Identification and Characterization of Two Penta-EF-Hand Ca^{2+}-Binding Proteins in *Dictyostelium discoideum*[1]

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Penta-EF-hand (PEF) proteins such as ALG-2 (apoptosis-linked gene 2 product) and the calpain small subunit are a newly classified family of Ca^{2+}-binding proteins that possess five EF-hand–like motifs. We identified two mutually homologous PEF proteins, designated DdPEF-1 and DdPEF-2 (64% amino acid residue identities), in the cellular slime mold *Dictyostelium discoideum*. Both PEF proteins showed a higher similarity to mammalian ALG-2 and peflin (Group I PEF proteins) than to calpain and sorcin subfamily (Group II PEF proteins) in the first EF-hand (EF-1) regions. Northern blot analyses revealed that DdPEF-1 and DdPEF-2 were constitutively expressed throughout development of *Dictyostelium*, but their levels of expression were developmentally regulated. *In situ* hybridization analyses demonstrated that DdPEF-1 was expressed in both the anterior prestalk and the posterior prespore regions of the tipped aggregate, slugs and early culminants. On the other hand, DdPEF-2 was dominantly expressed in the anterior tip region of these multicellular structures. Both PEF proteins were detected as 22–23-kDa proteins in soluble fractions in the presence of EGTA but in particulate fractions in the presence of Ca^{2+} by Western blotting using specific monoclonal antibodies. Together with the finding of PEF-like sequences in DNA databases of plants, fungi and protists, our results strongly suggest that Group I PEF proteins are ubiquitously present in all eukaryotes and play important roles in basic cellular functions.

Key words: ALG-2, calcium-binding protein, calpain, *Dictyostelium*, penta-EF-hand.

Calcium ions control a wide variety of cellular functions such as muscle contraction, secretion, fertilization, proliferation, differentiation, gene expression, cellular movement, and cell death (see Ref. *1* for review). Ca^{2+}-binding proteins are mediators of the signals and play pivotal roles in these cellular functions through different mechanisms. Some enzymes such as conventional protein kinase C and calpain contain Ca^{2+}-binding sites within the enzyme molecules, whereas calmodulin kinases and calcineurin require Ca^{2+}-bound calmodulin for activation. Based on primary and 3-D structures of the Ca^{2+}-binding domains, the intracellular Ca^{2+}-binding proteins are classified into four groups: EF-hand proteins, C2-domain–containing proteins, annexins and acidic Ca^{2+} -storage proteins (*2*). The EF-hand motif, the Ca^{2+}-binding helix-loop-helix structure, has been identified in numerous Ca^{2+}-binding proteins (*3*). The number of repetitive EF-hand motifs in protein molecules, regardless of whether they are capable of Ca^{2+}-binding, ranges from two to eight (*4*).

Recently, we classified a new family of Ca^{2+}-binding proteins with five EF-hand–like motifs, and we proposed the name "penta-EF-hand (PEF)" as a collective name for these domains (*5*). The PEF domains were found in the large and small subunits of conventional calpains (*6*), sorcin (*7*), grancalcin (*8*), apoptosis-linked gene 2 (ALG-2) (*9*), and peflin (*10*). The PEF domain emerged through the analyses of the Ca^{2+}-binding domains of the small subunits of rat and pig calpains by X-ray crystallography (*11, 12*). The recombinant Ca^{2+}-binding domains of the small subunits were shown to form homodimers through a pair of C-terminal fifth EF-hands (EF-5s) that have lost their Ca^{2+}-binding capacities due to two-residue insertions. The unique feature of the PEF domains in dimerization through EF-5s is supported by recently resolved crystal structures of rat and human m-calpain heterodimers and a grancalcin homodimer (*13–15*). Peflin was shown to form a heterodimer with ALG-2 by biochemical analyses (*16*).

The presence of PEF proteins in lower eukaryotes was suggested by the finding of a similar sequence in a hypothetical protein named YG25 in the budding yeast genome database (*5, 17*). However, the biochemical properties of such proteins in simple organisms have not yet been reported. The cellular slime mold *Dictyostelium discoideum* is

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Abbreviations Acc No, accession numbers of the DDBJ/GenBank/EMBL databases; CBB, Coomassie Brilliant Blue R250; CBP, Ca^{2+}-binding protein, EST, expressed sequence tags, PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, PCD, programmed cell death, PEF, penta-EF-hand.

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a useful model organism to study many basic problems in cell biology such as cell adhesion, intracellular signaling, pattern formation, and cell differentiation. *Dictyostelium* cells proliferate as haploid unicellular amoeboid cells (vegetative growth phase). Upon food starvation, they aggregate using relayed pulses of cAMP as a chemoattractant and form a multicellular mass of about 10^5 cells (developmental stage). These cells form a migrating slug that then becomes a fruiting body consisting of a mass of spore cells supported by a thin column of stalk cells. Cytoplasmic Ca^{2+} and H^+ concentrations determine the cell fate (*18*). A slow sustained increase in cytosolic Ca^{2+} levels mediates stalk gene induction by a low-molecular-weight signaling molecule named differentiation-inducing factor DIF-1 (*19*). During *Dictyostelium* stalk cell differentiation, amoebae undergo programmed cell death (PCD) accompanied by acquisition of plant cell–like properties such as large vacuoles and cellulosic cell walls. Although the nucleosomal DNA fragmentation and the formation of apoptotic bodies are not observed, the stalk cell differentiation may represent a core mechanism of PCD that arose during early eukaryote evolution (*20*).

ALG-2 and its interacting proteins are suggested to be involved in apoptosis and cell signaling in various types of cells (*9*, *21*, *22*). To gain more insight into the strucure and function of PEF proteins, we attempted to identify and characterize PEF proteins in the model eukaryote *Dictyostelium*, first by searching for homologous genes in the established cDNA databases of the organism (*23*), and then by detecting the proteins with specific monoclonal antibodies. Here we report that at least two different PEF proteins similar to ALG-2 are present in *Dictyostelium*.

EXPERIMENTAL PROCEDURES

Cell Culture—*Dictyostelium discoideum* strain Ax2 was cultured axenically in HL5 medium using Proteose Peptone No. 3 (Difco Laboratories, Detroit, MI, USA) at 22°C as described previously (*24*). The culture medium was supplemented with penicillin G (6 units/ml) and streptomycin sulfate (6 μg/ml). Mouse myeloma cell line Sp2 cells were cultured in RPMI1640 supplemented with 15% heat-inactivated fetal bovine serum (FBS), L-glutamine (0.3 mg/ml), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) at 37°C under humidified air containing 5% CO_2.

cDNA Cloning—The *Dictyostelium* cDNA sequencing project database Dicty-cDB (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) was searched using the "tblastn" search engine for clones with homology to mammalian PEF proteins. Two cDNA clones, SSA625 and SSB886, that showed high scores were obtained from the project and the full DNA sequences were determined with an automated fluorescent sequencer, ABI PRISM 310 (PE Applied Biosystems), using a BigDye™ terminator cycle sequencing ready reaction kit (PE Applied Biosystems).

Expression of DdPEF-1 and -2 in E. coli—Polymerase chain reactions were performed to obtain cDNA fragments encoding only protein regions using the following primers: 5′-GTCATATGGATACCAACAAACACC-3′ and 5′- AAACTAAGCTAATAATATCATAAATGTG-3′ for DdPEF-1 and 5′-GTACGGATACGGATATACACCAG-3′ and 5′-AAACTAAAGCAATGATATCATAAAGTTG-3′ for DdPEF-2, respectively. The amplified DNAs were subcloned into pCR2.1TOPO TA cloning vector (Invitrogen), then fragments were excised with *Eco*RI located in the vector and inserted into the *Eco*RI site of pET-24d (Novagen, Madison, WI, USA) that had been modified to contain a hexahistidine (6xHis)-tag sequence between *Nco*I and *Bam*HI. *E. coli* BL21(DE3)-pLysS was transformed with the resultant plasmid pETHisDdPEF-1 or pETHisDdPEF-2 and cultured as described by Studier *et al.* (*25*). The *E. coli* lysates were used as positive controls for Western blot analyses. The recombinant proteins HisDdPEF-1 and HisDdPEF-2 were recovered in pellets from the *E. coli* lysates. After dissolving the inclusion bodies with 8 M guanidine hydrochloride, the recombinant PEF proteins were affinity-purified using Talon™ (Clontech) and eluted with SDS-PAGE sample buffer (63 mM Tris-HCl, pH6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) containing 50 mM EDTA. The eluted proteins were further purified by electrophoresis on preparative 12.5% polyacrylamide slab gels, followed by staining with cold 0.1 M KCl. White turbid bands were excised and proteins were extracted with PBS.

Monoclonal Antibodies—BALB/c female mice were immunized at footpads three times with a mixture of purified HisDdPEF-1 and HisDdPEF-2. Lymphocytes from popliteal lymph nodes of immunized mice were used for hybridoma preparation. Cell fusion, cloning of positive cells, and preparation of IgG fractions from mouse ascites were performed as described previously (*16*). Immunosignals in Western blotting were detected by the color development method as described previously (*10*).

Subcellular Fractionation—*Dictyostelium* cells were harvested and washed twice with PBS and suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol) containing protease inhibitors (0.1 mM pefabloc, 5 μg/ml leupeptin, 10 μM E-64, 1 μM pepstatin) in the presence of either 0.1 mM $CaCl_2$ or 5 mM EGTA. Cells were lysed by the nitrogen cavitation method using a cell disruption chamber (Kontes Mini-Bomb K-881455, Kontes, New Jersey, USA) under 30 atm for 30 min at 4°C according to the instruction manual provided. Alternatively, cells were lysed by addition of Triton X-100 to 1% and gently mixed for 15 min. Cell lysates were centrifuged at 1,000 $\times g$ (4,000 rpm by a TOMY TMP-11 rotor) for 10 min at 4°C (pellet, P1) The supernatants were centrifuged at 10,000 $\times g$ (13,000 rpm by a Tomy TMP-11 rotor) for 10 min at 4°C (pellet, P2), and the obtained supernatants were further centrifuged at 100,000 $\times g$ (60,000 rpm by a Beckman TLA 100 rotor) for 30 min at 4°C (pellet, P3; supernatant, S).

Northern Blotting—Total RNA was prepared from 5 × 10^7 cells that were developing on a sheet of Whatman No. 50 filter paper (4.2 cm in diameter) at 4-h intervals after starvation as described previously (*26*). Trizol reagent (Gibco BRL) was used for total RNA extraction according to the manufacture's instruction. After electrophoresis at 10 μg per lane in 1.0 % denaturing agarose, total RNA was transferred to a nylon membrane (Hybond-N+, Amersham-Pharmacia) and subjected to UV-cross-linking (TL-2000 Ultraviolet Transliker, Ultraviolet Products). Hybridization and detection of the signals were conducted with a digoxigenin (DIG) DNA labeling and detection kit (Roche Diagnostics), according to the manufacturer's protocol. The cDNA clones, SSA625 and SSB886, which encode DdPEF-1 and DdPEF-2, respectively, were used as the templates for probe preparation.

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In Situ Hybridization—Whole mount *in situ* hybridization analyses were performed according to the method of Escalante and Loomis (*27*) with a slight modification (*28*). DIG-labeled RNA probes were prepared by use of a DIG RNA labeling kit (Roche Diagnostics) for *in vitro* transcription according to the manufacturer's protocol. T7-polymerase was used to prepare antisense RNA probes by using a *Sal*I-digested pBluescript II KS(−) (Stratagene) harboring a cDNA clone, SSA625, SSB886, and SSJ770, as a template. In order to prepare sense RNA probes, T3-polymerase was used and each *Not*I-digested cDNA was used as a template. All probes were used at 25–50 μg/ml for hybridization. The color development reaction was performed for 30 min to 2 h. Pictures were taken with a digital camera set (Olympus HC-300) attached to a stereomicroscope (Olympus SZX12).

Computer Analyses—Homology search was performed using the "tblastn" program of the translated BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was performed with the Clustal X program released from the European Bioinformatics Institute (*29*), and a phylogenetic tree was drawn with the tree-viewing program TreeView (*30*) (http://taxonomy.zoology.gla.ac.uk/rod/treeview/). Motifs in the deduced amino acid sequence were searched using a motif database Prosite (http://expasy.ch/sprot/prosite.html).

RESULTS

Identification and Cloning of the Dictyostelium PEF cDNAs—The *Dictyostelium* cDNA project database Dicty-cDB was searched with the tblastn search engine to identify cDNAs with homology to the mammalian PEF proteins. Two different sets of partial cDNA sequences encoding potential PEF proteins were found. Full DNA sequencing of two representative clones, SSA625 and SSB886, revealed that they have N-terminal regions rich in hydrophobic residues (Fig. 1A) and that they contain five

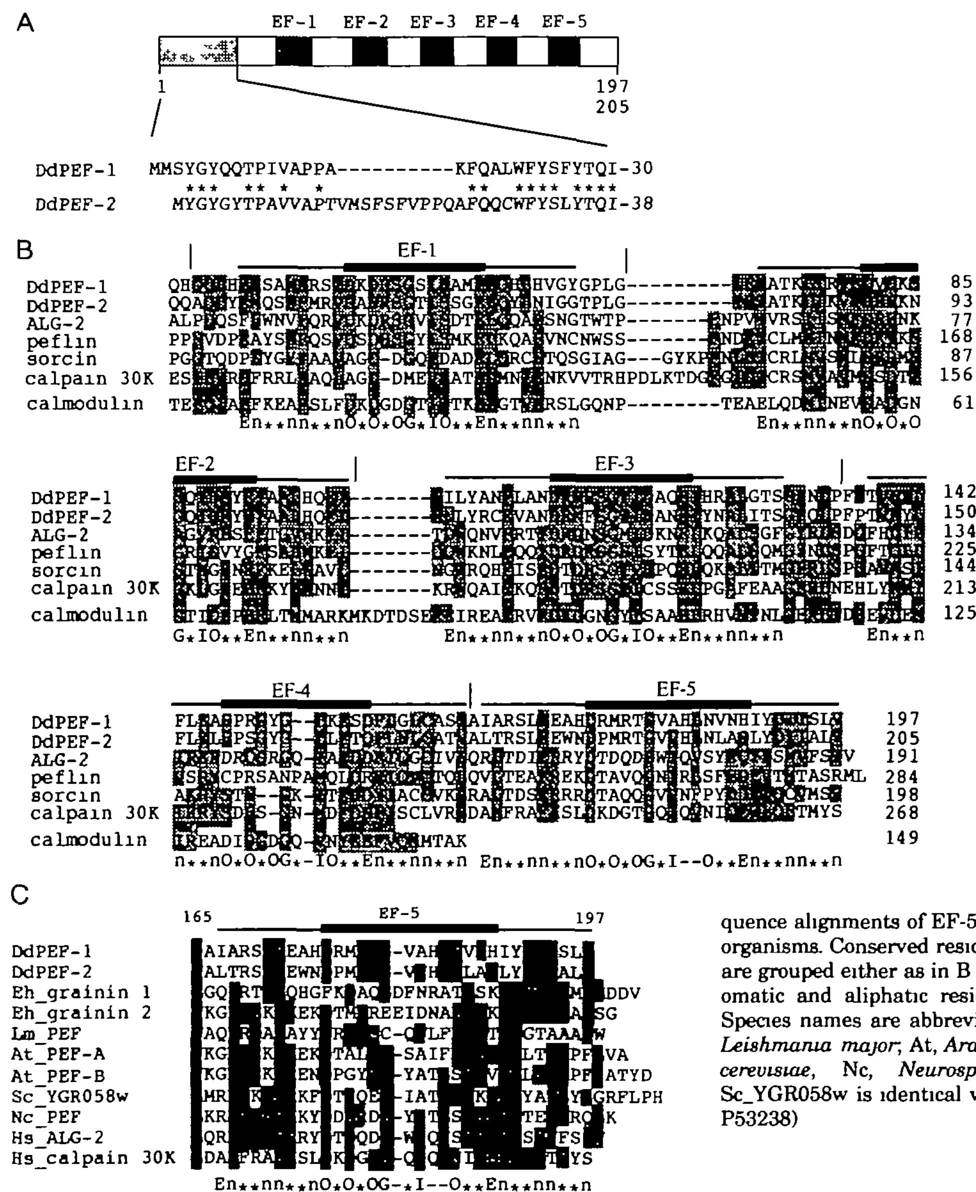

Fig 1 **Primary structures of *Dictyostelium* PEF proteins.** A. Schematic structures of DdPEF-1 and DdPEF-2. EF-hand–like motifs are indicated with diagonally hatched boxes Amino acid sequences of N-terminal hydrophobic regions are shown Identical residues are indicated with asterisks. B Multiple sequence alignments of the PEF proteins and calmodulin Sequences were aligned by use of the Clustal X program Potential Ca^{2+}-binding loops and α-helices of EF-hand structures are indicated with thick and thin bars above the DdPEF-1 sequence. Amino acid similarity groupings are aromatic (F, Y, and W), hydrophobic large side chain (L, I, V, and M), acid and amide (E, D, Q, and N), hydroxy small side chain (S and T); small side chain (G and A) Boundaries of EF-hand regions are indicated with vertical lines The consensus sequence of EF-hand, modified from Ref. *4*, has preferred residues at n (hydrophobic), O (oxygen containing), I (Ile, Val, Leu or Met), G (Gly), and E (acidic or amide) Asterisks indicate variable residues (often hydrophilic) Hyphens in consensus sequences in EF-4 and EF-5 regions indicate insertions. C Multiple sequence alignments of EF-5 regions of PEF proteins from various organisms. Conserved residues are stippled Amino acid residues are grouped either as in B (stippled) or hydrophobic residues (aromatic and aliphatic residues including Ala)(lightly stippled) Species names are abbreviated Eh, *Entamoeba histolytica*, Lm, *Leishmania major*, At, *Arabidopsis thaliana*, Sc, *Saccharomyces cerevisiae*, Nc, *Neurospora crassa*, Hs, *homo sapiens*. Sc_YGR058w is identical with YG25_YEAST (SwissProt Acc No P53238)

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EF-hand–like sequences (Fig. 1B). DdPEF-1 (clone SSA625) and DdPEF-2 (SSB886) are mutually homologous with 64 or 69% amino acid residue identities including or excluding the N-terminal regions Both *Dictyostelium* proteins lack seven residues seen in calmodulin between EF-2 and EF-3, and they contain C-terminal regions similar to the EF-5 sequences of other PEF proteins (Fig. 1B).

Comparison with the canonical EF-hand sequence (*4*) suggests that EF-4 and 5 of both *Dictyostelium* PEF proteins do not bind Ca^{2+} due to the presence of unfavorable residues at the Ca^{2+}-coordinating positions. Sequences in EF-1, EF-2 and EF-3 in DdPEF-1 and EF-1 and EF-2, in DdPEF-2 match with a 13-residue consensus pattern of EF-hand motif registered in Prosite (Motif, EF-HAND; Acc No PS00018).

Amino Acid Sequence Comparison with Other PEF Proteins—Both *Dictyostelium* PEF proteins showed similarities to mammalian ALG-2 and peflin in the EF-1 regions (28–38% amino acid identities). Similarities to sorcin (22–27% identities) and the calpain small subunit (calpain 30K) (11–17% identities) were even lower than to calmodulin (24–30% identities) This is due to the divergence of the latter group (Group II PEF proteins: sorcin, grancalcin, and calpain family members) in EF-1s. They lack one residue and contain unconserved residues in the Ca^{2+}-binding loops, whereas the former group (Group I PEF proteins) contains canonical 12-residue Ca^{2+}-binding loops as in the

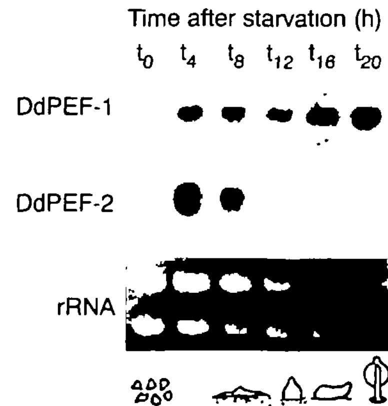

Fig 3 **Developmentally regulated expression of DdPEF-1 and DdPEF-2.** Total RNA was extracted at 4-h intervals after starvation from 5×10^7 *Dictyostelium* cells that were developing on separate sheets of Whatman No.50 filter paper supported by 1% non-nutrient agar The RNA was then subjected to Northern blot analyses of DdPEF-1 (top panel) and DdPEF-2 (middle panel) mRNAs. Each lane contains 10 μg total RNA. Bottom panel, ethidium bromide staining after agarose gel electrophoresis of isolated total RNA where rRNAs are major constituents. The stages t_0, t_4, t_8, t_{12}, t_{16}, and t_{20} correspond respectively to the vegetative, preaggregative, aggregation, tipped aggregate, migrating slug, and culmination stages

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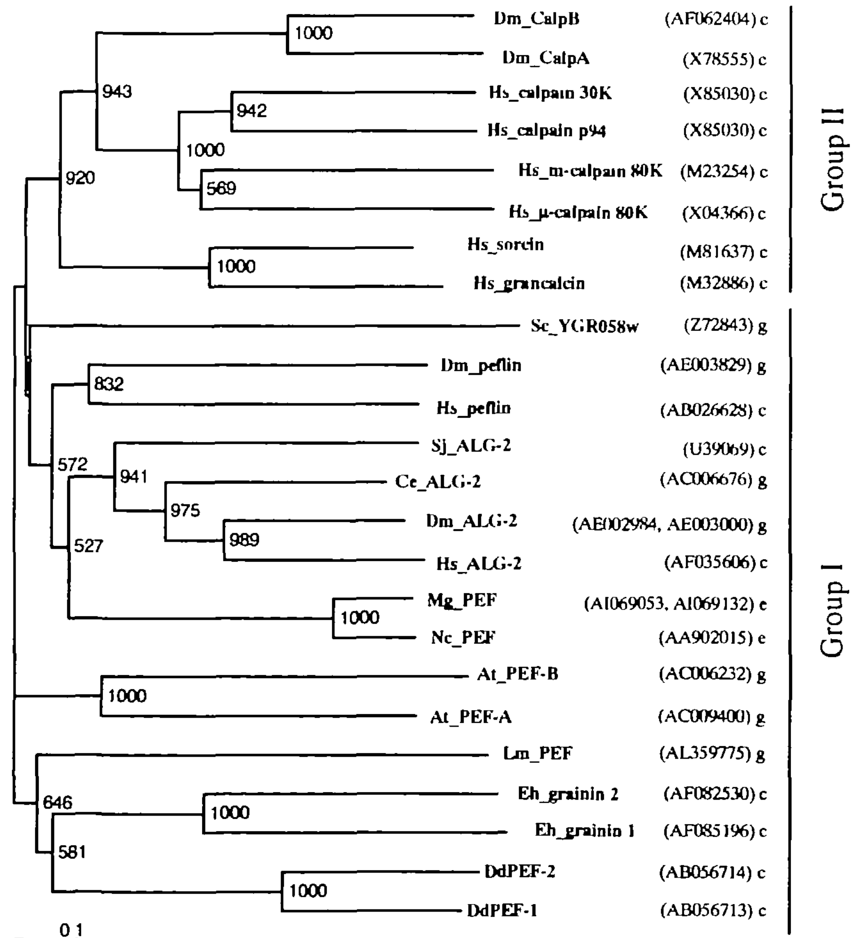

Fig 2 **Phylogenetic tree of the PEF protein family.** The PEF protein sequences were aligned by use of the CLUSTAL X program, and a bootstrap tree file was created. The phylogenetic tree was drawn with the provided tree-viewing program TreeView. The values indicate the number of times that branches are clustered together out of 1,000 bootstrap trials (values greater than 500 are labeled). Species names are abbreviated as in Fig 1C and as follows: Dm, *Drosophila melanogaster*, Ce, *Caenorhabditis elegans*, Sj, *Schistosoma japonicum*, Mg, *Magnaporthe grisea* Names of the PEF proteins are tentatively given. GenBank/EMBL/DDBJ accession numbers (Acc No) are indicated in parentheses. Letters c, e, and g indicate that the PEF amino acid sequences were predicted from full-length cDNAs, partial EST cDNAs, and genomic DNA sequences, respectively. The DNA sequence of GenBank Acc No U39069, deposited as a cDNA of the *S japonicum* sorcin mRNA, is attributed to an ortholog of ALG-2 (Sj_ALG-2)

case of calmodulin (Fig 1B and Ref. *4*). In contrast, the EF-3 regions of *Dictyostelium* PEF proteins showed an equal or even higher similarities to those of the calpain small subunit or sorcin (29–46% identities) than those of ALG-2 and peflin (31–37% identities). Recently, Nickel *et al.* (*31*) reported two calcium-binding proteins, designated grainin 1 and 2, from cytoplasmic granules of the protozoan parasite *Entamoeba histolytica*. These proteins have four EF-hand–like sequences and carboxy-terminal extensions. Surprisingly, alignment of the C-terminal regions of grainins with EF-5s of *Dictyostelium* PEF proteins revealed a moderate similarity (6% identical residues, 44–50% similar residues) (Fig 1C). By DNA database searches, PEF-like sequences were also found in potential Ca^{2+}-binding proteins of a parasitic protist *Leishmania major*, a model plant *Arabidopsis thaliana*, and fungi *Neurospora crassa* and *Magnaporthe grisea*.

To estimate evolutional distances among PEF proteins, a phylogenetic tree was constructed by comparing amino sequences of the PEF domains by use of the CLUSTAL X program. As shown in Fig. 2, both *Dictyostelium* PEF proteins are closer to protist PEF proteins and approximately equally distant from animal/fungi and plant PEF proteins. Partial sequences of the PEF proteins similar to *Arabidopsis* PEF-A and PEF-B were also found in other plants [EST Acc Nos are: Type A, BE040838 (rice), AW396962 (soy-

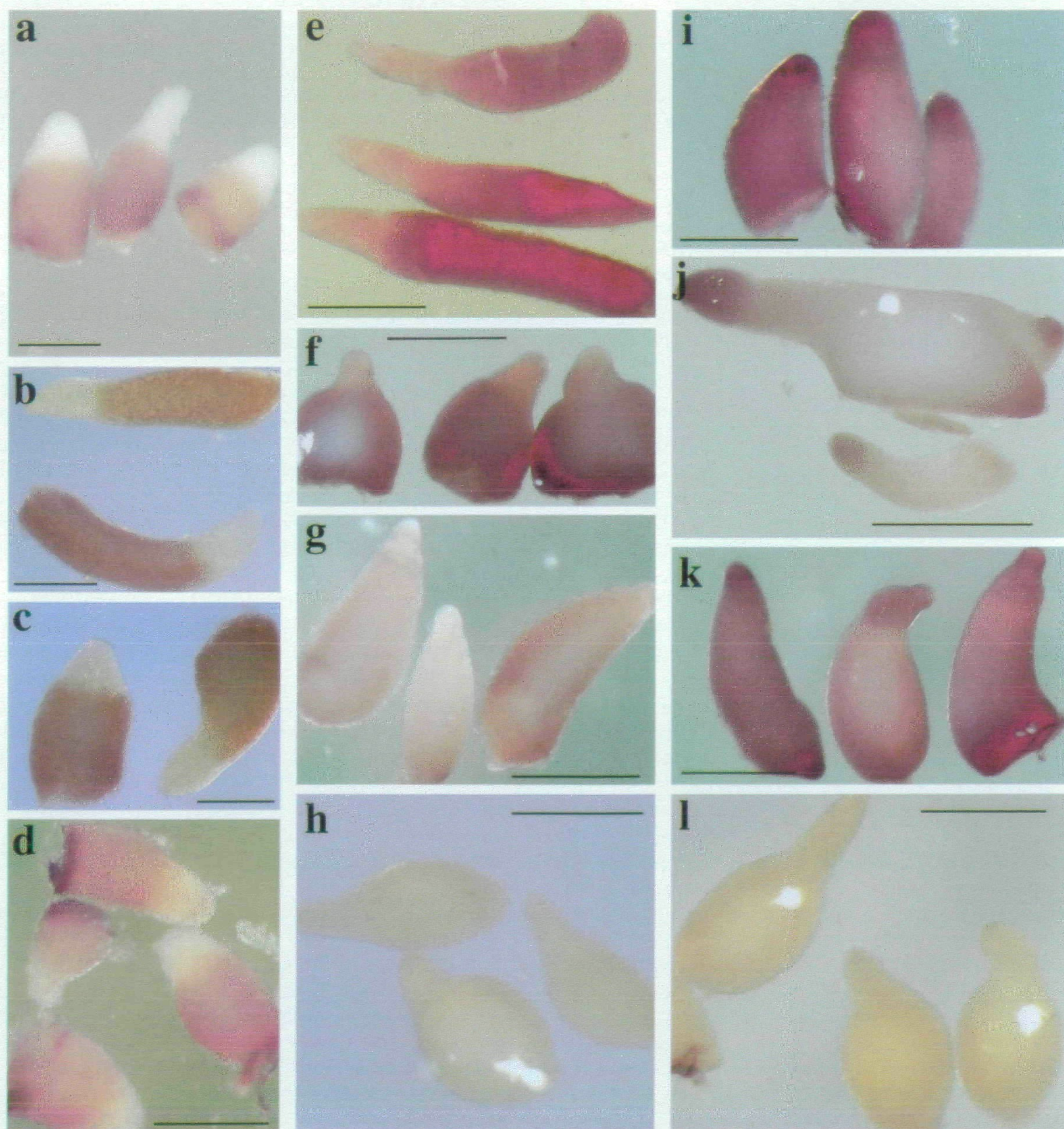

Fig. 4 ***In situ* hybridization analysis.** Spatial expression patterns of a pre-spore specific gene *pspA* (a–c), *pefA* (DdPEF-1, d–h), and *pefB* (DdPEF-2, i–l) during *Dictyostelium* development were analyzed by *in situ* hybridization as described in "EXPERIMENTAL PROCEDURES" Antisense RNAs for *pspA* (a–c), *pefA* (d–g), and *pefB* (i–k), and sense RNAs for *pefA* (h) and *pefB* (l) were used as probes. Developmental stages tipped aggregate (a, d, h, i, l), slugs (b, e, j), early culminants (c, f, g, k). Bars, 300 μm

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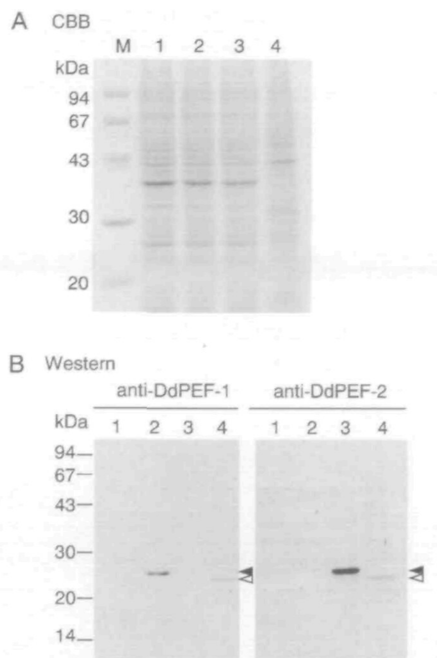

Fig 5 **Detection of DdPEF-1 and DdPEF-2 with monoclonal antibodies.** Lysates of *E coli* harboring a vector pET-24d (lanes 1) or PEF expressing plasmids (pETHisDdPEF-1, lanes 2, pETHisDdPEF-2, lanes 3) and *Dictyostelium* (lanes 4) were subjected to SDS-PAGE, followed by CBB staining (panel A) or Western blotting analysis using anti–DdPEF-1 (panel B left) or anti–DdPEF-2 (panel B right) monoclonal antibodies To reduce the recombinant PEF protein concentrations in the lysate samples (lanes 2 and 3), lysates of *E coli* expressing PEF (~5% of the total cell protein) were diluted 100-fold with that of *E coli* harboring pET-24d (negative control) Recombinant hexahistidine-tagged and endogenous *Dictyostelium* PEF proteins are indicated by closed and open arrowheads, respectively

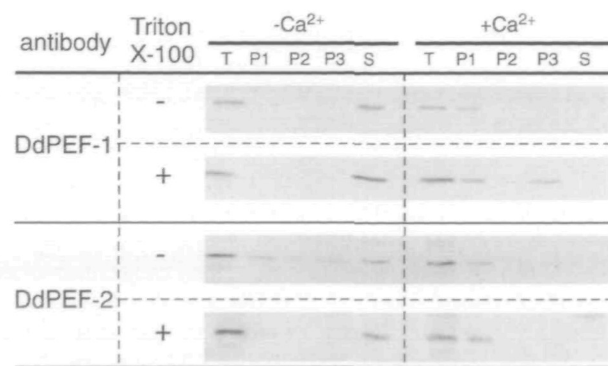

Fig 6. **Subcellular fractionation of DdPEF-1 and DdPEF-2.** *Dictyostelium* cells were suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM KCl, 0 1 mM pefabloc, 5 μg/ml leupeptin, 10 μM E-64, 1 μM pepstatin, 1 mM dithiothreitol) in the presence of either 0 1 mM $CaCl_2$ ($+Ca^{2+}$) or 5 mM EGTA ($-Ca^{2+}$), and lysed by nitrogen cavitation (–Triton X-100) or by gentle mixing in the presence of 1% Triton X-100 as decribed in "EXPERIMENTAL PROCEDURES" Subcellular fractionation was performed by sequential centrifugations T, total lysate, P1, 1,000 ×*g* pellet, P2, 10,000 ×*g* pellet, P3, 100, 000 ×*g* pellet, S, supernatant. DdPEF-1 and DdPEF-2 proteins were detected by Western blotting using the specific antibodies (anti–DdPEF-1 and anti–DdPEF-2)

bean); Type B, AU068303 (rice), AI930911(soybean)].

Expression of DdPEF-1 and DdPEF-2—Northern blot analyses revealed that both DdPEF-1 and DdPEF-2 were constitutively expressed throughout development (Fig. 3). The level of the DdPEF-1 gene (designated *pefA*) transcript (1.6 kb) gradually increased, while that of the DdPEF-2 gene (designated *pefB*) transcript (0 9 kb) reached a peak by the aggregation stage (4–8 h after starvation) and then abruptly decreased at the tipped aggregate (12 h after starvation) and remained at a low level in the later developmental stages. To obtain a clue to the biological roles of DdPEF-1 and DdPEF-2, the spatial expression patterns of the genes encoding these proteins were analyzed against the tipped aggregate (12 h after starvation), slugs (15 h after starvation), and early culminants (18 h after starvation) by *in situ* hybridization. As a positive control, SSJ770, a cDNA clone, which encodes a prespore-specific gene *D19* or *pspA* (32) was analyzed first, demonstrating that *pspA* was specifically expressed in the posterior prespore region of tipped aggregate, slugs and early culminants (Fig. 6, a–c). The gene *pefA* encoding DdPEF-1 was expressed more abundantly in the posterior prespore region than in the anterior prestalk region of tipped aggregate, slugs, and early culminants (Fig. 4, d–g). On the other hand, the *pefB* mRNA encoding DdPEF-2 was enriched in the extreme tip of the prestalk region, slugs, and early culminants (Fig. 4, i–k) more than in the posterior prespore region of these structures. Sense probes for both genes did not give any significant signal (Fig. 4, h and l).

Detection of DdPEF-1 and DdPEF-2 Using Specific Monoclonal Antibodies—Specificities of monoclonal antibodies against DdPEF-1 and DdPEF-2 were confirmed by Western blotting analysis of *E. coli* expressing the recombinant 6xHis-tagged *Dictyostelium* PEF proteins (HisDdPEF-1, HisDdPEF-2) (Fig. 5). Anti–DdPEF-1 antibody reacted with a band of 23 kDa protein in the lysates of *E. coli* expressing HisDdPEF-1 (panel B left, lane 2), but did not react with proteins in the lysates of *E. coli* harboring either control plasmid (lane 1) or HisDdPEF-2 expressing plasmid (lane 3). The antibody detected a 22-kDa protein in the lysates of *Dictyostelium* cells (lane 4). The slightly slower migration rate of HisDdPEF-1 appears to be due to the presence of a hexahistidine (6xHis)-tag in the recombinant protein. On the other hand, anti–DdPEF-2 antibody reacted with HisDdPEF-2 (panel B right, lane 3, closed arrowhead) but did not cross-react with HisDdPEF-1 (lane 2). This antibody also detected a 22-kDa protein in the *Dictyostelium* lysates (lane 4, open arrowhead).

Subcellular Localization—Subcellular localization of each *Dictyostelium* PEF protein was investigated by the differential centrifugation method followed by Western blotting using the obtained specific monoclonal antibodies (Fig. 6). In the absence of Ca^{2+}, both DdPEF-1 and DdPEF-2 were detected mostly in the cytosolic fractions (100,000 ×*g* supernatants, *S*). In the presence of Ca^{2+}, however, they were recovered mostly in the 1,000 ×*g* pellets (*P1*), and partially in the 10,000 ×*g* pellets (*P2*) and 100,000 ×*g* pellets (*P3*). Addition of the non-ionic detergent Triton X-100 (final concentration, 1%) into the lysis buffer containing Ca^{2+} was not effective in solubilization of the PEF proteins.

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DISCUSSION

We identified two new potential Ca^{2+}-binding proteins with five EF-hand-like motifs (penta-EF–hand), designated DdPEF-1 and DdPEF-2, in the cellular slime mold *Dictyostelium discoideum*. Based on the differences in the primary structures of EF-1 Ca^{2+}-binding loops, the *Dictyostelium* PEF proteins belong to a Group I PEF protein subfamily (ALG-2, peflin, and their fungal, plant, and protist homologs: possessing canonical 12-residue loops) rather than to a Group II PEF protein subfamily (sorcin, grancalcin and calpains possessing non-canonical 11-residue loops) (Fig. 1B). Homology searches of the genome databases of human and *Drosophila* revealed the existence of two Group I PEF proteins, ALG-2 and peflin. We also identified two PEF proteins in *Dictyostelium*. Results of the phyologenetic tree analyses indicate that relation between DdPEF-1 and DdPEF-2 is closer than that between ALG-2 and peflin (Fig. 2). The peflin gene diverged more rapidly than ALG-2 (human *vs. Drosophila*) and is absent in *C. elegans*. Both DdPEF-1 and DdPEF-2 may correpond to ALG-2 and may play more basic roles in eukaryotes, whereas peflin may function more specifically in higher eukaryotes.

Previously we reported that Ca^{2+} induced a conformational change of recombinant ALG-2 to expose a hydrophobic surface, and that ALG-2 precipitated at higher protein concentrations. Truncation of the N-terminal hydrophobic domain increased the solubility of the mutant recombinant protein in the presence of the ion (*33*). N-terminal domains of the mammalian PEF proteins other than the calpain large subunit are rich in Gly and hydrophobic residues. In the case of the calpain small subunit, the hydrophobic N-terminal domain was reported to bind membranes and play an important role in the change of subcellular localization induced by Ca^{2+} (*34*). Sorcin requires its N-terminal region to interact with annexin VII, which translocates to membranes in a Ca^{2+}-dependent manner (*35*). Thus, the N-terminal regions of PEF proteins seem to be involved in the interaction with phospholipids and/or target proteins on membranes. DdPEF-1 and DdPEF-2 also have clusters of hydrophobic residues in their N-terminal regions (Fig. 1A). The *Dictyostelium* PEF proteins were recovered in cytosolic fractions in the presence of a Ca^{2+} chelator EGTA, but recovered in particulate fractions in the presence of Ca^{2+} (Fig. 6). The effect of Ca^{2+} on the recovery of PEF proteins by the cellular fractionation was similarly observed in the case of peflin and ALG-2 (*16*). While peflin and ALG-2 were partially solubilized in the presence of 0.1% Triton X-100 in our previous studies, the *Dictyostelium* PEF proteins could not be solubilized by even 1% of the detergent (Fig. 6). They may bind to cytoskeletal proteins and/or signal-transducing proteins localized to detergent-resistant membranes named lipid rafts (*36*).

The presence of EF-5 is a unique feature of the PEF proteins. X-ray crystallographical analyses of the PEF domains have revealed that the helix-loop-helix strucure of EF-5 forms a new interface for dimerization (*11–15*). Stabilities and properties of PEF protein dimers vary among the PEF protein family members. Sorcin and grancalcin each form a tight homodimer that is eluted as such in gel filtration chromatography (*37, 38*). The *Dictyostelium* PEF proteins (22–23 kDa) were eluted in fractions corresponding to 24–35 kDa by gel filtration (data not shown). They may be present as monomers or weak homo- or heterodimers like ALG-2 that dissociate during gel filtration (*16, 33*).

In *Dictyostelium*, about 20% of cells comprising the anterior region of slugs are the precursors of stalk cells that are programmed to die at the terminal development. Morphological and biochemical characteristics of such developmentally regulated death of the stalk cells resemble those of the recently described alternative, nonapoptotic form of PCD (paraptosis) in animal cells (*39, 40*). Such PCD of *Dictyostelium* seems to be closely related to Ca^{2+}-signaling. In this connection, it should be noted that when cultured and allowed to develop in the presence of $^{45}Ca^{2+}$, resulting slugs and culminants accumulated more $^{45}Ca^{2+}$ in the anterior prestalk region than in the posterior prespore region (*41*). Also, *Dictyostelium* cells expressing jellyfish apoaequaporin revealed that intracellular free Ca^{2+} level was higher in prestalk cells than in prespore cells (*42, 43*). On the other hand, a chlorinated signal molecule, DIF-1 (differentiation-inducing factor 1 for stalk cells), has been shown to play an essential role in determination of the cell fate leading to the PCD (*44, 45*) It has been shown that DIF-1 causes a rise in the intracellular Ca^{2+} level (*19, 46*). These results are likely to show that Ca^{2+} signaling plays an essential role in *Dictyostelium* PCD, which is probably mediated by certain Ca^{2+} binding proteins (CBP). So far, many CBPs containing four EF-hands such as calmodulin, CBP-1, -2, -3, -4a, and –4b and CAF-1 in *Dictyostelium* have been reported (*47–52*). These proteins are believed to act as Ca^{2+} sensors to transduce Ca^{2+} signals to downstream components of the signaling pathways. However, their involvement in PCD remains unknown. On the other hand, DdPEF-1 and DdPEF-2, newly identified CBPs having five EF-hands, are homologous to mammalian ALG-2 (apoptosis-linked gene 2 product), and thus these proteins could serve as a mediator for Ca^{2+} signaling-related *Dictyostelium* PCD. DdPEF-2, whose expression was enriched in the anterior prestalk region of slugs and culminants, is particularly interesting in this regard (Fig. 4, i–k). Although it is premature to speculate about the biological functions of the *Dictyostelium* PEF proteins, they might have specific roles in the development of this organism. By taking advantage of facilitated gene disruption experiments in the haploid multi-celluar organism *Dictyostelium*, physiological functions of DdPEF-1 and DdPEF-2 should be clarified in the near future.

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