Identification of a Phosphatidylinositol 4,5-Bisphosphate-binding Site in Chicken Skeletal Muscle α-Actinin*

(Received for publication, August 24, 1995, and in revised form, November 17, 1995)

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We previously reported that phosphatidylinositol 4,5-bisphosphate (PIP$_2$) dramatically increases the gelating activity of smooth muscle α-actinin (Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992) Nature 359, 150–152) and that the hydrolysis of PIP$_2$ on α-actinin by tyrosine kinase activation may be important in cytoskeletal reorganization (Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) J. Biol. Chem. 269, 1518–1522). Here we report that a proteolytic fragment with lysylendopeptidase comprising amino acids 168–184 (TAPYRNVIQNPHLSWK) from striated muscle α-actinin contains a PIP$_2$-binding site. A synthetic peptide composed of the 17 amino acids remarkably inhibited the activities of phospholipase C (PLC)-γ1 and -δ1. Furthermore, we detected an interaction between PIP$_2$ and a bacterially expressed α-actinin fragment (amino acids 137–259) by PLC inhibition assay. Point mutants in which arginine 172 or lysine 184 of α-actinin were replaced by isoleucine reduced the inhibitory effect on PLC activity by nearly half. Direct interactions between PIP$_2$ and the peptide (amino acids 168–184) or the bacterially expressed protein (amino acids 137–259) were confirmed by enzyme-linked immunosorbent assay. We also found this region homologous to the sequence of the PIP$_2$-binding site in spectrin and the pleckstrin homology domains of PLC-δ1 and Grb7. Synthetic peptides from the homologous regions in spectrin and PLC-δ1 inhibited PLC activities. These results indicate that residues 168–184 comprise a binding site for PIP$_2$ in α-actinin and that similar sequences found in spectrin and PLC-δ1 may be involved in the interaction with PIP$_2$.

Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is a trace phospholipid, which generates two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol that respond to phospholipase C (PLC) activation by a variety of physiological stimuli. Inositol 1,4,5-trisphosphate and diacylglycerol are known to mobilize Ca$^{2+}$ from the endoplasmic reticulum and to activate protein kinase C (PKC), respectively (3, 4).

In addition to its role as a signal-generating lipid, PIP$_2$ has been shown to modulate the functions of various proteins such as PKC (5, 6), μ-calpain (7), ADP-ribosylation factor 1 (8), and phospholipase D (9). PIP$_2$ also binds to actin-regulating proteins such as profilin (10), coflin (11), gelsolin (12), gCap (13), and α-actinin (1) and regulates the functions of these proteins. When PIP$_2$ binds to α-actinin, which is an actin cross-linking protein, it further activates actin gelation by α-actinin (1). It is noteworthy that profilin plays crucial roles in tyrosine kinase-coupled PIP$_2$ hydrolysis. Under resting conditions, PLC-γ1 causes little hydrolysis of profilin-bound PIP$_2$, but PLC-γ1 phosphorylated by tyrosine kinases overcomes the inhibitory effect by profilin and hydrolyzes bound PIP$_2$ (14). It has also been shown that the decrease in PIP$_2$ bound to α-actinin and vinculin by treatment with platelet-derived growth factor correlates with the depolymerization of actin (2). All these data suggest that the amount of PIP$_2$ in the actin-binding protein regulates the development of stress fibers when the cells are stimulated.

α-Actinin was originally discovered in skeletal muscle as a protein factor promoting the superprecipitation of actomyosin and inducing the formation of actin fibers (15). The fact that α-actinin is found at focal contacts where actin is anchored to a variety of intercellular structures in non-muscle cells suggests that α-actinin plays some role in the linkage between the plasma membrane and actin. We previously reported that α-actinin from skeletal muscle contains large amounts of PIP$_2$, whereas that from smooth muscle contains little (1). Interestingly, the addition of PIP$_2$ to smooth muscle α-actinin increases the gelation activity of actin to the level produced by skeletal muscle α-actinin, suggesting that PIP$_2$ plays important roles in the organization of the cytoskeleton.

Recently, the preckstrin homology (PH) domain has been found in a variety of functional proteins (16), including protein kinases, substrates for kinases, regulators of small G proteins, PLC isozymes, and cytoskeletal proteins. This domain has been reported to bind to PIP$_2$ (17), although it also associates with the βγ subunit of trimeric G proteins (18, 19) and PKC (20). In that case, PIP$_2$ is thought to act as a target for PH domain-containing proteins in membranes.

To understand the role of PIP$_2$ in protein functioning or in protein-protein interactions, it is important to identify the PIP$_2$-binding site in proteins. We describe here that amino acids 168–184 in chicken skeletal muscle α-actinin comprise a PIP$_2$-binding site that and basic amino acids, arginine 172 and lysine 184, are important for this interaction. A region homologous to the PIP$_2$-binding site in α-actinin is also found in spectrin and the PH domains of several proteins including PLC-δ1 and Grb7.

EXPERIMENTAL PROCEDURES

Materials—Striated muscle α-actinin was purified from chicken pectoralis muscle according to the methods described by Feramisco and Burridge (21). Mouse monoclonal antibody to PIP$_2$ (18) was developed as described previously (22). Transformers site-directed mutagenesis kit and QIAexpress vector system were obtained from Clontech (Palo Alto, CA) and Qiagen. PIP$_2$ was prepared from bovine spinal cords by the method of Schacht (23). [3H]PIP$_2$ (7.6 Ci/mmol) was from DuPont NEN. DEAE-cellulose (Whatman), cellulose phosphate (Whatman), the Hi-
Sequences of the Proteolytic Digestion Fragments of α-Actinin—

Purified striated α-actinin was digested with α-chymotrypsin at an enzyme-to-substrate ratio of 1:200 (mol/mol) in 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 1 mM 2-mercaptoethanol at 37 °C for the indicated times. The proteolytic fragments of α-actinin were electrophoresed on 8.5% SDS-polyacrylamide gels and the gels were subjected to Western blot analysis with anti-PIP2 antibody as described previously (2).

Cleavage with lysylendopeptidase was carried out as follows. α-Actinin (1 mg/ml) was digested with 1:200 (mol/mol) lysylendopeptidase at 37 °C. The digest was separated by high performance liquid chromatography on a C18 reverse-phase column. For the detection of PIP2-bound peptide, every fraction, including the front, was lyophilized, dot-blotted on nitrocellulose, and stained with anti-PIP2 antibody. The amino acid sequence of the PIP2-containing fragment was determined with a protein sequencer (ABI 477A/210A).

Peptide Synthesis—Four of the following sequences were synthesized. Peptide I, WKEQKQRTK; peptide II, GERLKPDPGR-KMRFHK; peptide III, HHRPPIPILIDYSKLNKDD; and peptide IV, TAPYRNVIQNFHLSWK.

Preparation of PLC Enzymes—PLC-γ and PLC-ε were partially purified by the methods described previously (25). Bovine thymus (900 g) was homogenized in 1.8 liters of buffer containing 50 mM Tris-HCl, pH 7.6, 0.25 mM sucrose, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM diisopropyl fluorophosphate, 10 μM leupeptin, 2 μM bestatin, and 2 μM pepstatin, and centrifuged at 35,000 x g at 4 °C. The resulting supernatants were chromatographed on five chromatographic steps, including DEAE-cellulose (8 x 40 cm), cellulose phosphate (4 x 25 cm), HiTrap-heparin (10 ml), Sephadex 26/60, and MonoQ 5/5. PLC-γ or PLC-ε was purified by DEAE-cellulose and HiTrap-heparin.

PLC Assay—PLC activity was assayed by the methods described previously (25). In brief, a reaction mixture containing 50 mM Mes buffer, pH 6.5, 400 μM CaCl2, 1 mM bovine serum albumin, 20 μM PIP2, 10,000 cpm of [3H]PIP2, and PLC-γ or PLC-ε was incubated at 37 °C for 10 min in the presence or absence of various proteins or peptides. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1) and radioactive insoluble trisphosphate was extracted with 1 M HCl. The radioactivity was measured by a scintillation counter.

Construction of α-Actinin Mutants—Four point mutants, with mutations of Arg166, Arg172, Lys184, and Arg195 (designated as αAn.166.1, αAn.172.2, αAn.184.2, and αAn.195.1, respectively). The mutated nucleotide sequences were confirmed by the methods described previously (29) are shown in C (24) (PEP-spectrin), KLFQVKGRR.

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Determination of a PIP2-Binding Site in Chicken Skeletal α-Actinin—First, we examined whether the PIP2-binding site was located in the N-terminal actin-binding domain or the C-terminal tails of α-actinin, where two homodimers bind to each other. Cleavage of α-actinin with α-chymotrypsin for the indicated times revealed that the 102-kDa α-actinin was converted to 88-, 68-, 55-, and 34-kDa fragments as previously reported (27) (Fig. 1, A and B). Two positive peaks at 37 °C were obtained. The sizes of the α-chymotryptic cleavage fragments as reported previously (29) are shown in C. Expressed α-Actinin Fragments by Enzyme-linked Immunosorbent Assay—Peptides (I-IV) or proteins were coated on the 96-well multiplates overnight at room temperature. After the plates were blocked with 2% bovine serum albumin in phosphate-buffered saline, various amounts of PIP2 were added to each well and incubated at room temperature for 30 min. After washing the plates with phosphate-buffered saline containing 0.05% Tween 20, antibody against PIP2 was added to wells, followed by the treatment with peroxidase-conjugated anti-mouse immunoglobulins. The interactions were visualized with 0.4 mg/ml orthophenylene diamine in 100 mM citrate buffer, pH 5.0.

RESULTS

Fig. 1. α-Chymotryptic cleavage pattern of chicken skeletal α-actinin. 50 μg of skeletal muscle α-actinin was digested with α-chymotrypsin (molar ratio, 1:200) at 37 °C for the indicated times as described under “Experimental Procedures.” The digests were subjected to 8.5% acrylamide gels and stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose and immunostained with anti-PIP2 antibody (B). The sizes of the α-chymotryptic cleavage fragments as reported previously (29) are shown in C. The sizes of the α-chymotryptic cleavage fragments as reported previously (29) are shown in C.
Inhibited the activities of both PLC-α1 and PLC-α2, respectively. 5K-α-actinin, chicken skeletal muscle α-actinin; SM-α-actinin, chicken smooth muscle α-actinin; spectrin, human spectrin β-chain; integrin, mouse integrin β7 subunit precursor; mRNA.CP, yeast mRNA capping protein; PLC-α1.PH, Grb7.PH, plec(N).PH, plec(C).PH, Ras-GAP.PH, and racKβ.PH are the PH domains of various proteins. Amino acids alignment was done on the basis of RXXXXXXX(H/R/K)XX(X)(W/K/R).

A computer-assisted sequence homology search revealed that this sequence is homologous to chicken smooth muscle α-actinin (88.8%), human spectrin β-chain (58.8%), yeast mRNA capping protein (37.5%), and mouse integrin β7 subunit precursor (35.7%). Moreover, we found the homologous regions in the PH domains of several proteins including PLC-α1, Grb7, pleckstrin, Ras-GAP, and racKβ (Fig. 3).

Inhibition of Phospholipase C Activity by Synthetic Peptides—To examine whether a peptide that includes a PIP2-binding site inhibits the activity of PLC, we synthesized the corresponding peptides (Fig. 4A) of α-actinin. We used 20 μM recombinant peptides as maximal soluble concentration. As shown in Fig. 5, αAn.0.1 partially inhibited the activities of PLC-γ1 (Fig. 5A) and PLC-α1 (Fig. 5B) to about 69 and 63% of the control level, respectively. These values are comparative to those obtained for α-actinin and more effective than that for peptide IV. Two point-mutated proteins, αAn.166.1 and αAn.195.1, also inhibited the activities almost as identically to αAn.0.1, while αAn.172.2 and αAn.184.2 inhibited the activity of PLC-γ1 to about 80–85% and PLC-α1 to about 82–87% of control, respectively. These results show that the basic amino acids arginine 173 and lysine 184 play important roles in the interaction between PIP2 and the α-actinin PH-binding site.

Direct Interactions of PIP2 with Peptides and Bacterially Expressed α-Actinin Fragments—To confirm whether the residues 168–184 of α-actinin contains a PIP2-binding site, we examined the effect of α-actinin purified from chicken smooth muscle on PLC activities (Fig. 4C). At maximum soluble concentration, 19.2 μM, α-actinin caused a decrease in activity down to about 70% of control. Since we found that there are regions homologous to the PIP2-binding site of α-actinin in the spectrin β-chain and the PH domains of several proteins, we also synthesized the corresponding peptides (Fig. 4A) and examined the effects of these peptides on PLC activities. We chose PLC-α1 and Grab7 on the basis of alignment, RXXXXXXX(H/R/K)XX(X)(W/K/R). A peptide from gelsolin, which is a known PIP2-binding site (24), was also synthesized. PEP-PLC-α1 strongly inhibited the activity of PLC-γ1 in a dose-dependent manner and 200 μM PEP-PLC-α1 caused a decrease in activity down to 23% of control. PEP-spectrin also caused a decrease in the activity of PLC-γ1 to 53%. On the other hand, 100 μM PEP-Grb7 and PEP-gelsolin had no significant effect on PLC-γ1 activity. Similar effects were observed when PLC-α1 was used, but the degrees of inhibition or stimulation of PLC-α1 activity were weaker than those of PLC-γ1 (Fig. 4D).

Inhibition of PLC Activity by Bacterial Expression Proteins—To clarify the precise mechanism of the interaction between PIP2 and the PIP2-binding site in α-actinin, we produced various bacterial histidine tag proteins. We examined whether these proteins inhibited PLC activities as strongly as peptide IV or α-actinin. We used 20 μM recombinant peptides as maximal soluble concentration. As shown in Fig. 5, αAn.0.1 partially inhibited the activities of PLC-γ1 (Fig. 5A) and PLC-α1 (Fig. 5B) to about 69 and 63% of the control level, respectively. These values are comparative to those obtained for α-actinin and more effective than that for peptide IV. Two point-mutated proteins, αAn.166.1 and αAn.195.1, also inhibited the activities almost as identically to αAn.0.1, while αAn.172.2 and αAn.184.2 inhibited the activity of PLC-γ1 to about 80–85% and PLC-α1 to about 82–87% of control, respectively. These results show that the basic amino acids arginine 173 and lysine 184 play important roles in the interaction between PIP2 and the α-actinin PH-binding site.

DISCUSSION

There are many reports of specific interactions between phospholipids and proteins. The C2 domains of PKC (5, 6),

![Diagram](image-url)
phospholipase A2 (28), PLC, Ras-GTPase activating protein (29), rabphilin (30), and synaptotagmin I (31, 32) have been proposed to contain phospholipid binding domains. ADP-ribosylation factor I (8), dynamin (33), myristoylated alanine-rich prokintiC substrate (34), m-calpain (7), and many actin-regulating proteins (1, 10–14) have also been shown to interact with acidic phospholipids including PIP2. These interactions induce the translocation of PKC, synaptotagmin I, and dynamin to the plasma membrane, or activate phospholipase D, ADP-ribosylation factor I, and m-calpain. Synaptotagmin I is thought to be involved in the docking and fusion steps in calcium-dependent exocytosis. Interestingly, it has become clear that PIP2 synthesis by phosphatidylinositol 4-phosphate 5-kinase is also concerned in exocytosis (35). Additional evidence for a role of PIP2 in vesicular trafficking was provided by Cantley et al. (36). They reported that PIP2 stimulates in vitro the activity of partially purified membrane phospholipase D, in which PIP2 functions as a phospholipase D cofactor (9). These results suggest that phospholipids by themselves play important roles in modulating enzyme activities and targeting for translocation.

We have shown that α-actinin from chicken striated muscle contains large amounts of PIP2 while α-actinin from chicken smooth muscle has little PIP2, but that the latter can bind to exogenous PIP2. In vitro, the addition of PIP2 dramatically stimulates the gelating activity of actin by smooth muscle α-actinin (1). Furthermore, it has been shown that the amount of PIP2 bound to α-actinin and vinculin decrease in response to platelet-derived growth factor stimulation in vivo (2). These facts suggest that α-actinin-bound PIP2 is dynamically metabolized under physiological conditions and that PIP2 by itself regulates the organization of stress fibers. Thus, we tried to clarify the binding site of PIP2 in α-actinin.

Amino acid sequences which contain PIP2-binding site in skeletal muscle α-actinin are homologous to that in chicken smooth muscle α-actinin (Fig. 3), except for the substitution of a basic amino acid, arginine, to another basic amino acid, lysine. This substitution may have no effect on PIP2-binding, but these basic amino acids seem to be very important for binding, because mutants in which either arginine 172 or lysine 184 is replaced by isoleucine partially lose their inhibitory effect on PLC activities (Fig. 5). Sequences homologous to the PIP2-binding domain in α-actinin also exist in some cytoskeletal-related proteins such as spectrin β-chain or integrin β-7 subunit precursor, although these are not yet reported as PIP2-binding proteins. On the other hand, we found no homologous sequence in gelsolin or coflin, which have been reported previously to be

![Image](4)

**Fig. 4.** Inhibition of PLC-γ1 and PLC-δ1 activities by synthetic peptides and α-actinin. Synthetic peptide sequences are shown in A. The inhibition of PLC-γ1 and PLC-δ1 activities by peptide I ( ), peptide II ( ), peptide III ( ), and peptide IV ( ) at various doses is shown in B. PLC activities were measured as described under "Experimental Procedures" using 20 μM PIP2 as a substrate. The total activity of PLC-γ1 is 5400 dpm and that of PLC-δ1 is 8600 dpm. Inhibition by peptide IV ( ) in the presence of 0.5% octyl glucoside was also examined (B). C shows the inhibition of PLC-γ1 and PLC-δ1 activities by smooth muscle α-actinin at concentrations of 0.38, and 13.1 μM. Inhibition of PLC-γ1 and PLC-δ1 by peptides corresponding to PEP-spectrin ( ), PLC-δ1 ( ), PEP-Grb7 ( ), and PEP-gelsolin ( ) is shown in D.

![Image](5)

**Fig. 5.** Inhibition of PLC-γ1 and PLC-δ1 activities by bacterial expression of PIP2-binding sites on α-actinin. Activities of PLC-γ1 (A) and PLC-δ1 (B) were measured in the presence of 20 μM various point mutants or a non-mutant as described under "Experimental Procedures."
domain is reported to associate not only with PIP2 (17), but also protein-protein or lipid-protein interactions, because the PH domain is involved in signaling of Bruton’s tyrosine kinase (20).

An arginine to cysteine substitution in the N-terminal PH domain may play a critical role in the signaling of Bruton’s tyrosine kinase. This problem remains to be solved in future.

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