Phosphoinositides differentially regulate α -actinin flexibility and function

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α -Actinin is a cell-adhesion and cytoskeletal protein that bundles actin microfilaments and links these filaments directly to integrin-adhesion receptors. Phosphoinositides bind to and regulate the interaction of α -actinin with actin filaments and integrin receptors. In the present study, we demonstrate that PtdIns(3,4,5) P_3 inhibits and disrupts α -actinin-bundling activity, whereas PtdIns(4,5) P_2 can only inhibit activity. In addition, a protease-sensitivity assay was developed to examine the flexibility of the linker region between the actin-binding domain and the spectrin repeats of α -actinin. Both phosphoinositides influenced the extent of proteolysis and the cleavage sites. PtdIns(4,5) P_2 binding decreased the proteolysis of α -actinin, suggesting a role in stabiliz-

ing the structure of the protein. In contrast, PtdIns(3,4,5) P_3 binding enhanced α -actinin proteolysis, indicating an increase in the flexibility of the protein. Furthermore, phosphoinositide binding influenced the proteolysis of the N- and C-terminal domains of α -actinin, indicating regulation of structure within both domains. These results support the hypothesis that PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 differentially regulate α -actinin function by modulating the structure and flexibility of the protein.

Key words: α -actinin, actin bundling, cytoskeleton, focal adhesion, phosphoinositide, protein flexibility.

INTRODUCTION

α -Actinin is an actin-bundling protein that plays an important role in the regulation of cell adhesion by linking actin filaments directly to integrin receptors [1–3]. The interaction of α -actinin with actin filaments and integrins must be regulated precisely for cells to carry out various processes, such as adhesion, migration and growth. α -Actinin exists as an anti-parallel homodimer, which orients the actin-binding domains at opposite ends (Figure 1), allowing each dimer to bind two actin filaments, leading to the formation of bundles [4]. The N-terminal actin-binding domain of α -actinin consists of two calponin homology (CH) domains, both of which interact directly with the actin filament [5,6]. The actin-binding domain is connected, by a flexible linker, to four spectrin repeats which mediate the interaction between the two α -actinin molecules, forming the dimer [7]. The C-terminal domain of α -actinin also appears to be connected to the spectrin repeats by a flexible region which allows this domain to bind to the linker region between the actin-binding domain and the spectrin repeats of the adjacent molecule [8].

Although the regulation of α -actinin function is not yet understood, evidence indicates that phosphoinositides play a role in controlling its activity. Phosphoinositides bind to the CH2 domain, residues 159–175 of chicken α -actinin 1, inhibiting α -actinin-bundling activity by blocking the interaction of the actin-binding domain with actin filaments [9,10]. In adherent fibroblasts, expression of an α -actinin mutant with reduced affinity for phosphoinositides resulted in an increase in cellular bundling, altering the organization of the actin cytoskeleton [10]. In addition, we demonstrated that α -actinin binds predominantly to PtdIns(4,5) P_2 in these unstimulated cells [11], and proposed that the regulation of α -actinin by PtdIns(4,5) P_2 modulates the extent of stress fibre formation [10]. In PDGF (platelet-derived growth factor)-treated fibroblasts, α -actinin binding to PtdIns(3,4,5) P_3 was also observed, correlating with the redistribution of α -actinin from focal adhesions and actin stress fibres to the Triton-X-100 soluble fraction [11]. PtdIns(3,4,5) P_3 binding was found to disrupt the interaction between α -actinin and integrins, whereas

PtdIns(4,5) P_2 had no effect [11]. This was the first evidence of differential regulation of α -actinin by phosphoinositide binding, and helps to explain how cells can maintain focal adhesions in the presence of high cellular concentrations of PtdIns(4,5) P_2 . We proposed that localized production of PtdIns(3,4,5) P_3 within the focal adhesions of stimulated cells results in the disruption of α -actinin interaction with actin filaments and integrin receptors [10,11]. Although we have demonstrated that phosphoinositide binding inhibits α -actinin-bundling activity, it is not known whether these phosphoinositides disrupt actin-filament bundles formed by α -actinin cross-linking. Therefore, in the present study, we examined the ability of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 to disrupt α -actinin-bundled actin filaments and the mechanisms by which phosphoinositides regulate the structure of α -actinin.

EXPERIMENTAL

Proteins and reagents

Chicken gizzard α -actinin, glutathione S-transferase (GST)- α -actinin fragments 1–269, 218–749 and 713–887, and His-tagged CH2 domain (His-CH2) were prepared as described previously [10]. Phosphoinositides and inositol phosphates were purchased from Matreya (State College, PA, U.S.A.) or Echelon Research Laboratories (Salt Lake City, UT, U.S.A.). Thermolysin, chymotrypsin and anti- α -actinin IgM (BM-75.2) were from Sigma. Anti- α -actinin IgG (AT6.172) was from Chemicon.

Actin-filament-bundling assays

Bundling assays were carried out as described previously [10]. Briefly, actin filaments (10.4 μ M) were incubated with α -actinin (1 μ M) for 30 min at room temperature (22 °C). Phosphoinositides (50 μ M) were added and incubated for an additional 30 min. The samples were then centrifuged at 10 000 *g* for 30 min. The supernatant and pellet were separated, and the proteins were analysed by electrophoresis. Proteins were detected by Gelcode

Abbreviations used: CH, calponin homology; GST, glutathione S-transferase.

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Figure 1 Structure of the α -actinin homodimer

The relative positions of the actin-binding domain, the four spectrin-repeats and the EF-hands motifs are shown [4].

Blue (Pierce) staining and quantified using a Kodak Image Station 440CF.

Actin-filament-binding assays

The binding of GST- α -actinin (1–269) to actin filaments was carried out as described previously [10]. Briefly, actin filaments (10.4 μ M) were incubated with GST- α -actinin (1–269) (1 μ M) for 30 min at room temperature. Phosphoinositides (50 μ M) were added and incubated for an additional 30 min. The samples were then centrifuged at 100 000 *g* for 30 min. The supernatant and pellet were separated, and the proteins were analysed by electrophoresis. Proteins were detected by Gelcode Blue staining and quantified using a Kodak Image Station 440CF.

CD spectral analysis

The CD spectra of α -actinin or the 27 kDa fragment were determined at a concentration of 5 μ M in a 1 mm path-length cell. The total concentration of amide bonds was calculated based on the protein concentration determined by measuring the A_{280} [12]. The CD of each sample was measured from 260 to 203 nm with total $A_{280} < 1.0$ to ensure sufficient light transmission. Spectra were measured at 25 °C using a model J-720 JASCO spectrometer calibrated with (+)-10-camphorsulphonic acid at two points to ensure reliability [13]. The data were collected at 1 nm intervals using an on-line PC computer, and at least six spectra were averaged, corrected by subtraction of buffer spectra (usually negligible), and then smoothed.

Proteolytic-sensitivity assay

α -Actinin, GST- α -actinin (1–269) or His-CH2 (1 μ M) were incubated in the absence or presence of phosphoinositides (0–50 μ M) for 15 min in 10 mM Hepes, pH 7.0, and 0.1 mM EDTA. Thermolysin (1:30 ratio) and 1 mM CaCl_2 were added and the incubation was continued at 30 °C for the indicated time. Experiments were also carried out by incubating with chymotrypsin (1:30) for 30 min at 30 °C. The reaction was stopped by the addition of 2 $\times$ SDS Stop buffer (250 mM Tris, pH 6.8, 2% SDS, 5 mM EGTA, 5 mM EDTA, 25 mM dithiothreitol and 10% glycerol), followed by incubation at 100 °C for 5 min. Proteins were separated by electrophoresis and stained with Gelcode Blue or transferred on to nitrocellulose and immunoblotted.

N-terminal sequencing

Following thermolysin proteolysis and electrophoresis, proteins were transferred on to PVDF membranes (Millipore). Bands were identified by Ponceau S staining, excised, and N-terminal protein sequence analysis carried out by the Genomics and Proteomics Facility in the Institute of Molecular Biology at the University of Oregon, Eugene, OR, U.S.A.

RESULTS AND DISCUSSION

PtdIns(3,4,5) P_3 , but not PtdIns(4,5) P_2 , disrupts α -actinin-bundled actin filaments

Previously, we demonstrated that phosphoinositides bind to and inhibit the bundling activity of α -actinin by blocking its interaction with actin filaments [10]. For these experiments, α -actinin was allowed to bind to the phosphoinositides before incubation with the actin filaments. In the present study, we asked whether PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 could disrupt bundles of actin filaments cross-linked by α -actinin. Bundling assays were carried out exactly as described previously [10], except that the phosphoinositides were added following the incubation of α -actinin with actin filaments and the formation of bundles (Figure 2). As a control, inhibition assays were run alongside the disruption assays. Consistent with previous results [10], both PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 inhibited the bundling of actin filaments by α -actinin, with PtdIns(3,4,5) P_3 being more effective. However, PtdIns(4,5) P_2 (12.5–100 μ M) had no significant effect on the α -actinin-bundled actin filaments (Figures 2A–2C). In contrast, PtdIns(3,4,5) P_3 (50 μ M) disrupted the α -actinin-bundled actin filaments almost as effectively as it inhibited their formation (Figures 2A and 2B). In addition, PtdIns(3,4,5) P_3 -disruption of α -actinin-bundled actin filaments was concentration-dependent (Figures 2D and 2E). These results are similar to those observed for phosphoinositide regulation of α -actinin binding to integrins; PtdIns(3,4,5) P_3 disrupted the interaction, whereas PtdIns(4,5) P_2 had no effect [11]. These data support the hypothesis that the stimulation of PtdIns(3,4,5) P_3 within focal adhesions results in the disruption of the α -actinin link between actin filaments and integrin receptors, leading to the restructuring of focal adhesions and reorganization of the actin cytoskeleton.

Phosphoinositides do not disrupt the interaction between the isolated α -actinin actin-binding domain and actin filaments

In a previous report, we also demonstrated that PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 inhibit the bundling activity of α -actinin by blocking the interaction of the actin-binding domain with actin filaments [10]. The binding assay experiments were carried out by pre-incubating GST- α -actinin (1–269), a fusion protein containing the actin-binding domain, with the phosphoinositides before the addition of actin filaments [10]. Since PtdIns(3,4,5) P_3 was able to disrupt almost as well as it inhibited α -actinin-bundling activity, we expected that PtdIns(3,4,5) P_3 would also be able to disrupt the interaction of the actin-binding domain with actin filaments. Binding assays were carried out exactly as described previously [10], except that the phosphoinositides were added following the incubation of GST- α -actinin (1–269) with actin filaments (Figure 3). To our surprise, PtdIns(3,4,5) P_3 had little effect on the interaction of GST- α -actinin (1–269) with actin filaments. As expected, PtdIns(4,5) P_2 was also unable to disrupt the binding of GST- α -actinin (1–269) to actin filaments. Inhibition assays run alongside the disruption assays demonstrated that, as previously reported, PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 inhibit the binding of GST- α -actinin (1–269) to actin filaments [10]. These results suggest that the isolated actin-binding domain interacts with actin filaments differently from the full-length α -actinin dimer. The inability of PtdIns(3,4,5) P_3 to disrupt the interaction between the isolated actin-binding domain and actin filaments can be explained by two possibilities: (i) the phosphoinositide-binding domain is not accessible when the isolated actin-binding domain is bound to actin filaments, or (ii) PtdIns(3,4,5) P_3 disrupts α -actinin-bundled actin filaments by influencing the structure of regions of α -actinin outside the actin-binding domain. In order to understand

Figure 2 PtdIns(3,4,5)P₃ inhibits and disrupts α -actinin-bundling activity, whereas PtdIns(4,5)P₂ only inhibits

(A) Actin filaments (10.4 μ M) were incubated with α -actinin (1 μ M) for 30 min, followed by centrifugation at 10 000 *g* for 30 min. Proteins from the supernatant (S) and the pellet (P) were

Figure 3 Phosphoinositides do not disrupt the interaction of the α -actinin actin-binding domain with actin filaments

(A) Actin filaments (10.4 μ M) were incubated with GST- α -actinin (1-269) (1 μ M) for 30 min, followed by centrifugation at 100 000 *g* for 30 min. Proteins from the supernatant (S) and the pellet (P) were separated by electrophoresis and were stained. For the inhibition assays, GST- α -actinin (1-269) was pre-incubated with the phosphoinositides (50 μ M) for 15 min. To measure disruption, the phosphoinositides (50 μ M) were added following incubation of the actin filaments with GST- α -actinin (1-269). Molecular mass (in kDa) is indicated on the left of the gel. (B) Actin bands were quantified by densitometry, and the percentage of total actin in the pellet was calculated to represent binding. Results are means $\pm$ S.E.M. (*n* = 4).

how PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ were differentially regulating the function of α -actinin, we next examined the influence of phosphoinositide binding on the structure of the protein.

Phosphoinositide regulation of α -actinin secondary structure

PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ have been demonstrated to bind to the same site on neurogranin and profilin, differentially regulating secondary structure [14,15]. In order to study the influence of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ binding on α -actinin, the secondary structure was examined using CD spectroscopy. Consistent with previous studies [16], α -actinin was observed to contain approx. 56% α -helical structure (results not shown). However, the presence of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ did not have any effect on the CD spectra of the full-length protein (results not shown). In order to enhance the sensitivity of this assay, the actin-binding domain was isolated following proteolysis of full-length α -actinin with thermolysin [1] and examined by

separated by electrophoresis and were stained. For the inhibition assays, α -actinin was pre-incubated with the phosphoinositides (50 μ M) for 15 min. To measure disruption, the phosphoinositides (50 μ M) were added following incubation of the actin filaments with α -actinin. Molecular mass (in kDa) is indicated on the left of the gel. (B) Actin bands were quantified by densitometry, and the percentage of total actin in the pellet was calculated to represent bundling activity. Results are means $\pm$ S.E.M. (*n* = 5) (C) PtdIns(4,5)P₂ (12.5–100 μ M) had no effect on α -actinin-bundled actin filaments. Molecular mass (in kDa) is indicated on the left of the gel. Results are representative of three to four separate experiments. (D) and (E) PtdIns(3,4,5)P₃ disruption of α -actinin-bundling activity was concentration-dependent. In (D), molecular mass (in kDa) is indicated on the left of the gel. Results in (E) are means $\pm$ S.E.M. (*n* = 3). **P* < 0.05 by ANOVA.

Figure 4 Influence of phosphoinositides on the secondary structure of α -actinin

CD spectra of the 27 kDa actin-binding domain of α -actinin (3.7 μ M) in the absence and presence of 100 μ M PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 . Results are the mean of six separate experiments.

CD spectroscopy. Consistent with the structure of the actin-binding domains of utrophin [5] and dystrophin [6], the CD spectra of the 27 kDa actin-binding domain of α -actinin was indicative of α -helical structure (Figure 4). Analysis of the spectrum by the variable selection method [17] indicated approx. 62% α -helical structure. PtdIns(4,5) P_2 did not appear to affect the secondary structure of the actin-binding domain. PtdIns(3,4,5) P_3 consistently induced a small decrease in α -helical content; however, the magnitude of the decrease suggests that phosphoinositide regulation of α -actinin function does not occur through modulation of secondary structure.

Phosphoinositides differentially regulate α -actinin flexibility

Electron microscopy of purified α -actinin has demonstrated that the link between the actin-binding domain and the spectrin repeats is flexible, allowing the actin-binding domain to assume various orientations relative to the spectrin repeats [7]. Limited proteolysis of α -actinin results in stable breakdown products representing the actin-binding domain and the spectrin repeats [1,16]. The high susceptibility to proteolysis demonstrates further the flexibility of the link between the domains. Since the phosphoinositide-binding site lies in close proximity to the linker region, we proposed that phosphoinositide binding regulates the flexibility of the actin-binding domain. The susceptibility of the linker region to thermolysin proteolysis [1] provided a method to assay flexibility in the absence and presence of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 . After 60 min of incubation with thermolysin under control conditions, we observed partial proteolysis of α -actinin with major breakdown products of 74, 68, 34 and 27 kDa (Figure 5A). To characterize further the breakdown products, we immunoblotted with antibodies (Figure 6) recognizing the N-terminus (AT6.172) and the C-terminus (BM-75.2) of α -actinin (Figures 5B and 5C). The 34 and 27 kDa breakdown products reacted with the N-terminal antibody, demonstrating that these fragments contain the actin-binding domain. The C-terminal antibody recognized the 74 kDa breakdown product, showing that this fragment contained the EF hands domain and the spectrin repeats. The 68 kDa breakdown product did not react with either antibody, indicating that both

Figure 5 PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 influence the sensitivity of α -actinin to thermolysin proteolysis differently

α -Actinin (1 μ M) was pre-incubated with PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 (50 μ M) for 15 min at 30 °C. Thermolysin was added (at 1:30) and incubated for 1 h at 30 °C. The proteins were separated by electrophoresis and were stained with Gelcode Blue for total protein (A) or were immunoblotted with antibodies recognizing the N-terminus (B) or C-terminus (C) of α -actinin. Results are representative of six separate experiments. Positions of molecular-mass markers (in kDa) are indicated on the left of the gels, with sizes of specific bands (in kDa) indicated on the right.

the N- and C-termini of α -actinin had been cleaved from this polypeptide.

In the presence of PtdIns(3,4,5) P_3 , we not only observed an increase in α -actinin proteolysis, but also differences in the size of the breakdown products, indicating cleavage at different sites (Figure 5). The major breakdown products produced by thermolysin cleavage in the presence of PtdIns(3,4,5) P_3 consisted of doublets migrating at 75 and 61 kDa. Immunoblotting clearly showed that PtdIns(3,4,5) P_3 enhanced the proteolysis of α -actinin by thermolysin (Figures 5B and 5C). Differences in the pattern of breakdown products were also verified by immunoblotting analysis. The 75 kDa doublet reacted with the C-terminal-recognizing antibody; however, the 61 kDa doublet did not react with either antibody. Furthermore, no polypeptides were observed that reacted with the N-terminal-recognizing antibody, indicating that

Figure 6 α -Actinin antibodies recognize the N- or C-terminal domain

GST- α -actinin fusion proteins were separated by electrophoresis and stained for total protein (Gelcode Blue) or immunoblotted with anti- α -actinin antibodies AT6.172 and BM-75.2, as indicated. Positions of molecular-mass markers (in kDa) are indicated on the left-hand side.

Figure 7 PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 influence the sensitivity of α -actinin to chymotrypsin proteolysis differently

α -Actinin (1 μ M) was pre-incubated with PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 (50 μ M) for 15 min at 30 °C. Chymotrypsin was added (at 1:30) and incubated for 30 min at 30 °C. The proteins were separated by electrophoresis and were stained. Results are representative of two separate experiments. Positions of molecular-mass markers (in kDa) are indicated on the left of the gel.

thermolysin proteolysis in the presence of PtdIns(3,4,5) P_3 resulted in no stable breakdown products containing the actin-binding domain. These results suggest that PtdIns(3,4,5) P_3 increases the protease susceptibility and thus flexibility of the actin-binding domain.

In contrast, the presence of PtdIns(4,5) P_2 did not appear to affect the extent of α -actinin proteolysis by thermolysin (Figure 5A). However, the pattern of breakdown products, 74, 68, 60 and 34 kDa, observed in the presence of PtdIns(4,5) P_2 differed from that of control and PtdIns(3,4,5) P_3 -bound α -actinin. The 74 and 34 kDa breakdown products appear to be the same as those observed under control conditions, reacting with the C- and N-terminal antibodies respectively (Figure 5B and 5C). However, the 68 and 60 kDa breakdown products did not react with either antibody. Phosphoinositide-induced modification of α -actinin proteolysis was observed at concentrations as low as 12.5 μ M with saturation occurring at approx. 50 μ M (results not shown).

To demonstrate that the influence on α -actinin proteolysis by thermolysin was not the result of the phosphoinositides acting upon the enzyme, we repeated the experiment using chymotrypsin. Consistent with previous reports [16], we observed complete proteolysis of α -actinin following 30 min of incubation with chymotrypsin under control conditions (Figure 7). Complete pro-

Figure 8 PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 binding to α -actinin alters the sites of thermolysin cleavage

The major breakdown products resulting from the proteolysis of α -actinin by thermolysin were analysed by N-terminal sequencing (see Figure 4 for reference). A diagram of chicken α -actinin 1 [2,21] is shown, identifying the sites of phosphoinositide [9,10] and antibody binding. The N-terminal residue at which α -actinin was cleaved is indicated to the left of each of the fragments diagrammed.

teolysis of α -actinin by chymotrypsin was also observed in the presence of PtdIns(3,4,5) P_3 ; however, the pattern of breakdown products differed compared with control conditions. In contrast, PtdIns(4,5) P_2 appeared to inhibit proteolysis of full-length α -actinin by chymotrypsin. Consistent with the thermolysin experiments, the breakdown products resulting from chymotrypsin cleavage in the presence of PtdIns(4,5) P_2 differed from that of control and PtdIns(3,4,5) P_3 reactions. These experiments verify that the alterations in protease sensitivity are a result of phosphoinositide binding to α -actinin.

To determine the influence of the acyl chains on the interaction and regulation of α -actinin structure by phosphoinositides, thermolysin proteolysis was examined in the presence of diC₈ derivatives of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 , Ins(1,4,5) P_2 , and Ins(1,3,4,5) P_3 . These molecules only affected thermolysin proteolysis of α -actinin at concentrations of 500 μ M (results not shown), consistent with their lesser effect on the binding of GST- α -actinin (1–269) to actin filaments [10]. Ching et al. [18] also observed decreased binding and regulation of diC₈-phosphoinositides and inositol phosphates to the Src homology 2 domain of phosphoinositide 3-kinase. These results suggest that the acyl chains of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 play an important role in the binding and regulation of α -actinin structure.

In order to determine more precisely where within the α -actinin dimer phosphoinositide binding was influencing structure, N-terminal sequencing was carried out on the major breakdown products. A diagram showing the thermolysin cleavage sites of α -actinin in the absence and presence of PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 is shown in Figure 8. The 68 and 74 kDa breakdown products produced by thermolysin under control conditions were cleaved at Leu²⁶⁷, Ala²⁶⁸ or Val²⁶⁹. These results are consistent with the specificity of thermolysin cleavage at the N-terminus of hydrophobic residues [19,20]. In the presence of PtdIns(4,5) P_2 , thermolysin was observed to cleave α -actinin at these same residues to produce breakdown products of 60, 68 and 74 kDa. The 60 kDa breakdown product indicates that, in the presence of PtdIns(4,5) P_2 , thermolysin is cleaving a site within spectrin

Figure 9 PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 influence the sensitivity of GST- α -actinin (1–269) and His-CH2 to thermolysin proteolysis differently

GST- α -actinin (1–269) or His-CH2 (1 μ M) was pre-incubated with PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 (50 μ M) for 15 min. Thermolysin was added (at 1:30) and incubated for 30 min at 30 °C. The proteins were separated by electrophoresis and were stained with Gelcode Blue. Results are representative of four separate experiments. Positions of molecular-mass markers (in kDa) are indicated on the left of the gels.

repeat 4. Furthermore, in the presence of PtdIns(3,4,5) P_3 , thermolysin was found to cleave α -actinin at Phe²⁵⁰ as well as Leu²⁶⁷. Therefore phosphoinositide binding alters the structure of α -actinin, allowing thermolysin to access new cleavage sites at Phe²⁵⁰ and within spectrin repeat 4.

To examine further the regulation of α -actinin structure by phosphoinositides, thermolysin proteolysis experiments were carried out with GST- α -actinin (1–269) and His-CH2. Although the phosphoinositide-induced modification in protease sensitivity and breakdown products were not as dramatic for the α -actinin fragments as observed for the full-length protein, consistent results were obtained (Figure 9). Phosphoinositide binding altered the proteolytic rate and cleavage sites for GST- α -actinin (1–269) and His-CH2, with PtdIns(3,4,5) P_3 being more effective. These results are consistent with the hypothesis that phosphoinositide binding is modifying the structure of α -actinin within the CH2 domain as well as within other regions of the protein.

The data demonstrate that PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 binding to α -actinin induce distinct structural conformations. Furthermore, the cleavage sites, the size of the breakdown products and the loss of immunoreactivity demonstrate that phosphoinositide binding influences the structure of both the N- and C-terminal domains of α -actinin within the antiparallel homodimer. These results are consistent with those of Young and Gautel [8], demonstrating that PtdIns(4,5) P_2 binding to the actin-binding domain of skeletal α -actinin stimulated the interaction of titin with the C-terminal domain. We propose that the structural differences induced by phosphoinositide binding account for the differential regulation α -actinin function by PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 .

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