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Phosphatidylinositol 4,5-Bisphosphate Phosphatase Regulates the Rearrangement of Actin Filaments

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Phosphatidylinositol 4,5-bisphosphate (PIP_2) reorganizes actin filaments by modulating the functions of a variety of actin-regulatory proteins. Until now, it was thought that bound PIP_2 is hydrolyzed only by tyrosine-phosphorylated phospholipase Cγ (PLCγ) after the activation of tyrosine kinases. Here, we show a new mechanism for the hydrolysis of bound PIP_2 and the regulation of actin filaments by PIP_2 phosphatase (synaptojanin). We isolated a 150-kDa protein (p150) from brains that binds the SH3 domains of Ash/Grb2. The sequence of this protein was found to be homologous to that of synaptojanin. The expression of p150 in COS 7 cells produces a decrease in the number of actin stress fibers in the center of the cells and causes the cells to become multinuclear. On the other hand, the expression of a PIP_2 phosphatase-negative mutant does not disrupt actin stress fibers or produce the multinuclear phenotype. We have also shown that p150 forms the complexes with Ash/Grb2 and epidermal growth factor (EGF) receptors only when the cells are treated with EGF and that it reorganizes actin filaments in an EGF-dependent manner. Moreover, the PIP_2 phosphatase activity of native p150 purified from bovine brains is not inhibited by profilin, cofilin, or α-actinin, although PLCδ1 activity is markedly inhibited by these proteins. Furthermore, p150 suppresses actin gelation, which is induced by smooth muscle α-actinin. All these data suggest that p150 (synaptojanin) hydrolyzes PIP_2 bound to actin regulatory proteins, resulting in the rearrangement of actin filaments downstream of tyrosine kinase and Ash/Grb2.

Phosphatidylinositol 4,5-bisphosphate (PIP_2) plays important roles in generating two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, by phospholipase C (PLC) activation in response to a variety of physiological stimuli. IP_3 mobilizes Ca^{2+} from the endoplasmic reticulum, and diacylglycerol activates protein kinase C (2, 32).

In addition to its roles as a signal-generating lipid, PIP_2 modulates the functions of various proteins such as protein kinase C (5, 15), μ-calpain (36), ADP-ribosylation factor (34), and PLD (20). PIP_2 binds to actin-regulating proteins such as profilin, cofilin, gelsolin, and gCap and suppresses the functions of these proteins (16, 18, 38, 39). On the other hand, PIP_2 also binds to α-actinin (9, 10), which is an actin-cross-linking protein, and further activates the actin-gelating activity of α-actinin. Therefore, it seems likely that PIP_2 regulates actin polymerization and depolymerization through its action on actin-regulatory proteins. It has also been shown that the decrease in the amount of PIP_2 bound to α-actinin and vinculin produced by treatment with PDGF correlates with the actin depolymerization (10). In the case of profilin, since PIP_2 binding inhibits the formation of profilin-actin complexes, it is presumed that PIP_2 sequesters profilin from actin in resting cells and that profilin is released from PIP_2 upon growth factor stimulation through hydrolysis by the attack of activated PLC (37). Furthermore, Hartwig et al. (14) used permeabilized platelets to demonstrate a signaling pathway for actin assembly involving Rac in which the final message is phosphoinositide-mediated F-actin uncapping. All these data suggest that an increase in the amount of PIP_2 bound to actin-regulatory proteins stimulates the development of stress fibers while a decrease leads to actin depolymerization. Therefore, it is very important to know how PIP_2 bound to actin regulatory proteins is hydrolyzed in response to stimuli. It has been demonstrated that PIP_2 bound to actin-regulatory proteins is hydrolyzed in vivo in response to stimuli (10). Considering the metabolizing pathways of PIP_2, there are three possible routes, the conversion to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by phosphatidylinositol (PI) 3-kinase, hydrolysis to IP_3 and diacylglycerol by PLC, or hydrolysis to phosphatidylinositol 4-phosphate (PIP) by PIP_2 phosphatase. Until now, it was thought that the hydrolysis of PIP_2 bound to actin-regulatory proteins in response to receptor tyrosine kinase activation is caused mainly by tyrosine-phosphorylated PLCγ1 (12), because PIP_2 bound to actin-regulatory proteins is not hydrolyzed by PLCs while PLCγ1 phosphorylated by tyrosine kinase overcomes the inhibitory effect of profilin and hydrolyzes bound PIP_2.

Inositol polyphosphate 5-phosphatases are classified into three groups by their substrate specificity (22). Group 1 enzymes hydrolyze inositol phosphates such as IP_3 and inositol 1,3,4,5-tetrakisphosphate but not lipid substrates. Group 2 enzymes hydrolyze not only inositol phosphates but also lipids such as PIP_2 and PIP_3. This group includes the inositol 5-phosphatase mutated in Lowe's oculocerebrorenal syndrome (1), which is caused by a mutation in the X chromosome-encoded OCRL gene. OCRL prefers lipid substrates rather than inositol phosphates and functions as a PIP_2 5-phosphatase (40). Group 3 enzymes hydrolyze only the 3-phosphate-containing substrates such as PIP_3 and inositol 1,3,4,5-tetrakisphosphate (22). On the other hand, several PIP_2 phosphatases have been purified and characterized from human platelets and bovine brains (22, 25, 33). However, the involvement of PIP_2 phosphatase in the regulation of actin filaments has not been studied.

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The Src homology 2 and 3 (SH2 and SH3) domains have been demonstrated to play crucial roles in the tyrosine kinase signalling system. One protein, Ash/Grb2, composed of one SH2 and two SH3 domains (21, 23), has been found to link the signal between receptor tyrosine kinase and Sos (Ras GDP/GTP exchange protein), resulting in Ras activation. This is probably due to an interaction between the SH3 of Ash/Grb2 and a proline-rich sequence in Sos (3, 4, 7, 19, 35). A recent study has shown that the Ash SH3 binds to a variety of proline-rich proteins including dynamin, Cbl, Abl, and N-WASP (8, 13, 26, 27–29). In addition, it has been found that injecting an antibody against Ash/Grb2 into cells abolishes the reorganization of actin stress fibers, suggesting that some signals lead to cytoskeleton reorganization (24).

We tried to identify proteins bound to the Ash SH3 domains from bovine brains to clarify the downstream signals of Ash to the cytoskeleton. Four major proteins, of 180, 150, 100, and 65 kDa, were found to bind specifically to glutathione-*S*-transferase (GST)–Ash/Grb2 fusion proteins. Of these, the 180-, 100-, and 65-kDa proteins were shown to be Sos, dynamin, and N-WASP, respectively (29, 30).

Here, we purified the 150-kDa protein and isolated its cDNA from bovine brain cDNA libraries. The protein encodes PIP_2 phosphatase (synaptojanin), which diminishes actin stress fibers in the central area. Epidermal growth factor (EGF) stimulation causes the localization of actin filaments in peripheral membranes in overexpressed COS 7 cells. In addition, we demonstrate that p150 hydrolyzes PIP_2 bound to such actin-regulatory proteins as profilin, cofilin, and α-actinin. The PIP_2-dependent gelating activity of α-actinin decreases upon treatment by p150. These data show that PIP_2 phosphatase participates in the rearrangement of actin filaments that occurs in growth factor-stimulated cells.

MATERIALS AND METHODS

Purification of p150 and the cloning of its cDNA. GST-Ash/Grb2 fusion proteins expressed in *Escherichia coli* were used for the affinity purification of Ash/Grb2 binding proteins from the bovine brain cytosol fraction as described previously (28). The bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The proteins were stained with Ponceau S, and the 150-kDa region was cut out and treated with lysylendopeptidase. The resulting peptides were separated on a reverse-phase C_{18} column with a 0 to 60% gradient of acetonitrile in 0.1% trifluoroacetic acid, and several peptides were sequenced with an Applied Biosystems peptide sequencer. Two peptide sequences, N-DGARSVSRTIQNNFFD-C and N-ASEHAADIQMVNFDYYHQMV-C, were obtained. Two synthetic oligonucleotides, 5-ACIATICARAAWAAWTTTWT TWGA-3 and 5-AAWTTWGAWTAWCAWCARATGG-3 (R indicates A or G, W indicates C or T), corresponding to each peptide, were then synthesized and used as probes for screening the λgt10 bovine brain library. Positive plaques were isolated and sequenced by the conventional dideoxy-termination method. One clone was found to contain a full-length open reading frame.

Antibodies. An anti-p150 antibody was prepared by injecting the proteins purified by GST-Ash/Grb2 affinity chromatography and SDS-PAGE into rabbits (30). The antibodies were purified from the sera with strips of polyvinylidene difluoride membrane containing the 150-kDa protein. The anti-Ash/Grb2 antibody was prepared as described previously (28). The anti-actin antibody was a gift from T. Endo (Chiba University School of Science). The anti-EGF receptor antibody and anti-phosphotyrosine antibody PY20 were purchased from Transduction Laboratories, and Upstate Biotechnology, Inc., respectively.

Metabolic labelling of PC12 cells. For the preparation of [^{35}S]methionine-labelled PC12 cell lysates, the culture medium of semiconfluent cells was replaced with methionine-free medium and the cells were incubated with 100 μCi of [^{35}S]methionine per 150-mm-diameter dish for 3 h. After treatment, the cells were harvested and subjected to in vitro binding assays with several GST fusion constructs of p150.

In vitro binding assays with GST fusion proteins. Several GST fusion proteins expressed in *E. coli* were immobilized on glutathione-Sepharose beads and mixed with crude protein samples (cell lysates or brain homogenates). After being washed, the beads were suspended in SDS sample buffer and subjected to Western blot analysis.

Immunoprecipitation. Protein samples including bovine brain cytosol fractions and COS 7 cell lysates were precleared with protein A-agarose beads. The supernatants were mixed with anti-p150 antibody or preimmune rabbit serum (negative control) for 2 h. Protein A-agarose beads were then added, and the mixtures were incubated for an additional 2 h. After being washed, the immunoprecipitates were analyzed by Western blot analysis.

GST fusion proteins. GST-Ash/Grb2 was constructed as described previously (28). Several GST fusion proteins of p150 were prepared as follows. The DNA fragments encoding the regions shown in Fig. 1 (see below) were amplified by PCR with synthetic oligonucleotide primers and then inserted into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia LKB Biotechnology Inc.). The recombinant plasmids were transformed into *E. coli* JM-109 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to express GST fusion proteins. The bacteria were collected by centrifugation and resuspended in *E. coli* lysis buffer (40 mM Tris-HCl [pH 7.2], 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM diisopropylfluorophosphate [DIFP], 1% Triton X-100). Vigorous sonication was performed prior to centrifugation at 100,000 × *g* for 30 min. The resulting supernatants were saved as crude extracts containing GST fusion proteins. The proteins were purified with glutathione-Sepharose beads.

Transient expression of p150 and its mutants in COS 7 cells. Full-length p150 and a PIP_2 phosphatase activity-negative mutant (ΔPhos) construct, in which amino acids (aa) 725 to 734 (FWCGDFNYRI) and aa 801 to 809 (PAWT DRVLW) were deleted, were prepared as follows. We divided the DNAs encoding the expressed regions into three portions (those encoding aa 1 to 724, 735 to 800, and 810 to 1211). All three fragments were amplified by PCR with primers designed for the ΔPhos construct. First, each amplified fragment was ligated into the *Eco*RI, *Eco*RI-*Bam*HI, and *Bam*HI sites of pBS (Bluescript KS) and verified by restriction enzyme digestion and DNA sequencing. Then they were ligated into a pcDL-SRαII mammalian expression vector. The recombinant plasmids for transfection were prepared from *E. coli* by the conventional alkali lysis method and polyethylene glycol precipitation. A 20-μg portion of each plasmid was mixed with 10^7 cells, and the mixtures were subjected to electroporation (Gene Pulser; Bio-Rad). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 72 h, the cells were analyzed by Western blot analysis and immunofluorescence microscopy. For the analysis of EGF signaling, the cells electroporated with full-length constructs were serum starved for 24 h prior to stimulation with 100 ng of human recombinant EGF (Gibco) per ml for 10 min.

Immunofluorescence microscopy. Cells cultured on coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline PBS. After incubation with the anti-p150 antibody followed by anti-rabbit antibody linked to fluorescein isothiocyanate (FITC) and rhodamine-conjugated phalloidin or DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), the cells were observed by fluorescence microscopy (Axioskop; Zeiss).

Purification of p150/bovine synaptojanin. Bovine brains were homogenized in 20 mM Tris-HCl (pH 7.6) containing 10% glycerol (buffer A), 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 100 μM DIFP, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml and centrifuged at 100,000 × *g* for 1 h. The resultant supernatants were fractionated with saturated ammonium sulfate (45%). The precipitated fractions were applied to a HiLoad Q-Sepharose high-performance column and eluted with a linear gradient of 0 to 1 M NaCl after dialysis with buffer A. p150 was detected with anti-p150 antibody. The p150 eluted at around 0.3 M NaCl. Fractions were collected, applied to Blue Sepharose after twofold dilution in 20 mM HEPES–NaOH (pH 6.8) containing 10% glycerol (buffer B), and eluted with a gradient of 1 to 2 M NaCl in buffer B. Anti-p150 antibody-reactive proteins eluted at 1.25 to 1.8 M NaCl. Next, the samples were applied to a HiLoad Superdex 200 column equilibrated with buffer A containing 150 mM NaCl. Fractions containing p150 (fractions 68 to 75) were applied to heparin-Sepharose after twofold dilution in buffer B and eluted with a gradient of 0 to 1 M NaCl in buffer B. p150 eluted at around 0.3 M NaCl. To remove contaminating proteins, the samples were reapplied to the HiLoad Sephadex 200 and heparin-Sepharose columns. Purified p150 which eluted at 0.3 M NaCl from the heparin-Sepharose column was stored at −80°C. After the final step, the sample appeared as a single band in SDS-PAGE.

Assay of PIP_2 phosphatase activity. PIP_2 phosphatase activity was measured as previously described by Matzaris et al. (25). Briefly, the reaction was carried out in a volume of 50 μl of Tris-HCl (pH 7.2)–150 mM NaCl–200 μg of bovine serum albumin per ml–3 mM $MgCl_2$–2 mM cetyltriethylammonium bromide–20 μM [^{3}H]PIP_2 for 10 min at 37°C.

Purification of actin-regulatory proteins. Profilin was purified from bovine spleen (17), and α-actinin was purified from chicken gizzard (9); cofilin was prepared from recombinant DNA (donated by T. Obinata, Chiba University School of Science) and purified by the method described by Nagaoka et al. (31).

Gelating activity of α-actinin. Various concentrations of PIP_2 and smooth muscle α-actinin (60 μl of 0.25 mg/ml) were mixed and incubated at 25°C for 1 h in buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EGTA, 10 mM β-mercaptoethanol, and 0.1 mM PMSF. Then 60 μl of 0.75-mg/ml rabbit skeletal G-actin and 1 μg of p150 in buffer containing 5 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 0.1 mM ATP and 0.2 mM dithiothreitol were added to the solution, followed by 100 mM KCl and 2 mM $MgCl_2$. Falling-ball viscometry was performed at 25°C (9).

PLC activity. PLCδ1 was partially purified from bovine thymus, and PLC activity was measured as previously described (11). Briefly, reaction mixtures containing various concentrations of actin-regulatory proteins and PLC in 20 mM morpho-

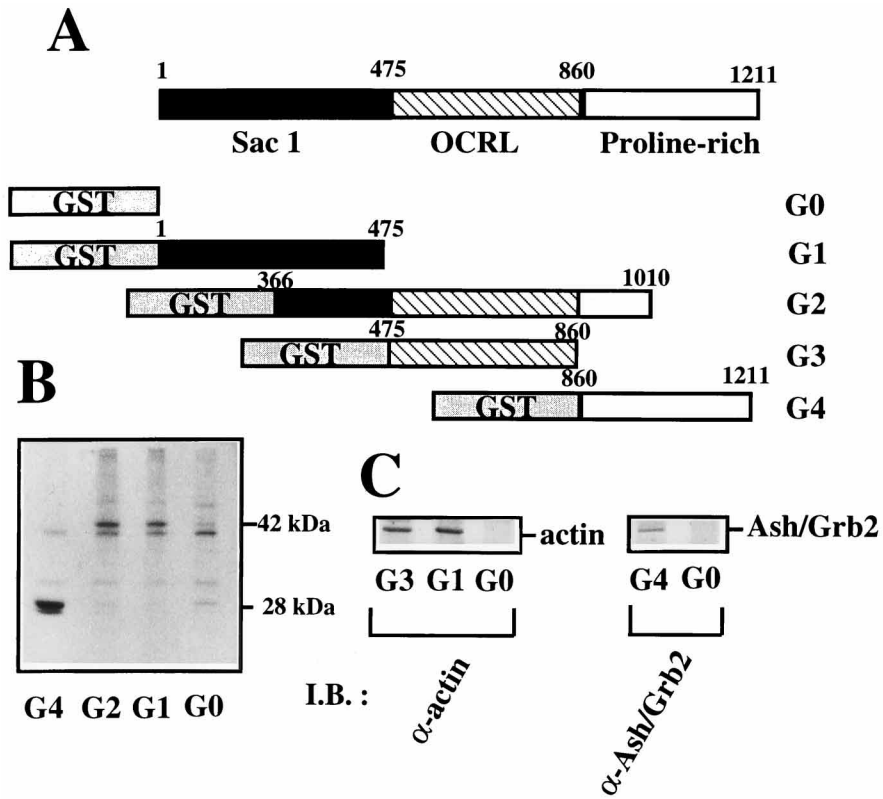


FIG. 1. Detection of p150-associated proteins. (A) Deletion constructs of p150 GST fusion proteins. (B) Detection of binding proteins to GST fusion proteins. Several GST fusion proteins expressed in *E. coli* were immobilized on glutathione-Sepharose beads and mixed with ^{35}S-labelled PC12 cell lysates. After being washed, the beads were suspended in SDS sample buffer and subjected to autoradiography. (C) Binding of actin and Ash/Grb2. Proteins bound to the GST fusion proteins were analyzed by Western blotting with anti-actin and anti-Ash/Grb2 antibodies. I.B., immunoblotting.

lineethanesulfonic acid (MES; pH 6.0)–400 μM $CaCl_2$–20 μM [^{3}H]PIP_2 were incubated for 10 min at 37°C, and the reactions were stopped by the addition of chloroform-methanol (2:1). The radioactivity of the formed IP_3 was measured.

RESULTS

Cloning of Ash/Grb2-associated p150. We purified the 150-kDa protein as a binding protein to GST-Ash/Grb2 fusion proteins and digested it with lysylendopeptidase. The resulting peptides were separated on a reverse-phase C_{18} column, and the partial amino acid sequences of two peptides (DGARSVSR TIQNNFFD and ASEHAADIQMVNFDYYHQMV) were determined. At that time, the proteins containing these sequences were not registered among the GenBank databases. Therefore, we isolated a cDNA clone encoding p150. Degenerate oligonucleotides based on two of the peptide sequences were used to screen a bovine λgt10 cDNA library, and several positive clones were obtained. The isolated DNA fragments were used as probes to isolate a full-length clone (clone 3). The amino acid sequence of p150 determined from clone 3 (accession no. D85682 in the DDBJ/EMBL/GenBank database) is homologous to that of synaptojanin (30), recently isolated as inositol 5-phosphatase, suggesting that p150 is the bovine homolog of rat synaptojanin. GST-p150 fusion proteins expressed in *E. coli* were recognized by an antibody to p150 prepared with purified bovine brain p150 (30), showing that this clone encodes an Ash/Grb2 binding p150.

Association with other proteins. To investigate p150 signalling, we prepared deletion constructs of p150 as GST fusion proteins and expressed them in *E. coli* (Fig. 1A). Since synaptojanin consists of three major domains, i.e., the N-terminal Sac 1 homologous domain, the OCRL homologous domain, and the C-terminal proline-rich region, various GST fusion proteins containing each domain were made and mixed with [^{35}S]labelled PC12 cell lysates. As shown in Fig. 1B, several proteins were found to bind specifically to these recombinant

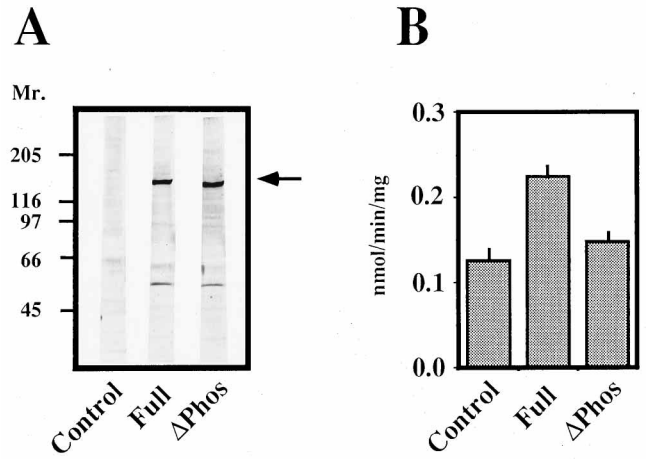


FIG. 2. PIP_2 phosphatase activity in full-length p150- and PIP_2 phosphatase activity-negative mutant (ΔPhos)-expressing COS 7 cells. Full-length p150 and ΔPhos were transfected into COS 7 cells. (A) Amounts of p150 and ΔPhos expressed in COS 7 cells. Cell lysates of full-length p150- and ΔPhos-expressing COS 7 cells were analyzed by Western blotting with anti-p150 antibody. (B) PIP_2 phosphatase activity in full-length p150- and ΔPhos-expressing COS 7 cells. PIP_2 phosphatase activity was measured with cell lysates from full-length p150- and ΔPhos-expressing COS 7 cells.

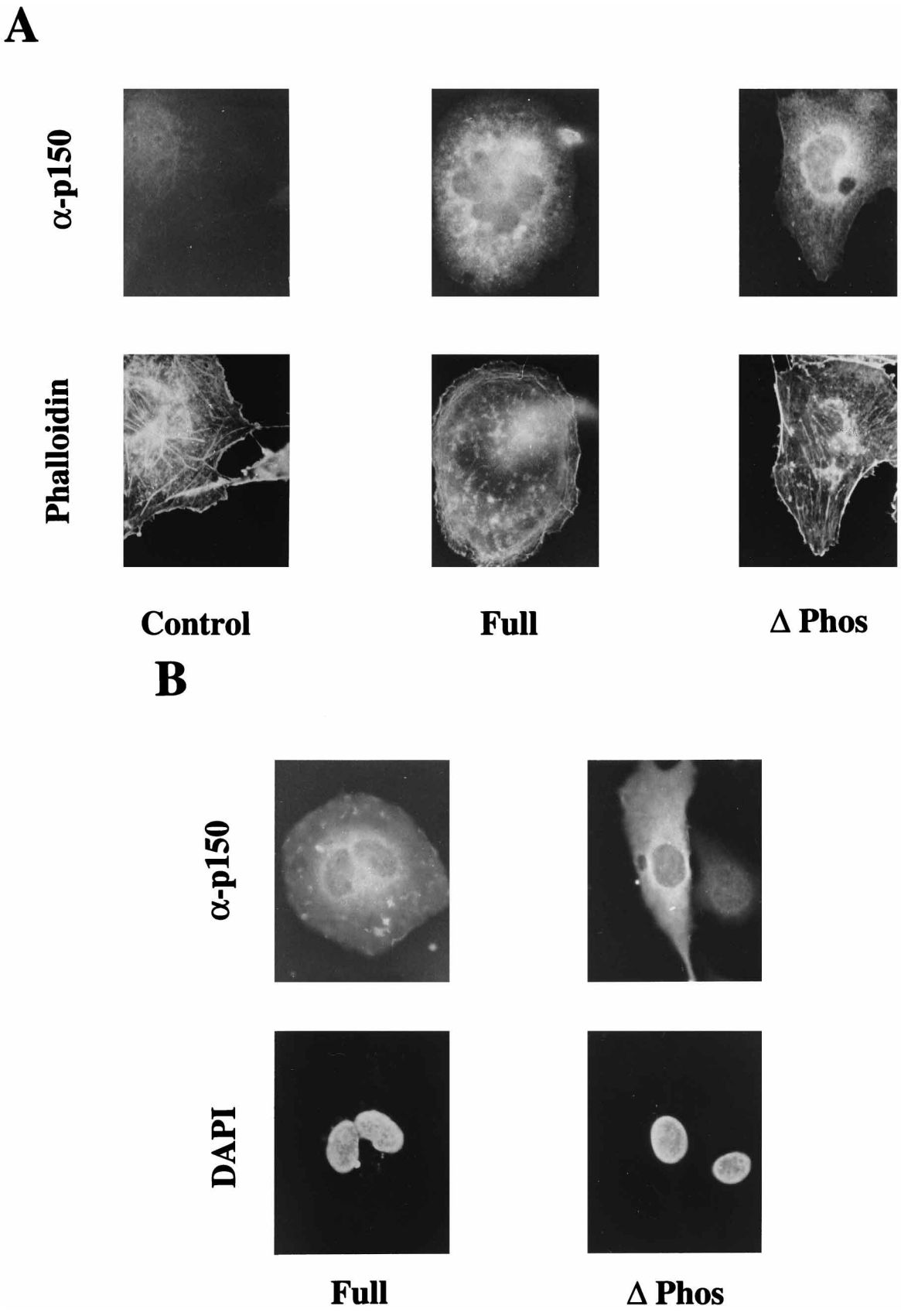


FIG. 3. Expression of p150 and ΔPhos in COS 7 cells. (A) Full-length p150 and ΔPhos constructs were expressed in COS 7 cells and immunostained with anti-p150 antibody–FITC and rhodamine-phalloidin. (B) The cells were stained with DAPI and anti-p150 antibody.

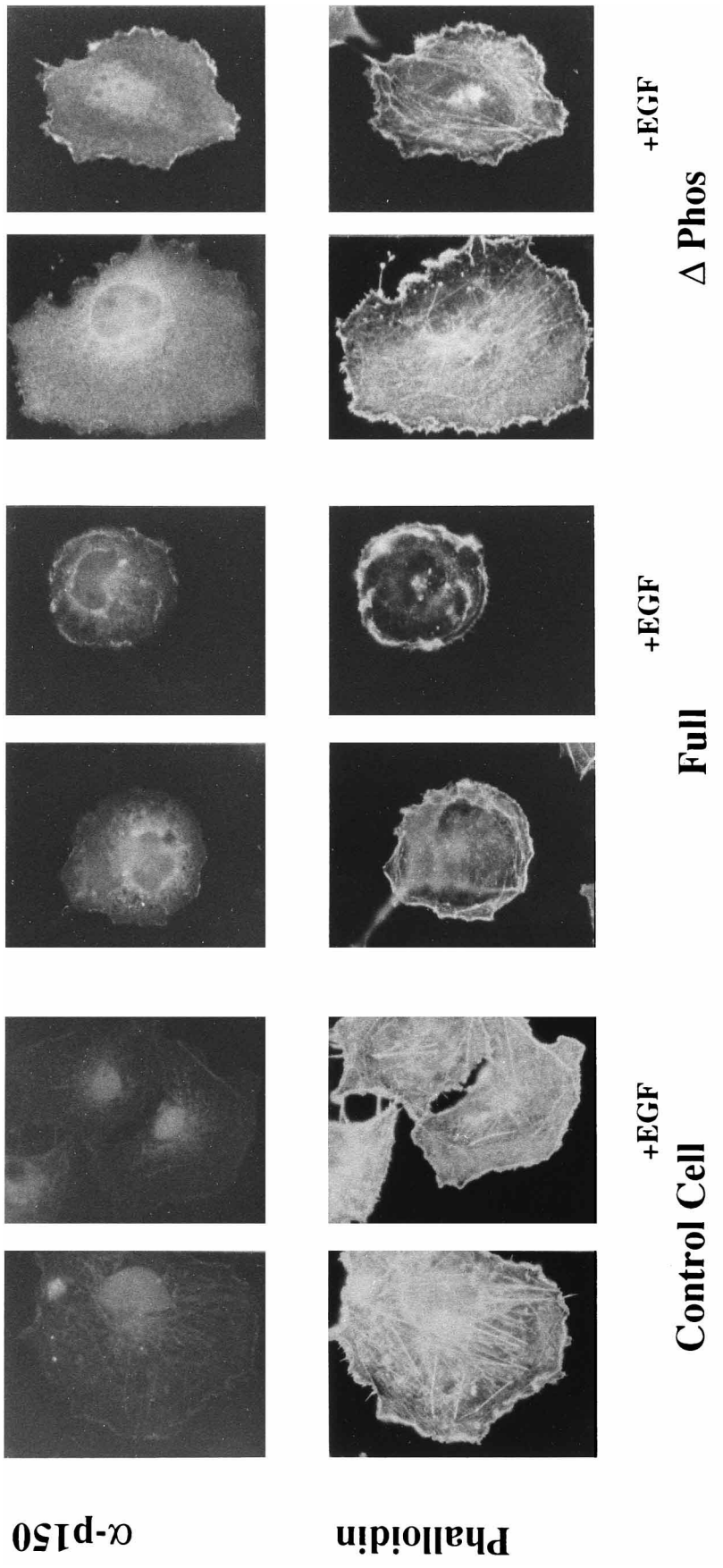


FIG. 4. Full-length p150- and ΔPhos-transfected COS 7 cells were stimulated with EGF and doubly stained with anti-p150 antibody–FITC and rhodamine-phalloidin.

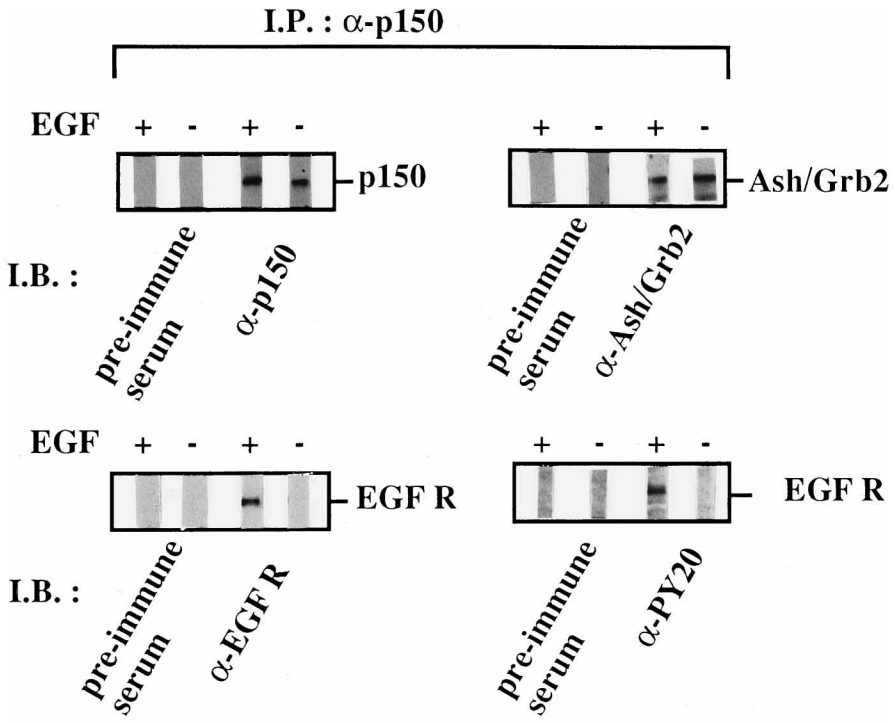


FIG. 5. Association of p150 with the EGF receptor. Lysates of full-length p150-expressing COS 7 cells were immunoprecipitated (I.P.) with anti-p150 antibody (α-p150). The precipitates were immunoblotted (I.B.) with anti-p150, anti-Ash/Grb2, anti-EGF receptor (α-EGF R), and anti-phosphotyrosine antibodies.

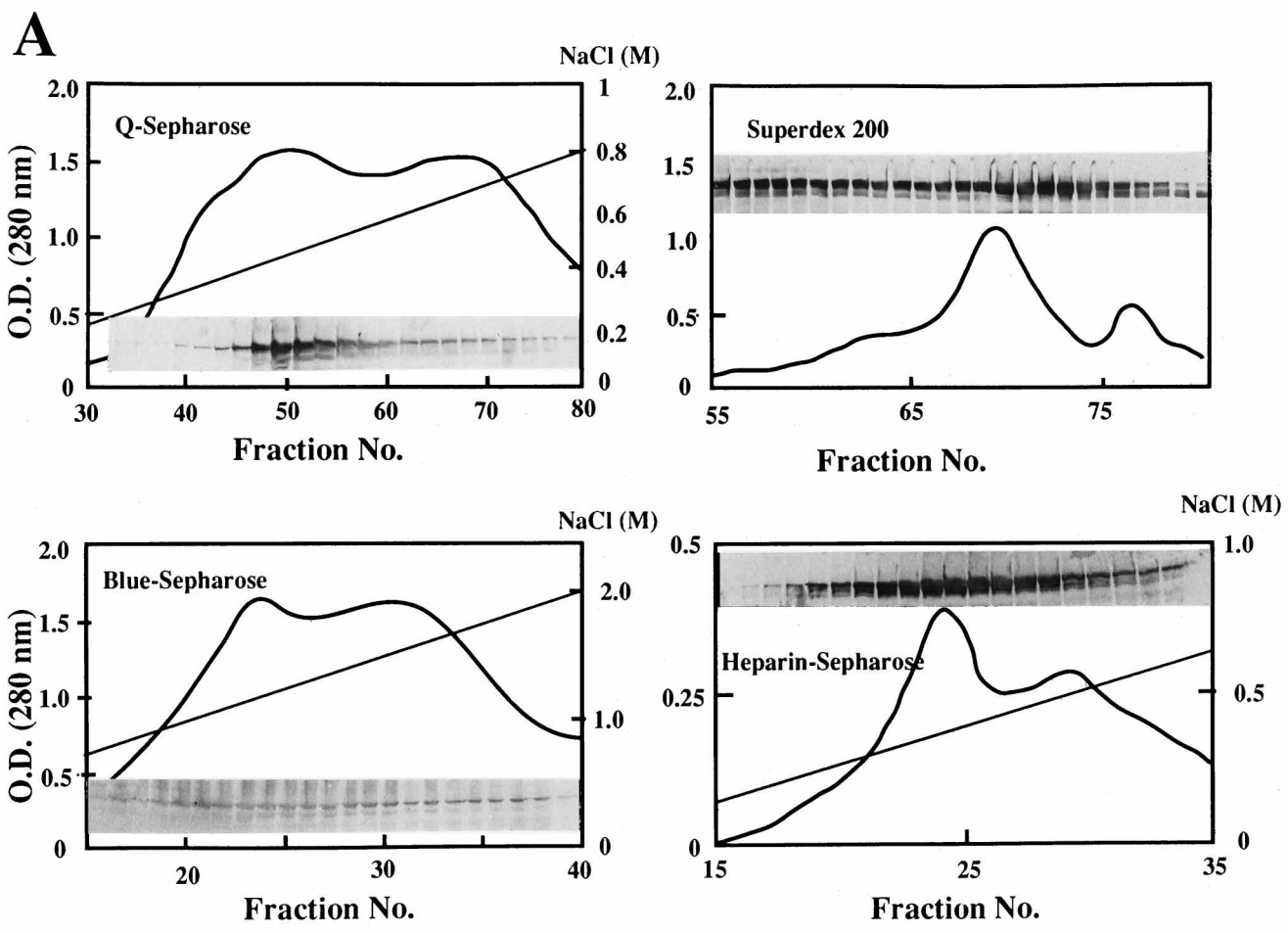
proteins. G1, G2, and G3 proteins bound to a 42-kDa protein. G4 (proline-rich region) protein associated with a 24-kDa protein. Since Sac 1 is thought to participate in cytoskeletal rearrangement (6), it seemed likely that the 42-kDa protein is actin. We determined that the p42 and p24 proteins were actin and Ash/Grb2, respectively, by immunoblotting (Fig. 1C). However, G3, which does not include the Sac 1 region, also bound to actin, suggesting that G3 still contains an actin binding site in addition to the catalytic region of phosphatase activity.

p150 is a PIP_2 phosphatase and regulates actin filaments. Since p150 contains an OCRL homologous region, it is expected to have PIP_2 phosphatase activity. Therefore, full-length p150 and ΔPhos, in which amino acids (FWCGDFNYRI and PAWTDRVLW, corresponding to aa 725 to 734 and 801 to 809, respectively) in the highly conserved catalytic domain of PIP_2 phosphatase are deleted, were transfected into COS 7 cells. Figure 2A shows the amounts of p150 and ΔPhos expressed in COS 7 cells, as determined by Western blotting, to be the same. On the other hand, we found little p150 in COS 7 cells transfected with the control vector alone. By using total-cell lysates from these transfected COS 7 cells, PIP_2 phosphatase activity was measured. Although the PIP_2 phosphatase activity was low in control vector- and ΔPhos-transfected COS 7 cell lysates, a significant increase was detected in p150-transfected cell lysates (Fig. 2B).

Next, the cells were immunostained with anti-p150 antibody. When the full-length construct was transfected into cells under serum-containing conditions, about 15% of the cells were stained strongly by the anti-p150 antibody (Fig. 3A). The perinuclear areas were especially strongly stained, and other areas, except the nucleus, also showed positive signals. It appears that p150 is present in structural components as short string or dot shapes (about 80% of p150-expressing cells). It is also noteworthy that cells expressing p150 become multinuclear (Fig. 3B), suggesting that nuclear division occurs but cell division is inhibited. Although the surrounding cells contain endogenous p150, its level is too low for the detection of immunostaining signals. The effect of p150 on actin filaments was determined by double staining with rhodamine-phalloidin. Normal COS 7 cells develop actin stress fibers that cross the entire cell. However, actin stress fibers disappear in the central area of p150-expressing cells, while cortical actin filaments in the membrane area and circular actin rings remain. Thus, p150 expression results in the loss of central actin stress fibers and the appearance of string or dot-like actin instead. When the phosphatase-negative mutant (ΔPhos) was transfected, ΔPhos also distributed predominantly in the perinuclear areas. However, the cells, in contrast to full-length p150-expressing cells, had only one nucleus, showing that the PIP_2 phosphatase activity of p150 plays an important role in p150-induced inhibition of cell division. In these cells, actin stress fibers similar to those in normal COS 7 cells were observed, suggesting that PIP_2 phosphatase disrupts actin filaments.

To examine the effect of EGF, full-length p150-expressing cells were stimulated for 10 min with EGF after 24 h of starvation. Upon EGF stimulation, p150 was seen to move to the peripheral membrane area, with actin filaments also transferring to the same area and forming strange membrane ruffles (Fig. 4). These phenomena were observed in more than 80% of the p150-expressing cells. On the other hand, when ΔPhos-expressing cells were treated with EGF, ΔPhos also moved to the plasma membranes with some actin filaments colocalizing in the plasma membranes, but actin stress fibers in the center of the cells were still observed.

p150 associates with the EGF receptor through Ash/Grb2. To clarify whether p150 can associate with the EGF receptor



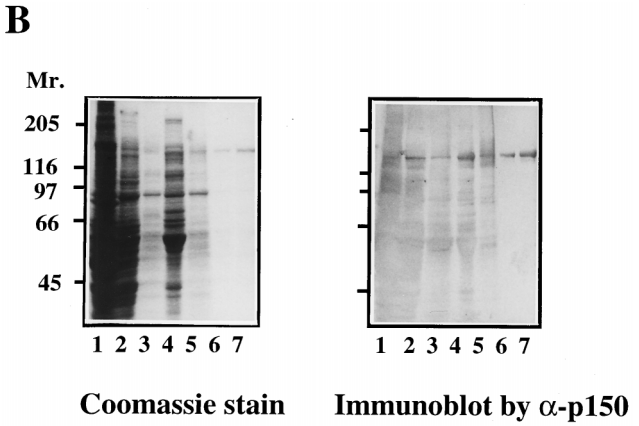


FIG. 6. Purification of native p150 from bovine brains. Native p150 was purified from bovine brains by sequential chromatography to a single band in SDS-PAGE. (A) Elution patterns from HiLoad Q-Sepharose, Blue-Sepharose, first HiLoad Superdex 200, and first heparin-Sepharose columns. The Western blotting results for each fraction by anti-p150 antibody are also shown. (B) After each purification step, the sample was subjected to SDS-PAGE and stained with Coomassie brilliant blue. A portion of each sample was analyzed by Western blotting with anti-p150 antibody. Lanes: 1, ammonium sulfate precipitation; 2, HiLoad Q-Sepharose; 3, Blue-Sepharose; 4, HiLoad Superdex 200; 5, heparin-Sepharose; 6, HiLoad Superdex 200; 7, heparin-Sepharose. The purified p150 showed a specific PIP$_2$ phosphatase activity of 8.82 μmol/min/mg of protein. O.D., optical density.

through Ash/Grb2, immunoprecipitation experiments were carried out with lysates of p150-transfected COS 7 cells. As shown in Fig. 5, Ash/Grb2 and p150 coimmunoprecipitated with anti-p150 antibodies irrespective of EGF stimulation. However, they did not form complexes with the EGF receptor without EGF treatment. On the other hand, when COS 7 cells were stimulated with EGF, the EGF receptor coimmunoprecipitated with p150 and Ash/Grb2, suggesting that the complexes of p150 and Ash/Grb2 are recruited to the EGF receptor by EGF treatment.

p150 hydrolyzes PIP$_2$ bound to actin-regulatory proteins. It is known that PIP$_2$ bound to actin-regulatory proteins is not hydrolyzed by PLC (12). Therefore, we examined whether p150 can hydrolyze PIP$_2$ bound to such actin-regulatory proteins as profilin, cofilin, or α-actinin. Since GST-p150 expressed in *E. coli* does not show PIP$_2$ phosphatase activity, we purified native p150 from bovine brains. The anti-p150 antibody-positive 150-kDa proteins were purified from bovine brains by HiLoad Q-Sepharose, Blue-Sepharose, HiLoad Superdex 200, and heparin-Sepharose column chromatography (Fig. 6A). The purified p150 appeared as a single band in SDS-PAGE (Fig. 6B) and Western blots with anti-p150 antibody. The purified p150 showed a remarkably high PIP$_2$ phosphatase activity (specific activity, 8.82 μmol/min/mg of protein). Using the purified p150, we studied whether p150 can hydrolyze PIP$_2$ bound to actin-regulatory proteins. As shown in

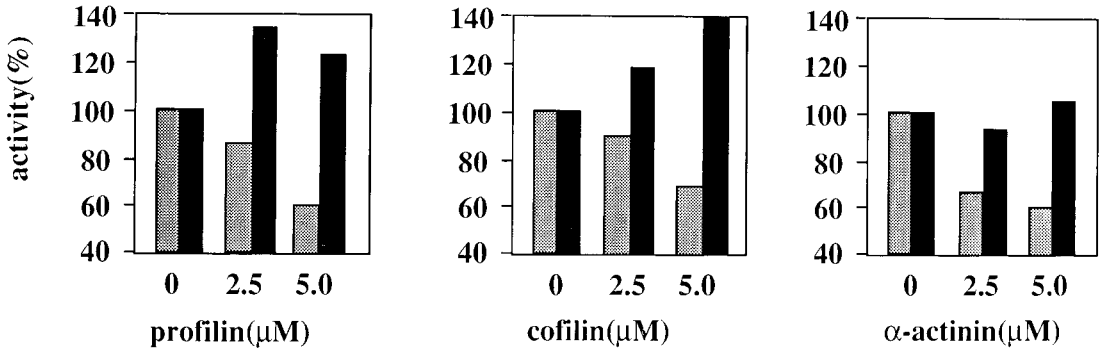


FIG. 7. Hydrolysis of PIP_2 bound to actin-regulatory proteins. In the presence of various concentrations of profilin, cofilin, or α-actinin, the PLC (▨) and PIP_2 phosphatase (■) activities were measured by using purified PLCδ1 and p150.

Fig. 7, actin-regulatory proteins including cofilin, profilin, and α-actinin inhibit PIP_2 hydrolysis by PLCδ1 as shown previously (11), while PIP_2 phosphatase activity is not inhibited but, rather, activated by these proteins.

We previously demonstrated that PIP_2 is required for α-actinin-induced actin gelation (9). Therefore, we examined the effect of p150 on actin gelation by α-actinin by the falling-ball method with purified p150. As shown in Fig. 8, chicken gizzard smooth muscle α-actinin did not gelate actin at all. However, the exogenous addition of PIP_2 caused actin gelation by α-actinin in a concentration-dependent manner. The further addition of p150 (1 μg of protein) to this system suppressed actin gelation. However, when the PIP_2 concentrations were increased, this inhibitory effect disappeared, suggesting that PIP_2 phosphatase can regulate the rearrangement of actin filaments.

DISCUSSION

It is well known that PIP_2 plays important roles not only as a second messenger-generating lipid but also as a regulator of actin filament reorganization. PIP_2 binds to actin-regulatory proteins such as profilin, cofilin, gelsolin, and gCap (16, 18, 38, 39), resulting in the inhibition of the functions of these proteins. PIP_2 also binds to α-actinin and enhances the actin-gelating activity of α-actinin (9, 11). All these data suggest that PIP_2 regulates actin polymerization and depolymerization through actin-regulatory proteins. In this case, one important problem is how the level of PIP_2 bound to proteins is controlled. PIP_2 is synthesized from PI through two phosphate addition steps involving PI 4-kinase and PIP 5-kinase. The PIP_2 formed is hydrolyzed by PLC to IP_3 and DG or by PIP_2 phosphatase to PIP. However, since PIP_2 bound to actin-regulatory proteins cannot be hydrolyzed by PLC, another mechanism has been thought to exist for the hydrolysis of bound PIP_2. Goldschmidt-Clermont et al. (12) provided evidence that PIP_2 bound to profilin is not hydrolyzed by PLCγ1 but that PLCγ1 phosphorylated by tyrosine kinases overcomes the inhibitory effect of profilin and hydrolyzes profilin-bound PIP_2, suggesting that bound PIP_2 is degraded when the tyrosine kinase-PLCγ1 pathway is activated. Until now, it was thought that this was the sole pathway to hydrolyze bound PIP_2. On the other hand, it has not been shown whether PIP_2 phosphatase hydrolyzes bound PIP_2. We show here that PIP_2 phosphatase (p150) can hydrolyze PIP_2 bound to such actin-regulatory proteins as profilin, cofilin, and α-actinin, indicating that there is an another pathway for the hydrolysis of bound PIP_2.

Overexpression studies revealed that p150 distributes predominantly in the perinuclear areas, where actin stress fibers disappear and string- or dot-like actin filaments appear instead, suggesting that p150 plays important roles in the rearrangement of actin filaments in vivo. In addition, cells expressing p150 become multinuclear. Failure of cell division may be caused by a defect in the reorganization of actin filaments. However, when a PIP_2 phosphatase activity-negative mutant was transfected, the cells showed actin stress fibers similar to those in normal COS 7 cells and had one nucleus, suggesting that PIP_2 phosphatase activity is required for these events.

We also demonstrated that p150 forms complexes with Ash/Grb2 and binds to EGF receptor only when cells are treated with EGF. Moreover, p150 is expressed ubiquitously, although it is present predominantly in brain (data not shown). Thus, it appears likely that p150 and its homologs bind to tyrosine kinase receptors through adaptor proteins such as Ash/Grb2 when tyrosine kinases are stimulated and then hydrolyzes PIP_2 bound to actin-regulatory proteins, resulting in the rearrangement of actin filaments. In nerve cells, amphiphysin may be the most abundant partner for p150 (26). It is possible that p150 is present downstream of tyrosine kinase receptors and upon receptor stimulation is transferred to membrane areas, where it hydrolyzes PIP_2 bound to actin-regulatory proteins. In fact, p150 is present mainly in perinuclear areas of COS 7 cells and

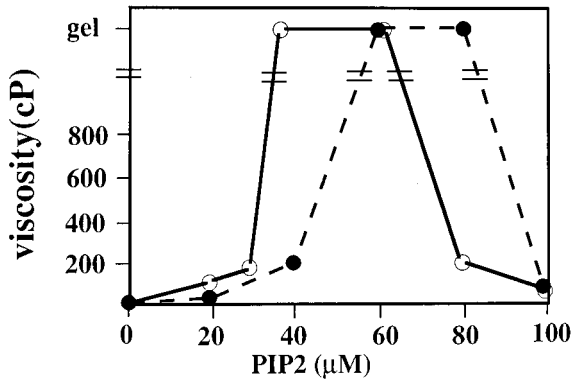


FIG. 8. Inhibition of α-actinin-induced actin gelation by p150. Chicken gizzard α-actinin-induced actin gelation was measured by the falling-ball method. Actin gelation by α-actinin was measured in the presence of various concentrations of PIP2 (○). p150 (1 μg of protein) was added to this system, and the effect on actin gelation was examined (●).

moves to the membranes upon EGF treatment, as shown in Fig. 4.

Since PIP_2 phosphatase activity is not changed by the binding of p150 to Ash/Grb2 (data not shown), another factor may be necessary for PIP_2 phosphatase activation. However, it seems likely that the topological change, from the cytosolic area to the membrane, is important for the regulation of regional actin filaments. Since p150 can bind to actin filaments, as shown in Fig. 1, p150 may effectively hydrolyze PIP_2 bound to cytoskeletal components. In addition, p150 contains a Sac 1 homology domain that may play an important role in protein distribution (6) and proline-rich domains where Ash/Grb2 binds. These domains may be important for recruiting p150 to the membrane area and colocalizing with actin filaments when tyrosine kinase receptors are activated. The phosphatase activity-negative mutant also translocates into the membrane area, as seen in cells expressing full-length p150 upon tyrosine kinase receptor stimulation, without affecting actin stress fibers, suggesting that PIP_2 phosphatase activity is necessary for the rearrangement of actin filaments. All these data show that p150 (synaptojanin) hydrolyzes PIP_2 bound to actin-regulatory proteins and causes the rearrangement of actin filaments.

REFERENCES

1. **Attree, O., I. M. Olivos, I. Okabe, L. C. Bailey, D. L. Nelson, R. A. Lewis, R. R. McInners, and R. L. Nussbaum.** 1992. The Lowe's oculocerebrorenal syndrome gene encodes a protein highly homologous to inositol polyphosphate-5-phosphatase. Nature **358:**239–242.
2. **Berridge, M. J.** 1993. Inositol trisphosphate and calcium signalling. Nature **361:**315–325.
3. **Buday, L., and J. Downward.** 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell **73:**611–620.
4. **Chardin, P., J. H. Camonis, N. W. Gale, A. L. Van, J. Schlessinger, M. H. Wigler, and D. Bar-Sagi.** 1993. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science **260:**1338–1343.
5. **Chauhan, A., H. Brockerhoff, H. M. Wisniewski, and V. P. S. Chauhan.** 1991. Interaction of protein kinase C with phosphoinositides. Arch. Biochem. Biophys. **287:**283–287.
6. **Cleves, A. E., P. J. Novick, and V. A. Bankaitis.** 1989. Mutations in the SAC1 gene suppress defects in yeast Golgi and yeast actin function. J. Cell Biol. **109:**2939–2950.
7. **Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Siezland, and R. A. Weinberg.** 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature **363:**45–51.
8. **Feller, S. M., R. Ren, H. Hanafusa, and D. Baltimore.** 1994. SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl. Trends Biochem. Sci. **19:**453–458.
9. **Fukami, K., K. Furuhashi, M. Inagaki, T. Endo, S. Hatano, and T. Takenawa.** 1992. Requirement of phosphatidylinositol 4,5-bisphosphate for alpha-actinin function. Nature **359:**150–152.
10. **Fukami, K., T. Endo, M. Inamura, and T. Takenawa.** 1994. Alpha-actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. J. Biol. Chem. **269:**1518–1522.
11. **Fukami, K., N. Sawada, T. Endo, and T. Takenawa.** 1996. Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle α-actinin. J. Biol. Chem. **271:**2646–2650.
12. **Goldschmidt-Clermont, P. J., J. W. Kim, L. M. Machesky, S. G. Rhee, and T. D. Pollard.** 1991. Regulation of phospholipase C-gamma 1 by profilin and tyrosine phosphorylation. Science **251:**1231–1233.
13. **Gout, I., R. Dhand, I. D. Hiles, M. J. Fry, G. Panayotou, P. Das, O. Truong, F. Totty, J. Hsuan, G. W. Booker, and M. D. Waterfield.** 1993. The GTPase dynamin binds to and is activated by a subset of SH3 domains. Cell **75:**25–36.
14. **Hartwig, J. H., G. M. Bokoch, C. L. Carpenter, P. A. Janmey, L. A. Tayor, A. Toker, and T. P. Stossel.** 1995. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell **82:**643–653.
15. **Huang, F. L., and K. P. Huang.** 1991. Interaction of protein kinase C isozymes with phosphatidylinositol 4,5-bisphosphate. J. Biol. Chem. **266:**8727–8733.
16. **Janmey, P. A., and T. P. Stossel.** 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. Nature **325:**362–364.
17. **Janmey, P. A.** 1991. Polyproline affinity method for purification of platelet profilin and modification with pyrene-maleimide. Methods Enzymol. **196:**92–99.
18. **Lassing, I., and U. Lindberg.** 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature **314:**472–474.
19. **Li, N., A. Batzer, R. Daly, V. Yajnik, E. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger.** 1993. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature **363:**85–88.
20. **Liscovitch, M., and L. C. Cantley.** 1995. Signal transduction and membrane traffic: the PITP/phosphoinositide connection. Cell **81:**659–662.
21. **Lowenstein, E. J., R. J. Daly, A. G. Batzer, B. Margolis, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. The SH2 and SH3 domain-containing protein Grb2 links tyrosine kinases to ras signaling. Cell **70:**431–442.
22. **Majerus, P. W.** 1996. Inositols do it all. Genes Dev. **10:**1051–1053.
23. **Matuoka, K., M. Shibata, A. Yamakawa, and T. Takenawa.** 1992. Cloning of ASH, a ubiquitous protein composed of one Src homology region (SH) 2 and two SH3 domains, from human and rat cDNA libraries. Proc. Natl. Acad. Sci. USA **89:**9015–9019.
24. **Matuoka, K., F. Shibasaki, M. Shibata, and T. Takenawa.** 1993. Ash/Grb-2, a SH2/SH3-containing protein, couples to signaling for mitogenesis and cytoskeletal reorganization by EGF and PDGF. EMBO J. **12:**3467–3473.
25. **Matzaris, M., S. P. Jackson, K. M. Laxminarayan, C. J. Speed, and C. A. Mitchell.** 1994. Identification and characterization of the phosphatidylinositol 4,5-bisphosphate 5-phosphatase in human platelets. J. Biol. Chem. **269:**3397–3402.
26. **McPherson, P. S., E. P. Garcia, V. I. Slepnev, C. David, X. Zhang, D. Grabs, W. S. Sossin, R. Bauerfreind, Y. Nemoto, and P. D. Camilli.** 1996. A presynaptic inositol-5-phosphatase. Nature **379:**353–356.
27. **Meisner, H., and M. P. Czech.** 1995. Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. J. Biol. Chem. **270:**25332–25353.
28. **Miki, H., K. Miura, K. Matuoka, T. Nakata, N. Hirokawa, S. Orita, K. Kaibuchi, Y. Takai, and T. Takenawa.** 1994. Association of Ash/Grb2 with dynamin through the Src homology 3 domain. J. Biol. Chem. **269:**5489–5492.
29. **Miki, H., K. Miura, and T. Takenawa.** 1996. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J. **15:**5325–5335.
30. **Miura, K., H. Miki, K. Shimazaki, N. Kawai, and T. Takenawa.** 1996. Interaction of Ash/Grb2 via its SH3 domains with neuron-specific p150 and p65. Biochem. J. **316:**639–645.
31. **Nagaoka, R., K. Kusano, H. Abe, and T. Obinata.** 1995. Effects of cofilin on actin filamentous structures in cultured muscle cells. Intracellular regulation of cofilin action. J. Cell Sci. **108:**581–593.
32. **Nishizuka, Y.** 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature **308:**693–698.
33. **Palmer, F. B. S., R. Theolis, H. W. Cook, and D. Byers.** 1994. Purification of two immunologically related phosphatidylinositol 4,5-bisphosphate phosphatase from bovine brain cytosol. J. Biol. Chem. **269:**3403–3410.
34. **Randazzo, P. A., and R. A. Kahn.** 1994. GTP hydrolysis by ADP-ribosylation factor is dependent on both an ADP-ribosylation factor GTPase-activating protein and acid phospholipids. J. Biol. Chem. **269:**10758–10763.
35. **Rozakis-Adocock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell.** 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature **363:**83–85.
36. **Saido, T. C., H. Suzuki, H. Yamazaki, K. Tanoue, and K. Suzuki.** 1993. Positive regulation of mu-calpain action by polyphosphoinositides. J. Biol. Chem. **267:**24585–24590.
37. **Sohn, R. H., J. Chen, K. S. Koblan, P. F. Bray, and P. J. Goldschmidt-Clermont.** 1995. Localization of a binding site for phosphatidylinositol 4,5-bisphosphate on human profilin. J. Biol. Chem. **270:**21114–21120.
38. **Yonezawa, N., Y. Homma, I. Yahara, H. Sakai, and E. Nishida.** 1991. A short sequence responsible for both phosphoinositide binding and actin binding activities of cofilin. J. Biol. Chem. **266:**17218–17221.
39. **Yu, F. X., P. A. Johnston, T. C. Sudhof, and H. L. Yin.** 1990. gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. Science **250:**1413–1415.
40. **Zhang, X., A. B. Jefferson, V. Auethavekiat, and P. W. Majerus.** 1995. The protein deficient in Lowe syndrome is a phosphatidylinositol-4,5-bisphosphate 5-phosphatase. Proc. Natl. Acad. Sci. USA **92:**4853–4856.