# Regulation of Integrin $\alpha_{IIb}\beta_3$ Activation by Distinct Regions of Its Cytoplasmic Tails†

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ABSTRACT: The short cytoplasmic tails regulate activation of integrin adhesion receptors via clasping/unclasping of their membrane-proximal helices. Using integrin $\alpha_{IIb}\beta_3$ as a model, we show that a previously reported activating mutation $\alpha_{IIb}(R^{995}D)$ that perturbs the electrostatic interface in the clasp only partially activates $\alpha_{IIb}\beta_3$ and that extensive activation of the receptor is achieved by complete deletion of $\alpha_{IIb}$ CT or triple mutations in $\alpha_{IIb}(V^{990}A/F^{992}A/R^{995}D)$ that disrupt both electrostatic and hydrophobic interfaces in the clasp. The results provide quantitative evidence for an equilibrium-based integrin activation process where shifting the equilibrium to the fully activated state requires total unclasping of the cytoplasmic tails. We further demonstrate that while the C-terminal region of the $\alpha_{IIb}$ tail minimally influences $\alpha_{IIb}\beta_3$ activation, the C-terminal region of the $\beta_3$ tail is critically involved. A disease-causing mutation of $S^{752}P$ in this region, but not $S^{752}A$, suppressed partial activation induced by $R^{995}D$ or the talin head domain but did not affect activation induced by $\alpha_{IIb}$ truncation. NMR spectroscopy revealed that $S^{752}P$ but not the $S^{752}A$ mutation disrupted a C-terminal helix within the $\beta_3$ tail, suggesting that the C-terminal helix may regulate the equilibrium-based clasping/unclasping process. Together, these data provide molecular insights into how distinct regions of the cytoplasmic tails differentially and cooperatively regulate integrin activation.

Platelet adhesion and aggregation contribute to the preservation of vascular integrity after injury and are involved in cardiovascular diseases, contributing to the initiation and progression of atherosclerotic lesions (*1*). Integrin $\alpha_{IIb}\beta_3$, the major membrane protein on the surface of platelets, plays critical roles in these processes. Central to the function of $\alpha_{IIb}\beta_3$ is its capacity to undergo activation, a transition from a resting to an active state in which it becomes competent to bind extracellular ligands that can support platelet responses (*2*, *3*). The process of activation is initiated at the two cytoplasmic tails (CTs)[1] of the heterodimeric integrin subunits, which then triggers a conformational change in the extracellular domain of the receptor to enhance ligand binding, affinity modulation, a process referred to as *inside-out signaling*. As a consequence of ligand occupancy, *outside-in signaling* is induced, which mediates a series of intracellular responses. These molecular events appear to be broadly applicable to the integrin family; the membrane-proximal regions of all integrins are highly conserved, and formation of an $\alpha/\beta$ clasp has been demonstrated for several integrin family members (*4*).

Compared to other signaling receptors, the CTs of integrins are very small. In the case of $\alpha_{IIb}\beta_3$, the $\alpha_{IIb}$ CT contains 20 amino acids ($K^{989}-E^{1008}$) and the $\beta_3$ CT ($K^{716}-T^{762}$) 47 residues. However, inside-out and outside-in signals must be received and transmitted through these structures. The CTs are thought to mediate these signaling events through modifications of their structural and spatial organization and/or through interactions with specific cytoplasmic components (*4*). The NMR structure of the $\alpha_{IIb}$ tail demonstrates a membrane-proximal helical feature followed by a turn structure formed by $NRP^{998}P^{999}$ residues (*5*). The $\beta_3$ CT exhibits α-helical structures both in its membrane-proximal $K^{716}-R^{734}$ sequence and in its C-terminal region $K^{748}-T^{755}$ (*6*). Multiple studies have indicated that the $\alpha_{IIb}$ and $\beta_3$ CTs form a complex, and different mechanisms have been proposed as to how the CT complex may be perturbed for initiating the inside-out activation (*7*−*11*). Hughes et al. (*7*) suggested a hinge model where disruption of a particular salt bridge between $R^{995}$ of $\alpha_{IIb}$ and $D^{723}$ of $\beta_3$ leads to the activation of the integrin. An NMR study (*9*) using truncated $\alpha_{IIb}$ and $\beta_3$ CT peptides suggested the existence of two heterodimeric conformers and the importance of hydrophobic bonds in maintaining the heterodimers. On the other hand, another NMR study (*8*) revealed that only one single averaged heterodimeric conformer exists on the basis of full-length $\alpha_{IIb}$ and $\beta_3$ CTs, in which the particular salt bridge between $\alpha_{IIb}(R^{995})$ and $\beta_3(D^{723})$ was just one of several electrostatic as well as hydrophobic contacts that clasp the membrane-proximal helices of the two CTs together. Studies by Takagi et al. (*12*) and Kim et al. (*13*) demonstrated that a full separation of the CT complex is necessary for the activation of the integrin, which would require disruption of hydrophobic as well as electrostatic bonds. The membrane-distal regions of the $\alpha_{IIb}$ and $\beta_3$ CT might also be involved in controlling integrin activation: (1) the turn structure of $\alpha_{IIb}$ CT facilitated by interactions of the C-terminus and the positively charged membrane-proximal region probably stabilizes the $\alpha_{IIb}$ CT in a "closed" conformation, and the double mutation of $P^{998}AP^{999}A$ in the $\alpha_{IIb}$ CT, which

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[1] Abbreviations: CT, cytoplasmic tail; talin-H, talin head domain; NOE, nuclear Overhauser effect; MBP, maltose-binding protein; DPC, dodecylphosphocholine; NMR, nuclear magnetic resonance.

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destabilizes this folded structure (*5*), is reported to activate $\alpha_{IIb}\beta_3$ (*14*); (2) in the $\beta_3$ CT, a point mutation at $Y^{747}A$, which would disturb its turn motif, or at $S^{752}P$, a naturally occurring point mutant which impairs bidirectional signaling of $\alpha_{IIb}\beta_3$, or truncations at $F^{754}$, $Y^{747}$, or $T^{741}$ sites, all well beyond the membrane-proximal helical domain of $\beta_3$ CT, silence integrin activation (*15*−*17*). In addition, the talin head domain (talin-H) binds to the $NPLY^{747}$ motif and an upstream region of the $\beta_3$ CT to disrupt the membrane-proximal CT complex and induces integrin activation (*8*, *18*, *19*).

To further understand how the structures of the CTs regulate integrin activation, structure-based mutational analyses have been conducted in transfected CHO cells, a commonly used cell type for such studies (*7*, *15*). We find that integrin $\alpha_{IIb}\beta_3$ can exist in different active states, supporting the notion that both the electrostatic and the hydrophobic interactions between the membrane-proximal CT complex regulate activation. Interestingly, while the C-terminal region of the $\alpha_{IIb}$ CT has a minor effect on influencing integrin activation, the C-terminal region of the $\beta_3$ CT is critically involved in a cooperative and structure-based regulation of integrin function.

## EXPERIMENTAL PROCEDURES

*Proteins, Monoclonal Antibodies, and Plasmids.* $\alpha_{IIb}$ and $\alpha_{IIb}$ ($P^{998}A/P^{999}A$) CT peptides were synthesized in the Biotechnology Core of the Cleveland Clinic Foundation. Talin-H and MBP/His-fused $\beta_3$ CT were expressed and purified as previously described (*6*, *8*). PAC1, a mAb specific for the active conformer of $\alpha_{IIb}\beta_3$ extracellular domain, was from Becton Dickinson Immunocytometry Systems (San Jose, CA). 2G12, a mAb against the extracellular complex of $\alpha_{IIb}\beta_3$, was developed by Dr. Woods (*20*) and labeled with Alexa Fluor 488 using the Alexa Fluor 488 protein labeling kit (Molecular Probes, Inc., Eugene, OR). Goat anti-mouse IgM conjugated with Alexa Fluor 488 was from Molecular Probes. Human $\alpha_{IIb}$ and $\beta_3$ cDNA and $\alpha_{IIb}(R^{995}D)$ cDNA were provided by J. Fox (Cleveland Clinic Foundation) and Takaaki Hato (Ehime University, Japan), respectively.

*Site-Directed Mutagenesis and Transient Transfection.* The human cDNA of $\alpha_{IIb}$ and $\beta_3$ were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). The 3′ nucleotide sequences including the cytoplasmic tails of $\alpha_{IIb}$ and $\beta_3$ were subcloned into pBluescript II SK(+) vector. Substitutions or truncations were introduced into $\alpha_{IIb}$ and $\beta_3$ using QuickChange site-directed mutagenesis kits (Stratagene, La Jolla, CA). The nucleotide sequences of all mutants were confirmed. Different combinations of the $\alpha_{IIb}$ and $\beta_3$ cDNA were cotransfected into CHO-K1 cells using lipofectamine 2000 (Invitrogen). The transfected cells were cultured for at least 24 h prior to further analyses.

*Flow Cytometry.* Transfected cells were detached and suspended at $1 \times 10^7$ cells/mL in Hank's BSS buffer with 1.2 mM $Ca^{2+}$, 0.8 mM $Mg^{2+}$ and 0.1% BSA. Aliquots of each cell suspension were incubated for 30 min at room temperature with 10 $\mu$g/mL PAC1 IgM or 10 $\mu$g/mL Alexa Fluor 488-labeled 2G12 IgG. Then, the cells incubated with PAC1 IgM were resuspended in the same buffer with 10 $\mu$g/mL Alexa Fluor 488 goat anti-mouse IgM conjugate and incubated for 30 min on ice. After washing, the cells were fixed with 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

To evaluate the extent of integrin activation, the ratio of PAC1 binding, which interacts only with activated $\alpha_{IIb}\beta_3$, to 2G12 binding, which measures total surface expression of $\alpha_{IIb}\beta_3$, was determined. Activation (*a*) is defined as

$$a = (F_i - F_v)/(F'_i - F'_v)$$

where $F_i$ and $F_v$ are the mean fluorescence intensities (MFI) of PAC-1 binding to CHO cells transfected with integrin or corresponding empty vectors, respectively; similarly, $F'_i$ and $F'_v$ are the MFI of 2G12 binding to CHO cells transfected with integrin or empty vectors, respectively. We defined the active state of wild-type (WT) $\alpha_{IIb}\beta_3$ as 1, and the active state of a mutant (*I*) is expressed relative to WT as

$$I = a(\text{mutant})/a(\text{WT})$$

Surface expression of WT and all $\alpha_{IIb}\beta_3$ mutants was similar as monitored by the 2G12 signal with the exception of $\alpha_{IIb}(\Delta 991)$, which was only expressed at low levels on the CHO cells; however, the *I* value of this mutant was similar to that of another truncation mutant, $\beta_3(\Delta 717)$, supporting the validity of the quantification system used.

*Surface Plasmon Resonance.* The interaction of the talin-H (F2-F3) with the $\beta_3$ CT and its mutants, $S^{752}A$ and $S^{752}P$, was quantified by SPR. The association and dissociation rate constants ($k_a$ and $k_d$) and the dissociation constant ($K_d$) were measured using the BIAcore 3000 biosensor (BIAcore AB, Uppsala, Sweden). The MBP-$\beta_3$ CT and its mutants were immobilized onto the CM5 sensor chip via an amine coupling kit (BIAcore AB, Uppsala, Sweden) according to the manufacturer's instructions. Experiments were performed at 37 °C in 10 mM Hepes buffer, pH 7.4. Talin-H was injected at the concentration range 0.25−2 $\mu$M, and the flow rate was 20 $\mu$L/min. Experimental data were analyzed using BIAevaluation 3.2 software supplied by BIAcore AB.

*NMR Spectroscopy.* To examine $\alpha_{IIb}/\beta_3$ tail association in transferred NOE experiments, a solution of 1 mM unlabeled $\alpha_{IIb}$ CT or its mutants was prepared in the absence or presence of 0.1 mM MBP-$\beta_3(S^{752}P)$ in 20 mM phosphate buffer and 5 mM $Ca^{2+}$, pH 6.3. To examine how talin-H perturbs the $\alpha_{IIb}/\beta_3(S^{752}P)$ CT interaction, a solution of 1 mM unlabeled $\alpha_{IIb}$ tail was prepared in the presence of 0.1 mM MBP-$\beta_3(S^{752}P)$ and 0.2 mM talin-H (F1-F2-F3) in 20 mM phosphate buffer and 5 mM $Ca^{2+}$, pH 6.3. To characterize the structures of $\beta_3(S^{752}P)$ or $\beta_3(S^{752}A)$ mutants, 1 mM $^{15}N/^{13}C$-labeled mutant was dissolved in 300 mM deuterated DPC in 20 mM phosphate buffer and 5 mM $Ca^{2+}$, pH 6.3. All NMR experiments were performed as previously described (*6*).

## RESULTS

Our previous structural studies demonstrated that multiple hydrophobic and electrostatic contacts within the membrane-proximal helical regions form the $\alpha_{IIb}$ and $\beta_3$ CT complex (Figure 1A). To evaluate the contribution of these contacts in regulating the receptor function, two sets of structure-based point mutants, $\alpha_{IIb}(R^{995}D)$, which abolishes the $\alpha_{IIb}(R^{995})/\beta_3(D^{723})$ salt bridge, and $\alpha_{IIb}(V^{990}AF^{992}A)$, which impairs a hydrophobic interface of the complex, were prepared and analyzed. As shown in Figure 1B, both mutations induced activation of $\alpha_{IIb}\beta_3$, but the extent of the activation was substantially lower than that induced by complete

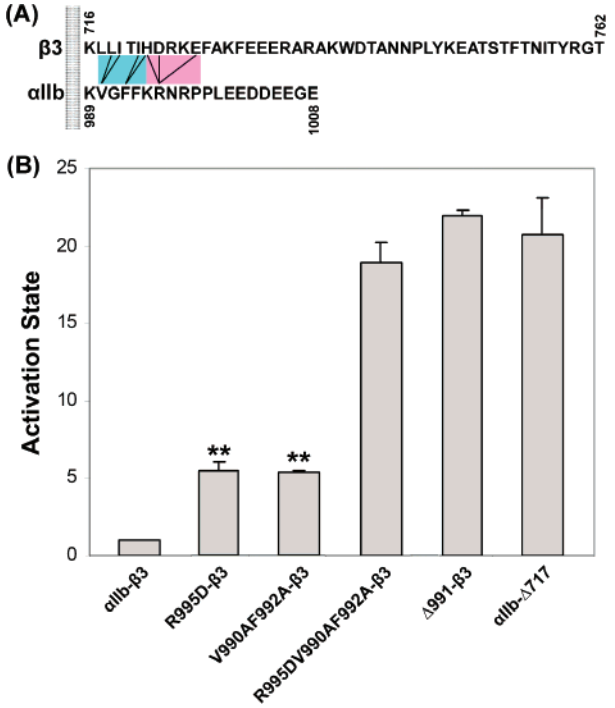


FIGURE 1: The membrane-proximal complex controls integrin activation states. (A) Cytoplasmic tail sequences of the $\alpha_{IIb}$ and $\beta_3$ subunits and membrane-proximal complex formed by electrostatic (purple) and hydrophobic (blue) contacts. (B) The activation state of wild-type $\alpha_{IIb}\beta_3$ and each of the indicated mutants was determined on the basis of the ratio of PAC-1 to 2G12 binding as measured by FACS. This ratio was assigned a value of 1 for WT, and the activation state of each mutant is compared to WT (see Experimental Procedures). Results presented are means ± SD from three independent experiments. **, $P < 0.01$ by Student's *t*-test.

truncation of either CT, $\alpha_{IIb}(\Delta991)$, or $\beta_3(\Delta717)$. PAC-1 binding to $\alpha_{IIb}(R^{995}D)\beta_3$- or $\alpha_{IIb}(V^{990}AF^{992}A)\beta_3$-transfected CHO cells was increased about 5-fold compared to WT $\alpha_{IIb}\beta_3$ while deletion of either CT, $\alpha_{IIb}(\Delta991)\beta_3$, or $\alpha_{IIb}\beta_3(\Delta717)$ induced more than 20-fold PAC-1 binding compared to WT $\alpha_{IIb}\beta_3$ (Figure 1B). In a mutant in which both the hydrophobic and electrostatic bonds that maintained the CT complex were eliminated, $\alpha_{IIb}(R^{995}DV^{990}AF^{992}A)$, activation was higher than that induced by either $\alpha_{IIb}(R^{995}D)$ or $\alpha_{IIb}(V^{990}AF^{992}A)$ individually and was similar to that caused by truncation of either CT, $\alpha_{IIb}(\Delta991)$, or $\beta_3(\Delta717)$ (Figure 1B). These experiments were performed using PAC-1 antibody as an $\alpha_{IIb}\beta_3$ ligand mimetic. Similar results were obtained using Alexa Fluor 488-labeled fibrinogen as a ligand (data not shown). Together, these data indicate that different degrees of perturbation/unclasping on the membrane-proximal complex of $\alpha_{IIb}\beta_3$ CT can lead to apparent differences in the extent of integrin activation. Destruction of the bond maintaining the membrane-proximal complex is as efficient in inducing activation as deletion of the entire CT.

The acidic C-terminal region of the $\alpha_{IIb}$ CT was previously shown to interact with the positively charged N-terminal region but not with residues involved in the formation of the membrane-proximal clasp (*5*, *8*). Nevertheless, Leisner et al. reported that the double mutation of $P^{998}A/P^{999}A$ led to activation of $\alpha_{IIb}\beta_3$ (*14*). Our previous NMR studies further suggested that these mutations destabilized the $\alpha_{IIb}$ turn structure but did not alter the membrane-proximal helix (*5*) and, therefore, should not impair the membrane-proximal CT complex. This prediction was verified by the transferred NOE experiment shown in Figure 2B−D. As compared to the

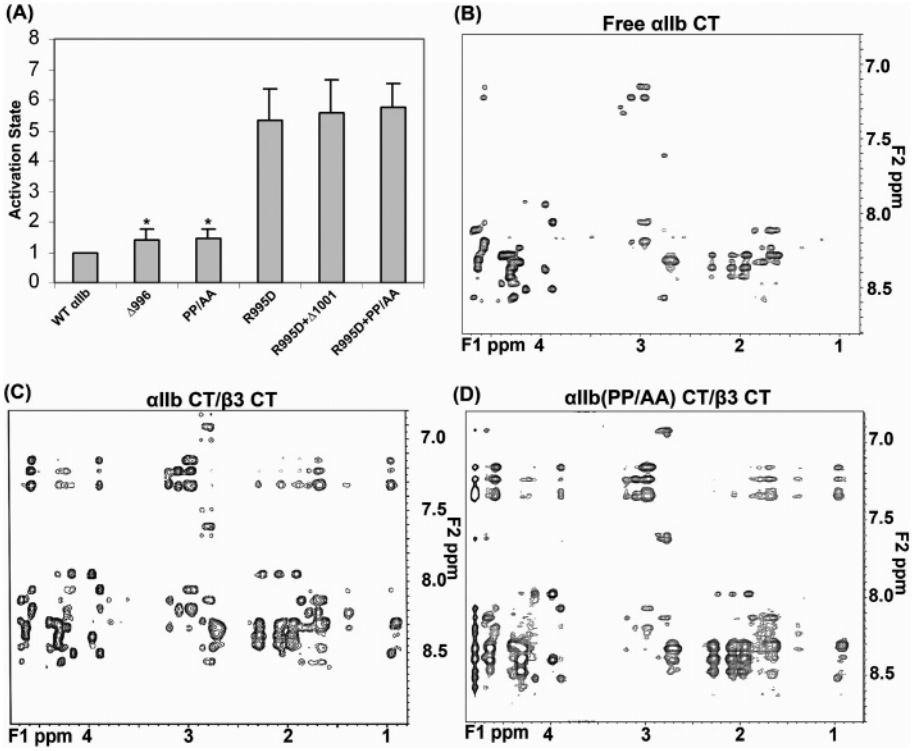


FIGURE 2: The membrane-distal region of $\alpha_{IIb}$ CT fails to disturb the membrane-proximal complex and has only a minor effect on $\alpha_{IIb}\beta_3$ activation. (A) Effect of the C-terminal region of the $\alpha_{IIb}$ CT on integrin activation. CHO cells were transiently transfected with cDNA for WT $\beta_3$ and $\alpha_{IIb}$ bearing the indicated mutations. The activation state of each was evaluated as described in Figure 1. Results presented are means ± SD for three independent experiments. *, $P < 0.05$. (B−D) 2D NOESY spectra of the $\alpha_{IIb}$ tail in the free form (top panel) as compared to the WT $\alpha_{IIb}$ tail (left) and $\alpha_{IIb}$ ($P^{998}A/P^{999}A$) (right) in the presence of MBP-$\beta_3$ ($\alpha_{IIb}$:MBP-$\beta_3$ = 1 mM:0.1 mM). Substantial transferred NOEs are seen in the latter two cases, indicating that $P^{998}A/P^{999}A$ had minimal impact on the membrane-proximal $\alpha_{IIb}/\beta_3$ complex.

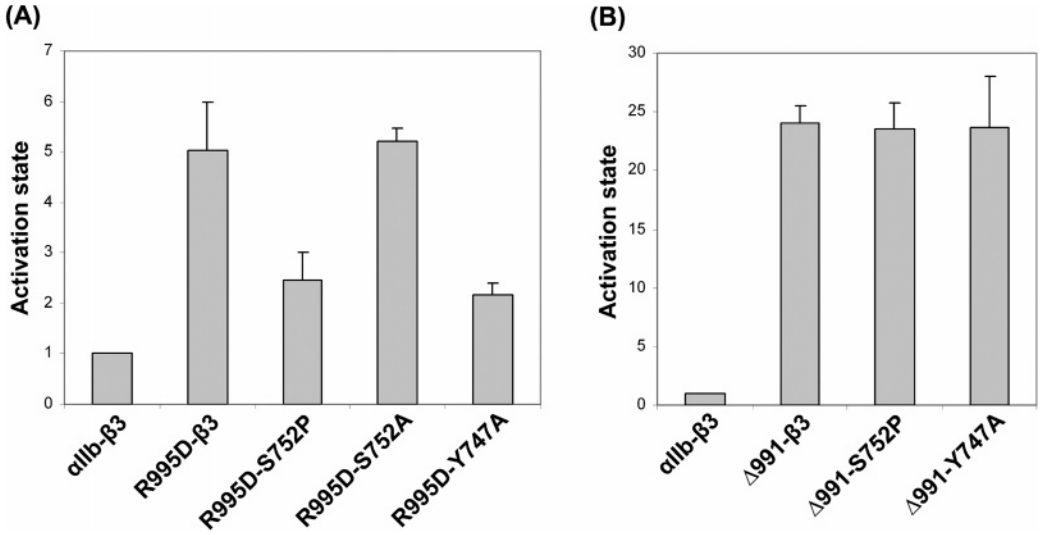


FIGURE 3: Mutations in the C-terminal region of $\beta_3$ CT differentially influence partially activated (A) and highly activated $\alpha_{IIb}\beta_3$ (B). CHO cells were transfected with WT $\alpha_{IIb}\beta_3$ or its mutant forms, and their activation states were evaluated. Results presented are means ± SD from three independent experiments. The difference between $\alpha_{IIb}(R^{995}D)\beta_3(S^{752}P)$ or $\alpha_{IIb}(R^{995}D)\beta_3(Y^{747}A)$ and $\alpha_{IIb}(R^{995}D)\beta_3$ is statistically significant ($P < 0.01$).

NOE spectrum of the free $\alpha_{IIb}$ CT, substantially increased transferred NOE peaks were induced for the $\alpha_{IIb}$ tail upon binding to $\beta_3$ CT. The transferred NOEs induced by addition of the $\beta_3$ CT were similar between wild-type and $P^{998}A/P^{999}A$ $\alpha_{IIb}$ CT, indicating that the mutation had minimal impact on the membrane-proximal clasp. The minimal effect of the $P^{998}A/P^{999}A$ mutation on the membrane clasp suggests that the extent of activation of the mutant should be modest. Indeed, as shown in Figure 2A, substitution of $P^{998}P^{999}$ with AA or elimination of the entire turn by truncation at $N^{996}$ only weakly induced integrin activation. Although the extent of activation was only ~1.5-fold, it was significantly enhanced compared to WT ($P < 0.05$). The extent of activation of the $\alpha_{IIb}$ C-terminal mutation/truncation was substantially less than the partial activation (>5-fold) induced by $\alpha_{IIb}(R^{995}D)$ (see Figure 2A). Deletion of the acidic C-terminal tail from $E^{1001}$ or $P^{998}A/P^{999}A$ substitutions had no additive effect on the $R^{995}D$-activated integrin $\alpha_{IIb}\beta_3$ (Figure 2A). These findings suggest that the membrane-distal region of $\alpha_{IIb}$ CT has only a minor role in controlling $\alpha_{IIb}\beta_3$ activation.

The $\beta_3$ CT can be divided into the membrane-proximal helix, residues $K^{716}-R^{734}$, a mid-segment region, residues $A^{735}-Y^{747}$, which includes a reverse turn structure formed by $N^{744}$PLY and a C-terminal region, residues $K^{748}-T^{762}$, which is composed of the second helix structure ($K^{748}-T^{755}$), and a second turn motif ($N^{756}$ITY). While the midsegment NPLY is a major site for talin-H binding during integrin activation (*18*, *21*, *22*), the mechanism as to how the C-terminal region may govern integrin activation is poorly understood. For example, substitution of $Ser^{752}$ with proline but not alanine significantly impairs integrin activation (*23*), and different deletions at the C-terminal region can block $\alpha_{IIb}\beta_3$ activation by vWF-GPIb-IX engagement (*17*). To dissect the molecular mechanism by which the C-terminal region of $\beta_3$ CT regulates integrin activation, we examined the effect of the $S^{752}P$ mutation on integrin activity and structure. Substitution of $S^{752}P$ markedly suppressed the partial activation of $\alpha_{IIb}\beta_3$ induced by the $\alpha_{IIb}(R^{995}D)$ mutation (Figure 3A). In contrast, this mutation did not affect the highly activated state induced by complete disruption of the membrane-proximal clasp, i.e., truncation of $\alpha_{IIb}$ at 991, $\alpha_{IIb}(\Delta 991)$ (Figure 3B). $S^{752}A$ behaved as WT $\beta_3$ CT and had no apparent inhibitory effect on the $R^{995}D$-induced partial activation of $\alpha_{IIb}\beta_3$ (Figure 3A). As a comparison, $Y^{747}A$ mutation in the midsegment region had a similar effect as $S^{752}P$ on $R^{995}D$- and Δ991-induced integrin activation (Figure 3).

Since $Y^{747}A$ is known to disrupt a talin-H binding site, we assessed whether the effect of the $S^{752}P$ might also be due to reduced talin-H binding. Accordingly, we performed transferred NOE experiments to assess the effects of $S^{752}P$ on dissociation of the membrane-proximal complex by talin-H. As shown in Figure 4A, talin-H was capable of diminishing the transferred NOEs arising from $\alpha_{IIb}/\beta_3(S^{752}P)$ tail association to a similar extent as observed with the WT $\alpha_{IIb}/\beta_3$ complex (*8*). Moreover, we directly measured the affinities of talin-H for WT $\beta_3$ CT, the $S^{752}P$ and the $S^{752}A$ mutants, by surface plasmon resonance and found them to be very similar [$K_d = (2.5 \pm 0.4) \times 10^{-6}$, $(2.2 \pm 0.4) \times 10^{-6}$, and $(2.3 \pm 0.03) \times 10^{-6}$ M of talin-H for WT, $S^{752}P$, and $S^{752}A$, respectively; $n = 3-5$ determinations for each]. Thus, $S^{752}P$ does not directly influence talin-H binding to the $\beta_3$ CT.

Next, we examined the effects of the $S^{752}$ on $\alpha_{IIb}\beta_3$ activation by talin-H. In cotransfection experiments, talin-H induced integrin $\alpha_{IIb}\beta_3$ activation to an extent very similar to that induced by the partial unclasping mutation $\alpha_{IIb}(R^{995}D)$ (compare Figure 4B and Figure 1B), suggesting the possibility that talin-H may partially unclasp the membrane-proximal complex in the context of the intact integrin. Point mutation $\beta_3(Y^{747}A)$, which perturbs talin-H binding to $\beta_3$ CT (*19*, *21*, *22*), dramatically reduced talin-H-induced $\alpha_{IIb}\beta_3$ activation. Point mutation $\beta_3(S^{752}P)$, but not $\beta_3(S^{752}A)$, significantly blunted talin-H-induced integrin activation, although its suppressive function was weaker than the $Y^{747}A$ mutation. Since $S^{752}P$ itself fails to impair talin-H binding to $\beta_3$ CT, the suppressive role of the $S^{752}P$ mutation on talin-H-induced integrin activation must involve an indirect mechanism.

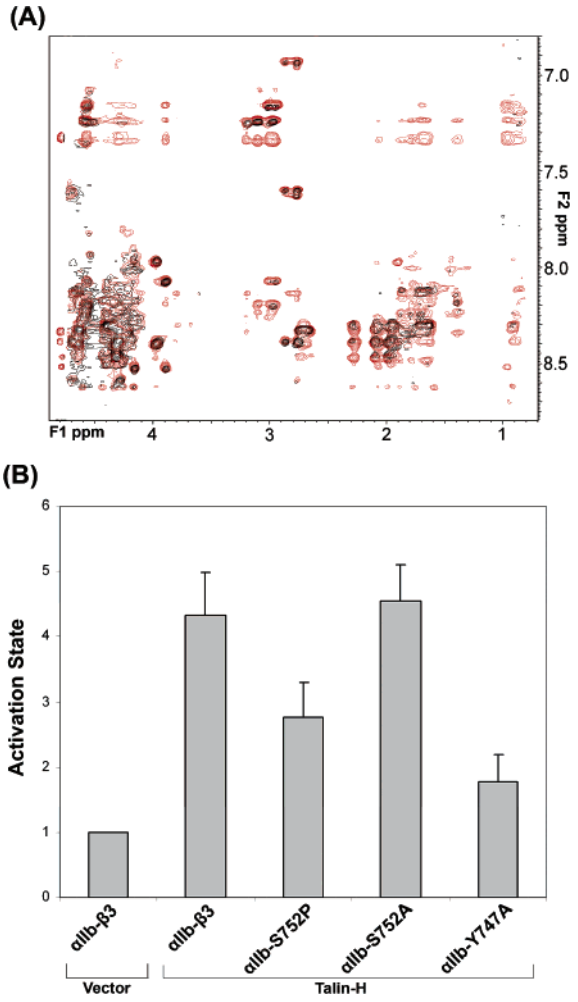


FIGURE 4: $S^{752}P$ impairs talin-H-induced integrin activation but does not affect unclasping by talin-H. (A) 2D NOESY spectrum of the $\alpha_{IIb}$ tail in the presence of MBP-$\beta_3$($S^{752}P$) ($\alpha_{IIb}$:MBP-$\beta_3$($S^{752}P$) = 1 mM:0.1 mM) showing substantial transferred NOEs due to $\alpha_{IIb}/\beta_3$ tail association (red). Addition of talin-H (0.2 mM) diminished the transferred NOEs (black). The same effect was observed for the wild-type $\alpha_{IIb}/\beta_3$ complex (*8*). (B) The influences of $S^{752}P$, $S^{752}A$, and $Y^{747}A$ substitutions on talin-H-induced integrin activation were evaluated using transfected CHO cells. Different combinations of WT or mutant $\alpha_{IIb}$ and the $\beta_3$ subunits were cotransfected with talin-H or empty vector, and the activation state of each was determined. With talin-H, the difference between $\alpha_{IIb}\beta_3$($S^{752}P$) and $\alpha_{IIb}\beta_3$ was statistically significant ($P < 0.01$).

The differential effects of the $S^{752}P$ and $S^{752}A$ mutations on integrin activation led us to consider how they affected the structure of the CT. $S^{752}$ is part of a C-terminal helix when WT $\beta_3$ CT is placed in a membrane environment (*6*). The $S^{752}P$ and $S^{752}A$ mutants of $\beta_3$ CT were dissolved in membrane-mimic DPC micelles and analyzed by NMR. As shown in Figure 5, the C-terminal helix between the NPLY and NITY motifs was abolished by the $S^{752}P$ substitution. In contrast, the helix found in the WT ($S^{752}$) form was fully preserved in the $S^{752}A$ mutant. Taken together, the differential effects of the $S^{752}P$ and $S^{752}A$ mutations suggest a role of this C-terminal helical region in regulating integrin $\alpha_{IIb}\beta_3$ activation.

## DISCUSSION

Our study on the role of CTs in regulating integrin activation has led to several notable findings. First, we found

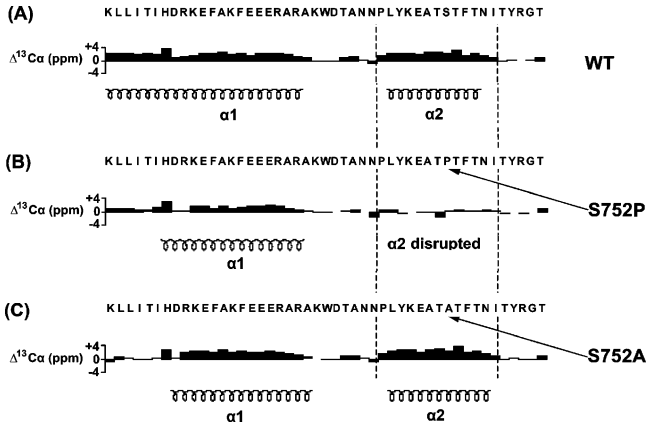


FIGURE 5: Secondary structure of WT $\beta_3$ CT and its mutants $S^{752}P$ and $S^{752}A$ in DPC micelles. Chemical shift differences of backbone $^{13}C\alpha$ and their corresponding random coil values are shown, which provide secondary structure information. Dotted lines highlight regions of structural changes reflected by the $^{13}C\alpha$ chemical shifts; i.e., while WT (A) and $S^{752}A$ (C) adopt the α helix, the $S^{752}P$ (B) mutation disrupts the α helix at the C-terminus of the $\beta_3$ CT.

that differences in the perturbation of the membrane-proximal clasp can affect the extent of the integrin activation; i.e., complete truncation of the $\alpha_{IIb}$ CT led to the high activation of the receptor, whereas point mutations, such as $\alpha_{IIb}$ $R^{995}D$ and $\alpha_{IIb}$ $V^{990}AF^{992}A$, which perturb individual contact interactions within the membrane-proximal clasp, only partially activated the receptor. This observation is consistent with our previously proposed population-shifted equilibrium model for integrin activation (*4*), where a tightly clasped CT complex, established by all of the hydrophobic and electrostatic contacts, maintains the inactive state of $\alpha_{IIb}\beta_3$, and a fully unclasped CT represents the fully active state of the receptor. On the basis of this model, point mutations or limited deletions can shift the equilibrium between the fully clasped and unclasped states, leading to apparent partial activation. More disruptive mutations, such as multiple point mutations at clasp residues, would shift the equilibrium still further and lead to still greater apparent activation. Complete truncation of either CT or disruption of all electrostatic and hydrophobic interactions by mutation leads to the entire population of $\alpha_{IIb}\beta_3$ in the unclasped state and, hence, full activation of the receptors. We recognize that other studies have not documented a partially activated state of the receptor despite evaluation of some of the same mutations (*7*, *24*). This difference may reflect the approaches used to define full activation or differences in the intrinsic stability of the clasp in the cell expression systems used.

A second finding of our study is that $\alpha_{IIb}$ $P^{998}A/P^{999}A$, previously described as an activated mutant by Leisner et al. (*14*), only weakly activates the $\alpha_{IIb}\beta_3$. This conclusion is consistent with previous data in which deletion of the C-terminal membrane-distal region at $N^{996}$ hardly induces integrin activation (*23*). On the basis of this observation, even though $P^{998}/P^{999}$ perturbs the stability of the turn structure in $\alpha_{IIb}$ CT, which in turn disrupts the interactions between the acidic C-terminal region and the positively charged N-terminal residues (*5*), the effect on integrin activation is more likely to enhance the embedding of the membrane-proximal helix of the $\alpha_{IIb}$ CT once it is unclasped rather than unclasping of the CT complex per se (*6*). The former event appears to stabilize the unclasped state and may, therefore,

in itself lead to only weak activation of the receptor, i.e., a subtle shift in the equilibrium between the resting and fully active states.

The third and surprising finding of our study was that the $S^{752}P$ but not $S^{752}A$ mutation in the C-terminal region of $\beta_3$ CT markedly suppressed the integrin activation induced by the $\alpha_{IIb}$ $R^{995}D$ mutation or talin-H but not by the truncation of the entire $\alpha_{IIb}$ CT. Since $S^{752}$ is distant from the membrane-proximal clasp (*8*) and did not affect the complex formation of the isolated CTs (ref *10* and Figure 4A), the $S^{752}P$ mutation must exert its effect indirectly on the $R^{995}D$- or talin-H-induced membrane-proximal clasping/unclasping process. This is strongly supported by the fact that $S^{752}P$ had no inhibitory effect on the $\alpha_{IIb}(\Delta 991)$-induced high activation state of $\alpha_{IIb}\beta_3$ (Figure 3B) in which the membrane-proximal clasp is completely obliterated. All of these data suggest that the clasping/unclasping of the membrane-proximal complex is the final intracellular switch to control integrin activation, whereas the $S^{752}$-containing C-terminal $\beta_3$ tail plays a positive role in fine-tuning the clasping/unclasping equilibrium. Since the $S^{752}P$ mutation but not the WT and $S^{752}A$ disrupted the C-terminal helix (Figure 5), such structural perturbation may be detrimental for stabilizing the unclasped activated state. On the basis of our population-shifted model (*4*), the membrane detachment process may suppress the integrin activation by promoting the equilibrium shift from the unclasped active state (induced by either $R^{995}D$ or talin-H) back to the clasped inactive state. Consistently, the $S^{752}P$ mutation did not suppress the $\alpha_{IIb}(\Delta 991)$-induced high activation state because the complete deletion of the $\alpha_{IIb}$ CT eliminated the possibility to re-form the membrane-proximal clasp. It should be noted that, as compared to the $\alpha_{IIb}(\Delta 991)$-induced or agonist-induced high activation state, talin-H induced only modest activation, which suggests that other coregulators may be involved in inside-out signaling. The C-terminal helix and the second membrane anchor may be required for binding of the $\beta_3$ CT to another protein/domain that coregulates integrin activation. Alternatively, it remains a possibility that $S^{752}P$ mutation and its structural consequence do not affect the unclasping events but may favor the reassociation of the disrupted clasp. In an equilibrium-based model of integrin activation, enhanced reassociation would also be detected as a suppression of $\alpha_{IIb}\beta_3$ activation. It is noteworthy that the $P^{752}$ substitution partially but did not fully suppress talin-H-induced $\alpha_{IIb}\beta_3$ activation (Figures 3A and 4B), whereas in platelets this mutation completely inhibits ligand binding to the integrin (*25*). This difference suggesting that the postulated coregulators or equilibrium between the clasp and unclasp states may be similar but not identical in platelets and CHO cells. Once the clasp is severed, it remains unclear how this separation then alters the transmembrane domains of $\alpha_{IIb}\beta_3$ remains. The coiled-coil structures of the $\alpha_{IIb}$ and $\beta_3$ transmembrane regions appear to be associated in the resting state and may themselves separate or may undergo a twisting or piston motion while remaining associated, thereby propagating the inside-out signal (*26*−*29*).

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