Tyrosine Phosphorylation of the Integrin β3 Subunit Regulates β3 Cleavage by Calpain*

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Outside-in signaling of β3 integrins induces and requires phosphorylation at tyrosine 747 (Tyr747) and tyrosine 759 (Tyr759) of the β3 subunit, but the mechanism for this requirement is unclear. On the other hand, a key consequence of integrin signaling, cell spreading, is inhibited by calpain cleavage of β3 cytoplasmic domain. Here we show that β3 tyrosine phosphorylation inhibits calpain cleavage. Mutating both tyrosines to phenylalanine sensitizes β3 to calpain cleavage. Furthermore, phosphorylation at Tyr747 and Tyr759 of β3 in the focal adhesion sites and the leading edge of spreading platelets was differentially regulated. Selective dephosphorylation of Tyr759 is associated with calpain cleavage at Tyr759. Thus, one mechanism by which tyrosine phosphorylation promotes integrin signaling and cell spreading is its inhibition of calpain cleavage of the β3 cytoplasmic domain.

Integrins mediate cell adhesion and transduce signals that are critical in the dynamic regulation of cell adhesion, spreading, migration, and proliferation (1, 2). Integrin signaling is a two-way process exemplified by inside-out and outside-in signaling of the platelet integrin, αIIbβ3. Inside-out signaling is believed to be transduced by talin binding to the cytoplasmic domain of αIIbβ3 (3–7), and consequent conformational changes (5, 6), which propagate to the ligand binding domain of αIIbβ3, activating ligand binding function (8, 9). Ligand binding to αIIbβ3 not only forms adhesive bond but also induces outside-in signaling, leading to cell spreading, secretion, stabilization of platelet adhesion, and amplification of platelet aggregation (10, 11).

The cytoplasmic domain of β3 is critical in bidirectional signaling (12–15). Inside-out signaling requires the membrane proximal region and the two NXXY motifs in the β3 cytoplasmic domain (3–6, 15–19). Outside-in signaling requires the intact cytoplasmic domain of β3 (15) and also requires tyrosine phosphorylation in NXXY motifs (20, 21). However, the mechanism responsible for the role of tyrosine phosphorylation of β3 in outside-in signaling is unclear. On the other hand, the cytoplasmic domain of β3 is cleaved by the calcium-dependent proteases (calpain) at sites flanking two NXXY motifs, preferentially at the C-terminal side of Tyr759 (15, 22, 23). A consequence of calpain cleavage of β3 at Tyr759 is the inhibition of β3-dependent cell spreading, which is an outside-in signaling event (15). In studying the relationship between these two seemingly unrelated β3 modifications that regulate the function of the cytoplasmic domain of β3, we found that tyrosine phosphorylation in β3 cytoplasmic domain inhibits cleavage of β3 by calpain. Since calpain cleavage negatively regulates outside-in signaling-mediated cell spreading, our finding provides a mechanism by which tyrosine phosphorylation of β3 promotes integrin outside-in signaling.

EXPERIMENTAL PROCEDURES

Peptides—Peptides were synthesized by Protein Chemistry Laboratory, University of Illinois at Chicago, purified by reverse phase-high performance liquid chromatography, and correct molecular weights verified by electrospray ion-trap mass spectrometry. Double tyrosine-phosphorylated β3 cytoplasmic domain peptides were verified by SDS-PAGE and immunoblots with anti-β3 cytoplasmic domain antibodies.

Antibodies—To generate anti-peptide antibodies, synthetic peptides conjugated to keyhole limpet hemocyanin were used to immunize rabbits (24). The antibody pY759 was generated using the peptide CTpYRGT with a linker cystein and a 5-residue sequence corresponding to tyrosine-phosphorylated β3 C terminus. The anti-serum was absorbed (three times) with Sepharose 4B coupled with a non-phosphorylated CTYRGT peptide to remove phosphorylation-independent reactivity. Rabbit antibodies specific for β3 with a phospho-tyrosine at Tyr747 or Tyr759 were also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit antibody, 8053, and a mouse monoclonal antibody, mAb15, against the extracellular domain of β3, were generous gifts from Dr. Mark Ginsberg, University of California, San Diego, CA. The antibody against the β3 C-terminal TYRGT sequence, Ab762, or antibodies recognizing the calpain cleavage-generated new C terminus at each of the calpain cleavage sites, Ab759, Ab754, Ab747, and Ab741, were described previously (15, 22). Purified μ-calpain and an antibody specific for calpain-cleaved fodrin were generous gifts from Dr. T. Saijo (25).

Calpain Cleavage of Synthetic β3 Cytoplasmic Domain Peptides—The β3-C-pY peptide (1 mg/ml, 0.1 ml) solubilized in 0.05 M Tris, 0.15 M NaCl, 1 mM CaCl2, and 1 mM dithiothreitol (pH 7.4) were incubated with 1 μg of purified μ-calpain at 30 °C for 30 min. After adding an equal volume of 2 × SDS-PAGE sample buffer containing 5 mM EDTA and 0.1 mM E64 (a calpain inhibitor), the samples were subjected to SDS-PAGE

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using 10–20% gradient gels and immunoblotted with antibodies recognizing intact β3 C terminus or calpain cleaved fragments of β3.

Detection of Tyrosine Phosphorylation and Calpain Cleavage of Integrin β3 in Platelets—Blood from healthy human donors or from wild type and knock-in mice with both Tyr759 and Tyr747 mutated to phenolalanine (20, 21) was anticoagulated with 1/7 volume of ACD (2.5% trisodium citrate, 2.0% d-glucose, 1.5% citric acid) (26). Washed platelets in Tyrode’s buffer were allowed to stay at 25 °C for 1 h (26). Platelet aggregation was induced by 0.1 unit/ml of α-thrombin in a Chronolog aggregometer stirring at 1000 rpm for 3 min. In some cases, 1 mM calcium ionophore A23187 was added 2 min after adding α-thrombin. In phosphatase inhibition experiments, platelets were incubated with 0.5 mM sodium vanadate at 37 °C for 5 min prior to addition of agonists. Platelets were solubilized in SDS-PAGE sample buffer containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM E64, 0.1% Triton X-100, 1% bovine serum albumin; analyzed by SDS-PAGE using 4–15% gradient gels; and immunoblotted with various antibodies. Results were visualized with the enhanced chemiluminescence reagent (Amersham Biosciences).

Localization of Calpain-cleaved or Tyrosine-phosphorylated β3 Molecules in Spreading Platelets—Lab-Tek® chamber slides (Nalgen Nunc, Naperville, IL) were precoated with 10 µg/ml fibrinogen followed by incubation with 5% bovine serum albumin. Platelet suspension in Tyrode’s buffer (100 µl, 10^⁵/ml) was added to the wells and incubated at 37 °C for 90 and 180 min. The plates were rinsed, and adherent platelets were fixed with 4% paraformaldehyde and permeabilized with 0.1 M Tris, 10 mM EGTA, 0.15 M NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 1% bovine serum albumin (pH 7.5). The samples were incubated with a mouse anti-β3 antibody, mAb15, and one of the rabbit anti-β3 antibodies. After washing, platelets were stained with Alexa Fluor® 488-conjugated goat anti-mouse IgG and Alexa Fluor® 594-conjugated goat anti-rabbit IgG. Data were collected using a Zeiss LSM 5 software. The area of each field was quantitated in randomly chosen fields by using the colocalization tool in Zeiss LSM 5 software and expressed as average number of pixels/platelet. Statistical significance was determined using a t test.

RESULTS AND DISCUSSION

To determine whether tyrosine phosphorylation of the integrin β3 cytoplasmic domain regulates calpain cleavage of β3, a 43-residue phosphopeptide (β3-C-pY) corresponding to the sequence of the β3 cytoplasmic domain was synthesized with both Y747 and Y759 phosphorylated (Fig. 1A). As a control, we also synthesized a non-phosphorylated peptide with the sequence identical to β3-C-pY (β3-C). These peptides were treated with purified human µ-calpain and then immunoblotted with antibodies that recognize the β3 cytoplasmic domain only when β3 is cleaved at the previously characterized calpain cleavage sites (cleavage indicator antibodies Ab754 and Ab759) (22), and with Ab762, an antibody that recognizes the β3 C-terminal TYRGT sequence (15). Ab762 still reacts with the β3 cytoplasmic domain when Tyr759 is phosphorylated but at a reduced affinity (Fig. 1B). However, the reactivity of this antibody is totally abolished with calpain-cleaved β3 (15) (Fig. 1B). Treatment of non-phosphorylated β3-C peptide with µ-calpain caused the loss of reactivity with Ab762 and gain of reactivity with cleavage indicator antibodies, indicating that calpain cleaved the peptide at specific sites. In contrast, the tyrosine-phosphorylated β3 cytoplasmic domain peptide, β3-C-pY, showed no loss of reactivity with Ab762 following calpain treatment and no gain of reactivity with the cleavage indicator antibodies. Thus, tyrosine phosphorylation protects β3 cytoplasmic domain from calpain cleavage in the in vitro assays using purified calpain and β3 peptides.

To detect β3 tyrosine phosphorylation in vitro and in platelets, we developed an anti-peptide antiserum (Ab pY759) specific for the phosphopeptide, C-TpYRG, corresponding to the β3 C-terminal sequence. The Ab pY759 reacted with the phosphorylated β3-C-pY peptide but not the nonphosphorylated β3-C peptide, further verifying the β3-C-pY phosphopeptide (Fig. 1B). These results also show that the reactivity of Ab pY759 with β3 is phosphorylation-dependent and thus specifically indicates β3 tyrosine phosphorylation. In immunoblot, Ab pY759 had essentially no reactivity with β3 from resting platelets but reacted strongly with β3 from platelets aggregated in response to thrombin, which induces β3 tyrosine phosphorylation. Prevention of platelet aggregation by the integrin inhibitor, RGDS, inhibited the reaction of Ab pY759 with β3, confirming that β3 phosphorylation is induced by integrin outside-in signaling (20).

To investigate whether tyrosine phosphorylation protects integrin from cleavage by calpain in platelets, platelets were treated with thrombin to induce tyrosine phosphorylation of β3. Platelets were also treated with tyrosine phosphatase inhibitor, sodium vanadate (Fig. 2) to prevent dephosphorylation of...
\(\beta_3\) cytoplasmic domain by tyrosine phosphatases and thus further enhance \(\beta_3\) phosphorylation. Phosphorylation at Tyr\(^{747}\) and Tyr\(^{759}\) induced by thrombin and their further enhancement by sodium vanadate were indicated by immunoblotting with Ab pY759 and an anti-pY747 antibody (Fig. 2). Platelets were then treated with the calcium ionophore, A23187, to induce cleavage of \(\beta_3\). Consistent with our previous results, A23187 induced cleavages of \(\beta_3\) at the C-terminal side of residues 747, 754, and 759 in platelets. However, A23187-induced \(\beta_3\) cleavage is substantially reduced in thrombin-treated platelets and further reduced in platelets treated with both thrombin and sodium vanadate, in correlation with the increased \(\beta_3\) tyrosine phosphorylation. The effects of thrombin and sodium vanadate are unlikely to be related to changes in expression levels of \(\beta_3\) because immunoblotting with the anti-\(\beta_3\) extracellular domain antibody, 8053, showed similar levels of \(\beta_3\) in platelets treated with or without thrombin or/and sodium vanadate. To exclude the possibility that the effects of thrombin and sodium vanadate in inhibiting calpain cleavage of \(\beta_3\) may be caused by nonspecific effect of thrombin or sodium vanadate on calpain activity, we also examined whether thrombin or/and sodium vanadate may inhibit calpain cleavage of another calpain substrate, fodrin. A23187 induced calpain cleavage of fodrin as indicated by reactivity with a calpain cleavage-specific antibody against fodrin (25) (Fig. 2). Since this cleavage was not affected by the treatment of platelets with thrombin and/or sodium vanadate, it is unlikely that thrombin and/or sodium vanadate had nonspecific effect on calpain activity. Rather, their effect on \(\beta_3\) cleavage is likely to be specifically caused by reducing \(\beta_3\) susceptibility to calpain cleavage. Since we showed that thrombin induced tyrosine phosphorylation at Tyr\(^{759}\) and Tyr\(^{747}\) of \(\beta_3\), which is enhanced by sodium vanadate, our results suggest that tyrosine phosphorylation of \(\beta_3\) inhibited A23187-induced calpain cleavage of \(\beta_3\) cytoplasmic domain in platelets.

It is known that calpain is activated following platelet aggregation induced by thrombin. However, unlike A23187 that induces cleavage of \(\sim 70\%\) of \(\beta_3\) molecules in 5 min, thrombin only induces calpain cleavage of a small population of \(\beta_3\) during platelet aggregation (15). Fig. 2 shows that thrombin, but not A23187, induced tyrosine phosphorylation of \(\beta_3\). To further examine whether thrombin-induced tyrosine phosphorylation protects \(\beta_3\) from calpain cleavage without adding calcium ionophore, platelets were pretreated with or without sodium vanadate and then stimulated with thrombin. Fig. 3 shows that thrombin-induced calpain cleavage of a population of integrin molecules mainly occurs at Tyr\(^{759}\) site, and this cleavage is inhibited by sodium vanadate. To exclude the possible nonspecific effect of sodium vanadate on calpain activity, we show that sodium vanadate failed to affect thrombin-induced calpain cleavage of fodrin. These results suggest that thrombin-induced phosphorylation of \(\beta_3\) inhibited thrombin-induced calpain cleavage of \(\beta_3\) during platelet aggregation. To further support the protective effect of tyrosine phosphorylation against calpain cleavage, we show that mouse \(\beta_3\) (DiYF) with both Tyr\(^{747}\) and Tyr\(^{759}\) mutated to phenylalanine is more susceptible to calpain cleavage than wild type during thrombin-induced platelet aggregation (Fig. 3B).

Tyrosine phosphorylation of \(\beta_3\) also occurs in platelets spread on fibrinogen surfaces (Fig. 4) as indicated by staining with antibodies specifically recognizing \(\beta_3\) molecules that are phosphorylated at Tyr\(^{759}\) or Tyr\(^{747}\) (Fig. 4). At an earlier time point (90 min), staining of both pY759 and pY747 is strong and forms punctate focal adhesion-like structures mainly in the leading edge of spreading platelets particularly at the tips of pseudopods (arrows). At a later time point, while pY747 stain-
Phosphorylation Regulates Integrin Cleavage by Calpain

**A**

|       | Red  | Green: MAAb15 | Overlay |
|-------|------|---------------|---------|
| 90 min| pY759|               |         |
|       | pY747|               |         |
|       | Ab759|               |         |
|       | NC   |               |         |
| 180 min| pY759|               |         |
|       | pY747|               |         |
|       | Ab759|               |         |
|       | NC   |               |         |

**B**

![Diagram](image)

**FIGURE 4.** β₃ tyrosine phosphorylation and calpain cleavage in platelets spreading on fibrinogen. A, platelets were allowed to spread on fibrinogen-coated slides for 90 and 180 min, fixed, and permeabilized. The slides were stained with a mouse anti-β₃ extracellular domain antibody, mAb15 (green) and one of the tyrosine phosphorylation-specific (pY747 and pY759) or calpain cleavage-specific (Ab759) antibodies (red). Affinity-depleted Ab 759 antiserum was used as negative control (NC). Data were collected with a Zeiss confocal microscope (63× lens). B, quantitation of area (pixel number/platelet) of β₃ tyrosine phosphorylation and calpain cleavage in six random fields of 6 slide wells from three experiments (mean ± S.E., platelet numbers are marked above each column; *p < 0.001). Quantitation of negative control (NC) is from 4 slide wells from two separate experiments.

Phosphorylation becomes even stronger and forms focal adhesion-like structures ringing the platelets, pY759 staining becomes decreased and more diffuse, and its focal adhesion-like structures are almost lost, suggesting pY759 dephosphorylation at focal adhesion sites and leading edge. These data indicate that phosphorylation at Tyr⁷⁵⁹ and Tyr⁷⁴⁷ is differentially regulated in spreading platelets, and that pY759 is preferentially dephosphorylated at a later stage of platelet spreading. To determine whether tyrosine phosphorylation affected calpain cleavage of β₃ in spreading platelets, we also stained spreading platelets with the antibody Ab759 that recognizes the calpain cleavage site at Tyr⁷⁵⁹ (the major calpain cleavage site in platelets, see Fig. 3A). At 90 min, Ab759 staining was weak in spreading platelets. Interestingly, Ab759 stain was even weaker in the margin of platelets where the punctated ring of tyrosine-phosphorylated β₃ was strong. These results are consistent with the above observation that tyrosine phosphorylation inhibits calpain cleavage of β₃. At 180 min, more β₃ molecules are cleaved at Tyr⁷⁵⁹, which correlated well with the decrease in pY759 staining. Calpain cleavage at sites near pY747 was barely detectable in spreading platelets (data not shown). These results suggest that calpain cleavage mainly occurs to a population of dephosphorylated integrin molecules. The differential dephosphorylation of pY747 and pY759 also explains why calpain preferentially cleaves β₃ at the Tyr⁷⁵⁹ site in platelets. Furthermore, we previously reported that cells expressing a β₃ truncation mutant mimicking calpain cleavage at Tyr⁷⁵⁹ showed reduced spreading on fibrinogen, suggesting that the calpain-cleaved form of β₃ is defective in mediating spreading (15). Thus, our results suggest that one mechanism by which tyrosine phosphorylation of β₃ facilitates outside-in signaling and platelet spreading is its inhibition of calpain cleavage of β₃.

It is known that the intact β₃ C terminus is required for outside-in signal leading to cell spreading (15). This requirement is consistent with the findings that c-Src binds to the β₃ C-terminal domain and that inhibition of Src family of protein kinases inhibited cell spreading (27–29). Calpain cleavage of β₃ cytoplasmic domain disrupts the c-Src binding site in the C-terminal domain of β₃, which potentially explains why calpain cleavage of integrins plays important roles in detaching the rear end of a cell during migration (30), and in cell detachment during apoptosis (31). Calpain is abundant in the cell focal adhesion sites (32) and is activated by integrin-mediated calcium elevation (33). Thus, cell spreading and firm adhesion would not be possible without protection of β₃ cytoplasmic domain from calpain cleavage. Furthermore, calpain also regulates the functions of several other focal adhesion proteins including talin (34), focal adhesion kinase (35), protein-tyrosine phosphatase 1B (36), and Rho A (37). Thus, phosphorylation-dependent regul-
Phosphorylation regulates integrin cleavage by calpain. Our results provide an important mechanism by which tyrosine phosphorylation of β3 promotes integrin outside-in signaling and by which the timing and location of calpain cleavage of integrins are dynamically regulated.

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