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Pharbin, a Novel Inositol Polyphosphate 5-Phosphatase, Induces Dendritic Appearances in Fibroblasts

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We have cloned a cDNA encoding a novel protein pharbin with a homology to inositol polyphosphate 5-phosphatases. Pharbin contains relatively well-conserved catalytic motifs for 5-phosphatase, a proline-rich sequence corresponding to the SH3-binding motif, and a sequence consistent with the CaaX motif at the C-terminus. COS-7 cells transfected with pharbin exhibited elevated hydrolytic activity on the 5-phosphate group of inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, and phosphatidylinositol 4,5-bisphosphate. Thus, pharbin indeed serves as an inositol polyphosphate 5-phosphatase. When pharbin was transfected to C3H/10T1/2 fibroblasts, it was located to the plasma membrane-associated structures including membrane ruffles. The cells were converted to dendritic forms within 24 h. The protein with deleted or point-mutated CaaX motif hardly induced the dendritic forms but remained associated with the membranes. These results imply that the CaaX motif is required for the morphological alteration but that some other structural element is likely to also be responsible for the membrane localization.

Inositol phospholipids and their metabolites inositol polyphosphates play essential roles in a variety of cellular functions (1–3). Inositol polyphosphate 5-phosphatases (IP5Pases) hydrolyze 5-position phosphate group of water-soluble inositol phosphates and corresponding phospholipids. They are categorized into three groups according to their substrate specificity (4, 5). Group 1 includes the type I IP5Pases that hydrolyze only water-soluble inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4) (6, 7). Considering the roles of these substrates in Ca^{2+} mobilization from Ca^{2+} stores, primary function of the enzymes is likely to terminate the Ca^{2+} mobilization signaling. Group 2 enzymes hydrolyze the above soluble inositol polyphosphates and their corresponding lipids phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) and PtdIns 3,4,5-P_3. These enzymes include type II IP5Pases (8–10), OCRL (11, 12), and synaptojanin (13–15). PtdIns 4,5-P_2 and PtdIns 3,4,5-P_3 are implicated in signaling in many aspects not only through their regulated hydrolysis to generate second messengers but also by interaction with various proteins (2, 3). Thus, the group 2 enzymes may regulate a variety of these functions. The type II enzymes as well as the type I phosphatases seem to be associated with membranes presumably through an isoprenyl group attached to the cysteine residue in the C-terminal CaaX motif (9, 10, 16). Group 3 includes SHIP and related enzymes, which hydrolyze exclusively the substrates with 3-position phosphate, PtdIns 3,4,5-P_3 and Ins 1,3,4,5-P_4 (17–20). The substrate specificity suggests that the enzymes participate in the regulation of Ca^{2+}-independent protein kinase C activity and Akt/PKB pathway.

We have cloned a cDNA encoding a protein with a homology to known IP5Pases and conserved 5-phosphatase motifs. This protein designated as pharbin also contains a putative SH3-binding motif and a putative CaaX motif. As pharbin hydrolyzed the 5-phosphate group of Ins 1,4,5-P_3, Ins 1,3,4,5-P_4, and PtdIns 4,5-P_2, it indeed acts as an IP5Pase. Remarkably, overexpression of this protein resulted in a dendritic cell morphology. The CaaX motif seems to be required for the morphological alteration.

MATERIALS AND METHODS

cDNA cloning and sequencing. The constitutively activated form of the small GTPase M-Ras^{G22V} was expressed as the glutathione *S*-transferase (GST)-fusion protein in *E. coli* (21). The rat brain cDNA expression library constructed in λZAPII (presented by Dr. K. Watanabe) was screened with the [α]^{32}P]GTP-labeled GST-M-Ras^{G22V}

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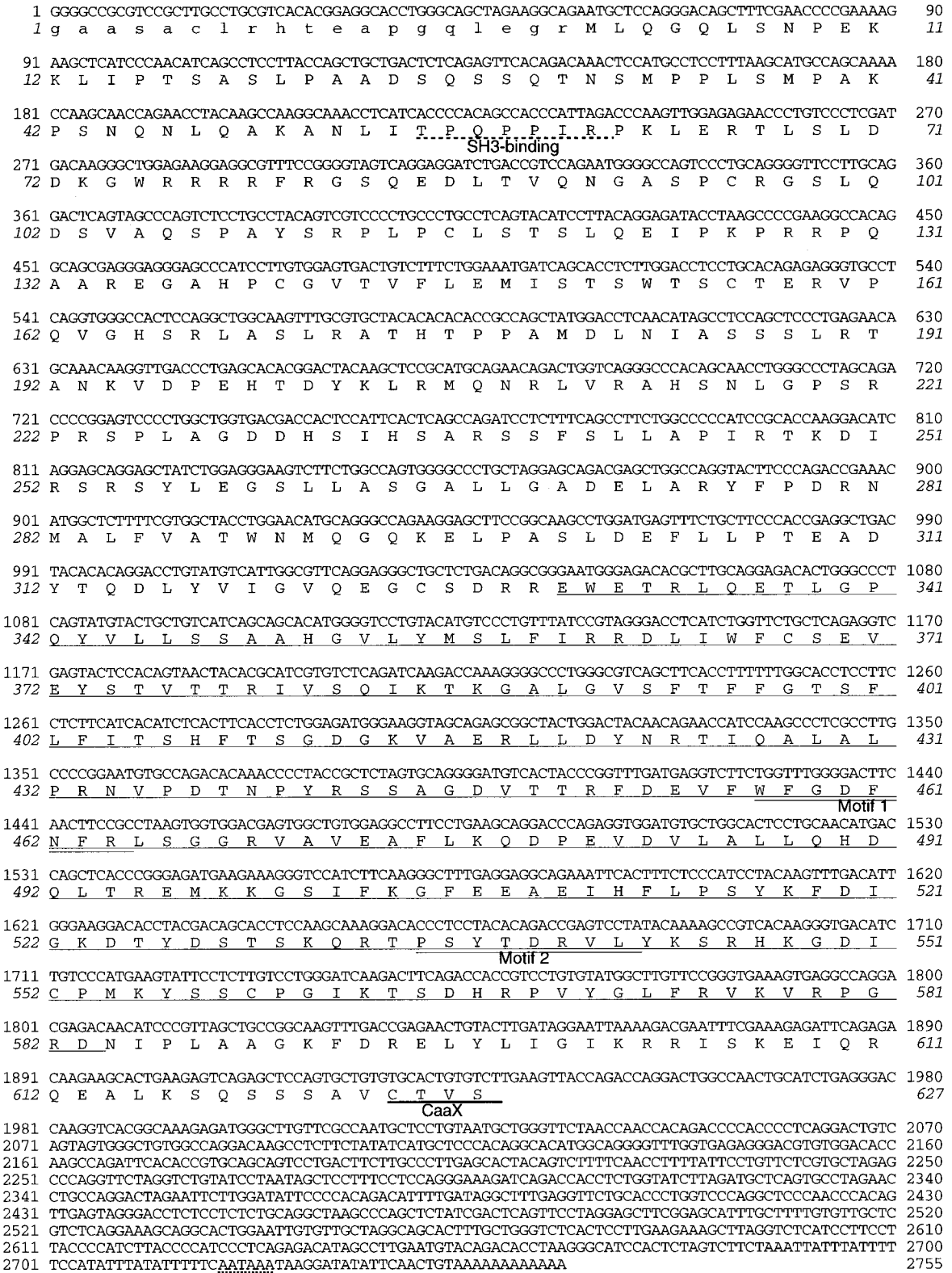

FIG. 1. Nucleotide sequence and predicted amino acid sequence of rat pharbin. The amino acid sequences downstream and upstream of the putative initiation codon ($A^{58}TG$) are inscribed with capital and lowercase letters, respectively. The amino acid representing the putative initiation codon is numbered as 1. Single underlines represent the region homologous to type II IP5Pase. Double underlines indicate the motifs 1 and 2 defining 5-phosphatase domain. A dashed and a thick single underlines denote the SH3-binding and the CaaX motifs, respectively. A dotted underline in the 3′ untranslated region indicates the poly(A) addition signal. The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB026288.

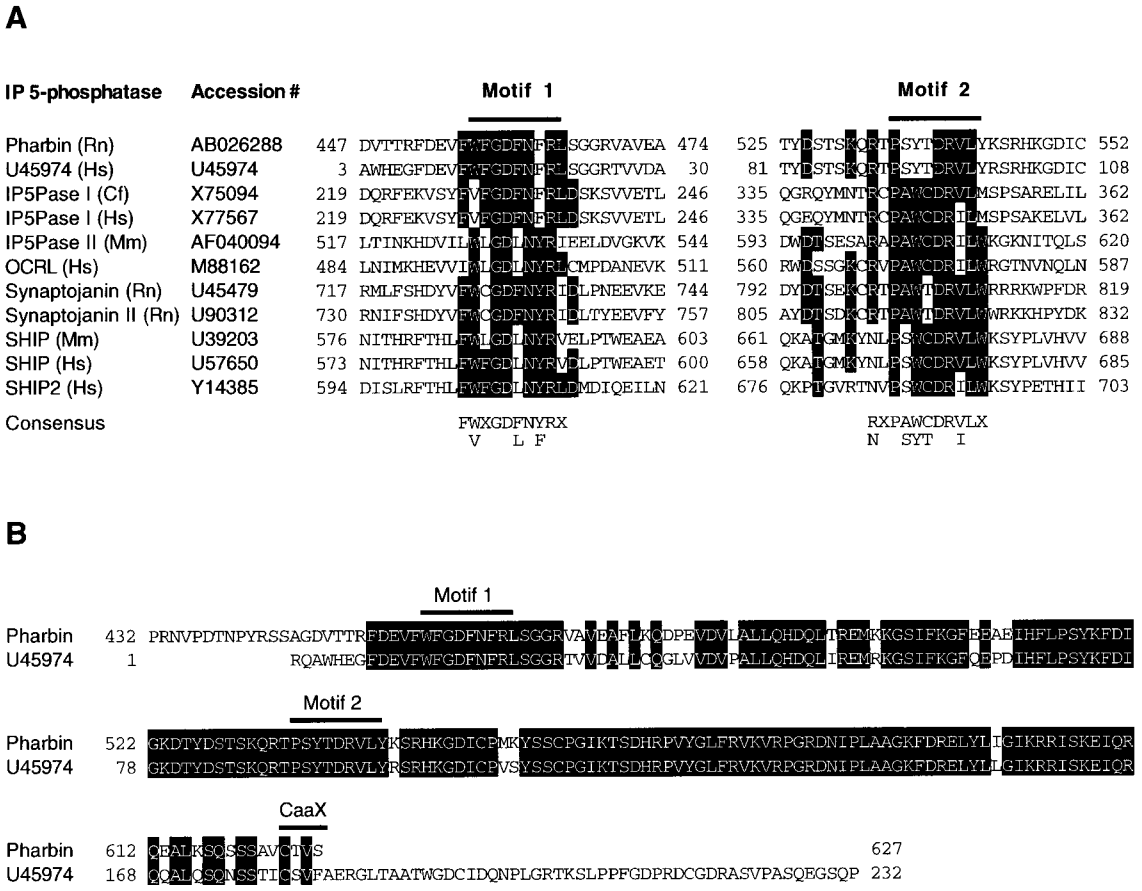

FIG. 2. Comparison of the amino acid sequence of pharbin with those of known IP5Pases. (A) Amino acid sequences around the motifs 1 and 2. Amino acids at positions of >50% identity are shown in white on black. Abbreviations of animal species are: Rn, rat; Hs, human; Cf, dog; Mm, mouse. (B) Amino acid sequences of pharbin and a putative IP5Pase U45974. Amino acids identical between the two proteins are shown in white on black. Note that the C-terminal CaaX motif is present in pharbin but not in U45974.

according to Manser *et al.* (22). Nucleotide sequence of cloned cDNAs was determined with LI-COR 4000 automated DNA sequencing system. The nucleotide and amino acid sequences were analyzed by GENETYX-Mac softwares (Ver. 10.1, Software Development Co.).

Phosphatase assay. The pharbin cDNA was subcloned in frame to the Myc-epitope-tag in *Sma*I site of pCMVmyc vector (21). COS-7 cells were transfected with the recombinant plasmid or the empty vector by electroporation with BioRad Gene Pulser II System. Two days after the transfection, the cells were harvested and lysed with the lysis buffer (150 mM NaCl, 40 mM Tris-HCl (pH 7.6), 2 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The cells were briefly sonicated and centrifuged at 10,000*g* for 20 min. The supernatant was applied for immunoprecipitation with the anti-Myc monoclonal antibody (mAb) Myc1-9E10 (23) and ImmunoPure Immobilized Protein A (Pierce). Assay for IP5Pase was carried out as described (24) by using [^{3}H]Ins 1,4,5-P_3 and [^{3}H]Ins 1,3,4,5-P_4. The inositol polyphosphates were fractionated by HPLC according to Zhang and Buxton (25), and the radioactivity of each fraction was quantitated by liquid scintillation counting. For the assay of [^{3}H]Ins 1,3,4-P_3 hydrolyzing activity, the 5-phosphate of [^{3}H]Ins 1,3,4,5-P_4 was hydrolyzed with recombinant SHIP protein and the resulting [^{3}H]Ins 1,3,4-P_3 was used as a substrate. Phosphatase activity to [^{3}H]PtdInd 4,5-P_2 was determined as described (26). Radiolabeled lipids were extracted, resolved by thin-layer chromatography (TLC), and detected by fluorography after treating the TLC plate with ENH^{3}ANCE Spray (NEN Life Science Products, Inc.).

Epitope tagging and transfection. To delete the CaaX motif, the 3′ *Sac*I fragment containing the DNA sequence corresponding to the C-terminal 9 amino acids was removed from the pharbin cDNA. A point mutation of the Cys residue in the CaaX motif to Ser was introduced in the cDNA by using Transformer site-directed mutagenesis kit (Clontech Laboratories, Inc.). The cDNAs of the deletion mutant (pharbinΔCaaX) and the point mutant (pharbin^{C624S}) were subcloned in frame to the Myc-epitope-tag in *Sma*I site of the pCMVmyc vector. C3H/10T1/2 cells cultured on glass coverslips were transfected with the recombinant plasmids by calcium phosphate-mediated method as described (27). The cells were doubly stained with the Myc1-9E10 to detect the Myc-epitope-tag and with rhodamine-phalloidin. They were examined with a Zeiss Axioskop microscope equipped with phase-contrast and epifluorescence optics (21, 27).

RESULTS AND DISCUSSION

Structural properties of pharbin. During an attempt to identify proteins interacting with M-Ras, a recently identified Ras family small GTPase (21), several cDNAs encoding novel proteins were cloned from the rat brain cDNA expression library. One of the cDNAs was 2.75 kb and its longest open reading frame coded for a novel 627-amino acid protein with a calcu-

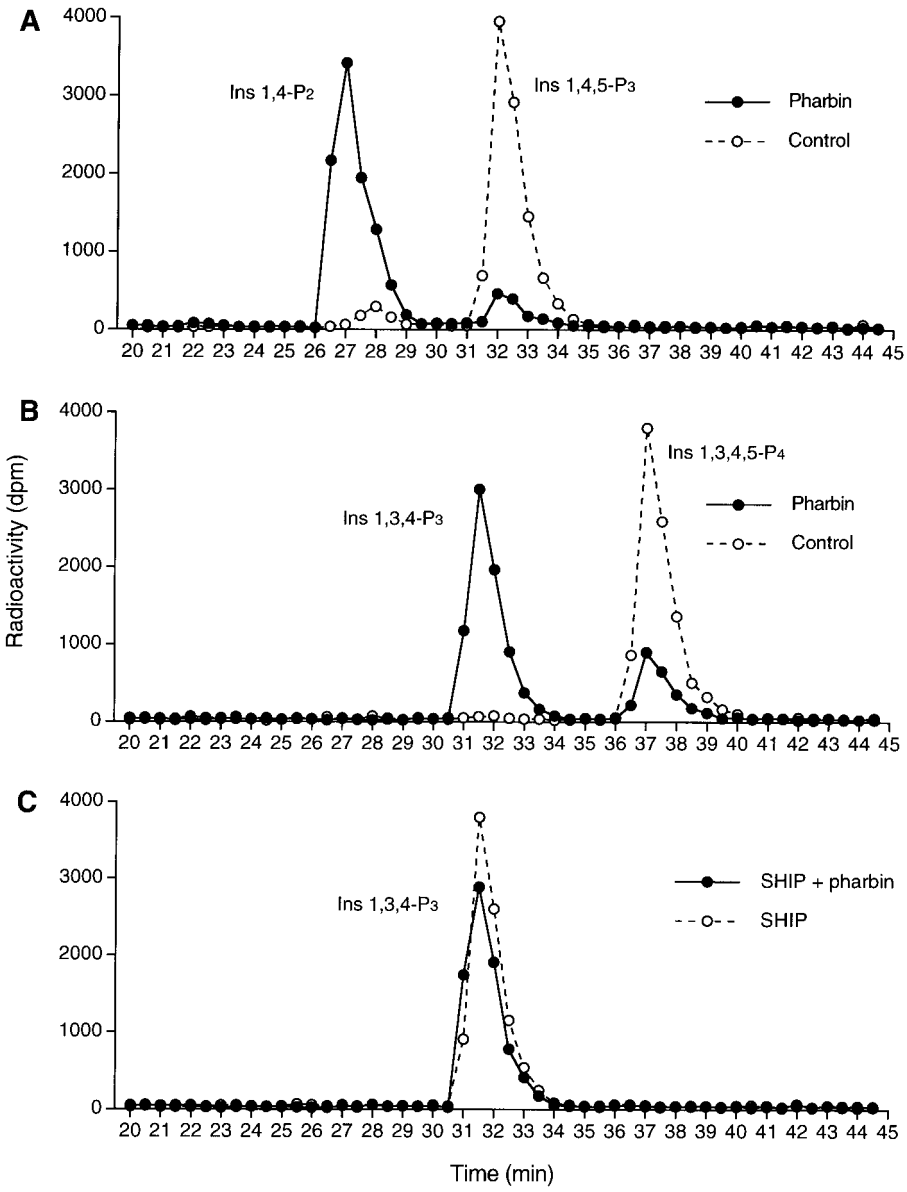

FIG. 3. IP5Pase activity of pharbin to soluble inositol polyphosphates. [^{3}H]Ins 1,4,5-P_3 or [^{3}H]Ins 1,3,4,5-P_4 was incubated with the immunoprecipitated Myc-tagged pharbin, Myc-tagged SHIP, or Myc-tag. The products were fractionated by HPLC and quantitated by liquid scintillation counting. (A) Phosphatase activity to Ins 1,4,5-P_3. [^{3}H]Ins 1,4,5-P_3 was incubated with immunoprecipitated Myc-tagged pharbin (solid circles and solid lines) or with immunoprecipitated Myc-tag (control, open circles and broken lines). The two peaks represented the positions of Ins 1,4-P_2 and Ins 1,4,5-P_3, respectively. (B) Phosphatase activity to Ins 1,3,4,5-P_4. [^{3}H]Ins 1,3,4,5-P_4 was incubated with immunoprecipitated Myc-tagged pharbin (solid circles and solid lines) or with immunoprecipitated Myc-tag (control, open circles and broken lines). The two peaks represented the positions of Ins 1,3,4-P_3 and Ins 1,3,4,5-P_4, respectively. (C) Phosphatase activity to Ins 1,3,4-P_3. [^{3}H]Ins 1,3,4,5-P_4 was incubated with immunoprecipitated Myc-tagged SHIP and pharbin (solid circles and solid lines) or with immunoprecipitated Myc-tagged SHIP (open circles and broken lines). The peak represented the position of Ins 1,3,4-P_3. Pharbin did not exhibit phosphatase activity to Ins 1,3,4-P_3.

lated molecular mass of 70,087 Da (Fig. 1) (DDBJ/EMBL/GenBank accession number AB026288). We further screened $\sim 1 \times 10^6$ plaques of the cDNA library with this cDNA to obtain longer cDNAs. Among the clones that we acquired, three clones were identical to the above one, and they contained the longest 5′ untranslated region. Accordingly, although no inframe termination codon was present upstream of the first A^{58}TG in these clones, this might represent the initiation codon. The amino acid sequence of the open reading frame exhibited 27–36% identity over 220–290 amino acids to the known IP5Pases except for type I IP5Pases. The conserved two motifs, WXGDXNXR and PXWCDRXL, defining 5-phosphatase domain (28)

were also detected in this homology region (Fig. 2A). Thus, we designated this protein as pharbin (a 5-*ph*osphatase that induces *arbo*rization, see below). The human EST clone U45974 contains a partial coding sequence for a putative IP5Pase lacking the N-terminal region. A part of its amino acid sequence was very similar (86.3% identical over 175 amino acids) to that of pharbin, and the two phosphatase domain motifs were identical between these two proteins (Fig. 2B). Only pharbin and U45974 possessed specific Tyr in the motif 2 instead of Trp among IP5Pases (Fig. 2A).

Pharbin contained upstream of the 5-phosphatase homology region a Pro-rich sequence corresponding to the Class II consensus of Src homology 3 (SH3)-binding motif (29, 30). The C-terminus of pharbin ended with -CTVS, which seems to correspond to CaaX motif present in type I and II IP5Pases. The CaaX motif (C, Cys; a, aliphatic amino acid; X, any amino acid) is a signal for three types of posttranslational modifications, i.e., isoprenylation, proteolytic processing, and methylation (31, 32). An isoprenoid (either farnesyl or geranylgeranyl group) added to the Cys residue is responsible for membrane association. The C-terminal residue of the CaaX motif, or the "X", in general determines which isoprenoid is added to the Cys residue. When "X" is Ser, Met, or Gln, the CaaX motif is recognized by farnesyltransferase, whereas Leu at this position results in modification by geranylgeranyltransferase-I (31, 32). Thus, pharbin is likely to be farnesylated as is type I IP5Pase, which ends with -CVVQ (16). On the other hand, type II IP5Pase with the C-terminus of -CNPL is postulated to be geranylgeranylated (9).

Although pharbin and U45974 exhibited high homology in the 5-phosphatase homology region, they were diverged upstream of the motif 1 (Fig. 2B). In addition, the C-terminus of U45974 was 49 amino acids longer than that of pharbin and did not contain CaaX motif. These facts suggest that U45974 is not a human ortholog of pharbin but that they are distinct proteins even if they share substrate specificity. The relationship between pharbin and U45974 appears to be similar to that between type II IP5Pase and OCRL because the latter two enzymes show high homology only in the 5-phosphatase homology region and the type II enzyme but not OCRL contains CaaX motif.

IP5Pase activity of pharbin. To address whether pharbin possessed IP5Pase activity, Myc-tagged pharbin was exogenously expressed in COS-7 cells by transfection. The Myc-tagged pharbin was immunoprecipitated with the anti-Myc mAb Myc1-9E10 and analyzed for phosphatase activity to Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 by fractionating through HPLC (Figs. 3A and 3B). Pharbin hydrolyzed 5-position phosphates of both the inositol polyphosphates, whereas immunoprecipitate of the transfectant with the empty vector did not exhibit the phosphatase activity. Ins 1,3,4-P_3, which was produced by hydrolyzing Ins 1,3,4,5-P_4 with the group 3 5-phosphatase SHIP, was not hydrolyzed by pharbin (Fig. 3C). These results indicate that pharbin indeed exhibits IP5Pase activity and acts exclusively on a 5-position phosphate group.

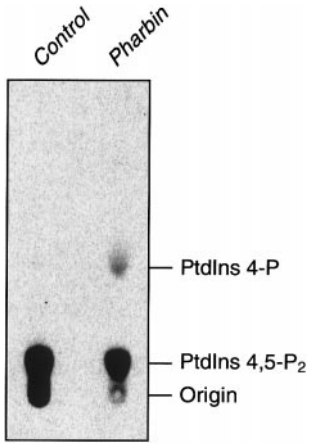

FIG. 4. IP5Pase activity of pharbin to PtdIns 4,5-P_2. [^{3}H]PtdIns 4,5-P_2 was incubated with immunoprecipitated Myc-tagged pharbin or Myc-tag (control). The products were resolved by TLC and detected by fluorography.

Next, 5-phosphatase activity of the immunoprecipitated pharbin to the lipid PtdIns 4,5-P_2 was determined by TLC. Pharbin but not a mock transfectant hydrolyzed PtdIns 4,5-P_2 to generate PtdIns 4-P (Fig. 4). Thus, pharbin acts as a 5-phosphatase on not only the water-soluble but also the lipid substrates, and consequently may belong to the group 2. Considering the diversity in the two phosphatase domain motifs between pharbin and the known group 2 enzymes, however, catalytic efficiency to the four substrates might be varying between pharbin and the known group 2 enzymes. In fact, relative catalytic efficiency to the four substrates is distinct even among the known group 2 enzymes (12). It is of interest to determine whether U45974, which has two phosphatase domain motifs identical to those of pharbin, exhibits substrate specificity identical or very similar to that of pharbin.

Localization and a cellular effect of pharbin. Since pharbin contains a putative CaaX motif at the C-terminus, we examined whether pharbin was located to cellular membranes. The recombinant plasmid expressing N-terminal Myc-epitope-tagged pharbin was transfected to 10T1/2 fibroblasts. In addition to a diffused cytoplasmic distribution, pharbin was characteristically concentrated on the plasma membrane-associated structures including membrane ruffles by 8 h after the transfection (Fig. 5a). Actin was colocalized with pharbin on these membrane-associated structures as determined by rhodamine-phalloidin staining (Fig. 5b). The cells tended to be elongated and

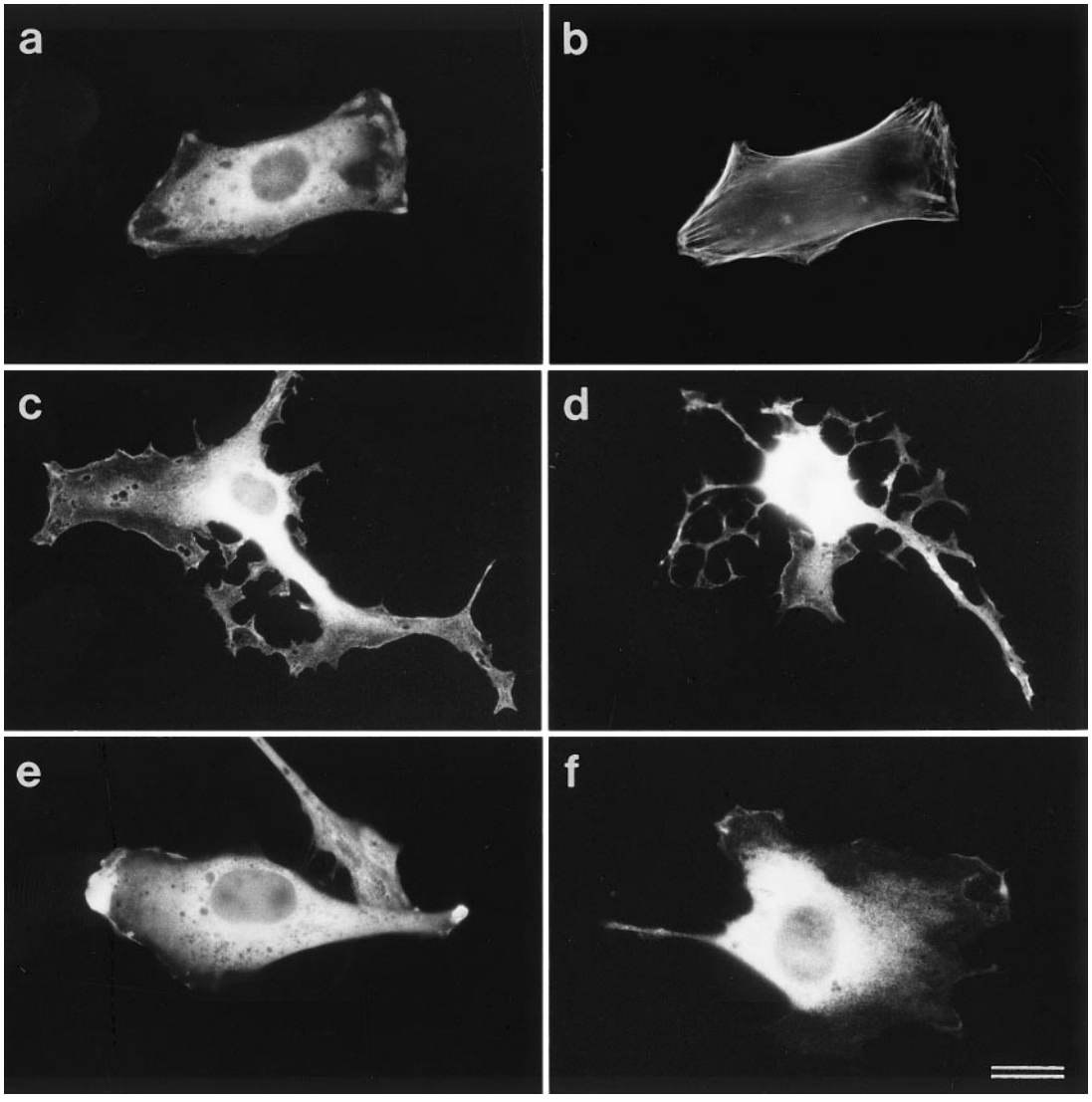

FIG. 5. Localization of pharbin and induction of dendritic forms by pharbin. The recombinant plasmids expressing Myc-epitope-tagged pharbin, pharbinΔCaaX, and pharbin^{C624S} were transfected to 10T1/2 fibroblasts. Cells expressing the exogenous pharbin were detected by immunostaining with the mAb Myc1-9E10. (a and b) Cells expressing pharbin, 8 h after the transfection. The cells were doubly stained with Myc1-9E10 (a) and rhodamine–phalloidin (b). (c) Cells expressing pharbin, 12 h after the transfection. (d) Cells expressing pharbin, 24 h after the transfection. (e) Cells expressing pharbinΔCaaX, 24 h after the transfection. (f) Cells expressing pharbin^{C624S}, 24 h after the transfection. Bar, 20 μm.

retracted (Fig. 5c) and showed arborized or dendritic figures by 24 h after the transfection (Fig. 5d). The arborization became more prominent with the lapse of time, and the whole cell bodies turned to long and thin branched protrusions. Pharbin remained associated with the plasma membrane in the arborized cells (Figs. 5c and 5d). Pharbin diffusely distributed in the cytoplasm and that located to the membrane-associated structures may be responsible for the hydrolysis of the soluble inositol polyphosphates and that of the membrane-associated lipid substrates, respectively. This postulation is corroborated by the finding that elimination of the membrane-targeting motifs of type II IP5Pase results in the shift of the activity from membranes to the cytosol (10).

To determine whether the CaaX motif was responsible for the membrane localization and the morphological alteration, pharbin with the deletion mutation (pharbinΔCaaX) and that with the point mutation (pharbin^{C624S}) in the CaaX motif were expressed. Both the mutant proteins remained associated with the plasma membrane but scarcely affected the cell shape (Figs. 5e and 5f). Expression of the wild-type and the mutated pharbin tagged with green fluorescent protein (GFP) at the N-terminus showed the localization and the cell shape indistinguishable from those induced by

the Myc-tagged protein (data not shown). These results seem to imply that a mechanism other than isoprenylation on the CaaX motif is also responsible for the membrane localization but that the CaaX motif is essential for the alteration in the cell shape.

Different from pharbin, type I IP5Pase with mutations in the CaaX motif loses its membrane-association potential (16). This implies that the CaaX motif is the sole membrane-targeting motif for the type I enzyme. On the other hand, membrane association of the 115-kDa type II IP5Pase is mediated not only by the CaaX motif but also by the N-terminal region (10). The sequence rich in Leu or the sequence homologous to those in several membrane receptor proteins is postulated to be responsible for the membrane-association ability of the N-terminal region (10). However, pharbin does not contain such sequences. One possible explanation for the membrane localization of the pharbin mutated in the CaaX motif is that pharbin binds to some membrane-associated protein. In this context, the putative class II SH3-binding motif in pharbin should be noted because the SH3-containing protein Grb2/Ash is located to the membrane ruffles (33) as is pharbin. Since the mutated pharbin hardly induced the dendritic form, the CaaX motif is likely to be really functional. Both the CaaX and the SH3-binding motifs in pharbin might play important roles in the localization and several functions including IP5Pase activity to particular substrates.

Overexpression of the group 2 IP5Pase synaptojanin causes disassembly of the actin stress fibers presumably by hydrolyzing PtdIns 4,5-P_2 bound to α-actinin resulting in retardation of the actin gelation ability of α-actinin (14). Since pharbin was colocalized with actin on the membrane-associated structures, it may also hydrolyze PtdIns 4,5-P_2 bound to certain actin-binding regulatory proteins such as α-actinin, vinculin, and profilin (34–38). Modulation of these actin regulatory proteins by hydrolyzing bound PtdIns 4,5-P_2 might cause the arborization of the cells. Pharbin with mutations in the CaaX motif scarcely induced the dendritic form. This might imply that the CaaX motif but not the other membrane-targeting motif can make pharbin access PtdIns 4,5-P_2 bound to the actin-binding proteins. Although pharbin was cloned during the ligand overlay screening of the cDNA expression library with the constitutively activated M-Ras, direct interaction between M-Ras and pharbin has not yet been demonstrated. However, the dendritic cell shape induced by overexpressed pharbin is reminiscent of that induced by the constitutively activated M-Ras (21). It remains to be determined whether the morphological alteration is induced by pharbin targeted directly or indirectly by M-Ras or whether it is induced independently by M-Ras and pharbin.

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