A Novel Human Phosphatidylethanolamine-binding Protein Resists Tumor Necrosis Factor α-induced Apoptosis by Inhibiting Mitogen-activated Protein Kinase Pathway Activation and Phosphatidylethanolamine Externalization*

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The phosphatidylethanolamine (PE)-binding proteins (PEBPs) are an evolutionarily conserved family of proteins with pivotal biological functions. Here we describe the cloning and functional characterization of a novel family member, human phosphatidylethanolamine-binding protein 4 (hPEBP4). hPEBP4 is expressed in most human tissues and highly expressed in tumor cells. Its expression in tumor cells is further enhanced upon tumor necrosis factor (TNF) α treatment, whereas hPEBP4 normally co-localizes with lysosomes, TNFα stimulation triggers its transfer to the cell membrane, where it binds to Raf-1 and MEK1. L929 cells overexpressing hPEBP4 are resistant to both TNF-α-induced ERK1/2, MEK1, and JNK activation and TNFα-mediated apoptosis. Co-precipitation and in vitro protein binding assay demonstrated that hPEBP4 interacts with Raf-1 and MEK1. A truncated form of hPEBP4, lacking the PE-binding domain, maintains lysosomal co-localization but has no effect on cellular responses to TNFα. Given that MCF-7 breast cancer cells expressed hPEBP4 at a high level, small interfering RNA was used to silence the expression of hPEBP4. We demonstrated that down-regulation of hPEBP4 expression sensitizes MCF-7 breast cancer cells to TNFα-induced apoptosis. hPEBP4 appears to promote cellular resistance to TNF-induced apoptosis by inhibiting activation of the Raf-1/MEK/ERK pathway, JNK, and PE externalization, and the conserved region of PE-binding domain appears to play a vital role in this biological activity of hPEBP4.

The phosphatidylethanolamine-binding protein (PEBP) family consists of a number of 21–23-kDa basic proteins, first identified in bovine brain, with preferential in vitro affinity for phosphatidylethanolamine, a component of the cell membrane. This family is an evolutionarily conserved group found in species of flowering plants (Antirrhinum) (1), parasites (Plasmodium falciparium) (2), nematodes (Toxocara canis) (3), insects (Drosophila melanogaster) (4), and mammals, including cattle, monkeys, and humans (5). A number of functions have been suggested for the mammalian PEBP proteins, including lipid binding and inhibition of serine proteases (6). These proteins can also act as precursors for a bioactive peptide HCNP (hippocampal cholinergeric neurostimulating peptide), important in hippocampus development (5). Plant PEBP homologues are involved in the control of a morphogenic switch between shoot growth and flower structures (7). Yeast two-hybrid screen analysis has shown that human PEBP1 (hPEBP1, also called Raf kinase inhibitory protein or RKIP) acts as a suppressor of Raf-1 kinase activity and mitogen-activated protein kinase signaling in fibroblasts via its ability to sequester and inactivate Raf-1 and MEK1 (8, 9). Both Raf-1 and MEK bind to the highly conserved phosphatidylethanolamine-binding domain of hPEBP; hPEBP induces dissociation of Raf-1-MEK complexes and behaves as a competitive inhibitor of MEK phosphorylation. Mapping of the binding domains has shown that MEK and Raf-1 bind to overlapping sites in hPEBP1, whereas MEK and hPEBP1 associate with different domains in Raf-1, and Raf-1 and hPEBP1 bind to different sites in MEK. In addition, mPEBP2, a recently characterized novel testis-specific member of the phosphatidylethanolamine-binding protein family, co-localizes with members of the mitogen-activated protein kinase pathway in late spermatocytes and spermatids and on the midpiece of epididymal sperm (10). Most aminophospholipids, including phosphatidylserine (PS) and, to a lesser extent, phosphatidylethanolamine (PE), are located in the inner leaflet of the plasma membrane (11, 12). One of the earliest events during apoptosis is the loss of membrane phospholipid asymmetry, leading to the exposure of PS and PE on the outer leaflet (13), a phenomenon called “phospholipid flip-flop”. This mechanism facilitates the recognition of apoptotic cells; Ro, a 19-amino acid tetracyclic polypeptide with a molecular mass of 2041 Da isolated from Streptocystis...
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cilium griseovorticalatum, can be used to monitor the transbilayer movement of PE in biological membranes during cell division and apoptosis (14–18). PE is usually exposed on the surface of apoptotic cells (15, 19–21), and the fluorescence-labeled Ro peptide, FL-SA-Ro, has proven to be a useful tool for detecting apoptotic cells via specific PE binding.

In the present study, we report the molecular cloning and characterization of a new member of the PEBP family derived from human bone marrow stromal cells (BMSCs). This protein contains a typical phosphatidylethanolamine-binding domain and has been designated as human phosphatidylethanolamine-binding protein 4 (hPEBP4). The ability of hPEBP4 to function as an anti-apoptotic molecule and the mechanisms that underlie hPEBP4 effects were investigated.

MATERIALS AND METHODS

Reagents and Cell Culture—All of the cells were grown in RPMI 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 4.5 g/liter d-glucose, nonessential amino acids (100 μM each) (PAA Laboratories), 100 μg/ml streptomycin, and 2 mM glutamine at 37 °C in a 5% CO₂ atmosphere.

Isolation of hPEBP4 cDNA—Full-length hPEBP4 cDNA was directly isolated from a human BMSC cDNA library by random sequencing as described previously (22). A plasmid cDNA library of pCMV SPORT6.0 vector (Invitrogen) was constructed using the Superscript plasmid system (Invitrogen) for cDNA synthesis. The full-length cDNA of clone HNC7B1 appeared to encode a protein with a typical phosphatidylethanolamine-binding domain and was hence designated as hPEBP4. The full-length sequence is available in the GenBank™ data base under accession number AY0937148.

Isolation of Human Blood Cells— Buffy coats from healthy donors were obtained from the transfusion center of Changhai Hospital (Shanghai, China). Human peripheral blood mononuclear cells were prepared by Ficoll-Hypaque (PAA Laboratories) density gradient centrifugation. Peripheral monocytes, CD4⁺ T cells, CD8⁺ T cells, and B cells were directly isolated from human peripheral blood mononuclear cells using anti-CD14, anti-CD3, anti-CD4, anti-CD8, and anti-CD19 monoclonal antibody-conjugated magnetic microbeads (Miltenyi Biotech), respectively. Some monocyte samples were stimulated with lipopolysaccharide (1 μg/ml for 24 h), and hPEBP4 expression examined in all groups by RT-PCR, as described below. The purity of each cell population was generally 90–95%.

RT-PCR and Northern Blot Analysis of hPEBP4 mRNA Expression—RT-PCR and Northern blotting were performed as described previously (23). Primers specific for hPEBP4 were 5'-GGGTTGGAACATGAGG-GCTG-3' (sense) and 5'-TCCTGGCTGGGTCCTCCG-3' (antisense). Northern blot filters containing human poly(A)+ RNA (2 μg/ lane) were probed with hPEBP4 probe. The filters were hybridized with the 5'-labeled hPEBP4 cDNA probe in ExpressionHyb hybridization solution (Clontech).

Quantitation of hPEBP4 Expression by Real Time Fluorescence Monitored PCR—Quantitation of inductive expression patterns of hPEBP4 in A549 and LoVo cells was performed on a Cycler fitted with an optical assembly unit (Bio-Rad). For TaqMan real-time PCR of hPEBP4, hPEBP4-specific primers used were 5'-CCACCATCACGGATAT-AGG-3' (forward) and 5'-GGGACTTACCGGCTGTC-3' (reverse). The hPEBP4 probe 5'-CCTGGATGGAGCCGATATCGAAGTTC-3' (antisense). Northern blot filters containing human poly(A)+ RNA (2 μg/lane) were probed with hPEBP4 probe. The filters were hybridized with the 32P-labeled hPEBP4 cDNA probe in ExpressionHyb hybridization solution (Clontech).

Analysis of the primary amino acid sequences of the varied PEBP family members revealed that the N terminus is poorly conserved in hPEBP4 and other members of the PEBP family. In contrast, the C terminus of hPEBP4 contains several cysteine residues (14). The hPEBP4 probes used were 5'-CCTGGATGGAGCCGATATCGAAGTTC-3' (antisense). Northern blot filters containing human poly(A)+ RNA (2 μg/lane) were probed with hPEBP4 probe. The filters were hybridized with the 32P-labeled hPEBP4 cDNA probe in ExpressionHyb hybridization solution (Clontech).

Protein and Generation of Anti-hPEBP4 Polyclonal Antibody—Analysis of the primary amino acid sequences of the varied PEBP family members revealed that the N terminus is poorly conserved in this family. The N-terminal 99-amino acid section of hPEBP4 was thus expressed, and rabbit antibodies that specifically recognize the N terminus of hPEBP4 were generated. cDNA encoding the 99 N-terminal amino acids of hPEBP4 was cloned into pGEX-2T (Amersham Biosciences). The soluble glutathione S-transferase fusion protein was obtained under isopropyl β-D-thiogalactopyranoside induction (0.2 mM) at 37 °C for 4 h with Escherichia coli strain BL21 as a host and purified by glutathione-Sepharose 4B affinity chromatography (Pierce). The purified fusion protein was used to immunize rabbits according to a conventional procedure, as described (24). Anti-hPEBP4 serum was purified using protein A affinity chromatography (Pierce), and titration analysis was performed by Western blot.

Cell Transfection—The expression vectors hPEBP4-B and p75PEBP4-B were transiently transfected into L929 cells using Lipo-fectAMINE reagent (Invitrogen) for 40 h with pcDNA3.1/Myc-His (−) B as a mock control. After serum starvation for 24 h, the cells were exposed to 20 ng/ml TNFα overnight and then analyzed by Western blot or apoptosis assay. In some experiments, 1 μg of hPEBP4-B, p75PEBP4-B, or control vector were co-transfected together with the Ras-B expression vector (1 μg) into L929 cells, and the cells were harvested 48 h later for subsequent analysis.

Cytoskeleton of hPEBP4 by Fluorescence Confocal Microscopy—HEP2 cells transiently transfected with hPEBP4-GFP, p75PEBP4-GFP, or GFP control vector, growing on glass coverslides, were placed in 6-well plates, treated with 20 ng/ml TNFα (Sigma) for 10 min, and then incubated with LysoTracker Red DND-99 (Molecular Probes) for 15 min at room temperature in the dark. At the same time, GFP, hPEBP4-GFP, or p75PEBP4-GFP vector were co-transfected into some cells with either pDsRed-mem (encoding a membrane-localized form of red fluorescent protein) or pEGFP-N1, or pEGFP-C1. The samples were washed briefly in phosphate-buffered saline and fixed in 4% polyformaldehyde prior to observation with a fluorescence confocal microscope (LSM confocal microscope; Carl Zeiss).

Co-precipitation and in Vitro Protein Binding Assay— 36 h after transfection, transiently transfected L929 cells were serum-starved for 24 h, stimulated with 20 ng/ml TNFα for 10 min, harvested, and analyzed by Western blot (lysate buffer containing 1× SDS-PAGE, and transferred to Protein nitrocellulose membranes (Schleicher & Schuell). The blots were probed with antibodies specific for Myc (Oncogene), phospho-ERK1/2, phospho-MEK-1, phospho-Raf-1, or MEK1-FLAG, or Raf-1-FLAG-transfected cells for 2–3 h at 4 °C, the samples were washed four times in lysis buffer containing 10 μM amiodazole and 0.1% Triton X-100, and Western blotting was performed. For in vitro protein binding assay, the extract of 293HEK cells transiently transfected with hPEBP4-B or p75PEBP4-B for 48 h were mixed with Ni-NTA beads as a mock control. Co-precipitated proteins with cell lysate was analyzed by Western blot (lysate buffer containing 1× SDS-PAGE, and transferred to Protein nitrocellulose membranes (Schleicher & Schuell). The blots were probed with antibodies specific for Myc (Oncogene), phospho-ERK1/2, phospho-MEK-1, phospho-Raf-1, or MEK1-FLAG, or Raf-1-FLAG-transfected cells for 2–3 h at 4 °C, the samples were washed four times in phosphate-buffered saline and analyzed by Western blot.

Western Blot Analysis— A BCA protein assay reagent kit (Pierce) was used to measure protein concentration. Samples containing equal amounts of protein were prepared as above, separated by 12% SDS-PAGE, and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). The blots were probed with antibodies specific for Myc (Oncogene), phospho-ERK1/2, phospho-MEK-1, phospho-Raf-1, or MEK1-FLAG, or Raf-1-FLAG, or MeK1-FLAG, or Raf-1-FLAG-transfected cells for 2–3 h at 4 °C, the samples were washed four times in phosphate-buffered saline and analyzed by Western blot.

Construction of FL-SA-Ro—Ro 09-0198, kindly provided by Roche Applied Science, was biotinylated using Biotin (Long Arm) NHS (Vector Laboratories) (14). FL-SA (Vector Laboratories) was mixed with biotinylated Ro 09-0198, and the resulting FL-SA-Ro complexes were purified by gel filtration, as described (16).

Apoptosis Assay—The cells were washed, resuspended in staining buffer, and stained with PI and either annexin V (Apoptosis kit, Becton Dickinson) or R123 (R-302; Molecular Probes), according to the manufacturer’s instructions, or FL-SA-Ro, as described (17). Stained cells were analyzed by fluorescence-activated cell sorter (FACScalibur, Becton Dickinson).

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hPEBP4 siRNA Assay—21-nucleotide sequences of hPEBP4 siRNA were synthesized by Proligo: 5'/H11032-GGAAAAGUCAUCUCUCCTT (sense) and 5'/H11032-GGAGAGAGAUGACUUUUCCTT (antisense). hPEBP4 mutation control siRNA oligonucleotides were 5'/H11032-GGAAAAUCUACUCUCCTT (sense) and 5'/H11032-GGAGAGAGUAGACUUUUCCTT (antisense). For annealing, 20 μM single-stranded 21-nucleotide RNAs were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C and then for 1 h at 37 °C. siRNA duplexes were transfected into MCF-7 breast cancer cells using Oligofectamine reagent (Invitrogen), as described (25). All of the data shown in this article are representative of at least three independent experiments.

RESULTS

Identification and Sequence Analysis of hPEBP4—A novel cDNA, 874 bp in length and containing a complete open reading frame of 684 bp with an upstream in-frame stop codon (TAA) and a putative polyadenylation signal located 18 bases upstream of the poly(A) stretch, was directly isolated from a human BMSC cDNA library. The cDNA potentially encoded a 227-residue protein, with a calculated molecular mass of 25,734 Daltons and an isoelectric point of 5.71. Homology analysis revealed close similarity (~30–60%) to other known mem-

Fig. 1. Multiple alignment of hPEBP4 with closely related PEBP family members. Alignment was performed with the GCG package and minimally adjusted manually. Identical residues are boxed in black, and similar residues are in gray. The phosphatidylethanolamine-binding domain is indicated by a bold arrow spanning residues 84–191 of hPEBP4. Shaded circles over residues indicate those that are important for PE binding. The accession numbers for the PEBPs and PEBP-like proteins are as follows: hPEBP4, AY037148; hPEBP1, P30086; monkey, S46485; rat, CAA57088; mouse, AA926535; bovine, P13696; Onchocerca volvulus, P31729; and Caenorhabditis elegans, NP_505930.
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Members of PEBP family from human, bovine, mouse, rat, monkey, D. melanogaster, and others (Fig. 1). At the primary amino acid level, it shared the highest homology with an unnamed murine PEBP-like protein, BAB24810 (45% identity and 57% similarity). As such, the two appeared to represent interspecies orthologues, whereas the other PEBPs represented paralogues, exhibiting significantly lower sequence homology. Based on its sequence similarity with other PEBP family members, the conservation of PEBP features, and its putative capacity to bind phosphatidylethanolamine, the novel molecule was designated hPEBP4. The human PEBP4 cDNA corresponded to Unigene cluster Hs.352388, located on human chromosome 8p21.2.

Based on sequence analysis and existing PEBP structures, a number of features characteristic of the PEBP family, with likely functional importance, were identified in hPEBP4. As with all PEBP family members, human PEBP4 was predicted to contain a PE-binding domain, in this case between amino acids 84 and 191 (Fig. 1). Residues previously demonstrated to be conserved among all members of the family (Pro97, Asp98, Pro100, His112, and Arg149) were also found in hPEBP4. These residues are thought to be involved in determining the local structure of a biologically important ligand-binding site in PEBPs (26). The two main regions of high sequence conservation among hPEBP4 and other PEBPs occurred between residues 91–117 and residues 141–153 (based on hPEBP4 numbering). Residues 91–101 (including cis-Pro100) lined one side of the putative PE-binding site, with residues 96–98 and 100 forming an Asp-Pro-Asp-Xaa-Pro motif that is universally conserved in all family members. The first aspartate of this motif (Asp96), along with His112, lay at the base of the binding pocket. Arg149 is part of a second highly conserved region running between residues 146 and 149, with the motif Gly-Xaa-His-Arg. The 141–153 conserved region formed part of the second side of the binding pocket, as well as a loop adjacent to this site. These structural features suggest that hPEBP4 might possess PE binding properties similar to those seen in other PEBPs, whose structures have been determined by x-ray crystallography.

Expression Pattern of hPEBP4 mRNA—RT-PCR analysis revealed that hPEBP4 mRNA is expressed in a variety of tumor cells and freshly isolated cells, including Daudi (Burkitt’s lymphoma), NAMALWA (Burkitt’s lymphoma), MCF-7 (breast carcinoma), PC-3 (prostate carcinoma), and CaoV-3 (ovarian carcinoma), NAMALWA (Burkitt’s lymphoma), MCF-7 (breast carcinoma), HT-29 (colon carcinoma), or LoVo (colorectal carcinoma) cells, human BMSCs, and human CD19+ B lymphocytes (Fig. 2, A–C). Message was not detected in HuT-78 (cutaneous T-cell lymphoma), HL-60 (myelomonocytic), U937 (promonocytic), THP-1 (monocytic), SMMC 7721 (hepatocellular carcinoma), HeLa (cervical carcinoma), A549 (lung carcinoma), HT-29 (colon carcinoma), or LoVo (colorectal carcinoma) cells nor in freshly isolated human peripheral blood monocytes, lipopolysaccharide-stimulated monocytes, or human CD4+ or CD8+ T lymphocytes. The mRNA expression pattern of hPEBP4 in normal human tissues was examined by Northern blot analysis. Clontech human MTN blots revealed the presence of a single hPEBP4 mRNA as a strong 1.2-kb message, expressed strongly in testis, heart, skeletal muscle, and thyroid and weakly in lung, liver, spinal cord, brain, adrenal gland, and bone marrow (Fig. 2D). This expression pattern in normal human tissues is consistent with those previously demonstrated for human, mouse, rat, and bovine PEBPs, which are particularly abundant in heart, brain, and testis.

The effects of TNFα on hPEBP4 mRNA expression in A549 and LoVo cells, which do not normally express hPEBP4, were examined by quantitative real time PCR analysis. Following TNFα stimulation, hPEBP4 expression increased in the cells, reaching a maximum at 12 h and then decreasing (Fig. 2E). The inducible, time-dependent, hPEBP4 expression pattern suggests that hPEBP4 might play roles in cellular responses to extracellular stimuli and in self-protection from damage.

Cytolocalization of hPEBP4—The cytolocalization of GFP-fused hPEBP4 protein in HEK293 cells was examined by fluorescence confocal microscopy. 48 h after transfection with hPEBP4-GFP (full-length) or p75PEBP-GFP (75 N-terminal...
residues of hPEBP4, lacking the PE-binding domain), GFP fluorescence displayed a cytoplasmic distribution in transfected cells, with signal particularly strong in the perinuclear region. Further subcellular localization analysis was performed using specific probes for cell organelles including mitochondria, endoplasmic reticulum, Golgi apparatus, and lysosomes. The majority of hPEBP4-GFP and p75PEBP-GFP green staining correlated with the red lysosome signal (LysoTracker), as displayed by yellow fluorescence in overlaid images (Fig. 3A), but did not correlate with red staining associated with mitochondria, endoplasmic reticulum, or Golgi apparatus (data not shown). Cells transfected only with GFP showed fluorescence in both cytoplasm and nucleus and no evidence of colocalization with lysosomes. The results suggest that hPEBP4 localizes primarily to lysosomes, in a PE-binding domain-independent manner.

**TNFα-induced hPEBP4 Translocation from Lysosome to Cell Membrane**—Because TNFα could induce hPEBP4 expression, we examined its influence on hPEBP4 cytolocalization. hPEBP4-GFP, p75PEBP-GFP, or GFP were co-transfected into HEK293 cells together with pDsRed-mem, which encoded a membrane-localized form of red fluorescent protein. When transfected cells were stimulated with TNFα (20 ng/ml) for 10 min, hPEBP4-GFP translocated from lysosomes to the cell membrane (Fig. 3B). In contrast, p75PEBP-GFP, which lacked the PE-binding domain, did not demonstrate translocation. Expression of GFP, GFP-fused p75PEBP4, GFP-fused hPEBP4 proteins in transfected cells was confirmed by Western blot (Fig. 3C). These results provide evidence that hPEBP4 might be induced to translocate from lysosomes to the plasma membrane following cellular exposure to extracellular stimuli such as TNFα and that this translocation requires the PE-binding domain.

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![Fig. 3. Cytolocalization of hPEBP4 proteins in transfected HEK 293 cells.](http://www.jbc.org/)

A. transfected HEK 293 cells without TNFα simulation. HEK 293 cells were transiently transfected with hPEBP4-GFP, p75PEBP4-GFP, or control GFP vector. 48 h after transfection, the cells were fixed and stained with lysosome-specific LysoTracker Red DND-99. Co-localization of hPEBP4-GFP (green) and lysosomes (red) is demonstrated by yellow fluorescence in overlay images.

B. transfected HEK 293 cells with TNFα simulation. HEK 293 cells were transiently transfected with hPEBP4-GFP, p75PEBP4-GFP, or control GFP vector, together with pDsRed-mem, which encoded a membrane-localized form of red fluorescent protein. 40 h after transfection, the cells were stimulated with 20 ng/ml TNFα for 10 min.

C. cell lysates of GFP vector, p75PEBP4-GFP, hPEBP4-GFP, hPEBP4-B, pCNA3.1/Myc-His (-)B, or p75PEBP4-B transfected HEK 293 cells were analyzed by Western blot using either anti-GFP antibody or anti-Myc antibody.
L929 cells was blocked by overexpression of hPEBP4 but not by overexpression of PE-binding domain-deficient p75PEBP4. Also, when hPEBP4-B was co-transfected with the Ras expression vector Ras-B, Ras-mediated activation of MEK1 and ERK1/2 was also inhibited (Fig. 4B). In contrast, Raf-1 phosphorylation triggered by TNFα stimulation or co-transfection with Ras-B was not influenced by either form of hPEBP4.

hPEBP4 Associates with Raf-1 and MEK1—To investigate whether hPEBP4 could interact with endogenous Raf-1 and MEK1, lysates of L929 cells transiently transfected with hPEBP4-B, p75PEBP4-B, or control vector and then treated with TNFα were precipitated using Ni-NTA beads. The precipitates were resolved by SDS-PAGE and probed for both Raf-1 and MEK1, but not ERK1/2, which is downstream of MEK1 and unlikely to directly interact with hPEBP. As shown in Fig. 4C, hPEBP4 co-precipitated with Raf-1 and MEK1 after TNFα stimulation, whereas p75PEBP4 did not, indicating that TNFα prompts hPEBP4 to associate with Raf-1 and MEK1 and that this association requires the PE-binding domain of hPEBP4.

An in vitro protein binding assay was carried out to further demonstrate this interaction. Cell lysates of hPEBP4-B-, p75PEBP4-B-, or control vector-transfected L929 cells were immobilized on Ni-NTA beads and incubated with cell extracts of HEK293 cells transfected with Raf-1-FLAG or MEK1-FLAG, respectively. Fig. 4D showed Western blot of protein-protein interaction. hPEBP4 specially bound to Raf-1 and MEK1. No binding was detected of p75PEBP4 and control. These results were further confirmed using confocal microscopy (Fig. 4E).

hPEBP4 translocated from lysosomes to the membrane and co-localized with Raf-1 and MEK1 upon TNFα treatment, whereas p75PEBP4 did not. Given that the Ras/Raf-1/MEK/ERK signaling pathway activated by TNFα was inhibited in hPEBP4-overexpressing cells, it appears that extracellular stimuli such as TNFα cause hPEBP4 to depart from its usual lysosomal location, traveling to a membrane proximal position where it binds to Raf-1 or MEK-1, dissociating the Raf-1/MEK complex and thus behaving as a competitive inhibitor of MEK phosphorylation. Notably, p75PEBP4, a truncated form lacking a PE-binding domain, had no effect on any of the above described events, save for co-localization with lysosomes, indicating that it is the PE-binding domain that binds Raf-1 and MEK-1 and exerts a negative effect on the Raf-1/MEK1/ERK cascade.

hPEBP4 Inhibits TNFα-induced Apoptosis—Phospholipids such as PE and PS are located on the inner leaflet of the plasma membrane, and loss of membrane phospholipid asymmetry is one of the earliest events during apoptosis. PE, as well as PS, can be detected on the cell surface during the

with 10 ng/ml TNFα for 10 min. Equivalent protein loadings of each lysate were immunoblotted with antibodies recognizing the phosphorylated, active forms of pp42/pp44 ERK, MEK1, or Raf-1. B, Ras-B was co-transfected together with hPEBP4-B, p75PEBP4-B, or mock control vector into L929 cells. 48 h after transfection, the cells were lysed and subjected to Western blot analysis with the same antibody as described in A. C, hPEBP4 binding to Raf-1 and MEK1 in vivo. Lysates of His-labeled hPEBP transfected L929 cells stimulated with TNFα for 10 min were incubated with Ni-NTA beads. The beads were collected by centrifugation, and samples were immunoblotted using Raf-1 or MEK1 or anti-hPEBP4 antibody. D, hPEBP4 binding with Raf-1 and MEK1 in vitro. Lysates of hPEBP4-B or p75PEBP4-B transfected cells were immobilized with Ni-NTA beads. After incubation with cell extracts of Raf-1-FLAG or MEK1-FLAG transfectants for 2–3 h at 4°C, agarose pellets were washed and subjected to SDS-PAGE. E, hPEBP4 co-localization with Raf-1 and MEK1 upon TNFα treatment. HEK293 cells were transiently transfected with hPEBP4-GFP, p75PEBP4-GFP, or control GFP vector, together with Raf-1-RFP or MEK1-RFP vectors and then stimulated with 20 ng/ml TNFα for 10 min. Co-localization of hPEBP4-GFP (green) and Raf-1-RFP or MEK1-RFP (red) is demonstrated by yellow fluorescence in overlay images.

Fig. 4. Effects of hPEBP4 overexpression on Ras/Raf-1/MEK1/ERK1/2 signaling pathway and interaction of hPEBP4 with Raf-1 and MEK1. A, hPEBP4-B, p75PEBP4-B, or mock control vector transfected L929 cells were serum-starved for 24 h and then treated with 10 ng/ml TNFα for 10 min. Equivalent protein loadings of each lysate were immunoblotted with antibodies recognizing the phosphorylated, active forms of pp42/pp44 ERK, MEK1, or Raf-1. B, Ras-B was co-transfected together with hPEBP4-B, p75PEBP4-B, or mock control vector into L929 cells. 48 h after transfection, the cells were lysed and subjected to Western blot analysis with the same antibody as described in A. C, hPEBP4 binding to Raf-1 and MEK1 in vivo. Lysates of His-labeled hPEBP transfected L929 cells stimulated with TNFα for 10 min were incubated with Ni-NTA beads. The beads were collected by centrifugation, and samples were immunoblotted using Raf-1 or MEK1 or anti-hPEBP4 antibody. D, hPEBP4 binding with Raf-1 and MEK1 in vitro. Lysates of hPEBP4-B or p75PEBP4-B transfected cells were immobilized with Ni-NTA beads. After incubation with cell extracts of Raf-1-FLAG or MEK1-FLAG transfectants for 2–3 h at 4°C, agarose pellets were washed and subjected to SDS-PAGE. E, hPEBP4 co-localization with Raf-1 and MEK1 upon TNFα treatment. HEK293 cells were transiently transfected with hPEBP4-GFP, p75PEBP4-GFP, or control GFP vector, together with Raf-1-RFP or MEK1-RFP vectors and then stimulated with 20 ng/ml TNFα for 10 min. Co-localization of hPEBP4-GFP (green) and Raf-1-RFP or MEK1-RFP (red) is demonstrated by yellow fluorescence in overlay images.
early stages of apoptosis, indicating a loss of asymmetric distribution of aminophospholipids. Because hPEBP4 contained a conserved PE-binding domain and translocated from lysosomes to the membrane upon TNFα treatment, we wondered whether it played a role in the process of cellular apoptosis. To investigate this possibility, L929 cells were transiently transfected with hPEBP4-B, p75PEBP4-B, or control vector, stimulated with the indicated concentrations of TNFα for 20 h and apoptosis assessed. Loss of mitochondrial inner transmembrane potential is also often associated with the early stages of apoptosis and may be one of the central features of the process (27). The green fluorescent cationic dye Rhodamine 123 (R-302) binds selectively to the inner mitochondrial membrane and accumulates in the charged membrane compartments of living cells; apoptotic cells exhibit a loss of R123 binding. As shown in Fig. 5A, hPEBP4 overexpression in L929 cells transiently transfected with hPEBP4-B transfectants did not differ in their baseline level of apoptosis, when compared with p75PEBP4-B or mock transfectants but did exhibit lower levels of apoptosis following TNFα treatment. Fluorescein isothiocyanate-conjugated annexin V/PI staining was also performed to detect apoptotic cells. Cells acquire annexin V-binding sites during apoptosis, providing another method for detecting cells undergoing apoptosis. Apoptotic cells are annexin V-positive (including PI or PI+), whereas necrotic cells are annexin V-negative and PI-positive. As shown in Fig. 5B, the percentage of hPEBP4-overexpressing L929 cells that were annexin V positive (apoptotic) was much less than that of p75PEBP4-overexpressing cells, mock control, or untransfected cells following exposure to TNFα. The same result was obtained in human lung carcinoma cells A549 (data not shown). Together these results suggest that hPEBP4 might be involved in cellular resistance to TNFα-induced apoptosis and that the conserved region of PE-binding domain appears to play a vital role in this biological activity of hPEBP4.

Overexpression of hPEBP4 Inhibits TNFα-induced Activation of JNK and PE Externalization—TNF receptor-associated factor 2 (TRAF2) in TNF signaling activates a mitogen-activated protein kinase kinase kinase, which then activates ERK or JNK. The roles of JNK activation in apoptosis are highly controversial, with reports suggesting pro-apoptotic, anti-apoptotic, and neutral roles (28–31). Because hPEBP4 plays a negative role in TNF-induced apoptosis, its effects on TNF-induced JNK activation were tested. L929 cells were transiently transfected with mock, hPEBP4-B, or p75PEBP4-B vectors. After serum starvation for 24 h and stimulation with TNFα for various lengths of time, the phosphorylation levels of JNK were examined. As shown in Fig. 6A, TNFα-induced phosphorylation of JNK in L929 cells was inhibited by overexpression of hPEBP4 but not by overexpression of p75PEBP4. However, no binding between hPEBP4 with TNFR1, TNFR2, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, TRADD, or FADD was detected, as assayed by immunoprecipitation with Ni-NTA beads from lysates of L929 cells stably transfected with hPEBP4 vector (data not shown). Meanwhile, TNF-mediated activation of caspase-3 (Fig. 6B), caspase-8, and p38 (data not shown) were not influenced by hPEBP4 overexpression.
Ro 09-0198 (cinnamycin), a tetracyclic peptide antibiotic that recognizes the structure of phosphatidylethanolamine in a highly specific manner, forming a tight equimolar complex with PE on biological membranes, has been used to monitor the transbilayer movement of PE in biological membranes during cell division and apoptosis. Because hPEBP4 had a PE-binding domain and translocates from lysosomes to the cell membrane after TNFα/H9251 stimulation, we wondered whether it could associate with PE and inhibit its exposure, thus rendering the cell insensitive to apoptotic stimuli. We conjugated biotinylated Ro to FL-SA to study the molecular movement of PE in L929 cells, prepared as described for the apoptosis assay. As shown in Fig. 6, no difference was observed among the groups in the absence of TNFα/H9251. However, when cells were subjected to incubation with 20 ng/ml TNFα/H9251 for 20 h, PE exposure was suppressed only by overexpression of hPEBP4. This result demonstrates that the insensitivity of hPEBP4 transfectants to TNFα-induced apoptosis is at least partly due to inhibition of PE externalization.

**DISCUSSION**

The PEBP family is a highly conserved group of proteins with homologues in a wide variety of organisms. We report here the molecular cloning and characterization of a new member of the PEBP family, hPEBP4, from human BMSCs. hPEBP4 appears to promote cellular resistance to TNFα-induced apoptosis. The result further demonstrates that hPEBP4 promotes cellular resistance to TNFα-induced apoptosis.

**Silencing of hPEBP4 Expression Sensitizes MCF-7 Breast Cancer Cells to TNFα-induced Apoptosis**—Overexpression of hPEBP4 in L929 cells showed that this protein represents a potent survival molecule. Given its high expression in breast cancer cells, the possibility that silencing hPEBP4 expression could sensitize MCF-7 breast cancer cells to apoptosis induction was investigated. siRNA was used to silence the expression of hPEBP4 protein in MCF-7 breast cancer cells, with a mutant siRNA duplex and no siRNA as controls. Western blotting and RT-PCR confirmed the complete silencing of hPEBP4 expression in MCF-7 cells (Fig. 7A). Annexin V/PI staining was used to detect apoptotic cells after exposure to 20 ng/ml TNFα for 20 h. As shown in Fig. 7B, hPEBP4-silenced MCF-7 cells were more sensitive to TNFα-induced apoptosis. The result further demonstrates that hPEBP4 promotes cellular resistance to TNFα-induced apoptosis.
than 220 residues in length and contain at least two insertions and one deletion in their protein sequence, when compared with proteins belonging to the three existing subfamilies. Therefore, hPEBP4 belongs to the fourth PEBP subfamily; this is represented in its nomenclature, hPEBP4. Although the N-terminal sequence of hPEBP4 varied notably from those of known PEBPs, and the two insertions (between residues 55 and 56 and residues 102 and 103) and the deletion (residues 131–134 of hPEBP numbering) were all located in regions identified as PE-binding structures, it seems that the overall functions of hPEBP4 were not affected because it could associate with Raf-1 and MEK1 and inhibit TNFα/H9251-induced activation of the Ras/Raf-1/MEK/ERK signaling pathway, all PEBP family member attributes (8–10).

Sequence analysis and preliminary homology modeling based on the crystal structure of bovine and human PEBPs (26, 32) suggest that hPEBP4 also contains a putative PE-binding pocket, which could potentially allow anchoring of this protein to the inner leaflet of membranes. In addition, hPEBP4 has high sequence and predicted topology homology with analogous Raf-1- and MEK-binding regions predicted in deletion studies (9). In this article we observed that hPEBP4 was involved in the regulation of mitogen-activated protein kinase signaling.

Fig. 7. hPEBP4 RNA interference in MCF-7 breast cancer cells enhances TNFα-induced apoptosis. A, functional silencing of endogenous hPEBP4 expression by RNA interference in MCF-7 cells. MCF-7 cells were transfected with hPEBP-specific siRNA (hPEBP4 siRNA) or mutated hPEBP4 siRNA control using Oligofectamine reagent. 48 h after transfection, the cells were harvested, and hPEBP4 expression was detected by Western blotting with anti-hPEBP4 antibody (upper panels) and RT-PCR analysis (lower panels). B, MCF-7 cells transfected with specific hPEBP4 siRNA or hPEBP4 mutation siRNA control were stained with annexin V and PI following incubation with TNFα for 20 h. The percentages of apoptotic cells in each sample are indicated.

hPEBP4 from lysosomes to cell membrane and co-localization with Raf-1 or MEK1 following TNFα treatment was observed by confocal microscopy. Because the highly conserved PE-binding domain of other PEBPs has been proven to bind to Raf-1 and MEK1 (8, 9), we constructed vectors expressing a truncated form of hPEBP4, p75PEBP4, which lacked a PE-binding domain, and found that it could not inhibit the Raf-1/MEK/ERK pathway. p75PEBP4 was also unable to associate with either Raf-1 or MEK. These data are in agreement with the hypothesis put forward by Yeung et al. (8, 9), derived from their studies on known PEBPs, suggesting that both Raf-1 and MEK bind to phosphatidylethanolamine-binding domains (accession number PS01220) and that PEBPs are targeted to the cell membrane following mitogenic stimulation.

PE and PS, located on the cytoplasmic surface of the cell membrane, play important roles in maintaining plasma membrane asymmetry. Translocation of both PE and PS from the inner to the outer leaflet of the plasma membrane precedes apoptosis, suggesting that redistribution of PS and PE may be an early symptom of apoptotic cell death. Cells overexpressing hPEBP4 were resistant to apoptosis induced by TNFα, whereas silencing of hPEBP4 using siRNA sensitized the cells to apoptotic stimuli, consistent with the previous observation that TNFα-resistant cKDH-8/11 cells had increased levels of hPEBP compared with TNFα-sensitive KDH-8/YK cells (33). Using FL-SA-Ro, which stringently recognizes the structure of PE, we found that TNFα-induced PE exposure on the surface of cells was significantly increased during hPEBP4 mRNA silencing and decreased during hPEBP4 overexpression. The data suggest that hPEBP4 is targeted to the cell membrane and binds to PE situated on the inner leaflet of the plasma membrane,
preventing PE externalization, and maintaining membrane phospholipid asymmetry.

The TNFR1 signaling complex is composed of the trimerized receptor, among which the FADD recruits and activates procaspase 8, initiating the apoptotic pathway, and the TNF receptor-associated factors 2 and 5 (TRAF2 and TRAF5) and the receptor-interacting protein are involved in the activation of JNK (34, 35). The contribution of JNK activation to apoptosis depends on both cell type and stimulus (35), and its precise role in TNF-induced apoptosis is unclear (36, 37). JNK has recently been shown to positively regulate TNF-induced apoptosis (38). We found that up-regulation of hPEBP4 expression in L929 cells inhibited TNFα-induced ERK and JNK activation, as well as apoptosis. However, hPEBP4 had no effect on p38, caspase-3, or caspase-8 activation, and did not bind to TNFR1, TNFR2, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, TRADD, or FADD (data not shown). hPEBP4-mediated suppression of ERK and JNK activation thus appears to be partially responsible for the observed inhibition of TNFα-induced apoptosis; further investigation is required to identify other mechanisms by which hPEBP promotes apoptosis resistance.

In sum, we have cloned and characterized hPEBP4, a novel member of the PEBP family that potentially functions as a survival-enhancing molecule, inhibiting TNFα-induced apoptosis by interfering with Ras/Raf/MEK/ERK signaling, JNK activation, and PE externalization via its phosphatidylethanolamine-binding domain.

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REFERENCES
1. Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997) Science 275, 80–83
2. Trottstein, F., and Cowman, A. F. (1995) Mol. Biochem. Parasitol. 70, 235–239
3. Gens, D., Ferguson, C. J., Robertson, B. D., Nieves, R., Page, A. P., Blaxter, M. L., and Maizels, R. M. (1995) J. Biol. Chem. 270, 18517–18522
4. Pikielny, C. W., Hasen, G., Rouyer, F., and Rosbash, M. (1994) Neuron 12, 35–49
5. Tsuchiya, T., Tojo, N., Agui, H., and Ojika, K. (1995) Mol. Brain. Res. 30, 381–384
6. Hengst, U., Albrecht, H., Hess, D., and Monard, D. (2001) J. Biol. Chem. 276, 525–540
7. Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S., and Coen, E. (1996) Nature 379, 791–797
8. Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K. D., Rose, D. W., Mischak, H., Sedivy, J. M., and Kolch, W. (1999) Nature 401, 173–177
9. Yeung, K., Janosch, P., McFerran, B., Rose, D. W., Mischak, H., Sedivy, J. M., and Kolch, W. (2000) Mol. Cell. Biol. 20, 3079–3085
10. Hickox, D. M., Gibbs, G., Morrison, J. B., Sehgal, K., Edgar, K., Keah, H. H., Alter, K., Loveland, K. L., Hearn, M. T., de Kretser, D. M., and O'Bryan, M. K. (2002) Biol. Reprod. 67, 917–927
11. Seigneuret, M., and Devaux, P. P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3753–3755
12. Zwaal, R. F., and Schroit, A. J. (1997) Blood 89, 1121–1132
13. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFave, D. M., and Green, D. R. (1995) J. Exp. Med. 182, 1545–1556
14. Aoki, Y., Uenaka, T., Aoki, J., Umeda, K., and Inoue, K. (1994) J. Biochem. (Tokyo) 116, 291–297
15. Umeda, M., and Emoto, K. (1999) Chem. Phys. Lipids 101, 81–91
16. Emoto, K., Kobayashi, T., Yamaji, A., Aizawa, H., Yahara, I., Inoue, K., and Umeda, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12867–12872
17. Emoto, K., Toyama-Sorimachi, N., Karasuyama, H., Inoue, K., and Umeda, M. (1997) Exp. Cell Res. 233, 430–434
18. Machaidze, G., Ziegler, A., and Seeleg, J. (2002) Biochemistry 41, 1965–1971
19. Barnett, Foster, D., Abul-Milh, M., Huesca, M., and Lingwood, C. A. (2000) Infect. Immun. 68, 3108–3115
20. Shir, G., Ficht, J. D., Maassarian, G., Matzner, W., Ching, W., and Chong, P. (2000) Hum. Reprod. 15, 1932–1936
21. Familiar, A., Zwart, B., Huisman, H. G., Rensink, I., Roen, D., Hordijk, P. L., Aarden, L. A., and Hack, C. E. (2001) J. Immunol. 167, 647–654
22. Liu, S., Yu, Y., An, H., Lin, N., Wang, W., Zhang, W., Wan, T., and Cao, X. (2003) Immunol. Lett. 86, 169–175
23. Sun, H., Li, N., Wang, X., Lin, S., Chen, T., Zhang, L., Wan, T., and Cao, X. (2005) Biochem. Biophys. Res. Commun. 301, 176–182
24. Li, N., Zhang, W., Wan, T., Zhang, J., Chen, T., Yu, Y., Wang, J., and Cao, X. (2001) J. Biol. Chem. 276, 28106–28112
25. Bashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) Methods 26, 199–213
26. Serre, L., Vallier, B., Bureaud, N., Schoentgen, F., and Zelwer, C. (1998). Structure 6, 1265–1268
27. Huang, T. J., Price, S. A., Chilton, L., Calcutt, N. A., Tomlinson, D. R., Price, S. A., Chilton, L., Calcutt, N. A., Tomlinson, D. R., Verkhratsky, A., and Fernyhough, P. (2003) Diabetes 52, 2129–2136
28. Aisaki, K., Kanno, H., Oyaizu, N., Hara, Y., Miwa, S., and Ikawa, Y. (1999) Jpn. J. Cancer Res. 90, 171–179
29. Christ, O., Seiter, S., Matzu, S., Burge, C., and Zoller, M. (2001) Clin. Cancer Res. 7, 985–998
30. Bergmann, A., Agapite, J., Calle, K., and Steller, H. (1998) Cell 95, 331–341
31. Tang, G., Minemoto, Y., Dibling, B., Purocell, N. H., Li, Z., Karrin, M., and Liu, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
32. Kuramitsu, Y., Fujimoto, M., Tanaka, T., Ohata, H., and Nakamura, K. (2000) Electrophoresis 21, 660–664
33. Baud, V., and Karin, M. (2001) Trends Cell Biol. 11, 372–377
34. Locksley, R. M., Killeen N., and Lenardo, M. J. (2001) Cell 104, 487–501
35. Matsuo, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Saffidi, C., Breit, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
36. Chang, L., and Karin, M. (2001) Nature 410, 37–40
37. Davis, R. J. (2000) Cell 103, 239–252
A Novel Human Phosphatidylethanolamine-binding Protein Resists Tumor Necrosis Factor α-induced Apoptosis by Inhibiting Mitogen-activated Protein Kinase Pathway Activation and Phosphatidylethanolamine Externalization

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