The Lysosome-associated Apoptosis-inducing Protein Containing the Pleckstrin Homology (PH) and FYVE Domains (LAPF), Representative of a Novel Family of PH and FYVE Domain-containing Proteins, Induces Caspase-independent Apoptosis via the Lysosomal-Mitochondrial Pathway

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Lysosomes have recently been identified as important apoptotic signal integrators in response to various stimuli. Here we report the functional characterization of LAPF, a novel lysosome-associated apoptosis-inducing protein containing PH and FYVE domains. LAPF is a representative of a new protein family, the Phafins (protein containing both PH and FYVE domains), which consists of 14 unidentified proteins from various species. Overexpression of LAPF in L929 cells induces apoptosis and also increases cell sensitivity to TNFα-induced apoptosis, concomitant with its translocation to lysosomes. Two mutants of LAPF, either lacking the PH or FYVE domain, failed to induce cell death and translocate to lysosomes, suggesting that both domains are required for its apoptosis-inducing activity and relocation. We demonstrate that LAPF may induce apoptosis via the following steps: LAPF translocation to lysosomes, lysosomal membrane permeabilization (LMP), release of cathepsin (cath) D and L, mitochondrial membrane permeabilization (MMP), release of apoptosis-inducing factor (AIF), and caspase-independent apoptosis. The cath D-specific inhibitor attenuates LAPF-induced apoptosis, indicating a pivotal role of lysosomes in LAPF-initiated apoptosis. We also demonstrate that the lysosomal pathway was employed in the typical apoptotic model in which high dose TNFα was used to stimulate L929 cells. Silencing of LAPF expression by small RNA interference protected L929 cells from hTNFα-induced apoptosis by impairing hTNFα-triggered LMP and MMP. Therefore, LAPF may launch caspase-independent apoptosis through the lysosomal-mitochondrial pathway.

Apoptosis can be induced either through the extrinsic pathway, initiated by the ligation of death receptors, or the intrinsic pathway, triggered by perturbation of intracellular homeostasis (1, 2). Both pathways converge on a common "central executioner," a self-amplifying mechanism that involves mitochondrial membrane permeabilization (MMP) and/or caspase activation cascades (3–5). Despite the central role of mitochondria in apoptosis, mounting evidence suggests that other organelles, including lysosomes, the endoplasmic reticulum, and the Golgi apparatus, are also points of pro-apoptotic signaling integration (1).

Recent studies have shown that lysosomal membrane permeabilization (LMP) is an early and, perhaps, initiating event in apoptosis triggered by ligation of death receptors, lysosomalotropic agents, oxidative stresses, or serum withdrawal (6–9). The specific release of cathepsins from lysosomes into the cytosol is critical for lysosome-mediated cell death and is responsible for activation of downstream signal pathways. Cathepsins can indirectly induce MMP by modifying cytosolic proteins, including Bax, Bak, or Bid, conferring upon them the ability to translocate to the outer mitochondrial membrane, and can also directly activate effector caspases, in particular caspase-3 (2, 7, 10, 11). However, direct activation of caspases is controversial because other investigators (2, 12) hold opposite opinions. The dominant roles of LMP and cathepsins in apoptosis have been demonstrated by several studies. Hepatocytes isolated from cathepsin (cath) B gene knock-out mice (cath B−/−) are relatively resistant to TNFα-induced apoptosis, as downstream cytochrome c release from the mitochondria is blocked (13). The inhibitor of LMP, Bafilomycin A1, also inhibits all hallmarks of apoptosis in HeLa cells usually induced by nortoxacinc (a lysosomalotropic agent) plus UV light, including loss of mitochondrial transmembrane potential (∆Ψm) and release of cytochrome c (7). Furthermore, pretreatment with pepstatin A, a specific inhibitor of cath D, protects L929 cells from TNFα cytotoxicity, indicating that the lysosomal pathway may be important to TNFα-induced apoptosis of L929 cells despite the existence of multiple death signaling pathways (14).

Although significant progress has been made in illuminating the role of lysosomes in apoptosis and identifying the downstream signal pathways triggered by lysosomal cathepsins, the mechanisms that account for lysosomal membrane permeabilization triggered by apoptotic stimuli remain to be elucidated.

The abbreviations used are: MMP, mitochondrial membrane permeabilization; AO, acridine orange; AIF, apoptosis-inducing factor; BMSC, bone marrow stromal cells; cath, cathepsin; ∆Ψm, mitochondrial transmembrane potential; E64d, epoxysaccinyl-γ-l-leucylamido-3-methyl-butane ethyl ester; TNFα, human tumor necrosis factor α; LAPF, lysosome-associated and apoptosis-inducing protein containing PH and FYVE domains; LMP, lysosomal membrane permeabilization; MEFs, murine embryonic fibroblasts; PH, pleckstrin homology; Phafin, protein containing both PH and FYVE domains; PI, propidium iodide; R123, rhodamine 123; TNFR, TNF receptor; TRADD, TNFR-associated protein with death domain; zVAD, benzoyloxy carbonyl-Val-Ala-Asp-CHO; PBS, phosphate-buffered saline; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, short interfering RNA.
LAPF Induces Apoptosis via the Lysosomal-Mitochondrial Pathway

In the current study, we report the identification and characterization of the novel protein LAPF, a lysosome-associated and apoptosis-inducing protein containing PH (pleckstrin homology) and FYVE (Fab1, YGLO23, Yip27, and EEA1) domains that is representative of a novel protein family, the PhaFins. LAPF acts as a pro-apoptotic protein via translocating to lysosomes, facilitating apoptosis induction through a lysosomal-mitochondrial apoptotic pathway.

EXPERIMENTAL PROCEDURES

Reagents—Peptatin A, E64d, leupeptin, aprotinin, and ALLN were from Sigma-Aldrich; hTNFα from R&D Systems Inc.; zVAD from Promega; annexin V, propidium iodide (PI), Lyso Tracker Red, Mito Tracker Red, acridine orange (AO), Rhodamine123 (R123), Avidin-Texas Red, and fluorescein isothiocyanate-labeled anti-mouse secondary antibody from Molecular Probes; mouse monoclonal anti-AIF antibody (E-1), mouse monoclonal anti-cytochrome c antibody (6H2), and polyclonal goat antibodies against cath D (G-19), cath L (S-20), and cath B (S-12) from Santa Cruz Biotechnology; biotinylated secondary anti-IgG antibodies, and cell lysis buffer from Cell Signaling Technology.

cDNA Cloning and Expression Vector Construction—The full-length cDNA of human LAPF (hLAPF) was directly isolated from a cDNA library of human bone marrow stromal cells (BMSC) by random sequencing, as described previously (15). The full-length sequence of hLAPF is available in the GenBankTM data base under accession number Y0037145. Mouse LAPF (mLAPF) cDNA was derived from mouse BMSC by RT-PCR using primers corresponding to the sequence of hLAPF. The full-length coding region of hLAPF or mLAPF cDNA was also cloned into the pEGFP-N1 vector (Clontech) for C-terminal GFP fusion protein expression.

Northern Blot and RT-PCR Assay—The mRNA expression patterns of LAPF were analyzed by Northern blot and RT-PCR as described previously (16). Probes corresponding to the full-length cDNA sequence of hLAPF were labeled with [32P]dCTP using a random primer DNA labeling kit (Takara). Total cellular RNA was extracted using the TRIzol reagent (Invitrogen), and first strand cDNA prepared using the Superscript II system with an oligo(dT)15 primer (Invitrogen). The full-length cDNA sequence of hLAPF was labeled with [32P]dCTP using a random primer DNA labeling kit (Takara). Total cellular RNA was extracted using the TRIzol reagent (Invitrogen), and first strand cDNA prepared using the Superscript II system with an oligo(dT)15 primer (Invitrogen). cDNA synthesis was checked by PCR, with human or mouse β-actin primers as a positive control. Specific primers for RT-PCR were: 5'-ATGGTG-GACCACCTTGCCAC-3' (forward) and, 5'-TCAGCTGTGGAAG-GCGAGACCC-3' (reverse) for hLAPF, 5'-ATGGTGACCTTG-GCAAAC-3' (forward) and, 5'-TCAGCTGTGGAAG-GCGAGACC-3' (reverse) for mLAPF. The reactions were subjected to denaturation (94 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 90 s) for 30 cycles.

Cell Culture and Transfection—L929 (mouse fibrosarcoma) cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfection with Polyfect reagent (Qiagen) was performed as recommended by the manufacturer. Stable cell lines overexpressing hLAPF or mLAPF were selected by 600–1000 μg/ml G418 for 2–3 weeks, cloned by limiting dilution, and designated as L929-hLAPF and L929-mLAPF, respectively. Stable expression of LAPF was confirmed by RT-PCR or Western blot.

Antibody Preparation and Western Blot—A polyclonal antibody against the C-terminal 84 residues of hLAPF was generated by immunizing rabbits with glutathione S-transferase fusion proteins with routine methods. The anti-hLAPF serum was purified using protein A affinity chromatography (Pierce). Western blot results revealed that the anti-hLAPF antibody recognized both full-length hLAPF and mLAPF. Western blot analysis was performed as described previously (16).

Apoptosis Assay—Apoptotic cells were analyzed by annexin V-PI staining according to the manufacturer’s instructions. After washing in PBS, cells were resuspended in prediluted binding buffer and stained with fluorescein isothiocyanate-conjugated annexin V for 10 min at room temperature, protected from light. Cells were then washed and resuspended in binding buffer. PI (1 μg/ml) was added, and the cell suspension was immediately analyzed by flow cytometry. The percentages of viable and dead cells were determined from 10,000 cells per sample.

MTT Assay—The MTT assay is based on the activity of mitochondrial dehydrogenases in live cells, which oxidize the tetrazolium salt (MTT, Sigma) to a blue formazan salt. Approximately 1 × 10⁴ cells were plated onto each well of a 96-well plate and incubated with 50 ng/ml hTNFα for the indicated time periods. Then cells were washed and incubated with MTT (100 μg/ml) at 37 °C for 4 h. The supernatant was disposed, and 150 μl of Me₆SO was added to each well. The absorbance of the reaction solution at 570 nm was recorded. The absorbance from the wells of L929 cells cultured without hTNFα (control) was taken as the 100% viability value. The percent viability of the treated cells was calculated by the formula (A sample/A control) × 100%.

Transmission Electron Microscopy—Apoptosis of L929-LAPF cells was confirmed by transmission electron microscopy as described previously (17). Cells were washed in PBS, fixed in 2.5% glutaraldehyde for 2 h at room temperature, and then postfixed in 1% osmium tetroxide for 1 h at room temperature. After dehydration in a graded series of ethanol and infiltration in propylene oxide, cells were embedded in Epon-812. Ultrathin sections were stained with uranyl acetate and lead citrate and cell morphology observed by TEM (PHILIPS TECNAI 10) at 80 kV. Micrographs were taken randomly from different sections of three cultures for each experimental condition.

Immunofluorescence and Confocal Laser Scanning Microscopy—Transiently transfected L929 cells expressing LAPF-GFP, hLAPFΔPH-GFP, or hLAPFΔFY-GFP were cultured on coverslips, stained with 200 nM Lyso Tracker Red or 100 nM Mito Tracker Red for 15 min at 37 °C, fixed in 4% polyformaldehyde for 15 min, washed two times, and observed by fluorescence confocal microscopy (LSM ConfoScan Microscope, Carl Zeiss).

Alternatively, cells were processed for immunofluorescence as described earlier (18). Briefly, the cells were incubated with primary antibodies, including cat D antibody, cath L antibody, AIF antibody, and then rinsed and incubated with biotinylated secondary anti-IgG antibodies and subsequently Avidin-Texas Red conjugate. Finally, the cells were rinsed in PBS and viewed under fluorescence confocal microscope. Controls incubated without cat D antibody, cath L antibody, or AIF antibody did not stain. Images were processed with Adobe Photoshop software 7.0.

Lysosomal Stability Assay—Cells were assessed for lysosomal stability using Lyso Tracker Red-uptake and AO uptake assays (17). These molecules accumulate in lysosomes on the basis of low pH or proton trapping, respectively. Cells were incubated with 50 nM Lyso Tracker Red for 30 min or with 5 μM AO for 30 min at 37 °C, then red fluorescence of 10,000 cells per sample was determined by flow cytometry. The percentage of cells with low intensity red fluorescence (pale cells) was used as a marker of the extent of lysosomal destabilization.

Mitochondrial Membrane Potential (∆Ψm) Assessment—R123 acts as a voltage-sensitive probe. Decrease in the mitochondrial membrane potential, and thus the transmembrane proton gradient, is paralleled by
a reduction of R123-induced fluorescence. Cells were incubated with 1 nM R123 for 30 min in culture medium at 37 °C. The cells were washed with PBS, and FL1 channel fluorescence assed by flow cytometry.

Preparation of S-100 Fractions—A previously established procedure was used to isolate 100,000 × g supernatant from soluble extracts (designated as S-100) for analysis of cathepsins and AIF release (19). Cells (5 × 10⁶) were harvested, washed with ice-cold PBS, and the pellets suspended in 5 volumes of ice-cold buffer A (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose), supplemented with protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml ALLN). After sitting on ice for 15 min, cells were homogenized with 15 strokes of a Wheaton Dounce homogenizer (no. 358005). The homogenate was centrifuged at successive higher speeds; 750 × g for 10 min, 10,000 × g for 15 min, then 100,000 × g for 1 h. The supernatant (S-100) was stored at −80 °C until use in Western blot assay.

RNA Interference—The GeneSuppressor System (Imgenex) was used for RNA interference. Cells transfected with the short interfering RNA (siRNA) expression vector (pSuppressorNeo) experience steady, long term mRNA inhibition after selection for 2–3 weeks under 600–1000 μg/ml G418. A 21-nucleotide siRNA duplex (GGTACTGCAACAGGA GTGACC) corresponding to mLAPF was synthesized and inserted into this vector. The sequence TCAGTCACGTTAATGGTCGTT, with no homology to any human or mouse mRNA, was used as a non-specific RNAi control (CTRLi).

RESULTS

Identification of the LAPF and Phafin Family—The hLAPF cDNA clone, directly isolated from a human BMSC cDNA library by large scale random sequencing, contained an open reading frame of 840 bp with an in-frame stop codon upstream of the initiating methionine and a 3’-poly(A) tail, indicating that the hLAPF cDNA was of full-length. It potentially encoded a 279-amino acid protein with a predicted molecular mass of 31.2 kDa and isoelectric point of 8.59. The putative protein might be a non-secretory soluble protein lacking an N-terminal signal peptide and transmembrane regions, as predicted by the SOSUI (signal) program and the Tmpred program. Based on the primary amino acid sequence, an unknown mouse protein (NP_077724) and an unknown rat protein (ENSRNOP00000020352), sharing about 90% overall identity to hLAPF (Fig. 1A), were designated mouse and rat LAPF (mLAPF and rLAPF), respectively. Basic local alignment analysis against a genomic data base showed that hLAPF was located on human chromosome 19q11, and mLAPF was located on mouse chromosome 7B1 (data not shown).

Homology analysis revealed that LAPF shares close similarities (about 34–91% similarity) to other 11 unrecognized proteins originating from various species. Phylogenetic analysis of these proteins was performed using ClustalW and TreeView software (Fig. 1B). Structural analysis based on multiple alignments showed that all of these proteins were predicted to contain two common structural characteristics, an N-terminal PH domain and a C-terminal FYVE domain (Fig. 1C). We thus classified these proteins as a novel family, designated the Phafins (protein containing both PH and FYVE domains). Members are predicted to contain six conserved blocks within the PH domain (residues 1–11, 15–27, 28–39, 40–59, 66–77, 79–103), which comprise a β-sandwich and a C-terminal α-helix possessing a highly conserved three-dimensional organization. A basic FYVE domain consisting of eight conserved cysteine residues is universally present in all members, two of these residues are part of the core motif R+HHC+XCG (where + is a positively charged residue and X is any amino acid) (Fig. 1A).

Based on homology comparison, similarity in structural features and extent of evolutionary conservation, members of the Phafin family may be grouped into two subfamilies: Phafin-1, which includes hLAPF, mLAPF (279 residues), and rLAPF (279 residues); Phafin-2, including hPhafin-2 (Homo sapiens, 249 residues), mLPhafin-2 (Mus musculus, 249 residues), dPhafin-2 (Danio rerio, 247 residues), rPhafin-2 (Fugu rubripes, 225 residues). The remainder form an unclassified group: AAH13319 (H. sapiens, 376 residues), AAH53138 (D. rerio, 293 residues), XP_314634 (Anopheles gambiae, 193 residues), NP_569923 (Drosophila melanogaster, 316 residues), AAL28825 (D. melanogaster, 204 residues), NP_499183 (Caenorhabditis elegans, 266 residues), and S40944 (C. elegans, 254 residues) (Fig. 1B). However, whether these disparate proteins share similar functions still needs to be further examined.

Expression Patterns of LAPF—To examine the tissue distribution of LAPF, Northern blotting was performed using mRNA blots from multiple human adult tissues. An ~1.8-kb hLAPF transcript was abundant in heart and skeletal muscle, whereas expression in brain, thymus, spleen, kidney, liver, small intestine, placenta, lung, and PBL was weak (Fig. 2A). mLAPF was also widely expressed in adult mouse tissues, as detected by RT-PCR (Fig. 2B).

RT-PCR analysis revealed differences in LAPF expression levels between various cell types. hLAPF expression was observed in freshly isolated BMSC, dendritic cells (DC) and a variety of leukemia cell lines, including U937 (promonocytic), MOLT-4 (T lymphoma), Raji (B lymphoma), HL60 (myelomonocytic), K562 (erythroid/megakaryocytic), but not in solid tumor cell lines (Fig. 2C). In contrast, mLAPF mRNA could also be detected in some solid tumor cell lines including L929 (fibrosarcoma), CT-26 (colon carcinoma), in addition to BMSC, DC and leukemia cell lines (Fig. 2D).

The effects of hTNFα, LPS, and PMA treatments on LAPF expression in U937 and L929 cells, which express low levels of LAPF, were examined by RT-PCR. Expression of hLAPF mRNA in U937 cells increased 12 h after an apoptosis-triggering dose (50 ng/ml) of hTNFα, and was maintained at high levels until 24 h, whereas mLAPF mRNA levels increased within 8 h in hTNFα-treated L929 cells (Fig. 2E). Simultaneously, the protein expression of endogenous LAPF in these cells was examined by an anti-LAPF antibody, which recognized both full-length hLAPF and mLAPF. In parallel with mRNA expression profiles, hLAPF and mLAPF protein expression in U937 and L929 cells was induced by hTNFα at 36 and 24 h, respectively, and maintained until at least 48 h (Fig. 2F). However, neither lipopolysaccharides (2 μg/ml) nor phorbol 12-myristate 13-acetate (20 μM) up-regulated LAPF gene expression (data not shown). These data indicated a time-dependent increase in LAPF expression in response to apoptotic stimuli such as hTNFα.

LAPF Induces Apoptosis of L929 Cells—To investigate the biological functions of LAPF, we constructed eukaryotic expression vectors of LAPF and screened some platforms for biological function analysis including cellular growth inhibition or stimulation by transfection, which had been established in our laboratory (15, 16, 20). We found that TNFα-sensitive L929 cells transiently transfected with LAPF expression vectors underwent apoptotic cell death. Approximately 14% of L929 cells overexpressing hLAPF or mLAPF displayed annexin V-positive staining in the absence of any apoptotic stimuli, whereas only 3% of control vector transfected cells (mock) and parental L929 cells (control) were annexin V-positive (Fig. 3A). To examine the respective roles of the PH and FYVE domains in LAPF-induced cellular apoptosis, we generated two LAPF mutant vectors, LAPFΔPH (lacking the N-terminal PH domain) and LAPFΔFY (lacking the C-terminal FYVE domain) (Fig.
Transfection with these two LAPF mutants did not induce apoptosis, suggesting that both domains of LAPF are required for its apoptosis-inducing activity (Fig. 3A).

To further investigate whether stably expressed LAPF displayed apoptotic activity, L929 cells were stably transfected with LAPF expression vectors. Several subclones of L929-LAPF cells stably expressing...
LAPF Induces Apoptosis via the Lysosomal-Mitochondrial Pathway

hLAPF or mLAPF were established, as confirmed by Western blot, but no significant spontaneous apoptosis was observed (Fig. 3, B and C). Exposure to different doses of hTNFα, which selectively binds murine TNFR-1 but not TNFR-2, revealed that L929-LAPF cells were more sensitive to hTNFα than controls (Fig. 3C). The relatively low concentration of 10 ng/ml hTNFα efficiently elicited L929-LAPF apoptosis, but not that of controls. Stimulation with 10 ng/ml hTNFα resulted in a time-dependent increase in apoptosis of L929-LAPF cells (Fig. 3D). Furthermore, hTNFα-stimulated L929-hLAPF cells displayed many apoptotic hallmarks, including a reduction in nuclear DNA (Fig. 3E), and chromatin condensation and margination, determined by transmission electron microscopy (Fig. 3F). However, DNA cleavage in dying L929-hLAPF cells was limited to the generation of larger 50–200-kb fragments rather than complete nucleosome laddering, indicating that apoptosis was caspase-independent (Fig. 3G). On the basis of the high sequence homology between hLAPF and mLAPF, and their similar apoptosis-inducing capacity, we selected hLAPF to investigate the mechanism by which LAPF induces apoptosis in L929 cells.

LAPF Translocates to Lysosomes during Apoptosis—To determine the subcellular localization of LAPF, we constructed plasmids encoding green fluorescent protein (GFP)-fused LAPF or LAPF mutants. Both human and mouse full-length LAPF-GFP exhibited a diffuse cytosolic and nuclear distribution 24 h after transient transfection, which became concentrated in the perinuclear region and partially colocalized with the lysosome-specific fluorescent dye LysoTracker at 48 h, and was primarily colocalized with clustering lysosomes as cells became rounded and experienced apoptotic shrinkage at 60–72 h (Fig. 4, A and B). However, the two mutants of hLAPF, hLAPFΔPH and hLAPFΔFY, failed to colocalize to lysosomes. The former maintained a diffuse expression pattern while the latter was membrane-localized, indicating that both domains were required for lysosomal location of LAPF (Fig. 4A). LAPF did not translocate to mitochondria, a crucial apoptosis-related organelle (Fig. 4C). These results suggested that the translocation of LAPF to lysosomes was parallel to its apoptosis-inducing activity.

LAPF Initiates Lysosomal Membrane Permeabilization—Initially thought to be merely the home of hydrolytic proteases, the lysosome is now also known as a key organelle upon which various apoptotic signals converge (1). In view of the apoptotic activity of LAPF and its translocation to lysosomes, we were interested in whether lysosomes participated in LAPF-induced cell demise. To determine the effect of LAPF activity on lysosomal membrane stabilization, two lysosomotropic fluorescence probes, Lyso Tracker Red and AO, were used on the basis of either low pH or proton trapping. Lysosomal destabilization of L929-hLAPF cells was detected at 12 h after hTNFα treatment and gradually increased until 48 h, as evidenced by an increased number of "pale cells" (cells with reduced numbers of Lyso Tracker and AO-accumulating lysosomes), whereas this change was very weak in control cells (Fig. 5, A and B).

It has been demonstrated that the lysosomal cathepsins are readily released into the cytosol after lysosomal destabilization (21). We therefore examined the subcellular distribution of cath D, an aspartic protease, and cath B and L, two cysteine proteases. All these three proteases are abundant in lysosomes. The release of cath D and L, but not cath B, from lysosomes into the cytosol was detected in the 100,000 × g supernatant of soluble extracts (S-100) 12 h after hTNFα treatment in L929-hLAPF cells, and increased over time, whereas the release was observed until 36 h after hTNFα treatment in control cells (Fig. 5C and data not shown). Immunofluorescence analysis also revealed that the largely granular staining of cath D and cath L in the perinuclear region was changed to a diffuse pattern throughout the cells after 24 h of stimula-
FIGURE 3. LAPF induces apoptosis of L929 cells. A, LAPF induced apoptosis of L929 cells. L929 cells were transiently transfected with the indicated plasmids, harvested 60 h later, and stained with annexin V/PI. The percentage of gated cells that were apoptotic (annexin V single positive and annexin V/PI double positive) was assessed. B, stable expression of hLAPF and mLAPF in L929 cells, as confirmed by Western blot using anti-LAPF Ab. C and D, sensitivity of L929-LAPF cells to hTNFα-induced apoptosis. Transfected cells were treated with different dosages of hTNFα for 24 h (C), or with 10 ng/ml hTNFα for various lengths of time (D). Apoptosis is represented by the percentage of cells that were annexin V-positive, and results are presented as means ± S.D. from three independent experiments. E, DNA hypodiploidy of apoptotic L929 cells induced by 10 ng/ml hTNFα stimulation was stained with 2.5 μg/ml PI for 30 min at 37 °C. Percentages of cells exhibiting hypodiploidy DNA content (region marked by horizontal marker) are shown. F, chromatin condensation of L929 cells stimulated with 10 ng/ml hTNFα was analyzed by electron microscopy at 0 h and 48 h. G, genomic DNA cleavage in transfected and control L929 cells following stimulation with 10 ng/ml hTNFα for the indicated times, as analyzed by agarose gel electrophoresis. L929 cells treated with 100 ng/ml TNFα + 4 μg/ml Act D for 7 h served as a positive control (PC).
LAPF Induces Apoptosis via the Lysosomal-Mitochondrial Pathway

A translocation of hLAPF to lysosomes. L929 cells were transiently transfected with hLAPF-GFP expression vectors, and two-color confocal microscopy analysis of lysosome (LysoTracker staining, red) and GFP fusion proteins (green) was performed at the indicated times following transfection. L929 cells transfected with hLAPFΔPH-GFP or hLAPFΔFY-GFP vectors were analyzed 60 h after transfection. B, translocation of mLAPF to lysosomes. L929 cells were transiently transfected with mLAPF-GFP expression vectors, and stained with Lyso Tracker Red at the indicated times following transfection, and viewed under a confocal microscope. C, ability of LAPF to translocate to mitochondria was assessed by transfecting cells with hLAPF- or mLAPF-GFP fusion vectors, then staining with Mito Tracker Red 60 h after transfection.

FIGURE 4. LAPF translocates to lysosomes during apoptosis. A, translocation of hLAPF to lysosomes. L929 cells were transiently transfected with hLAPF-GFP expression vectors, and two-color confocal microscopy analysis of lysosome (LysoTracker staining, red) and GFP fusion proteins (green) was performed at the indicated times following transfection. L929 cells transfected with hLAPFΔPH-GFP or hLAPFΔFY-GFP vectors were analyzed 60 h after transfection. B, translocation of mLAPF to lysosomes. L929 cells were transiently transfected with mLAPF-GFP expression vectors, and stained with Lyso Tracker Red at the indicated times following transfection, and viewed under a confocal microscope. C, ability of LAPF to translocate to mitochondria was assessed by transfecting cells with hLAPF- or mLAPF-GFP fusion vectors, then staining with Mito Tracker Red 60 h after transfection.

LAPF Triggers Caspase-independent Apoptosis—We found that not only did the pan-caspase inhibitor zVAD fail to rescue L929-LAPF cells from apoptosis, but that it actually increased cellular sensitivity to hTNFα cytotoxicity in a dose-dependent manner (Fig. 6A). Furthermore, zVAD also facilitated the translocation of LAPF to lysosomes in a dose-dependent manner (Fig. 6B). Given the observation that cleavage of capase-3, capase-8 and -9 was not detected in L929-LAPF cells (data not shown), it could be speculated that hTNFα-induced apoptosis of L929-LAPF cells is caspase-independent.

LAPF induced signs of MMP, such as the dissipation of ΔΨm, demonstrated by a reduction in R123 incorporation, and release of AIF 24 h after hTNFα treatment, ~12-h later than it induced LMP (Figs. 6C and 5C). The release of AIF from mitochondria into the cytosol was further demonstrated by immunofluorescence analysis, as indicated by the disappearance of the strong perinuclear pattern (Fig. 6D). Compared with AIF, the release of cytochrome c was observed at more advanced stages of apoptosis, 36 h after hTNFα treatment (Fig. 5C). It has been demonstrated that the partial release of AIF at early stages provokes higher release level of AIF and subsequent release of cytochrome c, but AIF alone is sufficient to induce the caspase-independent apoptosis (22–24). Moreover, AIF translocates from the intermembrane space of mitochondria to the nucleus, where it subsequently results in peripheral chromatin condensation and large scale DNA fragmentation (22, 24). The peripheral chromatin condensation and 50–200 kb DNA fragmentation observed in hTNFα-induced apoptotic L929-LAPF cells were also indicative that AIF might be the executor of LAPF-induced apoptosis (Fig. 3, F and G). These results suggested that LAPF triggered caspase-independent apoptosis by altering mitochondrial membrane permeabilization.

Inhibition of Cath D Activity Impairs LAPF-induced Apoptosis and MMP—It has been established that lysosomal cathepsins may trigger MMP by inducing conformational changes in Bax and Bak or cleavage of Bid, which in turn accumulate to mitochondria and mediate MMP (2, 7). To determine the relation between LMP and MMP, cells were pre-incubated with cathepsin inhibitors for 24 h so as to render intralysosomal accumulation of the inhibitors (14). Pepstatin A, a specific inhibitor of the aspartic protease cath D, significantly protected L929-LAPF cells from hTNFα-triggered apoptosis, relieved the decrease in ΔΨm, and delayed the release of AIF from mitochondria (Fig. 6, E and F, p < 0.01, and G). E64d, an inhibitor of the cysteine proteases including cath B and L, had no significant effect on LAPF-induced apoptosis and MMP (Fig. 6, E and F, p > 0.05). Furthermore, combined use of E64d could not significantly enhance the protecting effect of pepstatin A on LAPF-induced apoptosis and MMP (Fig. 6, E and F). The ability of pepstatin A to inhibit AIF release was further supported by immunofluorescence results (Fig. 6D). Taken together, MMP was mainly triggered by the release of cath D from lysosomes in LAPF-initiated apoptosis.

Silencing of LAPF Impairs hTNFα-induced LMP and Apoptosis—To further determine the role of LAPF in cellular apoptosis under physiological conditions, small RNA interference was used to silence the expression of LAPF protein in L929 cells. Almost no LAPF protein was detectable in stable mLAPF-silenced cells (L929-mLAPFi) by immunoblot, compared with that in a nonspecific control RNAi sequence transfected cells (L929-CTRL) and parental L929 cells (Fig. 7A).

In a typical apoptotic model, in which L929 cells are stimulated with hTNFα, further confirming the release of these lysosomal cathepsins into the cytosol (Fig. 5D). All together, these data indicated that LAPF elicited hallmarks of LMP including destabilization of lysosomal membrane and release of cath D and cath L.
FIGURE 5. LAPF initiates lysosomal membrane permeabilization. A and B, destabilization of lysosomal membranes. L929-hLAPF cells and mock-transfected cells were harvested after hTNFα stimulation (10 ng/ml), as indicated, and stained with Lyso Tracker Red (A) or AO (B) for flow cytometry analysis. Cells with decreased red fluorescence (pale cells) were gated, and their percentages are defined by the horizontal marker. C, release of cathepsin D, cathepsin L, AIF, and cytochrome c (cyt c). S-100 was extracted from L929-hLAPF cells and mock-transfected cells treated with 10 ng/ml hTNFα as indicated, for the detection of cathepsins, AIF, and cyt c release by immunoblot analysis. D, immunofluorescence confocal microscopy of cathepsin D and cathepsin L localization. L929-hLAPF cells were treated with 10 ng/ml hTNFα, as indicated, and subjected to immunofluorescence staining for cathepsin D (upper panel) or cathepsin L (lower panel).
FIGURE 6. LAPF triggers caspase-independent but cathepsin D-dependent apoptosis. A, effect of zVAD on LAPF-initiated apoptosis. L929-hLAPF cells were pretreated with different doses of zVAD for 1 h, stimulated with hTNFα (10 ng/ml) for 24 h, and stained with annexin V/PI. Results are expressed as the percentage of cells that were annexin V-positive, and are presented as means ± S.D. from three independent experiments. B, effect of zVAD on the lysosomal translocation of LAPF. L929 cells were transiently transfected with hLAPF-GFP expression vectors. Twenty-four hours after transfection, cells were treated with different doses of zVAD for 1 h, and hTNFα was added (final concentration 10 ng/ml) for another 12 h. Cells were stained with Lyso Tracker Red and viewed under a confocal microscope. C, effect of LAPF-initiated apoptosis on MMP. Cells were stimulated with 10 ng/ml hTNFα for the indicated time, stained with R123 (1 μM) and analyzed by fluorescence activated cell sorting (FACS). Results are expressed as percentage of cells that were R123low, marked by a horizontal bar on histograms. D, release of AIF, detected by immunofluorescence. L929-hLAPF cells were pretreated with pepstatin A (Pst A) (30 μM, specific for cathepsin D) or E64d (10 μM, specific for the cysteine proteases) for 24 h, stimulated with hTNFα (10 ng/ml) as indicated, or were treated with 10 ng/ml hTNFα alone as indicated, then subjected to immunofluorescence staining for AIF. E, pepstatin A influence on hLAPF-induced apoptosis. Cells were pretreated with pepstatin A (30 μM), E64d (10 μM), and both (Pst A/E64d) for 24 h, respectively, stimulated with hTNFα (10 ng/ml) as indicated. Apoptosis was evaluated by annexin V/PI staining, and results are expressed as mean percentage of cells that are annexin V-positive ± S.D. *, p < 0.01 versus L929-hLAPF cells treated with hTNFα at indicated times. F and G, effect of pepstatin A on MMP (R123 staining, F) and AIF release from mitochondria (Western blot assay of AIF contained in S-100 fraction, G), indicating inhibition of MMP. Cells were treated as in E, then either stained with R123 (F) or processed to extract the S-100 fraction (G). Results in F were expressed as mean percentage of cells that were R123low ± S.D. *, p < 0.01 versus L929-hLAPF cells treated with hTNFα at indicated times.
LAPF induces apoptosis via the lysosomal-mitochondrial pathway

LMP in L929 cells induced by 50 ng/ml hTNFα were also observed. Given the observation that LAPF enhanced the sensitivity of L929 cells to hTNFα cytotoxicity through a lysosomal-mitochondrial pathway, we postulated that LAPF might play a role in this typical apoptosis model. As expected, 50 ng/ml hTNFα-induced cell death was significantly inhibited in L929-mLAPFi cells, compared with that in L929-CTRLi and parental L929 cells, as measured by the annexin V/PI staining and the MTT assay (Fig. 7, B and D). Furthermore, hTNFα-induced LMP and MMP were noticeably inhibited in L929-mLAPFi cells (Fig. 7, C and E). Collectively, these data suggested that LAPF contributed to hTNFα-induced apoptosis via a lysosomal-mitochondrial apoptotic pathway initiated by lysosomal destabilization.

DISCUSSION

In the present work we describe a novel conserved family, the Phafins, which consists of 14 unidentified proteins from various species that share common structures, namely a PH domain and a FYVE domain. Sequence analysis reveals that Phafins do not resemble any identified proteins, but share significant homology among themselves, suggesting that they may represent a novel protein family. The universal distribution of Phafins in mammals, fish, insects, and even the phylogenetically less evolved organism C. elegans, underscores the highly evolutionary conservation among Phafins and points to possible roles in organism development and/or important cell signaling. The potential functions of Phafins are implicated by the PH and FYVE domains, both of which act as binding domains, but lack catalytic activity (25, 26). The PH domain can interact with some proteins, including the βγ subunits of G proteins and PKC, and also bind phosphorylated phosphatidylinositol (PtdIns) such as phosphatidylinositol 4,5-bisphosphate (PIP2) and inositol 1,4,5-trisphosphate (IP3) (27–30). The FYVE domain, a zinc-finger-like domain, has a high degree of specificity for phosphatidylinositol 3-phosphate (PtdIns3P), with binding affinity sufficient to mediate membrane association (31, 32). Based on the characteristics of the PH and FYVE domains, it is postulated that Phafins may act as adaptor proteins, via recruiting specific proteins to membrane structures for functional achievement. In the case of the LAPF-initiated lysosomal-mitochondrial apoptotic pathway, it is instructive to further determine LAPF-binding proteins.

The data from the present study revealed a novel LAPF-lysosomal-mitochondrial apoptotic pathway. In this signal cascade, the translocation of LAPF to lysosomes and the subsequent lysosomal rupture were thought as early and initiating events. We have illustrated a functional linkage between LAPF and lysosomes, based on the following evidence. First, the translocation of LAPF to lysosomes was correlatively increased with the progression of apoptosis. Second, the changes of lysosomes, including destabilization of lysosomes and the release of catheps D and L, also showed a likewise time-dependent pattern during apoptosis. Finally, RNA interference of LAPF expression could protect L929 cells from hTNFα-induced apoptosis by stabilizing lysosomal membrane. Taken together, these data suggest that the translocation of LAPF to lysosomes may contribute to the destabilization of hTNFα-induced LMP.
tation of lysosomal membrane, which subsequently activates the downstream apoptotic cascades. Although the lysosomal-mitochondrial pathway has been found in more cellular apoptotic models, it is still not fully understood how the LMP triggers MMP (2, 17, 33). It appears that a specific translocation process of lysosomal cathepsins might be a key to understand this (2, 7, 34). In our study, we observed the translocation of cath D to cytosol after the damage of lysosomes. Moreover, the cath D-specific inhibitor, pepstatin A, could attenuate downstream MMP. These findings indicate the important role of cath D in launching MMP in our LAPF-lysosomal-mitochondrial pathway. It has been revealed that in pre-apoptotic-activated T cells released cath D is required for activation of the pro-apoptotic protein Bax, which subsequently undergoes conformational change and is inserted into mitochondrial membrane, hence resulting in MMP (23, 35). It is hypothesized that released cath D might degrade cytosolic chaperones that sequesters Bax in an inactive conformation, including certain isoforms of the cytosolic 14–3–3 proteins and the Ku70 protein (23, 36, 37). There exist two possible pathways after MMP is triggered. 1) MMP could lead to classical mitochondrial pathway with cytochrome c release and caspase-dependent apoptosis (2, 7, 17). 2) MMP triggers the release of another mitochondrial protein, AIF, which then results in caspase-independent death, as described in our research. Our results were strongly supported by the finding of Bidere et al. (23) who presented a lysosome-cath D-mitochondria-AIF apoptotic pathway in staurosporine-induced apoptosis of T lymphocytes.

The apoptotic effects of TNFαs are mainly mediated by the TNF receptor 1 (TNFR1). Following ligation, TNFR1 is recruited with adapter proteins TRADD and then FADD, which binds to and activates caspase-8 in turn, triggering the extrinsic pathway (38). However, it has been accepted recently that death receptors, which were originally believed to induce apoptosis only via the direct activation of the caspase cascade, may simultaneously activate lysosome-involved death pathways. TNFαs could induce the breakdown of lysosomes and release of different cathepsins, such as cath B and D, depending on the various cell type (6, 13, 14, 39–42). The translocation of cath D presented an early event in the death of L929 cells induced by TNFαs (14). Cath B was implicated as an effector pro tease in several prime and tumor cells, namely isolated hepatocytes, murine embryonic fibroblasts (MEFs), MCF-7, MCF-7SI human breast, and WEHI-S fibrosarcoma cells (6, 13, 40–42). How does TNFα induce LMP and the release of cathepsins? Guicciardi et al. (2003) demonstrated that phosphorylation of the important role of cath D in launching MMP in our LAPF-lysosomal-mitochondrial pathway. Our results were strongly supported by the finding of Bidere et al. (23) who presented a lysosome-cath D-mitochondria-AIF apoptotic pathway in staurosporine-induced apoptosis of T lymphocytes.

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