Phosphatidylinositol 3-kinase binds to α-actinin through the p85 subunit

Futoshi SHIBASAKI,* Kiyoko FUKAMI,* Yasuhisa FUKUI† and Tadaomi TAKENAWA*‡

*Department of Molecular Oncology, The Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan and †Department of Biochemistry, Faculty of Agriculture, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

Phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to play an important role in the signal transduction of cell growth. It is also suggested that it is involved in cytoskeletal reorganization. We have found that α-actinin copurifies with PI 3-kinase from bovine thymus. The antibody against PI 3-kinase 85 kDa subunit (p85) also co-immunoprecipitates α-actinin from lysates of NIH/3T3 cells. In addition, anti-α-actinin antibody coprecipitates PI 3-kinase activity. This coprecipitation was observed even after depolymerization of actin fibres, suggesting that PI 3-kinase binds directly to α-actinin. As α-actinin is a phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$)-binding protein, binding experiments using various constructs of truncated p85 were carried out in the presence or absence of $PI4,5P_2$. In the absence of $PI4,5P_2$, chicken gizzard α-actinin binds only to the whole p85 construct, but it binds to the proline-rich region of p85 fragments in the presence of $PI4,5P_2$. This binding is enhanced with increased concentrations of $PI4,5P_2$ up to 10 μM, whereas phosphatidylinositol and phosphatidylinositol 4-phosphate were not good activators of α-actinin binding. These results suggest that PI 3-kinase binds to α-actinin and regulates cytoskeletal reorganization.

INTRODUCTION

Phosphatidylinositol (PI) kinase plays an important role in polyphosphoinositide turnover which produces two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, because it is thought that the phosphorylation of PI is a rate-limiting step in phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) synthesis. This PI kinase catalyses the formation of phosphatidylinositol 4-phosphate via phosphorylation of the 4-position of the inositol ring of PI. This is further phosphorylated to $PI4,5P_2$ by PIP kinase. The resultant lipid is hydrolysed by phospholipase C in response to various hormones and mitogens [1,2].

In addition to this type of PI kinase (PI 4-kinase), a new type of PI kinase has been shown to be present in complexes with middle T/$pp60^{c\text{-}src}$, $pp60^{v\text{-}src}$, platelet-derived growth factor (PDGF) receptor, colony-stimulating factor (CSF)-1 receptor and insulin receptor [3–10]. This enzyme catalyses the formation of new lipids, phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$) [9,11] and its activity is correlated with the appearance of an 85 kDa protein that is phosphorylated on tyrosine by tyrosine kinase [3,4]. This evidence strongly suggests that a new type of PI kinase plays an important role in mitogenic and oncogenic signals. Recently, we [12] and Carpenter et al. [13] have succeeded in purifying PI 3-kinase, showing that it consists of two subunits, 85 kDa (p85) and 110 kDa (p110). We also demonstrated that p110 has PI 3-kinase catalytic activity. More recently, cDNA cloning of both subunits has been achieved [14–17]. This showed that the p110 subunit has PI 3-kinase activity and that the p85 subunit contains SH2 and SH3 domains. The SH2 and SH3 domains have been observed in several proteins involved in the signal-transduction pathway, including phospholipase C_γ [18], rasGAP [19] and oncogene products such as Crk [20], Nck [21] and Vav [22]. Several experiments have revealed that the SH2 domain recognizes a specific peptide sequence containing phosphotyrosine and forms complexes with tyrosine-phosphorylated proteins [23,24]. PI 3-kinase has also been demonstrated to bind tyrosine-phosphorylated sites of PDGF, epidermal growth factor and insulin receptor substrate 1, through the SH2 domain of p85 (reviewed in [24]). On the other hand, SH3 domains are thought to have an affinity for proline-rich residues, as first defined by 3BP1 protein [25,26]. Several cytoskeletal proteins, including α-spectrin [27], myosin 1b [28] and yeast actin-binding protein [29], have been found to have only the SH3 domain without the SH2 domain. This suggests a role for SH3 in the regulation of cytoskeletal organization but it is not clear how it achieves this.

In the present paper, we show that α-actinin copurifies with PI 3-kinase and is co-immunoprecipitated with p85 by anti-p85 antibody. Furthermore, the anti-α-actinin antibody co-precipitates PI 3-kinase activity. Binding experiments using various constructs of truncated p85 reveal that the SH3 C-terminal region of p85 plays an important role in the binding of α-actinin.

EXPERIMENTAL

Materials

Phosphatidylinositol (porcine liver) and phosphatidylserine (porcine liver) were purchased from Serdary Research Laboratory (London, Ontario, Canada). Phosphatidylinositol 4-monophosphate (bovine brain) was from Sigma. $PI4,5P_2$ was purified from bovine spinal cords by the method of Schacht [30]. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Du Pont–New England Nuclear. Di-isopropyl fluorophosphate and phenylmethanesulphonyl fluoride were from Sigma. Leupeptin was obtained from Peptide Research Laboratory (Tokyo, Japan). The HiTrapTM Heparin column and glutathione–Sepharose 4B beads were purchased from Pharmacia. GSH was from Wako Chemicals (Tokyo, Japan). Alkaline phosphatase-conjugated

Abbreviations used: IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; $PI4,5P_2$, phosphatidylinositol 4,5-bisphosphate; PI3P, phosphatidylinositol 3-phosphate; $PI3,4P_2$, phosphatidylinositol 3,4-bisphosphate; $PI3,4,5P_3$, phosphatidylinositol 3,4,5-trisphosphate; PDGF, platelet-derived growth factor; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; CSF, colony-stimulating factor; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate.

‡ To whom correspondence should be addressed.

anti-mouse or anti-rabbit antibody and alkaline phosphatase substrates were from Promega. Restriction endonucleases (*Sna*B2, *Hin*dIII, *Sca*I and *Eco*RV) were from Takara (Tokyo, Japan). Adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA) was from Boehringer-Mannheim Biochemicals. Anti-actin monoclonal antibody was obtained from Oncogene Science. Anti-α-actinin antibody and anti-vinculin antibody were gifts from Dr. T. Endo (Faculty of Science, Chiba University, Chiba, Japan). Anti-PI4,5P_2 antibody was made as described previously [31]. Chicken gizzard α-actinin was a gift from Dr. M. Inagaki (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan).

Cell culture and immunoprecipitation

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Cell Culture Laboratory), penicillin (100 units/ml) and streptomycin sulphate (100 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Confluent NIH/3T3 cells were washed twice with PBS, and then lysed in a modified RIPA buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM di-isopropyl fluorophosphate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1% Triton X-100, 1% deoxycholate and 0.1% SDS. The lysates were sonicated and kept on ice for 1 h. After centrifugation, the supernatants were incubated with anti-p85 antibody or anti-α-actinin antibody for 1 h at 4 °C. The samples were then incubated for 1 h at 4 °C with Protein A–Sepharose. The immunoprecipitates were recovered by centrifugation and washed once with 0.5 M LiCl/20 mM Tris/HCl (pH 7.4), once with PBS, and finally twice with 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100.

Immunoblotting

The collected immunoprecipitates were lysed in SDS/sample buffer, subjected to SDS/PAGE and then transferred to a nylon filter (Clear Blot Membrane-p, ATTO, Tokyo, Japan). The membrane was incubated with 5% skimmed milk overnight at room temperature, and then washed with PBS containing 0.1% Tween 20. The filter was incubated with each antibody, and then incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibody for an additional 1 h at room temperature.

Immunoprecipitation of PI 3-kinase activity

NIH/3T3 cells were washed twice with PBS and lysed with lysis buffer containing 40 mM Tris/HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1 mM phenylmethanesulphonyl fluoride, 0.1 mM di-isopropyl fluorophosphate, 10 μg/ml leupeptin and 1% dodecylsucrose. After centrifugation, the supernatant was incubated with anti-p85 antibody or anti-α-actinin antibody for 4 h, followed by shaking with Protein A–Sepharose for an additional 1 h. The immunoprecipitates were collected by centrifugation, and then washed once with the lysis buffer, once with 0.5 mM LiCl/20 mM Tris/HCl (pH 7.4), once with PBS, and finally twice with 20 mM Tris/HCl (pH 7.6), containing 150 mM NaCl, 10% glycerol and 1 mM EGTA.

PI 3-kinase assay

The PI 3-kinase activity of the immunoprecipitates was assayed as described previously [32] in a final volume of 50 μl containing 50 mM Tris/HCl (pH 7.6), 5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM NaCl, 1 mM phosphatidylinositol, 400 μM phosphatidylserine, 10 μM ATP, [γ-^{32}P]ATP (1–2 μCi) and enzymes. After incubation for 10 min at 30 °C, the reaction was stopped by the addition of 2 ml of chloroform/methanol/conc. HCl (200:100:1, by vol.). Then 0.5 ml of 1 M HCl was added, and the mixture was vortexed and separated into two phases by centrifugation. The upper phase was discarded and the lower phase washed with 0.5 ml of synthetic upper phase. After centrifugation, the resultant lower phase was spotted on t.l.c. plates (silica gel 60; Merck) and developed in methanol/chloroform/ammonia/water (100:70:15:25, by vol.). The plates were visualized by autoradiography. The spots of PI3P were scraped off and counted by Čerenkov's method.

Expression and purification of various p85 constructs

A full-length cDNA encoding the p85 of PI 3-kinase was cloned from HeLa cells as described previously [33]. Each restricted fragment of p85 was ligated into bacterial expression plasmid pGEX-2T. Detailed descriptions of each construct are given in the legend to Figure 4(a). Plasmid-bearing *Escherichia coli* (JM109) was induced by 100 μM isopropyl β-D-thiogalactoside (IPTG) for 2 h at 37 °C after 3 h preincubation, collected by centrifugation, and lysed with RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS. The lysates were sonicated for 5 × 1 min, and centrifuged at 105000 *g* for 30 min at 2 °C. The expressed glutathione S-transferase (GST) fusion protein was purified by affinity chromatography with glutathione–Sepharose beads. The beads were collected by centrifugation and washed once with RIPA buffer, once with 0.5 M LiCl/20 mM Tris/HCl (pH 7.4), once with PBS and finally twice with 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100. These fusion-protein-coupled beads were used in α-actinin-binding experiments.

Binding of various constructs to α-actinin

Purified α-actinin from smooth muscle was added to various kinds of GST-fusion proteins beads, including 85 kDa constructs, in 1 ml of reaction buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS, and then gently shaken overnight at 4 °C. The beads collected by centrifugation were washed once with reaction buffer, once with 0.5 M LiCl/20 mM Tris/HCl (pH 7.4), once with PBS and twice with 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100. Fusion proteins bound to α-actinin were eluted with 50 mM GSH in 0.1 M Tris/HCl (pH 8.0). The collected supernatants obtained by centrifugation were boiled in SDS/sample buffer, subjected to SDS/PAGE (7.5% gels) and then transferred to nylon filter.

Production of anti-p85 polyclonal antibody

A full-length protein from the 85 kDa subunit expressed as a GST fusion protein from *E. coli* was purified with glutathione–Sepharose beads. The protein eluted with 100 μg/ml thrombin (human thrombin; Sigma) from glutathione–Sepharose beads was subjected to chromatography on a HiTrapTM Heparin column pre-equilibrated with 20 mM Tris/HCl (pH 7.6), 1 mM EGTA and 10% glycerol. The column was developed with a 30 ml linear gradient of 0–0.5 M NaCl in the same buffer. Each fraction was examined by SDS/PAGE.

A female New Zealand White rabbit was immunized with this purified 85 kDa protein emulsified in Freund's complete adjuvant. The polyclonal anti-p85 antibody reacted with PI-3-kinase p85 purified as described previously [33].

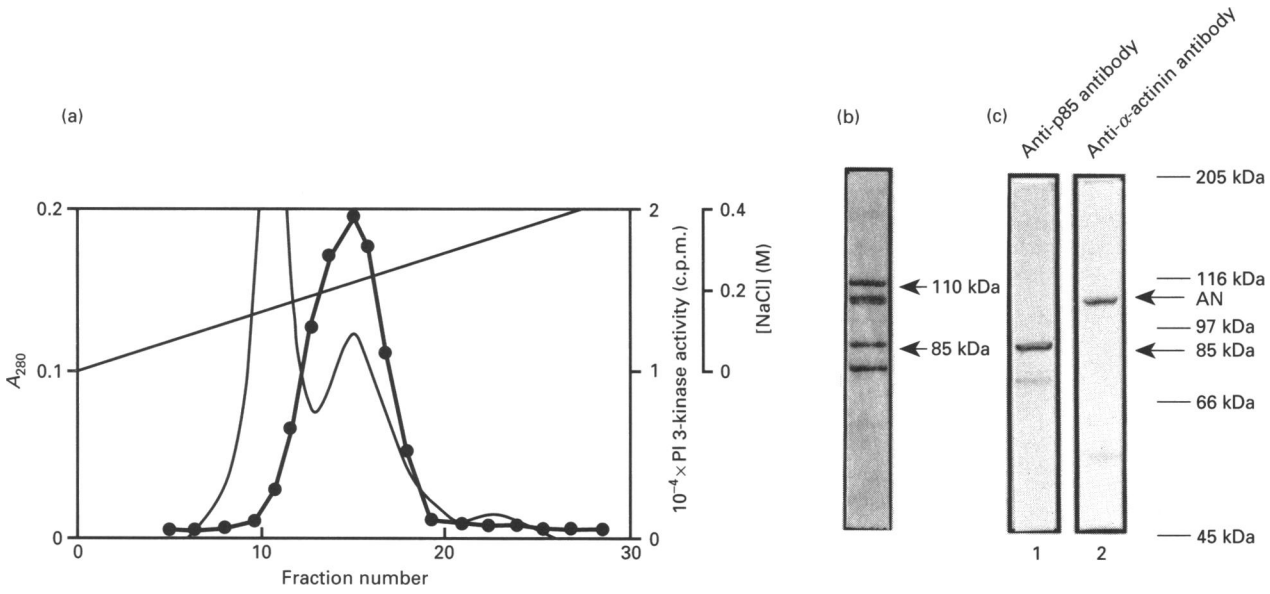

Figure 1 Co-purification of α-actinin with PI 3-kinase

(**a**) Elution of protein and PI 3-kinase activity from a heparin column after a five-step purification. The PI 3-kinase activity peak is in fraction number 15. —, A_{280}; ●, PI 3-kinase activity. (**b**) Coomassie Brilliant Blue staining of fraction number 15 after SDS/PAGE (7.5% gels). Arrows show the heterodimer of PI 3-kinase (110 kDa and 85 kDa). (**c**) The peak fraction of PI 3-kinase activity was subjected to SDS/PAGE (7.5% gels), transferred to a nylon filter, and stained with anti-p85 antibody (lane 1) or anti-α-actinin antibody (lane 2). An arrow (AN) shows α-actinin. Molecular-mass markers (205 kDa, 116 kDa, 97 kDa, 66 kDa and 45 kDa) are shown from top to bottom.

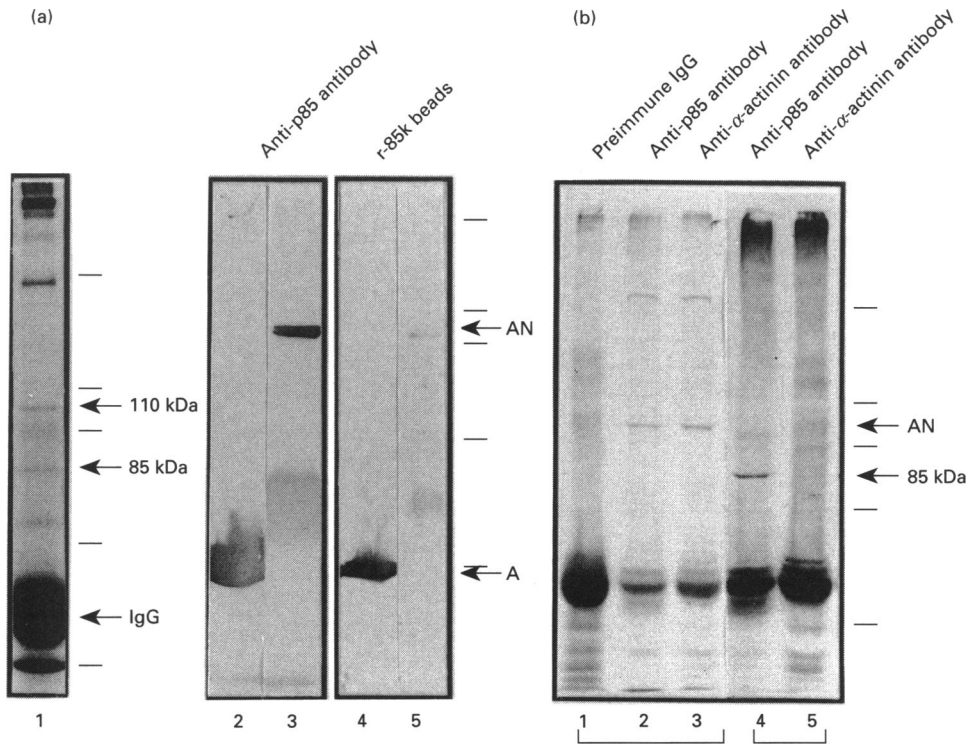

Figure 2 α-Actinin binds to the 85 kDa subunit of PI 3-kinase *in vivo*

(**a**) NIH/3T3 cell lysates were immunoprecipitated with anti-p85 antibody and subjected to SDS/PAGE (7.5% gels). Proteins were stained with Coomassie Brilliant Blue (lane 1). The immunoprecipitates prepared with anti-p85 antibody or the coprecipitated proteins with recombinant 85 kDa–GST fusion protein–Sepharose (r-85 k beads) were subjected to SDS/PAGE (10% gels), transferred to a nylon filter, and stained with anti-actin antibody (lanes 2 and 4) or anti-α-actinin antibody (lanes 3 and 5). (**b**) Immunoprecipitates prepared with preimmune IgG (lane 1), anti-p85 antibody (lane 2) or anti-α-actinin antibody (lane 3) were subjected to SDS/PAGE (7.5% gels), transferred to a nylon filter, and stained with anti-α-actinin antibody. The immunoprecipitates prepared with anti-p85 antibody (lane 4) or anti-α-actinin antibody (lane 5) were stained with anti-p85 kDa antibody. Molecular-mass markers (205 kDa, 116 kDa, 97 kDa, 66 kDa and 45 kDa) are shown from top to bottom. Abbreviations: A, actin; AN, α-actinin.

Purification of PI 3-kinase and α-actinin

The PI 3-kinase heterodimer was purified as described previously [12]. The starting material was obtained from the 105000 *g* supernatant of the cytosol fraction from bovine thymus. The PI 3-kinase heterodimer fractions were used as immunoprecipitation samples after purification through five column chromatography steps including DEAE-cellulose, cellulose phosphate, Mono Q, Superdex and HiTrapTM Heparin columns. α-Actinin was purified from bovine thymus by the method of Feramisco and Burridge [34].

RESULTS

Copurification of PI 3-kinase with α-actinin

PI 3-kinase was purified from bovine thymus by six successive chromatography steps, including DEAE-cellulose, cellulose phosphate, Mono Q, Superdex, Heparin and Mono S columns, as described previously [12]. We found that a 100 kDa protein copurified with PI 3-kinase through five of the steps. This protein was removed only by the final Mono S column, suggesting a very close association with PI 3-kinase. Figure 1 shows the results of the sixth Hitrap TM Heparin column chromatography step. PI 3-kinase activity was eluted at around 0.24 M NaCl, peaking in fraction 15. This fraction was subjected to SDS/PAGE and the gel was stained with Coomassie Brilliant Blue (Figure 1b). Four protein bands were strongly stained and several bands (180, 97 and 60 kDa) were faintly stained. Among the strongly stained bands, the 110 and 85 kDa proteins have already been demonstrated to be the catalytic and regulatory subunits of PI 3-kinase respectively. Therefore the 100 and 75 kDa proteins appear to be associated with PI 3-kinase. However, as the 75 kDa protein was stained by the antibody to the 85 kDa subunit (Figure 1c, lane 1), it may be a degradation product of this subunit. We were interested in the remaining 100 kDa protein because it may provide information about the interaction of PI 3-kinase with other proteins. PI 3-kinase p85 contains the SH3 domain which is thought to interact with the cytoskeleton. Therefore we tried staining with antibodies against cytoskeletal components (α-actinin and vinculin) with molecular masses around 100 kDa. Surprisingly, the 100 kDa protein was strongly stained by the anti-α-actinin antibody (Figure 1c, lane 2). This shows that α-actinin is copurified with PI 3-kinase. The dissociation of α-actinin from PI 3-kinase in the final Mono S column may be the result of treatment at low pH (6.3); the other chromatography steps were performed in pH 7.6 buffer.

Association of α-actinin with the PI 3-kinase 85 kDa subunit

NIH/3T3 cells were lysed with RIPA buffer and immunoprecipitated with anti-p85 antibody and protein A–Sepharose. The precipitates were subjected to SDS/PAGE and the gels stained with Coomassie Brilliant Blue (Figure 2a). The immunoprecipitates contained several proteins including 200, 100, 75 and 42 kDa proteins, in addition to the PI 3 kinase p85 and 110 kDa subunits. The 42 kDa protein was found to be actin by precipitation with anti-actin antibody (Figure 2a, lane 2) and the 100 kDa protein was found to be α-actinin (Figure 2a, lane 3). However, vinculin was not detected in the immunoprecipitates by Western blot with anti-vinculin antibody. These data suggest that PI 3-kinase is closely associated with α-actinin. Next, we examined whether α-actinin from NIH/3T3 cell lysates could bind to p85-subunit-conjugated beads. As shown in Figure 2(a), lanes 4 and 5, actin and α-actinin bound to the 85 kDa subunit beads. Although the anti-p85 antibody immunoprecipitated significant amounts of α-actinin, p85 was detected in small amounts in the anti-α-actinin immunoprecipitates (Figure 2b, lane 5). This may be due to the different amounts of α-actinin and PI 3-kinase present; the amounts of α-actinin are much larger than those of PI 3-kinase.

Table 1 PI 3-kinase activity co-immunoprecipitates with anti-α-actinin antibody

Lysates of NIH/3T3 cells were immunoprecipitated with preimmume rabbit serum or various amounts of anti-α-actinin antibody coupled to Protein A–Sepharose. The immunoprecipitates were washed, treated with or without 2 mM p[NH]ppA for 10 min at room temperature, and washed again with cold PBS. The PI 3-kinase activity of the immunoprecipitates was assayed as described in the Experimental section. The reaction products were separated on silica gel t.l.c. plates and visualized by autoradiography. The spots of PI3P were scraped off and counted by the Čerenkov's method. The results are presented as means ± S.E.M. of triplicate determinations of two separate experiments.

Volume of antibody (μl)	PI 3-kinase activity (c.p.m.)
Preimmune serum	
10	20 ± 3
Anti-α-actinin antibody	
1	53 ± 4
5	580 ± 16
10	810 ± 15
10	870 ± 18*

* Experiment was carried out in the presence of 2 mM p[NH]ppA.

Co-immunoprecipitation of PI 3-kinase activity with α-actinin

Lysates of NIH/3T3 cells were immunoprecipitated with various amounts of α-actinin antibody and Protein A–Sepharose. The immunoprecipitates were assayed for PI 3-kinase activity. As shown in Table 1, PI 3-kinase activity is coprecipitated with α-actinin; however, it is not clear whether PI 3-kinase binds directly to α-actinin or indirectly via cytoskeletal components. Thus we examined whether PI 3-kinase is also coprecipitated with α-actinin after the actin fibre is depolymerized by the addition of p[NH]ppA. PI 3-kinase activity was still coprecipitated with α-actinin even after actin fibre depolymerization, suggesting that it binds directly to α-actinin. Next, we examined whether purified α-actinin fractions also contain PI 3-kinase. Western-blot analysis with anti-(PI 3-kinase 85 kDa subunit) antibody revealed that the purified α-actinin fractions from bovine thymus contained PI 3-kinase. However, the amount was very small compared with α-actinin in purified PI 3-kinase fractions (results not shown). These results may reflect the fact that α-actinin is present in much larger amounts than PI 3-kinase protein in bovine thymus.

α-Actinin binds to the proline-rich region of p85

We have previously demonstrated that α-actinin is present as a PI4,5P_2-bound form [35]. First, to clarify which part of the p85 is necessary for binding to α-actinin, various truncated constructs of p85 subunit were produced (Figure 3a) as GST fusion proteins in *E. coli*. Using these truncated proteins, we examined the binding activity of chicken gizzard α-actinin in the presence or absence of PI4,5P_2. In these experiments, smooth-muscle α-actinin was used to examine the effect of PI4,5P_2 on α-actinin binding, because it contains very little PI4,5P_2 [35]. In the absence of exogenous PI4,5P_2 α-actinin bound only to whole p85 and not to the truncated fragments containing SH3 (Figure 3b).

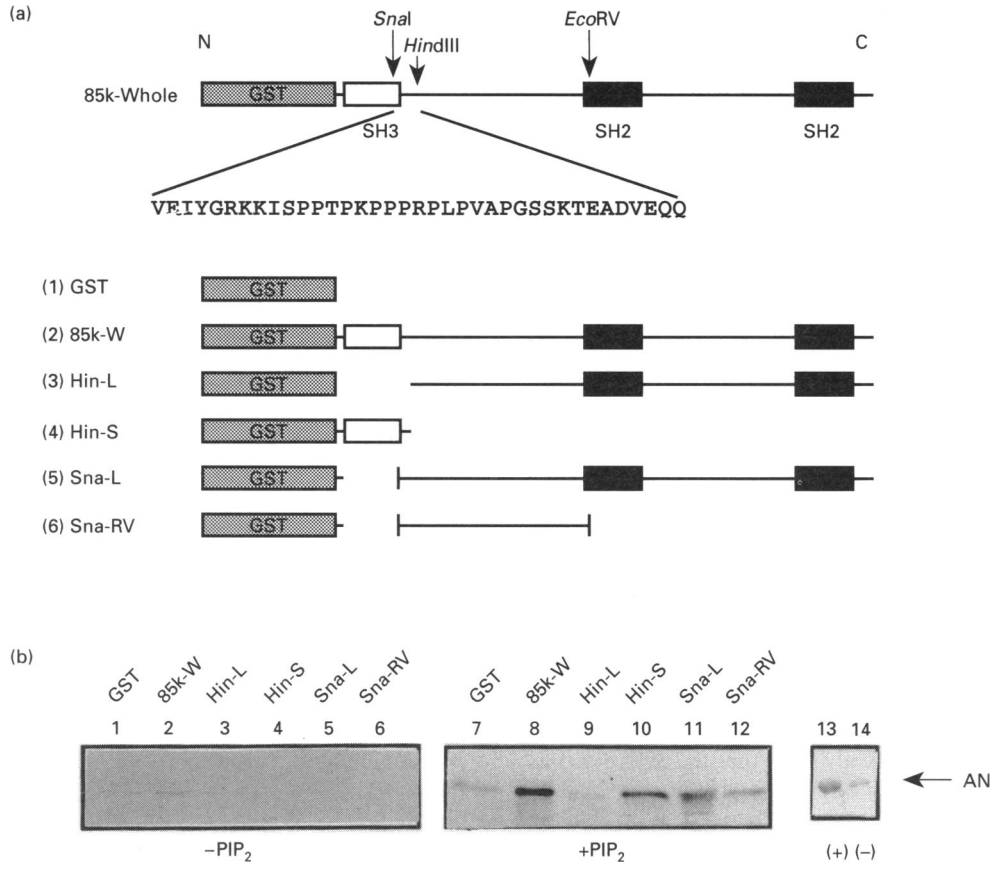

Figure 3 α-Actinin binds directly to recombinant 85 kDa–GST fusion protein

(**a**) Schematic representation of the various SH2/SH3-containing constructs of brain PI 3-kinase 85 kDa subunits (α-type). Each mutant of the 85 kDa–GST fusion protein was expressed and purified as described in the Experimental section. Breaks in the line representing each mutant indicate the extent of deletion by each restriction enzyme. Construct (1) GST alone; construct (2) (85 k-W) encompasses the whole length of the 85 kDa subunit; construct (3) (Hin-L) encompasses the *Hin*dIII restriction fragment containing residue 388 to the C-terminus; construct (4) (Hin-S) encompasses the *Hin*dIII restriction fragment containing residue 388 to the N-terminus; construct (5) (Sna-L) encompasses the *Sna*B2 restriction fragment containing residue 261 to the C-terminus; construct (6) (Sna-RV) encompasses the *Sna*B2 and *Eco*RV restriction fragment containing residues 261–1134. Open and closed boxes indicate the SH3 and SH2 regions respectively. (**b**) α-Actinin binds directly to 85 kDa mutants *in vitro*. α-Actinin (1 μg) and each 85 kDa mutant (1 μg of protein) were added to the reaction buffer containing 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS, and then gently shaken for 2 h at 4 °C without (lanes 1–6) or with (lanes 7–12) 10 μM $PI4,5P_2$. The α-actinin bound to each 85 kDa mutant–GST fusion protein–Sepharose was washed, subjected to SDS/PAGE (7.5% gels), transferred to a nylon filter, and then stained with anti-α-actinin antibody. Lanes 13 and 14 show the PIP_2 binding activity of α-actinin. α-Actinin (1 μg) was preincubated with (lane 13) or without (lane 14) 10 μM $PI4,5P_2$ in the reaction buffer for 10 min at room temperature, subjected to SDS/PAGE (7.5% gels), transferred to a nylon filter, and stained with anti-$PI4,5P_2$ antibody. AN indicates α-actinin.

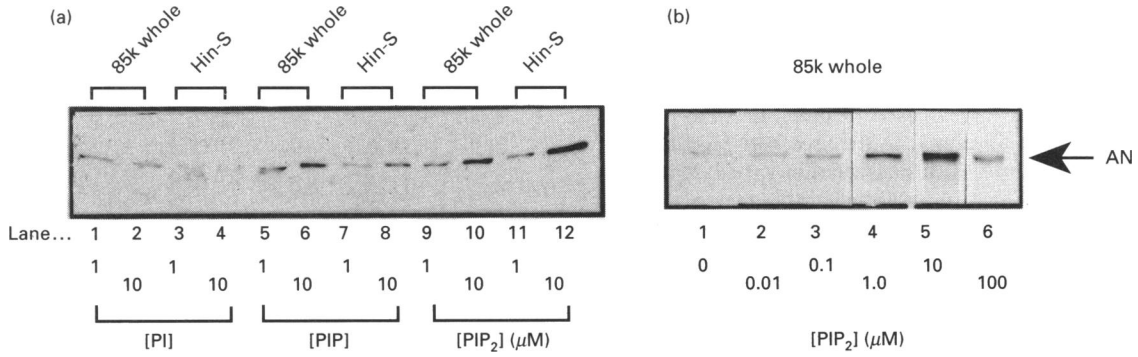

Figure 4 Effect of inositol phospholipids on the binding of 85 kDa mutants to α-actinin

(**a**) Effect of PI, PI4P and $PI4,5P_2$. Whole 85 kDa (lanes 1, 2, 5, 6, 9 and 10), or Hin-S (lanes 3, 4, 7, 8, 11 and 12) and 1 μg of α-actinin were added to the reaction buffer with 1 μM or 10 μM PI (lanes 1–4), PI4P (lanes 5–8) or $PI4,5P_2$ (lanes 9–12). (**b**) Dose-dependence of $PI4,5P_2$. Whole 85 kDa–GST fusion protein (1 μg) and α-actinin (1 μg) were added to the reaction buffer containing various concentrations of $PI4,5P_2$. Coprecipitated α-actinin was detected by anti-α-actinin antibody on nylon filter. AN indicates α-actinin.

In the presence of $PI4,5P_2$, all SH3-containing constructs, even incomplete SH3 fragments bound to α-actinin. Sna-L and Sna-RV, which contain only small fragments of SH3, bound to α-actinin. These results suggest that the area between the SH3 C-terminal region and SH2 plays an important role in the recognition of α-actinin.

Next, using whole p85 and Hin-S fragment, the effect of inositol phospholipids was examined. Phosphatidylinositol and phosphatidylinositol 4-phosphate were not good activators of α-actinin binding, but $PI4,5P_2$ stimulated the binding of α-actinin (Figure 4a). This activation increased with increasing $PI4,5P_2$ concentrations up to 10 μM and then decreased at concentrations above 10 μM (Figure 4b).

DISCUSSION

PI 3-kinase catalyses a new pathway producing PI3P, $PI3,4P_2$ and $PI3,4,5P_3$. These lipids are formed in growth-factor-stimulated and transformed cells by *src* family oncogenes (reviewed in [24]). However, as they have recently been found to be formed in activated platelets and neutrophils, it has been suggested that this kinase regulates cytoskeletal organization. Therefore it is interesting to clarify whether or not PI 3-kinase associates with the cytoskeleton. Indeed, PI 3-kinase is present in the cytoskeleton [36]. PI 3-kinase p85 contains two SH2 domains and one SH3 domain. It is already clear that the SH2 domains recognize a sequence containing phosphotyrosine and bind to activated PDGF and epidermal growth factor receptors. On the other hand, SH3 domains have been shown to bind proline-rich residues, and an interaction with the cytoskeleton has been assumed because several cytoskeletal proteins, such as myosin 1b, spectrin and yeast actin-binding protein, contain SH3 domains.

During the purification of PI 3-kinase, we found a 100 kDa protein that copurified with it until the final step. This protein was demonstrated to be α-actinin. Immunoprecipitates prepared with anti-p85 antibody also contained α-actinin. Furthermore, anti-α-actinin antibody precipitated PI 3-kinase activity. These data demonstrate that PI 3-kinase recognizes and binds α-actinin. This association was still observed even after actin filaments were disrupted by the addition of p[NH]ppA, suggesting that PI 3-kinase binds directly to α-actinin, rather than through other actin-binding proteins. PI 3-kinase is considered to play an important role in the modification of the cytoskeleton [37–39]. It has been shown that activated PI 3-kinase and focal adhesion components become associated with the platelet's integrin-rich cytoskeleton [37]. Therefore PI 3-kinase may localize in the cytoskeleton bound to α-actinin and effectively reorganize the cytoskeleton for membrane trafficking when cells are stimulated.

Truncated p85 proteins were used to determine the binding site of α-actinin. α-Actinin bound to the area between SH3 and SH2, although the features of this association varied depending on the presence of $PI4,5P_2$. In its absence, this region was not able to bind α-actinin because of the absence of a long arm connecting to the SH2 domain that seemed to be necessary for binding. This region contains two proline-rich sequences. Recently, the SH3 region of v-Src has been demonstrated to bind proline-rich residues of p85 [40]. The SH2 region did not contribute to the binding to α-actinin at all. $PI4,5P_2$ is involved in the generation of the second messengers, IP_3 and diacylglycerol, in response to agonists. Simultaneously, it plays an important role in the reorganization of the cytoskeleton. Recently, it has been found that actin-binding proteins, such as profilin [41], gelsolin [42], and cofilin [43] and gCap32 [44], contain $PI4,5P_2$ as bound lipid and that it regulates actin polymerization and depolymerization. α-Actinin is also an actin-binding protein which is bound to $PI4,5P_2$, and the addition of $PI4,5P_2$ dramatically increases actin polymerization by α-actinin [35]. This indicates that $PI4,5P_2$ causes a conformational change in α-actinin. On the basis of these findings, we tested the effect of $PI4,5P_2$ on the binding of α-actinin to the SH3 domain of p85. The addition of $PI4,5P_2$ caused the binding of α-actinin to the proline-rich sequence near SH3, but shorter constructs involving this sequence did not bind α-actinin in the absence of $PI4,5P_2$. Therefore $PI4,5P_2$ appears to influence the conformation of α-actinin. However, it still remains to be determined whether $PI4,5P_2$ actually affects PI 3-kinase binding to α-actinin in intact cells.

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