Cooperative Role of the Membrane-proximal and -distal Residues of the Integrin β3 Cytoplasmic Domain in Regulation of Talin-mediated αIIbβ3 Activation*

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Integrin cytoplasmic tails regulate integrin activation that is required for high affinity binding with ligands. The interaction of the integrin β subunit tail with a cytoplasmic protein, talin, largely contributes to integrin activation. Here we report the cooperative interaction of the β3 membrane-proximal and -distal residues in regulation of talin-mediated αIIbβ3 activation. Because a chimeric integrin, αIIbβ3/β1, in which the β3 tail was replaced with the β1 tail was constitutively active, we searched for the residues responsible for integrin activation among the residues that differed between the β3 and β1 tails. Single amino acid substitutions of Ile-719 and Glu-749 in the β3 membrane-proximal and -distal regions, respectively, with the corresponding β1 residues or alanine rendered αIIbβ3 constitutively active. The I719M/E749S double mutant had the same ligand binding activity as αIIbβ3/β1. These β3 mutations also induced αVβ3 activation. Conversely, substitution of Met-719 or Ser-749 in the β1 tail with the corresponding β3 tail residue (M719I or S749E) inhibited αIIbβ3/β1 activation, and the M719I/S749E double mutant inhibited ligand binding to a level comparable with that of the wild-type αIIbβ3. Knock down of talin by short hairpin RNA inhibited the I719M- and E749S-induced αIIbβ3 activation. These results suggest that the β3 membrane-proximal and -distal residues cooperatively regulate talin-mediated αIIbβ3 activation.

Platelet integrin αIIbβ3 is a transmembrane receptor that mediates platelet adhesion and aggregation (1, 2). αIIbβ3 exists in a low affinity state in resting platelets and requires activation for high affinity binding with soluble ligands (1, 2). Activation of αIIbβ3 is tightly linked to structural rearrangements of the αIIbβ3 molecule. Recent crystal and NMR structure studies have revealed the precise conformational changes that occur during these rearrangements, including separation of the cytoplasmic, transmembrane, and extracellular leg domains of the αIIb and β3 subunits, a switchblade-like extension from a bent to an extended conformation of the extracellular domains, outward swing of the hybrid domain, and structural rearrangements of the head domain containing a ligand binding pocket (3–8). This long range conformational transduction is initiated from the cytoplasmic tails of the αIIb and β3 subunits in the integrin activation process referred to as inside-out signaling.

Several NMR analyses have identified multiple hydrophobic and electrostatic contacts within the membrane-proximal helices of the αIIb and β3 cytoplasmic tails (3, 9). Deletion or mutation of the membrane-proximal helix of the αIIb GFFKR or β3 LLITIHD sequence renders αIIbβ3 constitutively active (10, 11). Therefore, it is considered that the membrane-proximal regions of the αIIb and β3 cytoplasmic tails associate to form a clasp, maintaining αIIbβ3 in a low affinity state. On the other hand, the structural basis of the membrane-distal regions of the αIIb and β3 tails is less obvious.

The structure of the αIIb membrane-distal region has been explored by NMR. Analysis of the myristoylated peptide containing the entire αIIb tail sequence revealed that the membrane-distal loop folded back toward the membrane-proximal helix, while this conformation was not observed in recent analysis of the αIIb tail in a membrane-embedding environment (5, 12). A lipid-modified peptide containing the membrane-distal sequence (RPPLEED) inhibits agonist-induced αIIbβ3 activation (13). Mutation of the αIIb RPP distal sequence activates αIIbβ3 in a metabolic energy-dependent manner (14, 15). These findings suggest that the membrane-distal region of the αIIb tail suppresses energy-dependent αIIbβ3 activation, although the mechanism responsible for activation of the αIIbβ3 mutants remains unclear.

The β3 membrane-distal region has been shown to interact with many signaling and cytoskeletal proteins transmitting inside-out signals (1, 2). One of them, talin, directly interacts with the membrane-proximal and -distal regions of the β3 tail (16, 17), and any defect of this interaction strongly inhibits inside-out signaling (17, 18). Mutation of S752P and truncation at Arg-724 in the β3 membrane-distal region abolish platelet agonist-induced αIIbβ3 activation (19, 20). Mutations of the β3 EFAKFEEE, NPLY, and NITY sequences inhibit αIIbβ3 inside-out signaling (21, 22). These findings suggest that the β3 membrane-distal region plays a stimulatory, and not inhibitory, role in αIIbβ3 activation.

In view of the link between integrin activation and allosteric structural rearrangements of the extracellular segments of integrins (23), one would expect that structural changes in the β3 membrane-distal region containing binding sites for intracellular proteins would be relayed to the membrane-proximal...
region, leading to αIIbβ3 activation. However, there has been no evidence that structural rearrangement of the β3 membrane-distal region is directly linked to integrin activation. Except for the talin binding site, the structure of the β3 membrane-distal region is ill-defined, and no activating mutation has so far been reported in the β3 membrane-distal region. In this context, we considered that a previously reported αIIbβ3 mutant in which the β3 tail was replaced by the β1 tail was noteworthy (24). This chimeric integrin, αIIbβ3/β1, was constitutively active. Because the β1 and β3 subunits have relatively high sequence homology, we reasoned that the residues differing between the β1 and β3 tails are responsible for αIIbβ3 activation. The β3 and β1 tails have 20 residues that differ, and 18 of them are located in the membrane-distal region, raising the possibility that the membrane-distal residue(s) may contribute to αIIbβ3 activation.

In this study, we attempted to identify the critical residue(s) for regulation of αIIbβ3 activation using a model of activated αIIbβ3, αIIbβ3/β1. We obtained evidence that structural perturbation of specific residues in the β3 membrane-distal region can induce αIIbβ3 activation and that the membrane-proximal and -distal residues in the β3 tail cooperatively regulate talin-mediated αIIbβ3 activation.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies and Plasmids—PAC1, a monoclonal antibody specifically recognizing the active conformation of αIIbβ3, was a generous gift from Dr. S. J. Shattil (University of California, San Diego). 4 B10 is a non-function-blocking anti-αIIbβ3 complex-specific monoclonal antibody produced in our laboratory (15, 25). AP3, a non-function-blocking anti-β3 monoclonal antibody, was obtained from GTI, Brookfield, WI. Human αIIb, αV, and β3 cDNAs were kindly provided by Dr. S. J. Shattil. Human β1A cDNA was kindly provided by Dr. Y. Takada (UC Davis Medical Center, Sacramento, CA). The plasms expressing short hairpin RNAs (shRNA)2 were generous gifts from Dr. S. J. Shattil (18).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by overlap extension polymerase chain reaction (PCR) or by using a QuikChange site-directed mutagenesis kit (Stratagene) as described previously (15, 25). Two recombinant β3 fragments were generated from a full-length β3 cDNA template by PCR with Pfu polymerase and complementary primers containing the desired mutations. These two fragments were combined and subjected to PCR using primers containing HindIII and XbaI restriction sites at the 5'- and 3'-ends of β3, respectively. The amplified PCR product was digested with HindIII/XbaI and cloned into a mammalian expression vector, pCDNA3 (Invitrogen). The nucleotide sequences of the inserts were confirmed by sequence analysis.

Expression of Recombinant Integrins—The mutated β3 and wild-type αIIb cDNAs were cotransfected into Chinese hamster ovary (CHO) cells with Lipofectamine (Invitrogen) in accordance with the manufacturer’s instructions. Human 293T cells were also used for transfection. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 48 h to achieve cell surface expression of the recombinant integrins.

Ligand Binding Assay—PAC1 and fibrinogen binding assays were performed as described previously (15, 25, 26). CHO cells were suspended in Tyrode’s buffer (137 mM NaCl, 12 mM NaHCO3, 2.6 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 5 mM Hepes, 5.5 mM glucose, and 1 mg/ml bovine serum albumin, pH 7.4) and incubated for 30 min at room temperature with 10 μg/ml PAC1 and 5 μg/ml biotin-labeled 4B10 in the presence of 2 mM RGDS peptide or buffer. The cells were then washed and resuspended in a mixture of 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgM antibody (BIOSOURCE, Camarillo, CA) and a 1:50 dilution of phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) for 25 min on ice. Then, 1 μl of 1 mg/ml propidium iodide (Sigma) was added. After 5 min the cells were washed and resuspended in 500 μl of ice-cold Tyrode’s buffer and analyzed on a FACSCalibur flow cytometer (BD Biosciences). After electronic compensation, PAC1 binding (FL1) was analyzed on the gated subset of live cells (propidium iodide-negative, FL3) that were positive for fibrinogen binding (FL2). In the assays for fibrinogen binding to αIIbβ3, fluorescein isothiocyanate-conjugated fibrinogen was used instead of PAC1. The binding of fibrinogen to αβ3 in CHO cells was measured as described previously (25).

Talin Knock Down—Knock down of talin expression in CHO cells was performed by the small interfering RNA technique according to the method of Tadokoro et al. (18). CHO cells were transfected with plasmids encoding αIIb cDNA, β3 cDNA, and shRNAs targeting talin-1. Scrambled shRNA was used as a control. A preparation without shRNA transfection was included as another control. The cells were cultured for 72 h to allow the shRNA to exert its maximal inhibitory effect (18). The transfected cells were then subjected to ligand binding assay as described above. To assess talin expression, CHO cells were transfected with shRNA and pEFGP-C1 (BD Biosciences Clontech). The cells were then fixed with 0.5% paraformaldehyde, permeabilized with 0.05% saponin, and incubated for 30 min at room temperature with anti-talin-1 and -2 monoclonal antibody 8d4 (Sigma). After washing, the cells were incubated for 30 min on ice with phycoerythrin-conjugated goat anti-mouse IgG (BIOSOURCE). They were then washed and resuspended in phosphate-buffered saline containing 0.025% saponin, 0.25% bovine serum albumin, and 1 μg/ml propidium iodide. Binding of 8d4 antibody to talin was analyzed using flow cytometry by gating propiom iodide-negative live cells that were positive for green fluorescent protein expression.

RESULTS

Mutation of the Ile-719 and Glu-749 Residues in the β3 Tail Activates β3 Integrins—To identify the residues critical for regulation of αIIbβ3 activation in the β3 tail, we focused on amino acids in the β3 tail that differed from those in the β1 tail as the αIIbβ3/β1 chimeric integrin, in which the β3 tail is replaced with the β1 tail, has been reported to be constitutively active (24). We produced 13 αIIbβ3 mutants in which the individual or group residues in the β3 tail were substituted with the corresponding β1 tail residues (Fig. 1). The αIIbβ3 mutants were

2 The abbreviations used are: shRNA, short hairpin RNA; CHO, Chinese hamster ovary; WT, wild type.
Activation of αllbβ3 by β3 Tail Mutations

The expression of αllbβ3 mutants on the cell surface was 93–136% that of the wild-type αllbβ3, indicating almost equivalent expression of the mutated integrins (Fig. 2A). Among these mutants, αllbβ3 bearing the β3I719M or β3E749S mutation bound significantly higher levels of PAC1 than wild-type αllbβ3 without any stimulation and the RGDS peptide abolished PAC1 binding to the mutants, indicating a constitutively active state (Fig. 2B). Other mutants, as well as wild-type αllbβ3, were in a low affinity state. To determine whether activation of αllbβ3 by I719M and E749S mutation is due to loss of Ile-719 and Glu-749 residues or insertion of methionine and serine into the β3 tail, we examined the effects on PAC1 binding of I719A and E749A mutants in which the residues concerned were replaced with alanine. I719A and E749A bound the same level of PAC1 as I719M and E749S did, suggesting that loss of the side chains of Ile-719 and Glu-749 residues is responsible for αllbβ3 activation (Fig. 2B).

Although the I719M and E749S mutants were constitutively active, each exhibited less PAC1 binding than the αllbβ3/β1-activating integrin in which the entire β3 tail was replaced by the β1 tail (Fig. 3). Because we screened all the β3 tail residues that were different from the β1 residues, it is unlikely that tail residues other than Ile-719 and Glu-749 are involved in αllbβ3/β1 activation. To clarify whether the combination of I719M and E749S mutations was able to reproduce an activating state comparable with αllbβ3/β1, we produced the I719M/E749S double mutant. This mutant was expressed normally on the surface of CHO cells and showed more PAC1 binding than the single mutants, reaching the same level of PAC1 binding as αllbβ3/β1 (Fig. 3). Similar effects were observed when soluble fibrinogen, a physiological ligand for αllbβ3, was used instead of PAC1 (Fig. 3). Similar results were also obtained when human 293T cells were used instead of CHO cells (data not shown). These results suggest that mutation of the membrane-proximal Ile-719 and membrane-distal Glu-749 residues in the β3 tail has an additive effect on αllbβ3 activation and that activation of the chimeric integrin αllbβ3/β1 can be recapitulated by double mutations of Ile-719 and Glu-749 residues in the β3 tail.

The β3 subunit can associate with αllb and αV subunits, forming αllbβ3 and αVβ3 integrins. To

![FIGURE 1. Amino acid sequences of β3 and β1A cytoplasmic tails. The residues differing between β3 and β1 are boxed. Individual or group residues within the box are substituted with the corresponding β1 tail residues.](image)

![FIGURE 2. Homology-scanning mutagenesis of the β3 tail residues. A, expression of αllbβ3 mutants in CHO cells. CHO cells were transfected with cDNAs for wild-type (WT) αllb and β3 bearing the indicated mutation or WT β3. Binding of 4B10 anti-αllbβ3 monoclonal antibody to cells was measured by flow cytometry. The amounts bound were expressed as percentages of the value for 4B10 binding to cells expressing WT αllbβ3. B, PAC1 binding to αllbβ3 mutants. PAC1 and 4B10 binding to cells was measured simultaneously by flow cytometry. The mean fluorescence intensity (mfi) of specific (RGDS-inhibitable) PAC1 binding was divided by that of 4B10 binding for assessment on a per-αllbβ3 molecule basis. The data presented are means ± S.E. of three independent experiments. The asterisks denote a significant difference (p < 0.05) compared with the WT value.](image)
Introduction of the β3 I719 and β3E749 Residues into the β1 Tail Suppresses Integrin Activation—The foregoing results showed that the I719M and E749S mutations were responsible for activation of the αIββ3/β1 chimeric integrin. We next examined whether the converse mutations, M719I and S749E, prevented αIββ3/β1 activation. The M719I and S749E αIββ3/β1 mutants reduced PAC1 binding significantly, even though expression of these mutants was sufficient (Fig. 5). The M719I/S749E double mutant reduced PAC1 binding to a level comparable with wild-type αIββ3. These results are consistent with the foregoing results obtained with αIββ3I719M and αIββ3E749S mutations and further suggest the cooperative role of the β3I719 and β3E749 residues in regulation of αIββ3 activation.

The β3I719 and β3E749 Residues Suppress Talin-dependent αIββ3 Activation—To explore the mechanism of αIββ3 activation by the Ile-719 and Glu-749 mutations, we examined whether activation of these mutant integrins is mediated by talin, a final common element of signaling pathways to integrin activation (18). Knock down of talin was carried out by the use of shRNA and was confirmed by inhibition of talin expression in CHO cells by 66% (Fig. 6A). This inhibitory effect was the same as that shown in the previous study (18). Talin knock down significantly reduced PAC1 binding to the CHO cells expressing the mutant αIββ3, whereas control shRNA had no effect (Fig. 6B). This result indicates that both the I719M and E749S mutations induce αIββ3 activation in a talin-dependent manner, suggesting that the β3I719 and β3E749 residues suppress talin-mediated αIββ3 activation to retain αIββ3 in a default low affinity state.

DISCUSSION

In this study, we have identified two residues in the β3 tail, Ile-719 and Glu-749, that are critical for maintaining αIββ3 in a low affinity state. Ile-719 is located in the membrane-proximal region of the β3 tail and binds to the αIββ3 tail in NMR...
structure models (3, 9). Because the membrane-proximal inter-
face between the α and β tails forms a clasp to maintain inte-
grins at a low affinity state (23), one would expect that muta-
tions within the membrane-proximal clasping region would
activate integrins. A simple explanation for αIIIβ3 activation
by Ile-719 mutation is that loss of the bond between the β3I719
and the αIII tail directly disrupts the membrane-proximal
clasp. Although the Ile-719-mediated contact is only part of the
multiple hydrophobic and electrostatic interactions within the
clasp, disturbing this specific contact may weaken the interac-
tion and facilitate αIIIβ3 activation. However, the Ile-719
mutation-induced αIIIβ3 activation is talin-dependent, as
demonstrated in this study. A similar mutational effect on
αIIIβ3 activation has been demonstrated for the Asp-723 res-
due in the β3 membrane-proximal region (18, 27). Talin binds
to not only the β3 membrane-distal region but also the β3 membrane-
proximal region, and a recent study has shown that binding of talin to
the latter is critical for αIIbβ3 activation (17). Therefore, it is possible
that structural changes around the β3 membrane-proximal Ile-719 and
Asp-723 residues give access to the talin protein, thereby activating
αIIbβ3.

Another activating mutation of the β3 tail residue, Glu-749, is
intriguing. Unlike Ile-719, Glu-749 is located in the β3 membrane-dis-
tal region. This region directly interacts with many signaling and
cytoskeletal molecules, including tyrosine kinases and talin, that are
involved in the inside-out signaling pathway (18, 28, 29). Deletion or
mutation of the β3 membrane-distal region has been reported to
result in failure of αIIbβ3 activation by inside-out signaling (19–22).
These studies clearly demonstrate that the β3 membrane-distal region
functions as a receiver of intracellular signals activating αIIbβ3.
Because integrin activation is brought about by allosteric struc-
tural rearrangement of each extracellular segment of integrins (23),
it is likely that the structural changes in the β3 membrane-dis-
tal region following interaction with intracellular molecules are
replied to the membrane-proxi-
mal region, leading to αIIbβ3 activa-
tion. However, there is no direct
evidence that the structural re-
arangement of the β3 membrane-
distal region induces integrin acti-

![Graph A](image1)

**FIGURE 6. Inhibition of β3 tail mutation-induced αIIbβ3 activation by talin knock down.** A, expression of
talin in CHO cells. CHO cells were transfected with plasmids encoding green fluorescent protein and talin-
targeted or scrambled shRNA. The cells were fixed, permeabilized, and incubated with anti-talin antibody.
Antibody binding to green fluorescent protein-positive cells was measured by flow cytometry and expressed
as a percentage of the value for antibody binding to cells not transfected with shRNA. B, effect of shRNA on
PAC1 binding to αIIbβ3 mutants. CHO cells were transfected with cDNAs for WT αIIb and β3 bearing the
indicated mutation or WT β3, with or without shRNA containing talin-targeted or scrambled sequence. Specific
PAC1 binding is expressed on a per-αIIbβ3 molecule basis as the mean fluorescence intensity (mfi) of PAC1
binding divided by that of 4B10 binding. The data presented are means ± S.E. of three independent
experiments.
Talin binds to the WDTANNPLYK sequence that is outside Glu-749 (16). However, Glu-749 is adjacent to this talin binding site and NMR perturbation of Glu-749 by interaction with talin has been observed (16). It is therefore possible that the Glu-749 mutation has a direct effect on talin binding and thus affects αIIbβ3 activation through talin-mediated mechanism. To demonstrate this hypothesis, a direct binding assay for interaction of talin with recombinant β3 tails is necessary. Unfortunately, we were unable to perform such an assay because detection of low affinity integrin-talin interactions requires integrin tails connected with specially designed model proteins bearing coiled-coil and glycine spacer structures that were not commercially available (30).

Talin was recently reported to compete with filamin for binding to the β3 tail, and this competition may regulate integrin activation (31). Filamin binds to the PLYKEATSTFTN sequence encompassing Glu-749 (31). It is therefore possible that reduced binding of filamin to the E749S mutant results in more talin binding, leading to αIIbβ3 activation. However, this mechanism is unlikely because the β1 tail bound much less filamin than the β3 tail although both tails have serine at the β3E749 position, indicating that E749S is not a determinant of filamin binding affinity (24).

A recent NMR analysis has demonstrated that the β3 membrane-distal NPLY motif is anchored to the membrane surface and that this membrane anchoring may restrict binding of talin to the β3 tail to constrain αIIbβ3 in a default low affinity state (5). Because Glu-749 lies within the helix flanking the NPLY loop (5), Glu-749 mutation may release the membrane anchoring of the NPLY, thus facilitating talin binding to the β3 tail. This hypothesis could account for talin-dependent activation of αIIbβ3 by Glu-749 mutation. Structural analysis of the talin/Glu-749 mutant complex at the atomic level will be required to verify this hypothesis.

Because the β3 membrane-distal region binds intracellular signaling molecules, it is possible that Glu-749 mutation affects the interaction of these proteins with the membrane-distal tail that regulates αIIbβ3 activation. The β3E749 residue is critical for binding of some phosphotyrosine-binding proteins, including Dok1 and Numb (32). Dok1 competes with talin for binding to the β3 tail and has the ability to inhibit αIIbβ3 activation (17). It is therefore possible that reduced binding of Dok1 to the β3E749S tail results in increased binding of talin, thereby activating αIIbβ3, although overexpression of Dok1 is required for partial inhibition of αIIbβ3 activation (17). The effect of Numb on integrin signaling is unknown. Another phosphotyrosine-binding protein, Dab2, binds to the β3 tail and functions as a negative regulator of αIIbβ3 activation (33). However, it is unlikely that Dab2 is involved in the mechanism of E749S-induced αIIbβ3 activation because the E749S mutation had no effect on Dab2 binding (32). Because the Glu-749 residue is specific for β3 among all the integrin β subunits, it may interact with some β3-specific partner to restrain αIIbβ3 activation. Based on the present study, screening of intracellular proteins competent to bind the wild-type but not E749S mutant β3 tail may be useful for detecting novel regulators of αIIbβ3 activation.

It should be noted that the mutational effects of the membrane-proximal Ile-719 and the membrane-distal Glu-749 residues were additive and talin-dependent. This result suggests that the conformational changes in both the membrane-proximal and -distal regions play a cooperative role in talin function. Very recently, Wegener et al. (17) demonstrated the cooperative roles of the membrane-proximal and -distal regions in the process of talin-mediated αIIbβ3 activation. They proposed a model of talin-induced integrin activation in which interaction of talin with the membrane-distal region anchors talin to the β subunit tail tightly and subsequent interaction of talin with the membrane-proximal region induces separation or reorientation of the integrin tails, leading to integrin activation. Disruption of either interaction with the membrane-proximal or -distal region by β3 tail mutations resulted in failure of αIIbβ3 activation (17). Our study showed that either Ile-719 or Glu-749 mutation induced talin-dependent αIIbβ3 activation, thus supporting this recent model of talin-induced integrin activation in terms of gain-of-function mutations. Wegener et al. (17) employed F2/F3 fragments of talin to activate αIIbβ3. In contrast, we observed the effects of the endogenous intact talin molecule on integrin activation. Therefore, our findings may indicate the β3 tail site critical for interaction with the intact talin molecule. Although we could not define the mechanism underlying the activating effects of the β3 tail mutations, identification of the β3 tail residues as regulators of talin-mediated αIIbβ3 activation will help to unravel the molecular basis for inside-out signaling of integrins.

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