Peflin and ALG-2, Members of the Penta-EF-Hand Protein Family, Form a Heterodimer that Dissociates in a Ca\(^{2+}\)-dependent Manner

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Running title: Heterodimer of Peflin and ALG-2
SUMMARY

Peflin, a newly identified 30-kDa Ca\(^{2+}\)-binding protein, belongs to the penta-EF-hand (PEF) protein family, which includes the calpain small subunit, sorcin, grancalcin, and ALG-2 (apoptosis-linked gene 2). We prepared a monoclonal antibody against human peflin. The antibody immunoprecipitated a 22-kDa protein as well as the 30-kDa protein from the lysate of Jurkat cells. Western blotting of the immunoprecipitates revealed that the 22-kDa protein corresponds to ALG-2. This was confirmed by Western blotting of the immunoprecipitates of epitope-tagged peflin or ALG-2 whose cDNA expression constructs were transfected to HEK293 cells. Gel filtration of the cytosolic fraction of Jurkat cells revealed co-elution of peflin and ALG-2 in fractions eluting earlier than recombinant ALG-2, further supporting the notion of heterodimerization of the two PEF proteins. Surprisingly, peflin dissociated from ALG-2 in the presence of Ca\(^{2+}\). Peflin and ALG-2 co-localized in the cytoplasm, but ALG-2 was also detected in the nuclei as revealed by immunofluorescent staining and subcellular fractionation. Peflin was recovered in the cytosolic fraction in the absence of Ca\(^{2+}\) but in the membrane/cytoskeletal fraction in the presence of Ca\(^{2+}\). These results suggest that peflin has features common to those of other PEF proteins (dimerization and translocation to membranes) and may modulate the function of ALG-2 in Ca\(^{2+}\) signaling.
INTRODUCTION

It is now accepted that Ca$^{2+}$ is one of the most versatile second messengers relaying information within cells to regulate their activities such as muscle contraction, secretory events, cell cycle, differentiation, gene expression and apoptosis. Ca$^{2+}$ plays its pivotal role through specific classes of Ca$^{2+}$-binding proteins, most of which possess Ca$^{2+}$-binding motifs such as endonexin folds, C2 regions or EF-hands. Many EF-hand type Ca$^{2+}$-binding proteins have been identified, and they have been classified into dozens of families based on amino acid sequence similarities and number of EF-hand motifs in their molecules (1).

Recently, we classified a new family of proteins possessing domains with five EF-hand-like motifs, and we proposed the name "penta-EF-hand (PEF)" as a collective name for these domains (2). The PEF domain was originally found in the Ca$^{2+}$-binding domain of the small subunit of calpain, an intracellular Ca$^{2+}$-dependent cysteine protease, by X-ray crystallography (3, 4). Later studies revealed that the PEF domains are present in several other Ca$^{2+}$-binding proteins such as the calpain large subunit, sorcin (5), grancalcin (6) and apoptosis-linked gene 2 (ALG-2) (7). While sorcin and grancalcin exist as homodimers (8, 9), calpains exist as heterodimers of the large catalytic and small regulatory subunits (10). The bacterially expressed recombinant PEF domain of the calpain small subunit forms a homodimer without the large subunit (11). X-ray crystallographic studies have also revealed that the dimers are formed through a pair of fifth EF-hands (EF-5s) that have lost their Ca$^{2+}$-binding capacities due to two-residue insertions (3, 4). Therefore, it has been proposed that PEF proteins may form dimers with each other through EF-5, which provides a new interface for the interaction with possible targets.

The calpain small subunit, sorcin, grancalcin and ALG-2 have hydrophobic domains with variable lengths in the amino-terminal regions.
In the case of calpains, the hydrophobic N-terminal domains bind to the membranes and play an important role in the change of subcellular localization induced by Ca\textsuperscript{2+} (12). The N-terminal region of sorcin is required to interact with the membrane-localized annexin VII in a Ca\textsuperscript{2+}-dependent manner (13). Thus, the N-terminal regions of PEF proteins are thought to interact with phospholipids and/or target proteins on membranes.

Previously, we reported a novel PEF protein, peflin (PEF protein with a long N-terminal hydrophobic domain), which was cloned after a homology search for other PEF proteins (14). Peflin is most similar to ALG-2 in the PEF domain and has the longest N-terminal hydrophobic region of proteins in the PEF family. Peflin is expressed in several human cell lines, but its target protein and function have not been determined yet.

In this study, using a monoclonal antibody (MoAb) specific to human peflin, we demonstrated that peflin was co-immunoprecipitated with ALG-2. The peflin/ALG-2 heterodimer dissociated in a Ca\textsuperscript{2+}-dependent manner. The N-terminal hydrophobic domain of peflin was not essential for the heterodimerization. Peflin co-localized with ALG-2 in the cytoplasm and changed the subcellular localization in a Ca\textsuperscript{2+}-dependent manner.

**EXPERIMENTAL PROCEDURES**

*Cell culture* - Jurkat cells were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (0.3 mg/ml), penicillin (100 units/ml) and streptomycin (100 \(\mu\)g/ml) at 37\(^\circ\)C under humidified air containing 5% CO\textsubscript{2}. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented as above.
Preparation of anti-peflin monoclonal antibody - Balb/c female mice were immunized three times with His-tagged N-terminal truncated peflin (His-peflinΔN) prepared as described previously (14). Hybridomas were generated by polyethylene glycol (PEG)-mediated fusion of donor splenocytes to the P3 myeloma cell line. Positive hybridomas were identified by enzyme-linked immunosorbent assay (ELISA) and cloned by limited dilution. Cloned hybridomas were transplanted intraperitoneally to Balb/c mice. The IgG fraction was prepared from ascites and purified by the ammonium sulfate precipitation method. Western blotting was performed as described previously (14).

Metabolic labeling and immunoprecipitation - Jurkat cells (1x10^7) were incubated in a 60-mm dish containing 1.5 ml of a methionine/cystine-free medium for metabolic labeling (Sigma) supplemented with PBS-dialized FBS to 10% and [35S]-labeled amino acid mixture (100 μCi/ml, 70% methionine and 30% cysteine) at 37 °C for 4 h under humidified air containing 5% CO2. Cells were washed with phosphate-buffered saline (PBS) and lysed in buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 0.2% NP-40, 0.1 mM pefabloc, 25 μM leupeptin, 10 μM E-64, and 1 μM pepstatin) containing 5 mM EGTA or 0.01 mM CaCl2. Aliquots were incubated with indicated antibodies for 4 h at 4 °C and further incubated with Protein G Sepharose 4FF (Amersham-Pharmacia) overnight. Immunocomplexes were washed three times with buffer A and subjected to SDS-PAGE and analyzed by autoradiography using a BAS 2000 system (Fuji Film, Kanagawa, Japan). Anti-human RECK monoclonal antibody (MoAb) 32C10A (15) was used as a negative control antibody for immunoprecipitation. Anti-Flag MoAb M2 was obtained from Stratagene (La Jolla, CA). Anti-mouse ALG-2 polyclonal antibody (PoAb) raised in rabbits was affinity-purified using recombinant
human ALG-2 as described previously (16).

**Expression vectors and transfection** - An EcoRI fragment of the full-length peflin cDNA was inserted into a eukaryotic expression vector, pCXN2 (a derivative of pCAGGS, Ref. 17; a kind gift from Dr. J. Miyazaki), and a BamHI fragment of either a full-length or an N-terminal truncated peflin (peflinΔN: amino acids 116-284) was inserted in-frame into a pCMV-tag2 vector (Stratagene) for expression as Flag-tagged protein. A human ALG-2 cDNA was cloned from Jurkat cells by the reverse transcription-polymerase chain reaction (RT-PCR) method and a BglII/BamHI fragment was inserted into pCXN2 and pCMV-tag2. One day after HEK293 cells (1x10^6 cells/60-mm dish) had been seeded, the cells were transfected with the expression plasmid DNAs by the conventional calcium phosphate precipitation method. After 48 h, cells were collected and analyzed by the immunoprecipitation and/or Western blotting methods where aliquots of immunoprecipitated proteins and cell lysates were subjected to SDS-PAGE using comparable amounts of the relevant samples. The DNA transfection efficiency monitored by the expression of a green fluorescent protein (GFP) construct, pCMV-EGFP (obtained from CLONTECH), was about 20% under a similar condition in separate experiments.

**Immunofluorescent staining** - Cytospin preparations of Jurkat cell suspension (2x10^5 cells/0.2 ml) were prepared by centrifugation using an SC-2 adapter (Tomy Seiko, Tokyo, Japan), fixed in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100/PBS. After blocking with 1% bovine serum albumin (BSA) in 0.1% Tween 20/PBS, cover glasses were incubated with primary antibodies (anti-peflin MoAb and anti-ALG-2 PoAb) at 4 °C overnight and secondary antibodies (fluorescein
isothiocyanate (FITC)-conjugated anti-mouse IgG for peflin and rhodamine-conjugated anti-rabbit IgG for ALG-2) at room temperature for 30 min. Immunofluorescences were analyzed by an MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad).

**Subcellular fractionation** - Subcellular fractionation was performed by lysing cells with a Dounce homogenizer in buffer B (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol, and the protease inhibitors as described above), followed by centrifugation at 1,000 x g (4,000 rpm by a Sakuma M-150 rotor) for 10 min at 4°C (pellet, $P_1$). The supernatant was further centrifuged either at 10,000 x g (13,000 rpm by a Sakuma M-150 rotor) for 10 min at 4°C (pellet, $P_2$) or directly at 100,000 x g (60,000 rpm by a Beckman TLA 100 rotor) for 30 min at 4°C (pellet, $P_3$; supernatant, $S$). Nuclei were purified essentially as described by Dignam et al. (18). Briefly, the crude nuclear fraction ($P_1$) was homogenized in buffer B containing 0.1% Triton X-100 and 0.2 M sucrose, layered onto a cushion of buffer A containing 2 M sucrose, and centrifuged at 20,000 x g (18,000 rpm by a Beckman TLS-55 rotor) for 30 min at 4°C.

**Gel filtration** - Jurkat cells ($2 \times 10^8$) were washed twice with PBS and lysed in buffer B containing 5 mM EGTA, and the cytosolic fractions (100,000 x g, supernatant) were prepared as above. The cytosolic proteins were fractionated by gel filtration using a Superdex-75 column (1.0 cm x 30 cm, Amersham-Pharmacia). Fractions (0.2 ml each) were collected and analyzed by Western blotting. Recombinant human ALG-2 was prepared essentially as described previously (16) and subjected to gel filtration using the same column.
RESULTS

Detection of peflin with monoclonal antibody - Previously, we prepared anti-peflin antiserum and detected a 30-kDa protein as a major band in the lysates of various cell lines (14). The antiserum, however, also cross-reacted with a protein of about 40 kDa, and it remained unknown whether the 30-kDa protein was processed from the 40-kDa protein. In the present study, we prepared a monoclonal antibody (MoAb), named P1G, which was more specific to the peflin protein. As shown in Fig. 1A, the prepared MoAb P1G recognized a 30-kDa protein as a single band in Jurkat cell lysates by Western blotting, whereas the antiserum reacted additionally with other proteins. The MoAb could detect Flag-tagged peflin (Flag-peflin) and Flag-tagged N-terminal truncated peflin (Flag-peflinΔN) exogenously expressed in HEK293 cells as differently migrating bands at expected positions. Thus, it was concluded that the 30-kDa protein detected with the antiserum corresponds to an unprocessed peflin molecule. To determine whether MoAb P1G could immunoprecipitate peflin, we performed immunoprecipitation followed by Western blotting. The peflin protein was immunoprecipitated with MoAb P1G (Fig. 1B, lane 3) but not with an irrelevant MoAb 32C10A against human RECK (Fig. 1B, lane 2), which is not expressed in Jurkat cells.

Co-immunoprecipitation of peflin with ALG-2 - To search for a peflin-interacting protein, we metabolically labeled Jurkat cells with [35S]-amino acids and immunoprecipitated peflin with MoAb P1G. An autoradiogram revealed a protein band of about 22 kDa, which was co-immunoprecipitated with peflin in the presence of the Ca2+ chelator EGTA but not in the presence of CaCl2 (Fig. 2). PEF proteins have the common feature of dimerization with each other (2). For example, sorcin and grancalcin form homodimers (8, 9), and calpains form heterodimers of the
large and small subunits (10). Interestingly, heterodimers of the calpain subunits have been reported to dissociate in a Ca\(^{2+}\)-dependent manner (19). We thought that peflin might also dimerize with itself or with other PEF proteins. We suspected the co-immunoprecipitated 22-kDa protein to be ALG-2 since it is the 22-kDa protein most similar to peflin in the PEF protein family.

To investigate the interaction of peflin with ALG-2 in Jurkat cells, we performed a combined immunoprecipitation-Western blotting analysis. As shown in Fig. 3, ALG-2 was detected in the immunoprecipitates using anti-peflin MoAb P1G in the presence of EGTA but not in the presence of CaCl\(_2\). Anti-ALG-2 polyclonal antibody (PoAb) also immunoprecipitated peflin in the presence of EGTA in a complementary experiment. The immunoprecipitation experiments were repeated at least three times under different conditions by varying the concentrations of antibodies and protein G. The figures show representative results. Efficiency of the immunoprecipitation of ALG-2 with anti-ALG-2 PoAb was poor, particularly in the presence of Ca\(^{2+}\) (see DISCUSSION below).

Next, we examined the interaction between the two PEF proteins using HEK293 cells co-transfected with Flag-tagged peflin and ALG-2 expression vectors. ALG-2 was detected in the immunoprecipitates of Flag-peflin using anti-Flag MoAb M2 (Fig. 4A). ALG-2 was also co-immunoprecipitated with N-terminal truncated peflin (Flag-peflinΔN), indicating that the PEF domain of peflin is the site of this interaction. This was confirmed by complementary co-immunoprecipitation of Flag-ALG-2 with untagged peflin (Fig. 4B). On the other hand, untagged peflin was not co-immunoprecipitated with Flag-peflin (Fig. 4C), suggesting no possibility of peflin/peflin interaction. In contrast, untagged ALG-2 was precipitated with Flag-tagged ALG-2 regardless of the presence of either EGTA or CaCl\(_2\) as reported previously (Fig. 4D and Ref. 20).
Gel filtration analysis of peflin/ALG-2 heterodimer - To further examine whether peflin forms a complex with ALG-2, we performed gel chromatography of the soluble fraction of Jurkat cells in the presence of EGTA. Peflin and ALG-2 were co-eluted in the fractions corresponding to 40-50 kDa (Fig. 5B, top and middle, fractions 9-14) greater than the calculated molecular masses (peflin, 30 kDa; ALG-2, 22 kDa). On the other hand, recombinant human ALG-2 (rALG-2) was detected in the fractions eluting later than in those by Jurkat ALG-2 (Fig. 5B, bottom, fractions 13-16). Recombinant human peflin was insoluble and could not be applied to the column.

Subcellular localization of peflin and ALG-2 - We investigated the localization of peflin and ALG-2 using Jurkat cells. Double-immunofluorescent staining was performed using both anti-peflin MoAb P1G and anti-ALG-2 PoAb, and analyzed by confocal laser scanning microscopy. Immunofluorescence was detected in the cytoplasm for peflin and in both the cytoplasm and the nucleus for ALG-2 (Fig. 6).

As shown in Fig. 7A, peflin was recovered in the cytosolic fraction (S) using a lysis buffer containing 3 mM MgCl₂ but containing neither EGTA nor CaCl₂ by subcellular fractionation based on the differential centrifugation method. In contrast, ALG-2 was recovered in the crude nuclear fraction (P1), as well as in the cytosolic fraction. The crude nuclear fraction was subjected to centrifugation on a 2 M sucrose cushion. ALG-2 was detected in the purified nuclei (N), agreeing with the result of immunofluorescent staining (Fig. 6). In the presence of 0.01 mM CaCl₂, however, almost all peflin and ALG-2 were recovered in the crude nuclear fraction (P1, Fig. 7B and 7C), but the purified nuclei contained a smaller amount of peflin, and most of the peflin protein was recovered in the membrane/cytoskeletal fraction above a 2 M sucrose cushion (data not
shown). In contrast, under the same conditions, roughly equal amounts of ALG-2 were recovered in the membrane/cytoskeletal fraction and in the purified nuclei (data not shown). Inclusion of 0.1% Triton X-100 in a buffer containing no or 0.01 mM CaCl$_2$ partially solubilized peflin and ALG-2 (Fig. 7B and 7C), but some of the PEF proteins were resistant to the detergent. A higher concentration of Triton X-100 (1%) gave similar results (data not shown). These results suggested that peflin and ALG-2 also changed their subcellular distribution with Ca$^{2+}$ as reported for other PEF proteins, probably from the cytosol to the membrane and detergent-insoluble (cytoskeletal) fractions.

DISCUSSION

We showed that peflin exists as a complex with ALG-2 in the absence of Ca$^{2+}$ but that the complex dissociates in the presence of the divalent cation. Peflin and ALG-2 seem to interact directly because the two proteins were eluted from the gel filtration column at the position of a heterodimer (Fig. 5). Previously, Missotten et al. showed that ALG-2 forms a homodimer by co-immunoprecipitation of transiently co-overexpressed Flag- and Myc-tagged proteins in HEK293 cells (20). In agreement with their result, we also detected a complex of exogenously expressed Flag-tagged ALG-2 and untagged ALG-2, but the efficiency of the dimer formation was lower than that of the heterodimer formation with peflin (Fig. 4B and 4D). Without co-transfection with untagged ALG-2, no ALG-2 immunoreactive band was detected in the immunoprecipitates of anti-Flag MoAb M2 (data not shown). In similar experiments, no Flag-peflin/peflin complex was observed (Fig. 4C). On the other hand, a peflin/ALG-2 complex was clearly observed in the co-transfection assays (Fig. 4A and 4B) and even in the endogenously expressing proteins in
Jurkat cells (Fig. 3). Thus, the formation of a peflin/ALG-2 heterodimer seems dominant over an ALG-2/ALG-2 homodimer.

Since the 22-kDa protein that was co-immunoprecipitated with peflin in [35S]-labeled Jurkat cells was identified as ALG-2 (Fig. 2), it became possible to estimate an approximate molar ratio between peflin and ALG-2 in the complex. Assuming that peflin and ALG-2 incorporate [35S]-labeled amino acids with similar efficiencies during de novo synthesis, relative specific radioactivities can be calculated from the numbers of methionine (excluding translation initiation Met) and cysteine residues in the proteins (peflin: 9 Met, 5 Cys; ALG-2: 3 Met, 1 Cys). Thus, the ratio of relative specific radioactivities of [35S]-peflin and [35S]-ALG-2 is 14 to 4. This ratio agrees well with that of the observed relative radioactivities of the 30-kDa band (peflin) and the 22-kDa band (ALG-2) in the autoradiogram analyzed by a bioimaging analyzer BAS 2000 system (3.5 : 1 vs 4.2 : 1, calculated from photostimulated luminescence units: peflin, 556; ALG-2, 132). This fact indicates the presence of an approximately equal molar ratio (1 : 0.83) of peflin and ALG-2 in the immunoprecipitates and suggests that the majority of peflin exists as a heterodimer with ALG-2 in the cytosol in the absence of Ca2+.

On the other hand, not all of ALG-2 forms a heterodimer with peflin. Approximately 50% of ALG-2 is present in the 100,000 x g supernatant fraction, and the rest is found in nuclei and membrane/cytoskeletal fractions (Figs. 6 and 7). Since the amounts of peflin in the latter fractions are quite low, non-cytosolic ALG-2 may exist either as a homodimer or complexed with unknown macromolecules. Co-elution of cytosolic ALG-2 with peflin in the gel filtration chromatography suggests that the majority of cytosolic ALG-2 forms a heterodimer with peflin (Fig. 5). The results of the immunoprecipitation experiments using anti-ALG-2 PoAb, however, do not support this notion (Fig. 3). While only a fraction (less
than 10%) of ALG-2 was immunoprecipitable from the lysate, more than half of peflin was co-immunoprecipitable with the antibody. The major cause of this inconsistency may be due to the nature of the anti-ALG-2 PoAb used in this study. The antibody was first raised in rabbits using denatured recombinant mouse ALG-2 and was later affinity-purified using recombinant human ALG-2 as a ligand. The obtained antibody may recognize only a fraction of ALG-2 that retains a specific conformation favoring interaction with peflin and may poorly recognize ALG-2 monomers and homodimers under undenatured conditions. Alternatively, the antibody may disrupt the protein-protein interaction under investigation. It is unlikely that the co-immunoprecipitation of peflin with anti-ALG-2 PoAb was due to a cross reactivity of the antibody with peflin, because the antibody did not react with peflin overexpressed in HEK293 cells by Western blotting (data not shown). Indeed, anti-Flag MoAb co-immunoprecipitated untagged peflin together with Flag-tagged ALG-2 from the lysates of HEK293 cells transfected with the tagged ALG-2 expressing construct (Fig. 4B).

X-ray crystallographic analyses of the PEF domains of the recombinant rat and pig calpain small subunits revealed homodimerization through EF-5 of each molecule (3, 4). Recently, the heterodimers of recombinant m-calpains of the large and small subunits and the homodimer of grancalcin have been crystallized, and PEF domains have been shown to form similar dimer structures through EF-5s (21-23). We assume that peflin forms a heterodimer with ALG-2 by a similar protein-protein interaction mechanism. The N-terminal hydrophobic region of peflin is not essential for heterodimer formation as revealed by Flag-peflinΔN (Fig. 4A). In the present study, however, we could not investigate the potential role of the EF-5 domains of peflin and ALG-2 in their interaction. A deletion mutant lacking EF-5 (peflinΔEF5) could not be
expressed in transient transfection experiments using HEK293 cells, suggesting the importance of heterodimerization with ALG-2 for stability and/or correct folding of peflin.

The results of cellular fractionation experiments suggested that peflin and ALG-2 translocate from the cytosolic fraction to the membrane and Triton X-100-insoluble (cytoskeletal) fractions in a Ca^{2+}-dependent manner (Fig. 7). In this study, however, we could not detect a change in the immunofluorescence after stimulation of Jurkat cells with Ca^{2+}-ionophore, and we could not obtain direct evidence of the Ca^{2+}-induced translocation of these proteins by immunofluorescent staining. Surprisingly, ALG-2 was also found to localize in nuclei, raising the possibility of a specific function in nuclear Ca^{2+} signaling. Recently, Krebs and Klemenz showed the nuclear localization of ALG-2 by immunofluorescent staining of breast cancer cells and observed disappearance of the nuclear localization of ALG-2 at the onset of mitosis (24).

Apoptotic pathways of ALG-2 have been partially clarified. ALG-2 was originally identified by the method called "death trap" in T-cell hybridoma using anti-CD3 antibody (7). An antisense ALG-2 cDNA expression prompted survival after a variety of apoptotic stimuli, but caspase activities were not affected (25). An ALG-2-interacting protein named either AIP1 (ALG-2-interacting protein 1) or Alix (ALG-2-interacting protein X) was cloned concurrently by two independent groups (20, 26). AIP1 is a 105-kDa protein with a proline-rich C-terminal region containing ten PXXP sequence motifs that potentially bind to SH3 domains. The N-terminal truncated AIP1 construct exerted dominant negative effects on the apoptosis of transfected cells induced by starvation of trophic factors or staurosporine (26). The interaction between AIP1 and ALG-2 requires Ca^{2+}. In addition, AIP1 has been reported to interact with SETA (SH3-containing protein expressed in tumorigenic astrocytes).
through its C-terminal proline-rich region, which binds to SH3-N (one of the two SH3 domains) of SETA in a Ca\(^{2+}\)-independent manner (27). Overexpressed SETA proteins capable of binding to AIP1 sensitized astrocytes to UV light-induced cell death. Thus, in resting cells, SETA/AIP1 and peflin/ALG-2 complexes may exist separately in the cytoplasm. After Ca\(^{2+}\)-mobilization, ALG-2 may dissociate from peflin and interact with SETA/AIP1 complex. In our preliminary experiments, however, peflin-overexpressed HEK293 cells did not show morphological changes and differences in apoptotic sensitivity upon stimulation with Ca\(^{2+}\)-ionophore or staurosporine compared to control transfectants. Studies are in progress to investigate the potential role of peflin in Ca\(^{2+}\)-dependent apoptosis under various conditions.

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REFERENCES
FOOTNOTES

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1 The abbreviations used are: ALG-2, apoptosis-linked gene 2; PEF, penta-EF-hand; MoAb, monoclonal antibody; PoAb, polyclonal antibody; FBS, fetal bovine serum; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue R250; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; AIP1, ALG-2-interacting protein; SETA, SH3 domain-containing expressed in tumorigenic astrocytes.
FIGURE LEGENDS

FIG. 1. **Characterization of peflin MoAb P1G.** A, Coomassie Brilliant Blue staining (CBB) and Western blotting (WB) of total Jurkat cell extract (*top left*) using anti-peflin PoAb (*Po*) or MoAb (*Mo*) P1G, and Western blotting of Flag-peflin (*lane 1*) or Flag-peflinΔN (*lane 2*) expressed in HEK293 cells using MoAb P1G (*top right*) are shown. Bands corresponding to peflin (30 kDa) and a non-specifically cross-reacting protein are indicated by an arrow and by an asterisk, respectively, in the top left panel. Schematic structures of peflin proteins are depicted (*bottom*). B, Lysates of Jurkat cells were immunoprecipitated with control MoAb 32C10A or anti-peflin MoAb P1G as described in EXPERIMENTAL PROCEDURES. Cell lysate (*lane 1*) and immunoprecipitated proteins (*lane 2, 32C10A; lane 3, P1G*) were analyzed by Western blotting using P1G. Mouse immunoglobulin heavy and light chains of immunoprecipitated antibodies (*IgG-H, IgG-L*) of MoAb P1G were also detected by subsequent Western blotting using peroxidase-conjugated anti-mouse IgG as a secondary antibody.

FIG. 2. **Co-immunoprecipitation of peflin with a 22-kDa protein using MoAb P1G.** After Jurkat cells had been labeled with [35S]-methionine and [35S]-cysteine for 4 h, the cells were lysed in the presence of 5 mM EGTA (*E*) or 0.01 mM CaCl$_2$ (*C*) as described in EXPERIMENTAL PROCEDURES. Whole cell extracts were immunoprecipitated with MoAb 32C10A (*control*) or MoAb P1G (*peflin*). Aliquots of immunoprecipitatated proteins (*Ppt*) and cell lysates (*Lysate*) were subjected to SDS-PAGE using comparable amounts of the relevant samples, autoradiographed by exposing on an imaging plate for 24 h (*Ppt*) or 1 h (*Lysate*), and analyzed by a FUJIX Bioimaging Analyzer Station BAS 2000. Asterisks and an arrow indicate non-specifically
precipitated proteins and a 22-kDa protein, respectively. Relative radioactivities of the 30-kDa and 22-kDa bands after subtraction of backgrounds are 556 and 132 photostimulated luminescence (PSL) units, respectively.

**FIG. 3. Identification of a 22-kDa peflin-interacting protein as ALG-2.** Whole Jurkat cell extracts in the presence of 5 mM EGTA (E) or 0.01 mM CaCl₂ (C) were immunoprecipitated with MoAb 32C10A (control) or MoAb P1G (peflin) or anti-ALG-2 PoAb (ALG-2). Co-immunoprecipitated proteins (Ppt) and cell lysates (Lysate) were detected by Western blotting using respective antibodies. Comparable amounts of the relevant samples were adjusted with volumes.

**FIG. 4. Examination of peflin/ALG-2 interaction.** HEK293 cells were co-transfected with expression vectors as indicated. A, Flag-peflin or Flag-peflinΔN, and ALG-2. B, Flag-ALG-2 and peflin. C, Flag-peflin and peflin. D, Flag-ALG-2 and ALG-2. After 48 h, cells were lysed in the presence of 5 mM EGTA (E) or 0.01 mM CaCl₂ (C) and immunoprecipitated with anti-Flag MoAb M2. The immunoprecipitates were analyzed by Western blotting with anti-peflin MoAb P1G or anti-ALG-2 PoAb. Mouse immunoglobulin light chains (IgG-L) of MoAb M2 in the immunoprecipitates were also detected by subsequent Western blotting using peroxidase-conjugated anti-mouse IgG as a secondary antibody.

**FIG. 5. Co-elution of peflin and ALG-2 in gel filtration column chromatography.** Preparation of the soluble fraction of Jurkat cells and recombinant ALG-2 (rALG-2), and gel filtration were performed as described in EXPERIMENTAL PROCEDURES. A, 100,000 x g
supernatant was applied to a Superdex-75 column (1.0 x 30 cm), and 0.2 ml-fractions were collected. The peak positions of three molecular mass markers are indicated with arrows. BSA, bovine serum albumin (67 kDa); OVA, ovalbumin (43 kDa); CHY, chymotrypsinogen (25 kDa). B, Western blotting of each fraction with anti-peflin MoAb P1G or anti-ALG-2 PoAb. Fraction numbers in panel A are indicated above each lane.

Fig. 6. **Subcellular localization of peflin and ALG-2 in indirect immunofluorescent staining.** Jurkat cells were double-immunostained for peflin (green) with anti-peflin MoAb P1G or for ALG-2 (red) with anti-ALG-2 PoAb by detecting with secondary FITC-conjugated anti-mouse IgG antibody and secondary rhodamine-conjugated anti-rabbit IgG antibody, respectively, as described in EXPERIMENTAL PROCEDURES. Immunofluorescences were visualized under a confocal laser-scanning microscope, and a merged image was obtained. Scale bar indicates 10 µm.

Fig. 7. **Subcellular fractionation of peflin and ALG-2.** A, Jurkat cells were homogenized in a low salt buffer and fractionated into cytosolic (S), light (P3) and heavy membrane (P2), and crude nuclear (P1) fractions by the differential centrifugation method as described in EXPERIMENTAL PROCEDURES. The crude nuclear fraction was re-homogenized and centrifuged on a 2 M sucrose cushion to obtain the purified nuclear fraction (N). Volumes for SDS-PAGE were adjusted to compare the relative amounts of the PEF proteins in the subcellular fractions. Peflin and ALG-2 were detected by Western blotting using anti-peflin P1G or anti-ALG-2 PoAb. B, Effects of Ca^{2+} on
subcellular fractionation of peflin and ALG-2 were examined. Cellular fractionation into cytosol (S), membrane (P2/P3), and crude nuclear (P1) fractions was performed in the presence of 5 mM EGTA or 0.01 mM CaCl2. Effects of a non-ionic detergent, 0.1% Triton X-100, on solubilization of peflin and ALG-2 were examined. C, The immunoblots shown in Fig. 7B were scanned with a flat-bed scanner (EPSON GT-7000 ART), and the densities were quantified using a computerized image analysis system for Macintosh (NIH Image software, version 1.55). Relative amounts of peflin and ALG-2 in each subcellular fraction were expressed in histograms, respectively, where the sum of each fraction (S, P2/P3 and P1) is 1.0.
Fig. 1. Kitaura et al.
Fig. 2. Kitaura et al.
Fig. 3. Kitaura et al.
Fig. 4. Kitaura et al.
Fig. 5. Kitauroa et al.
Peflin and ALG-2, Members of the Penta-EF-Hand Protein Family, Form a Heterodimer that Dissociates in a Ca\textsuperscript{2+}-Dependent Manner

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