Suppression of integrin activation by the membrane-distal sequence of the integrin α Ib cytoplasmic tail

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Integrin cytoplasmic tails regulate integrin activation including an increase in integrin affinity for ligands. Although there is ample evidence that the membrane-proximal regions of the α and β tails interact with each other to maintain integrins in a low-affinity state, little is known about the role of the membrane-distal region of the α tail in regulation of integrin activation. We report a critical sequence for regulation of integrin activation in the membrane-distal region of the α Ib tail. Alanine substitution of the RPP residues in the α Ib tail rendered α Ib β 3 constitutively active in a metabolic energy-dependent manner. Although an α Ib/ α 6A β 3 chimaeric integrin, in which the α Ib tail was replaced by the α 6A tail, was in an energy-dependent active state to bind soluble ligands, introduction of the RPP sequence into the α 6A tail inhibited binding of an activation-dependent antibody PAC1. In

α Ib/ α 6A β 3, deleting the TSDA sequence from the α 6A tail or single amino acid substitutions of the TSDA residues inhibited α Ib/ α 6A β 3 activation and replacing the membrane-distal region of the α Ib tail with TSDA rendered α Ib β 3 active, suggesting a stimulatory role of TSDA in energy-dependent integrin activation. However, adding TSDA to the α Ib tail containing the RPP sequence of the membrane-distal region failed to activate α Ib β 3. These results suggest that the RPP sequence after the GFFKR motif of the α Ib tail suppresses energy-dependent α Ib β 3 activation. These findings provide a molecular basis for the regulation of energy-dependent integrin activation by α subunit tails.

Key words: α Ib, α 6A, affinity modulation, cytoplasmic domain, integrin, metabolic energy.

INTRODUCTION

Integrins are α/β heterodimeric receptors, which mediate cell adhesion and signalling [1,2]. A fundamental function of integrins is ligand binding, which is rapidly and precisely regulated by a process referred to as ‘inside-out signalling’ or ‘integrin activation’ [3]. Transduction of intracellular signals to cytoplasmic domains of integrins leads to conformational changes in the extracellular domains that result in changes of the integrin affinity for ligands [4–6]. The cytoplasmic tails of integrin α and β subunits play a pivotal role in this process [7].

Integrin α Ib β 3 has served as a prototype for analysis of integrin activation [8]. The α Ib tail has a GFFKR sequence in its membrane-proximal region and this motif is conserved among all integrin α subunits. Deletion or mutation of the GFFKR sequence renders α Ib β 3 constitutively active [9,10]. The GFFKR sequence and its flanking region in the α Ib tail bind calcium integrin-binding protein and ancient ubiquitous protein 1 [11,12], and the interaction with the former protein activates α Ib β 3 [13]. The membrane-proximal regions of α Ib and β 3 cytoplasmic tails form α helical structures and resemble the two-stranded coiled-coil structure in which multiple hydrophobic and electrostatic interactions connect each other [14–16]. These findings suggest that the interaction between the membrane-proximal regions of α Ib and β 3 tails constrain α Ib β 3 in a default low-affinity state and perturbation of this interaction results in α Ib β 3 activation.

In addition to the membrane-proximal region, previous studies suggest that the membrane-distal region after the GFFKR sequence in the α Ib tail also regulates the affinity state of α Ib β 3. A lipid-modified peptide containing the membrane-distal sequence (RPPEED) penetrates the platelet membrane and inhibits agonist-induced α Ib β 3 activation [17], whereas a membrane-proximal heptapeptide (KVGFFKR) induces α Ib β 3 activation

[18]. A double mutation P998A (Pro⁹⁹⁸ → Ala)/P999A in the α Ib tail renders α Ib β 3 constitutively active to bind soluble ligands [19]. Although these findings suggest the involvement of the membrane-distal region of the α Ib tail in α Ib β 3 activation, a molecular basis for regulation of integrin activation by this region remains to be established.

In the present study, we conducted site-directed mutagenesis of the membrane-distal region of the α Ib tail, and examined the activation states of mutant α Ib β 3. Using a model of metabolic energy-dependent α Ib β 3 activation, we found the novel sequence critical for regulation of integrin activation in the α Ib tail.

MATERIALS AND METHODS

Monoclonal antibodies and plasmids

PAC1, a monoclonal antibody specific for an active conformation of α Ib β 3 [20], was a gift from Dr S. J. Shattil (The Scripps Research Institute, La Jolla, CA, U.S.A.). 4B10 is a non-function-blocking anti- α Ib β 3 complex-specific monoclonal antibody produced in our laboratory [21]. Human α Ib, α Ib/ α 6A and β 3 cDNAs were also provided by Dr S. J. Shattil. The α Ib/ α 6A cDNA encodes the extracellular and transmembrane domains of the α Ib subunit connected to the cytoplasmic domain of the α 6A subunit [22].

Site-directed mutagenesis

PCR with mutagenic primers was used to create cDNAs with amino acid sequence substitutions, deletions and additions. Substitutions were introduced into the tail sequences of α Ib and α Ib/ α 6A cDNAs by the PCR using primers containing the desired mutations or by using a Quik Change site-directed mutagenesis

Abbreviations used: CHO, Chinese-hamster ovary; GAIP, G α -interacting protein; GIPC, GAIP-interacting protein, C-terminus; TIP-2, tax interaction protein clone 2.

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kit (Stratagene, La Jolla, CA, U.S.A.). Deletion mutants of the $\alpha 6A$ tail sequence were generated from the $\alpha \text{IIb}/\alpha 6A$ cDNA template by the PCR using a common 5'-primer containing an *EcoRI* restriction site and a 3'-primer containing a stop codon and an *XbaI* restriction site. Additional mutants containing the TSDA sequence were generated from the αIIb or $\alpha \text{IIb}/\alpha 6A$ cDNA template by the PCR using a common 5'-primer containing an *EcoRI* site and a 3'-primer containing the nucleotide sequence encoding TSDA residues followed by a stop codon and an *XbaI* site. The amplified PCR products were digested with *EcoRI*-*XbaI* and cloned into a mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA, U.S.A.). The nucleotide sequences of the inserts were confirmed by sequence analysis. The amino acids in the $\alpha \text{IIb}/\alpha 6A$ chimaeric subunit were numbered sequentially from the N-terminus of the αIIb sequence.

Expression of recombinant integrins

Mutated αIIb or $\alpha \text{IIb}/\alpha 6A$ cDNA was co-transfected with wild-type $\beta 3$ cDNA into CHO (Chinese-hamster ovary) cells using LIPOFECTAMINETM (Invitrogen) or effectene (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Then the cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum for 48 h to achieve expression of recombinant integrins on the cell surfaces.

PAC1-binding assay

PAC1 is an anti- $\alpha \text{IIb}\beta 3$ monoclonal IgM antibody and recognizes only an active form of $\alpha \text{IIb}\beta 3$ [20]. PAC1 contains an RYD tract in its heavy-chain hypervariable region 3 (H-CDR3) and behaves like RGD-containing macromolecular ligands [23]. We used PAC1 to probe the activation states of $\alpha \text{IIb}\beta 3$ mutants. CHO cells were suspended at 1.5×10^7 cells/ml in Tyrode's buffer containing 137 mM NaCl, 12 mM NaHCO_3 , 2.6 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 5 mM Hepes, 5.5 mM glucose and 1 mg/ml BSA (pH 7.4). In the energy-depletion experiments, cells were suspended in glucose-free Tyrode's buffer containing 4 mg/ml 2-deoxy-D-glucose and 0.2% sodium azide and pre-incubated for 30 min at room temperature (22 °C). Aliquots of each cell suspension were incubated for 30 min at room temperature with a saturating amount of PAC1 IgM (0.4% PAC1 ascites at a final concentration) and 5 $\mu\text{g}/\text{ml}$ biotin-labelled 4B10 IgG in the presence of 2 mM RGDS peptide or buffer. RGDS peptide at this concentration can inhibit PAC1 binding completely [24]. Cells were then washed and resuspended in a mixture of a 1:20 dilution of FITC-conjugated goat anti-mouse IgM antibody (Biosource, Calmarillo, CA, U.S.A.) and a 1:50 dilution of phycoerythrin-streptavidin (Molecular Probes, Eugene, OR, U.S.A.) 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 analysed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). After electronic compensation, PAC1 binding (FL1) was analysed on the gated subset of live cells (propidium iodide-negative, FL3) that were positive for $\alpha \text{IIb}\beta 3$ expression (FL2).

RESULTS

Mutation of the RPP sequence in the αIIb tail renders $\alpha \text{IIb}\beta 3$ active in an energy-dependent manner

$\alpha \text{IIb}\beta 3$ is maintained in an inactive state probably by interaction between αIIb and $\beta 3$ tails. To explore residues critical for

Figure 1 Alanine-scanning mutagenesis of the membrane-distal αIIb tail residues

(A) Cytoplasmic tail sequence of the αIIb subunit. (B) Expression of integrins on CHO cell surfaces. CHO cells were transfected with cDNAs for WT (wild-type) $\beta 3$ and αIIb bearing the indicated mutation or WT αIIb . The PPR mutation represents substitution of ⁹⁹⁷RPP with PPR. Binding of the 4B10 anti- $\alpha \text{IIb}\beta 3$ 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 $\alpha \text{IIb}\beta 3$. (C) PAC1 binding to $\alpha \text{IIb}\beta 3$ mutants. PAC1 and 4B10 binding to cells were measured simultaneously by flow cytometry. The mfi (mean fluorescence intensity) of specific (RGDS-inhibitable) PAC1 binding was divided by that of 4B10 binding to assess on a per $\alpha \text{IIb}\beta 3$ molecule basis. Results presented are means $\pm$ S.E.M. for three independent experiments; *, a significant difference ($P < 0.01$) compared with the WT αIIb value.

regulation of $\alpha \text{IIb}\beta 3$ activation in the membrane-distal region of the αIIb tail, we applied alanine-scanning mutagenesis to all residues after the GFFKR sequence, and tested these mutants for PAC1 binding (Figure 1A). As the double mutation P998A/P999A was reported to activate $\alpha \text{IIb}\beta 3$, this mutant was included as a positive control [19]. The R995D mutant within the GFFKR sequence was included as another positive control [10]. Surface expression of $\alpha \text{IIb}\beta 3$ was determined by flow cytometry using a non-function-blocking anti- $\alpha \text{IIb}\beta 3$ monoclonal antibody 4B10 [21]. The mutants tested were expressed on the surface of CHO cells to 75–152% of wild-type $\alpha \text{IIb}\beta 3$ (Figure 1B). Then binding of PAC1 to CHO cells expressing $\alpha \text{IIb}\beta 3$ mutants was examined. Specific-PAC1 binding was defined as that which was inhibited by the RGDS peptide and was expressed relative to the amount of $\alpha \text{IIb}\beta 3$ on the cell surfaces, which was determined simultaneously using 4B10. 4B10 did not compete with PAC1 for binding to $\alpha \text{IIb}\beta 3$ (results not shown). Among all the mutations of the

Figure 2 Metabolic energy-dependent activation of α IIb β 3 mutants

CHO cells were transfected with cDNAs for wild-type β 3 and the indicated α IIb subunits. Cells were treated with metabolic inhibitors consisting of 4 mg/ml DOG (2-deoxy-D-glucose) and 0.2% NaN₃, and PAC1 and 4B10 binding was measured by flow cytometry. Specific PAC1 binding is expressed on a per α IIb β 3 molecule basis as the mfi (mean fluorescence intensity) of PAC1 binding divided by that of 4B10 binding. Results presented are the means $\pm$ S.E.M. for three independent experiments.

membrane-distal residues, only the R997A and P998A/P999A mutations showed significant PAC1 binding, implying activation of α IIb β 3 by mutation of the ⁹⁹⁷RPP sequence (Figure 1C). The α IIb β 3 mutant, in which the RPP sequence was substituted with a reversed sequence (PPR), was constitutively active (Figure 1C). These results suggest that the RPP sequence in the α IIb tail is involved in the regulation of α IIb β 3 activation.

In our assay using a transient transfection system, as shown in Figure 1(C), the R997A mutation showed a stronger phenotype when compared with the P998A/P999A mutation, but a weaker phenotype when compared with the R995D mutation, with respect to the ability to activate α IIb β 3. A distinctive feature of the R995D mutation is that α IIb β 3 activation by this mutation is independent of metabolic energy [22]. To see whether activation of the RPP sequence mutants was dependent on metabolic energy or not, these mutants were pretreated with 2-deoxy-D-glucose and sodium azide to inhibit oxidative metabolism and then tested for PAC1 binding. The R997A and P998A/P999A mutants showed decreased PAC1 binding under conditions of energy depletion (Figure 2). A similar result was obtained with the PPR mutant. In contrast, this treatment had no effect on PAC1 binding to the R995D mutant. These results suggest that mutation of the membrane-distal region of the α IIb tail activates α IIb β 3 through a mechanism distinct from that of the membrane-proximal region.

An RPP sequence inhibits activation of α IIb/ α 6A β 3 chimaeric integrin

To explore further the regulatory effect of the RPP sequence on metabolic energy-dependent α IIb β 3 activation, we took advantage of an α IIb/ α 6A β 3 chimaeric integrin in which the α IIb cytoplasmic tail was replaced with the α 6A tail. This integrin is constitutively active in a metabolic energy-dependent manner [22,25,26]. Since the α 6A tail has no RPP sequence (Figure 4A), we assumed that the absence of RPP sequence in the α 6A tail might be responsible for α IIb/ α 6A β 3 activation. To test this idea, we generated α IIb/ α 6A β 3 mutant in which the ⁹⁹⁷KKD sequence was substituted with the ⁹⁹⁷RPP sequence in the α IIb/ α 6A tail. This substitution strongly inhibited PAC1 binding, although the mutant integrin was sufficiently expressed on the cell surface (Figure 3). Substitution with an inversed sequence PPR had no

Figure 3 Inhibitory effect of the RPP sequence on α IIb/ α 6A β 3 activation

CHO cells were transfected with cDNAs for WT (wild-type) β 3 and α IIb/ α 6A bearing mutations or WT α IIb. RPP and PPR mutations represent replacement of the ⁹⁹⁷KKD sequence with RPP and PPR sequences in the α IIb/ α 6A tail respectively. Binding of 4B10 (white bars) and PAC1 (black bars) to cells was measured simultaneously by flow cytometry. Surface expression of integrins was expressed as percentages of the value for 4B10 binding to cells expressing intact α IIb/ α 6A β 3. The mean fluorescence intensity of specific PAC1 binding was divided by that of 4B10 binding, on a per α IIb/ α 6A β 3 molecule basis, and expressed as a percentage of the value for PAC1 binding to cells expressing intact α IIb/ α 6A β 3. Results presented are means $\pm$ S.E.M. for three independent experiments.

inhibitory effect on PAC1 binding. These results are consistent with the foregoing result, that mutation of the RPP sequence activated α IIb β 3.

TSDA sequence in the membrane-distal region of the α 6A tail renders α IIb/ α 6A β 3 active in an energy-dependent manner

Our results with RPP mutations suggest an inhibitory role of the RPP sequence in α IIb β 3 activation. However, a previous mutational study showed that deletion of the RPP sequences from the α IIb tail (α IIb Δ 996) keeps α IIb β 3 in an inactive state (Figure 6 and [22]), implying that loss of the RPP sequence from the α IIb tail is not sufficient for spontaneous α IIb β 3 activation. This result may be due to total loss of the membrane-distal region, which may comprise an energy-dependent integrin activation system. Since the membrane-proximal KXGFFKR sequence is conserved between α IIb and α 6A tails, we reasoned that some membrane-distal residue of the α 6A tail might have a stimulatory role in α IIb/ α 6A β 3 activation. To identify such residues, we produced a series of α IIb/ α 6A β 3 mutants with truncated α 6A tails. The truncation mutants tested were expressed on the surface of CHO cells to 98–196% of intact α IIb/ α 6A β 3 (Figure 4B). As expected, intact α IIb/ α 6A β 3 exhibited marked PAC1 binding. Although the α IIb/ α 6A Δ 1024, Δ 1023 and Δ 1022 deletions inhibited PAC1 binding slightly, α IIb/ α 6A Δ 1021 deletion and more proximal deletions decreased PAC1 binding to a low level comparable with that of an inactive form of wild-type α IIb β 3 (Figure 4C). This result indicates that deletion of the TSDA sequence from the C-terminus of the α 6A tail disrupts α IIb/ α 6A β 3 activation (Figures 4A and 4C). When all the deletion mutants were stimulated with an anti- α IIb β 3 activating monoclonal antibody, PT25-2 [27], they bound PAC1 as well as wild-type α IIb β 3 (results not shown), eliminating the possibility that long-range propagation of these mutations directly causes conformational disruption of an extracellular ligand-binding pocket.

We examined the effects of amino acid substitutions within the TSDA sequence on PAC1 binding. Substitution of a single residue with alanine or glutamine inhibited PAC1 binding significantly, whereas substitution of the Arg¹⁰¹⁹ residue outside the TSDA sequence with alanine had no effect (Figure 5). Substituting the TSDA sequence with an inverse sequence (ADST) caused significant inhibition. These results with TSDA-deletion and

Figure 4 Activation states of αIIb/α6Aβ3 truncation mutants

(A) Cytoplasmic tail sequence of the αIIb/α6A subunit. Amino acids in the αIIb/α6A chimaeric subunit are numbered sequentially from the N-terminus of the αIIb sequence. (B) Expression of integrins on CHO cell surfaces. CHO cells were transfected with cDNAs for WT (wild-type) β3 and αIIb/α6A bearing the indicated mutation or WT αIIb. Binding of the 4B10 anti-αIIbβ3 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 intact αIIb/α6Aβ3. (C) PAC1 binding to truncation mutants. The mean fluorescence intensity of specific PAC1 binding was divided by that of 4B10 binding, on a per αIIb/α6Aβ3 molecule basis, and expressed as a percentage of the value for PAC1 binding to cells expressing intact αIIb/α6Aβ3. Results presented are means ± S.E.M. for three independent experiments.

substitution mutants suggest that the TSDA sequence in the membrane-distal region of the α6A tail is critical for αIIb/α6Aβ3 activation, in addition to the absence of the RPP sequence from the α6A tail.

We next examined whether replacement of the αIIb membrane-distal region by the TSDA sequence activates αIIbβ3. We produced αIIbΔ996TSDA, an αIIbβ3 mutant in which the membrane-distal region of the αIIb tail after the GFFKR sequence was replaced by the TSDA sequence. The αIIbΔ996 deletion alone did not activate αIIbβ3, as reported previously [22]. In contrast, αIIbΔ996TSDA was in a constitutively active state to bind PAC1, although it bound less PAC1 when compared with αIIb/α6Aβ3 (Figure 6). To determine whether activation of this mutant requires metabolic energy as well as that of αIIb/α6Aβ3, αIIbΔ996TSDA was pretreated with 2-deoxy-D-glucose and sodium azide and then tested for PAC1 binding. αIIbΔ996TSDA showed reduced PAC1

Figure 5 Effects of amino acid substitution within the TSDA sequence of the α6A tail on PAC1 binding to αIIb/α6Aβ3

CHO cells were transfected with cDNAs for WT β3 and αIIb/α6A bearing the indicated mutation or WT αIIb. The mean fluorescence intensity of specific PAC1 binding was divided by that of 4B10 binding to assess on a per αIIb/α6Aβ3 molecule basis, and expressed as a percentage of the value for PAC1 binding to cells expressing intact αIIb/α6Aβ3. Results presented are the means ± S.E.M. for three independent experiments.

Figure 6 Effects of the TSDA sequence on PAC1 binding to αIIbβ3

CHO cells were transfected with cDNAs for WT (wild-type) β3 and the indicated α subunits. TSDA sequence was added to the C-terminus of the αIIbΔ996 or entire αIIb tail. The mean fluorescence intensity of specific PAC1 binding was divided by that of 4B10 binding, on a per αIIb/α6Aβ3 molecule basis, and expressed as a percentage of the value for PAC1 binding to cells expressing intact αIIb/α6Aβ3. Results presented are the means ± S.E.M. for three independent experiments.

binding under conditions of energy depletion, as did αIIb/α6Aβ3 (Figure 7); αIIbR995D had no effect. These results indicate that the αIIbΔ996TSDA mutant represents another model of metabolic energy-dependent αIIbβ3 activation.

An RPP sequence in the αIIb tail is critical for suppression of energy-dependent αIIbβ3 activation

We employed αIIbΔ996TSDA to explore the role of the membrane-distal region of the αIIb tail in energy-dependent αIIbβ3 activation. αIIbΔ996TSDA was obtained by adding TSDA to the αIIb tail lacking the membrane-distal region, and αIIbβ3 bearing this mutation was active. However, the addition of TSDA to the whole αIIb tail (αIIbTSDA) did not activate αIIbβ3 (Figure 6), despite adequate surface expression levels (results not shown). These results suggest that the membrane-distal region after the Arg⁹⁹⁵ residue in the αIIb tail suppresses TSDA-induced αIIbβ3 activation. To identify critical residues for suppression of αIIbβ3 activation in the αIIb tail, we produced a series of αIIbβ3 mutants in which the TSDA sequence was added to

Figure 7 Dependence on metabolic energy of constitutively active integrins

CHO cells were transfected with cDNAs for wild-type β 3 and the indicated α subunits. Cells were treated with metabolic inhibitors [4 mg/ml DOG (2-deoxy-D-glucose) and 0.2% NaN₃], and PAC1 and 4B10 binding was measured by flow cytometry. The mean fluorescence intensity of specific PAC1 binding was divided by that of 4B10 binding, on a per α IIB β 3 molecule basis, and expressed as a percentage of the value for PAC1 binding to cells expressing α IIB/ α 6A β 3. Results presented are the means $\pm$ S.E.M. for three independent experiments.

Figure 8 Effects of the membrane-distal α IIB tail residues on TSDA-induced α IIB β 3 activation

CHO cells were transfected with cDNAs for WT (wild-type) β 3 and the indicated α subunits. Specific PAC1 binding is expressed on a per α IIB β 3 molecule basis as the mfi (mean fluorescence intensity) of PAC1 binding divided by that of 4B10 binding. Results presented are the means $\pm$ S.E.M. for three independent experiments.

the C-terminus of the α IIB tail, containing residues after Arg⁹⁹⁵, and examined PAC1 binding to these mutants. The α IIB Δ 1000 mutant containing the ⁹⁹⁶NRPP sequence was not activated by adding TSDA (Figure 8). The α IIB Δ 998 mutant was partially activated by TSDA and the α IIB Δ 997 mutant containing ⁹⁹⁶N, but not ⁹⁹⁷RPP, was activated by TSDA to the same extent as the α IIB Δ 996 mutant. The α IIB/ α 6A Δ 1000 mutant containing the same length of sequence as the α IIB Δ 1000 mutant was activated by TSDA. These results indicate that the ⁹⁹⁷RPP sequence of the α IIB tail exerts dominant inhibition of TSDA-induced α IIB β 3 activation. This finding is consistent with the foregoing results showing the suppressive effects of the RPP sequence on α IIB β 3 and α IIB/ α 6A β 3 activation and, thus, it is suggested that the RPP sequence in the α IIB tail is critical for suppression of energy-dependent α IIB β 3 activation.

DISCUSSION

Identification of amino acid sequences critical for integrin activation and signalling in the cytoplasmic domains has contributed

to our understanding of the structural basis of integrin functions. The GFFKR sequence in integrin α tails and the NPXY motif in β tails have been identified as indispensable sites for integrin activation and signalling [28–30]. In the integrin α IIB β 3, the GFFKR forms a structural constraint that locks α IIB β 3 in a default low-affinity conformation. The NPXY motif directly interacts with a signalling adaptor protein Shc and two cytoskeletal proteins myosin and talin, and these interactions are essential for bidirectional signalling [30,31]. In the present study, we found that the RPP sequence in the α IIB tail serves as a suppressor of energy-dependent α IIB β 3 activation. The RPP sequence lies at the α IIB membrane-distal region following the GFFKR, and no functional motif has been identified in this region. The RPP sequence is conserved among α IIB, α V and α 8 subunits. The corresponding sequences in α L, α M, α X and α D are (Y/L)K(E/D), indicating sequence homology among α partners with β 2 subunit. Therefore this membrane-distal region of the α tails following GFFKR may be one of the structural determinants regulating integrin activation.

Recent NMR analyses of the peptides containing the entire α IIB tail sequence revealed that the N-terminal ⁹⁹¹GFFKRNRP residues form an α helix, terminating at Pro⁹⁹⁹, followed by a turn, which allows a closed conformation with the C-terminal loop to fold back towards the N-terminal helix [14,15]. Results from our study suggest that the RPP sequence of the α IIB tail, as well as the GFFKR sequence, plays a suppressive role in α IIB β 3 activation. However, in contrast with the metabolic energy-independent activation of α IIB β 3 by GFFKR mutations, activation of α IIB β 3 by RPP mutations was metabolically energy dependent. These findings suggest that the RPP sequence maintains α IIB β 3 in a low-affinity state through a mechanism distinct from that of the conserved GFFKR motif, even though both sequences are within the same α helix structure. Vinogradova et al. [14] proposed a hypothetical model of α IIB β 3 activation in which conformational changes in the position of the ⁹⁹⁸PP residues open the closed conformation between the N-terminal helix and C-terminal loop of the α IIB tail and thus allow access of some integrin activator to the α IIB β 3 tails. This model may explain the metabolic energy-dependent activation of α IIB β 3 by RPP mutations we observed. Recently, Tadokoro et al. [32] reported that knockdown of talin expression reduced energy-dependent integrin activation and that specific binding of talin to the membrane-distal residues of the integrin β tails led to integrin activation in an energy-dependent manner. Therefore it is probable that metabolic energy is required for regulation of this binding. Conformational changes in the RPP region of the α tails may allow talin to bind to β tails. In this context, it would be intriguing to determine whether talin knockdown reduces energy-dependent activation of the RPP mutant integrins.

In the present study, we used a chimaeric integrin α IIB/ α 6A β 3 as a model of metabolic energy-dependent activation of α IIB β 3. This integrin has been employed as a useful tool for studies on integrin signalling and molecular cloning of intracellular proteins involved in integrin activation [21,22,25,26,33–36]. However, the mechanism of α IIB/ α 6A β 3 activation remains poorly defined. Previous studies revealed that α IIB/ α 6A β 3 activation requires metabolic energy and is inhibited by overexpression of the isolated integrin β 1 or β 3 tail, suggesting the involvement of some intracellular component(s) in the process that activates this integrin [22,25]. The present study suggests that the absence of the RPP sequence and the presence of the TSDA sequence in the α 6A tail are involved in α IIB/ α 6A β 3 activation in an energy-dependent manner. Moreover, our result suggests that the presence of RPP inhibited TSDA-induced α IIB β 3 activation and indicates that the inhibitory function of RPP predominates over the stimulatory

function of TSDA. These findings raise the possibility that α IIb/ α 6A β 3 activation is induced by interaction of TSDA with some intracellular protein(s) concerned with integrin activation, which is prevented if the α tail contains RPP which interacts with the β 3 tail or other protein(s). A previous study showed that replacing the α IIb tail by an α 2, α 5 or α 6B tail also renders α IIb β 3 constitutively active, whereas replacing by α V does not [22]. These effects are correlated with the absence or presence of the RPP sequence in the α tails replaced. Moreover, the C-terminal TSDA sequence of the α 6A tail was recently reported to correspond to a type I PDZ recognition motif and to bind a PDZ-containing cytoplasmic protein TIP-2 (tax interaction protein clone 2)/GIPC [GAIP (G α -interacting protein)-interacting protein, C-terminus] [37,38]. Proteins of this family function as scaffold proteins for the assembly of signalling complexes [39] and TIP-2/GIPC may tether glucose transporter 1 to the cytoskeleton [40]. The α 5 tail also contains the TSDA sequence in its C-terminus and binds TIP-2/GIPC [37,38]. The α 6B tail can bind TIP-2/GIPC although it does not contain a conserved PDZ recognition sequence [37,38]. The α IIb/ α 5 β 3 and α IIb/ α 6B β 3 chimaeric integrins expressed in CHO cells are constitutively active [22] and TIP-2/GIPC is expressed by CHO cells [40]. Although these results raise the possibility that TIP-2/GIPC participates in the process of integrin activation, the differential effects of TSDA mutations on TIP-2/GIPC binding and α IIb/ α 6A β 3 activation do not support this. Substituting a serine or aspartate residue within the TSDA sequence with alanine and deleting the SDA sequence inhibited binding of TIP-2/GIPC to the α 6A tail completely [37], whereas the same mutations inhibited PAC1 binding to α IIb/ α 6A β 3 by 20–60 %, as shown in the present study (Figures 4 and 5). Moreover, although α IIb/ α 2 β 3 is constitutively active [22], TIP-2/GIPC did not bind to the α 2 tail [37,38]. However, these findings do not exclude the possibility that the interaction between TSDA and TIP-2/GIPC is one of the pathways to integrin activation and that the TSDA sequence interacts with as yet unknown intracellular proteins involved in integrin activation. Further studies will be required to identify components essential for α IIb/ α 6A β 3 activation and to determine the physiological significance of such components.

Evidence that integrin activation is regulated by an interaction between α and β tails is increasing [10,15–17,41,42], although this interaction was not detected under certain conditions [43,44]. The membrane-proximal regions of the α and β tails interact with each other to keep integrins in a low-affinity state and integrin activation is probably brought about by the removal of α cytoplasmic tail constraints from the β tail [10,16,41]. As for the membrane-distal regions, Ginsberg et al. [17] recently proposed that the ⁹⁹⁷RPPLEED region of the α IIb tail contacts the β 3 tail to maintain α IIb β 3 in a low-affinity state. This is consistent with our results for the ⁹⁹⁷RPP mutations, although the ¹⁰⁰⁰LEED mutations failed to activate α IIb β 3 (Figure 1C). Interestingly, another partner with the β 3 subunit, α V, has the RPP, but not the LEED, sequence in the corresponding position of the α IIb tail. Therefore it would be intriguing to determine whether the RPP sequence of the α V tail interacts with the β 3 tail to suppress α V β 3 activation. Our preliminary results showed that α V β 3 was activated by RPP mutations (J. Yamanouchi and T. Hato, unpublished work). Identification of these cytoplasmic tail sequences as regulators of integrin activation will help to unravel the molecular basis for energy-dependent integrin activation.

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REFERENCES

- Giancotti, F. G. and Ruoslahti, E. (1999) Transduction – integrin signaling. *Science* **285**, 1028–1032.
- Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* (Cambridge, Mass.) **110**, 673–687.
- Shattil, S. J. and Ginsberg, M. H. (1997) Integrin signaling in vascular biology. *J. Clin. Invest.* **100**, 1–5.
- Plow, E. F., Haas, T. K., Zhang, L., Loftus, J. and Smith, J. W. (2000) Ligand binding to integrins. *J. Biol. Chem.* **275**, 21785–21788.
- Liddington, R. C. and Ginsberg, M. H. (2002) Integrin activation takes shape. *J. Cell Biol.* **158**, 833–839.
- Shimaoka, M., Xiao, T., Liu, J. H., Yang, Y., Dong, Y., Jun, C. D., McCormack, A., Zhang, R., Joachimiak, A., Takagi, J. et al. (2003) Structures of the α L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. *Cell* (Cambridge, Mass.) **112**, 99–111.
- Woodsides, D. G., Liu, S. and Ginsberg, M. H. (2001) Integrin activation. *Thromb. Haemost.* **86**, 316–323.
- Shattil, S. J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. *Blood* **91**, 2645–2657.
- O'Toole, T. E., Mandelman, J., Forsyth, J., Shattil, S. J., Plow, E. F. and Ginsberg, M. H. (1991) Modulation of the affinity of integrin α IIb β 3 (GPIIb-IIIa) by the cytoplasmic domain of α IIb. *Science* **254**, 845–847.
- Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C. Y., McDonald, J. A., Shattil, S. J. and Ginsberg, M. H. (1996) Breaking the integrin hinge – a defined structural constraint regulates integrin signaling. *J. Biol. Chem.* **271**, 6571–6574.
- Barry, W. T., Boudignon-Proudhon, C., Shock, D. D., McFadden, A., Weiss, J. M., Sondek, J. and Parise, L. V. (2002) Molecular basis of CIB binding to the integrin α IIb cytoplasmic domain. *J. Biol. Chem.* **277**, 28877–28883.
- Kato, A., Kawamata, N., Tamayose, K., Egashira, M., Miura, R., Fujimura, T., Murayama, K. and Oshimi, K. (2002) Ancient ubiquitous protein 1 binds to the conserved membrane-proximal sequence of the cytoplasmic tail of the integrin α subunits that plays a crucial role in the inside-out signaling of α IIb β 3. *J. Biol. Chem.* **277**, 28934–28941.
- Tsuboi, S. (2002) Calcium integrin-binding protein activates platelet integrin α IIb β 3. *J. Biol. Chem.* **277**, 1919–1923.
- Vinogradova, O., Haas, T., Plow, E. F. and Qin, J. (2000) A structural basis for integrin activation by the cytoplasmic tail of the α IIb-subunit. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1450–1455.
- Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E. and Qin, J. (2002) A structural mechanism of integrin α IIb β 3 'inside-out' activation as regulated by its cytoplasmic face. *Cell* (Cambridge, Mass.) **110**, 587–597.
- Weljie, A. M., Hwang, P. M. and Vogel, H. J. (2002) Solution structures of the cytoplasmic tail complex from platelet integrin α IIb- and β 3-subunits. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 5878–5883.
- Ginsberg, M. H., Yaspan, B., Forsyth, J., Ulmer, T. S., Campbell, I. D. and Slepak, M. (2001) A membrane-distal segment of the integrin α IIb cytoplasmic domain regulates integrin activation. *J. Biol. Chem.* **276**, 22514–22521.
- Stephens, G., O'Lunaigh, N., Reilly, D., Harriott, P., Walker, B., Fitzgerald, D. and Moran, N. (1998) A sequence within the cytoplasmic tail of GPIIb independently activates platelet aggregation and thromboxane synthesis. *J. Biol. Chem.* **273**, 20317–20322.
- Leisner, T. M., Wencel-Drake, J. D., Wang, W. and Lam, S. C. T. (1999) Bidirectional transmembrane modulation of integrin α IIb β 3 conformations. *J. Biol. Chem.* **274**, 12945–12949.
- Shattil, S. J., Hoxie, J. A., Cunningham, M. and Brass, L. F. (1985) Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* **260**, 11107–11114.
- Yamanouchi, J., Hato, T., Tamura, T. and Fujita, S. (2002) Identification of critical residues for ligand binding in the integrin β 3 I-domain by site-directed mutagenesis. *Thromb. Haemost.* **87**, 756–762.
- O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J. and Ginsberg, M. H. (1994) Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* **124**, 1047–1059.
- Abrams, C., Deng, Y.-J., Steiner, B., O'Toole, T. E. and Shattil, S. J. (1994) Determinants of specificity of a baculovirus-expressed antibody Fab fragment that binds selectively to the activated form of integrin α IIb β 3. *J. Biol. Chem.* **269**, 18781–18788.
- Bennett, J. S., Shattil, S. J., Power, J. W. and Gartner, T. K. (1988) Interaction of fibrinogen with its platelet receptor. Differential effects of α and γ chain fibrinogen peptides on the glycoprotein IIb-IIIa complex. *J. Biol. Chem.* **263**, 12948–12953.

- 25 Chen, Y.-P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J. and Ginsberg, M. H. (1994) 'Inside-out' signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.* **269**, 18307–18310
- 26 O'Toole, T. E., Ylanne, J. and Culley, B. M. (1995) Regulation of integrin affinity states through an NPXY motif in the β subunit cytoplasmic domain. *J. Biol. Chem.* **270**, 8553–8558
- 27 Tokuhira, M., Handa, M., Kamata, T., Oda, A., Katayama, M., Tomiyama, Y., Murata, M., Kawai, Y., Watanabe, K. and Ikeda, Y. (1996) A novel regulatory epitope defined by a murine monoclonal antibody to the platelet GPIIb–IIIa complex (α IIb β 3 integrin). *Thromb. Haemost.* **76**, 1038–1046
- 28 Hughes, P. E. and Pfaff, M. (1998) Integrin affinity modulation. *Trends Cell Biol.* **8**, 359–364
- 29 Liu, S. C., Calderwood, D. A. and Ginsberg, M. H. (2000) Integrin cytoplasmic domain-binding proteins. *J. Cell Sci.* **113**, 3563–3571
- 30 Phillips, D. R., Nannizzi-Alamio, L. and Prasad, K. S. S. (2001) β 3 tyrosine phosphorylation in α IIb β 3 (platelet membrane GPIIb–IIIa) outside-in integrin signaling. *Thromb. Haemost.* **86**, 246–258
- 31 Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H. and Liddington, R. C. (2003) Structural determinants of integrin recognition by talin. *Mol. Cell* **11**, 49–58
- 32 Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H. and Calderwood, D. A. (2003) Talin binding to integrin β tails: a final common step in integrin activation. *Science* **302**, 103–106
- 33 Hughes, P. E., Renshaw, M. W., Pfaff, M., Forsyth, J., Keivens, V. M., Schwartz, M. A. and Ginsberg, M. H. (1997) Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell (Cambridge, Mass.)* **88**, 521–530
- 34 Fenczik, C. A., Sethi, T., Ramos, J. W., Hughes, P. E. and Ginsberg, M. H. (1997) Complementation of dominant suppression implicates CD98 in integrin activation. *Nature (London)* **390**, 81–85
- 35 Ramos, J. W., Kojima, T. K., Hughes, P. E., Fenczik, C. A. and Ginsberg, M. H. (1998) The death effector domain of PEA-15 is involved in its regulation of integrin activation. *J. Biol. Chem.* **273**, 33897–33900
- 36 Hato, T., Pampori, N. and Shattil, S. J. (1998) Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin α IIb β 3. *J. Cell Biol.* **141**, 1685–1695
- 37 Tani, T. T. and Mercurio, A. M. (2001) PDZ interaction sites in integrin α subunits. T14853, TIP/GIPC binds to a type I recognition sequence in α 6A/ α 5 and a novel sequence in α 6B. *J. Biol. Chem.* **276**, 36535–36542
- 38 El Mourabit, H., Poinat, P., Koster, J., Sondermann, H., Wixler, V., Wegener, E., Laplantine, E., Geerts, D., Georges-Labouesse, E., Sonnenberg, A. et al. (2002) The PDZ domain of TIP-2/GIPC interacts with the C-terminus of the integrin α 5 and α 6 subunits. *Matrix Biol.* **21**, 207–214
- 39 Harris, B. Z. and Lim, W. A. (2001) Mechanism and role of PDZ domains in signaling complex assembly. *J. Cell Sci.* **114**, 3219–3231
- 40 Bunn, R. C., Jensen, M. A. and Reed, B. C. (1999) Protein interactions with the glucose transporter binding protein GLUT1CBP that provide a link between GLUT1 and the cytoskeleton. *Mol. Biol. Cell* **10**, 819–832
- 41 Lu, C. F., Takagi, J. and Springer, T. A. (2001) Association of the membrane proximal regions of the α and β subunit cytoplasmic domains constrains an integrin in the inactive state. *J. Biol. Chem.* **276**, 14642–14648
- 42 Takagi, J., Erickson, H. P. and Springer, T. A. (2001) C-terminal opening mimics 'inside-out' activation of integrin α 5 β 1. *Nat. Struct. Biol.* **8**, 412–416
- 43 Ulmer, T. S., Yaspan, B., Ginsberg, M. H. and Campbell, I. D. (2001) NMR analysis of structure and dynamics of the cytosolic tails of integrin α IIb β 3 in aqueous solution. *Biochemistry* **40**, 7498–7508
- 44 Li, R., Babu, C. R., Lear, J. D., Wand, A. J., Bennett, J. S. and DeGrado, W. F. (2001) Oligomerization of the integrin α IIb β 3: roles of the transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12462–12467

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