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Cloning and Expression of Pleckstrin 2, a Novel Member of the Pleckstrin Family

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A novel member of the pleckstrin family has been identified and designated as mouse pleckstrin 2. The cDNA clone with an insert of 1588 bp contained a 1059-bp open reading frame encoding a polypeptide of 353 amino acid residues. The deduced amino acid sequence predicted that pleckstrin 2 has two pleckstrin homology (PH) domains at the N- and C-termini and a DEP (Dsh, egl-10, and pleckstrin) domain in the central region and showed 35% identity with the sequence of chicken pleckstrin. Northern blot and reverse-transcription polymerase chain reaction analysis revealed that pleckstrin 2 mRNA is ubiquitously expressed. Southern blot analysis indicated that the mouse pleckstrin 2 gene may consisit of two or more exons. To obtain information relative to natural ligand(s) for each of the PH domains *in vivo,* we employed the green fluorescent protein (GFP) tagged fusion protein system. Distributions of N-terminal and C-terminal PH domains of pleckstrin 2 were quite different from each other, suggesting that these PH domains may interact with distinct factor(s).

Pleckstrin, a 40-kDa protein which is expressed exclusively in hemopoietic cells, was originally identified as the major substrate for protein kinase C in platelets (1, 2). This protein contains two similar domains consisting of about 100 amino acid residues in the N- and C-terminal regions (3, 4). This represents the prototype of a module which is referred to as the pleckstrin homology (PH) domain, and which features an antiparallel β-sheet consisiting of seven strands and a C-terminal amphiphilic α-helix (5, 6). Since this discovery, such PH domains have been found in over 100 proteins including protein kinases, GTP-binding proteins, phospholipases and cytoskeletal proteins (3, 4). It has been established that the domains bind inositol phospholipids (7, 8), inositol phosphates (9, 10), $\beta\gamma$ subunits of heterotrimeric GTP-binding proteins (11) as well as protein kinase C (12, 13). As a result of this, the PH domains appear to be involved in various signaling processes via interactions with different factors.

The expression of pleckstrin is limited to hemopoietic cells (1, 2, 14) and spleen (15). In order to determine whether a novel member of the pleckstrin family is expressed in other cell types or tissues, we pursued a strategy involving the polymerase chain reaction (PCR) cloning and the expressed sequence tags (ESTs) database. Since the ESTs consist of partial "single pass" cDNA sequences from various species and tissues (16, 17), it permits us to look for new members of gene families.

In this study, we describe the cloning of pleckstrin 2 cDNA as a novel member of the pleckstrin family and its expression in living cells, as evidenced by the green fluorescent protein (GFP)-fusion protein system.

MATERIALS AND METHODS

EST database search. The dbEST was searched with the nucleotide sequences of human and chicken pleckstrin (2, 15) as queries using the BLAST search (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST).

5′ rapid amplification of cDNA ends (5′ RACE) and 3′ RACE. Balb/C male mouse liver Marathon ready cDNA (Clontech) was purchased. A cDNA was amplified by PCR with one of the gene-specific primers based on an EST sequence (GeneBank Accession No. AA 008011) (5′-RACE primer: 5′-GAACAGGCTTCCAGGAAG-TAC-3′) and AP1 primer (Clontech) were used in a first-round PCR condition, as recommended by the manufacture's protocol. The two primers were then removed and a second-round PCR was performed using a nested AP2 primer (Clontech) and 5′-RACE primer: 5′-ATGAACC-ATCGTGCCTTCCAG-3′ from Accession No. AA 008011.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AF170564.

Abbreviations: PH, pleckstrin homology; PCR, polymerase chain reaction; EST, expressed sequence tag; GFP, green fluorescent protein; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; DEP, dishevelled, egl-10, and pleckstrin; PIP_2, phosphatidylinositol 4, 5-bisphosphate; PI-3K, phosphatidylinositol 3-kinase; Btk, Bruton's tyrosine kinase.

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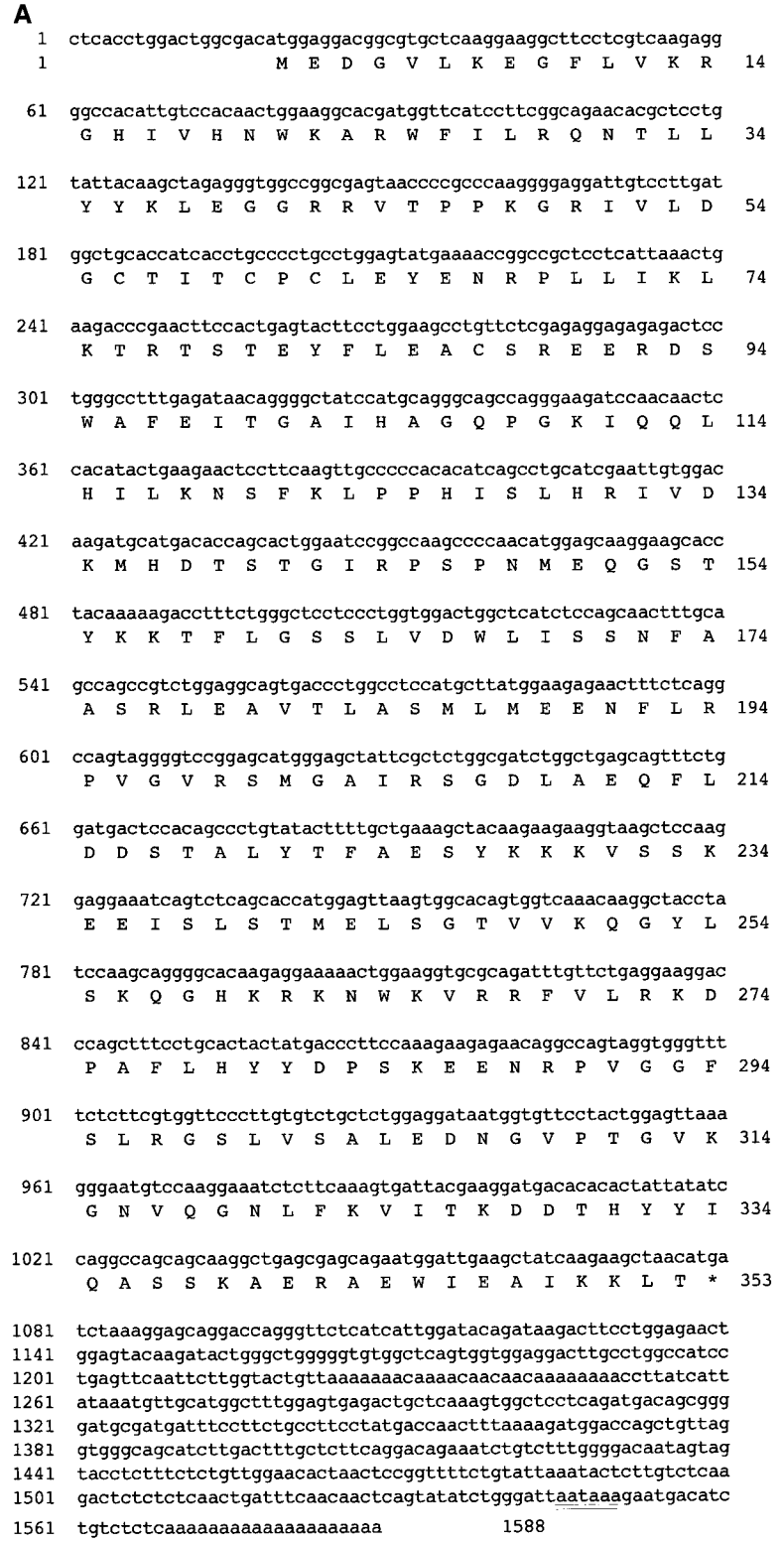

FIG. 1. (A) Nucleotide sequence of pleckstrin 2 cDNA and its deduced amino acid sequences. Nucleotide and amino acid residues are numbered from the 5′ end of the cDNA and from the initiation methionine residue, respectively. A stop codon is indicated by an asterisk. The putative polyadenylation signal is double-underlined. (B) Comparison of the deduced amino acid sequence of mouse pleckstrin 2 with chicken and human pleckstrin. *Asterisks* indicate where the deduced amino acid in the alignment is identical between mouse pleckstrin 2 and chicken pleckstrin. The PH domain is indicated by (+), and the DEP domain by (−).

FIG. 1—*Continued*

An amplified nested PCR product (about 200 bp) was cloned into pT7Blue T-vector (Novagen) and the resulting nucleotide sequences were determined.

3′-RACE PCR was performed by the following method. A first-round PCR was carried out with a combination of 3′-RACE primer; 5′-AACTTTGCAGCCAGCCGTCT-3′ from GeneBank Accession No. AA 403397 and AP1 primer. A second-round PCR was then performed using a combination of 3′-RACE primer; 5′-CCAAAAGAAGAGAA CAGGCCAG-3′ from AA823548 and AP2 primer. An amplified PCR product (about 800 bp) was cloned into pT7Blue T-vector and the resulting nucleotide sequences were determined.

cDNA cloning and sequencing. Total RNA was extracted from Balb/C mouse liver using TRIZOL reagent kit (Bethesda Research Laboratories) based on the method described by Chomczynski and Sacchi (18). The first strand cDNA was synthesized from 10 μg of total RNA using the Superscript II kit (Bethesda Research Laboratories) in conjunction with oligo dT primer. The PCR mixture included the sense primer, 5′-TGGACTGG CGACATGG-AGGAC- 3′ (corresponding to nt 7–28), the antisense primer, 5′-CCCTGGT-CCTGCT-CCTTTAGA-3′ (corresponding to nt 1081–1101), Ex Taq polymerase (TAKARA) and 200 ng of template cDNA from mouse liver. The nucleotide sequences of these primers were derived from the 5′ and 3′-RACE mentioned above. Each of the 30 cycles of the PCR included denaturing for 30 s at 95°C, annealing for 30 s at 62°C and an extension for 1 min at 72°C. The final extension time was 5 min. A PCR product (1095 bp in length) was subcloned into pT7Blue T-vector and at least 3 independent isolated clones were sequenced on both strands using a Dye terminator cycle sequencing kit (PE Applied Biosystems), and those cDNA inserts were found to be completely identical.

Northern, reverse-transcription polymerase chain reaction (RT-PCR) and Southern blot analysis. Total RNA (20 μg per each lane) from various Balb/c mouse tissues was subjected to 1% denaturing agarose gel electrophoresis and transferred onto a Hybond-N filter (Amersham). The filter was hybridized with a labeled probe at 50°C in 50% formamide, 5× SSC, 4× Denhart's solution, 50 mM sodium phosphate (pH 6.9) and 40 μg/ml salmon sperm DNA. After washing at 50°C for 1 h in 0.1× SSC and 0.1% SDS, the filter was exposed to an image plate (Fuji Co Ltd.) and analyzed with a FUJIX BAS 2000 system.

RT-PCR was performed as described above. Briefly, total RNA (10μg) from various mouse tissues and cell lines was reverse-transcribed with the Superscript II kit using an oligo dT primer. Then PCRs were done for pleckstrin 2 between a sense primer, 5′-AAGGCACGATGGTTCATCCT-3′ (corresponding to nt 83–102) and an antisense primer, 5′-GGATAGGTAGCCTTGTTTGA-3′ (corresponding to nt 765–784), and for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as an internal control between a sense primer, 5′-ACCACAGTCCATGCCATCAC-3′ (corresponding to nt 566–585) and an antisense primer, 5′-TCCACCACCCTGTT-GCTGTA-3′ (corresponding to nt 998–1017).

129/SV mouse genomic DNA was kindly provided by Dr. Vincent W. Keng (Nagoya University). For Southern blot analysis, 10 μg DNA was digested with *Eco*RI, *Bam*HI and *Pst*I, then electrophoresed on a 0.7% agarose gel and, finally, transferred onto a Hybond-N filter. Hybridization was performed in 6× SSC, 1% SDS, 10× Denhart's solution and 100 μg/ml salmom sperm DNA at 65°C. Washes were carried out in 0.1× SSC and 0.1% SDS at 65°C. Probes, including pleckstrin 2 (nt 19–1080 and nt 19–324), were labeled with [α-^{32}P]dCTP (111 TBq/mmol, Du Pont-New England Nuclear) using a random primed labelling system.

GFP expression plasmids. Synthesized oligonucleotides 5′-AGC-TTCGGAATTCG-3′ and 5′-TCGACGAATTCCGAAGCTT-3′ were annealed, phosphorylated, and ligated into the *Hind*III/*Sal*I sites of the pEGFP-C1 (Clontech) to produce the pEGFP-C1E1 vector. Pleckstrin 2 (coding for residues 1–353), the PH-N domain (residues 1–102 of pleckstrin 2), and the PH-C domain (residues 246–353 of pleck-

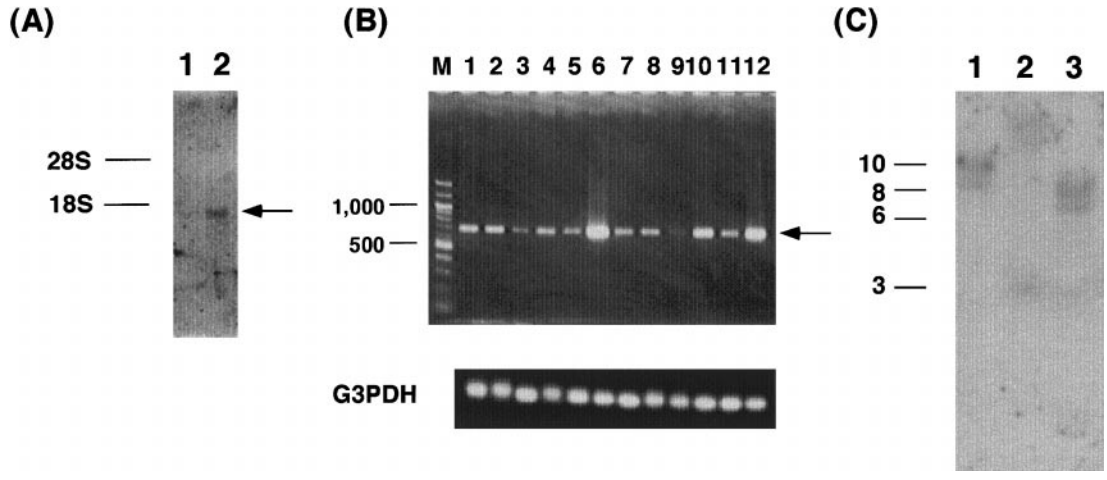

FIG. 2. (A) Northern blot analysis of pleckstrin 2 mRNA in mouse tissues. Total RNA (20 μg) from various mouse tissues were separated by electrophoresis on a 1.0% denaturing agarose gel. RNA was then transferred onto a nylon membrane and hybridized with ^{32}P-labelled pleckstrin 2 cDNA (nt 19–1080). Lane 1, kidney; lane 2, small intestine: *Arrow* indicates the position of pleckstrin 2 mRNA. (B) RT-PCR analysis of pleckstrin 2 mRNA in various mouse tissues and cell lines. *Upper* panel shows the PCR products of pleckstrin 2 (702 bp in size) which were separated by electrophoresis on a 2% agarose gel. *Arrow* indicates the position of pleckstrin 2. Marker sizes in bp are indicated to the *left*. *Lower* panel shows the PCR products of G3PDH (452 bp in size) as an internal control. M, molecular mass markers; lane 1, brain; lane 2, lung; lane 3, heart; lane 4, spleen; lane 5, liver; lane 6, small intestine; lane 7, kidney; lane 8, testis; lane 9, ovary; lane 10, F9 embryonal carcinoma cell; lane 11, NIH3T3 fibroblast; lane 12, RINm5F insulinoma cell. (C) Southern blot analysis. Mouse genomic DNA (10 μg) was digested with *Eco*RI, *Bam*HI, or *Pst*I, then electrophoresed, transfered, and hybridized with ^{32}P-labelled pleckstrin 2 cDNA (nt 19–324). Lane 1, *Eco*RI; lane 2, *Bam*HI; lane 3, *Pst*I: Marker sizes in kb are indicated to the *left*.

strin 2) were prepared by PCR using *Eco*RI sequence tagged primers mentioned below, respectively.

Pleckstrin 2:
sense primer, 5′-TAAGAATTCATGGAGGACGGCGTG-3′;
antisense primer, 5′-GGCGAATTCTCATGTTAGCTTCTT-3′

PH-N domain:
sense primer, 5′-TAAGAATTCATGGAGGACGGCGTG-3′;
antisense primer, 5′-GCGGAATTCTCAAGCCCCTGTTATCTC-3′

PH-C domain:
sense primer, 5′-GGCGAATTCGGCACAGTGGTCAAA-3′;
antisense primer, 5′-GGCGAATTCTCATGTTAGCTTCTT-3′

Each *Eco*RI fragment was inserted into the *Eco*RI site of the pEGFP-C1E1 to obtain GFP-Pleckstrin 2, GFP-PH-N, and GFP-PH-C, respectively.

Cell culture and DNA transfection of cells for laser microscopy. COS 7 cells were grown in Dulbecco's modified Eagles' medium containing 10% fetal calf serum. Cells were plated onto a 35 mm glass bottom microwell dish (MatTek corporation) the day before transfection. Transfection was performed using Lipofectamine PLUS (Life Tecnologies, Inc). 1 μg plasmid DNA was used. Twenty four h after transfection, cells were washed twice with a modified Krebs-Ringer buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 7 mM $MgSO_4$, 10 mM glucose and 10 mM Hepes–NaOH, PH 7.4) and observed by laser microscopy (Olympus).

RESULTS AND DISCUSSION

When chicken pleckstrin cDNA (15) was cloned, it was noticed that pleckstrin mRNA gave several bands on a northen blot under low stringent hybridization conditions. Based on this, we assumed that pleckstrin may constitute a family of proteins. We then searched dbEST with the nucleotide sequences of human and chicken pleckstrin as queries. As a result of the search, we obtained three useful EST sequences (AA008011, AA403397, and AA823548). Based on this information, we amplified the cDNA upstream or downstream of these clones by means of 5′- and 3′-RACE. Both the 5′-oriented cDNA extending to the 5′-noncoding region and the 3′-oriented cDNA extending to the 3′-noncoding region were obtained. In addition, we performed PCR cloning between the AA008011 and AA823548 EST sequences and obtained clones which include the entire coding region, including an initiation codon through the termination codon. Finally we isolated and analyzed three independent clones. All of these clones showed identical nucleotide sequences. The sequences are shown in Fig. 1A. The cDNA is 1588 bp in length and involves an open reading frame encoding a protein of 353 amino acids with a calculated molecular weight of 40,023 daltons. The 5′-noncoding nucleotide sequence around the first initiation codon was favorablly matched with Kozak's consensus sequence (19). The cDNA also contained a putative polyadenylation signal (AATAAA) which is followed by a poly A sequence. The deduced amino acid sequence showed a moderate similarity to those of human and chicken pleckstrin. The identity scores of the overall sequence were 37% to human and 40% to chicken pleckstrin, respectively (Fig. 1B). The predicted protein contains two PH domains which are located between L6 and A102, and between G246 and L352, respectively. Hereafter, each domain is designated as the N-

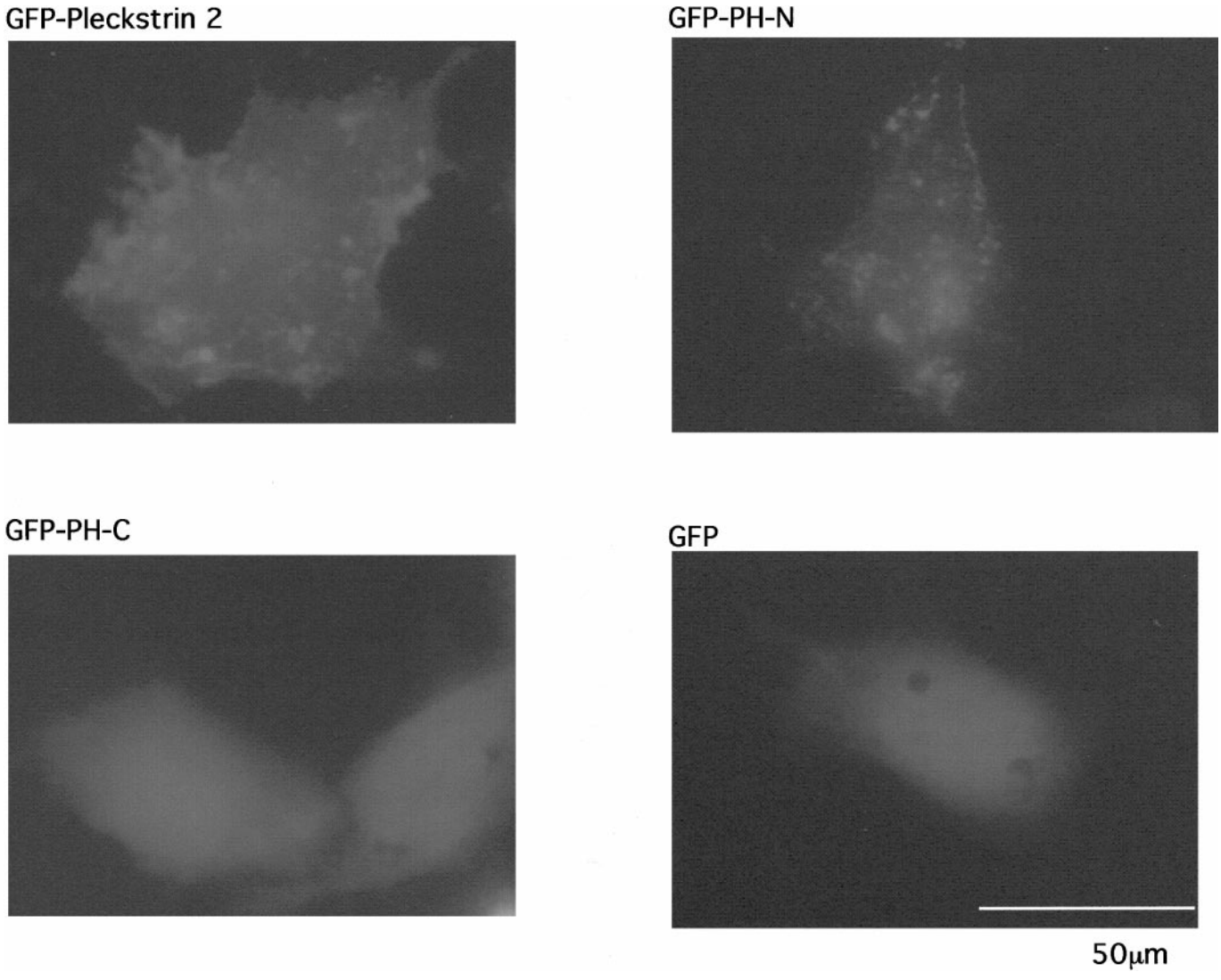

FIG. 3. Intracellular localization of the GFP-Pleckstrin 2 fusion protein and its mutants in COS 7 cells. Cells were transiently expressed GFP-Pleckstrin 2, GFP-PH-N, GFP-PH-C, and GFP. 1 μg plasmids DNA was transfected into COS 7 cells using the Lipofectamine PLUS. Twenty four h after transfection, the cells were analyzed by laser microscopy. Bar, 50 μm.

and the C-terminal PH domain. The amino acid sequences of the N- and C-terminal PH domains showed 35% and 57% identity with the corresponding domain of chicken pleckstrin, respectively. It has been reported that a new homologous and conserved domain, which is located between the two PH domains in pleckstrin exists in the case of three different proteins, dishevelled, egl-10 and pleckstrin (20). This domain is referred to as the DEP domain based on the initial letters of these 3 proteins. In the novel protein obtained in this study, this DEP domain was also present (from T138 to E 225; Fig. 1B) and showed a 34% identity with that of chicken pleckstrin. This domain may play an important role as a determinant of the activity of the protein. Indeed, in *Drosophila,* a mutation in the DEP domain in dishevelled has been recently reported to impair both membrane localization and the function of dishevelled in planar cell polarity signaling (21, 22). It would be very interesting to clarify the role of this domain in the signal transduction pathway of this protein. Therefore, we designated this novel pleckstrin family as pleckstrin 2, since this protein has two PH domains and belongs to a second member of the pleckstrin family.

In the case of pleckstrin, three potential phosphorylation sites for protein kinase C (e.g. S113, T114 and S117) were conserved between human and chicken (2, 15, 23, 24). Indeed, phosphorylation of pleckstrin on these sites could lead to the inhibition of agonist-induced phosphoinositide hydrolysis and the inhibition of the activity of phosphoinositide 3-kinase γ (25). Unlike pleckstrin, however, pleckstrin 2 has only one possible phosphorylation site (S120) for protein kinase C. This residue corresponds to S117 on human pleckstrin.

In our preliminary experiment, however, FLAG-tagged pleckstrin 2 did not mobilize slowly on Western blot analysis even after phorbol 12-myristate 13-acetate stimulation, as was observed for pleckstrin (26), suggesting that this protein may be phosphorylated weakly or not at all by protein kinase C (data not shown).

We next examined the expression of pleckstrin 2 mRNA in various mouse tissues by Northern blot analysis. We observed that pleckstrin 2 mRNA was approximately 1.7 kb in size and expressed in small intestine and kidney (Fig. 2A). However in other tissues, signals were very weak and not easily detectable. We then employed RT-PCR in various tissues and cell lines. As shown in Fig. 2B, pleckstrin 2 mRNA is expressed ubiquitously. Since pleckstrin was expressed exclusively in hemopoietic cells and spleen (2, 14, 15), the expression pattern of pleckstrin 2 was greatly different from that of pleckstrin. We next investigated the organization of the mouse pleckstrin 2 gene by Southern blot analysis. As shown in Fig. 2C, two digested fragments which were between about 3 to over 10 kb in size were observed in each lane. These results suggest that the gene may be a single copy and consist of two or more exons, or that a pseudogene may be exist.

Pleckstrin 2 contains two PH domains. It is now recognized that PH domains associate with phosphoinositide, GTP-binding protein, and protein kinase C (7–13). However, these results were obtained from *in vitro* binding assays. We therefore attempted to obtain a clue relative to the natural ligand(s) of the PH domains in living cells using a GFP fusion protein system. Four expression plasmids were used. These plasmids include the GFP gene alone or, the pleckstrin 2 genes encoding the full-length, N-terminal, and C-terminal PH domains, respectively, fused to the C-terminal of GFP. These constructs are designated as GFP, GFP-pleckstrin 2, GFP-PH-N, and GFP-PH-C, respectively. These plasmids were transiently transfected into COS 7 cells. We initially confirmed that the predicted fusion proteins were precisely produced by Western blot analysis using an anti GFP antibody (data not shown). We then observed the four GFP-fusion proteins by laser microscopy (Fig. 3). The control GFP was expressed in cytosol and nucleus. In contrast, GFP-pleckstrin 2 was observed diffusely in the cytosol and the plasma membrane. GFP-pleckstrin 2-expressed cells were flatter and broader than contol GFP-expressed ones. These observation were quite similar to that of cells which express pleckstrin (27), suggesting that cytoskeletal reorganization had occured. Moreover, GFP-PH-N had a prominent feature which was distinct from the others, in that it was localized diffusely and showed a dot-like pattern but partly localized in the plasma membrane. In addition, PH-N-GFP, N-terminal PH domain of pleckstrin 2 fused to N-terminus to GFP, showed a similar localization pattern (data not shown). The amino acid sequence at the N-terminal PH domain showed 35% identity and 54% similarity with chicken and human pleckstrin. In particular, a cluster of three lysine residues (K15, K16, and K24 in chicken) which have been shown to be critical in the association of the protein with phosphatidylinositol 4, 5-bisphosphate (PIP_2) (7, 8) was conserved in pleckstrin 2 (K13, R14 and K22 in pleckstrin 2). Although the N-terminal PH domain may be able to associate with plasma membrane through membrane anchoring PIP_2, the precise distribution is currently under investigation. On the other hand, GFP-PH-C was localized in cytosol. This suggests that each of the N- and C-terminal PH domains may be able to associate with distinct cellular factor(s). Since the amino acid sequence of pleckstrin 2, between K250 and R272 of the C-terminal PH domain is highly homologous to those of chicken and human pleckstrin, common factor(s) may interact with C-terminal PH domain of both pleckstrin 2 and pleckstrin. Isakoff *et al.* proposed that this region of the PH-C domain in pleckstrin may bind phosphatidyl inositol 3, 4-bisphosphate and phosphatidyl inositol 3, 4, 5-trisphosphate, which are generated by phosphatidyl inositol-3 kinase (PI-3K) activation initiated by growth factors (28). The PH domain of Bruton's tyrosine kinase (Btk) is capable of binding phosphatidyl inositol 3, 4-bisphosphate and phosphatidyl inositol 3, 4, 5-trisphosphate, and thereby GFP-Btk-PH-domain is translocated to the plasma membrane from the cytosol in parallel with PI-3K activation induced by growth factor (29). To further examine this prediction, we are currently investigating the issue of whether the pleckstrin 2 PH-C domain is capable of binding phosphatidyl inositol 3, 4-bisphosphate and phosphatidyl inositol 3, 4, 5-trisphosphate, and is translocated to the plasma membrane from the cytosol after growth factor stimulation.

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