Identification and Characterization of Unique Proline-rich Peptides Binding to the Mitochondrial Fission Protein hFis1*®

Mammalian mitochondrial fission requires at least two proteins, hFis1 and the dynamin-like GTPase DLP1/Drp1. The mitochondrial protein hFis1 is anchored at the outer membrane by a C-terminal transmembrane domain. The cytosolic domain of hFis1 contains six α helices [α1–α6] out of which [α2–α5] form tetratricopeptide repeat (TPR)-like motifs. DLP1 and possibly other proteins are thought to interact with the hFis1 TPR region during the fission process. It has also been suggested that the α1-helix regulates protein-protein interactions at the TPR. We performed random peptide phage display screening using the hFis1[α2–α6] as the target and identified ten different peptide sequences. Phage ELISA using mutant hFis1 indicates that the peptide binding requires the α2 and α3 helices and the intact TPR structure. Competition experiments and surface plasmon resonance analyses confirmed that a subset of free peptides enriched with proline residues directly bind to the target. Two of these peptides bind to the α1-containing intact cytosolic domain of hFis1 with decreased affinity. Peptide microinjection into cells abolished the mitochondrial swelling induced by overexpression of α1-deleted hFis1, and significantly decreased cytochrome c release from mitochondria upon apoptotic induction. Our data demonstrate that hFis1 can bind to multiple amino acid sequences selectively, and that the TPR constitutes the main binding region of hFis1, providing a first insight into the hFis1 TPR as a potential therapeutic target.

Mitochondria are dynamic organelles that undergo constant shape changes through processes including fission and fusion of the organelle tubules (1). Mitochondrial fission and fusion are evolutionarily conserved processes, suggesting their importance in basic cell function. It is presumed that fission and fusion of mitochondria allow the mixing of the mitochondrial genome as well as other mitochondrial components such as the respiratory chain complexes to maintain adequate organelle function (2–5). Mitochondrial dynamics is also required for the proper distribution of this organelle within cells especially in neurons (6–8) and to ensure correct inheritance of this organelle to daughter cells during cell division (9). Defects in the fission or fusion machinery result in human disease conditions, such as subtypes of Charcot-Marie-Tooth disease and autosomal dominant optic atrophy, indicating their functional importance (10–13). In addition, abnormal phenotypes of mitochondrial morphology have been reported in several neurodegenerative diseases such as Alzheimer, Parkinson, and Huntington diseases, and recent studies indicate the direct involvement of mitochondrial fission/fusion in these diseases (14–18).

The two major processes that govern mitochondrial morphology are fission and fusion of the organelle. Studies on mitochondrial fission in mammals have shown that dynamin-related large GTPases, mitofusin (Mfn) and OPA1, participate in the fusion of outer and inner mitochondrial membranes, respectively (19–24). Recent studies have revealed additional functions of these proteins, OPA1 participating in regulating the cristae junction structure and Mfn2 in tethering the mitochondria and the endoplasmic reticulum (ER) (25, 26).

Mitochondrial fission mediated by the two proteins, DLP1 and Fis1, is the most studied fission process in mammals (27–33). DLP1 also called Drp1 is a large GTPase of the dynamin family and is suggested to assemble into ring-like structures that constrict and sever the membrane tubules for mitochondrial fission (28, 29). Fis1 is a 17-kDa protein anchored at the outer membrane through a single C-terminal transmembrane domain (31, 33). Structural studies of human Fis1 (hFis1) revealed that the cytosolic domain of this protein has six α helices of which the helices α2–α3 and α4–α5 form two tandem TPR-like folds (34, 35). It is speculated that hFis1 acts as a receptor to recruit the cytosolic DLP1 to the mitochondrial surface (31). Mutational studies indicated that the DLP1 interaction takes place in the TPR domain of hFis1 and that the first helix α1 exerts a negative regulatory effect on this interaction (36). Disruption of the protein interaction through mutations to the hFis1 TPR domain was found to abolish mitochondrial fission (36). It is also reported that hFis1 forms oligomers through self-interaction, which plays a role in mitochondrial fission by DLP1 recruitment (37).

Fragmentation of mitochondrial tubules often occurs in cells undergoing apoptosis (38–41). In addition, excessive fission of mitochondria is observed in hyperglycemic conditions and this,

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in turn, causes overproduction of reactive oxygen species, leading to apoptotic cell death (42, 43). A similar event also takes place in conditions evoking high intracellular Ca\(^{2+}\) concentrations (44–46). Blocking mitochondrial fragmentation under these conditions by altering the fission or fusion activities prevents or delays apoptotic progression (39, 43–45, 47, 48), suggesting that manipulating mitochondrial morphology can potentially control certain pathological conditions involving apoptotic cell death. This notion indicates that molecular agents such as short peptides or small molecules binding to the fission machinery could inhibit mitochondrial fission and decrease apoptosis-associated pathologies.

The fission protein hFis1 is a limiting factor in mitochondrial fission and contains a well-defined binding motif through which protein interactions crucial for mitochondrial fission and apoptosis occur (31, 33–36). In this study, we used hFis1 as a target in random peptide phage display screening and identified 10 different peptide sequences binding to the TPR of hFis1. Binding analyses indicated that these peptides bind to a region within the TPR and that the extreme N-terminal region of hFis1 plays a negative role in ligand binding at the TPR. We found that a subset of these sequences binds to the target as synthetic free peptides. Microinjection of hFis1-binding peptides into cells revealed a significant effect on staurosporine-induced apoptosis. This study identifies, for the first time, novel hFis1-binding peptide sequences that will be useful tools for developing therapeutic agents targeting the mitochondrial fission process.

**EXPERIMENTAL PROCEDURES**

**Phage Display Screening**—The Ph.D.-12\textsuperscript{TM} library from New England Biolabs was used to pan against the target protein His\(_6\)-Myc hFis1\([32–122]\). The target protein was purified through the Probind Ni-resin (Invitrogen) from the *Escherichia coli* lysate and dialyzed in 0.1 M NaHCO\(_3\) buffer (pH 8.6). A single well of a 12-well polystyrene dish was coated with the purified protein at a 100 \(\mu\)g/ml concentration overnight. After the protein-coated well was blocked with 5 mg/ml bovine serum albumin (BSA), library phages (4.0 \(\times\) 10\(^{10}\)) were added to the well for target binding. Nonbinding phages were discarded, and the well was washed 10 times with Tris-buffered saline containing 0.1% Tween 20 (TBST). The bound phages were eluted with 0.2 M glycine, pH 2.2 for 10 min. The eluted solution was immediately neutralized using 1 M Tris-HCl, pH 9.1 and the eluted phages were amplified by infecting the *E. coli* strain ER 2738. The amplified phages were precipitated, purified, and titered for the next round of panning. A phage number of 1–2 \(\times\) 10\(^{11}\) pfu was used for the second and third round of panning using the same method described above. During the third round, the stringency of the wash was increased by using 0.5% Tween 20 in the wash buffer. At the end of the third round of panning, plate binders were eliminated by binding to a BSA-coated plate. The resulting phages were plated without amplification. Twenty phage plaques were isolated and amplified individually, and the phage DNA was purified for DNA sequencing to identify peptide sequences.

**Phage ELISA**—96-well plates were coated with the target protein and blocked with BSA. An additional set of uncoated wells was also blocked for the negative control. The amplified phages were added to each well. In experiments with different phage concentrations, serial dilutions of 5- and 10-fold were used. In competition ELISA, appropriate concentrations of synthetic free peptide were added along with phages. After the phage binding and washing, plates were incubated with horseradish peroxidase-conjugated anti-M13 antibody (GE Bioscience). Phage binding was measured at 415 nm after a color reaction with an HRP substrate.

**SPR Analyses**—Direct target binding of free synthetic peptides was assessed using a dual channel SPR instrument (Reichert, Depew, NY). Target proteins were immobilized to the sensor chip surface by free amine coupling with a mixture of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide injection followed by quenching the remaining activated carboxyl groups with 1 M ethanolamine, pH 8.5. A second reference cell was treated similarly, but the target protein was excluded. All working peptide dilutions were prepared in running buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 100 mM NaCl, and 0.1% C\(_{12}\)E\(_{10}\)) and injected for 4 min (association time) at a flow rate of 50 \(\mu\)l/min followed by a dissociation phase of 10 min. Nonspecific background binding was subtracted from each sensogram using SPR_V4017 Data Acquisition and Alignment Program (Reichert, Depew, NY). Binding rates and constants were independent of flow rate over a wide range. Best-fit kinetic parameters were obtained by global fitting analysis using Scrubber2 (Biologic Software, Australia).

**In Vitro DSP Cross-linking**—Dithiobis[succinimidyl]propionate (DSP) was used for the *in vitro* cross-linking. Purified His\(_6\)-Myc hFis1\([32–122]\) (100 \(\mu\)g/ml) was incubated with 50 \(\mu\)M peptides. Cross-linking was carried out for 15 min with 0, 0.1, and 0.2 mM DSP concentrations at room temperature. Cross-linking was stopped by 10 mM Tris-HCl, pH 7.5. The samples were prepared using SDS-PAGE sample buffer without \(\beta\)-mercaptoethanol. The cross-linked protein-peptide complex was detected by immunoblotting using anti-Myc antibody (Sigma).

**Cell Culture, Microinjection, and Indirect Immunofluorescence**—Clone 9 (ATCC CRL-1439) cells were used for all experiments. Cells were cultured in Ham’s F-12K medium, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin under 5% CO\(_2\) at 37 °C. Clone 9 cells stably expressing green fluorescent protein (GFP) in the mitochondrial matrix (31) were maintained in 200 \(\mu\)g/ml G418. For microinjection, peptides and DNA were diluted in reverse phosphate-buffered saline (4 mM Na\(_2\)HPO\(_4\), 1 mM KH\(_2\)PO\(_4\), 140 mM KCl, pH 7.3) and injected in 100 \(\mu\)M to 1 mM for peptide and 0.05 mg/ml for DNA. When necessary, Texas red-conjugated dextran (3,000 M.W., Molecular Probes) was added at 1–2 mg/ml as the microinjection marker. Cells were plated on the plate-etched grid coverslips in 35-mm dishes and were pressure-injected using the Narishige microinjection system. For indirect immunofluorescence, cells were fixed with 3.5% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.2% Triton X-100. Coverslips were then incubated in blocking buffer containing 5% horse serum. Mouse monoclonal anti-cytochrome c (BD Pharmingen) or anti-Myc
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(Sigma) was used for primary antibodies. For secondary antibodies, Alexa 488- or Alexa-594-conjugated anti-mouse antibodies (Molecular Probes Inc.) were used. Fluorescence images were acquired with an Olympus IX71 epifluorescence microscope through a CCD camera (Mediacybernetics, Inc.) driven by IPLab imaging software (Scanalytics, Inc.). Acquired images were adjusted using Adobe Photoshop (Adobe Systems Inc.) software.

**RESULTS**

**Phage Display Screening to Identify hFis1-binding Peptides**

To identify peptide sequences that bind to the mitochondrial fission protein hFis1, we employed random peptide phage display screening. A library of $\sim 2 \times 10^9$ diverse linear 12-mer sequences displayed on M13 phages was screened. The entire cytosolic domain of hFis1 (hFis1[1–122]) was initially used as the binding target for the screening. The target has the N-terminal His$_6$ and Myc tags and was overexpressed and purified from *E. coli*. However, our repeated attempts to identify peptides binding to the hFis1[1–122] resulted in selecting nonspecifically binding phages. Because previous studies suggest that the N-terminal sequence containing the hFis1 a$_1$-helix interferes with protein binding at the downstream site (36, 37), we next used the a$_1$-deleted cytosolic domain of hFis1 as a binding target (Fig. 1A). This contains the a$_2$–a$_6$ helices including the intact TPR region, and also the N-terminal His$_6$/Myc tags. Using this target, we were able to identify 10 different hFis1-binding peptide sequences. These peptide sequences were placed in three groups based on sequence characteristics (Fig. 1B). The most abundant and notable sequences (Group 1) include a potential consensus proline-leucine-proline (PLP).

**FIGURE 1.** Random peptide phage display screening identified ten peptide sequences specifically binding to the target with different binding strengths. A, hFis1 cytosolic domain contains six a$_i$ helices forming two TPR motifs. For the phage display screening, a$_1$-deleted cytosolic domain of hFis1 was used as the target protein (His$_6$-Myc-hFis1[32–122]). B, DNA sequencing from 20 selected phages identified 10 different peptide sequences. Some of the selected phages encoded redundant peptide sequences. Group 1 peptides have a potential consensus PLP (underlined). The group 2 peptides contain a leucine-rich sequence at their C termini. The last two peptides in the group 3 show a loosely conserved PXWX sequence. C, phage ELISA demonstrated that the all 10 phage-borne peptides bind to the target protein. Phage 13 showed the highest binding affinity, whereas phages 9 and 8 show $\sim 5$- and 20-fold lower affinities to the target.
be a stronger binder compared with the other two phages. Binding efficiency of Phage 13 (EC_{50} = 1.0 \times 10^5) was ~5 and 20 times higher than those of phage 9 (EC_{50} = 5.3 \times 10^4) and phage 8 (EC_{50} = 2.1 \times 10^5), respectively. Whereas these three phages do not represent their groups in terms of the binding characteristics of peptides, these data suggest that the phage display screening resulted in identification of peptide sequences with varied binding strengths.

**Phage-borne Peptides Bind to hFis1 Downstream of the α1-Helix and Require Intact TPR Helices for Binding**—To determine peptide binding regions in the hFis1 molecule, three different binding targets, hFis1[1–122], hFis1[32–122], and hFis1[61–122] were used for phage ELISA (Fig. 2A). hFis1[1–122], hFis1[32–122], and hFis1[61–122] contain [α1–α6], [α2–α6], and [α4–α6], respectively. Phage ELISA experiments demonstrated that all 10 phage-peptides bind to the original target [32–122] (α2–α6), whereas there is little binding to [1–122] (α1–α6) and [61–122] (α4–α6) (Fig. 2A). Although binding strengths of different phages varied at the given phage concentration, it is clear that these phages specifically bind to [α2–α6] and not to the others. These results indicate that binding of these peptide sequences requires the α2 and α3 helices and that all four TPR helices may be necessary for binding. The lack of phage binding to [1–122] that contains α1, despite the presence of the TPR region, further supports the notion that the α1-helix has an inhibitory role in the ligand binding at the TPR.

In addition to truncated hFis1, we also used binding targets in which each of the TPR helices contains a helix-breaking point mutation (leucine-to-proline) (36) for the phage ELISA experiments (Fig. 2B). We found that phage binding to these mutant hFis1 proteins was greatly reduced, almost to the control level (Fig. 2B), indicating that peptide binding requires the intact TPR structure. Interestingly, phage 9 showed reduced binding to the α2 and α4 mutants but normal or even increased binding to the α1 mutant hFis1.
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binding to the α3 and α5 mutants. Phage 7 also had an almost normal binding to the α5 mutant whereas binding to the other mutants was greatly reduced, suggesting that these two peptides have binding characteristics distinct from the others. The phage ELISA results using various truncations and point mutants of hFis1 indicate that the peptide sequences that we identified bind to hFis1 downstream of the α1-helix and require the intact TPR structure for binding.

PLP-containing Free Peptides Compete with Phage-borne Peptides for Target Binding—Our data indicate that the peptide sequences that we have identified likely bind to the TPR region of hFis1 as phage-borne peptides. To test whether the free peptides can bind to the hFis1 TPR, three synthetic 12-amino acid peptides corresponding to phages 2, 3, and 10 (Pep2, Pep3, and Pep10, respectively) from each group were made (Fig. 1B). We chose these peptides based on their relatively higher hydrophobicity for better solubility in aqueous solution. To assess the free peptide binding to the target hFis1[32–122], we included them in phage ELISA in a competition assay. In this assay, reduced phage binding indicates the binding of the free peptide to the target at a shared binding site. For the initial assessment of competition, 5, 10, and 50 μM concentrations of peptides were used against their own phages. We observed competition for target binding between the phage 2 and Pep2 in a concentration-dependent manner (Fig. 3A). However, no significant competition was found with Pep3, and only an insignificant overall decrease was observed with Pep10 (Fig. 3A).

To obtain more information about shared binding sites of the peptide sequences that we identified, we included each peptide in phage ELISA with different phages in competition assays. As expected based on initial competition results (Fig. 3A), Pep3 and Pep10 showed very little or no competition with all phages including their own phages (supplemental Fig. S1) (see “Discussion”). Unlike these two peptides, the Pep2 containing the putative “PLP” consensus competes with most of the phages for the target binding (Fig. 3B). Although the extent of binding inhibition varied among different phages, Pep2 markedly decreased phage binding except for the binding of phage 9. It is interesting that the binding of phage 9 was unaffected in the presence of the Pep2. As shown with the helix-breaking mutant hFis1 targets (Fig. 2B), it is possible that phage 9 occupies a binding site distinct from the others within the TPR. An additional PLP-containing synthetic peptide from phage 13 (Pep13) was also effective in competing with phage-borne peptides (Fig. 3C). Again, a minimal amount of competition of Pep13 was found with phage 9. These results suggest that 9 of 10 peptide sequences that we identified likely share the same or similar binding sites within the TPR.

In addition to their ability to inhibit the binding of multiple different phages, both Pep2 and Pep13 from the PLP-containing group showed prominent inhibition with their own phages (Fig. 3, B and E), suggesting that the conformational states of the PLP-containing free peptides are close to those of the phage-borne peptides and that they are good candidates for high affinity binders. Therefore, two additional PLP-containing free peptides, Pep1 and Pep4, were made, and the binding kinetics of these peptides was further tested. Through competition assays, we found that Pep2 showed a stronger inhibition of phage binding compared with the other three (Fig. 4B). The IC₅₀ value for Pep2 was ~5 μM whereas those for Pep1, Pep4, and Pep13 were in the 40–70 μM range in competition against the binding of corresponding phages (Fig. 4E).

To test the importance of the PLP sequence in target binding, three mutant peptides of Pep2 were made, in which Pro-6,
Leu-7, and Pro-8 were changed to alanine residues (Pep2-ALP, PAP, and PLA), and tested for target binding by competition ELISA against phage 2. We found that the P6A mutant peptide (Pep2-ALP) maintained the full capacity of competing with phage for target binding, indicating that the N-terminal proline residue of the PLP is not essential for binding (Fig. 4F). How-

ever, the mutations at the Leu-7 and Pro-8 abolished the competition. A complete abolition of binding was observed with the PAP peptide, whereas the PLA peptide showed a greatly reduced competition (Fig. 4F). These results suggest that the middle leucine and the C-terminal proline of the PLP play an important role in binding to hFis1.

PLP-containing Free Peptides Bind Directly to hFis1[32–122]—To assess direct binding of the PLP containing peptides to hFis1[32–122] and determine true affinity constants, we performed surface plasmon resonance (SPR) analyses. The target protein was immobilized onto the sensor chip and different concentrations of free peptides were injected into the flow channel for assessing peptide binding. SPR analyses demonstrated that PLP peptides directly bind to hFis1[32–122] (Fig. 5, A–D). Dissociation constants ($K_d$) calculated from SPR data revealed that Pep2 binds to the target with higher affinity ($K_d = 8.9 \mu M$) than the others (Fig. 5K), agreeing with the findings from competition ELISA. Pep13 also binds to the target with a relatively higher affinity ($K_d = 16.7 \mu M$) compared with Pep1 ($K_d = 97.8 \mu M$) and Pep4 ($K_d = 30.5 \mu M$). The Pep2 mutant Pep2-PAP did not show binding to hFis1[32–122] (Fig. 5E). Chemical cross-linking of the mixture of the target and peptide also verified that Pep2 and Pep13 bind directly to hFis1[32–122] (Fig. 5F). To confirm specific binding of these peptides to hFis1 TPR, we used a hFis1 TPR mutant, L42P (hFis1[32–122]-L42P), as a target for SPR analyses. As shown in Fig. 5, G and H, neither Pep2 nor Pep13 binds to the mutant target, indicating the binding specificity of these peptides for the intact hFis1 TPR. In addition, we also used Tom20 that contains the TPR motif and has a size similar to hFis1 for the SPR analyses. The SPR data indicate that both Pep2 and Pep13 did not bind to Tom20 (supplemental Fig. S2), further supporting the specificity of these peptides for the hFis1 TPR. This series of experimental data demonstrates that synthetic peptides carrying the sequences identified from the random peptide phage screening interact directly and specifically with the hFis1 TPR region.

PLP Peptides Bind to hFis1-containing hFis1 and Decrease Apoptotic Cell Death—Whereas our data demonstrate that the PLP peptides bind to the hFis1 TPR, the same peptide sequences displayed on the phage particles do not bind to hFis1[1–122] (Fig. 2A), raising the possi-
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**A** Pep1 / [32-122]

**B** Pep2 / [32-122]

**I** Pep2 / [1-122]

**C** Pep4 / [32-122]

**D** Pep13 / [32-122]

**J** Pep13 / [1-122]

**E** Pep2-PAP / [32-122]

**F** DSP 0 0.2 0.5 (mM)

![Image of Western blot analysis](Image)

**K**

| Peptide | K_d (µM) | K_d (mM) |
|---------|----------|----------|
| Pep1    | 97.8 ± 13.28 | ND       |
| Pep2    | 8.9 ± 0.17   | 14.6 ± 4.62 |
| Pep4    | 30.5 ± 4.54  | ND       |
| Pep13   | 16.7 ± 0.87  | 0.31 ± 0.02 |

ND: not determined
bility that these peptides may be unable to bind to the hFis1 protein in cells. Because the presence of bulky phage particles might sterically hinder the access of peptides to the hFis1 TPR in phage ELISA, we used SPR to test whether free PLP peptides bind to the α1-containing target. SPR analyses clearly indicated the binding of the two PLP peptides Pep2 and Pep13 to the hFis1[1–122] (Fig. 5, I and J). However, the calculated $K_d$ was ∼20-fold higher for Pep13 (310 μM) and was in the millimolar range for Pep2 (14.6 mM), indicating that they bind to this target with much lower affinities (Fig. 5K). Furthermore, binding of Pep1 and Pep4 was inconsistent, presumably because of their extremely low binding affinity. Possibly, the access of peptides to the binding site is limited in the α1-containing N-terminal region of hFis1, resulting in a decreased overall binding. In addition, whereas the peptide binding was unequivocal for Pep2 and Pep13, the SPR response of the hFis1[1–122] binding was much more noisy (Fig. 5, I and J) compared with that of the hFis1[32–122] binding, indicative of unstable and low affinity binding.

Because we detected binding of Pep13 and Pep2 to the α1-containing hFis1 albeit in low affinities, we evaluated the effect of these hFis1-binding peptides on mitochondrial morphology. Microinjection was used to introduce peptides into cells harboring GFP-labeled mitochondria. Upon microinjecting these peptides into cells, we observed the fission-defective elongated mitochondrial morphology in some of the injected cells (Fig. 6A). However, we found that the occurrence of this phenotype was inconsistent presumably because of their weak binding to the endogenous target. Nevertheless, Pep13 injection appeared to cause a stronger effect than the others, consistent with the relatively lower $K_d$ value of Pep13 for the α1-containing hFis1.

Overexpression of α1-deleted hFis1, hFis1[32–152], induces the formation of swollen ball-shaped mitochondria presumably through the increased interaction with DLP1 at the hFis1 TPR (36, 49). Because Pep2 and Pep13 have higher binding affinities for the hFis1 TPR in the absence of the α1-helix, we tested the peptide binding in cells by examining the effect of these peptides on the hFis1[32–152]-mediated mitochondrial swelling. We microinjected the plasmid DNA encoding Myc-hFis1[32–152] into the nuclei first and then the peptides into the cytoplasm, and evaluated mitochondrial morphology. We found that both Pep2 and Pep13 greatly reduced the formation of swollen mitochondria (Fig. 6, B–D). In control cells that were injected with DNA alone, ∼70% of injected cells contained swollen mitochondria. We found that the fraction of cells containing swollen mitochondria markedly decreased to 30 and 35% with Pep2 and Pep13, respectively (Fig. 6D). These results support the notion that these peptides bind to the α1-deleted hFis1 in cells and decrease the protein interaction at the hFis1 TPR.

Structural studies suggest that the N-terminal α1-containing region of hFis1 regulates the ligand access to the TPR (34, 35). It is speculated that a transient opening of TPR occurs during mitochondrial fission, which allows a regulated interaction with DLP1 (36). We found that the hFis1 TPR-binding peptides that we identified show a less significant effect on mitochondrial fission per se in normal conditions, possibly because of the low frequency of the binding site exposure in the resting state. In the early stage of apoptosis, the level of mitochondria-associated DLP1 is greatly increased presumably through the hFis1 function, which causes mitochondrial fragmentation and subsequent cell death (44), suggesting that the hFis1 TPR becomes more accessible for protein interactions during apoptosis. Therefore, we tested the effect of the hFis1-binding peptides on cytochrome c release in apoptotic conditions induced by staurosporine (STS). After a 4-h incubation with STS, more than 60% of control cells (uninjected or buffer-injected) released cytochrome c to the cytosol (Fig. 6, E, E′, and G). In contrast, we observed a marked decrease of cells showing cytosolic cytochrome c in the presence of the hFis1-binding peptides: ∼25 and 35% of Pep13- and Pep2-injected cells, respectively, released cytochrome c to the cytosol, indicating a protection from STS-induced apoptosis (Fig. 6, F, F′, and G). Nuclear condensation was also observed in STS-treated cells, which was blocked upon the peptide injection (Fig. 6, E′ and F′). It has been reported that, during the STS treatment, mitochondrial tubules become stretched, which is followed by mitochondrial fragmentation prior to the cytochrome c release (39). We observed the same temporal change of mitochondrial morphology in our cytochrome c staining of STS-treated cells. We found that none of the peptide-injected cells displaying mitochondrial cytochrome c had fragmented mitochondria. Mitochondria in these cells were held in the stretched state (Fig. 6F′), suggesting that the hFis1-binding peptides blocked mitochondrial fragmentation by inhibiting fission under this condition. This series of data indicate that Pep2 and Pep13 can bind to the target in cells and exert a protective effect in apoptotic conditions.

**DISCUSSION**

Disruption of mitochondrial morphology is associated with numerous disease states and recent studies indicate that alterations in mitochondrial fission/fusion proteins are linked to many pathological conditions (50). Although the cause-and-effect relationship between mitochondrial morphology and function is not fully understood yet, growing evidence indicates that morphological changes of mitochondria contribute to regulating mitochondrial function (42, 51). In addition, maintain-

**FIGURE 5.** PLP-containing peptides directly bind to the target. A–D, Four different concentrations of PLP-containing peptides, Pep1, Pep2, Pep4, and Pep13 were infused into the flow channel containing the hFis1[α2–α6] target for SPR analyses. All four peptides rapidly increased the refractory index unit (RIU), indicating increases in mass at the sensor chip by peptide binding to the target. 1 μRIU represents ∼1 pg of protein/mm². All experiments were done in triplicate for the associated $K_d$ values shown in K. Representative traces are shown. E, negative control peptide, Pep2-PAP does not bind to the target protein. F, purified His₆-Myc-hFis1[32–122] was mixed with 50 μM Pep2 or Pep13, and the protein-peptide complex was detected by DSP cross-linking. Immunoblotting with anti-Myc antibody shows a slightly higher molecular weight band (arrows), indicating the cross-linked product. Arrowheads, His₆-Myc-hFis1[132–122]. G and H, SPR analyses using the mutant hFis1[32–122]-L42P showing no binding of Pep2 and Pep13 to the TPR mutant. I and J, SPR analyses using hFis1[1–122] as the target. The increases of RIU are smaller for both Pep2 and Pep13, indicating low affinity binding to the α1-containing hFis1. Experiments were done in triplicate, and representative traces are shown. K, $K_d$ values calculated from SPR analyses. Pep2 and Pep13 showing higher affinities for hFis1[32–122] bind to the hFis1[1–122] with reduced affinities. Pep1 and Pep4 did not show consistent binding to the hFis1[1–122].
ing tubular mitochondria through genetic manipulation has been shown to prevent or delay apoptosis (39, 44, 47, 48), suggesting that modulating mitochondrial morphology can decrease apoptosis-associated tissue injury. A recent effort to use mitochondrial fission as a potential therapeutic target has resulted in the identification of a chemical compound named mdivi-1 (52). This compound has been shown to inhibit the self-assembly of Dnm1p (yeast homologue of DLP1) and to attenuate mitochondrial fission and apoptosis in mammalian cells (52). In the current study, we used the hFis1 protein that has a defined protein interaction site as a target and identified hFis1-binding peptides.

Whereas phage ELISA experiments indicated that the peptide sequences that we identified bind to the hFis1 TPR region, phage-borne peptides did not bind when the α1-helix was present (Fig. 2A). It is possible that the α1-helix participates in forming a binding site different from the one formed in the α1-deleted hFis1. In support of this notion, the Fis1p-Mdv1p and Fis1p-Caf4p co-crystallization study using yeast fission proteins indicated that the extreme N-terminal residues of Fis1p is packed against the hydrophobic groove in the concave surface of the Fis1p TPR and stabilizes the Mdv1p or Caf4p binding at the second hydrophobic groove within the TPR (53). However, it is unlikely that the same binding event occurs in hFis1 because the extreme N-terminal residues of yeast Fis1p are absent in hFis1, and there are no Mdv1p- or Caf4p-like proteins found in mammals. Furthermore, the initial library screening using the entire cytosolic domain of hFis1 that con-
tained the α1-helix was not able to identify hFis1-binding peptides, suggesting that the α1-helix does not participate in forming a stable binding site in the hFis1 molecule. Although mitochondrial fission and fusion are phylogenetically conserved processes, their underlying mechanisms at the protein level are likely evolved differentially in yeast and higher organisms. The more plausible hypothesis for the lack of phage binding to the α1-containing target is that the α1-helix acts as a negative factor for the ligand binding at the TPR as suggested before (36). In this hypothesis, the α1-helix of hFis1 forms a flexible arm that temporally regulates the ligand access by steric blocking the TPR region (34, 36). Subsequent studies support this notion, as deletion of the α1-helix enhanced hFis1 homo-oligomerization and the DLP1 interaction at the TPR (36, 37). A recent report also indicated that the deletion of the extreme N-terminal sequences of yeast Fis1p increased the Dnm1 binding at the TPR (54). In addition, the inability of the intact cytosolic domain of hFis1 to serve as a suitable target in the initial library screening suggests that the access to the TPR is blocked in the α1-containing hFis1.

Our competition ELISA experiments indicated that Pep3 and Pep10 did not bind to the target although they bind in phage-associated forms. Unlike phage-borne peptides, short linear peptides in solution have no structural constraint and can have multiple different conformations. It is likely that Pep3 and Pep10 take structures that are unfavorable for binding to the hFis1 TPR. It is possible that the phage particles associated with these peptides directly or indirectly render a level of structural stability to the peptides, allowing them to bind to the target in phage-associated forms. In contrast to these peptides, the PLP-containing free peptides bind to the target. Conformationally constrained peptides such as cyclic peptides generally bind with higher affinity (55). The presence of two or more proline residues in the PLP-containing peptides potentially restricts the peptide conformation, resulting in increased binding to the target. Among PLP peptides, Pep2 and Pep13 consistently show the ability to bind to both α1-deleted and α1-containing hFis1. However, their binding to the α1-containing hFis1 is markedly decreased, further supporting the negative role of the hFis1 N-terminal region in ligand binding at the TPR. Interestingly, Pep2 binds with higher affinity to [α2–α6] than Pep13, whereas Pep13 binds better to [α1–α6] than Pep2. The different binding characteristics of Pep2 and Pep13 to the α1-containing and α1-deleted targets were reflected in their effect on mitochondrial morphology (Fig. 6, A and D). Further tests using additional mutant peptides along with shorter peptides will provide useful information for modifying these peptides for improved binding.

Our results indicate that Pep2 and Pep13 bind to the hFis1 TPR in cells, and provide a protective effect in apoptosis-inducing conditions (Fig. 6). However, they were inconsistent in producing the fission-defective phenotype in resting conditions. One possibility is that the transient exposure of the TPR for mitochondrial fission occurs in a low frequency in normal conditions. During apoptosis, however, it appears that the hFis1 TPR becomes more accessible to other proteins or ligands, as the TPR-binding peptides effectively reduce STS-induced mitochondrial fragmentation and cytochrome c release. An interesting implication of this observation is that the hFis1 TPR-targeted peptides could be pathologically useful agents because they can act in abnormally increased fission but have a minimal effect in normal conditions. It is likely that peptide stability and binding affinities as well as the local concentration of the peptides in the vicinity of the fission microenvironment where hFis1 resides are important factors for these peptides to exert the pathologically relevant action. Further studies of these peptides including the characterization of the binding interface, and detailed structural analyses will be useful to improve and control binding properties of these peptides.

The field of studying mitochondrial dynamics is expanding rapidly as they play an important role in the development and progression of many pathological conditions. However, ill-defined mechanisms of mitochondrial dynamics as well as the scarceness of inhibitory reagents of mitochondrial fission/fusion have been hampering the progress of this research field. Although regulated protein interactions at the hFis1 are the key mechanisms for mitochondrial fission, the nature of these interactions has not been understood. The current study addresses these gaps by identifying sequence requirement for binding to the hFis1 TPR and, at the same time, by providing structural insight for hFis1-binding reagents and the therapeutic potential of targeting hFis1. The hFis1 TPR-binding peptides that we identified would provide a valuable structural platform on which their binding properties can be improved and modified to contribute to the development of potential therapeutic agents effective in apoptosis-associated pathological conditions.

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