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Critical roles for the COOH-terminal NITY and RGT sequences of the integrin β_3 cytoplasmic domain in inside-out and outside-in signaling

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Bidirectional signaling of integrin $\alpha_{IIb}\beta_3$ requires the β_3 cytoplasmic domain. To determine the sequence in the β_3 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 α_{IIb}, and mutants of β_3 with truncations at sites COOH terminal to T^{741}, Y^{747}, F^{754}, and Y^{759}. Truncation at Y^{759} 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 β_3 is important for outside-in signaling but not inside-out signaling. In contrast, truncation at F^{754}, Y^{747}, or T^{741} completely abolished integrin activation. A point mutation replacing Y^{759} with alanine also abolished integrin activation. Thus, the T^{755}NITY^{759} sequence of β_3, 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 Y^{759} in a population of β_3 during platelet aggregation and adhesion, suggesting that calpain may selectively regulate integrin outside-in signaling.

Introduction

The prototype integrin, $\alpha_{IIb}\beta_3$, plays critical roles in platelet adhesion and aggregation. Normally, $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ (Ginsberg et al., 1992; Shattil et al., 1998; Parise, 1999). Ligand binding to $\alpha_{IIb}\beta_3$ 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 Ca^{2+} (Pelletier et al., 1992), and cytoskeleton reorganization (Phillips et al., 1980), leading to cell spreading, stabilization of cell adhesion, and secondary wave of platelet aggregation (Shattil et al., 1998; Parise, 1999).

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 α_{IIb} and β_3 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 $\alpha_{IIb}\beta_3$ 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 β_3 endonexin (Kashiwagi et al., 1997) to β_3 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 β_3 cytoplasmic domain can be chemically modified during platelet activation. For example, T^{753} (Lerea et al., 1999), Y^{747}, and Y^{759} (Law et al., 1996) are phosphorylated by protein kinases, and the COOH-terminal region of β_3 is cleaved by calpain at the COOH-terminal side of Y^{741}, T^{747}, F^{754}, and Y^{759} (Du et al.,

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

Key words: integrin; platelet; calpain; signaling; platelet activation

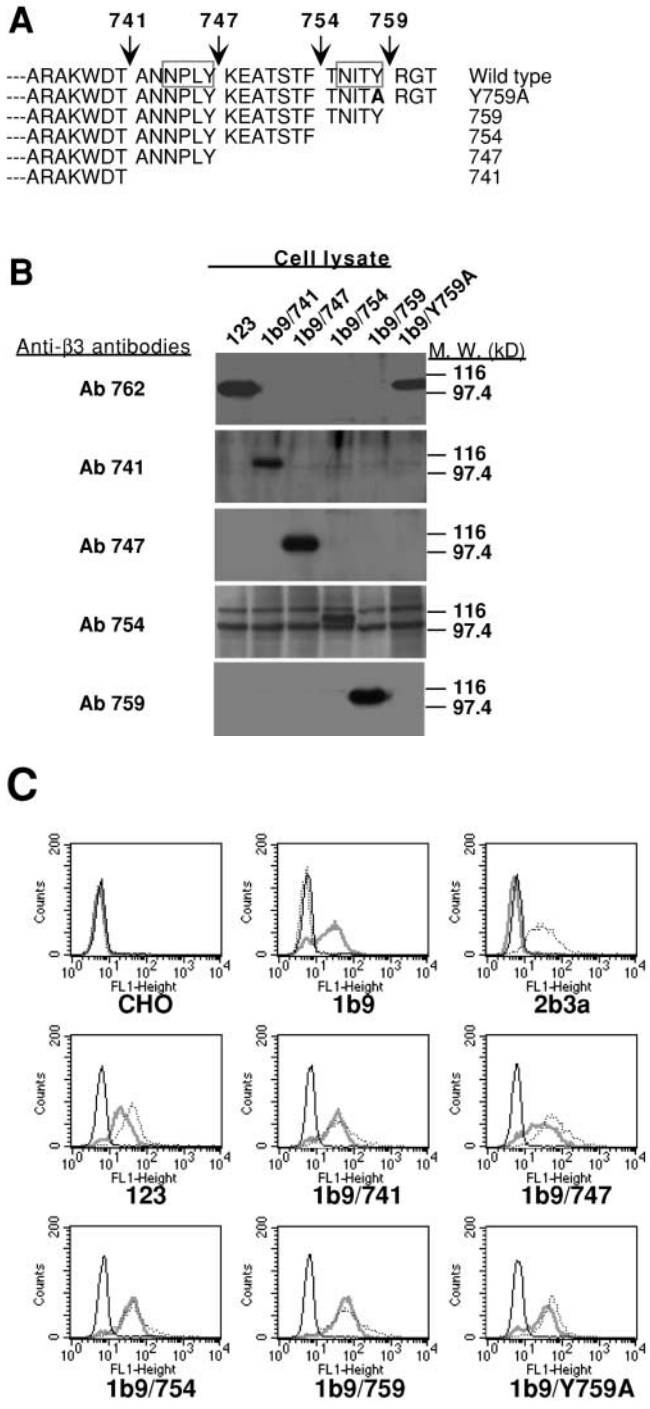


Figure 1. **Truncation mutants of $\alpha_{IIb}\beta_3$ coexpressed with GPIb-IX in CHO cells.** (A) Schematics showing truncation mutants of β_3 that mimic calpain cleavage at four previously identified sites. A Y759A point mutation is also depicted. (B) The mutants described in A were cotransfected with α_{IIb} 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-cleaved forms of β_3 (Ab 741, Ab 747, Ab 754, and Ab 759) and with an antibody recognizing the COOH terminus of β_3 (Ab 762). A cell line expressing wild-type $\alpha_{IIb}\beta_3$ and GPIb-IX (123) was also immunoblotted with these antibodies as a control. (C) Levels of surface expression of wild type or mutants of $\alpha_{IIb}\beta_3$ were examined by flow cytometry with an antibody recognizing $\alpha_{IIb}\beta_3$ complex (D57) (dotted lines), and levels of GPIb-IX expression were examined 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 $\alpha_{IIb}\beta_3$ alone (2b3a), respectively.

1995). Phosphorylation at both Y^{747} and Y^{759} is critical to outside-in signaling of the integrin (Law et al., 1999). The roles of calpain cleavage of the β_3 cytoplasmic domain in bidirectional signaling of the integrin, however, are not clear.

The requirement for specific sequences in the β_3 cytoplasmic domain in outside-in integrin signaling has been indicated by mutagenesis studies in transfected CHO cell models (Chen et al., 1994; Hughes et al., 1995; Ylanne et al., 1995; Patil et al., 1999) and transgenic mouse models (Law et al., 1999). Deletions or mutations that disrupted the two NXXY motifs in the cytoplasmic domain of the integrin abolished outside-in signal-dependent integrin functions such as stable cell adhesion and cell spreading (Ylanne et al., 1993, 1995; Chen et al., 1994; Patil et al., 1999). In a transgenic mouse model, mutations replacing both Y^{747} and Y^{759} in the NXXY motifs with phenylalanine to disrupt tyrosine phosphorylation inhibited outside-in signaling but had no significant effect on inside-out signaling (Law et al., 1999). The requirement for the cytoplasmic domain of β_3 in inside-out signaling has been indicated by defective integrin activation in variant Glanzmann's thrombasthenia patients whose β_3 cytoplasmic domain either has a point mutation replacing S^{752} with proline (Chen et al., 1992, 1994) or has a truncation mutation at R^{724} (Wang et al., 1997). However, mapping of functional sites important to inside-out signaling has been hampered by the lack of a suitable inside-out signaling model in cultured cells deficient in endogenous wild-type β_3, as recombinant integrin $\alpha_{IIb}\beta_3$ expressed in CHO cells is not activated by platelet agonists such as thrombin and ADP (O'Toole et al., 1990). Although constitutively active integrin mutants have been useful in characterizing the affinity regulation by integrin cytoplasmic domains (O'Toole et al., 1991, 1994; Hughes et al., 1995), these mutants obviously bypass the normal on–off switch mechanism of inside-out signaling. We and others have recently shown that integrin can be activated in a reconstituted CHO cell model via the GPIb-IX pathway (Gu et al., 1999; Zaffran et al., 2000; Li et al., 2001). In this model, and as shown here, binding of vWF to GPIb-IX induces inside-out signaling and results in activation of the fibrinogen-binding function of $\alpha_{IIb}\beta_3$, which replicates the GPIb-IX–induced inside-out signaling mechanism in platelets. To understand the structural requirement of the β_3 cytoplasmic domain in integrin inside-out signaling and the role of calpain cleavage in regulating integrin signaling, we have expressed different truncation mutants of β_3 in this reconstituted integrin activation model. We show that removal of the NITY sequence abolished inside-out signaling, whereas truncation of RGT sequence COOH terminal to the NITY motif reduced outside-in signaling without affecting inside-out signaling. Furthermore, a point mutation changing Y^{759} to alanine abolished inside-out signal-

ing and reduced outside-in signaling. Thus, the NITY sequence is essential for both inside-out and outside-in signaling of $\alpha_{IIb}\beta_3$, and the RGT sequence is important for outside-in signaling. Furthermore, localized calpain cleavage of β_3 during platelet activation mainly occurs at a site COOH terminal to Y^{759}, suggesting that calpain cleavage may selectively regulate outside-in signaling.

Results

CHO cell lines coexpressing mutants of the human integrin $\alpha_{IIb}\beta_3$ and GPIb-IX

To study the roles of the COOH-terminal region of β_3 in integrin signaling, we have generated four truncation mutants of the β_3 subunit with truncations at sites COOH terminal to T^{741}, Y^{747}, F^{754}, and Y^{759}. Truncations at these sites also coincide with previously identified calpain cleavage sites in β_3 (Du et al., 1995; Fig. 1 A). These mutants were cotransfected with the integrin α_{IIb} subunit into a CHO cell line that expresses recombinant human GPIb-IX complex (1b9). Complex formation between these β_3 mutants and α_{IIb} was verified by flow cytometry using an $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ bearing alanine substitution of Y^{759} in β_3 was also established (1b9/Y759A). Expression levels of GPIb-IX and integrin $\alpha_{IIb}\beta_3$ on the above mutant cell lines were comparable with a cell line expressing GPIb-IX and wild-type integrin $\alpha_{IIb}\beta_3$ (123 cells) at the time of experiments (Fig. 1 C).

Reconstituted CHO cell model of integrin $\alpha_{IIb}\beta_3$ activation

We recently reported the reconstitution of GPIb-IX–mediated integrin activation in CHO cells expressing recombinant human GPIb-IX and integrin $\alpha_{IIb}\beta_3$ (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 β_3-transfected CHO cells also express an $\alpha_v\beta_3$ integrin composed of endogenous α_v and recombinant β_3, Fig. 2 shows that vWF-induced fibrinogen binding to 123 cells is specifically inhibited by a monoclonal antibody that recognizes human $\alpha_{IIb}\beta_3$, but not by a blocking monoclonal antibody that recognizes $\alpha_v\beta_3$. Also, a cell line expressing GPIb-IX and β_3 without α_{IIb} failed to show RGDS-dependent fibrinogen binding under identical conditions (unpublished data). These results indicate that vWF-induced fibrinogen binding is mediated by $\alpha_{IIb}\beta_3$ but not $\alpha_v\beta_3$. Thus, vWF-induced fibrinogen binding to 123 cells specifically reflects the activation of the platelet integrin $\alpha_{IIb}\beta_3$.

Effects of β_3 mutations on integrin activation (inside-out signaling)

To understand the roles of the COOH-terminal region of β_3 in inside-out signaling of $\alpha_{IIb}\beta_3$, we examined vWF-induced fibrinogen binding to cell lines expressing GPIb-IX and truncation mutants of $\alpha_{IIb}\beta_3$ (1b9/741, 1b9/747, 1b9/754, and 1b9/759). Fig. 3 shows that truncation of the β_3 cytoplasmic domain at the site COOH terminal to Y^{759} did not affect vWF-induced activation of fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. In contrast, truncations at F^{754}, Y^{747}, or T^{741}

A

Reactivity of anti-αIIbβ3 and αvβ3 antibodies

Cell	Mouse IgG	2G12	anti-VNR1	D57
CHO	–	–	–	–
123	–	+	+	+
1b9/β3	–	–	+	–

B

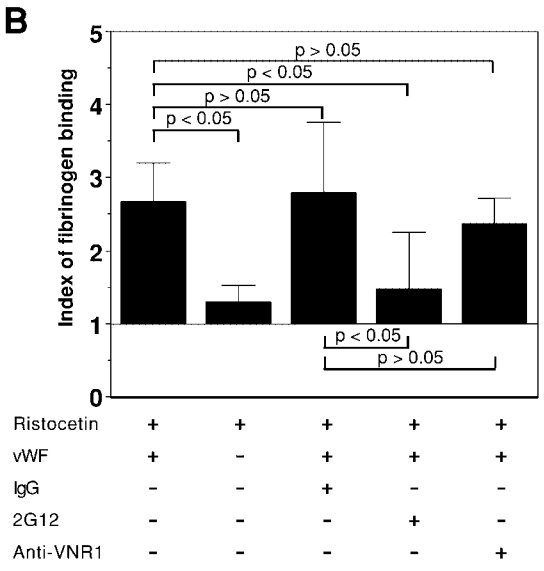


Figure 2. **vWF-induced activation of fibrinogen binding to cell lines coexpressing GPIb-IX and $\alpha_{IIb}\beta_3$ reflects specific activation of integrin $\alpha_{IIb}\beta_3$.** (A) CHO cells (CHO), CHO cells coexpressing GPIb-IX and integrin $\alpha_{IIb}\beta_3$ (123), and CHO cells coexpressing GPIb-IX and β_3 in the absence of α_{IIb} (1b9/β3) were incubated with control mouse IgG, $\alpha_{IIb}\beta_3$-specific antibodies, 2G12 and D57, and an antibody recognizing integrin $\alpha_v\beta_3$, anti-VNR1. The reactivity of these antibodies was detected by flow cytometry. (B) 123 cells were preincubated with 100 μg/ml of control mouse IgG, 2G12, or anti-VNR1 (previously shown to inhibit ligand-binding functions of $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$, respectively) for 10 min at 22°C, and then Oregon green–labeled fibrinogen (30 μg/ml) was added and activated with vWF in the presence of ristocetin. Nonspecific binding was estimated by adding the integrin inhibitor RGDS. Quantitative results from three experiments (means ± SD) are expressed as fibrinogen binding index (total bound fibrinogen/nonspecifically bound fibrinogen in the presence of RGDS). Statistical differences between samples were analyzed using the RRISM software and the Repeated Measures ANOVA method.

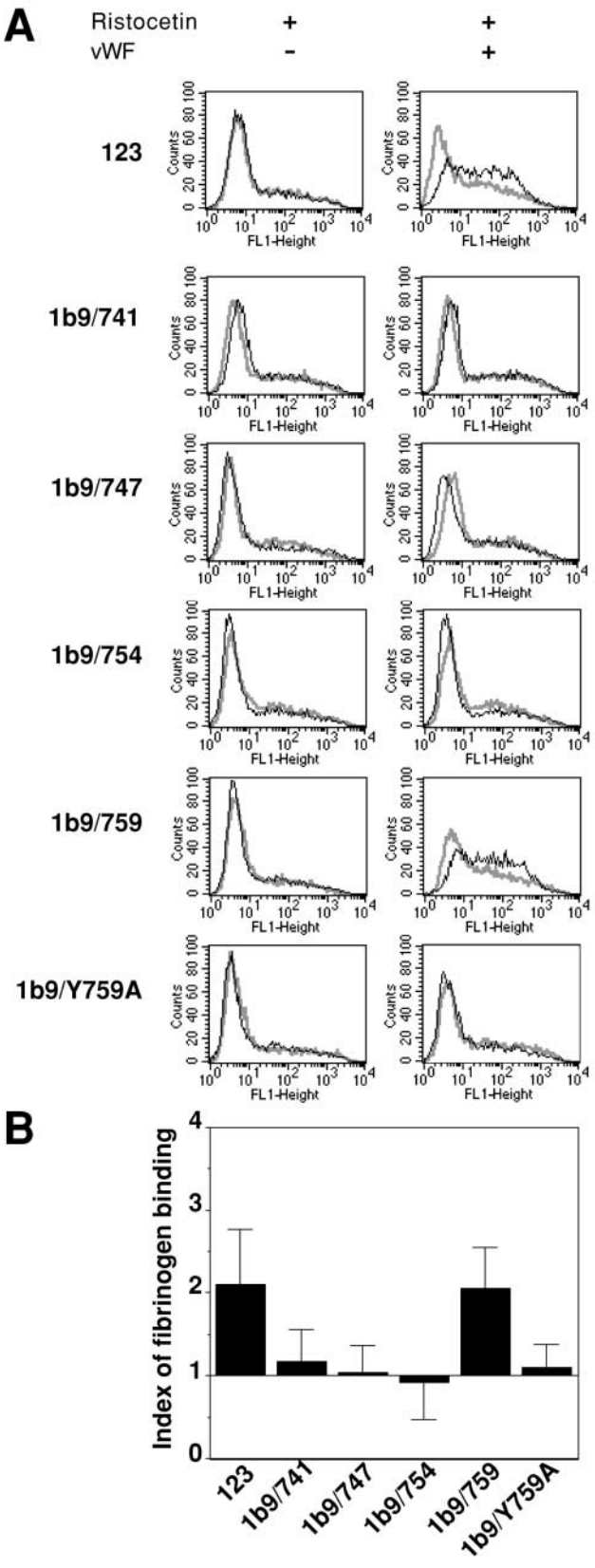


Figure 3. **vWF-induced fibrinogen binding to wild type or various mutants of $\alpha_{IIb}\beta_3$.** (A) Cells (123, 1b9/741, 1b9/747, 1b9/754, 1b9/759, and 1b9/Y759A) suspended in modified Tyrode's buffer were incubated at 22°C for 30 min with Oregon green–labeled fibrinogen (30 μg/ml) and 1 mg/ml of ristocetin with (right) or without (left) adding 25 μg/ml of vWF, and then were analyzed for fluorescence 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).

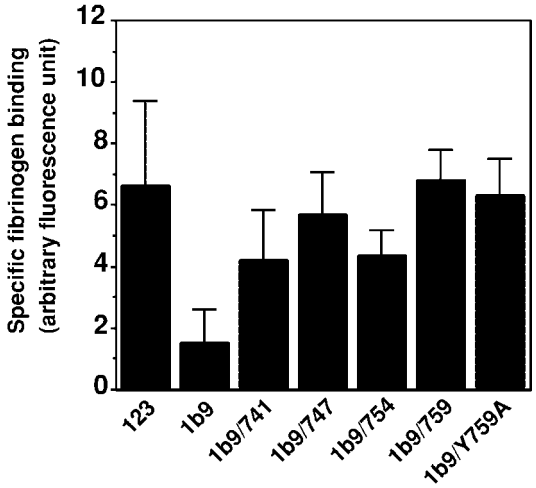


Figure 4. **Fibrinogen binding to mutants of $\alpha_{IIb}\beta_3$ pretreated with RGDS peptide.** Cells coexpressing GPIb-IX and wild-type $\alpha_{IIb}\beta_3$ (123) or integrin mutants were preincubated with or without 1 mM of RGDS peptide for 10 min and then fixed with paraformaldehyde (1% in PBS) for 30 min at 22°C. As a negative control, cells expressing GPIb-IX alone (1b9) were identically treated. The cells were washed, resuspended in modified Tyrode's buffer at a concentration of 5×10^5/ml, and then incubated with Oregon green–labeled fibrinogen for 30 min. Fibrinogen binding was detected by flow cytometry. Nonspecific binding was estimated by adding RGDS peptide to the assay. Specific fibrinogen binding was determined by subtracting the geometric means of fluorescence intensity of the nonspecific binding from that of total binding. Shown in the figure are the data (mean ± SD) from three experiments.

abolished vWF-induced integrin activation. Thus, it appears that the RGT sequence at the COOH terminus of the β_3 cytoplasmic domain is not required for integrin activation, whereas the sequence between F^{754} and Y^{759}, 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 $\alpha_{IIb}\beta_3$. Indeed, vWF-induced fibrinogen binding was abolished in the Y759A mutant, indicating that the NXXY motif, particularly Y^{759}, is required for vWF-induced activation of $\alpha_{IIb}\beta_3$.

Fibrinogen-binding function of the integrin $\alpha_{IIb}\beta_3$ mutants

A possible alternative interpretation to the above results is that the activation-defective mutants of $\alpha_{IIb}\beta_3$ (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 $\alpha_{IIb}\beta_3$ transforms the integrin into

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 $\alpha_{IIb}\beta_3$ (Fig. 4). All of the above mutant cell lines bound fibrinogen in a manner similar to wild-type integrin $\alpha_{IIb}\beta_3$ (123 cells). In contrast, 1b9 cells, in which no integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$–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 β_3 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 $\alpha_{IIb}\beta_3$) 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 β_3 integrin is required for cell spreading on vWF (Gu et al., 1999). As expected, CHO

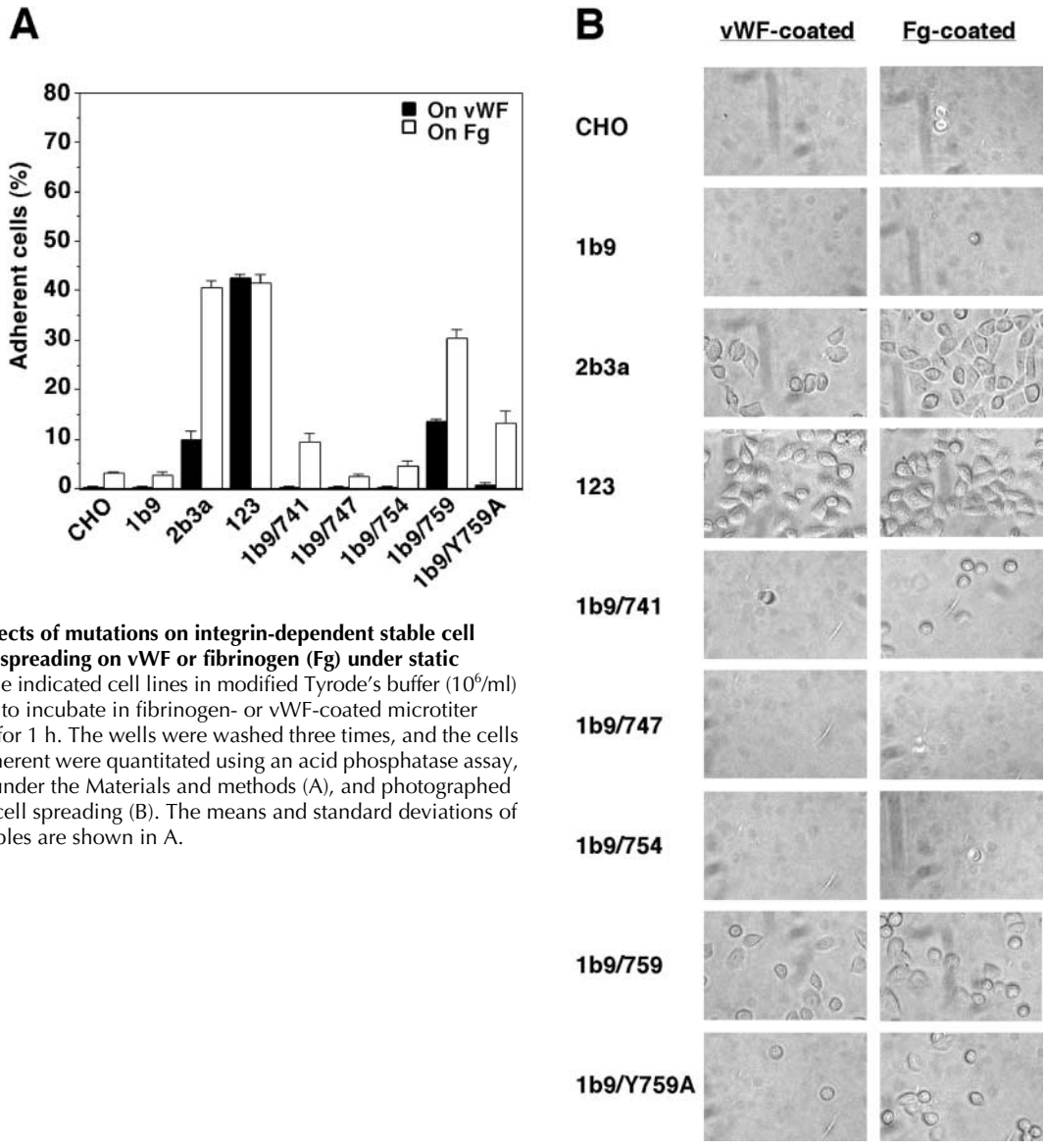


Figure 5. **Effects of mutations on integrin-dependent stable cell adhesion and spreading on vWF or fibrinogen (Fg) under static conditions.** The indicated cell lines in modified Tyrode's buffer (10^6/ml) were allowed to incubate in fibrinogen- or vWF-coated microtiter wells at 37°C for 1 h. The wells 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.

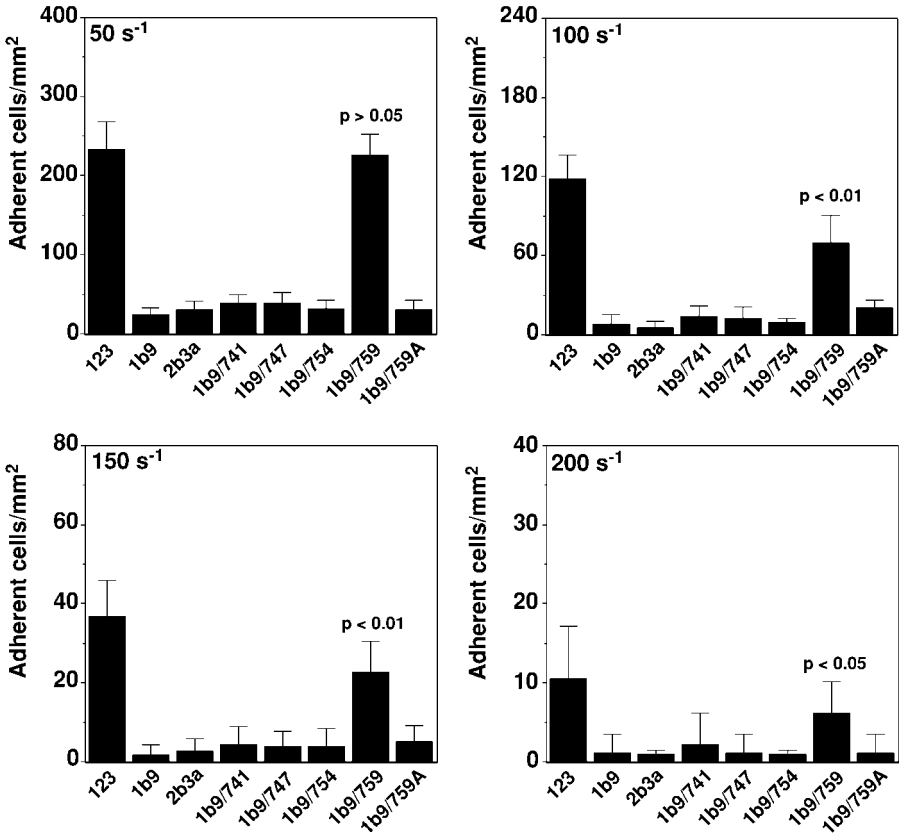


Figure 6. **Effects of mutations on GPIb-IX–induced, integrin-dependent cell adhesion to vWF under flow conditions.** Cells coexpressing GPIb-IX with wild type and different mutants of $\alpha_{IIb}\beta_3$ as well as control cells expressing GPIb-IX alone or $\alpha_{IIb}\beta_3$ alone (5×10^6/ml in modified Tyrode's buffer containing 5% BSA) were perfused though the capillary tubes precoated with vWF at indicated shear rates for 2 min, followed by a further 10-min perfusion with Tyrode's buffer at the same shear rate to wash out transient adherent cells. Cell interaction with immobilized vWF under flow conditions was observed under an inverted microscope and recorded on videotapes. The number of stable adherent cells on vWF was counted on images obtained at 10 randomly selected positions in each vWF-coated capillary tube. The difference between the control 123 cells with each of the other cell lines was analyzed by *t* test and shown to be highly significant ($P < 0.01$), unless otherwise indicated.

cells expressing integrin $\alpha_{IIb}\beta_3$ (2b3a) showed high levels of adhesion and spreading on fibrinogen but poorly adhered to vWF within the first hour of incubation (Fig. 5). However, with prolonged incubation, adhesion of 2b3a cells to vWF was slowly increased (unpublished data), suggesting a low affinity interaction between β_3 integrins and vWF. Coexpression of GPIb-IX with $\alpha_{IIb}\beta_3$ (123 cells) significantly enhanced and accelerated integrin-dependent cell adhesion and spreading on vWF, indicating that GPIb-IX activates integrin, leading to integrin-dependent cell spreading and stable adhesion. Cleavage of β_3 at or NH_2 terminal to F^{754} inhibited integrin-dependent stable cell adhesion and spreading on vWF and fibrinogen (Fig. 5), which is consistent with previous findings that this region of the integrin cytoplasmic domain is important for adhesion and spreading (Ylanne et al., 1993, 1995). Interestingly, truncation of β_3 at Y^{759} also showed a reduced stable adhesion to both vWF and fibrinogen, and most 1b9/759 cells that adhered to fibrinogen or vWF spread poorly (Fig. 5). Reduced stable adhesion in 1b9/759 cells was not caused by a defect in inside-out signaling, as $\alpha_{IIb}\beta_3$-dependent cell adhesion to fibrinogen under these conditions does not require inside-out signaling, and 1b9/759 cells showed normal inside-out signaling, as indicated by soluble fibrinogen binding (Fig. 3). Reduced stable adhesion of 1b9/759 cells is likely resultant from a decreased resistance to the washing procedure in the adhesion assay, as gentler washing can significantly increase 1b9/759 cell adhesion (unpublished data; Fig. 6). Thus, reduced cell adhesion and spreading in 1b9/759 cells suggest that integrin outside-in signaling in this truncation mutant is impaired.

Effects of integrin β_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 $\alpha_{IIb}\beta_3$ (Fig. 6). The amount of adherent 1b9/759 mutant cells on vWF, in contrast, was not reduced. Thus, the results in cell adhesion to vWF under this low 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 Δ759 mutant is normal, we conclude that the impaired outside-in signaling function of this mutant reduced the

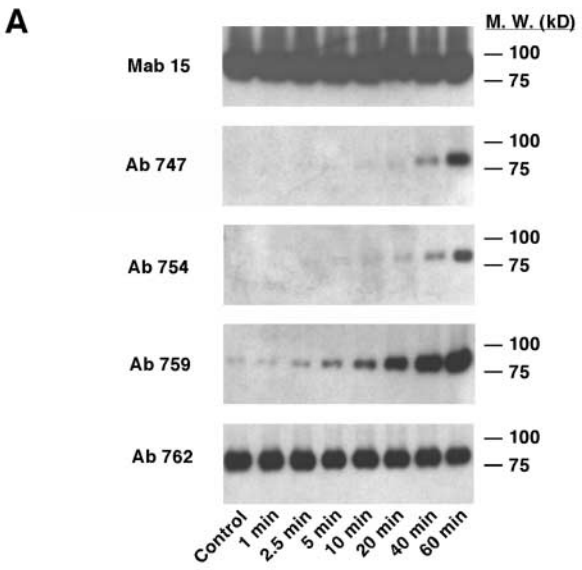
ability of 1b9/759 cells to resist shear stress. Together, these results indicate that the RGT sequence at the COOH terminus of β_3 is important for outside-in signaling of $\alpha_{IIb}\beta_3$, but the NITY sequence is important for both inside-out and outside-in signaling.

Differential cleavage at different sites of the β_3 cytoplasmic domain by calpain in platelets

We have shown previously that calpain may cleave the cytoplasmic domain of β_3 at sites COOH terminal to T^{741}, Y^{747}, F^{754}, and Y^{759}, which generates β_3 fragments identical to the above-described truncation mutants of β_3 (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 β_3 cytoplasmic domain at these different sites has the potential to differentially regulate outside-in and inside-out signaling of integrin $\alpha_{IIb}\beta_3$. To determine if calpain differentially cleaves the β_3 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 β_3 molecules with cleavage at Y^{759}, reacted with ~0.8% of the integrin $\alpha_{IIb}\beta_3$ molecules in washed "resting" platelets (Fig. 7). This reaction was unlikely to result from cross-reaction of this antibody with the intact β_3 subunit, because we showed that the antibody did not react with the intact β_3 subunit but reacted with the Δ759 mutant expressed in CHO cells (Fig. 2). Thus a very small percentage of the β_3 molecules in resting platelets has been cleaved at the Y^{759} site. Stimulation of platelets with thrombin caused a time-dependent and significant increase in the cleavage at Y^{759}. In contrast to the 759 site, calpain cleavage at T^{754} or Y^{747} (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 β_3 at Y^{759}. As we showed above that cleavage at Y^{759} 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 β_3 in spreading platelets

Calpain only cleaves a percentage of β_3 molecules in platelets stimulated with thrombin, suggesting that cleavage of β_3 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 (Bialkowska 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 β_3



B

Calpain cleavage of integrin β3 in thrombin-activated platelets (%)

		Incubation time (min)						
C-termini	Control	1	2.5	5	10	20	40	60
747	/	/	0.3	0.4	0.6	0.8	1.5	4.1
754	/	/	0.5	0.6	1.0	1.4	2.3	5.6
759	0.8	0.8	1.4	3.0	5.8	12.2	19.0	22.1
762	99.1	93.5	90.3	88.8	86.0	83.8	81.8	77.0

Figure 7. **Progressive cleavage at different sites of the β_3 cytoplasmic domain by calpain.** Washed human platelets (10^9/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 Y^{759}, F^{754}, T^{747}, and Y^{741} sites, with Ab762 to detect uncleaved β_3 and Mab 15 to detect total β_3 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.

that is not affected by calpain cleavage. Consistent with the above results in thrombin-stimulated platelets, calpain cleavage occurred mainly at Y^{759} (Fig. 8), but cleavages at F^{754} (Fig. 8) or the more NH_2-terminal sites (unpublished data) were significantly weaker. Whereas the β_3 molecules were distributed throughout the spreading platelets with particularly strong staining at the edges, calpain-cleaved β_3 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 β_3 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 Y^{759}) may

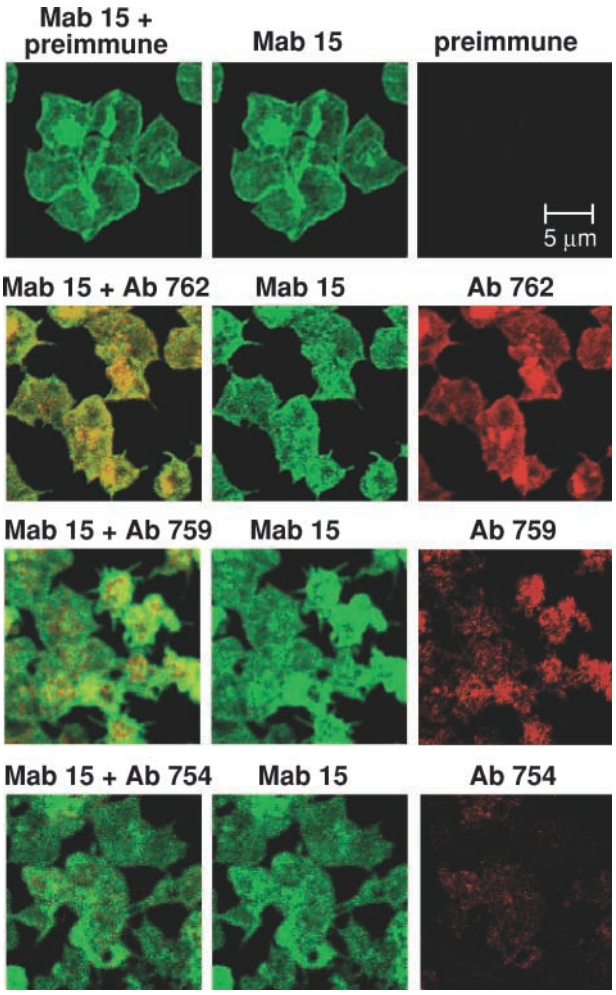


Figure 8. **Localization of calpain-cleaved β_3 in spreading platelets.** Platelets in Tyrode's buffer (1×10^8/ml) were incubated in the chamber slides precoated with 20 μg/ml fibrinogen for 2 h at 37°C. After three washes, the adherent platelets were fixed with 4% paraformaldehyde and permeabilized. The slides were incubated subsequently with Mab15, specific for integrin β_3 extracellular domain (green), and with one of the cleavage site–specific antibodies (Ab759 and Ab754) or an antibody against the COOH terminus of β_3 (Ab762) (red), and then with Alexa Fluor 488–conjugated anti–mouse IgG antibody and Alexa Fluor 546–conjugated anti–rabbit IgG antibodies. The slides were scanned under a confocal microscope (63× lens). Images of the green and red fluorescence overlay are shown in the left column. Green fluorescence (Mab15) from the same images is shown in the middle column. Red fluorescence (cleavage site–specific antibodies) from the same images is shown in the right column.

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 $\alpha_{IIb}\beta_3$, we show that the $N^{756}ITY^{759}$ motif of the β_3 cytoplasmic domain plays a critical role in inside-out signaling of the integrin $\alpha_{IIb}\beta_3$, and the COOH-terminal $R^{760}GT^{762}$ sequence of β_3 is important in outside-in signaling–dependent events. Furthermore, we show that calpain preferentially cleaves a population of β_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 β_3 cytoplasmic domain is critical to the inside-out signaling function of $\alpha_{IIb}\beta_3$. 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 $\alpha_{IIb}\beta_3$ and integrin-dependent stable cell adhesion and spreading. This is consistent with previous work showing that this region of the β_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 $\alpha_{IIb}\beta_3$.

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 mutation 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 β_3 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; Huttenlocher et al., 1997; Croce et al., 1999; Bialkowska et al., 2000; Azam et al., 2001). We have shown previously that the β_3 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 β_3 cytoplasmic domain also occurs during endothelial cell apoptosis (Meredith et al., 1998). The physiological roles of calpain cleavage of β_3 have been unclear. Calpain I knockout in mouse inhibited platelet aggregation but did not affect β_3 cleavage. This suggests that cleavage of the β_3 subunit is not involved in promoting platelet aggregation (Azam et al., 2001). Here we show that one of the functional consequences of calpain cleavage of β_3 is to negatively regulate the signaling functions of integrin $\alpha_{IIb}\beta_3$. Furthermore, we show that cleavage by calpain at different sites of β_3 may result in different regulatory effects. Cleavage at Y^{759} has no significant effect on inside-out signaling but significantly reduces the integrin functions associated with outside-in signaling. In contrast, cleavage at F^{754} or further NH_2-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 such cleavages are not important for the early phase of integrin activation. On the other hand, we found that calpain cleavage of integrin β_3 subunit in intact platelets mainly occurs at the most COOH-terminal Y^{759} site during platelet activation and adhesion (Figs. 7 and 8). Furthermore, cleavage of β_3 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 Y^{759} 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 $\alpha_v\beta_3$ (anti-VNR1) (O'Toole et al., 1990), against integrin β_3 (Mab15) (Frelinger et al., 1990), and against the integrin $\alpha_{IIb}\beta_3$ complex (D57 and 2G12) (Frojmovic 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 GPIb (SZ2) (Ruan et al., 1987) were a gift from C. Ruan (Jiangsu Institute of Hematology, Suzhou, China). Calpain cleavage site–specific antibodies were generated by immunizing rabbits with pentapeptides (AKWDT for Ab741, NNPLY for Ab747, ATSTF for Ab754, TNITY for Ab759, and TYRGT for Ab762) as described previously (Du et al., 1995). Oregon green 488–conjugated human fibrinogen, Alexa Fluor 488–conjugated goat anti–mouse IgG, and Alexa Fluor 546–conjugated goat anti–rabbit IgG were from Molecular Probes. Integrin α_{IIb} and β_3 cDNA clones in pCDM8 vector were provided by M. Ginsberg, and a mutant β_3 cDNA clone bearing the $Y^{759}A$ substitution was a gift from J. Ylänne (University of Helsinki, Helsinki, Finland). Cell lines expressing GPIb-IX complex (1b9), integrin $\alpha_{IIb}\beta_3$ (2b3a), or both GPIb-IX and $\alpha_{IIb}\beta_3$ (123) were described previously (Gu et al., 1999).

Construction of truncation mutants of β_3 subunit

Truncation mutagenesis was performed using PCR to introduce stop codons into integrin β_3 cDNA at sites corresponding to the carboxy side of amino acid residues 741, 747, 754, or 759, respectively. The forward primer has the sequence of AGAGCTTAAGGACAC at an AflII site of β_3 cDNA. The reverse primers contain an XhoI digestion site, a stop codon, and the 18-nucleotide β_3 sequences at the intended COOH terminus of each mutant. The PCR products were digested with restriction enzymes AspI and XhoI and ligated into a β_3 cDNA construct in a modified cDM8 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 β_3 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 β_3 cDNA was cotransfected together with wild-type α_{IIb} and pcDNA3.1/Hyg plasmid at a ratio of 5:5:1. Stable cell lines expressing mutant β_3 were selected in 0.2 mg/ml of hygromycin (Invitrogen). Expression of integrin $\alpha_{IIb}\beta_3$ was monitored by flow cytometry using D57. Expression of GPIb-IX was detected using SZ2. Cells expressing both GPIb-IX and integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ induced by ristocetin and vWF was examined by flow cytometric analysis of Oregon green 488–conjugated fibrinogen binding to $\alpha_{IIb}\beta_3$, as previously described (Gu et al., 1999; Li et al., 2001). In brief, transfected CHO cells were resuspended to 5×10^5/ml in modified Tyrode's solution (2.5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 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 mM).

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 NH_4Cl, 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

Microtiter wells were coated with 10 μg/ml human vWF or fibrinogen in 0.1 M $NaHCO_3$, pH 8.3, at 4°C overnight and then blocked with 5% BSA–PBS at 22°C for 2 h. Cell suspension (10^6/ml in modified Tyrode's buffer with 1% BSA) was added to ligand-coated microtiter wells and incubated for 60 min at 37°C in a CO_2 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 mM $NaHCO_3$, 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 (5×10^6/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 β_3 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, 1b9/747, 1b9/754, and 1b9/579 CHO cell lines were used as internal calibration standards for each of the cleavage-specific antibodies. The ratio of cleaved β_3 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 β_3 levels in the standard CHO cell lysates and the β_3 level in platelet lysates (as determined by immunoblotting with Mab15 directed against the extracellular domain of β_3).

Immunofluorescence analysis of calpain-cleaved β_3 in spreading platelets

Platelets in modified Tyrode's buffer (1×10^8/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, 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 0.1 mM E64, 0.1% Triton X-100, 1% BSA, pH 7.5. The samples were incubated first with Mab15 and one of the cleavage-specific antibodies and then with Alexa Fluor 488–conjugated goat anti–mouse IgG and Alexa Fluor 546–conjugated goat anti–rabbit IgG. The slides were then scanned under a Carl Zeiss MicroImaging, Inc. LSM510 confocal microscope (63× objective lens).

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