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Peflin, a Novel Member of the Five-EF-Hand-Protein Family, Is Similar to the Apoptosis-Linked Gene 2 (ALG-2) Protein but Possesses Nonapeptide Repeats in the N-Terminal Hydrophobic Region

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The calpain small subunits of sorcin, grancalcin, and ALG-2 constitute a family of the Ca^{2+}-binding proteins with five EF-hand-like motifs (penta-EF-hand domain or PEF domain) in their C-terminal regions and hydrophobic domains with variable lengths in their N-terminal regions. Searching the human DNA data base of expressed sequence tags (EST) revealed novel partial sequences similar to, but distinct from, the sequences of the previously known PEF proteins. We isolated a cDNA clone of near full length by 5′- and 3′-RACE (rapid amplification of cDNA end) methods and compared the predicted amino acid sequence (284 residues) of the novel EF-hand protein, named peflin, with those of known PEF proteins. The PEF domain of peflin is most similar to ALG-2 (40.9% identity) among the family, particularly in EF-1 (46.2%) and EF-3 (57.1%) regions. Peflin has a longer N-terminal hydrophobic domain than any other member of the family, and it contains nine nonapeptide (A/PPGGPYGGP) repeats. Western blot analysis demonstrated that peflin (30 kDa) was expressed in various nonadherent and adherent cultured human cell lines, including Jurkat, HL60, HeLa, and HT1080. Peflin may play basic roles in Ca^{2+} signaling irrespective of cell types.

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under Accession No. AB026628.

Abbreviations used: CBB, Coomassie brilliant blue R250; EST, expressed sequence tags; kb, kilobase(s); NHB, *N*-terminal hydrophobic; nt, length in nucleotides; PAGE, polyacrylamide gel electrophoresis; PEF, five (penta)-EF-hand; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcription–polymerase chain reaction.

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Ca^{2+} is one of the key second messengers and is involved in various cellular functions. Ca^{2+} signaling is mediated by a variety of Ca^{2+}-activated enzymes and Ca^{2+}-binding proteins (1). The EF-hand motif, i.e. the Ca^{2+}-binding helix-loop-helix structure, has been identified in numerous Ca^{2+}-binding proteins (2). The number of repetitive EF-hand motifs in protein molecules, whether or not capable of Ca^{2+}-binding, ranges from two to eight (see ref. 2 and references therein). From X-ray crystallographical analyses, we and others have independently revealed that the Ca^{2+}-binding domains of the pig and rat calpain small subunits have five instead of previously believed four EF-hand motifs, and that the calpain large subunits also possess the penta-EF-hand (PEF) domains based on the sequence alignment (3, 4). While EF-1 to EF-4 bind Ca^{2+} with high or low affinity, the *C*-terminal EF-5, having two-residue insertion, does not bind Ca^{2+}. PEF domains are also found in other mammalian Ca^{2+}-binding proteins (5) such as in sorcin, amplified together with the P-glycoprotein gene in multidrug-resistant cancer cells (6), in grancalcin which is supposed to be associated with granule-membrane fusion and degranulation of neutrophils (7), and in the product of the apoptosis-linked gene *ALG-2* (8).

While grancalcin and sorcin exist as homodimers (9, 10), calpain small subunits from various animals form heterodimers with large catalytic subunits (11). The recombinant Ca^{2+}-binding domain of the calpain small subunit, however, forms a homodimer in the absence of the catalytic subunit (12). One EF-5 pairs up with another EF-5 of the dimer counterpart as revealed by the X-ray crystallography (3, 4). Previously, we reported that recombinant ALG-2 (22 kDa) eluted slightly faster (at 28 kDa) than expected in a gel filtration chromatography (13). Although the result suggested a monomeric form of the recombinant protein, a

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possibility of dimer-monomer inter-conversion during the chromatography could not be ruled out. Recently Missotten *et al.* have showed that ALG-2 also forms a homodimer by the yeast two-hybrid method and by the co-immunoprecipitation of the tagged ALG-2 expressed transiently in human cells (14). Thus, formation of homodimer or heterodimer is the feature common to all the members of the PEF protein family.

The PEF proteins including ALG-2 are localized in cytosol, but Ca^{2+} induces the changes of subcellular localization (9, 11, 15–17). The PEF proteins are supposed to interact with target proteins on or near membranes in various aspects of cell regulation in response to Ca^{2+}. The *N*-terminal regions of the calpain small subunit, grancalcin, sorcin and ALG-2 are rich in Gly and hydrophobic residues regardless of the difference in their lengths (5). As suggested in the calpain small subunit (18), these hydrophobic *N*-terminal domains of the PEF proteins may also play important roles in their interactions with biological membranes.

To gain more insights into the nature of PEF protein structures, we searched for new members of the protein family. In the present report, we show that a newly identified EF-hand protein named peflin (*PEF* protein with *l*ong *N*-terminal hydrophobic doma*in*) is a novel member of the PEF protein family and is similar to ALG-2.

MATERIALS AND METHODS

Cell culture. HL60 and Jurkat cells were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C under humidified air containing 5% CO_2. HeLa and HT1080 cells were cultured similarly except using Dulbecco's modified Eagle medium (DMEM).

PCR. 5′- and 3′-RACE (rapid amplification of cDNA end) reactions were performed using a Marathon-Ready human fetus cDNA obtained from CLONTECH (Palo Alto, CA) and *Ex Taq* polymerase from Takara Shuzo (Kyoto, Japan). Gene specific primers used were designed from sequences of EST clones AA216424 for 5′-RACE and AA226371 for 3′-RACE, respectively. PCR products were cloned into a TA-cloning vector pCR-TOPO from Invitrogen (Carlsbad, CA). To obtain cDNA clones encoding an entire protein, PCR was performed with a forward primer containing the ATG codon and a reverse primer containing the stop codon using the Jurkat cell cDNA or fetus cDNA as a template. The nucleotide sequences of the isolated clones were determined with an automated fluorescent sequencer, ABI PRISM 310 (PE Applied Biosystems).

Northern blotting. Total RNA was isolated from cultured cells by the acid guanidine thiocyanate phenol/chloroform method (19). Approximately 5 μg of RNA was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, 40 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 10 mM sodium acetate and 1 mM EDTA, and transferred to a positively charged nylon membrane from Boehringer Mannheim. The cDNA clone designated as pRT-PCRFt was cut with *Bam*HI to delete the GC-rich region (NHB domain) (Fig. 1A). The resultant plasmid pTAdBmhpeflin was linearized with *Hin*dIII and used for *in vitro* transcription with T7 RNA polymerase to synthesize antisense RNA (959 nt), which was labeled with the psoralen-biotin system and hybridization signals were detected nonisotopically with the kits from Ambion (Austin, TX). The cDNA clones of pRT-PCRFt and p3′-RACE were fused at *Sac*I site and transferred to pBluescriptIIKS(+) to generate the full-length cDNA clone. The resultant plasmid pBShpeflin was linearized with *Eco*RV and used for *in vitro* transcription to synthesize sense RNA (1773 nt) containing short vector sequences (132 nt).

Expression of peflin in E. coli. A 0.9-kb cDNA fragment covering the entire protein coding region was excised from the clone pRT-PCRJk with *Nco*I containing the ATG codon and with *Eco*RI located in the vector, and was inserted into the *Nco*I/*Eco*RI sites of the T7 RNA polymerase expression vector pET-3d from Novagen (Madison, WI). *E. coli* BL21(DE3)pLysS was transformed with the resultant plasmid pET-hpeflin and cultured as described (20). The *E. coli* lysate was used as a positive control for Western analysis. For antigen preparation, a 0.6-kb *Bam*HI/*Eco*RI fragment covering EF-1 to 5 of peflin was subcloned into a vector pET-24His which has a 6xHis (hexahistidine) tag sequence between the *Nco*I and *Bam*HI sites of pET-24d. The recombinant protein HisΔNhpeflin was recovered in pellets from the *E. coli* lysates. After dissolving the inclusion bodies with 8 M guanidine hydrochloride, the recombinant protein was absorbed to the affinity resin TALON (CLONTECH) and eluted with 63 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 50 mM EDTA. Eluted HisΔNhpeflin was further purified by electrophoresing on a preparative 12.5% polyacrylamide slab gel, followed by staining with cold 0.1 M KCl and extraction with PBS.

Western blotting. Balb/c female mice were immunized with purified HisΔNhpeflin by the conventional method. Western blotting was performed essentially as described previously (21). After the membranes were blocked with PBS containing 0.1% Tween 20 and 5% skim milk, they were incubated with anti-serum diluted 2,000-fold with PBS containing 0.1% Tween 20 and 0.1% bovine serum albumin. Immuno-signals were detected by the color development method using 0.8 mg/ml diaminobenzidine (DAB) dissolved in 100 mM Tris-HCl, pH 7.5, containing 0.4 mg/ml $NiCl_2$ and 0.01% H_2O_2.

Computer analyses. Homology search was performed using a program of the advanced BLAST 2.0 search (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). Multiple sequence alignment was performed with a program CLUSTAL W ver 1.7 (22) and a phylogenetic tree was displayed with NJplot (23). Hydrophobicity profiles were obtained with the program, ProtScale (http://expasy.hcuge.ch/cgi-bin/protscale.pl), using the normalized consensus hydrophobicity scale (24). A window of 9 residues was selected. Motifs in the deduced amino acid sequence were searched by PROSITE (http://expasy.ch/sprot/prosite.html).

RESULTS

cDNA cloning. To find new members of the PEF protein family, we searched the human EST data bank using the BLAST program. Amino acid sequences of the EF-4/5 regions of the known PEF proteins were selected as query sequences because EF-5 is unique to the family. Among the low to medium scored EST clones, AA226371 displayed a novel sequence similar to but distinct from the known PEF protein sequences. Then, 5′ region of the nucleotide sequence of AA226371 was used as a query sequence to find overlapping clones. By the *in silico* gene walking method, EST clones covering the entire protein coding region were collected (Fig. 1A). The PCR-based methods of 5′- and 3′-RACEs as well as the conventional RT-PCR were performed to obtain a full-lengthed peflin cDNA. In the EST clone AA216424, residues upstream of the putative ATG codon is GACCCATGG. The cytosine residue

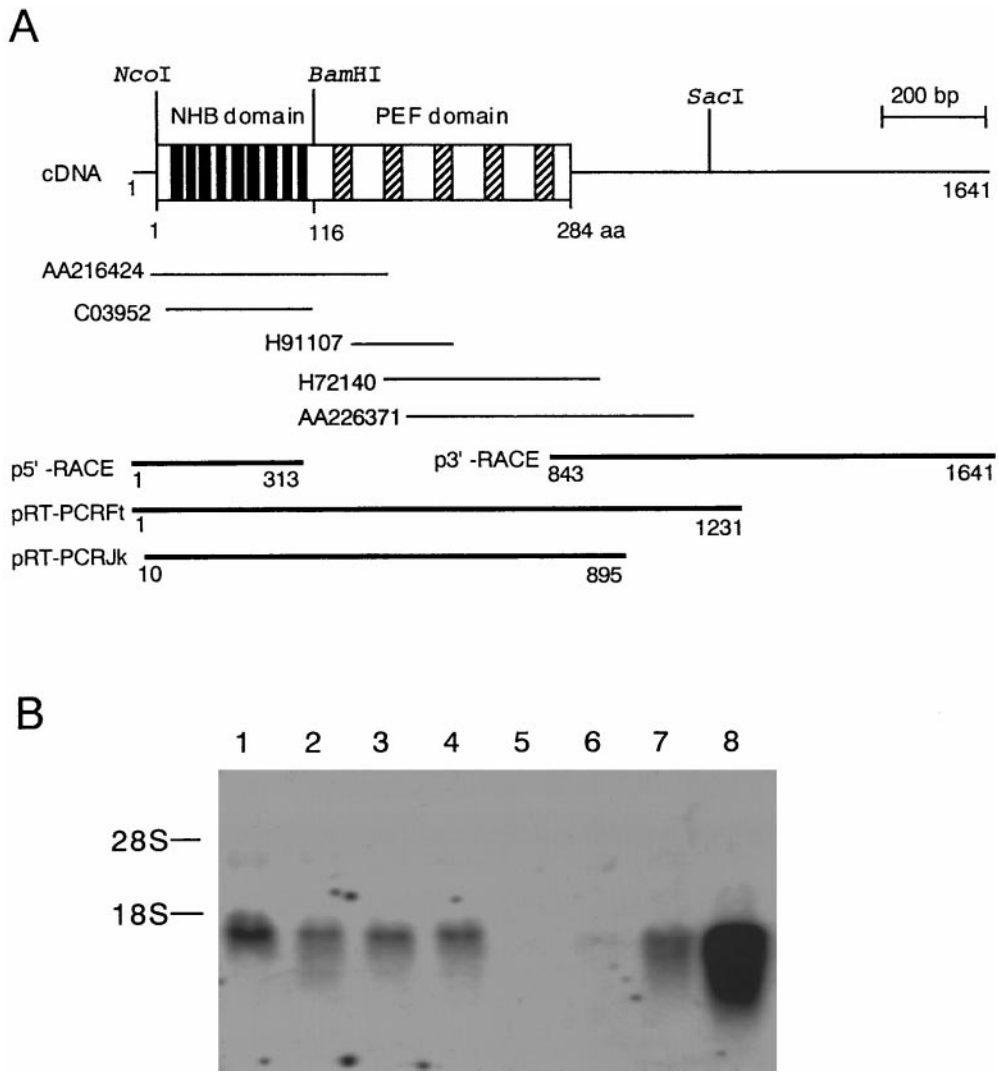

FIG. 1. Cloning of peflin cDNAs. (*A*) Schematic structure of peflin cDNA. Nonapeptide sequences and EF-hand-like sequences are indicated by solid boxes and diagonally hatched boxes, respectively. The cDNA sequence has 1641 nt including a poly(A) tail (30 nt). Lines are drawn to scale with the bar indicating 200 bp. Overlapping EST clones are shown with GenBank/EMBL/DDBJ accession numbers. The cDNA clones obtained in the present study are shown with thick solid lines, and the nucleotide residue numbers of the 5′ and 3′ ends are indicated. The clones were obtained from the human fetus cDNA library (pRT-PCRFt, p5′-RACE and p3′-RACE) and from Jurkat cells (pRT-PCRJk). (*B*) Northern blot analysis. Total RNA (5 μg) isolated from cultured cells (*lane 1,* Jurkat; *lane 2,* HL60; *lane 3,* HeLa; *lane 4,* HT1080) was electrophoresed on a 1% agarose gel containing formaldehyde and hybridization was performed with nonisotopically labeled riboprobe after transfer to a nylon membrane. The *in vitro* transcribed peflin RNA (1773 nt) containing short sequences derived from the vector was used as a positive control (*lane 6,* 1 pg; *lane 7,* 10 pg; *lane 8,* 100 pg). The control samples contained *in vitro* transcribed unrelated RNA (1900 nt, *Xenopus* elongation factor 1-α, 1 μg) as a carrier RNA. *Lane 5,* carrier RNA alone (1 μg). Ribosomal RNAs were used as size markers: 28S (4718 nt), 18S (1874 nt).

at −3 position disagrees with Kozak's consensus translation initiation site where residue at −3 should be purine (25). The obtained p5′-RACE clone, however, has a sequence ATCACCATGG in agreement with the consensus sequence. The number of the determined nucleotide residues of the peflin cDNA is 1641 including 30 residues from polyA tail. Assuming that mRNAs possess polyA tails of 100–200 residues in general, the size of the obtained cDNA agrees well with the estimated size of the peflin mRNA (1.8 kb) analysed by Northern blot analysis (Fig. 1B). The peflin mRNA was detected in various cultured adherent and non-adherent human cells including acute T-cell leukemia cells (Jurkat), promyelocytic leukemia cells (HL60), epitheloid carcinoma cells (HeLa) and fibrosarcoma cells (HT1080).

Deduced amino acid sequence. Peflin is similar to ALG-2 (29.9%: 85 amino acid residue identity out of 284 residues) both in the PEF domain (40.9% identity) and in the NHB (*N*-terminal *h*ydropho*b*ic) domain. In the latter domain, peflin has an insertion of nine re-

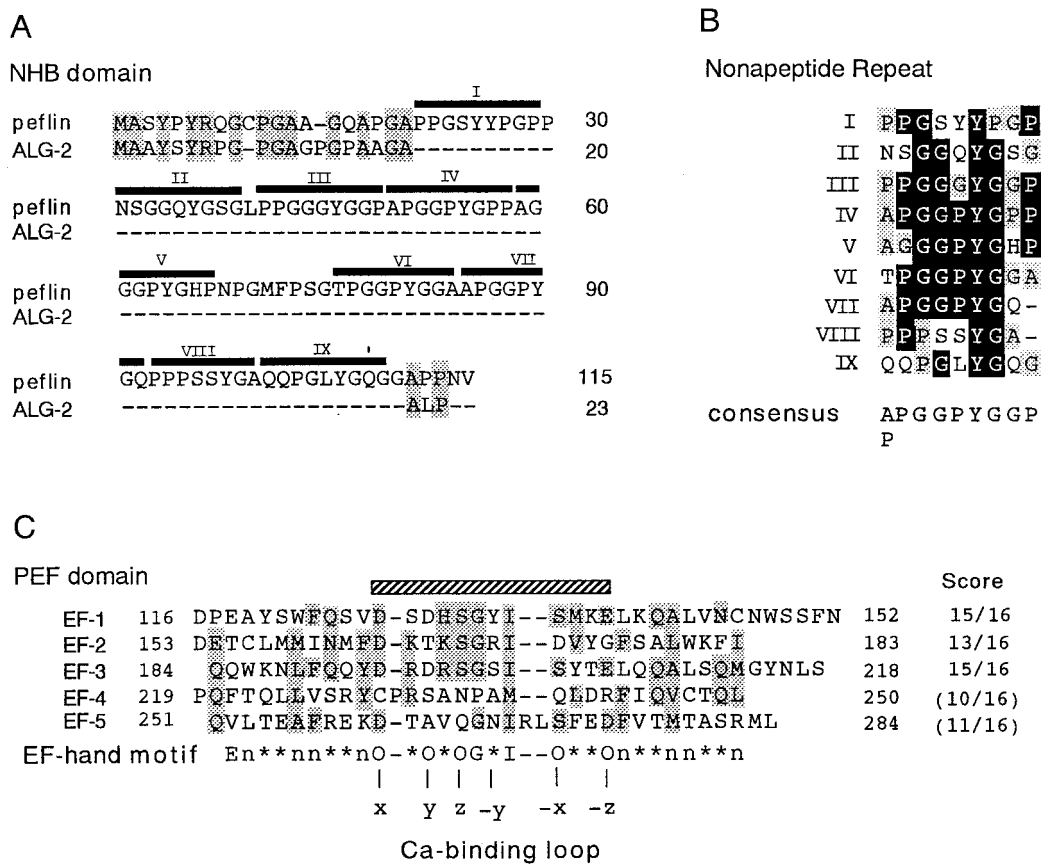

FIG. 2. Primary structure of peflin. (*A*) Alignment of the amino acid sequences of the NHB domains of peflin and ALG-2. Identical residues are stippled. The nonapeptide repeats are indicated with bars above the sequence. (*B*) Alignment of the nonapeptide repeats. Identical residues in at least four repeats are highlighted. Less conserved residues of G (Gly), P (Pro), and A (Ala) in the repeats are stippled. (*C*) Alignment of five EF-hand-like sequences (EF-1–EF-5) in the PEF domain. Identical or similar residues in at least three repeats are stippled. A potential Ca^{2+}-binding loop is indicated by a diagonally hatched bar. The EF-hand motif contains 16 preferred residues: E, acidic; n, hydrophobic; O, oxygen-containing; G, glycine; I, aliphatic side chains (Ile, Leu, Val, and Met); asterisks, variable residues (often hydrophilic); hyphens, gaps. EF-hand score: number of matched residues. Ca^{2+}-coordinating positions are indicated by x, y, z, −y, −x, and −z, where the oxygen atoms of side chains (x, y, z, −z), carbonyls (−y), and water molecules (−x) are usually ligands.

peats of nonapeptide A/PPGGPYGGP (Figs. 2A and 2B). The EF-hand scores in the PEF domain are high in EF-1 and EF-3 (Fig. 2C). EF-4 and EF-5 have one- and two-residue-insertion in the potential Ca^{2+}-binding loop, respectively. Potential phosphorylation sites are predicted with PROSITE: 135-SMK and 168-SGR (by protein kinase C); 135-SMKE, 150-SFND, 202-SYTE and 271-SFED (by casein kinase II).

Comparison with other PEF proteins. Multiple sequence alignment of the PEF proteins and calmodulin related proteins has revealed that peflin belongs to the PEF protein family (Fig. 3). Phylogenetic tree displays clearly that peflin is similar to ALG-2 and separated from calpain subfamily (Fig. 4). As shown in Table I, the conservation rate of amino acid residues varies in each EF-hand, among which EF-3 shows the highest value followed by EF-1. Interestingly, peflin is more similar to sorcin than to ALG-2 in EF-2. Compared with non-PEF proteins including calmodulin, peflin shows a moderate similarity from EF-1 to EF-3, but a low similarity in EF-4.

NHB domain. Although the lengths of the N-terminal domains of the PEF proteins are variable (23–113 residues), calpain small subunit, sorcin, grancalcin, ALG-2 and peflin have common features. Like other PEF proteins, peflin shows positive scores in the *N*-terminal domains in the hydrophobicity profiles (data not shown). The *N*-terminal hydrophobic domains (NHB domains) are rich in Gly, Pro, Ala and hydrophobic residues (Fig. 5). Prediction of secondary structures suggests that there are no α-helices nor β-sheets in the NHB domain of peflin (data not shown).

Detection of peflin protein. Antiserum was raised to immunologically detect the peflin protein in animal cells. The antiserum reacted with a band of 30 kDa in the lysate of *E. coli* expressing recombinant peflin, but not in that of control *E. coli* (Fig. 6A, *lanes 7* and *8*),

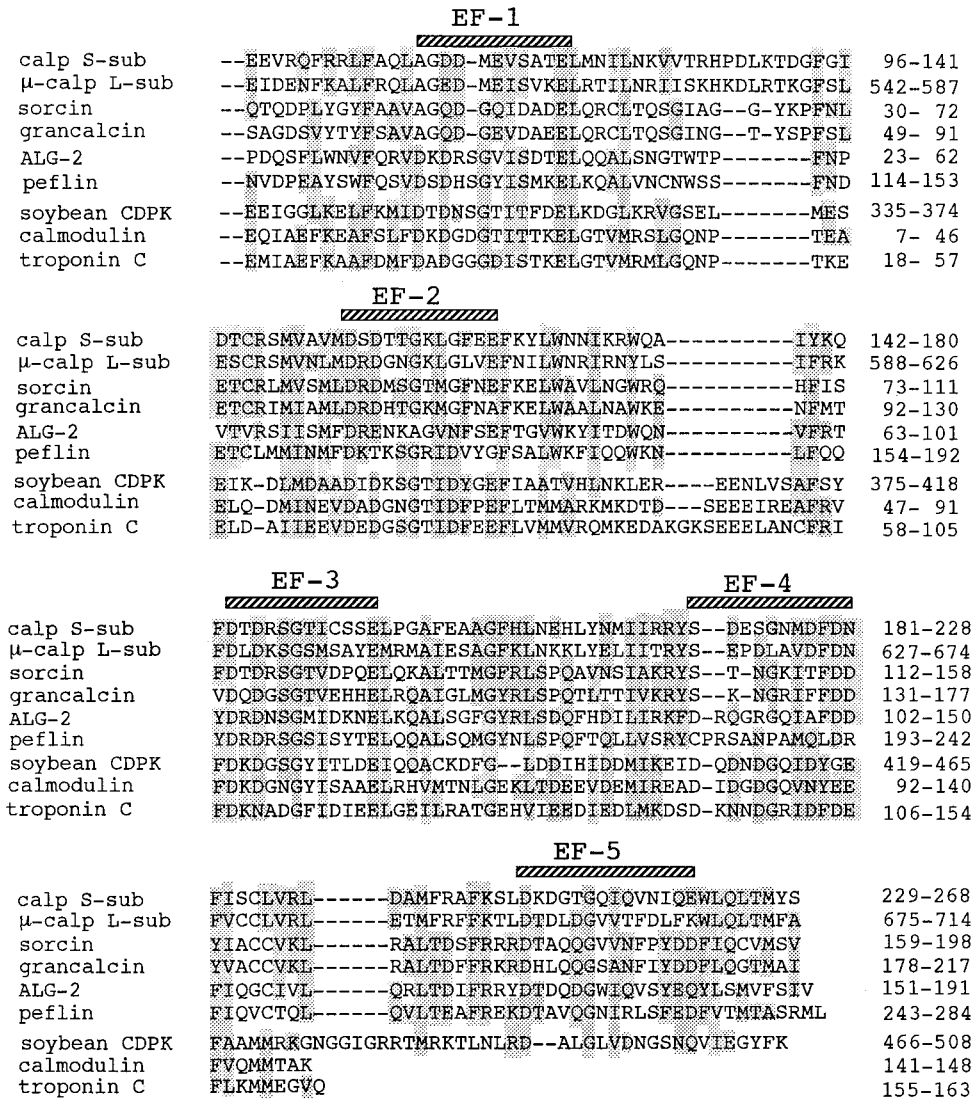

FIG. 3. Sequence alignment of the PEF domains. Identical or similar residues among the PEF proteins (top six sequences) and non-PEF proteins (soybean CDPK, mammalian and avian calmodulin, and chicken troponin C) are stippled. Amino acid similarity groupings are: aromatic (F, Y, W); aliphatic side chain (L, I, V, M, A); acidic and amide (E, D, Q, N); hydroxyl small side chain (S, T). The amino acid sequences are from GenBank/EMBL/DDBJ (human calpain small subunit, X04106; human μ-calpain large subunit, X04366; human ALG-2, AF035606; soybean CDPK, M64987) and SwissProt (human grancalcin, P28676; human sorcin, P30626; human calmodulin, P02593; chicken skeletal muscle troponin C, P02588). Potential Ca^{2+}-binding loops are indicated by diagonally hatched bars.

indicating that the prepared antiserum contained antibodies specific to peflin. When the lysate from Jurkat cells was subjected to Western blotting, a band of 30 kDa reacted with the immune serum but not with the pre-immune serum (Fig. 6A, *lanes 6* and *9*). Several faint bands of 40 kDa and greater molecular mass were detected with both immune and pre-immune sera, suggesting the results of non-specific immunological reactions for these bands. The molecular mass of peflin estimated by Western blot analysis, 30 kDa, agrees well with that calculated from the amino acid sequence of the open reading frame (30248.16 Da, translation initiation Met is excluded). Taken together, the band of 30-kDa protein corresponds to peflin and there occurs no major proteolytic processing of the protein in cells. As shown in Fig. 6B, the band of 30-kDa protein was detected also in promyelocytic leukemia cells (HL60), epitheloid carcinoma cells (HeLa) and fibrosarcoma cells (HT1080), in agreement with the expression of the peflin mRNA in these cells (Fig. 1B).

DISCUSSION

One of the most well known Ca^{2+}-binding proteins with multiple EF-hands is calmodulin which has four EF-hands and plays important roles in Ca^{2+} signaling by binding to enzymes, cytoskeletal proteins *etc.* (1, 2, 26). Previously we reported that a group of Ca^{2+}-

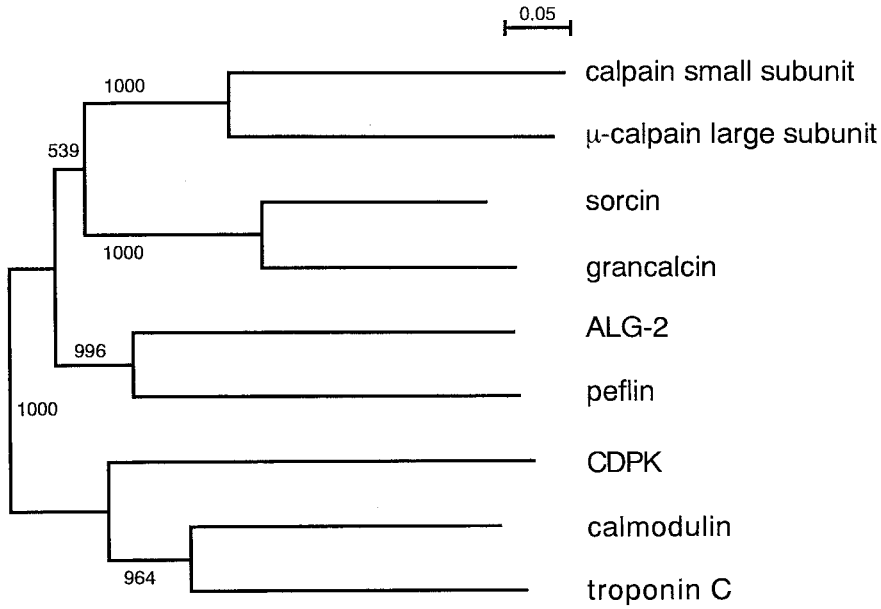

FIG. 4. Phylogenetic tree of the PEF protein family. Using the default setting of CLUSTAL W 1.7, the PEF domain sequences were aligned (shown in Fig. 3), and a bootstrap tree file was created. The phylogenetic tree was drawn with NJplot. The values indicate the number of times that branches are clustered together out of 1000 bootstrap trials. Horizontal branch lengths are drawn to scale with the bar indicating 0.05 amino acid replacement per site.

binding proteins possess five EF-hand-like sequences and show similarity to the sequence of the calpain small subunit (5). Although the loops of the fifth EF-hands (EF-5s) have two-residue-insertions and do not or may not bind Ca^{2+}, we propose the name PEF (penta-EF-hand) to collectively call the domains spanning from EF-1s to EF-5s of the Ca^{2+}-binding proteins (5, 13).

In addition to the presence of EF-5, a common feature of PEF domain is that all the members including peflin, the newly identified member in this report, have seven-residue-deletions between EF-2s and EF-3s when compared with the calmodulin sequence (Fig. 3). This region in calmodulin corresponds to a flexible central α-helix which links two spatially separated subdomains, i.e., N-lobe (EF-1 and EF-2) and C-lobe (EF-3 and EF-4) (27). Indeed, the 3-D structure of the calpain small subunit revealed by X-ray crystallography indicates that the EF-4 is in proximity to EF-1 and EF-2, and that the two lobes are not independent. Interestingly, while regions from EF-1s to EF-3s are relatively conserved between peflin and calmodulin, EF-4s are less conserved (Table I). This is also true when peflin is compared with soybean Ca^{2+}-dependent

TABLE I

Sequence Comparison of Peflin with PEF and Non-PEF Proteins

Regions in peflin:	EF-1	EF-2	EF-3	EF-4	EF-5	EF-1~EF-4	EF-1~EF-5
No. of residues compared:	39	31	35	32	34	137	171
PEF proteins							
Calpain S-subunit	17.9	29.0	**37.1**	21.9	17.6	26.3	24.6
Calpain L-subunit	23.1	**32.3**	**31.4**	21.9	14.7	27.0	24.6
Sorcin	25.6	**35.5**	**45.7**	28.1	**35.3**	**33.6**	**33.9**
Grancalcin	20.5	**35.5**	**45.7**	28.1	29.4	**32.1**	**31.6**
ALG-2	**46.2**	29.0	**57.1**	**31.3**	**38.2**	**41.6**	**40.9**
Non-PEF proteins							
CDPK	25.6	29.0	**34.3**	3.1	5.9	23.4	19.9
Calmodulin	25.6	25.8	22.9	9.4	—	21.2	—
Troponin C	23.1	29.0	28.6	9.4	—	22.6	—

Note. Numbers of identical amino acid residues in the indicated regions are counted and the degrees of similarities are expressed as percentage identities. Values greater than 30% are typed in boldface and boxed.

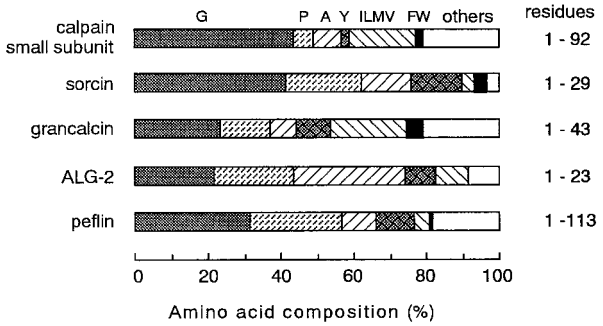

FIG. 5. Amino acid compositions of the NHB domains of the human PEF proteins. Numbers of amino acid residues used for calculation are indicated to the right. Amino acids are classified into seven groups: G, P, A, Y, ILMV, FW, and others.

protein kinase (CDPK) which has an extra region downstream of EF-4, giving the basis that CDPK does not belong to the PEF protein family. This fact supports the validity of our strategy where we used amino acid sequences ranging from EF-4s to EF-5s as query sequences for searching the EST data bases to find new members of the PEF protein family by the BLAST program. Using the sequences of EF-5s alone gave extremely low scores and it was difficult to evaluate the similarity between the hit sequences and those of the EF-hand proteins. No additional new members have been found in the updated data bases in animals, but distantly related PEF-like sequences have been found in plants and fungi (data not shown). Among the PEF proteins, peflin is most similar to ALG-2 (Fig. 4 and Table I). Although ALG-2 like sequence is found in the DNA data base of *C. elegans*, peflin homolog is not found in the invertebrate (data not shown). Peflin might play roles in Ca^{2+} signaling for events specific in higher vertebrates.

Whereas the calpain large subunit has a catalytic protease domain in the upstream of the PEF domain, the calpain small subunit, sorcin, grancalcin, ALG-2 and peflin have clusters of Gly, Pro, Ala and hydrophobic residues in their *N*-terminal domains (NHB domains) (Fig. 5). Among the PEF proteins, peflin is most similar to ALG-2 also in the NHB domain (Fig. 2A and data not shown). Interestingly, due to the insertion of nonapeptide repeats (A/PPGGPYGGP), peflin has the longest NHB domain while ALG-2 has the shortest one. The similar nonapeptide motif containing Tyr is not found in any other known proteins so far examined.

EF-hand scores in the PEF domain of peflin are high in EF-1 and EF-3, suggesting possible Ca^{2+}-binding at these sites (Fig. 2). In the present report, however, we could not show direct evidence of the Ca^{2+}-binding capacity of peflin. Neither full-lengthed peflin nor 6xHis-tagged PEF domain was recovered as soluble protein when expressed in *E. coli* even under low temperature, and the expressed insoluble proteins could not be used for Ca^{2+}-binding experiments. Fusion with thioredoxin resulted in solubilization of the recombinant protein, but the purified protein appeared at a void volume upon Superdex-200 gel filtration chromatography. The aggregated sample did not show Ca^{2+}-dependent conformational change by the assay using a fluorescent hydrophobicity probe which worked well for recombinant ALG-2 (13). An attempt of Ca^{2+}-overlay assay using proteins transferred to membranes after SDS-PAGE was also in failure, probably due to difficulty in renaturation of the denatured protein. To determine the ability of peflin to bind Ca^{2+}, the protein needs to be purified in the native form either from mammalian cells as naturally occurring peflin or from insect cells as recombinant peflin using baculovirus expression system. Subcellular fractionation of peflin in the presence and absence of Ca^{2+} suggests that peflin undergoes conformational change upon binding to Ca^{2+} and interacts with membranes or other macromolecules in the presence of the ion (data not shown).

Recently, an ALG-2-interacting protein named AIP1 or Alix was cloned by the yeast two-hybrid method (14, 15). AIP1/Alix is similar to BRO1, a yeast protein involved in the Pkc1p-MAP kinase cascade (29). BRO1 mutations result in a temperature-sensitive osmoremedial growth defect, which is suppressed by Ca^{2+}. *S.*

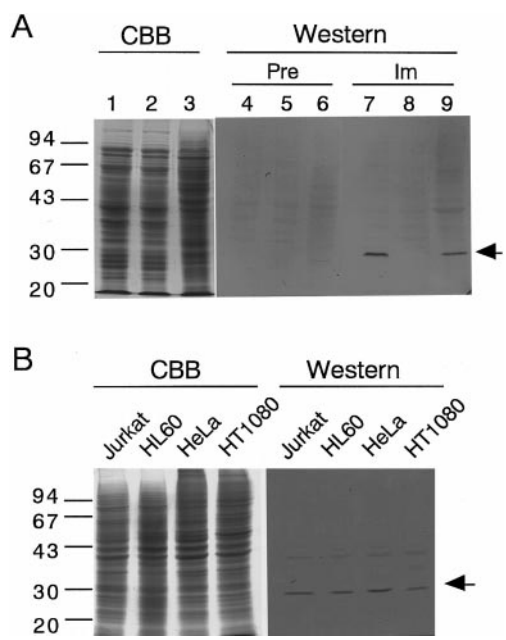

FIG. 6. Western blot analysis of peflin. (*A*) Examination of specificity of the anti-peflin antiserum. Mixed cell lysates from *E. coli* harboring pET-hpeflin (positive control) and *E. coli* harboring pET-3d in the ratio 1:49 (*lanes 1, 4,* and *7*), cell lysates from *E. coli* harboring pET-3d (*lanes 2, 5,* and *8*), and cell lysates from Jurkat (*lanes 3, 6,* and *9*) were electrophoresed on a 10% gel, followed by either staining with CBB (*lanes 1–3*) or Western blot analysis with preimmune serum (Pre, *lanes 4–6*) or immune serum (anti-peflin antiserum) (Im, *lanes 7–9*). Arrow indicates the immunoreactive bands for peflin (30 kDa). (*B*) Detection of peflin in several human cell lines by Western blotting.

cerevisiae genome contains a PEF-like sequence encoding a hypothetical 38-kDa protein YG-25 (5). It remains to be established whether YG-25 interacts with BRO1 and serves as an ALG-2-like protein. Future studies would clarify whether peflin acts like ALG-2 in a similar way in apoptotic pathways in mammalian cells by interacting with AIP1/Alix or its related proteins. Interestingly, the C-terminal region of AIP1/Alix is rich in Pro, Ala and Tyr as in the case of the NHB domain of peflin. Studies are in progress to search peflin-interacting proteins to infer physiological functions of the newly identified PEF protein.

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REFERENCES

1. Heizmann, C. W., and Hunziker, W. (1991) *Trends Biochem. Sci.* **16,** 98–103.
2. Persechini, A., Moncrief, N. D., and Kretsinger, R. H. (1996) *Trends Biochem. Sci.* **12,** 462–467.
3. Blanchard, H., Grochulski, P., Li, Y., Arthur, S. C., Kavies, P. L., Elce, J. S., and Cygler, M. (1997) *Nature Struct. Biol.* **4,** 532–538.
4. Lin, G., Chattopadhyay, D., Maki, M., Wang, K. K. W., Carson, M., Takano, E., Hatanaka, M., Delucas, L. J., and Narayana, S. V. L. (1997) *Nature Struct. Biol.* **4,** 539–545.
5. Maki, M., Narayana, S. V. L., and Hitomi, K. (1997) *Biochem. J.* **328,** 718–720.
6. Van der Bliek, A. M., Meyers, M. B., Biedler, J. L., Hes, E., and Borst, P. (1986) *EMBO J.* **5,** 3201–3208.
7. Boyhan, A., Casimir, C. M., French, J. K., Teahan, C. G., and Segal, A. W. (1992) *J. Biol. Chem.* **267,** 2928–2933.
8. Vito, P., Lacana', E., and D'Adamio, L. (1996) *Science* **271,** 521–525.
9. Teahan, C. G., Totty, N. F., and Segal, A. W. (1992) *Biochem. J.* **286,** 549–554.
10. Hamada, H., Okochi, E., Oh-hara, T., and Tsuruo, T. (1988) *Cancer Res.* **48,** 3173–3178.
11. Goll, D. E., Kleese, W. C., Okitani, A., Kumamoto, T., Cong, J., and Kapprell, H. (1990) *in* Intracellular Calcium-Dependent Proteolysis (Mellgren, R. L., and Murachi, T., Eds.), pp. 3–24, CRC Press, Boca Raton, FL.
12. Blanchard, H., Li, Y., Cygler, M., Kay, C. M., Arthur, J. S. C., Davies, P. L., and Elce, J. S. (1996) *Protein Sci.* **5,** 535–537.
13. Maki, M., Yamaguchi, K., Kitaura, Y., Satoh, H., and Hitomi, K. (1998) *J. Biochem.* **124,** 1170–1177.
14. Missotten, M., Nichols, A., Rieger, K., and Sadoul, R. (1999) *Cell Death Differ.* **6,** 124–129.
15. Vito, P., Pellegrini, L., Guiet, C., and D'Adamio, L. (1999) *J. Biol. Chem.* **3,** 1533–1540.
16. Meyers, M. B., Zamparelli, C., Verzili, D., Dicker, A. P., Blanck, T. J. J., and Chiancone, E. (1995) *FEBS Lett.* **357,** 230–234.
17. Mellgren, R. L. (1987) *FASEB J.* **1,** 110–115.
18. Imajoh, S., Kawasaki, H., and Suzuki, K. (1986) *J. Biochem.* **99,** 1281–1284.
19. Chomczynski, P., and Sacchi, N. (1986) *Anal. Biochem.* **162,** 156–159.
20. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
21. Hitomi, K., Uchiyama, Y., Ohkubo, I., Kunimatsu, M., Sasaki, M., and Maki, M. (1998) *Biochem. Biophys. Res. Commun.* **246,** 681–685.
22. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucl. Acids Res.* **22,** 4673–4680.
23. Perriere, G., and Gouy, M. (1996) *Biochimie* **78,** 364–369.
24. Eisenberg, D., Schwarz, E., Komarony, M., and Wall, R. (1984) *J. Mol. Biol.* **179,** 125–142.
25. Kozak, M. (1991) *Nucl. Acids Res.* **15,** 8125–8148.
26. O'Neil, K. T., and DeGrado, W. F. (1990) *Trends Biochem. Sci.* **15,** 59–65.
27. Ikura, M. (1996) *Trends Biochem. Sci.* **21,** 14–17.
28. Nickas, M. E., and Yaffe, M. P. (1996) *Mol. Cell. Biol.* **16,** 2585–2593.