α-Actinin is an abundant actin-bundling and adhesion protein that directly links actin filaments to integrin receptors. Previously, in platelet-derived growth factor-treated fibroblasts, we demonstrated that phosphoinositides bind to α-actinin, regulating its localization (Greenwood, J. A., Theibert, A. B., Prestwich, G. D., and Murphy-Ullrich, J. E. (2000) J. Cell Biol. 150, 627–642). In this study, phosphoinositide binding and regulation of α-actinin function is further characterized. Phosphoinositide binding specificity, determined using a protein-lipid overlay procedure, suggests that α-actinin interacts with phosphates on the 4th and 5th position of the inositol head group. Binding assays and mutational analyses demonstrate that phosphoinositides bind to the calponin homology domain 2 of α-actinin. Phosphoinositide binding inhibited the bundling activity of α-actinin by blocking the interaction of the actin-binding domain with actin filaments. Consistent with these results, excessive bundling of actin filaments was observed in fibroblasts expressing an α-actinin mutant with decreased phosphoinositide affinity. We conclude that the interaction of α-actinin with phosphoinositides regulates actin stress fibers in the cell by controlling the extent to which microfilaments are bundled.

The interaction of cells with their extracellular matrix is important for regulation of their growth, differentiation, and migration (1, 2). In cultured cells, the primary adhesive complex between the cell and the extracellular matrix substrate is called a focal adhesion (1). These plaque-like structures are composed of an elaborate network of interconnecting proteins that link the actin cytoskeleton to the extracellular matrix via integrin adhesion receptors (1). α-Actinin is an abundant actin-bundling and focal adhesion protein that provides a direct link between actin microfilaments and integrin receptors (3–5). Although it is clear that α-actinin function is important for modulating cell adhesion and motility (6, 7), it is not understood how α-actinin is regulated in the cell.

α-Actinin exists as an anti-parallel homodimer (Fig. 1), with each monomer composed of the following three regions: the actin-binding domain, the spectrin repeats, and the C-terminal EF hands domain (3). Although the crystal structure of full-length α-actinin has not been solved, cryoelectron microscopy and the crystal structure of the spectrin repeats suggest that the α-actinin dimer aligns as shown in Fig. 1 (8–10). α-Actinin belongs to a family of proteins, including dystrophin, utrophin, spectrin, ABP-120, and fibrin, in which the actin-binding domain consists of two tandem calponin homology (CH) domains (11). The CH1 domain from this family of proteins is critical for binding to actin filaments and can bind independently, whereas the CH2 domain enhances binding affinity but cannot bind alone (11–13). Based on various experimental approaches, it appears that the actin-binding domains of this family of proteins contain three sites that come in direct contact with actin filaments: sites 1 and 2 are within the CH1 domain, and site 3 is within the CH2 domain (12, 13).

The importance of α-actinin in integrin-mediated adhesion is illustrated by its localization within adherent cells. Intense immunostaining for α-actinin is observed in focal adhesions, reflecting its role in linking actin filaments to the cytoplasmic domain of integrins (14). Furthermore, Rajfur et al. (15) have used chromophore-assisted laser inactivation of GFP-α-actinin in a focal adhesion to demonstrate that loss of the α-actinin link between actin filaments and integrins results in the retraction of the stress fiber. As a key actin filament-bundling protein, α-actinin is also observed in a periodic distribution along stress fibers. Electron microscopy has shown that α-actinin is organized into dense bodies along the stress fiber that alternate with myosin filaments (14, 16). Although it is known that these contractile myosin filaments are necessary for the formation of stress fibers (17), it is not clear what role α-actinin plays or what mechanisms regulate the alternating distribution of these proteins along stress fibers.

PtdIns(4,5)-P$_2$ appears to be essential for the formation of focal adhesions and stress fibers (18, 19), and the type 1 PtdIns-P kinase has recently been localized in focal adhesions (20, 21). Moreover, PtdIns(4,5)-P$_2$ has been shown to affect the activity of a number of focal adhesion proteins in vitro including talin (22), vinculin (18, 23), syndecan-4 (24), and α-actinin (25). However, the role PtdIns(4,5)-P$_2$ plays in regulating the function of these proteins in cells remains to be determined. By using anti-PtdIns(4,5)-P$_2$, Fukushima et al. (25, 26) demonstrated that PtdIns(4,5)-P$_2$ bound α-actinin from chicken striated and smooth muscle as well as Balb/c 3T3 cells. This group mapped the PtdIns(4,5)-P$_2$-binding site in chicken skeletal α-actinin to residues 168–184 (27), adjacent to actin-binding site 3. The

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§ The abbreviations used are: CH, calponin homology; PtdIns, phosphatidylinositol; F-actin, filamentous actin; PDGF, platelet-derived growth factor; PI 3-kinase, phosphoinositide 3-kinase; GST, glutathione S-transferase; his-CH2, His-tagged CH2 domain; GFP, green fluorescent protein; Refs, rat embryonic fibroblasts; Ins, inositol.
Phosphoinositide Regulation of α-Antinin

Binding of PtdIns(4,5)-P$_2$ to α-actinin was found to increase the F-actin gelating activity of α-actinin indicating an increase in cross-linking (25). We have found that PtdIns(4,5)-P$_2$ binds to α-actinin in unstimulated fibroblasts, correlating with the localization of α-actinin to focal adhesions and actin stress fibers (28).

Activation of PI 3-kinase and production of 3-phosphorylated phosphoinositides also regulates cell adhesion and the actin cytoskeleton (29). Previously, we demonstrated that platelet-derived growth factor (PDGF) activation of PI 3-kinase induced the restructuring of focal adhesion plaques and reorganization of the actin cytoskeleton (28). The restructuring of focal adhesions was characterized by the loss of α-actinin and vinculin, whereas integrin receptors, talin and paxillin, remained localized to plaque structures. PtdIns(3,4,5)-P$_3$ was found to bind to α-actinin in PDGF-treated cells, correlating with redistribution from focal adhesions and reorganization of the actin cytoskeleton. Furthermore, the binding of PtdIns(3,4,5)-P$_3$ disrupted the interaction of α-actinin with the cyttoplasmic tail of the integrin β subunit. Based on the data described above, we proposed that PtdIns(4,5)-P$_2$ binding to α-actinin was involved in the formation of focal adhesions and stress fibers, whereas PtdIns(3,4,5)-P$_3$ binding resulted in the disassembly of these structures. In order to understand the contrasting roles of these two phosphoinositides, we investigated the regulation of α-actinin by PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ in vitro and in cultured cells. To our surprise, the results demonstrate that both PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ inhibit the bundling activity of α-actinin regulating stress fibers in the cell.

EXPERIMENTAL PROCEDURES

Reagents—Phosphoinositides were purchased from Echelon Research Laboratories (Salt Lake City, UT) and Matreya (State College, PA).

Proteins and DNA Constructs—α-Antinin was purified from chicken gizzard as described previously (30). GST-α-actinin fragments were expressed and purified as described previously (31). The expression plasmid encoding His-tagged CH2 domain (His-CH2) was constructed by amplifying the DNA encoding amino acids 138–269 of human α-actinin by PCR using the following primers, forward, 5'-TAA TCT AGA GAC ATC TCC GTG GAA GAG ACT TCA; backward, 5'-GTC AAA CTC GAG CTG GCC TCC AGA GAA GGA GTG; EGFP-α-actinin (15) was the template for amplification. The PCR fragment was then cloned into the XhoI and Xbal restriction sites of the His-expressed tagged sequence of pPROEX HTb (Invitrogen). Point mutants were produced in EGFP-α-actinin using QuikChange Site-directed Mutagenesis Kit (Stratagene) as described previously (27). The α-actinin gene was then subcloned into the HindIII restriction site of pPROEX HTb (Invitrogen). Nucleotide sequences were confirmed by sequence analysis. His-tagged proteins were expressed in BL21 bacteria and purified using nickel-nitrilotriacetic acid resin (Qiagen) following procedures described by the manufacturer.

Solid-phase Binding Assay—Phosphoinositide binding to α-actinin was assayed as described previously (33). Briefly, polystyrene 96-well microtiter plates were coated with 2 nmol of PtdIns(3,4,5)-P$_3$ or PtdIns(4,5)-P$_2$ in 85% ethanol and the solutions dried overnight. Plates were then blocked with 5% fatty acid-free BSA (Sigma) in TBS (20 mm Tris, pH 8.0, 0.15 M NaCl) for 1 h at room temperature. Wells were incubated with 1 μM α-actinin or GST-α-actinin fragment in blocking buffer for 1 h at room temperature. The wells were washed and incubated with monoclonal anti-α-actinin (Sigma) or anti-GST (Santa Cruz Biotechnology). The wells were then washed, incubated with the appropriate secondary antibody, washed, and developed with TMB substrate solution (Pierce). Absorbance at 450 nm was measured using a BIO-TEK Microplate Autoreader.

Protein-Lipid Overlay Assay—Nitrocellulose membranes containing immobilized phospholipids were purchased from Echelon Research Laboratories. Assays were carried out following manufacturer’s protocols incubating the membranes with 1 μg/ml α-actinin for 1 h at room temperature. Binding was detected by standard Western blotting procedures using polyclonal anti-α-actinin (Sigma) and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Membranes containing PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ were also used for the overlay assay.

In these experiments, PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ were immobilized by spotting 125 pmol of the phosphoinositides in 50% ethanol onto nitrocellulose (Hybond-C Extra, Amersham Biosciences). The membranes were allowed to dry for 1 h at room temperature prior to incubation with α-actinin.

Fluorescence Emissions Spectrometry—Fluorescence spectra were recorded using an SLM 8000C spectrofluorometer with protein concentrations of 2.5 μM in 10 mM HEPES, pH 7.0, 1 mM EDTA, at 30 °C. Excitation was carried out at 295 nm with a bandwidth of 8 nm, and the emission spectra were recorded with a bandwidth of 8 nm. The fluorescence spectrum of His-CH2 was first measured alone in a volume of 250 μl. Then 15 μl of phosphoinositide in water was added to achieve a final concentration of 30 μM, and measurement of the fluorescence spectrum was repeated at 120–140 °C. Phosphoinositides alone did not have any appreciable fluorescence.

F-actin Bundling Assay—The binding of F-actin was determined by sedimentation assays (34). Following a 15-min incubation with PtdIns(4,5)-P$_2$ or PtdIns(3,4,5)-P$_3$, α-actinin (0–5 μM) was added to the F-actin (10.4 μM), incubated for 30 min, and centrifuged at 10,000 × g for 30 min. All incubations were carried out at room temperature. The supernatant and pellet were separated, and the proteins were analyzed by electrophoresis. Actin was detected by Gelcode Blue (Pierce) staining and quantified by densitometry.

F-actin Binding Assay—The binding of GST-α-actinin (1–269) to F-actin was examined using the co-sedimentation assay described previously (35). Following a 15-min incubation with PtdIns(4,5)-P$_2$ or PtdIns(3,4,5)-P$_3$, GST-α-actinin (1–269) (1 μM) was added to the F-actin (10.4 μM), incubated for 30 min, and centrifuged at 100,000 × g for 30 min. All incubations were carried out at room temperature. The supernatant and pellet were separated, and the proteins were subjected to electrophoresis. GST-α-actinin (1–269) and actin were detected by Gelcode Blue staining and quantified by densitometry.

Cell Culture and Fluorescence Microscopy—Rat embryonic fibroblasts and U-87MG glioblastoma cells (ATCC) were cultured as described previously (28). Cells were transfected with pEGF-α-actinin wild-type and mutants using Tfx20 (Promega) or FuGENE 6 (Roche Applied Science) following the manufacturer’s protocols. One day after transfection, cells were prepared for fluorescence microscopy, cells were seeded into ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM Na$_3$VO$_4$, 1% Triton X-100, 0.5% Nonidet P-40, 30 mM sodium pyrophosphate, 50 mM Na$_3$F, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) as described previously (28). Digital images were captured using a Zeiss axiovert 100S microscope equipped with a Photometrics CoolSNAP HQ CCD camera controlled by MetaMorph software. The lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and protein from the supernatant and pellet were separated by electrophoresis and immunoblotted with anti-GFP (Santa Cruz Biotechnology), anti-α-actinin (Chemicon), or anti-actin (Sigma).

RESULTS

Select Phosphoinositides Bind the CH2 Domain of α-Antinin—In order to characterize the phosphoinositide-binding site on α-actinin, PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ binding was examined using GST fusion proteins containing various fragments of α-actinin (Fig. 1). By using a solid-phase binding assay, we found that only GST-α-actinin (1–269) bound specifically to PtdIns(4,5)-P$_2$ or PtdIns(3,4,5)-P$_3$ (Fig. 2A). In order to examine the affinity of α-actinin for PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$, the solid-phase binding assay was repeated in the presence of increasing concentrations of soluble phosphoinositides (Fig. 2B). Both PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ inhibited the binding of GST-α-actinin (1–269) to immobilized PtdIns(3,4,5)-P$_3$ in a concentration-dependent manner with an approximate IC$_{50}$ of 5 μM. The affinity determined for PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ binding to α-actinin is similar to that of the Src homology 2 domain of PI 3-kinase (K$_{d}$ = 23 μM for PtdIns(3,4,5)-P$_3$) which has a similar phosphoinositide binding sequence (36).

Although we have shown that α-actinin interacts primarily with PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ in the cell (28), phospholipid binding specificity was examined to further understanding of the chemistry of the interaction between the molecules. A protein-lipid overlay assay was used to probe an array of phospholipids immobilized to a nitrocellulose membrane.
α-Actinin interacted with the phospholipids in the following order of specificity: PtdIns (5)-P > PtdIns (3, 5)-P_2 > PtdIns (4)-P > PtdIns(3,4,5)-P_3 ~ PtdIns(4,5)-P_2 > phosphatidylserine > PtdIns (3, 4)-P_2 (Fig. 3A). Little or no binding was observed for lysophosphatidic acid, lysophosphocholine, PtdIns, PtdIns (3)-P, phosphatidylethanolamine, phosphatidylcholine, sphingosine 1-phosphate, and phosphatidic acid.

In order to examine the interaction of phosphoinositides with this site within the full-length protein, human α-actinin-1 was expressed as a His-tagged protein and purified. The putative PtdIns(4,5)-P_2 binding sequence in human α-actinin-1 is highly conserved corresponding to residues 158–174 (TAPYKN-VNIQNFHISWK) within the CH2 domain. By using site-directed mutagenesis, we replaced Lys162 (K162I) and Lys 174 (K174I) with isoleucine. We also produced a protein mutated at lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. B, the binding of His-α-actinin wild-type and mutants to PtdIns(4,5)-P_2 and PtdIns(3,4,5)-P_3 was quantified using standard densitometry techniques. n = 4 ± S.E. C, fluorescence spectra of His-CH2 (2.5 μM) in the absence and presence of PtdIns(4,5)-P_2 and PtdIns(3,4,5)-P_3 (50 μM). Phosphoinositides alone did not have any appreciable fluorescence. Results are the average of four separate experiments.
all three basic amino acid residues within the phosphoinositide-binding site of human α-actinin, replacing Lys$^{162}$ and Lys$^{174}$ with isoleucine and His$^{174}$ with leucine (K162I/H170L/K174I). Mutation of the basic residues within the proposed phosphoinositide-binding site resulted in inhibition of α-actinin binding to all of the phosphoinositides (Fig. 3A). Various concentrations (125 pmol to 1 nmol) of PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ were spotted onto nitrocellulose membranes and incubated with α-actinin. Concentration-dependent binding of α-actinin to immobilized PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ was observed (data not shown). The overlay assays were repeated quantifying the relative binding of wild-type and mutant α-actinin to PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ (Fig. 3B). Binding to PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ was inhibited by mutation of Lys$^{162}$ 40%, Lys$^{174}$ 30%, and Lys$^{162}$/His$^{170}$/Lys$^{174}$ 70%. For all the proteins, the mutations were more effective at inhibiting the binding to PtdIns(4,5)-P$_2$ compared with PtdIns(3,4,5)-P$_3$.

Because Trp$^{173}$ lies within the proposed binding motif, we hypothesized that phosphoinositide binding would influence the environment of this residue resulting in a change in fluorescence. Following excitation at 295 nm, His-CH$_2$ exhibited emission spectra typical for tryptophan with a maximum fluorescence at 330 nm (Fig. 3C). His-CH$_2$ fluorescence was quenched 20% upon addition of PtdIns(3,4,5)-P$_3$, whereas PtdIns(4,5)-P$_2$ was only quenched 10%. Increased fluorescence from 360 to 430 nm of the spectra was also observed in the presence of both phosphoinositides. These results confirm that PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ bind to the CH$_2$ domain of α-actinin. The difference in the ability of the phosphoinositides to quench fluorescence suggests that PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ differentially influence the structure of the CH$_2$ domain.

**Phosphoinositides Inhibit Bundling by Blocking the Binding of α-Actinin to F-Actin**—To assess the effects of phosphoinositides on α-actinin bundling activity, we used a low speed sedimentation assay (34). A preliminary study in the absence of phosphoinositides showed that the amount of actin bundled increased with the concentration of α-actinin, saturating at 5 μM (data not shown). Approximately 50% of the actin filaments bundled at 1 μM α-actinin, so this concentration was chosen for the assays to maximize sensitivity to both stimulation and inhibition of bundling activity by the phosphoinositides. Using this assay, both PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ inhibited α-actinin bundling activity in a concentration-dependent manner, with PtdIns(3,4,5)-P$_3$ being more effective (Fig. 4). In experiments comparing the influence of different phosphoinositides, α-actinin bundling activity was inhibited by 50 μM PtdIns(4)-P 34.7%, PtdIns(4,5)-P$_2$ 54.1%, and PtdIns(3,4,5)-P$_3$ 91.9% ($n=2$). To test whether inhibition of bundling was due to a direct effect on actin binding, we carried out co-sedimentation assays (35) using GST-α-actinin (1–269). As seen in Fig. 5, both PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ inhibited the binding of the GST fusion protein to actin filaments. The concentration dependence was similar to that seen for the inhibition of bundling, with PtdIns(3,4,5)-P$_3$ again being more effective. Finally, we found that the acyl chains are important for inhibition, as diC$_5$ derivatives of PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ did not inhibit the binding of GST-α-actinin (1–269) to F-actin until concentrations exceeded 50 μM (data not shown). Furthermore, the inositol head groups of the phosphoinositides, Ins(1,4,5)-P$_3$, and Ins(1,3,4,5)-P$_4$, did not have any effect on GST-α-actinin (1–269) binding to F-actin at concentrations as high as 500 μM (data not shown).

**Phosphoinositide Binding to α-Actinin Regulates Actin Stress Fibers**—To determine the influence of phosphoinositide binding on α-actinin function in mammalian cells, the wild-type and mutant α-actinin genes were expressed as GFP-tagged proteins in rat embryonic fibroblasts (REFs). Expression of the GFP-α-actinin was observed in −20% of the cells. As expected (37), wild-type GFP-α-actinin behaved as endogenous α-actinin, localizing to focal adhesions and, with a beaded staining pattern, along actin stress fibers (Fig. 6A). The K162I and K174I mutants were indistinguishable from wild type (data not shown). K162I/H170L/K174I GFP-α-actinin was also observed in focal adhesions, but staining along actin filament bundles was increased altering the organization of the actin cytoskeleton (Fig. 6, B and B”). Furthermore, abnormal tangles of bundled actin filaments were observed in the cytoplasm of some cells (Fig. 6, C and C”). Staining with rhodamine-phalloidin demonstrated that the wild-type and mutant GFP-α-actinin co-localized with F-actin (Fig. 6, A’ and C’). Based on these observations and the results of the bundling assays described above, it appeared that with a reduced affinity for phosphoinositides α-actinin was displaying excessive bundling activity. Because the amino acids replaced were located within the actin-binding domain, it was possible that the mutations in α-actinin were responsible for the increase in bundling activity observed in the cells. Therefore, the *in vitro* bundling activity of His-α-actinin wild-type and mutants was compared (Fig. 7). Similar amounts of actin were observed in the pellets for all four proteins demonstrating that the mutations did not directly alter α-actinin bundling activity.

Fig. 4. PtdIns(4,5)-P$_2$ and PtdIns(3,4,5)-P$_3$ inhibit α-actinin bundling activity. A, chicken gizzard α-actinin (1 μm) was preincubated with the specified concentration of PtdIns(4,5)-P$_2$ or PtdIns(3,4,5)-P$_3$ for 15 min at room temperature. α-Actinin was then added to a solution of actin filaments (10.4 μm), incubated for 30 min at room temperature, and centrifuged at 10,000 × g. Proteins from the supernatant (S) and the pellet (P) were separated by electrophoresis and detected by Gelcode Blue staining. B, the percentage of total actin in the pellet was quantified using standard densitometry techniques. $n=3$–4 ± S.E.
**Phosphoinositide Regulation of α-Actinin**

**DISCUSSION**

Several reports have demonstrated that α-actinin binds to specific phospholipids (25–27, 38–41); however, these studies did not determine the following: 1) the specificity of α-actinin interaction with phosphoinositides, 2) the location of the phosphoinositide-binding site, 3) the regulation of α-actinin function by phosphoinositide binding, and 4) the influence of phosphoinositide binding to α-actinin on the actin cytoskeleton and focal adhesions. In this study, we characterized the specificity of α-actinin binding to various phospholipids using a protein-lipid overlay procedure (Fig. 3A). α-Actinin appeared to bind to PtdIns(4,5)-P₂ and PtdIns(3,4,5)-P₃ with equal affinity, and a solid-phase binding assay yielded IC₅₀ ~5 μM for both phosphoinositides (Fig. 2B). Interestingly, α-actinin bound to PtdIns (5)-P, PtdIns (3, 5)-P₂, and PtdIns (4)-P, with the greatest affinity. Interaction of α-actinin with PtdIns (5)-P and PtdIns (3,5)-P₂ has not been demonstrated in cells; however, the high relative affinity suggests that further studies are warranted to determine the role of these phosphoinositides, which exist at low concentrations in the cell (29), in the regulation of α-actinin. Although we have detected PtdIns (4)-P binding to α-actinin in cells (28), the levels were significantly lower than PtdIns(4,5)-P₂ and were potentially the result of PtdIns(4,5)-P₂ dephosphorylation during the immunoprecipitation procedure. α-Actinin bound to phosphoinositides phosphorylated on the 4th and 5th positions of the inositol head group suggesting that the protein interacts with these phosphates. In the case of PtdIns (3,5)-P₂ and PtdIns(3,4,5)-P₃, phosphorylation on the 3rd position did not influence binding; however, no binding to PtdIns (3)-P was observed indicating that this phosphate is not sufficient for interaction with α-actinin.

GST fusion protein fragments and site-directed mutagenesis was used to demonstrate that phosphoinositides bind to the CH2 domain of α-actinin (Figs. 1–3). These results were consistent with those of Fukami et al. (27), who localized the PtdIns(4,5)-P₂ binding domain of α-actinin to amino acids 168–184 of the chicken protein. Presumably, the mutations inhibited phosphoinositide binding to α-actinin by directly replacing the amino acids required for electrostatic interactions between the two molecules or indirectly through structural modification which inhibited access of the phosphoinositides to the binding site.

Bundling and co-sedimentation assays were used to examine the effect of phosphoinositide binding on α-actinin function. We found that both PtdIns(4,5)-P₂ and PtdIns(3,4,5)-P₃ inhibited α-actinin bundling activity by blocking interaction of the actin-binding domain with actin filaments (Figs. 4 and 5), similar to that reported for vinculin (23), dystrophin (42), and filamin (32). But this contrasts with the report of Fukami et al. (27) that PtdIns(4,5)-P₂ significantly increased the cross-linking activity of smooth muscle α-actinin. Although different assays were used to measure α-actinin bundling activity, we do not know the reason for the discrepancy between the two studies. We also found that the ability to inhibit bundling activity increased with the number of phosphates on the inositol head group (PtdIns(3,4,5)-P₃ > PtdIns(4,5)-P₂ > PtdIns (4)-P). Because the 4- and 5-phosphate groups appear to be involved in binding as discussed above, we propose that the 3-phosphate group has the greatest influence on regulating α-actinin bundling activity. Further studies are necessary to understand how the binding of phosphoinositides regulates α-actinin structure.

**Fig. 5.** PtdIns(4,5)-P₂ and PtdIns(3,4,5)-P₃ inhibit α-actinin binding to F-actin. A, GST-α-actinin-(1–269) κ m was preincubated with the specified concentration of PtdIns(4,5)-P₂ or PtdIns(3,4,5)-P₃ for 15 min at room temperature. GST-α-actinin-(1–269) was then added to a solution of actin filaments (10.4 μM), incubated for 30 min at room temperature, and centrifuged at 100,000 × g. Proteins from the supernatant (S) and the pellet (P) were separated by electrophoresis and detected by Gelcode Blue staining. B, the percentage of total GST-α-actinin-(1–269) in the pellet was quantified using standard densitometry techniques. n = 2–9 ± S.E.
Electrophoresis and detected by immunostaining with anti-GFP. Proteins from the supernatant (WT and detected by Gelcode Blue staining. The supernatant (S) and the pellet (P) were separated by electrophoresis and detected by Gelcode Blue staining. WT, wild type. Results are representative of two separate experiments.

Mutations that reduce binding of phosphoinositides to α-actinin do not influence actin bundling. His-α-actinin (2.5 μM) was added to a solution of actin filaments (10.4 μM), incubated for 30 min at room temperature, and centrifuged at 10,000 × g. Proteins from the supernatant (S) and the pellet (P) were separated by electrophoresis and detected by Gelcode Blue staining. WT, wild type. Results are representative of two separate experiments.

Loss of phosphoinositide binding to α-actinin increases F-actin bundling. Transiently transfected REFs (A and B) or U-87MGs (C) expressing GFP-α-actinin wild-type or mutants were scraped into lysis buffer and centrifuged at 10,000 × g for 10 min. Proteins from the supernatant (S) and the pellet (P) were separated by electrophoresis and detected by immunostaining with anti-GFP (A), anti-α-actinin (B), or anti-actin (C). Results are representative of 3–7 separate experiments.

Mammalian cells expressing an α-actinin mutant with decreased phosphoinositide affinity were examined to determine the influence on the actin cytoskeleton and focal adhesions. The results demonstrated that inhibition of phosphoinositide binding to α-actinin resulted in excessive bundling of actin filaments in the cell (Figs. 6 and 8). The data suggest that phosphoinositide binding to α-actinin regulates actin stress fibers by controlling the extent of microfilaments bundling. We did not observe differences in the localization of wild-type and mutant GFP-α-actinin proteins to focal adhesions (Fig. 6, A–C), suggesting that phosphoinositides may not influence α-actinin within the focal adhesions of unstimulated fibroblasts.

Prior to this study, we and others had proposed that PtdIns(4,5)-P₂ was binding to α-actinin in unstimulated cells and regulating its localization and function within focal adhesions and along stress fibers. In contrast, the data reported here indicate that the population of α-actinin interacting with PtdIns(4,5)-P₂ is not localized to focal adhesions and along stress fibers. Instead, PtdIns(4,5)-P₂ binding was found to inhibit α-actinin binding to actin filaments and did not appear to be required for localization to focal adhesions. We propose that by inhibiting binding to actin filaments and localizing α-actinin to the plasma membrane, PtdIns(4,5)-P₂ controls the amount of α-actinin in the cytoplasm available for bundling, regulating organization of the actin cytoskeleton.

In PDGF-stimulated fibroblasts, we demonstrated previously (28) that PtdIns(3,4,5)-P₃ binding to α-actinin disrupted interaction with the integrin β subunit, correlating with the redistribution of α-actinin from focal adhesions. In the current study, we show that PtdIns(3,4,5)-P₃ binding also blocks the interaction of α-actinin with actin filaments inhibiting bundling activity (Figs. 4 and 5). Therefore, we propose that the binding of PtdIns(3,4,5)-P₃ to α-actinin plays an important role in PDGF-induced reorganization of the actin cytoskeleton by disrupting the link between integrins and α-actinin as well as the actin bundling activity of α-actinin.

Localization appears to be a critical factor for the regulation of proteins by phosphoinositides and may explain how PtdIns(4,5)-P₂ and PtdIns(3,4,5)-P₃ can both inhibit α-actinin binding to actin filaments yet differentially regulate the actin cytoskeleton. Based on the data presented in this and the previous report (28), PtdIns(4,5)-P₂ and PtdIns(3,4,5)-P₃ appear to be binding to and regulating different populations of α-actinin. We propose that α-actinin is interacting with PtdIns(4,5)-P₂ constitutively present in the plasma membrane in unstimulated cells, whereas PtdIns(3,4,5)-P₃ production occurs transiently and locally, binding to α-actinin in focal adhesions and along stress fibers. Further studies are required in order to determine the precise regulation of α-actinin by phosphoinositides within the cell. The α-actinin mutants with decreased affinity for phosphoinositides described in this study will aid in accomplishing this goal.
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REFERENCES
1. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–518
2. Gumbiner, B. M. (1996) Cell 84, 345–357
3. Blanchard, A., Ohanian, V., and Critchley, D. (1989) J. Muscle Res. Cell Motil. 10, 280–289
4. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) J. Cell Biol. 111, 721–729
5. Pavalko, F. M., Otey, C. A., Simon, K. O., and Burridge, K. (1991) Biochem. Soc. Trans. 19, 1065–1069
6. Gluck, U., Kwiatkowski, D. J., and Ben-Ze’ev, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 383–387
7. Gluck, U., and Ben-Ze’ev, A. (1994) J. Cell Sci. 107, 1773–1782
8. Yianne, J., Scheffzek, K., Young, P., and Saraste, M. (2001) Structure 9, 597–604
9. Djuricic-Carugo, K., Young, P., Gautel, M., and Saraste, M. (1999) Cell 98, 537–546
10. Tang, J., Taylor, D. W., and Taylor, K. A. (2001) J. Mol. Biol. 310, 845–858
11. Banuelos, S., Saraste, M., and Carugo, K. D. (1998) Structure 6, 1419–1431
12. Keep, N. H., Winder, S. J., Moore, C. A., Walker, S., Norwood, P. L., and Kendrick-Jones, J. (1999) Structure 7, 1539–1546
13. Norwood, F. L., Sutherland-Smith, A., Leplant, S., and Kendrick-Jones, J. (2000) Structure 8, 481–491
14. Lazarides, E., and Burridge, K. (1975) Cell 6, 289–298
15. Rajfur, Z., Roy, P., Otey, C., Romner, L., and Jacobson, K. (2002) Nat. Cell Biol. 4, 286–293
16. Langanger, G., Morremans, M., Danee ls, G., Sobieszek, A., De Brabander, M., and De Mey, J. (1986) J. Cell Biol. 102, 200–209
17. Chrzanowska-Wodnicka, M., and Burridge, K. (1996) J. Cell Biol. 133, 1403–1415
18. Craig, S. W., and Johnson, R. P. (1996) Curr. Opin. Cell Biol. 8, 74–85
19. Gilmore, A. P., and Burridge, K. (1996) Nature 381, 531–535
20. Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zencu, R., Veronese, S., Chang, S., Guo, J., Wenk, M. R., and De Camilli, P. (2002) Nature 420, 85–89
21. Ling, K., Doughman, R. L., Firestone, A. J., Bance, M. W., and Anderson, R. A. (2002) Nature 420, 89–93
22. Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A., Block, M. R., and Albigeos-Bizo, C. (2001) J. Biol. Chem. 276, 21217–21227
23. Steinle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999) J. Biol. Chem. 274, 18414–18420
24. Couchman, J. R., Vogt, S., Lim, S. T., Lim, Y., Oh, E. S., Prestwich, G. D., Theibert, A., Lee, W., and Woods, A. (2002) J. Biol. Chem. 277, 49296–49303
25. Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992) Nature 359, 150–152
26. Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) J. Biol. Chem. 269, 1518–1522
27. Fukami, K., Sawada, N., Endo, T., and Takenawa, T. (1996) J. Biol. Chem. 271, 2646–2650
28. Greenwood, J. A., Theibert, A. B., Prestwich, G. D., and Murphy-Ullrich, J. E. (2000) J. Cell Biol. 150, 627–642
29. Vanhaesebroeck, B., Levers, S. J., Ahmadi, K., Timms, J., Kato, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001) Annu. Rev. Biochem. 70, 535–602
30. Feramisco, J. R., and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199
31. McGreggor, A., Blanchard, A. D., Rowe, A. J., and Critchley, D. R. (1994) Biochem. J. 301, 225–233
32. Furuhashi, K., Inagaki, M., Hatano, S., Fukami, K., and Takenawa, T. (1992) Biochem. Biophys. Res. Commun. 184, 1261–1265
33. Chen, R., Kang, V. H., Chen, J., Shope, J. C., Tsuruhina, J., DeWald, D. B., and Prestwich, G. D. (2002) J. Histochem. Cytochem. 50, 697–708
34. Petrucci, T. C., and Morrow, J. S. (1997) J. Cell Biol. 105, 1355–1363
35. Way, M., Pope, B., and Weeds, A. G. (1992) J. Cell Biol. 119, 835–842
36. Ching, T. T., Lin, H. P., Yang, C. C., Oliveira, M., Lu, P. J., and Chen, C. S. (2001) J. Biol. Chem. 276, 43932–43938
37. Edlund, M., Lotano, M. A., and Otey, C. A. (2001) Cell Motil. Cytoskeleton 48, 190–200
38. Goldmann, W. H., Teodoridis, J. M., Sharma, C. P., Alonso, J. L., and Isenberg, G. (1999) Biochem. Biophys. Res. Commun. 264, 225–229
39. Han, X., Li, G., and Lin, K. (1997) Biochemistry 36, 10364–10371
40. Han, X., Li, G., and Lin, K. (1998) Biochemistry 37, 10730–10737
41. Burn, P., Rotman, A., Meyer, R. K., and Burger, M. R. (1985) Nature 314, 469–472
42. Mejean, C., Lebart, M. C., Roustan, C., and Benyamin, Y. (1995) Biochem. Biophys. Res. Commun. 216, 152–158