**Critical roles for the COOH-terminal NITY and RGT sequences of the integrin β₃ cytoplasmic domain in inside-out and outside-in signaling**

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Bidirectional signaling of integrin α₁β₃ requires the β₃ cytoplasmic domain. To determine the sequence in the β₃ cytoplasmic domain that is critical to integrin signaling, cell lines were established that coexpress the platelet receptor for von Willebrand factor (vWF), glycoprotein Ib-IX, integrin α₁β₃, and mutants of β₃ with truncations at sites COOH terminal to T⁷４¹, Y⁷４⁷, F⁷５⁴, and Y⁷⁵⁹. Truncation at Y⁷⁵⁹ did not affect integrin activation, as indicated by vWF-induced fibrinogen binding, but affected cell spreading and stable adhesion. Thus, the COOH-terminal RGT sequence of β₃ is important for outside-in signaling but not inside-out signaling. In contrast, truncation at F⁷⁵⁴, Y⁷⁴⁷, or T⁷⁴¹ completely abolished integrin activation. A point mutation replacing Y⁷⁵⁹ with alanine also abolished integrin activation. Thus, the T⁷⁵⁷NITY⁷⁵⁹ sequence of β₃ containing an NXXY motif, is critical to inside-out signaling, whereas the intact COOH terminus is important for outside-in signaling. In addition, we found that the calcium-dependent protease calpain preferentially cleaves at Y⁷⁵⁹ in a population of β₃ during platelet aggregation and adhesion, suggesting that calpain may selectively regulate integrin outside-in signaling.

**Introduction**

The prototype integrin, α₁β₃, plays critical roles in platelet adhesion and aggregation. Normally, α₁β₃ on circulating platelets is present in a “resting” form, with a low affinity for its ligands such as soluble fibrinogen and von Willebrand factor (vWF).* Upon vascular injury, exposure of platelets to soluble agonists, such as thrombin and ADP, or extracellular matrix adhesive proteins, such as collagen and vWF, induces “inside-out” signals activating the ligand-binding function of α₁β₃ (Ginsberg et al., 1992; Shattil et al., 1998; Parise, 1999). Ligand binding to α₁β₃ not only forms adhesive bonds between platelets and adhesive ligands, but also transmits “outside-in” signals to induce a series of cellular responses, such as protein phosphorylation (Ferrell and Martin, 1989; Golden et al., 1990; Lipfert et al., 1992; Clark et al., 1994; Law et al., 1996; Lerea et al., 1999), elevation of intracellular 

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*Abbreviation used in this paper: vWF, von Willebrand factor.

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Inside-out and outside-in signaling of integrins have been suggested to involve the interaction between the cytoplasmic domains of integrins and intracellular signaling molecules. Interactions between the membrane-proximal regions of the integrin α₁β₃ and β₃ cytoplasmic domains are important in maintaining a resting conformation of the integrin, and inside-out signaling is associated with disruption of this interaction (Hughes et al., 1996; Vinogradova et al., 2002). The cytoplasmic domain of α₁β₃ interacts with several intracellular proteins, including cytoskeletal proteins such as talin (Calderwood et al., 1999; Knezevic et al., 1996) and myosin (Phillips et al., 2001), calcium-binding protein CIB (Naik et al., 1997), phosphotyrosine-binding proteins SHC and GRB2 (Law et al., 1996), protein kinases such as integrin-linked protein kinase (Hannigan et al., 1996), and β₃ endonexin (Shattil et al., 1995). Binding of talin (Calderwood et al., 1999, 2002) and β₃ endonexin (Kashiwagi et al., 1997) to β₃ has been implicated in promoting integrin activation. Binding of phosphotyrosine-binding proteins and focal adhesion kinase has been suggested to play roles in outside-in signaling (Law et al., 1996). Also, the β₃ cytoplasmic domain can be chemically modified during platelet activation. For example, T⁷⁵⁵ (Lerea et al., 1999), Y⁷⁴⁷, and Y⁷⁵⁹ (Law et al., 1996) are phosphorylated by protein kinases, and the COOH-terminal region of β₃ is cleaved by calpain at the COOH-terminal side of Y⁷⁴¹, T⁷⁴⁷, F⁷⁵⁴, and Y⁷⁵⁹ (Du et al., 1998).
by flow cytometry with an antibody recognizing the COOH terminus of cleaved forms of GPIb-IX, and stable cell lines were established. Expression of correct truncation mutants of GPIb-IX (123) was also comparable with a cell line expressing GPIb-IX alone (1b9) and a cell line expressing α₁β₃, alone (2b3a), respectively.

Figure 1. Truncation mutants of α₁β₃ coexpressed with GPIb-IX in CHO cells. (A) Schematics showing truncation mutants of β₃ that mimic calpain cleavage at four previously identified sites. A Y759A point mutation is also depicted. (B) The mutants described in A were cotransfected with α₁ in a CHO cell line already expressing GPIb-IX, and stable cell lines were established. Expression of correct truncation mutants in each of the cell lines was verified by immunoblotting cell lysates with antibodies specifically recognizing calpain-truncation mutants in each of the cell lines was verified by immunoblotting cell lysates with antibodies specifically recognizing calpain-truncation mutants in each of the cell lines was verified by immuno-GPIb-IX, and stable cell lines were established. Expression of correct truncation mutants of GPIb-IX (123) was also comparable with a cell line expressing GPIb-IX alone (1b9) and a cell line expressing α₁β₃, alone (2b3a), respectively.

with an antibody against GPIbα, SZ2 (shaded lines). Nonspecific mouse IgG was used as a negative control (solid lines). Please note that levels of expression of each of these mutants were comparable with 123 cells. Expression of GPIb-IX and integrin was also comparable with a cell line expressing GPIb-IX alone (1b9) and a cell line expressing α₁β₃, alone (2b3a), respectively.

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ing and reduced outside-in signaling. Thus, the NITY sequence is essential for both inside-out and outside-in signaling of αIβ3, and the RGT sequence is important for outside-in signaling. Furthermore, localized calpain cleavage of β3 during platelet activation mainly occurs at a site COOH terminal to Y759, suggesting that calpain cleavage may selectively regulate outside-in signaling.

Results

CHO cell lines coexpressing mutants of the human integrin αIIIβ3 and GPIb-IX
To study the roles of the COOH-terminal region of β3 in integrin signaling, we have generated four truncation mutants of the β3 subunit with truncations at sites COOH terminal to T741, Y747, F754, and Y759. Truncations at these sites also coincide with previously identified calpain cleavage sites in β3 (Du et al., 1995; Fig. 1 A). These mutants were cotransfected with the integrin αIII subunit into a CHO cell line that expresses recombinant human GPIb-IX complex (1b9). Complex formation between these β3 mutants and αIII was verified by flow cytometry using an αIIIβ3 complex–specific monoclonal antibody, D57. Stable cell lines expressing both GPIb-IX and integrin mutants (1b9/741, 1b9/747, 1b9/754, and 1b9/759) were obtained by cell sorting with antibodies specific for integrin αIIIβ3 and GPIb-IX. Expression of correct mutants at each of the four sites was verified by immunoblotting with antibodies that specifically recognize each of these sites only when the site is truncated (Du et al., 1995; Fig. 1 B). In addition, a cell line coexpressing GPIb-IX and a mutant αIIIβ3 bearing alanine substitution of Y759 in β3 was also established (1b9/Y759A). Expression levels of GPIb-IX and integrin αIIIβ3 on the above mutant cell lines were comparable with a cell line expressing GPIb-IX and wild-type integrin αIIIβ3 (123 cells) at the time of experiments (Fig. 1 C).

Reconstituted CHO cell model of integrin αIIIβ3 activation
We recently reported the reconstitution of GPIb-IX–mediated integrin activation in CHO cells expressing recombinant human GPIb-IX and integrin αIIIβ3 (123 cells) (Gu et al., 1999). In this reconstituted integrin activation model, vWF binding to GPIb-IX in the presence of ristocetin activates fibrinogen binding to the 123 cells in an RGDS-dependent manner, thus allowing the study of integrin inside-out signaling using a specific recombinant DNA approach. Although β3-transfected CHO cells also express an αIβ3 integrin composed of endogenous αI and recombinant β3, Fig. 2 shows that vWF-induced fibrinogen binding to 123 cells is specifically inhibited by a monoclonal antibody that recognizes human αIIIβ3, but not by a blocking monoclonal antibody that recognizes αIβ3. Also, a cell line expressing GPIb-IX and β3 without αIII failed to show RGDS-dependent fibrinogen binding under identical conditions (unpublished data). These results indicate that vWF-induced fibrinogen binding is mediated by αIIIβ3 but not αIβ3. Thus, vWF-induced fibrinogen binding to 123 cells specifically reflects the activation of the platelet integrin αIIIβ3.

Effects of β3 mutations on integrin activation (inside-out signaling)
To understand the roles of the COOH-terminal region of β3 in inside-out signaling of αIIIβ3, we examined vWF-induced fibrinogen binding to cell lines expressing GPIb-IX and truncation mutants of αIIIβ3 (1b9/741, 1b9/747, 1b9/754, and 1b9/759). Fig. 3 shows that truncation of the β3 cytoplasmic domain at the site COOH terminal to Y759 did not affect vWF-induced activation of fibrinogen binding to integrin αIIIβ3. In contrast, truncations at F754, Y747, or T741...
abolished vWF-induced integrin activation. Thus, it appears that the RGT sequence at the COOH terminus of the cytoplasmic domain is not required for integrin activation, whereas the sequence between F754 and Y759, which contains an NXXY motif, is required for integrin activation. To further determine if the NXXY motif is important in integrin activation, we examined vWF-induced fibrinogen binding to CHO cells coexpressing GPIb-IX and the Y759A mutant of integrin αIIbβ3. Indeed, vWF-induced fibrinogen binding was abolished in the Y759A mutant, indicating that the NXXY motif, particularly Y759, is required for vWF-induced activation of αIIbβ3.

Fibrinogen-binding function of the integrin αIIbβ3 mutants

A possible alternative interpretation to the above results is that the activation-defective mutants of αIIbβ3 (754, 747, and 741) may have a loss of function in the extracellular ligand-binding site. To exclude this possibility, we examined the fibrinogen-binding function of these mutants. It has been demonstrated previously (Du et al., 1991) that binding of the ligand mimetic peptide, RGDS, to the extracellular ligand-binding domain of αIIbβ3 transforms the integrin into intensity by flow cytometry (solid lines). Nonspecific binding of fibrinogen was estimated by adding 1 mM RGDS (shaded lines). (B) Quantitative results from three experiments (means ± SD) are expressed as fibrinogen binding index (total bound fluorescence intensity/nonspecifically bound fluorescence intensity).
an active conformation. After fixation and removal of RGDS, the activated integrin is able to bind fibrinogen. Thus, we examined RGDS-induced fibrinogen binding to cells expressing mutants of α₁β₃ (Fig. 4). All of the above mutant cell lines bound fibrinogen in a manner similar to wild-type integrin α₁β₃ (123 cells). In contrast, 1b9 cells, in which no integrin α₁β₃ was expressed, did not bind fibrinogen after RGDS treatment. These data suggest that the activation-defective mutants used in this study retained fibrinogen-binding function. Therefore, the inability of these mutants to bind fibrinogen after vWF stimulation results from a deficiency in inside-out signaling.

Effect of mutations on cell adhesion and spreading under static conditions

An important function for α₁β₃ is to mediate stable platelet adhesion and spreading on immobilized integrin ligands such as vWF and fibrinogen. Integrin-dependent stable cell adhesion and spreading on vWF are significantly enhanced by inside-out signaling induced by GPIb-IX–vWF interaction (Savage et al., 1992) and also require integrin outside-in signaling. Integrin α₁β₃-mediated stable cell adhesion and spreading to immobilized fibrinogen, however, do not require inside-out signaling (Coller, 1980; Savage et al., 1992) but are dependent upon ligand-induced integrin activation and outside-in signaling (Du et al., 1991; Phillips et al., 1991; Ginsberg et al., 1995; Shattil et al., 1998). Thus, to determine the roles of the COOH-terminal region of β₃ in outside-in and inside-out signaling, we examined stable adhesion and spreading of the mutant cell lines to fibrinogen and vWF. As previously reported (Gu et al., 1999), CHO cells (without transfected GPIb-IX and integrin α₁β₃) poorly adhere to vWF or fibrinogen. CHO cells expressing GPIb-IX alone also poorly adhere to both vWF and fibrinogen (Fig. 5). Even in the presence of botrocetin, which enhances the GPIb-IX binding affinity of vWF and allows GPIb-IX-dependent stable cell adhesion to vWF, these cells poorly spread on vWF (unpublished data), indicating that β₃ integrin is required for cell spreading on vWF (Gu et al., 1999). As expected, CHO

Figure 5. Effects of mutations on integrin-dependent stable cell adhesion and spreading on vWF or fibrinogen (Fg) under static conditions. The indicated cell lines in modified Tyrode’s buffer (10⁵/ml) were allowed to incubate in fibrinogen- or vWF-coated microtiter wells at 37°C for 1 h. The wells were washed three times, and the cells remaining adherent were quantitated using an acid phosphatase assay, as described under the Materials and methods (A), and photographed to document cell spreading (B). The means and standard deviations of triplicate samples are shown in A.
Effects of integrin \( \beta_3 \) mutations on cell adhesion under flow

A major physiological role of GPIb-IX–induced integrin activation is to mediate stable platelet adhesion to immobilized vWF. Under flow conditions, initial transient platelet adhesion and rolling on vWF are mediated by GPIb-IX. Interaction of vWF with GPIb-IX induces integrin activation and integrin-dependent stable platelet adhesion. Thus, if the mutations of \( \alpha_{IIb} \beta_3 \) abolished integrin inside-out signaling, these mutants should also show defective stable adhesion to vWF under flow conditions. To determine this, the above-described cell lines were perfused through a capillary tube precoated with human vWF. At shear rates below 50 s\(^{-1}\), the cells expressing integrin mutants 1b9/754, 1b9/747, and 1b9/741 (all defective in inside-out signaling) were defective in stable cell adhesion to vWF compared with 123 cells expressing wild-type \( \alpha_{IIb} \beta_3 \). The shear rate condition mirrored integrin activation indicated by fibrinogen binding. However, when the flow shear rate was further increased to 100 s\(^{-1}\) or above, not only the integrin activation–deficient mutants, but also 1b9/759 cells showed significantly reduced adhesion to vWF, suggesting that the ability of 1b9/759 cells to resist shear force is impaired. This is consistent with the above results that 1b9/759 cells showed decreased adhesion in the static adhesion assay, which involves multiple washes with considerable shear force. As the vWF-induced ligand-binding function of the \( \Delta759 \) mutant is normal, we conclude that the impaired outside-in signaling function of this mutant reduced the...
ability of 1b9/759 cells to resist shear stress. Together, these results indicate that the RGT sequence at the COOH terminus of β3 is important for outside-in signaling of αIIbβ3, but the NITY sequence is important for both inside-out and outside-in signaling.

Differential cleavage at different sites of the β3 cytoplasmic domain by calpain in platelets
We have shown previously that calpain may cleave the cytoplasmic domain of β3 at sites COOH terminal to T741, Y747, F754, and Y759, which generates β3 fragments identical to the above-described truncation mutants of β3 (741, 747, 754, and 759). As we showed above that truncations at these different sites result in different functional effects on integrin signaling, our results also indicate that cleavage of the β3 cytoplasmic domain at these different sites has the potential to differentially regulate outside-in and inside-out signaling of integrin αIIbβ3. To determine if calpain differentially cleaves the β3 integrin at different sites during platelet activation, washed platelets were treated with or without thrombin (0.1 U/ml) and then immunoblotted for calpain cleavage by the cleavage-specific antibodies (Du et al., 1995). We found that anti-759 antibody, which only recognizes β3 molecules with cleavage at Y759, reacted with ~0.8% of the integrin αIIbβ3 molecules in washed “resting” platelets (Fig. 7). This reaction was unlikely to result from cross-reactivation of this antibody with the intact β3 subunit, because we showed that the antibody did not react with the intact β3 subunit but reacted with the A759 mutant expressed in CHO cells (Fig. 2). Thus a very small percentage of the β3 molecules in resting platelets has been cleaved at the Y759 site. Stimulation of platelets with thrombin caused a time-dependent and significant increase in the cleavage at Y759. In contrast to the 759 site, calpain cleavage at T741 or Y747 (Fig. 7) occurred to a much lesser degree and only after a much longer exposure to thrombin. Calpain cleavage at the 741 site was not detectable in thrombin-stimulated platelets (unpublished data) but was detected in platelets treated with calcium ionophore A23187 (Du et al., 1995). Thus, in thrombin-activated platelets, calpain preferentially cleaves β3 at Y759. As we showed above that cleavage at Y759 selectively reduced integrin outside-in signaling without affecting inside-out signaling, this result indicates that calpain cleavage has the potential to selectively regulate outside-in signaling during platelet activation.

Distribution of calpain-cleaved β3 in spreading platelets
Calpain only cleaves a percentage of β3 molecules in platelets stimulated with thrombin, suggesting that cleavage of β3 is likely to have only a localized effect on integrin function. It is known that during cell spreading on integrin ligands, integrin forms a localized signaling complex with cytoskeletal and signaling molecules that is dynamically regulated and involves calpain activity (Biakowska et al., 2000). To determine if selective integrin cleavage occurred at specific regions during platelet spreading on integrin ligands, platelets were allowed to spread on fibrinogen for 60 min and then double stained with calpain cleavage–specific antibodies and a monoclonal antibody against an extracellular epitope of β3 that is not affected by calpain cleavage. Consistent with the above results in thrombin-stimulated platelets, calpain cleavage occurred mainly at Y759 (Fig. 8), but cleavages at F754 (Fig. 8) or the more NH2-terminal sites (unpublished data) were significantly weaker. Whereas the β3 molecules were distributed throughout the spreading platelets with particularly strong staining at the edges, calpain-cleaved β3 was not seen at the edges (or in the pseudopods) but was concentrated in the central region of the spreading platelets as clusters. As integrin engagement with immobilized ligands starts from the central region where discoid platelets initially adhere, our data suggest that cleavage of β3 by calpain occurred only to the population of the integrin that is no longer at the leading edge of spreading platelets. This selective cleavage of the centrally located integrin population (mainly at Y759) may

Figure 7. Progressive cleavage at different sites of the β3 cytoplasmic domain by calpain. Washed human platelets (10⁷/ml) were directly solubilized in SDS-PAGE sample buffer containing EDTA and E64 (Control), or treated with thrombin (0.1 U/ml) for increasing lengths of time at 37°C before being solubilized. Platelet lysates were then immunoblotted with Ab759, Ab754, Ab747, and Ab741 to detect calpain cleavages at Y759, F754, T747, and Y741 sites, with Ab762 to detect uncleaved β3, and Mab 15 to detect total β3 levels. Quantification of antibody reactions was performed by scanning the immunoblots and analyzing using NIH Image as described under the Materials and methods. Percentages of the molecules cleaved (or uncleaved) at a particular site are shown in B.
serve to down-regulate local integrin outside-in signaling in these areas without affecting the activation state of the integrin. It is possible that selective cleavage of integrin at Y\(^{759}\) may serve to facilitate the reorganization of the integrin–cytoskeleton signaling complex during platelet spreading.

Discussion

By using a reconstituted integrin activation and adhesion model in transfected CHO cells and a panel of truncation mutants of the integrin \(\alpha_{\text{IIb}}\beta_3\), we show that the N\(^{760}\)ITY\(^{759}\) motif of the \(\beta_3\) cytoplasmic domain plays a critical role in inside-out signaling of the integrin \(\alpha_{\text{IIb}}\beta_3\), and the COOH-terminal R\(^{760}\)GT\(^{762}\) sequence of \(\beta_3\) is important in outside-in signaling–dependent events. Furthermore, we show that calpain preferentially cleaves a population of \(\beta_3\) at Y\(^{759}\) in thrombin-activated platelets as well as in platelets spreading on fibrinogen, suggesting that calpain may selectively regulate outside-in signaling of the integrin.

We conclude that the NITY sequence of the \(\beta_3\) cytoplasmic domain is critical to the inside-out signaling function of \(\alpha_{\text{IIb}}\beta_3\). This conclusion is supported by the finding that deletion of the RGT sequence COOH terminal to the NITY motif did not affect vWF-induced integrin activation, but deletion of the TNITY sequence completely abolished integrin activation. The importance of the NITY sequence in inside-out signaling is further supported by the data that the Y759A point mutation inhibited vWF-induced integrin activation. Also, we show that inhibition of vWF-induced fibrinogen binding is not caused by loss of the fibrinogen-binding function per se, as RGDS-induced fibrinogen binding to either 1b9/754 or 1b9/Y759A mutant cells was not different from the wild-type integrin. Furthermore, our data indicate that the NITY sequence and the COOH-terminal RGT sequence are both important for outside-in signaling of \(\alpha_{\text{IIb}}\beta_3\) and integrin-dependent stable cell adhesion and spreading. This is consistent with previous work showing that this region of the \(\beta_3\) cytoplasmic domain is important in cell spreading and focal adhesion formation (Ylanne et al., 1993, 1995). Thus, the NITY motif is important for both inside-out and outside-in signaling of the integrin \(\alpha_{\text{IIb}}\beta_3\).

The NITY motif contains a tyrosine residue that becomes phosphorylated during platelet aggregation (Law et al., 1996). The phosphorylated NXXY motifs have been shown to interact with several intracellular molecules, including myosin and phosphotyrosine-binding proteins such as GRB2 and SHC (Cowan et al., 2000), which are implicated in integrin outside-in signaling. It has been shown that muta-

tion of both tyrosine residues to phenolalanines selectively abolished outside-in signaling in transgenic mouse platelets (Law et al., 1999), suggesting that phosphorylation of one or both of these tyrosine residues is important for outside-in signaling but not for integrin activation (inside-out signaling). Thus, although we conclude that the NITY sequence is essential for inside-out signaling, phosphorylation at Y\(^{759}\) is unlikely to be involved in this process. In this regard, a functional difference between Y759F and Y759A mutations has been shown previously (Schaffner-Reckinger et al., 1998). Y759A, but not Y759F, inhibited integrin-dependent cell adhesion. Furthermore, tyrosine phosphorylation occurs only after platelet aggregation, suggesting that inside-out signaling does not involve tyrosine phosphorylation in the NXXY motifs (Law et al., 1996, 1999).

It is interesting to note that although the NITY sequence is important for both inside-out and outside-in signaling, there is a significant difference in the structural requirement between inside-out signaling (integrin activation) and outside-in signaling. Cleavage of the COOH-terminal three residues did not affect inside-out signaling but significantly inhibited outside-in signaling–dependent integrin function, such as cell spreading and stable cell adhesion. On the other hand, disruption of the NITY sequence by Y759A mutation abol-
ished vWF-induced integrin activation (as indicated by soluble fibrinogen binding) and partially (although significantly) inhibited cell spreading and stable cell adhesion on fibrinogen. In addition, previous work suggests that outside-in signaling, but not inside-out signaling, requires phosphorylation at tyrosine residues (Law et al., 1999). The difference in the structural requirements between inside-out and outside-in signals suggests that inside-out and outside-in signals may be mediated by different molecules (or mechanisms) that interact with the COOH-terminal region of β3 during platelet adhesion and aggregation. This also suggests that inside-out and outside-in signals can be differentially regulated.

The family of the calcium-dependent intracellular proteases, calpain, plays important roles in cytoskeletal reorganization, cell migration, platelet aggregation, and clot retraction (Fox et al., 1983; Huttonlocher et al., 1997; Croce et al., 1999; Bialkowska et al., 2000; Azam et al., 2001). We have shown previously that the β3 cytoplasmic domain is cleaved by either calpain I or calpain II at sites flanking two NXXY motifs in human platelets (Du et al., 1995; Pfaff et al., 1999). Calpain cleavage of the β3 cytoplasmic domain also occurs during endothelial cell apoptosis (Meredith et al., 1998). The physiological roles of calpain cleavage of β3 have been unclear. Calpain I knockout in mouse inhibited platelet activation and adhesion (Figs. 7 and 8). Furthermore, we show that cleavage by calpain at different sites of β3 may result in different regulatory effects. Cleavage at Y759 has no significant effect on inside-out signaling but significantly reduces the integrin functions associated with outside-in signaling. In contrast, cleavage at F754 or further NH2-terminal sites abolishes both inside-out and outside-in signaling. Nevertheless, cleavage at these sites occurs much later during platelet activation (Figs. 7 and 8), suggesting that these cleavages are not important for the early stage of integrin activation. On the other hand, we found that calpain cleavage of integrin β3 subunit in intact platelets mainly occurs at the most COOH-terminal Y759 site during platelet activation and adhesion (Figs. 7 and 8). Furthermore, cleavage of β3 does not appear to occur to the integrin molecules at the leading edge of spreading platelets but occurs to more centrally localized integrin molecules. Thus, it is likely that cleavage at Y759 serves to selectively down-regulate outside-in signaling in the integrin–cytoskeletal signaling complexes that have been formed during the earlier stage of platelet spreading, thereby facilitating the dynamic reorganization of the integrin–cytoskeleton signaling complex during platelet spreading.

Materials and methods

Reagents and cell lines

Human vWF was purified as described previously (Booth et al., 1984). Human α-thrombin was purchased from Enzyme Research Laboratories. RGDS peptide was from Bachem, and ristocetin was from Sigma-Aldrich. Monoclonal antibodies against integrin αβ₃ (anti-VNR1) (O’Toole et al., 1990), against integrin β₃ (Mab15) (Frelinger et al., 1990), and against the integrin α₈β₃ complex (D57 and 2G12) (Fojmovic et al., 1991) were provided by M. Ginsberg (The Scripps Research Institute, La Jolla, CA) and V. Woods (University of California, San Diego, CA). Monoclonal antibodies against GPIbα (SZ2) (Ruan et al., 1987) were a gift from C. Ruan (Institute of Hematology, Suzhou, China). Calpain cleavage site-specific antibodies were generated by immunizing rabbits with pentapeptides (AK-WDT for Ab741, NNPLY for Ab747, ATSTF for Ab754, TNITY for Ab759, and TVRGT for Ab762) as described previously (Du et al., 1995). Oregon green 488-conjugated goat anti-mouse IgG, and Alexa Fluor 546-conjugated goat anti-rabbit IgG were from Molecular Probes. Integrin α₈ and β₃ cDNA clones in pcDNA3 vector were provided by M. Ginsberg, and a mutant β₃ cDNA clone bearing the Y759A substitution was a gift from J. Ylänne (University of Helsinki, Helsinki, Finland). GPIb-IX cDNA expressing CHO cells expressing GPIbα and integrin α₈β₃ (2b3a), or both GPIb-IX and α₈β₃ (123) were described previously (Gu et al., 1999).

Construction of truncation mutants of β3 subunit

Truncation mutagenesis was performed using PCR to introduce stop codons into integrin β3 cDNA at sites corresponding to the carboxy side of amino acid residues 71, 747, 754, or 759, respectively. The forward primer has the sequence of AGAGCTTAAGGACAC at an AflII site of β3 cDNA. The reverse primers contain an XhoI digestion site, a stop codon, and the 18-nucleotide β3 sequences at the intended COOH terminus of each mutant. The PCR products were digested with restriction enzymes AspI and XhoI and ligated into a β3 cDNA construct in a modified pcDNA3 vector containing only the 3′-end XhoI site that was digested with the same restriction enzymes. All mutant constructs were verified by DNA sequencing.

Expression of mutant β3 cDNA constructs in CHO cells

CHO cells expressing GPIb-IX (1b9) were maintained in DEAE medium supplemented with 10% FBS, glutamine, and nonessential amino acid. Transfection was performed using LipofectAMINE 2000 (Life Technologies). Each mutant β3 cDNA was cotransfected together with wild-type α₈ and pcDNA3.1/Hyg plasmid at a ratio of 5:1. Stable cell lines expressing mutant β3 were selected in 0.2 μg/ml of hygromycin (Invitrogen). Expression of integrin α₈β₃, was monitored by flow cytometry using D57. Expression of GPIb-IX was detected using SZ2. Cells expressing both GPIb-IX and integrin α₈β₃ were selected by cell sorting. All cell lines were sorted using the expression levels of 123 cells as a gate until similar levels of expression were achieved. To verify correct expression of calpain cleavage-mimicking mutants, cells were also solubilized and electrophoresed on 7% polyacrylamide gels and then immunoblotted with various cleavage-specific antibodies followed by detection using the ECL kit from Amersham.

Fibrinogen binding to transfected CHO cells activated by vWF

Activation of α₈β₃ induced by ristocetin and vWF was examined by flow cytometric analysis of Oregon green 488–conjugated fibrinogen binding to α₈β₃, as previously described (Gu et al., 1999; Li et al., 2001). In brief, transfected CHO cells were resuspended to 5 × 10⁶/ml in modified Tyrode’s solution (2.5 mM Hepes, 130 mM NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 5.5 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, 0.1% BSA, pH 7.4), incubated with Oregon green 488–conjugated fibrinogen (30 μg/ml) and ristocetin (1 mg/ml in the presence or absence of purified human vWF (25 μg/ml) at 22°C for 30 min, and analyzed by flow cytometry. Nonspecific binding of fibrinogen was estimated by measuring fibrinogen binding in the presence of an integrin inhibitor, RGDS (1 μM).

Fibrinogen binding induced by RGDS peptide

RGDS pretreatment–induced fibrinogen binding was assayed according to a previously described method (Du et al., 1991). Cells in modified Tyrode’s solution were incubated with 1 mM RGDS peptide at 22°C for 10 min. Paraformaldehyde–PBS was then added (to a final concentration of 1%), and the mixture was incubated at 22°C for 1 h. After adding 250 mM NaCl, the fixed cells were washed, resuspended in modified Tyrode’s solution, and incubated with Oregon green 488–conjugated fibrinogen (30 μg/ml) for 30 min before flow cytometric analyses.

Cell adhesion under static conditions

Microtitre wells were coated with 10 μg/ml human vWF or fibrinogen in 0.1 M NaHCO₃, pH 8.3, at 4°C overnight and then blocked with 5% BSA–PBS at 22°C for 2 h. Cell suspension (10 μl/ml in modified Tyrode’s buffer with 1% BSA) was added to ligand-coated microtitre wells and incubated for 60 min at 37°C in a CO₂ incubator. After three washes, cell spreading was examined under an inverted microscope (20× objective lens). In quantitative assays, 50 μl of 0.3% p-nitrophenyl phosphate in 1% Triton X-100, 50 mM
sodium acetate, pH 5.0, was added to microtiter wells and incubated at 37°C for 1 h. The reaction was stopped by adding 50 μl of 1 M NaOH. Results were determined by reading the OD at a 405-nm wavelength.

Cell adhesion under flow

Purified human vWF (100 μg/ml with 0.1 M NaHCO3, pH 8.3) was added into a glass capillary tube (0.59 mm ID, 75 mm in length; Harvard Apparatus Inc.) and then incubated overnight in a humid environment at 4°C (Englund et al., 2001). The capillary tubes were rinsed with PBS and then blocked with 5% BSA in PBS. CHO cells expressing human platelet receptors (5 × 10^5/ml in modified Tyrode’s buffer containing 5% BSA) were perfused with a syringe pump (Harvard Apparatus Inc.) through the capillary tube at various shear rates for 2 min followed by perfusion for 10 min with cell-free buffer at the same shear rates. Shear rate was calculated as described by Slack and Turitto (1994). Cell interaction with immobilized vWF was observed in real time under an inverted microscope and recorded on videotapes. The number of stable adherent cells on immobilized vWF was counted on images obtained in 10 randomly selected fields in the vWF-coated tubes. The statistical difference between 123 cells and each one of the mutant cell lines was determined using the t test.

Calpain cleavage of integrin β3 subunit in human platelets

Preparation of washed human platelets has been described previously (Li et al., 2003). Platelet aggregation was induced in a Chronolog lumi-aggregometer by adding 0.1 U/ml thrombin with constant stirring at 1,000 rpm for various lengths of time. The reaction was stopped by adding an equal volume of 2× SDS-PAGE sample buffer containing 1 mM EDTA, 1 mM PMSF, and 0.1 mM E64. Samples were then analyzed by SDS-PAGE on a 7% gel and immunoblotted with cleavage-specific antibodies (Du et al., 1995). The densities of reactive bands were scanned and then quantitated using NIH Image. Lysates prepared from 123, 159/747, 159/754, and 159/579 CHO cell lines were used as internal calibration standards for each of the cleavage-specific antibodies. The ratio of cleaved β3 molecules was calculated as the ratio between the optical density of the reaction of an antibody with platelet lysates and the reaction of the same antibody with the corresponding standard CHO cell lysates multiplied by the ratio between the β3 levels in the standard CHO cell lysates and the β3 level in platelet lysates (as determined by immunoblotting with Mab15 directed against the extracellular domain of β3).

Immunofluorescence analysis of calpain-cleaved β3 in spreading platelets

Platelets in modified Tyrode’s buffer (1 × 10^5/ml) were allowed to adhere to the Lab-Tek chamber slides (Nunc) precoated with 20 μg/ml of fibrinogen at 37°C for 2 h as previously described (Bodnar et al., 1999). The chamber slides were rinsed three times. Adherent platelets were fixed with 1% paraformaldehyde and permeabilized with 0.1 M Tris, 10 mM EGTA, 1% BSA, pH 7.5. The samples were incubated first with Mab15 and one of the cleavage-specific antibodies. The ratio of cleaved integrin β3 subunit and defective activation of platelet integrin αIIbβ3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. Proc. Natl. Acad. Sci. USA 89:10169–10173.

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