PHOSPHOINOSITIDE BINDING TO THE SUBSTRATE REGULATES
SUSCEPTIBILITY TO PROTEOLYSIS BY CALPAIN*

Chelsea R. Sprague, Tamara S. Fraley, Hyo Sang Jang, Sangeet Lal, and Jeffrey A. Greenwood

From the Department of Biochemistry and Biophysics,
Oregon State University, Corvallis, Oregon 97331

Running head: Calpain Proteolysis of α-Actinin

Address correspondence to: Jeffrey A. Greenwood, Department of Biochemistry and Biophysics, ALS 2011, Oregon State University, Corvallis, Oregon 97331, Tel. 541 737-4997; Fax. 541 737-0481; E-mail: jeffrey.greenwood@orst.edu

Calpain-mediated proteolysis regulates cytoskeletal dynamics and is altered during aging and the progression of numerous diseases or pathological conditions. Although, several cytoskeletal proteins have been identified as substrates, it is not clear how localized calpain activity is regulated and the mechanisms controlling substrate recognition. In this study, we report that phosphoinositide binding regulates the susceptibility of the cytoskeletal adhesion protein α-actinin to proteolysis by calpain 1 and 2. At first, α-actinin did not appear to be a substrate for calpain 2, however, PtdIns (3,4,5)-P3 binding to α-actinin resulted in nearly complete proteolysis of the full-length protein producing stable breakdown products. Calpain 1 was able to cleave α-actinin in the absence of phosphoinositide binding, however, PtdIns (3,4,5)-P3 binding increased the rate of proteolysis and PtdIns (4,5)-P2 binding significantly inhibited cleavage. Phosphoinositide binding appeared to regulate calpain proteolysis of α-actinin by modulating the exposure of a highly sensitive cleavage site within the CH2 domain. In U87MG glioblastoma cells, which contain elevated levels of PtdIns (3,4,5)-P3, α-actinin co-localized with calpain within dynamic actin cytoskeletal structures. Furthermore, proteolysis of α-actinin producing stable breakdown products was observed in U87MG cells treated with calcium ionophore to activate the calcium-dependent calpains. Additional evidence of PtdIns (3,4,5)-P3 mediated calpain proteolysis of α-actinin was observed in rat embryonic fibroblasts. These results suggest that PtdIns (3,4,5)-P3 binding is a critical determinant for α-actinin proteolysis by calpain. In conclusion, phosphoinositide binding to the substrate is a potential mechanism for regulating susceptibility to proteolysis by calpain.

Calpain 1 and 2 are ubiquitous calcium-dependent proteases which play an important role in the signaling of various cellular processes and have been implicated in the degeneration observed in numerous pathological conditions (1). The requirement of calcium concentrations above physiological levels, micromolar for calpain 1 and millimolar for calpain 2, has stimulated much investigation for other factors involved in the activation of calpain. Autolysis lowers the concentration of calcium required for half-maximal activity from 7.1 µM to 0.6 µM for calpain 1 and from 1000 µM to 180 µM for calpain 2 (2). However, there is currently no evidence that autolysis is required for calpain modulation in cells. PtdIns (4,5)-P2 binding also lowers the concentration of calcium required for the activation of calpain (3-5) and is a potential mechanism for regulating calpain during cell migration (6). More recently, Glading et al. published a series of studies showing that phosphorylation on serine 50 by extracellular signal-regulated kinase activates calpain 2, mediating detachment of the rear of the cell during epidermal growth factor induced fibroblast migration (7-9). Although progress has been made in understanding how calpain activity is regulated in the cell, lack of knowledge regarding the mechanisms controlling substrate recognition limits the understanding of calpain function.

Calpain 1 and 2 cleave several adhesion proteins involved in cell motility and inhibition of calpain activity reduces cell migration (10). A role for calpain proteolysis in cell adhesion was first proposed in a 1987 study by Beckerle et al. (11) which localized calpain 2 to focal adhesions and demonstrated that talin was a sensitive substrate. In this study, the authors showed that purified α-actinin was not susceptible to calpain proteolysis (11). Using pharmacological inhibitors, the importance of calpain activity was
extended to cell migration, identifying a role for calpain in the detachment of the rear of the cell during migration (12,13). A role for calpain in the regulation of cell migration was further established when embryonic fibroblasts lacking calpain 1 and 2 activity were observed to have decreased rates of migration correlating with a loss of actin stress fibers and focal adhesions (14). Cell adhesion and migration has become a valuable model system for examining the physiological regulation and function of calpain proteolysis.

Although the work cited above provides convincing evidence that calpain 1 and 2 are necessary for the migration of various cell types, the precise mechanisms controlling substrate recognition during cell migration are not understood. Phosphoinositides are also important for cell migration, particularly during chemotaxis (15,16). PtdIns (3,4,5)-P_3 is required for establishing polarity and actin polymerization at the leading edge of migrating cells (17); and more recently, a role for PtdIns (3,4,5)-P_3 was identified at the rear of migrating cells (18). PtdIns (4,5)-P_2 also appears to play an important role at the leading edge and rear of migrating cells (19). Although the specific functions of PtdIns (4,5)-P_2 and PtdIns (3,4,5)-P_3 during cell migration are not clearly defined, both phosphoinositides bind to and regulate the structure and function of various cytoskeletal and adhesion proteins (20). Two studies have identified loose connections between phosphoinositide binding and calpain proteolysis. The first showed that overexpression of phosphoinositide 3-kinase (PI 3-kinase) enhanced calpain proteolysis of fodrin, a brain-specific isoform of spectrin (21). The second demonstrated that calpain cleaved α-actinin in T cells (22) stimulated under conditions which activate PI 3-kinase (23).

Based on these studies, we proposed the hypothesis that the susceptibility of adhesion proteins to calpain proteolysis is controlled by phosphoinositide binding to the substrate protein. In order to test this hypothesis, we examined the proteolysis of α-actinin by calpain in the absence and presence of PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 in vitro and in cultured glioblastoma cells. Our results demonstrate that phosphoinositide regulation of α-actinin structure controls access to the calpain cleavage site modulating susceptibility to proteolytic posttranslational modification.

**Experimental Procedures**

**Reagents and Proteins** – Phosphoinositides were purchased from Matreya (State College, PA). Anti-α-actinin IgM (BM-75.2) and anti-talin were from Sigma. Anti-calpain 2 was purchased from Triple Point Biologics (Forest Grove, Oregon). Anti-α-actinin IgG (AT6.172) and anti-calpain 1 were from Chemicon. Anti-GFP was purchased from Santa Cruz Biotechnology. Calpain 1, (purified from human erythrocytes, specific activity ≥ 1000 units/mg), calpain 2 (rat recombinant, specific activity ≥ 1500 units/mg), and A23187 were purchased from Calbiochem. α-Actinin dimer was purified from chicken gizzard as previously described (24). The concentrations reported for α-actinin are for the dimeric form. GST-α-actinin actin-binding domain and His-tagged CH2 domain (His-CH2) were expressed and purified as described (25,26).

**Calpain Proteolysis Assays** – α-Actinin (1 μM) was preincubated with phosphoinositides for 15 min at 30°C. Incubations were continued for an additional 1 hr in the absence or presence of calpain and 1 mM calcium. Reactions were stopped by the addition of modified gel loading buffer containing 250 mM Tris, pH 6.8, 2% SDS, 5 mM EGTA, 5 mM EDTA, 25 mM DTT, and 10% glycerol, followed by a 2 min incubation at 100°C. The proteins were separated by SDS-PAGE, stained with Gelcode Blue (Pierce) or transferred to nitrocellulose for Western Blotting, and quantified using a KODAK ImageStation 440CF.

**N-terminal Sequencing** – Following calpain proteolysis and electrophoresis, proteins were transferred to PVDF membranes (Millipore). Protein bands were identified by Ponceau S staining, excised, and N-terminal sequence analysis carried out by The University of Texas Medical Branch Biomolecular Resource Facility, Galveston, Texas.

**Cell Culture and Immunofluorescence Microscopy** – U87MG glioblastoma cells were cultured in DME containing 4.5 g/liter glucose, 2 mM glutamine, and 10% FBS following the protocols from the supplier (ATCC). Immunostaining was carried out using cells plated on fibronectin as described by (27) and fixed with 3% formaldehyde (Tousimis) in PBS for 30 min at 25°C. Cells were permeabilized with 0.5% Triton


X-100 in PBS for 5 min, blocked with 1% BSA in PBS overnight at 4°C, and incubated for 1 hr at 37°C with anti-α-actinin IgM (1:500), anti-calpain 1 IgG (1:20), or anti-calpain 2 IgG (1:20). Cells were then washed with PBS and incubated for 30 min at 37°C with the appropriate FITC or Texas Red labeled secondary antibody (The Jackson Laboratory, Bar Harbor, ME) and mounted using ProLong Gold antifade reagent (Invitrogen). Immunostaining was examined using a Zeiss Axiovert 100X microscope and images captured with a Photometrics CoolSNAP HQ CCD camera controlled by MetaMorph 6.3 Imaging Software.

Rat embryonic fibroblasts were cultured in DME containing 4.5 g/liter glucose, 2 mM glutamine, and 10% FBS, and treated with platelet-derived growth factor (PDGF) as previously described (28). Cells were co-transfected with pEGFP-α-actinin wild type or mutant and p110K227E 5′-Myc as previously reported (25,29).

RESULTS AND DISCUSSION

PtdIns (3,4,5)-P₃ binding increases the susceptibility of α-actinin to proteolysis by calpain 2. Although evidence has been reported that calpain is involved in the proteolysis of α-actinin in T cells (22), α-actinin was shown to be a poor substrate for calpain 2 in vitro (11). However, the in vitro assays were carried out in the absence of phosphoinositides. In the presence of PtdIns (3,4,5)-P₃, α-actinin is highly susceptible to proteolysis by calpain 2 (Fig. 1). Consistent with the previous report (11), less than 20% of the α-actinin protein is cleaved by calpain 2 in the absence of PtdIns (3,4,5)-P₃ (Fig. 1 – lane 2). However, when PtdIns (3,4,5)-P₃ is bound to α-actinin, almost complete proteolysis of the full-length protein is observed leaving stable breakdown products of ~80kDa and ~65kDa (Fig. 1 – lane 4). Although differences in the size of breakdown products were observed, PtdIns (4,5)-P₂ did not alter the extent of α-actinin proteolysis by calpain 2 (Fig. 1 – lane 6). The increase in susceptibility resulting from PtdIns (3,4,5)-P₃ binding to α-actinin was observed at low nM concentrations of calpain (Fig. 2). At a concentration of 10 nM calpain 2, the major breakdown product appears to be ~80kDa, consistent with the size of the α-actinin fragment resulting from the activation of calpain in anti-CD3 treated T cells (22). In addition, anti-CD3 also activates PI 3-kinase in T cells (23), further supporting the hypothesis that PtdIns (3,4,5)-P₃ binding regulates the susceptibility of α-actinin to proteolysis by calpain. The small increase in the size of the major calpain 2-induced breakdown product observed with increasing concentrations of PtdIns (3,4,5)-P₃ indicates that binding enhances the susceptibility of α-actinin by exposing a highly sensitive cleavage site (Fig. 3).

To determine if PtdIns (3,4,5)-P₃ influences the concentration of calcium required for calpain 2 cleavage of α-actinin, proteolysis assays were carried out in the presence of increasing calcium concentrations. PtdIns (3,4,5)-P₃ did not appear to alter the concentration of calcium required for calpain 2 to cleave α-actinin (Fig. 4).

PtdIns (4,5)-P₂ has been shown to bind to and influence the activity of calpain 2 (3,4), however, the presence of PtdIns (4,5)-P₂ did not enhance the proteolysis of α-actinin by calpain 2. In addition, the presence of PtdIns (4,5)-P₂ or PtdIns (3,4,5)-P₃ did not affect the proteolysis of GST, a moderate substrate for calpain 2 (data not shown). These results support the hypothesis that it is the direct binding of PtdIns (3,4,5)-P₃ to α-actinin that is regulating susceptibility to proteolysis by calpain.

Calpain 2 cleavage removes the actin-binding domain of α-actinin. Using monoclonal antibodies specific for the N- or C-terminus of α-actinin (30), we were able to deduce that calpain 2 cleaves within the N-terminal region of the protein (Fig. 5). Immunoblotting with anti-α-actinin clone AT6.172, recognizing the N-terminus (30), demonstrated that calpain proteolysis in the presence of PtdIns (3,4,5)-P₃ resulted in an almost complete loss of signal (Fig. 5A – lane 4). In contrast, immunoblotting with anti-α-actinin clone BM-75.2, recognizing the C-terminus (30), demonstrated that calpain proteolysis in the presence of PtdIns (3,4,5)-P₃ resulted in a stable breakdown product of ~80kDa which included the C-terminus (Fig. 5B – lane 4). Furthermore, proteolysis of a GST fusion protein containing the actin binding domain of α-actinin (Fig. 6) demonstrated that both PtdIns (4,5)-P₂ and PtdIns (3,4,5)-P₃ increased the susceptibility of the
isolated domain to cleavage by calpain 2 (Fig. 6). However, the extent of calpain cleavage of the isolated CH2 domain was independent of phosphoinositide binding (Fig. 7). A diagram of the full-length α-actinin homodimer is shown in Fig. 8.

The combined results of the proteolysis assays suggest that a highly sensitive calpain cleavage site resides within the CH2 domain of α-actinin. The susceptibility of the CH2 domain to calpain proteolysis suggests that this cleavage site is exposed in the isolated domain (Fig. 7). However, when the actin-binding domain was expressed with the CH1 and CH2 domains together, the highly sensitive calpain cleavage site is no longer accessible. The crystal structure of the isolated α-actinin actin-binding domain demonstrated interaction between the CH1 and CH2 domains (31), with the potential to restrict access to the highly sensitive calpain cleavage site. Both PtdIns (4,5)-P2 and PtdIns (3,4,5)-P3 increased the susceptibility of the isolated actin-binding domain to calpain proteolysis. Presumably, phosphoinositide binding disrupted the interaction between the CH domains increasing access to the highly sensitive calpain cleavage site. Previous studies have also presented evidence of interaction between the N- and C-terminal domains of the α-actinin homodimer (32,33), which would further restrict access to the highly sensitive calpain cleavage site. Interestingly, only the binding of PtdIns (3,4,5)-P3 could expose the highly sensitive cleavage site within the α-actinin homodimer increasing susceptibility to calpain proteolysis (Fig. 1).

Similarly, only PtdIns (3,4,5)-P3 binding can disrupt α-actinin bundled actin filaments (30). As we have previously proposed, the phosphates on the fourth and fifth position of the inositol head group of PtdIns (4,5)-P2 and PtdIns (3,4,5)-P3 appear to mediate binding with the CH2 domain, whereas the phosphate on the third position of the inositol head group appears to disrupt interaction between the N- and C-terminal domains of the α-actinin homodimer (25,30).

To further understand phosphoinositide regulation of α-actinin proteolysis by calpain, it was important to identify the location of the highly sensitive calpain cleavage site. N-terminal sequencing of the ~80kDa breakdown product resulting from calpain 2 proteolysis of PtdIns (3,4,5)-P3 bound α-actinin revealed that cleavage occurred after tyrosine 246 (Fig. 8) within the final helix of the CH2 domain (31). Although the signal was significantly lower, evidence for a secondary cleavage site after serine 243 was also observed. PtdIns (3,4,5)-P3 dependent cleavage of α-actinin within the CH2 domain is consistent with the binding of phosphoinositides to this domain (25).

PtdIns (3,4,5)-P3 binding increases while PtdIns (4,5)-P2 binding decreases the susceptibility of α-actinin to proteolysis by calpain 1. Similar to the calpain 2 isoform, calpain 1 is a ubiquitous calcium-activated protease involved in the regulation of cell migration (1,10,34). Although calpain 1 is activated by micromolar levels of calcium compared to the millimolar concentrations required for calpain 2, both isoforms appear to have the same substrate specificity. To determine if phosphoinositide binding influenced α-actinin susceptibility to cleavage by calpain 1, the proteolysis assay described above was repeated. Some cleavage of α-actinin by calpain 1 was observed in the absence of phosphoinositides (Fig. 9 – lane 2). However, consistent with calpain 2, PtdIns (3,4,5)-P3 increased the susceptibility of α-actinin to calpain 1 proteolysis by altering the primary site of cleavage to produce a breakdown product of ~80kDa (Fig. 9 – lane 4). In contrast to calpain 2, PtdIns (4,5)-P2 binding inhibited calpain 1 proteolysis of α-actinin (Fig. 9 – lane 6). N-terminal sequencing of the ~80kDa breakdown product showed that calpain 1 cleaved PtdIns (3,4,5)-P3 bound α-actinin after tyrosine 246, the same site as calpain 2 (Fig. 8). By exposing the cleavage site at tyrosine 246, PtdIns (3,4,5)-P3 not only increased the susceptibility of α-actinin, but restricted calpain 1 to the production of one primary breakdown product. Thus far, the results suggest that phosphoinositide binding to the substrate influences calpain proteolysis by 1) regulating the rate or extent of proteolysis and 2) controlling the cleavage site and production of breakdown products.

To determine if PtdIns (3,4,5)-P3 influences the concentration of calcium required for calpain 1 cleavage of α-actinin, proteolysis assays were carried out in the presence of increasing calcium concentrations. Surprisingly, the presence of PtdIns (3,4,5)-P3 increased the in...
in vitro concentration of calcium required for calpain 1 cleavage of α-actinin from 125μM to 250μM (Fig. 10). However, the negatively charged phosphate groups on phosphoinositides have been demonstrated to bind divalent cations in vitro (35), providing one explanation for the increased calcium requirement.

Calcium dependent proteolysis of α-actinin in U87MG glioblastoma cells. Although, calpain proteolysis of α-actinin has been shown in T cells (22) under conditions which potentially activated PI 3-kinase (23), it was important to verify these results in cells known to be regulated by PtdIns (3,4,5)-P_3. U87MG glioblastoma cells are deficient for the PtdIns (3,4,5)-P_3 phosphatase PTEN and therefore have relatively high basal levels of PtdIns (3,4,5)-P_3 (36,37), providing a good system to test if α-actinin was a substrate for calpain in adherent cells. Since co-localization is required for an enzyme to act upon its substrate in a cellular system, we used immunofluorescence microscopy to determine the localization of α-actinin and calpain 1 and 2 in U87MG glioblastoma cells induced to migrate by plating on fibronectin as previously described (27). Strong staining for calpain 1 was observed in the perinuclear region of U87MG cells (Fig. 11A). However, a distinct population of calpain 1 was also observed at the leading edge of this migrating cell (Fig. 11A – arrows). α-Actinin staining was observed in the perinuclear region and within the adhesion complexes at the leading edge and rear of the cell (Fig. 11A'). A beaded staining pattern was also observed representing the population of α-actinin localized along actin stress fibers. Most importantly, all of the staining observed for calpain 1 at the leading edge of the cell co-localized with α-actinin containing adhesion complexes (Fig. 11A – arrows). However, not all α-actinin staining adhesion complexes co-stained for calpain 1. For example, calpain 1 staining was not observed in the α-actinin containing adhesion complex at the rear of the cell (Fig. 11A – arrowheads). Similar to calpain 1, the strongest staining for calpain 2 was observed in the perinuclear region (Fig. 11B). However, distinct populations of calpain 2 staining were also observed to co-localize with α-actinin within membrane ruffles at the cell edge (Fig. 11B – arrows). These results demonstrate that calpain 1 and 2 co-localize with α-actinin within highly dynamic actin cytoskeletal structures of U87MG glioblastoma cells. However, the percent of the total α-actinin population localized to these dynamic structures is small; suggesting that only a small fraction of the total α-actinin population is subject to proteolysis by calpain.

Protein from U87MG glioblastoma total cell lysates were immunoblotted to assay for α-actinin proteolysis. The calcium ionophore A23187 was added to the cells to rapidly increase the intracellular concentrations of calcium and activate calpain. Activation of the calpain-dependent calpain proteases was verified by immunoblotting for talin, an established calpain substrate and adhesion protein (11,38). As expected, a time-dependent increase in the previously reported talin breakdown product (11,38) was observed following treatment with A23187 (Fig. 12). Similar to talin, a time-dependent increase in α-actinin breakdown products was observed in the A23187 treated cells. In addition, α-actinin breakdown products were observed in untreated cells (t = 0), suggesting that a basal level of calpain activity exists in U87MG cells. Interestingly, no talin proteolysis was observed in the untreated cells. Franco et al. have reported that calpain 2 is responsible for the proteolysis of talin in fibroblasts (39). Therefore, it is possible that calpain 2 was responsible for the A23187 stimulated proteolysis of α-actinin and talin in the U87MG cells, whereas calpain 1 was responsible for the proteolysis of α-actinin observed in the untreated cells (Fig. 12).

PDGF-induced proteolysis of α-actinin in fibroblasts. Numerous studies have reported that PDGF induces cell motility in a PI 3-kinase dependent manner (40). Previously, we demonstrated that PDGF treatment of fibroblasts induced PtdIns (3,4,5)-P_3 binding to α-actinin resulting in the restructuring of focal adhesion plaques (28). Lysates from PDGF treated fibroblasts were immunoblotted to determine if α-actinin proteolysis correlated with focal adhesion restructuring. Time-dependent increases in α-actinin breakdown products were observed following 10 and 30 minute treatments with PDGF (Fig. 13). The breakdown products migrated as a doublet of ~80kDa consistent with that observed for α-actinin proteolysis by calpain in vitro (Figs. 1-3, 9). These results suggest that PtdIns (3,4,5)-P_3 mediated calpain proteolysis of α-actinin may
play a role in the regulation of cell adhesion during migration.

**Phosphoinositide binding regulates the susceptibility of α-actinin to proteolysis in fibroblasts.** To test the hypothesis that PtdIns(3,4,5)-P$_3$ binding regulates the susceptibility of α-actinin to calpain proteolysis in a cellular system, constitutively active PI 3-kinase was co-expressed with GFP-α-actinin wildtype or a GFP-α-actinin mutant with decreased affinity for phosphoinositides (25,29). Co-expression of GFP-α-actinin wildtype with constitutively active PI 3-kinase resulted in an increase in α-actinin breakdown products (Fig. 14 – lanes 1 and 2). In contrast, an increase in α-actinin breakdown products was not observed when the GFP-α-actinin mutant was co-expressed with constitutively active PI 3-kinase (Fig. 14 – lanes 4 and 5). Co-expression of GFP-α-actinin with constitutively active PI 3-kinase was confirmed by immunostaining as shown in (29). Previously, we used this experimental system to demonstrate that PtdIns (3,4,5)-P$_3$ binding to α-actinin played a role in regulating the disassembly of focal adhesions and reorganization of the actin cytoskeleton (29). Results of the current study suggest that the mechanism by which PtdIns (3,4,5)-P$_3$ binding mediates α-actinin function involves regulation of susceptibility to proteolysis by calpain.

The requirement for calpain activity during cell adhesion and migration is well established with influence on the processes of cell spreading, membrane protrusion, adhesion complex turnover, and tail retraction (12,38,39,41-44). In addition to α-actinin, talin, vinculin, filamin, spectrin, and ezrin are involved in the formation, maintenance, and turnover of adhesion complexes and are regulated by phosphoinositide binding (20,45). Interestingly, all of these adhesion proteins are also substrates for calpain (10,22,41,46-49). Although calpain cleavage of adhesion proteins is an important regulatory mechanism, it is not clear how the localization, activation, and substrate selection for calpain 1 and 2 are modulated during cell adhesion and migration. This study addresses the cellular regulation of the calpain system from the point-of-view of the substrate, identifying a role for phosphoinositide binding in modulating the susceptibility of α-actinin to calpain proteolysis. Further studies are necessary to understand the role of PtdIns (3,4,5)-P$_3$ regulated proteolysis of α-actinin by calpain in cell adhesion and migration. In addition, it will be important to determine if calpain proteolysis of other proteins are influenced by phosphoinositide binding.

**REFERENCES**

FOOTNOTES

*This work was supported by Grant GM 63711 to J.A.G. from the National Institute of General Medical Sciences, National Institutes of Health. This publication was made possible in part by the Cell Imaging and Analysis Facility and Services Core of the Environmental Health Sciences Center at Oregon State University from Grant P30 ES00210, National Institute of Environmental Health Sciences, National Institutes of Health. C.R.S. was supported in part by a summer undergraduate research fellowship funded by Howard Hughes Medical Institute Grant 52003741 awarded to Oregon State University.

The abbreviations used are: CH, calponin homology; PtdIns, phosphatidylinositol; PI 3-kinase, phosphoinositide 3-kinase; GST, glutathione S-transferase; his-CH2, his-tagged CH2 domain; PDGF, platelet-derived growth factor.

FIGURE LEGENDS

Fig. 1. PtdIns (3,4,5)-P3 binding increases the susceptibility of α-actinin to proteolysis by calpain 2. α-Actinin (1 µM) was pre-incubated with 50 µM PtdIns (4,5)-P2 or PtdIns (3,4,5)-P3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 2 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE, stained with Gelcode Blue, and quantified by densitometry. All of the protein bands visible on the gel represent the uncleaved protein or the breakdown products of α-actinin. n=6-13 ± SE.

Fig. 2. Calpain 2 cleaves PtdIns (3,4,5)-P3 bound α-actinin at low nM concentrations. α-Actinin (1 µM) was pre-incubated in the absence or presence of 50 µM PtdIns (3,4,5)-P3 for 15 min at 30°C. Increasing concentrations of calpain 2 were added and incubated for an additional 60 min. All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE and stained with Gelcode Blue.
Fig. 3. PtdIns (3,4,5)-P_3 binding to α-actinin exposes a highly sensitive calpain 2 cleavage site. α-Actinin (1 µM) was pre-incubated in the presence of increasing concentrations of PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 2 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE and stained with Gelcode Blue.

Fig. 4. PtdIns (3,4,5)-P_3 binding does not alter the concentration of calcium required for calpain 2 to cleave α-actinin. α-Actinin (1 µM) was pre-incubated in the absence (♦) or presence (■) of 50 µM PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the presence of calpain 2 (0.25 µM) and increasing concentrations of calcium. The proteins were separated by SDS-PAGE, stained with Gelcode Blue, and quantified by densitometry.

Fig. 5. Calpain 2 cleavage removes the N-terminal actin-binding domain of α-actinin. α-Actinin (1 µM) was pre-incubated with 50 µM PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 2 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies recognizing the N-terminus (A) or C-terminus (B) of α-actinin.

Fig. 6. Binding of both PtdIns (3,4,5)-P_3 and PtdIns (4,5)-P_2 increases the susceptibility of the isolated actin-binding domain of α-actinin to proteolysis by calpain 2. GST-actin-binding domain (1 µM) was pre-incubated with 50 µM PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 2 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE, stained with Gelcode Blue, and quantified by densitometry. n=3 ± SE.

Fig. 7. Phosphoinositide binding is not required for calpain 2 proteolysis of the isolated CH2 domain of α-actinin. Hist-CH2 domain (1 µM) was pre-incubated with 50 µM PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 2 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE, stained with Gelcode Blue, and quantified by densitometry. n=3 ± SE.

Fig. 8. Calpain cleaves α-actinin after tyrosine 246 within the last α-helix of the CH2 domain. The diagram of the α-actinin homodimer identifies the three regions of the protein: actin-binding domain (ABD), the spectrin repeats, and the EF hands domain. The location of the phosphoinositide binding site is represented by the symbol within the CH2 domain (25). The amino acid sequence surrounding the calpain cleavage site is magnified. The primary cleavage site determined by N-terminal sequencing is marked by the arrowhead; a secondary cleavage site is identified by the arrow.

Fig. 9. PtdIns (4,5)-P_2 and PtdIns (3,4,5)-P_3 differentially regulate α-actinin proteolysis by calpain 1. α-Actinin (1 µM) was pre-incubated with 50 µM PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the absence or presence of calpain 1 (0.25 µM). All samples contained 1 mM calcium. The proteins were separated by SDS-PAGE and stained with Gelcode Blue. Results are representative of four separate experiments.

Fig. 10. Calcium concentration curve for calpain 1 proteolysis of α-actinin. α-Actinin (1 µM) was pre-incubated in the absence (♦) or presence (■) of 50 µM PtdIns (3,4,5)-P_3 for 15 min at 30°C. Incubations were continued for an additional 60 min in the presence of calpain 1 (0.25 µM) and increasing concentrations of calcium. The proteins were separated by SDS-PAGE, stained with Gelcode Blue, and quantified by densitometry.
**Figure 11.** Calpain 1 and 2 co-localize with α-actinin in U87MG glioblastoma cells. Cells were stained with antibodies specific for calpain 1 (A) or calpain 2 (B) and co-stained with antibodies recognizing α-actinin (A' and B'). Merged image is shown with α-actinin staining pseudo-colored red and calpain staining green (A'' and B''). Arrows identify regions of co-localization. Arrowheads show lack of co-localization within the adhesion complexes of the tail of a migrating cell. Results are representative of three separate experiments. Bar = 10 µm.

**Fig. 12.** Calcium induces proteolysis of α-actinin in U87MG glioblastoma cells. The calcium ionophore A23187 (10 µM) was added to the cells for the indicated times and total cell lysates immunoblotted for α-actinin or talin. Results are representative of two separate experiments.

**Fig. 13.** PDGF induces proteolysis of α-actinin in fibroblasts. Total cell lysates from PDGF (30 ng/ml) treated fibroblasts were immunoblotted for α-actinin. The signal from the full-length α-actinin protein was so strong that in order to clearly image the breakdown products the immunoblot was cut and the portion containing the uncleaved protein was developed separately from the cleavage products. Results are representative of two separate experiments.

**Fig. 14.** PtdIns (3,4,5)-P₃ binding increases the susceptibility of α-actinin to calpain proteolysis in fibroblasts. Fibroblasts were co-transfected to express constitutively active PI 3-kinase (p110K227E 5'-Myc) with wildtype GFP-α-actinin (lanes 1 and 2) or the GFP-α-actinin mutant with decreased affinity for phosphoinositides (lanes 4 and 5). Total cell lysates were immunoblotted with anti-GFP. Lane 3 contains lysate from untransfected cells. Results are representative of two separate experiments.
Figure 1

![Western blot experiment results](image)

**Legend:**
- **Calpain 2:**
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6

- **PtdIns (3,4,5)-P₃:**
  - -
  - -
  - +
  - +
  - -
  - -

- **PtdIns (4,5)-P₂:**
  - -
  - -
  - -
  - -
  - +
  - +

**α-actinin (as a percent of control):**
- 100
- 80
- 60
- 40
- 20
- 0
Figure 2

-PtdIns (3,4,5)-P$_3$

+PtdIns (3,4,5)-P$_3$

α-actinin

Calpain (nM)

0 1 10 100 1000
Figure 3

[Image: Gel electrophoresis showing the effect of PtdIns (3,4,5)-P₃ (µM) on -Calpain activity.

- Calpain
- 0
- 6.25
- 12.5
- 25
- 50

PtdIns (3,4,5)-P₃ (µM)

Molecular weight markers: 100 kDa and 72 kDa

→ α-actinin]
Figure 5

A. N-terminal Antibody AT6.172

B. C-terminal Antibody BM-75.2

Calpain 2: - + - + - + +
PtdIns (3,4,5)-P₃: - - + + - - -
PtdIns (4,5)-P₂: - - - - + + +

α-actinin
Figure 6

GST-α-actinin 1-269

α-actinin (% of control)

Calpain 2: - + - + - + +
PtdIns (3,4,5)-P₃: - + + + - - -
PtdIns (4,5)-P₂: - - - + + +
Figure 7

[Image of a gel electrophoresis analysis with molecular weight markers (24, 17, 11) and a Coomassie blue stained protein band at His-CH2.

A bar graph showing α-actinin expression (% of control) in response to different treatments:

- Calpain 2:
  - -
  - +
  - -
  - +
  - -
  - +

- PtdIns (3,4,5)-P₃:
  - -
  - -
  - +
  - +
  - -
  - -

- PtdIns (4,5)-P₂:
  - -
  - -
  - -
  - +
  - +
  - +

Error bars are present for the conditions with +, indicating variability in the measurements.
Figure 9

Calpain 1:
- - + - + - +

PtdIns (3,4,5)-P₃:
- - - + + - -
PtdIns (4,5)-P₂:
- - - - + + +

α-actinin:
-
Figure 10

\[ \text{\(\alpha\)-actinin (\% of control)} \]

\[ \text{Calcium concentration (mM)} \]
Figure 12

- α-actinin
- talin

Time (min)
Figure 13

![Image of Western blot analysis showing time-dependent changes under PDGF treatment.](http://www.jbc.org/)

- **α-actinin**
- **cleavage products**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDGF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(by guest on October 5, 2017)
Figure 14

GFP-α-actinin

PI 3-kinase: 
- +

Wildtype

Mutant
Phosphoinositide binding to the substrate regulates susceptibility to proteolysis by calpain
Chelsea R. Sprague, Tamara S. Fraley, Hyo Sang Jang, Sangeet Lal and Jeffrey A. Greenwood

*J. Biol. Chem.* published online February 6, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M707436200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/02/06/jbc.M707436200.citation.full.html#ref-list-1