments. Cells were maintained at 37°C, 5% CO_2 in Ham's F-12K medium (Clone 9) or in DMEM (Dulbecco's modified eagle's medium; BHK-21), supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. A stable cell line (Clone 9) carrying GFP or RFP in mitochondrial matrix was maintained in 200 μg ml^{-1} G418. Mutant constructs were generated by standard molecular biology techniques using PCR. cDNAs were subcloned into the pcDNA3 vector (Invitrogen Inc.) for expression in mammalian cells. Absence of unwanted mutations after PCR were verified by DNA sequencing. For cell transfection, DNA constructs were purified using plasmid purification columns (Qiagen). Cells were plated 16-24 hours before transfection either on glass coverslips in 35 mm tissue culture dishes for immunofluorescence or in 100 mm Petri dishes for immunoprecipitation. Transfections were performed using LipofectAMINE® (Invitrogen, Inc.) per the manufacturer's instructions. Transfected cells were allowed to recover for 16-24 hours before processing for either immunofluorescence or immunoprecipitation.

Immunoprecipitation

BHK-21 cells transfected with hFis1 cDNA constructs were rinsed with cold Buffer A (20 mM Hepes pH 7.2, 100 mM KCl, 2 mM $MgCl_2$) and scraped in Buffer A containing 1.0% Triton X-100 plus protease inhibitor cocktail (Sigma). After removing insoluble debris by centrifugation, anti-DLP1 antibodies (DLP-MID) (Yoon et al., 1998) were added to the cell extract and incubated for 2 hours at 4°C with continuous mixing. Immune complexes were isolated using protein A-sepharose beads (Sigma). Beads were rinsed four times with Buffer A containing 0.5% Triton X-100 and the immune complexes were then eluted with SDS-PAGE sample buffer.

Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously (Henley and McNiven, 1996). Briefly, cells were fixed and permeabilized, and then incubated in blocking buffer containing 5% horse serum for 1 hour at 37°C. The rabbit polyclonal anti-DLP-N (Yoon et al., 1998), mouse monoclonal anti-c-Myc (Clone 9E10: Sigma), mouse monoclonal anti-cytochrome *c* (Pharmingen), and rabbit polyclonal anti-Bcl-2 (Santa Cruz Biotech.) were used for primary antibodies. For secondary antibodies, Alexa 488 or 594-conjugated anti-mouse or rabbit antibodies (Molecular Probes) were used. After appropriate rinsing, coverslips were mounted in ProLong antifade reagent (Molecular Probes) on glass slides and cells were viewed with an Olympus IX71 epifluorescence microscope. Fluorescence images were acquired with an Evolution QEi camera (Mediacybernetics, Inc.) driven by IPLab imaging software (Scanalytics, Inc.). Acquired images were adjusted using Adobe Photoshop (Adobe Systems Inc.) software.

Cyclosporin A and bongkrekic acid treatments, Bcl-2 co-expression, cytochrome *c* assay

Clone 9 cells transfected with Myc-hFis1[32-152] were treated with mitochondrial permeability transition (MPT) inhibitors, cyclosporin A (5 μM, Calbiochem) or bongkrekic acid (25 μM, Calbiochem) at 4 hours post transfection. After further incubation for 20 hours, cells were fixed for indirect immunofluorescence. For testing the effect of Bcl-2 on mitochondrial swelling, Bcl-2 and Myc-hFis1[32-152] were co-transfected into Clone 9 cell and fixed 24 hours after transfection and stained for Bcl-2 and Myc. Bcl-2 cDNA was generously provided by the late Stanley Korsmeyer (Dana-Farber Cancer Institute). Because Myc-hFis1[32-152] was completely co-localized with mitochondria, mitochondrial morphology was judged by anti-Myc staining. For cytochrome *c* staining, Myc-hFis1[32-152] or Myc-hFis1-WT were co-transfected with pEGFP-C1 (Clonetech) as a transfection marker and cells were stained for cytochrome *c*.

Cobalt-quenched calcein measurement

The mitochondrial permeability transition was assayed by measuring cobalt-quenched calcein fluorescence in mitochondria as described previously (Scorrano et al., 1999). Clone 9 cells transfected with Myc-hFis1[32-152] were loaded with 1.0 μM calcein-acetomethoxy ester (Molecular Probes/Invitrogen Inc.) and 1.0 mM $CoCl_2$ in Hank's balanced salt solution (Invitrogen) for 20 minutes at 37°C. At the end of the incubation, mitochondria were labeled with 25 nM MitoTracker Red CMXRos (Molecular Probes) for 10 minutes. Fluorescence images were acquired for both MitoTracker and calcein, and fluorescence intensity was measured in mitochondrial regions by imaging software (IPLab, Scanalytics).

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation (Almeida and Medina, 1997). BHK-21 cells were plated in 100 mm Petri dishes and either mock transfected or transfected with appropriate DNA constructs. At 16 hours post transfection, cells were rinsed once with cold isolation buffer (IB:10 mM Hepes pH 7.2, 1 mM EDTA, 320 mM sucrose) and harvested in 1.0 ml IB per dish. Cells were centrifuged at 700 ***g*** for 5 minutes and the pellet was resuspended in the IB. After cells were homogenized with 20 strokes in a Dounce homogenizer, homogenate was centrifuged at 700 ***g*** for 8 minutes. While the supernatant (S1) was kept in ice, the pellet was homogenized and centrifuged again. The pellet was discarded and the supernatant was pooled with S1 (PNS: post-nuclear supernatant). PNS was centrifuged at 17,000 ***g*** for 15 minutes. The supernatant was collected as the cytosol and the brown mitochondrial pellet was resuspended in IB after whitish material in the pellet was carefully discarded.

Results

α1-helix of hFis1 is necessary for mitochondrial fission

It has been shown that overexpression of hFis1 induces mitochondrial fragmentation (James et al., 2003; Stojanovski et al., 2004; Yoon et al., 2003). We have previously shown that deletion of the N-terminal region of hFis1 abolished the mitochondrial fragmentation but did not alter the mitochondrial localization of hFis1 (Yoon et al., 2003). To further define the role of the N-terminal region of hFis1, we transfected cells with Myc-tagged hFis1 deletion constructs (Fig. 1) and assessed the effects on mitochondrial morphology. The short 10 amino acid Myc-tag at the N-terminus does not interfere with the normal function of hFis1 (Yoon et al., 2003). Upon transfection of Clone 9 cells harboring fluorescently labeled mitochondria, approximately 90% of cells overexpressing full-length hFis1 (Myc-hFis1-WT) contained fragmented mitochondria, as shown previously (Yoon et al., 2003) (Fig. 2A). Deletion of the first 10 amino acids of hFis1

(Myc-hFis1[11-152]) reduced the number of cells with fragmented mitochondria to 55%, and greater reductions of the fragmented phenotype was observed upon further deletions (Fig. 2A). Among Myc-hFis1[21-152] overexpressing cells, 28% showed fragmented mitochondria, whereas the frequency of cells with fragmented mitochondria was markedly reduced to less than 5% upon overexpression of Myc-hFis1[32-152], Myc-hFis1[61-152], or Myc-hFis1[92-152]. The cytosolic domain of hFis1 has been shown to contain six α-helices (α1-α6) (Dohm et al., 2004; Suzuki et al., 2003). The first helix (α1) is included in the first 27 amino acids that are deleted in the Myc-hFis1[32-152]. Therefore, our data indicate that the α1-helix of hFis1 is important for mitochondrial fission. Unexpectedly, we observed a third phenotype of mitochondrial morphology. In more than 70% of the cells overexpressing Myc-hFis1[32-152], individual mitochondria were swollen and enlarged, showing ball-shape appearance (Fig. 2A). Deletion of the first 60 amino acids of hFis1 (Myc-hFis1[61-152]) still induced mitochondrial swelling (40%). However, the 91 amino acid deletion (Myc-hFis1[92-152]) did not cause any swelling, showing the tubular mitochondrial phenotype in the majority of the transfected cells (Fig. 2A).

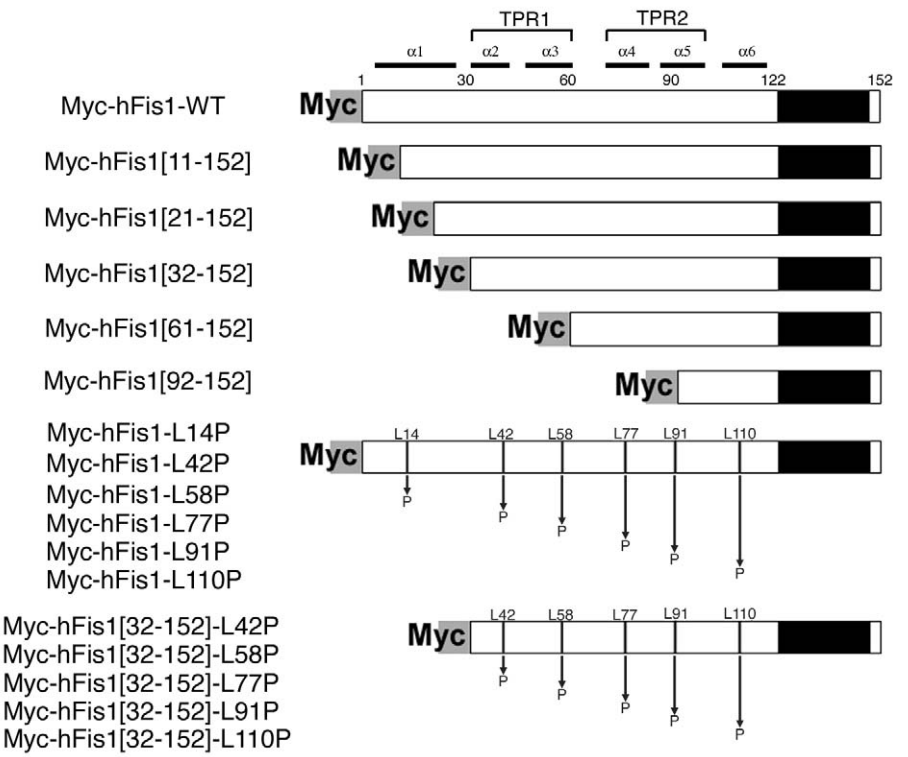


Fig. 1. Illustration of hFis1 constructs used in this study. All hFis1 constructs were tagged with the Myc-epitope at the N-terminus. The hFis1 transmembrane domain spanning the mitochondrial outer membrane is depicted in black near the C-terminal end. Wild-type hFis1 (hFis1-WT) contains six α-helices (α1-α6) in its cytosolic domain. Helices α2-α3 and α4-α5 form the two TPR motifs, TPR1 and TPR2, respectively.

DLP1 or DLP1-containing complex binds to the TPR region of hFis1

Previous studies in yeast indicate that Fis1p recruits Dnm1p to the mitochondrial surface for the fission reaction (Mozdy et al., 2000; Shaw and Nunnari, 2002). Because overexpression of the α1-deleted hFis1 greatly decreases mitochondrial fission, this implicates that the α1 is a potential domain of hFis1 that interacts with the DLP1 complex. In our previous report, the interaction between DLP1 and the full-length hFis1 appeared extremely weak and transient, and it was detectable only after chemical crosslinking (Yoon et al., 2003). However, we found increased interactions between DLP1 and the truncated hFis1 proteins, as they were co-immunoprecipitated without crosslinking (Fig. 2B). While very little or no interaction with DLP1 was observed using the full length (FL) or the 10 amino acid deletion, Myc-hFis1[21-152], Myc-hFis1[32-152] and Myc-hFis1[61-152] were consistently co-precipitated with DLP1 (Fig. 2B). However, no interaction was observed between Myc-hFis1[92-152] and DLP1. These results demonstrate that the N-terminal α1 of hFis1 does not contain the DLP1-binding site. Furthermore, this result indicates that removal of the N-terminal α1 of hFis1 increases or stabilizes the interaction between hFis1 and DLP1.

The crystal structure of the cytosolic domain of hFis1 revealed the presence of six α-helices (α1-α6) that form two TPR motifs (Dohm et al., 2004; Suzuki et al., 2003). The first TPR motif (TPR1) is composed of α2 and α3 (aa32-61), and the second (TPR2) α4 and α5 (aa66-101) (Dohm et al., 2004; Suzuki et al., 2003). TPR1 and 2 form a concave structure, providing a potential binding surface for its partners (Dohm et al., 2004). Our co-immunoprecipitation experiments indicated that DLP1 interacts with 20, 31 and 60 amino acid deletions, but does not interact with the 91 amino acid deletion that eliminates the majority of the TPR motifs (Fig. 2B), strongly suggesting that the TPR region binds to DLP1 or DLP1-containing complex. To assess the role of the six helices in DLP1 binding and mitochondrial fission, we made helix-breaking point mutations (leucine-to-proline) in each helix (α1, L14P; α2, L42P; α3, L58P; α4, L77P; α5, L91P; α6, L110P). If these mutations interfere with DLP1 binding, it is expected that hFis1-induced mitochondrial fragmentation would be abolished. As shown previously, fragmented mitochondria as a result of extensive fission were observed in majority of the cells overexpressing the wild-type hFis1. By contrast, the mutations in α2 (L42P), α3 (L58P), α4 (L77P) and α5 (L91P), which form TPR motifs, effectively reduced the extensive fission, showing that less than 15% of the mutant overexpressing cells contained fragmented mitochondria (Fig. 3A). However, in cells overexpressing Myc-hFis1-L14P or Myc-hFis1-L110P, approximately 40% of the cells still displayed fragmented mitochondria, indicating that these point mutations were less effective in abolishing mitochondrial fragmentation. This data indicates that correct conformations of the four helices forming

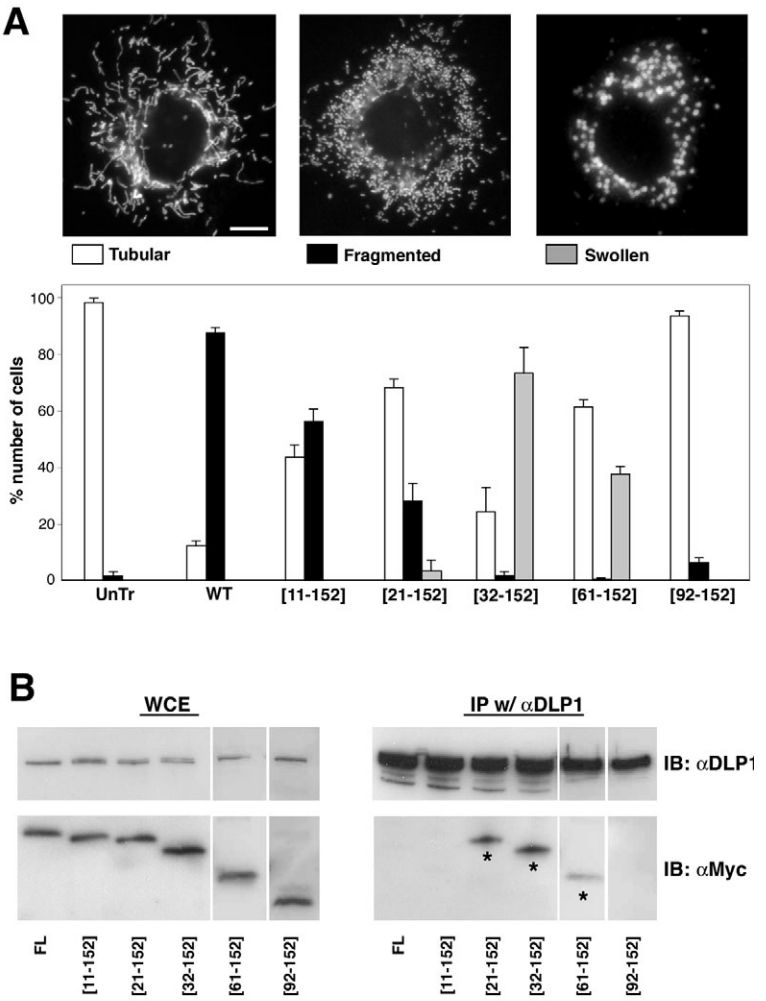


Fig. 2. Deletion of α1-helix of hFis1 abolishes mitochondrial fragmentation and increases the DLP1-hFis1 interaction. (A) Deletions in the N-terminal cytosolic domain of hFis1 abolished mitochondrial fragmentation. Myc-tagged hFis1 full length (WT) and deletion constructs, hFis1[11-152], hFis1[21-152], hFis1[32-152], hFis1[61-152] and hFis1[92-152] were transfected into Clone 9 cells harboring GFP-labeled mitochondria and cells overexpressing the Myc-tagged proteins were detected by immunofluorescence using the anti-Myc antibody. Two-hundred to 300 untransfected cells and cells transfected with each construct were counted to score mitochondrial morphology. The upper three panels represent the three mitochondrial morphologies used for scoring the different mitochondrial phenotypes. Mitochondria were detected by GFP fluorescence and anti-Myc staining completely overlapped with the GFP signal. While untransfected cells contained normal tubular mitochondria (top left, bottom UnTr), mitochondria in cells overexpressing hFis1-WT were finely fragmented, showing a punctate mitochondrial phenotype (top middle, bottom WT). The number of cells containing fragmented mitochondria decreased as N-terminal amino acids were further deleted. The third phenotype that shows swollen, ball-shape mitochondria (top right) were prevalent in cells overexpressing Myc-hFis1[32-152]. Scale bar, 10 μm. (B) Deletion of the N-terminal region of hFis1 enhances the hFis1-DLP1 interaction. BHK-21 cells were transfected with full length (FL) or N-terminally truncated hFis1 constructs and whole cell extract (WCE) was subjected to immunoprecipitation by anti-DLP1 antibodies. Immunoprecipitated proteins were analyzed by immunoblotting with anti-DLP1 and anti-Myc antibodies. All Myc-tagged hFis1 constructs, FL and deletion mutants, were expressed in cells as detected in the WCE. Myc-hFis1[21-152], Myc-hFis1[32-152], and Myc-hFis1[61-152] were readily detected in immune complexes isolated by the anti-DLP1 antibody while little binding was obtained with cells expressing FL and Myc-hFis1[11-152]. No binding was detected with Myc-hFis1[92-152]. 1% of starting materials and 50% of immunoprecipitated materials were loaded onto the gel for immunoblot analyses.

TPR motifs (α2–α5) are essential for mitochondrial fission, presumably providing a DLP1 binding site.

In addition, cells overexpressing hFis1 containing the TPR mutations frequently showed a fission-defective mitochondrial phenotype, indicating that theses mutations exert the dominant-negative effect (Fig. 3B). These mitochondria were overly elongated and often severely entangled around the nucleus. Cells with mitochondrial morphologies shown in Fig. 3B were found throughout cells overexpressing four TPR mutants. Cell counting demonstrated that 25 to 60% of TPR mutant cells containing tubular mitochondria showed the fission-defective mitochondrial phenotype, whereas non-TPR mutants showed no mitochondrial elongation (Fig. 3C). For more qualitative analyses, we measured pixel lengths of mitochondrial tubules. Because it was difficult to quantify severely entangled mitochondria by systemized morphometrical analyses, we selected cells with less severe phenotype and measured pixel lengths of only discernible mitochondria. Although these values are gross underestimations of the elongation in mutant cells due to excluding perinuclear entanglement, results in Fig. 3D clearly showed overall increase of mitochondrial length in these cells. Possible dimerization of yeast Fis1p has been previously reported (Mozdy et al., 2000) and dimers of hFis1 have been observed in the crystal structure (Dohm et al., 2004). The dominant-negative effect by TPR mutants suggests that dimerization or oligomerization of hFis1 may be important for forming the DLP1 binding site. Little or no dominant-negative effect by L14P and L110P suggests that α1 and α6 of hFis1 are not a major part of the binding site for DLP1, although structural changes of these helices do affect mitochondrial fission (Fig. 3A).

Deletion of the first three α-helices (hFis1[61-152]) eliminates TPR1 but still interacts with DLP1, albeit in a reduced extent, suggesting a participation of the three downstream helices (α4-α6) in binding to DLP1 or DLP1-containing complex (Fig. 2B). To assess the role of the three downstream helices in DLP1 binding, we performed immunoprecipitation with three hFis1 mutants L77P (α4), L91P (α5), and L110P (α6) in Myc-hFis1[32-152] that readily co-immunoprecipitates with DLP1. As shown in Fig. 3E, L77P and L91P mutations in Myc-hFis1[32-152] showed a greatly decreased binding to DLP1, whereas the L110P mutation maintained the binding to a level similar to the simple deletion Myc-hFis1[32-152] upon co-immunoprecipitation. It is possible that these mutations may

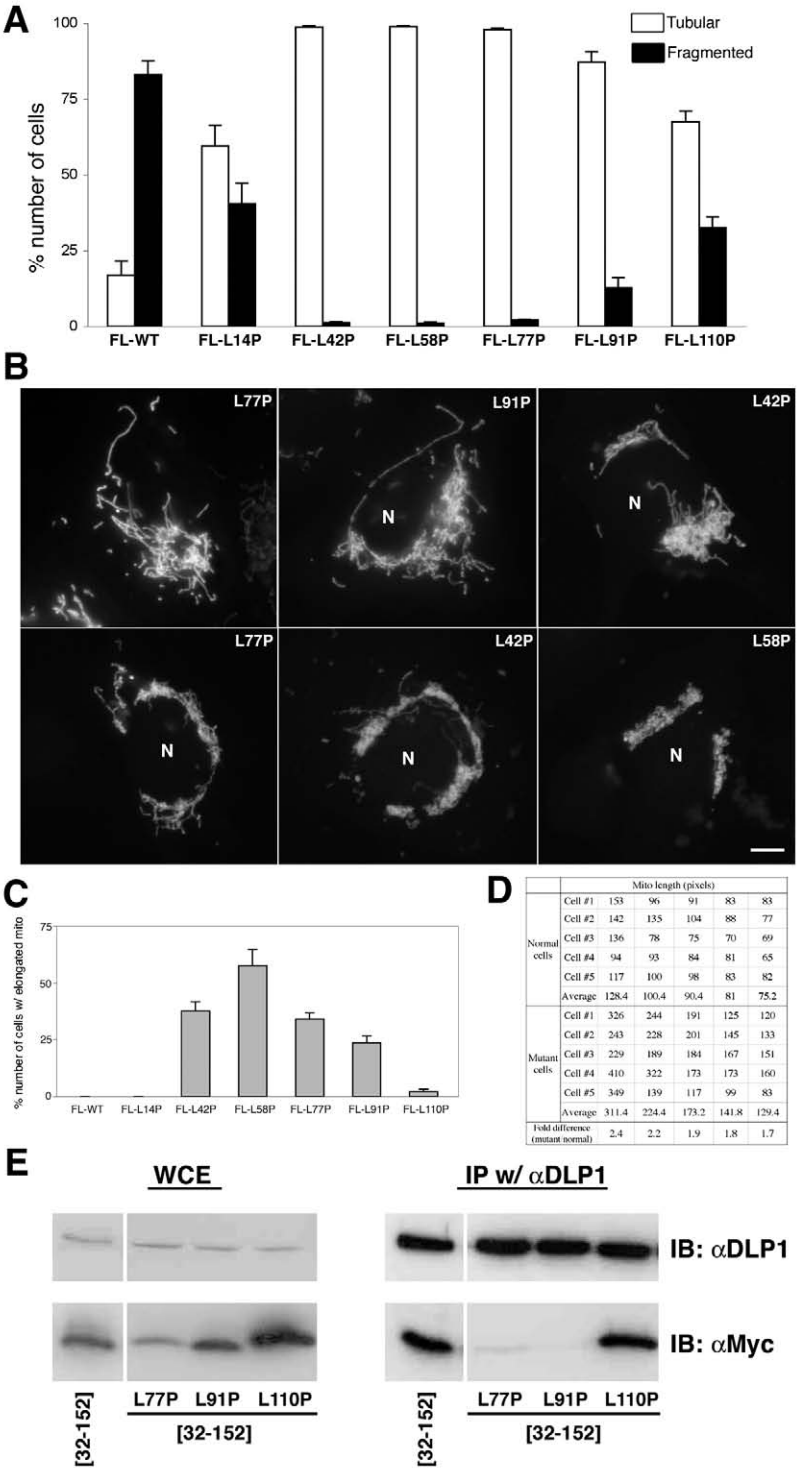


Fig. 3. Two TPR motifs are important for interaction with DLP1 or DLP1-containing complex. (A) Helix-breaking point mutations in α1 (L14P), α2 (L42P), and α3 (L58P), α4 (L77P), α5 (L91P), and α6 (L110P) were made in full-length hFis1 and overexpressed in Clone 9 cells to test their effects on mitochondrial fragmentation. The L42P, L58P, L77P, and L91P point mutations in helices forming TPR abolished the mitochondrial fragmentation effectively, showing that less than 15% of the point mutant-expressing cells contained fragmented mitochondria. L14P and L110P point mutations in non-TPR helices were less effective in abolishing mitochondrial fragmentation. (B) Gallery of cells expressing TPR mutations showing the dominant-negative mitochondrial phenotype. These mitochondrial morphologies were found throughout cells overexpressing four TPR mutants. Mitochondria are elongated and often severely entangled around the nucleus indicative of a fission-defective mitochondrial phenotype, suggesting that these point mutants exert a dominant-negative effect. N, nucleus; scale bar, 10 μm. (C,D) Morphometric analyses of mitochondrial elongation in cells expressing TPR mutants. Cell counting revealed that 25-60% of cells expressing TPR mutants displayed the dominant-negative mitochondrial phenotype whereas non-TPR mutants showed no mitochondrial elongation (C). For morphometric analysis, five mutant cells with less severely entangled mitochondria and five untransfected normal cells were selected, and pixel lengths were measured only in clearly discernible mitochondria. Pixel values representing the length of mitochondria were sorted from the longest to the shortest, and the five longest mitochondrial tubules from each cell are presented (D). Note that overall lengths of mitochondrial tubules are longer in mutant cells, showing approximately two-fold increase. (E) α4 and α5 participate in binding DLP1 or DLP1-containing complex. Three helix-breaking mutants L77P, L91P, and L110P in Myc-hFis1[32-152] were tested for DLP1 binding by co-immunoprecipitations. Both L77P and L91P mutations greatly reduced the binding to DLP1, whereas the L110P mutant showed the DLP1 interaction to the level similar to Myc-hFis1[32-152]. 1% of starting materials and 50% of immunoprecipitated materials were loaded onto the gel for immunoblot analyses.

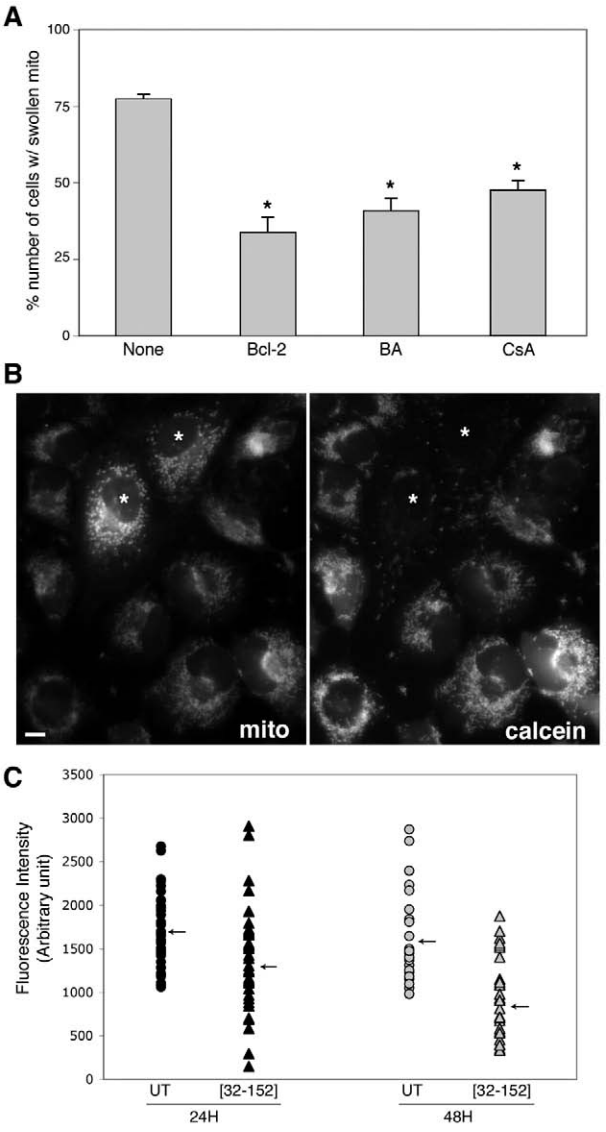
affect proper protein folding and alter the different region of hFis1 that binds to DLP1. However, expressed mutant proteins were correctly targeted to mitochondria, which requires chaperon binding and the mitochondrial import process, suggesting that overall folding was likely unaffected by these mutations. Our data indicate that the correct conformation of TPR motifs of hFis1 is necessary for interacting with DLP1 or DLP1-containing complex. The biochemical and morphological evidence above suggests that DLP1 or a DLP1-containing complex interacts with hFis1 through TPR motifs.

Inhibitors of mitochondrial permeability transition reduce mitochondrial swelling by overexpression of the α1-deleted hFis1

Participation of mitochondrial fission proteins in apoptosis has previously been reported (Frank et al., 2001; James et al., 2003; Lee et al., 2004). However, the precise role of the fission proteins during apoptosis is largely unknown. We observed abnormal swelling of mitochondria by overexpressing α1-deletion of hFis1 (Myc-hFis1[32-152]) (Fig. 2A). Because mitochondrial swelling often occurs by the mitochondrial permeability transition (MPT) in the early stage of apoptosis, we tested if the mitochondrial swelling induced by the α1 deletion of hFis1 is mediated by the MPT.

The anti-apoptotic protein Bcl-2 prevents the MPT by sequestering pro-apoptotic factors including BH3-only protein or by regulating the calcium homeostasis (Green and Kroemer, 2004; Orrenius, 2004; Pinton et al., 2000; Pinton et al., 2001). We tested the effect of the Bcl-2 overexpression on mitochondrial swelling induced by the Myc-hFis1[32-152] expression. As shown in Fig. 4A, co-expression of Bcl-2 and Myc-hFis1[32-152] resulted in marked decrease of mitochondrial swelling. Overexpression of Myc-hFis1[32-152] alone consistently induced the formation of swollen mitochondria in more than 75% of the transfected cells. However, co-expression of Bcl-2 reduced mitochondrial swelling to 30%. We also tested the effect of bongkrekic acid (BA) and cyclosporin A (CsA) that are known to prevent the MPT and found a decrease of mitochondrial swelling (40-45%) as well (Fig. 4A).

To test directly whether the MPT was involved in mitochondrial swelling upon overexpression of Myc-hFis1[32-152], we performed the cobalt-quenched calcein measurement (Scorrano et al., 1999). In this assay, whereas cytoplasmic calcein is quenched by cobalt ions, normal mitochondria are impermeable to cobalt ions, resulting in mitochondrial calcein staining. Upon the MPT, cobalt ions move into mitochondria where they quench and reduce mitochondrial calcein fluorescence (Fig. 4B). As shown in Fig. 4C, we measured calcein fluorescence intensity in mitochondria and observed overall reduction of calcein fluorescence in swollen mitochondria. While levels of calcein staining in mitochondria varied from cell to cell presumably due to variations in timing and level of expression during transient transfection, the median value of calcein fluorescence was reduced approximately two fold in swollen mitochondria in 48 hours post transfection. In addition, we tested mitochondrial inner membrane potential by using tetramethyl rhodamine ethyl ester because the MPT is accompanied with decrease of the membrane potential, and found that swollen mitochondria had markedly reduced membrane potential (results not shown). These results indicate that the MPT contributes to mitochondrial swelling induced by overexpression of the α1-deleted hFis1.



DLP1 binding to the hFis1 TPR is necessary for the mitochondrial swelling induced by α1-deleted hFis1

Because mitochondrial swelling is promoted by the α1 deletion that increases DLP1 binding at the TPR region of hFis1, DLP1 is likely to participate in this process. In support of this notion, we observed the increased association of DLP1 with swollen mitochondria by immunofluorescence. Endogenous DLP1 under normal conditions distributes throughout the cytoplasm and a small population associates with mitochondrial tubules (Pitts et al., 1999; Smirnova et al., 2001; Smirnova et al., 1998).

Fig. 4. The mitochondrial permeability transition contributes to mitochondrial swelling caused by overexpression of α1-deleted hFis1. (A) Clone 9 cells were transfected with Myc-hFis1[32-152] alone or co-transfected with Bcl-2 and Myc-hFis1[32-152], and scored for the swollen mitochondrial morphology. Co-expression of Bcl-2 with Myc-hFis1[32-152] reduced the number of cells containing swollen mitochondria by more than twofold, indicating that the mitochondrial permeability transition (MPT) played a role in mitochondrial swelling. Incubating cells transfected with Myc-hFis1[32-152] with MPT inhibitors bongkrekic acid (BA) or cyclosporin A (CsA) also decreased mitochondrial swelling. (B) Cobalt-quenched calcein measurement. Clone 9 cells transfected with Myc-hFis1[32-152] were loaded with calcein and $CoCl_2$ and stained with MitoTracker Red. (Left panel: mito) Swollen mitochondria by overexpression of Myc-hFis1[32-152] were visible in cells with asterisks. (Right panel: calcein) Calcein fluorescence was greatly reduced in swollen mitochondria (cells with asterisks). Cobalt ions entered mitochondria and quenched calcein fluorescence, indicating that the MPT occurred in swollen mitochondria. (C) Quantitation of calcein fluorescence in mitochondria. Calcein fluorescence in the mitochondrial regions was quantified from digital images. Mitochondrial regions were identified based on MitoTracker staining. Because of cell-to-cell variations of calcein fluorescence, intensity values from each cell were plotted to show the distribution, and median values were indicated as arrows. Fluorescence intensity was measured in 43 cells each for untransfected (UT) and transfected ([32-152]) at 24 hours post transfection (black circle and triangle, respectively), and in 28 cells each for UT and [32-152] at 48 hours post transfection (gray symbols). Experiments were repeated three times and similar quantitative results were obtained in all three experiments.

In cells containing fragmented mitochondria by overexpression of full-length hFis1, distribution of endogenous DLP1 showed little difference from that of untransfected control cells. Fluorescent DLP1 spots were present throughout the cytoplasm as well as were associated with hFis1-decorated punctate mitochondria (Fig. 5A-a,a′). However, we frequently observed accumulation of DLP1 on swollen ball-shape mitochondria induced by overexpression of hFis1[32-152] (Fig. 5A-b,b′). In addition to punctate spots, DLP1 forms patches around swollen mitochondrial spheres coated with hFis1, indicating the increased association of DLP1 with hFis1[32-152].

To further test the increased DLP1 binding to mitochondria in cells overexpressing α1-deleted hFis1, we isolated mitochondrial fractions from untransfected normal cells and cells overexpressing hFis1[32-152] by differential centrifugation and performed immunoblot analyses for DLP1. As shown previously, DLP1 was found mostly in the cytosolic fraction (Fig. 5B). We found that the mitochondrial fraction from cells transfected with hFis1[32-152] contained increased DLP1 compared with that of untransfected cells after normalization (Fig. 5B). This data supports that swollen mitochondria in cells overexpressing hFis1[32-152] contain more DLP1 on their surface. We also found that the co-expression of Bcl-2 with hFis1[32-152] reduced the amount of DLP1 in the mitochondrial fraction to the level in untransfected normal cells (Fig. 5C). These data indicate that mitochondrial swelling induced by overexpression of hFis1[32-152] is likely mediated by increased DLP1 binding to the mitochondrial surface and that Bcl-2 reduces DLP1 accumulation in mitochondria. These results suggest that Bcl-2 may directly or indirectly participate in recruitment of DLP1 to mitochondria.

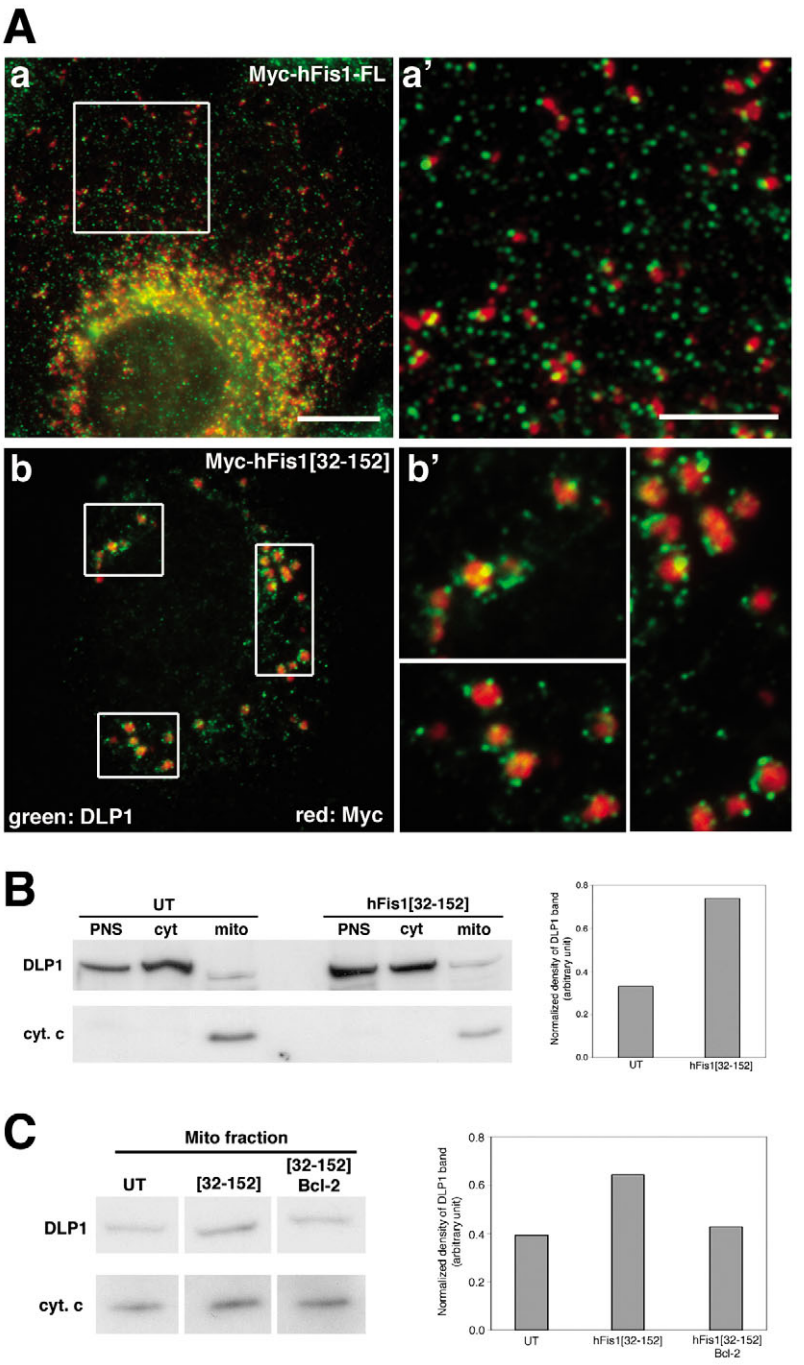
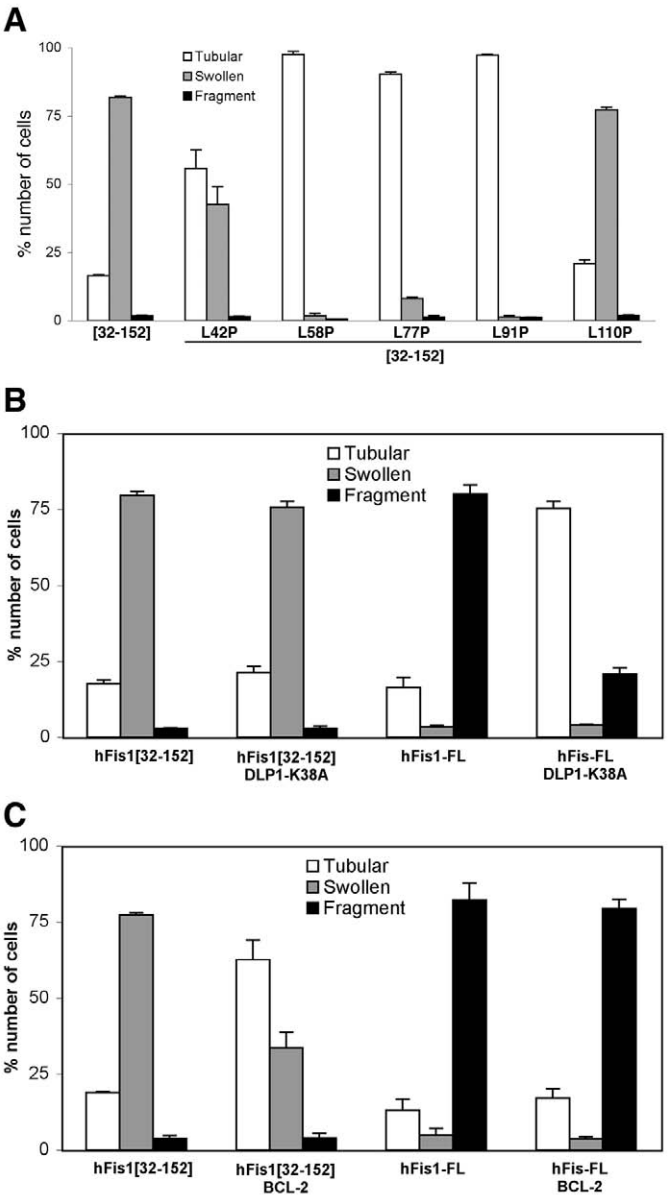


Fig. 5. Swollen mitochondria induced by overexpression of α1-deleted hFis1 contain increased DLP1. (A) Clone 9 cells transfected with Myc-hFis1-FL or Myc-hFis1[32-152] were immunostained with anti-Myc and anti-DLP1 antibodies. DLP1 puncta (green) distributed throughout the cytoplasm in cells expressing Myc-hFis1-FL (a) and associated with fragmented mitochondria stained with anti-Myc (red) shown in enlarged image (a′). However, in cells expressing Myc-hFis1[32-152] (b), the non-mitochondrial distribution of DLP1 appeared to be reduced while more DLP1 (green) associated with Myc-hFis1[32-152] (red) of swollen mitochondria, forming lager spots and patches of DLP1 (b′). Scale bar, 20 μm for a,b, and 10 μm for a′,b′. (B) Cellular fractions isolated from untransfected cells and cells transfected with Myc-hFis1[32-152] were analyzed by immunoblotting. Each lane was loaded with 15-20 μg total protein and probed for DLP1 and cytochrome *c* for a mitochondrial marker. Note that no cytochrome *c* was detected in the cytosol from cells overexpressing Myc-hFis1[32-152], indicating that cytochrome *c* release did not occur at the time of fractionation (16 hours post-transfection). Most DLP1 was found in the cytosolic fraction and mitochondria contained a low level of DLP1 (left panel). PNS, post-nuclear supernatant; cyt, cytosolic fraction; mito, mitochondrial fraction. Densitometry was performed using ImageJ (NIH) software, and density of the DLP1 band in mitochondrial fraction was normalized against the cytochrome *c* band. DLP1 band density after normalization showed increased DLP1 in the mitochondrial fraction from the cells overexpressing Myc-hFis1[32-152] (right panel). (C) Mitochondrial fractions from untransfected cells, Myc-hFis1[32-152]-transfected cells, and cells co-transfected with Bcl-2 and Myc-hFis1[32-152] were immunoblotted for DLP1. Whereas more DLP1 was found in the mitochondrial fraction from cells transfected with Myc-hFis1[32-152], co-expression with Bcl-2 reduced the mitochondrial DLP1 to the level of untransfected cells (left panel). The right panel shows densitometric analysis.

To test whether DLP1 interaction with hFis1 is necessary for mitochondrial swelling, we transfected cells with five helix-breaking mutations in Myc-hFis1[32-152] and assessed their effects on mitochondrial swelling. The number of cells containing swollen mitochondria was dramatically reduced in cells expressing Myc-hFis1[32-152]-L58P, Myc-hFis1[32-152]-L77P, or Myc-hFis1[32-152]-L91P with a concomitant increase of cells containing tubular mitochondria, whereas the L110P mutation had no effect on reducing mitochondrial swelling (Fig. 6A). The L42P mutation in α2 helix was less effective in abolishing mitochondrial swelling, showing only two-fold reduction, although the same mutation in the full-length background almost completely abolished mitochondrial fragmentation. It is currently unclear how the same mutation has differential effects on mitochondrial fragmentation and swelling; however, it is possible that α2 helix participates in initial binding of DLP1 in the presence of α1 helix, but only in part in the stable interaction with DLP1 without the α1. Based on our data that the α1 deletion increases the DLP1 binding at the TPR region and that the mutations abolishing DLP1 binding decrease mitochondrial swelling, it is likely that aberrantly stabilized or prolonged interaction between DLP1 and hFis1 causes mitochondrial swelling partly via the MPT.

To further address the role of DLP1 in mitochondrial swelling, we tested whether the GTPase activity of DLP1 is required for this process by co-expressing Myc-hFis1[32-152] with a dominant-negative DLP1 mutant, DLP1-K38A. DLP1-K38A has been shown to be GTPase-defective and have greatly reduced binding to GTP (Yoon et al., 2001). DLP1-K38A markedly reduced mitochondrial fragmentation induced by full-length hFis1, indicating that hFis1-mediated mitochondrial fission requires GTPase activity of DLP1 (Fig. 6B). However, we found that overexpression of DLP1-K38A had little effect on mitochondrial swelling induced by Myc-hFis1[32-152] (Fig. 6B). This indicates that the hFis1-DLP1 interaction is independent of the DLP1 GTPase activity, suggesting that mitochondrial fission may occur in a two-step process (see discussion). Interestingly, co-expression of Bcl-2 with Myc-hFis1-FL did not affect the mitochondrial fragmentation, whereas Bcl-2 expression reduced the mitochondrial swelling caused by Myc-hFis1[32-152] (Fig. 6C), suggesting that Bcl-2 is not directly involved in the DLP1/hFis1-mediated mitochondrial fission but possibly in the early interaction of hFis1 and DLP1.



Overexpression of α1-deleted hFis1 delays apoptotic progression

Because mitochondrial permeability transition leads to apoptosis, we tested whether cells containing swollen mitochondria induced by overexpression of α1-deleted hFis1 release cytochrome *c* and undergo cell death. However, we found that many of the cells overexpressing Myc-hFis1[32-152] retained cytochrome *c* in swollen mitochondria (Fig. 7A-b,b′), whereas the cytochrome *c* staining was greatly reduced and diffuse in cells expressing Myc-hFis1-WT (a,a′). Among cells transfected with hFis1[32-152], approximately 20% showed diffuse cytochrome *c* staining up to 72 hours post transfection, whereas 70% of cells expressing full-length hFis1 released cytochrome *c* by 72 hours (Fig. 7B). We also observed

Fig. 6. DLP1 binding to hFis1, but not the DLP1-GTPase activity, is necessary for mitochondrial swelling induced by overexpression of α1-deleted hFis1. (A) Leucine-to-proline mutants (L42P, L58P, L77P, L91P, and L110P) in Myc-hFis1[32-152] were expressed in Clone 9 cells and mitochondrial morphologies were analyzed. The TPR mutants (L42P, L58P, L77P, and L91P) in Myc-hFis1[32-152] significantly decreased the mitochondrial swelling phenotype of Myc-hFis1[32-152] although L42P was less effective, indicating that DLP1 binding to the hFis1 TPR is necessary for mitochondrial swelling. The L110P mutation had no effect on mitochondrial swelling. (B) Co-expression of the GTPase-defective DLP1-K38A with Myc-hFis1[32-152] did not reduce the mitochondrial swelling, but mitochondrial fragmentation by Myc-hFis1-FL was greatly decreased by DLP1-K38A, indicating that GTP hydrolysis by DLP1 is required for mitochondrial fission but not for the swelling. (C) Although mitochondrial swelling via α1-deletion (Myc-hFis1[32-152]) was reduced by the Bcl-2 co-expression, Bcl-2 had no effect on mitochondrial fragmentation induced by Myc-hFis1-FL, indicating that Bcl-2 does not directly participate in DLP1/hFis1-mediated mitochondrial fission.

a similar trend of reduced apoptosis in cells overexpressing Myc-hFis1[32-152] judged by nuclear condensation (results not shown).

In addition, we found that the number of cells overexpressing full-length hFis1 greatly decreased to 20% of the initial survival after 72 hours, indicating hFis1-mediated apoptosis as reported previously (James et al., 2003). However, we observed 75-80% of the initial survival in cells overexpressing hFis1[32-152] at 72 hours post transfection (Fig. 7C). These results suggest that apoptotic progression is partially arrested or delayed in cells overexpressing α1-deleted hFis1. To test whether there is a direct link between mitochondrial swelling and cell death, we co-expressed Myc-hFis1[32-152] with Bcl-2 and assessed cell viability. We found that co-expression of Bcl-2, which reduces mitochondrial swelling, increased the cell survival in cells overexpressing Myc-hFis1[32-152]. Approximately 95% of cells co-transfected with Bcl-2 and Myc-hFis1[32-152] survived in 72 hours (Fig. 7C), indicating that Bcl-2 can further decrease cell death presumably by decreasing mitochondrial swelling in cells expressing α1-deleted hFis1.

Discussion

Our data demonstrate that deletion of the N-terminal α1-helix of hFis1 abolishes the ability of hFis1 to promote mitochondrial fission, indicating that α1 is necessary for mitochondrial fission. However, we found that the α1 deletion increases the binding of DLP1-containg complex to the TPR region of hFis1. These observations suggest that the α1 of hFis1 may serve as a regulatory element that destabilizes the DLP1-hFis1 interaction during mitochondrial fission. Why would such a destabilization or dissociation of DLP1 from hFis1 be necessary for mitochondrial fission? It has been observed that more DLP1 is present in the cytosol than on membranes at steady state (Smirnova et al., 2001; Yoon et al., 1998), indicating that the period DLP1 resides on membranes for the fission reaction is short. In the yeast mutant lacking Fis1p, most Dnm1p lost mitochondrial localization, indicating that Fis1p recruits Dnm1p for mitochondrial fission (Mozdy et al., 2000). We found that the interaction between full-length hFis1 and DLP1 is extremely week and/or transient (Yoon et al., 2003), and that, in this report, deletion of the first α-helix of hFis1 increases the interaction with DLP1 while losing the ability to promote mitochondrial fission. It has also been found that purified dynamin or DLP1 alone has a full capacity of constricting membranes in vitro (Sweitzer and Hinshaw, 1998; Yoon et al., 2001). Based on these observations, we speculate that hFis1 mediates DLP1 assembly on the mitochondrial surface via a two-step process. DLP1 would initially bind to the TPR motif of hFis1 resulting in recruitment of DLP1 to the

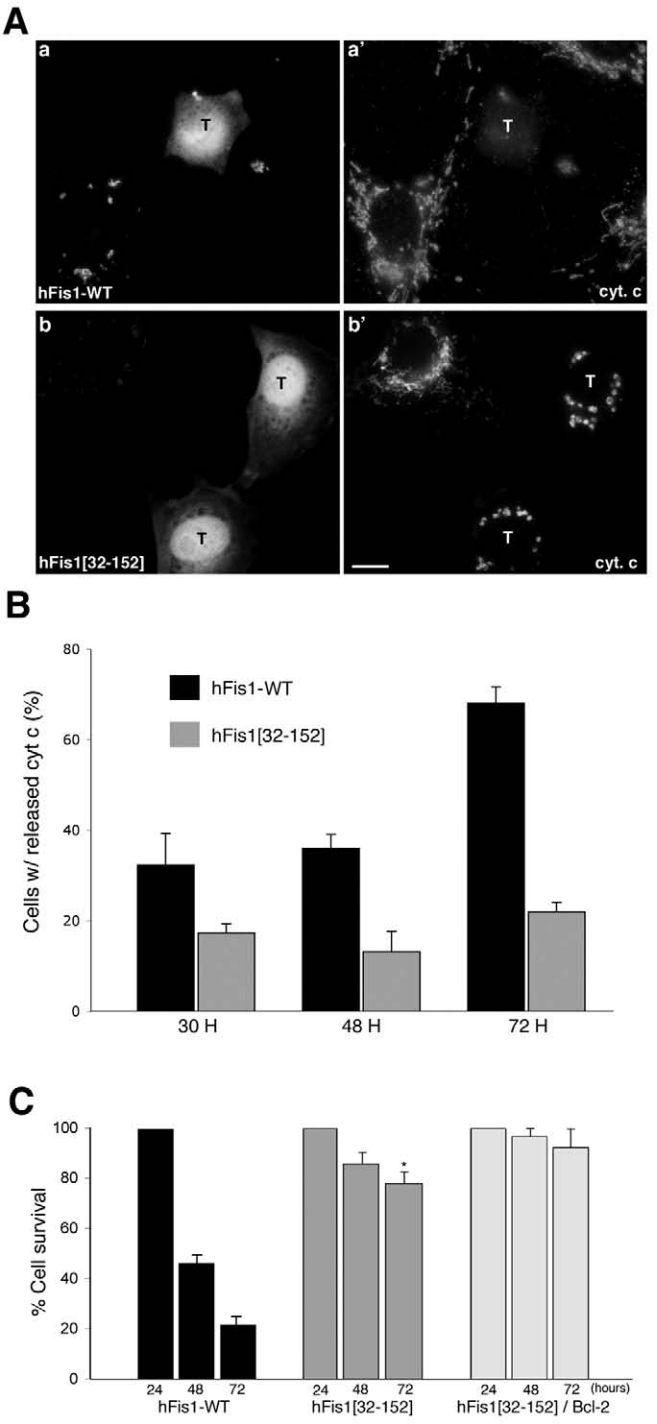


Fig. 7. Cytochrome *c* release and cell death are delayed in cells overexpressing Myc-hFis1[32-152]. Myc-hFis1[32-152] or Myc-hFis1-FL was co-expressed with GFP as a transfection marker and distributions of cytochrome *c* were analyzed by immunofluorescence. (A) Cytochrome *c* staining was greatly reduced and diffuse in cells expressing Myc-hFis1-WT, indicating cytochrome *c* release from mitochondria (a,a′). Cells expressing Myc-hFis1[32-152] contained cytochrome *c* in their swollen mitochondria (b,b′). T: transfected cells. Scale bar, 10 μm. (B) Cells with diffuse cytochrome *c* staining were scored at different times after transfection. Less than 25% of cells transfected with Myc-hFis1[32-152] released cytochrome *c* up to 72 hours post transfection, whereas 70% of cells expressing full-length hFis1 released cytochrome *c* at 72 hours. (C) Frequencies of cells expressing full-length hFis1, hFis1[32-152], or both Bcl-2 and hFis1[32-152] were scored at 24, 48 and 72 hours post transfection and presented as percent cell survival at each time point. The frequency at 24 hours was given as initial survival (100%). Survival of cells expressing full-length hFis1 decreased to 20% after 72 hours, whereas that of hFis1[32-152] cells were 75-80% at the same time point. Co-expression of Bcl-2 with Myc-hFis1[32-152] increased cell survival to 95% after 72 hours.

mitochondrial surface. This initial recruiting step may be necessary for not only determining the fission site but also for achieving a local DLP1 concentration high enough for self-assembly. Subsequently, the α1 would mediate release of DLP1 from the TPR allowing direct binding of DLP1 to the mitochondrial membrane surface for GTP-mediated constriction and fission. This releasing step mediated by the α1-helix could be regulated to ensure that DLP1 is properly assembled for the fission reaction. Alternatively, destabilization of the hFis1-DLP1 interaction during mitochondrial fission could be to facilitate DLP1 recycling to the cytosol after the fission reaction. It is possible that the α1 of hFis1 may serve to release DLP1 to the cytosol post-fission so that DLP1 can be recharged with GTP and form a new fission complex with hFis1.

Our data indicate that the α1 of hFis1 may regulate the DLP1 interaction with the hFis1 TPR. In support of this, molecular modeling studies have predicted that the α1-helix of hFis1 can form a flexible arm (Dohm et al., 2004; Suzuki et al., 2003) and may serve as a switch to regulate protein-protein interactions by modulating accessibility to binding sites (Dohm et al., 2004). Structural analyses of yeast Fis1p also suggest that the N-terminal tail sequence binds to the TPR region, indicating a potential regulatory mechanism by the N-terminal sequence (Suzuki et al., 2005). Immunolocalization studies indicate that endogenous hFis1 appears to be evenly distributed along the mitochondrial surface (James et al., 2003; Suzuki et al., 2003), which is in contrast to DLP1 that localizes in foci. Possibly, a fission signal may change conformation of a subset of hFis1 at fission sites potentially by post-translational modifications, which leads to DLP1 recruitment to the specific location on the mitochondrial surface. It is possible that hFis1 interacts with a third, yet unknown protein to regulate the DLP1-hFis1 interaction via α1. In yeast, Mdv1p was found to interact with Fis1p and Dnm1p, the yeast homologues of hFis1 and DLP1, respectively (Cerveny and Jensen, 2003; Tieu and Nunnari, 2000; Tieu et al., 2002). Recently, the protein structure of yeast Fis1p was determined using NMR spectroscopy (Suzuki et al., 2005). In that study, it was found that the unique N-terminal amino acid sequence present prior to the α1-helix was predicted to interact with the concave surface formed by TPR folds and was required for Mdv1p recruitment to mitochondria (Suzuki et al., 2005). However, this N-terminal sequence in yeast Fis1p is absent in hFis1 and the Mdv1p ortholog has not been found in mammals, indicating different regulatory mechanisms in human and yeast mitochondrial fission processes.

While no Mdv1p-like protein has been identified in mammalian cells, anti-apoptotic proteins Bcl-x_L and Bcl-2 have been reported to interact with hFis1 (James et al., 2003; Kong et al., 2005). Potential interaction of hFis1 with anti-apoptotic proteins is interesting regarding our data that overexpression of α1-deleted hFis1 induces formation of swollen spherical mitochondria in part by the MPT, which can be reduced by overexpression of Bcl-2, BA, and CsA (Fig. 4). Although it is possible that the mitochondrial swelling reflects an abnormal process artifactually induced by overexpression of the mutant protein, the following evidence suggests that the swelling is not likely to be the random phenomenon caused by overexpression of an abnormal protein: (1) The swelling phenomenon is specifically associated with the overexpression of mutants lacking the α1 helix (hFis1[32-152] and [61-152] to some extent) but not with the overexpression of other deletions; (2) Single point mutations in the TPR of hFis1[32-152] almost completely abrogate mitochondrial swelling; and (3) DLP1 and hFis1 have been shown to participate in the apoptotic process prior to cytochrome *c* release (Lee et al., 2004) in which the MPT often occurs as an upstream event.

We observed accumulation of DLP1 on swollen mitochondria by immunofluorescence and immunoblotting (Fig. 5), and an increased interaction of DLP1 with α1-deleted hFis1 by immunoprecipitation (Fig. 2B). Our results further demonstrated that Bcl-2 reduced mitochondrial swelling via decreasing DLP1 binding to mitochondria (Fig. 5C), suggesting that Bcl-2 may participate directly or indirectly in the DLP1-hFis1 interaction during the early DLP1 recruitment stage. At present, it is not clear how the prolonged or stabilized interaction between DLP1 and hFis1 leads to the MPT. It is possible that DLP1 and/or hFis1 interact with components [voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT)] or regulators (Bcl-2 family proteins) of the PT pore and contribute to the MPT. In support of this, DLP1 and Mfn, fission and fusion proteins, respectively, have been found in close proximity to the MPT inducer Bax (Karbowski et al., 2002) and, as discussed, anti-apoptotic proteins Bcl-2 and Bcl-x_L interact with hFis1 (James et al., 2003; Kong et al., 2005). Alternatively, given that PT pore is an ill-defined hypothetical structure and that mitochondrial swelling in cultured hepatocytes still occurs in the absence of ANT (Kokoszka et al., 2004), it is intriguing to speculate that mitochondrial fission process itself causes a transient MPT without involving the defined pore system. A small compromise of membrane integrity during mitochondrial fission could serve as a 'pore' that is quickly closed upon completion of fission. Possibly, stalling mitochondrial fission by the increased interaction between hFis1 and DLP1 through α1 deletion may cause sustained opening of the 'pore', inducing MPT and mitochondrial swelling. Partial prevention of mitochondrial swelling by BA or CsA that closes conventional pores (Fig. 4A) may suggest existence of the alternative identity of PT pore.

Interestingly, mitochondrial swelling induced by hFis1 α1 deletion greatly delays cytochrome *c* release and downstream apoptotic events. It is currently unclear how the apoptotic delay occurs after the MPT and before the cytochrome *c* release in cells overexpressing α1-deleted hFis1. Because DLP1-mediated mitochondrial fission is required for cytochrome *c* release (Frank et al., 2001; Lee et al., 2004), it is possible that perturbation of correct DLP1 assembly by α1-deleted hFis1 inhibits fission thus delays cytochrome *c* release. It is also possible that overexpressed α1-deleted hFis1 may artifactually coat the mitochondrial surface and physically prevent the outer membrane permeabilization required for cytochrome *c* release. Regardless, our data suggest that the α1 deletion in hFis1 may halt mitochondrial fission after the DLP1 recruitment and before the GTP-mediated fission reaction via prolonged DLP1 binding at the hFis1 TPR, and this arrest may cause MPT. While it is unclear how the apoptotic delay is mediated after the MPT, our results suggest that correctly regulated interaction between DLP1 and hFis1 is necessary for not only the normal fission process but also the progression of apoptosis.

In this study, we have identified two domains of hFis1

important for mitochondrial fission with each domain serving a different role. First, the TPR of hFis1 is involved in the DLP1 binding and, second, the N-terminal α1-helix of hFis1 in regulating the interaction between DLP1 and hFis1. Our data suggest that hFis1 is a main regulator of mitochondrial fission by orchestrating the recruitment and assembly of the fission protein DLP1 during both normal and apoptotic fission processes. As discussed, more proteins are likely to be involved in this process and it will be of great interest to identify how these proteins interact and what upstream signals regulate the interactions between these mitochondrial fission factors.

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