Calpin Cleavage of the Cytoplasmic Domain of the Integrin \( \beta_3 \) Subunit*

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The cytoplasmic domains of integrin \( \beta \) subunits are involved in bidirectional transmembrane signaling. We report that the cytoplasmic domain of the integrin \( \beta_3 \) subunit undergoes limited proteolysis by calpain, an intracellular calcium-dependent protease. Calpain cleavage occurs during platelet aggregation induced by agonists such as thrombin. Five cleavage sites have been identified. Four of these sites (C-terminal to Thr743, Tyr747, Phe754, and Tyr759) are utilized in intact platelets and flank two NXXY motifs (Asn744-Pro-Leu-Tyr747 and Asn756-Ile-Thr-Tyr759). The fifth site (Ala735) is accessible to calpain after EDTA treatment of the \( \alpha_{IIb}\beta_3 \) heterodimer. The NXXY motif is critical to the bidirectional signaling functions of \( \beta_3 \) integrins and their association with the cytoskeleton. Thus, calpain cleavage of the \( \beta_3 \) cytoplasmic domain may provide a means to regulate integrin signaling functions.

Integrins, a family of adhesion receptors, play important roles in cellular functions such as adhesion, migration, cell proliferation, and differentiation (1). The functions of integrins are modulated by bidirectional transmembrane signaling as exemplified by platelet integrin \( \alpha_{IIb}\beta_3 \) (glycoprotein IIb-IIIa) (2). When platelets are stimulated, as happens during platelet aggregation, the cytoplasmic domain may provide a means to regulate integrin signaling functions.

Numerous cytoplasmic proteins are colocalized with integrins in focal adhesion sites and thus may be involved in intracellular signaling to and from integrins (11). Calpain is among these proteins (12). Calpain represents a family of intracellular calcium-dependent neutral proteases (13). Of the two classic members of this family, \( \mu \)-calpain and m-calpain, \( \mu \)-calpain is probably the predominant form in platelets (14). These two isoforms of calpain have no major difference in substrate specificity, but differ in calcium sensitivity (14). Calpain can be activated during platelet aggregation by a rise in the cytoplasmic calcium level and/or by its translocation to the membrane (13, 14). Furthermore, calpain activation promotes the shedding of procoagulant membrane vesicles from aggregated platelets (15). While the mechanisms of calpain regulation are still not fully understood, calpain cleavages can regulate a variety of intracellular processes (16–19). We now report that calpain may regulate the function of \( \beta_3 \) integrins by limited cleavage of the cytoplasmic domain of \( \beta_3 \) at specific sites flanking two NXXY motifs.

EXPERIMENTAL PROCEDURES

Purified Proteins and Peptides—Integrin \( \alpha_{IIb}\beta_3 \) was purified as described previously (20) by sequential chromatography of platelet lysates on heparin, concanavalin A, and gel filtration columns. \( \mu \)-Calpain was purified from rabbit skeletal muscle and characterized as described previously (21). The specific activity of the enzyme was 650 units/mg. The synthetic peptide TIDHRKEFAKFEEERARAKWDTANNPLYK-EATSTFTNITYRG, corresponding to the cytoplasmic domain of \( \beta_3 \), was kindly provided by Dr. E. F. Plow (Department of Cardiovascular Biology, Cleveland Clinic Foundation, Cleveland, OH). All other peptides were synthesized using an Applied Biosystems Model 430A automated peptide synthesizer and were subsequently purified by high performance liquid chromatography (HPLC).1 The mass of all synthetic peptides was verified by ion-spray mass spectrometry.

Antibodies—The monoclonal antibodies PMI-1 (against the \( \alpha_{IIb} \) heavy chain) and 15 (anti-\( \beta_3 \) heavy chain) were produced and characterized as described previously (22, 23). The rabbit antipeptide antibodies anti-IIBC (2276) (against the C-terminal 20 residues of the \( \alpha_{IIb} \) light chain cytoplasmic domain), anti-\( \beta_3 \) (8275) (against the C-terminal 20 residues of the \( \beta_3 \) cytoplasmic domain), and anti-V41 (against the N-terminal 13 residues of the \( \alpha_{IIb} \) light chain) have been described previously (24, 25). The specificity of anti-IIBC and anti-\( \beta_3 \)C was verified using recombinant integrin \( \alpha_{IIb}\beta_3 \) with truncations in the cytoplasmic domain of \( \alpha_{IIb} \) and \( \beta_3 \) respectively (26). To produce antibodies recognizing specific calpain cleavage sites, pentapeptides corresponding to C-terminal sequences identified by calpain digestion of the synthetic \( \beta_3 \) cytoplasmic domain peptide were synthesized with a cysteine incorporated at their N termini. The peptides were conjugated with keyhole limpet hemocyanin (Sigma) by a cysteine-specific cross-linking reagent, m-maleimido-benzoyl-N-hydroxysuccinimide ester (Pierce). The conjugates were then used to immunize rabbits as described previously (26). In some cases, the antipeptide antibodies were affinity-purified using

1 The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; Ab, antibody.
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**RESULTS**

Preferential Cleavage of the \(\beta_3\) Cytoplasmic Domain by \(\mu\)-Calpain—To investigate whether integrin \(\alpha_{11}\beta_3\) is a substrate for calpain, purified \(\alpha_{11}\beta_3\) and calpain were incubated together in vitro. Cleavage of the integrin was then assessed by immunoblotting with site-specific antibodies. Calpain digestion abolished reactivity of an antipeptide antibody against the C-terminal 20 residues of the \(\beta_3\) subunit (Fig. 1). In contrast, the reactivities of antibodies against the entire subunit or against its extracellular domain were not affected by calpain treatment. Moreover, there was no major reduction in mass of the \(\beta_3\) subunit, indicating that the bulk of \(\beta_3\) is calpain-resistant. Thus, calpain cleavage sites in \(\beta_3\) are located primarily within the cytoplasmic domain.

In contrast, the cytoplasmic domain of \(\alpha_{11}\beta_3\) did not appear to be cleaved by calpain, as indicated by the preservation of reactivity with an antibody to the C terminus of the \(\alpha_{11}\beta_3\) cytoplasmic domain (anti-\(\beta_3\)C). In addition, reactivity with an antibody to the N terminus of the \(\alpha_{11}\beta_3\) heavy chain (PMI-1) was also unaffected by calpain. The N terminus of the \(\alpha_{11}\beta_3\) light chain appeared to be cleaved since a second more mobile band, reactive with the antibody against the C terminus of the \(\alpha_{11}\beta_3\) light chain, became evident after calpain treatment (Fig. 1). This more mobile band did not react with antibody anti-V41 (specific for the N terminus of the \(\alpha_{11}\beta_3\) light chain, and anti-V41 staining of the intact \(\alpha_{11}\beta_3\) light chain was decreased by calpain treatment. Thus, the \(\alpha_{11}\beta_3\) cytoplasmic domain resists calpain cleavage, while the N terminus of the light chain is susceptible. Consequently, the cytoplasmic domain of integrin \(\beta_3\) appears to be a preferred intracellular substrate for calpain.

To determine if cleavage of the \(\beta_3\) cytoplasmic domain by calpain occurs in intact cells, washed platelets were treated with a calcium ionophore, A23187 (1 \(\muM\)), for 3 min at 37°C to activate intracellular calpain. After solubilization, intact \(\beta_3\) subunit was depleted by sequential immunoprecipitation with anti-\(\beta_3\)C, and residual \(\beta_3\) was detected by Western blotting with an antibody to the extracellular domain of \(\beta_3\) (monoclonal antibody 15). Densitometry showed that >70% of \(\beta_3\) from A23187-treated platelets remained in the supernatant after quantitative immunoadsorption by anti-\(\beta_3\)C. This result indicates that most of the \(\beta_3\) subunit in A23187-activated platelets lacks the C-terminal portion. In contrast, >99% of \(\beta_3\) in resting

**FIG. 1. Cleavage of purified integrin \(\alpha_{11}\beta_3\) by \(\mu\)-calpain.** Integrin \(\alpha_{11}\beta_3\) was incubated with \(\mu\)-calpain at 30°C for 5 min and then analyzed by SDS-PAGE and Western blotting. A, control or calpain-treated integrin \(\alpha_{11}\beta_3\) was immunoblotted with rabbit antipeptide antibody anti-\(\beta_3\)C (against the C-terminal 20 amino acid residues of the \(\beta_3\) cytoplasmic domain), antibody 8053 (against the entire \(\beta_3\) subunit), monoclonal antibody PMI-1 (against the \(\alpha_{11}\) heavy chain), and rabbit antipeptide antibodies anti-IIBC (against the C terminus of the cytoplasmic domain of the \(\alpha_{11}\) light chain) and anti-V41 (against the N terminus of the \(\alpha_{11}\) light chain). B, shown is a schematic of the localization of epitopes recognized by the various anti-\(\alpha_{11}\beta_3\) antibodies used in A. MAb, monoclonal antibody.
platelets was depleted by immunoprecipitation with anti-E-64d (a membrane-permeable calpain inhibitor) (0.2 mM E-64, 1 mM PMSF, and 5 mM EDTA. 50 μl of supernatant were fractionated by SDS-PAGE and immunoblotted with a monoclonal anti-β3 antibody, 15. B, platelets were stirred at 37 °C for 3 min after adding buffer (0), 1 μM A23187 (A), or A23187 with 0.1 mM cytoplasmic domain peptide, Glu731–Thr762. 

FIG. 2. Calpain cleavage of β3 in platelets. A, washed platelets in modified Tyrode’s buffer (0.4 ml) were incubated at 37 °C with (A23187) or without (control) 1 μM calcium ionophore A23187 for 3 min with continuous stirring at 1000 rpm. The platelets were solubilized by addition of an equal volume of solubilization buffer (see “Experimental Procedures”) and immunoprecipitated twice with 50 μl of preimmune serum (control (Ctrl)), anti-β3 antibody 8053 (β3), or the anti-β3 C-terminal peptide antibody, anti-β3C (β3C). After removing the antibody-bound proteins with protein A-conjugated Sepharose beads, the 50 μl of supernatant were fractionated by SDS-PAGE and immunoblotted with preimmune serum (Ctrl) or anti-β3C.

Identification of the Calpain Cleavage Sites in the Cytoplasmic Domain of β3—To map calpain cleavage sites in the cytoplasmic domain of the β3 subunit, a 43-residue synthetic peptide based on the sequence of the cytoplasmic domain was incubated with purified calpain, and the products were separated by reverse-phase HPLC (Fig. 3). The molecular masses of peptide fragments from major HPLC peaks were determined by ion-spray mass spectrometry (Table I). The identities of the fragments were assigned from their masses using the MacProMass program. The majority of calpain digestion-generated fragments were unambiguously assigned (Table I). For fragment d (mass = 1376 Da), two assignments (Lys748–Tyr759 or Tyr747–Thr758) were possible. Lys748 was predicted to be the N terminus of fragment a, and Tyr759 was the C terminus of fragment g. Thus, Lys748–Tyr759 is likely to account for fragment d. The cleavage sites identified are depicted schematically in Fig. 3C.

Three of the cleavage site assignments were confirmed by N-terminal sequence analysis of the products of calpain digestion of the β3 cytoplasmic domain peptide, Glu731–Thr762. Three HPLC peaks were found to contain peptides with N-terminal sequences differing from the intact peptide: A742NNPLYKEA..., K748EATSTF..., and T755TNITYRT. Confident three of the calpain cleavage sites identified by mass spectrometry (Fig. 3C). Thus, five calpain cleavage sites were identified C-terminal to Ala735, Thr741, Tyr747, Phe754, and Tyr759 of the β3 subunit.

Calpain Cleavage Site Utilization in Intact Platelets—To determine whether the calpain cleavage sites identified in vitro were used in platelets, we generated cleavage site-specific antibodies. To generate such antibodies, we immunized rabbits with pentapeptides EEERA (Ab 735), AKWDT (Ab 741), NNPLY (Ab 747), ATSTF (Ab 754), and TNITY (Ab 759). In each case, the antibody is identified by the number of the new C-terminal residue generated by the predicted cleavage. Western blot analysis indicated that these antibodies either did not react (Ab 735, Ab 741, Ab 747, and Ab 754) or reacted weakly (Ab 759) with β3 from resting platelets (Fig. 4). When the platelets were treated with the calcium ionophore A23187, there was a marked increase in staining with Ab 741, Ab 747, Ab 754, and Ab 759 (Fig. 4). This indicates the exposure of new C termini in β3 corresponding to calpain cleavage sites C-
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A synthetic peptide corresponding to the \( \beta_3 \) cytoplasmic domain was incubated with calpain at 30 °C for 30 min, and the proteolytic fragment was separated by reverse-phase HPLC. Samples from each peak were analyzed by ion-spray mass spectrometry as described under “Experimental Procedures.” Sequences were assigned on the basis of determined molecular mass and the sequence of the parent peptide using MacProMass as described under “Experimental Procedures.”

### Table I

| HPLC peaks | Retention time | Molecular mass | Sequence assignment | Designation |
|------------|----------------|----------------|---------------------|-------------|
| a          | 9.02           | 783            | KEATSTTF            | Lys748-Phe754 |
| b          | 18.20          | 926            | TNITYRGT\(^a\)      | Thr755-Thr762 |
| c          | 22.37          | 2006           | TTDHREAPFEEERA      | Thr720-Ala738 |
| d          | 24.07          | 1376           | KEATSTFTNITY\(^a\) | Lys748-Tyr759 |
| e          | 25.68          | 1457           | YKEATSTFTNIT\(^b\) | Tyr747-Tyr758 |
| f          | 27.18          | 2213           | ANNPLYKEATSTTF\(^a\) | Ala742-Phe754 |
| g          | 28.13          | 2048           | ANNPLYKEATSTFTNIT\(^a\) | Ala742-Tyr754 |

\(^a\) Also found by N-terminal sequencing.

\(^b\) Alternative assignment for fragment d, but not observed by N-terminal sequencing.

![Calpain cleavage site utilization in integrin \( \beta_3 \) in intact platelets](image)

Washed platelets resuspended in Tyrode’s buffer (10⁹/ml) were treated with 1 µM A23187 in the absence (A) or presence (A + E) of 1 mM E-64d. Alternatively, no A23187 was added (0). After 3 min at 37 °C, platelets were solubilized by addition of an equal volume of SDS sample buffer containing 0.2 mM E-64, 0.5 mM leupeptin, 1 mM PMSF, and 5 mM EDTA. The proteins were separated by SDS-PAGE and then immunoblotted with the indicated calpain cleavage site-specific antibodies. Antibody binding was visualized by peroxidase-conjugated goat anti-rabbit antibodies and ECL.

Terminal reactivity with antibodies specific for the calpain digestion sites of \( \beta_3 \). As shown in Fig. 6, the cleavage site-specific antibody, Ab 754, did not bind to \( \beta_3 \) from resting platelets (lane 0). In contrast, binding of the antibody was observed as early as 1 min after thrombin addition, before the platelets were fully aggregated. Reaction with Ab 754 increased with time (Fig. 6) and was inhibited by adding E-64d (data not shown), indicating that continuing calpain cleavage occurred. Similarly increased reactivities of the antibodies against the other calpain cleavage sites (Ab 741, Ab 747, and Ab 759) were also observed during thrombin-induced platelet aggregation (data not shown).
DISCUSSION

We have found that the cytoplasmic domain of the integrin \( \beta_3 \) subunit is cleaved by calpain and have mapped five cleavage sites. Four of these sites (C-terminal to Thr\(^{741} \), Tyr\(^{747} \), Phe\(^{754} \), and Tyr\(^{759} \)) are utilized in intact platelets. The fifth site is accessible only after treatment known to dissociate the \( \alpha_{11b}\beta_3 \) heterodimer. These calpain cleavages remove residues critical for the attachment of the integrin to the cytoskeleton and bidirectional transmembrane signaling. Thus, calpain cleavage may regulate functions of the \( \beta_3 \) integrins.

The conclusion that calpain cleaves the cytoplasmic domain of integrin \( \beta_3 \) comes from three lines of evidence: 1) in vitro cleavage of the \( \beta_3 \) cytoplasmic domain of purified integrin \( \alpha_{11b}\beta_3 \) by purified \( \mu \)-calpain, 2) cleavage of synthetic \( \beta_3 \) cytoplasmic domain peptides by purified \( \mu \)-calpain, and 3) limited cleavage of the cytoplasmic domain of the integrin in intact platelets stimulated by A23187 or thrombin. Although the conditions differ significantly, the results from these experiments are highly consistent, indicating that primary structure-defined structures of the \( \beta_3 \) cytoplasmic domain are recognized by calpain. Calpain cleavages released only small peptide fragments from the C-terminal region of \( \beta_3 \), resulting in no significant shift in its mobility on SDS-PAGE. This may explain why calpain cleavage of the \( \beta_3 \) subunit was not noted previously (30).

Calpain cleavage of \( \beta_3 \) occurs during platelet aggregation, suggesting that it may regulate platelet function. Immunoblot with an antibody specific for the \( \beta_3 \) C terminus almost completely depleted \( \beta_3 \) from resting platelets. In comparison, \( \sim70\% \) of \( \beta_3 \) in A23187-activated platelets did not react with this antibody (Fig. 2A). This indicates that the bulk of \( \beta_3 \) in resting platelets is intact. However, calpain cleavage of \( \beta_3 \) was observed as early as 1 min after adding a physiological platelet agonist (thrombin) and increased with a time course similar to that of calpain activation in thrombin-activated platelets (Fig. 2B) (7). Platelet aggregation leads to calpain cleavage of cytoskeletal proteins such as talin (16) and signaling molecules such as protein-tyrosine phosphatase IB (18), pp60c-src (19). Interestingly, the \( \beta \) subunit cytoplasmic domain is believed to interact with talin (31, 32), and integrins regulate the functions of the other signaling molecules (11, 17, 18). Thus, it is possible that calpain cleavage of both the cytoplasmic domain of an integrin and its downstream signaling partners may be a coordinated process. In vitro digestion of the purified integrin by calpain also established cleavage at the N-terminal domain of the \( \alpha_{11b} \) light chain, which is consistent with an earlier observation that \( \alpha_{11b} \) may be proteolytically modified (24). This cleavage is not regulated by platelet activation (24).

Calpain cleavage site utilization in intact cells was detected with antipeptide antibodies specific for the cleavage sites. Each antigenic peptide had a 5-residue sequence (normally a minimum required for an epitope) corresponding to the C terminus generated by a predicted calpain cleavage. The antipeptide antibodies reacted preferentially with calpain-cleaved \( \beta_3 \) (Fig. 4). As described previously (26) and in this study (Fig. 4), this strategy can thus be used to identify protease cleavage site utilization in vivo.

The \( \alpha_{11b} \) subunit may affect calpain access to cleavage sites in the \( \beta_3 \) cytoplasmic domain. Four of five calpain cleavage sites in \( \beta_3 \) were cleaved by calpain in intact platelets. However, the most membrane-proximal cleavage site (Ala\(^{735} \)) became susceptible to calpain cleavage only after pretreatment of the integrin with EDTA. As EDTA is known to dissociate the calcium-dependent complex of \( \alpha_{11b} \) and \( \beta_3 \) subunits (28), this result suggests that the region near Ala\(^{735} \) was shielded from calpain cleavage in the complexed \( \alpha_{11b}\beta_3 \) heterodimer. Thus, either the interaction of \( \alpha_{11b} \) with \( \beta_3 \) may regulate the conformation of the \( \beta_3 \) cytoplasmic domain, or the cytoplasmic domain of \( \alpha_{11b} \) may directly interact with the cytoplasmic domain of \( \beta_3 \) at a site close to Ala\(^{735} \).

Calpain specificity is not defined solely by the amino acid residues flanking the scissile bonds (14). Thus, the secondary and tertiary structures of the protein in the vicinity of the scissile bond may be important determinants of cleavage. Peptide-derived calpain inhibitors such as chloromethyl ketones (Leu-Leu-Tyr-CH\(_2\)Cl and Leu-Leu-Phe-CH\(_2\)Cl) contain the hydrophobic residues (leucine) N-terminal to an aromatic amino acid residue (Tyr or Phe) (33). Moreover, a hydrophobic residue (corresponding to P2 and P3 positions) N-terminal to an aromatic or a positively charged residue at the cleavage site is a pattern frequently present in calpain substrates (14). The four calpain cleavage sites in the \( \beta_3 \) cytoplasmic domain identified in intact platelets (A\(^{742} \)NNPLY\(^{747} \) ↓ and T\(^{755} \)NITY\(^{759} \) ↓) flank two NXXY motifs. The two NXXY motifs in the \( \beta_3 \) cytoplasmic domain each contain a leucine or isoleucine at one of the X positions, N-terminal to a tyrosine at the calpain cleavage site. Thus, the NXXY motifs are similar to cleavage sites found in other calpain substrates. Calpain cleavage also occurs on the N-terminal side of the NXXY sequence. In the low density lipoprotein receptor, the NXXY motif forms a tight turn so that both N- and C-terminal flanks are sterically adjacent (34). It is possible that such turns could exist in the \( \beta_3 \) cytoplasmic domain.

Cleavage of the \( \beta_3 \) cytoplasmic domain by calpain near two NXXY sites may be an important mechanism for the regulation of its bidirectional signaling and \( \alpha_{11b}\beta_3 \) attachment to the cytoskeleton following ligand binding. The more N-terminal NXXY motif of the \( \beta_3 \) cytoplasmic domain has the sequence NPLY, similar to the NPXY internalization signal identified in the low density lipoprotein receptor (35). Mutations disrupting this motif abolish the capacity of the \( \beta_3 \) cytoplasmic domain to regulate the affinity state of the receptor (inside-out signaling) (9), to associate with the cytoskeleton at focal adhesion sites (10, 36), and to mediate cell migration (37). Furthermore, NXXY motifs containing hydrophobic residues at one of the X residues are highly conserved in the cytoplasmic domain of most integrin \( \beta \) subunits, including \( \beta_1 \), \( \beta_2 \), \( \beta_3 \), \( \beta_4 \), and \( \beta_7 \), and have been implicated in the functions of some of these subunits (38, 39). Thus, it is possible that calpain cleavage may also occur to these integrins near the NXXY motif and regulate the functions of these integrins. So far, only the \( \beta_4 \) cytoplasmic domain has been reported to be a substrate for calpain (40, 41). The \( \beta_3 \) cytoplasmic domain is very different from that of other integrin \( \beta \) subunits in primary sequence, size (1019 residues), and function (42, 43). Although the \( \beta_3 \) cytoplasmic domain also contains an NXXY sequence (42), calpain cleavage sites in the \( \beta_3 \) cytoplasmic domain have not been accurately identified and thus cannot be compared with cleavage sites in \( \beta_3 \) as described in this study.

The work presented here provides the first evidence of physiologically regulated calpain cleavage of an integrin cytoplasmic domain. In platelets, cleavage of the \( \beta_3 \) subunit could limit or reverse platelet aggregation and could permit relaxation of contracted fibrin clots. It may also be important to the calpain-dependent shedding of integrin-containing, procoagulant membrane vesicles during platelet aggregation (44). As calpain co-localizes with integrins in focal adhesion sites, calpain cleavage at these sites may also serve as a mechanism to detach migrating cells from the extracellular matrix while leaving an integrin "trail" behind (45).

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