

# Tests of the Extension and Deadbolt Models of Integrin Activation\*

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Despite extensive evidence that integrin conformational changes between bent and extended conformations regulate affinity for ligands, an alternative hypothesis has been proposed in which a “deadbolt” can regulate affinity for ligand in the absence of extension. Here, we tested both the deadbolt and the extension models. According to the deadbolt model, a hairpin loop in the  $\beta 3$  tail domain could act as a deadbolt to restrain the displacement of the  $\beta 3$  I domain  $\beta 6$ - $\alpha 7$  loop and maintain integrin in the low affinity state. We found that mutating or deleting the  $\beta 3$  tail domain loop has no effect on ligand binding by either  $\alpha \text{IIb}\beta 3$  or  $\alpha \text{V}\beta 3$  integrins. In contrast, we found that mutations that lock integrins in the bent conformation with disulfide bonds resist inside-out activation induced by cytoplasmic domain mutation. Furthermore, we demonstrated that extension is required for accessibility to fibronectin but not smaller fragments. The data demonstrate that integrin extension is required for ligand binding during integrin inside-out signaling and that the deadbolt does not regulate integrin activation.

Integrins are cell adhesion molecules that transmit bidirectional signals across the plasma membrane and regulate many biological functions, including cell differentiation, cell migration, and wound healing (1, 2). A bent conformation observed by x-ray crystallography (3) and electron microscopy (4, 5) represents the physiological low affinity state. Integrin activation is regulated by binding of intracellular proteins such as talin to the integrin  $\beta$ -subunit cytoplasmic tail (6), leading to separation between the  $\alpha$ - and  $\beta$ -subunits at their transmembrane and cytoplasmic domains (7–9) and propagation of conformational changes from the transmembrane domains to the ligand binding headpiece, which increases integrin affinity for ligand (10, 11). These long range conformational changes involve integrin extension. Conversely, ligand binding also induces integrin extension, which leads to the separation of the  $\alpha$ - and  $\beta$ -subunit legs and the transmembrane and cytoplasmic regions (11).

Recent co-crystal structures of integrin  $\alpha \text{IIb}\beta 3$  headpiece bound to ligands revealed the molecular basis for the large

conformational changes that accompany ligand binding (12). The conformational changes in the ligand binding site that increase affinity for ligand are allosterically linked by a crankshaft-like  $\alpha 7$ -helix displacement in the  $\beta 3$  I domain to a  $60^\circ$  change in the angle between the  $\beta 3$  I and hybrid domains and to a  $70 \text{ \AA}$  increase in separation between the  $\alpha$ - and  $\beta$ -subunits at their knees. Since the hybrid domain and connecting  $\beta$ -leg domains are central in the interface between the integrin headpiece and legs in the bent conformation, integrin extension enables hybrid domain swing-out. The crystal structures are in excellent agreement with studies using electron microscopy (4, 5, 13, 14), NMR (15), small angle x-ray scattering (16), and mutational studies and mapping of the epitopes of allosteric activating and inhibiting antibodies (17–20), which all support the switchblade model for integrin activation, in which integrin extension and leg separation are coupled with swing-out of the hybrid domain and the displacement of the  $\beta$ -I domain  $\alpha 7$  helix.

Two effects of extension are important for integrin activation. First, extension places the ligand binding site  $150$ – $200 \text{ \AA}$  further above the cell surface and orients it pointing away from, rather than toward, the surface of the cell on which it expressed. This favors accessibility to ligands on other cells and in the extracellular matrix. Second, extension enables hybrid domain swing-out and conversion of the headpiece from the closed, low affinity to the open high affinity state (11).

There is controversy about the requirement of extension for integrin activation. An electron microscopy (EM)<sup>2</sup> study reported that soluble  $\alpha \text{V}\beta 3$  bound to a fibronectin fragment was still in the bent conformation (21). As a supplement or alternative to integrin extension, regulation of integrin affinity by a “deadbolt” has been proposed. This proposal is based on the observation of an interaction at a small  $60 \text{ \AA}^2$  interface between the  $\beta$ -tail domain CD loop (the deadbolt) and the  $\beta$ -I domain  $\beta 6$  strand- $\alpha 7$  helix region in the unliganded  $\alpha \text{V}\beta 3$  crystal structure (22). It was hypothesized that 1) this interaction restricts the displacement of the  $\beta$ -I domain  $\beta 6$ - $\alpha 7$  loop and thus keeps the integrin in the low affinity state and 2) that loss of this interaction would induce inside-out activation without extension. Here, we directly tested the deadbolt model by mutating or deleting the  $\beta$ -tail domain CD loop and show that

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<sup>2</sup> The abbreviations used are: EM, electron microscopy; mAb, monoclonal antibody; I-EGF, integrin-epidermal growth factor-like; LIBS, ligand-induced binding site; Fn, fibronectin; Fg, fibrinogen; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; CHO, Chinese hamster ovary.

these mutations have no effect on ligand binding by  $\alpha$ IIb $\beta$ 3 or  $\alpha$ V $\beta$ 3 integrins. We further demonstrate that integrins locked in the bent conformation are resistant to inside-out activation and that extension is important to promote accessibility of cell surface integrins for soluble ligands.

## EXPERIMENTAL PROCEDURES

**Plasmid Construction, Transient Transfection, and Immunoprecipitation**—Plasmids coding for full-length human  $\alpha$ IIb or  $\alpha$ V and  $\beta$ 3 were subcloned into pEF/V5-HisA and pcDNA3.1/Myc-His (+), respectively (4). Cysteine substitutions (G307C in  $\alpha$ V and R563C in  $\beta$ 3) and the N305T mutant of  $\beta$ 3 were described previously (4, 23). Other mutants were made using site-directed mutagenesis with the QuikChange kit (Stratagene, La Jolla, CA). Constructs were transfected into 293T cells using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instruction. Transfected cells were metabolically labeled with [<sup>35</sup>S]cysteine/methionine (PerkinElmer Life Science) and immunoprecipitated with the conformation-independent  $\beta$ 3 mAb AP3, which recognizes residues in the plexin-semaphorin-integrin (PSI) and hybrid domains (24–26) as described (7).

**Soluble Ligand Binding**—The fragments of human fibronectin (Fn) type III domains 7–10 (Fn7–10) and 9–10 (Fn9–10) were prepared as described previously (27). The purified fragments were labeled by Alexa Fluor 488 using Alexa Fluor 488 protein labeling kit A10235 (Molecular Probes, Eugene, OR). Binding of fluorescein isothiocyanate (FITC)-labeled human fibrinogen (Fg) (Enzyme Research Laboratories, South Bend, IN), FITC-labeled human Fn (Sigma), and Alexa Fluor 488-labeled Fn7–10 and Fn9–10 and PAC-1 IgM, which recognizes activated  $\alpha$ IIb $\beta$ 3 at its ligand binding site (28, 29) (BD Biosciences), was determined as described (23). Briefly, transfected cells suspended in 20 mM HEPES-buffered saline (pH 7.4) supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated with 50  $\mu$ g/ml fluorescently labeled ligands or 10  $\mu$ g/ml PAC-1 in the presence of 5 mM EDTA, 1 mM Ca<sup>2+</sup>/1 mM Mg<sup>2+</sup>, 1 mM Ca<sup>2+</sup>/1 mM Mn<sup>2+</sup>, 1 mM Ca<sup>2+</sup>/1 mM Mn<sup>2+</sup> plus either 10  $\mu$ g/ml activating mAb PT25-2 to the  $\alpha$ IIb  $\beta$ -propeller domain (29, 30) or 10  $\mu$ g/ml activating mAb LIBS1 to the  $\beta$ 3 leg domain (31) at room temperature for 30 min and then stained with Cy3-labeled anti- $\beta$ 3 mAb AP3 on ice for 30 min. For PAC-1 binding, cells were washed and stained with FITC-conjugated anti-mouse IgM and Cy3-labeled AP3 on ice for another 30 min before being subjected to flow cytometry. Binding activity is presented as the mean fluorescence intensity (MFI) of FITC-conjugated anti-mouse IgM or ligands, after subtraction of background MFI in EDTA, expressed as a percentage of the MFI of the Cy3-AP3.

**LIBS Epitope Expression**—Anti-LIBS mAb LIBS1 and D3 to the  $\beta$ 3 leg domain (31) were kindly provided by Drs. M. H. Ginsberg (University of California, San Diego, La Jolla, CA) (32) and L. K. Jennings (University of Tennessee Health Science Center, Memphis, TN) (33). LIBS epitope expression was determined as described previously (23). In brief, transfected cells suspended in HEPES-buffered saline supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated with or without 25  $\mu$ M cyclo-(Arg-Gly-Asp-D-Phe-Val) (cyclo-RGDfv)

(Bachem Bioscience, Inc., King of Prussia, PA) peptide or 1 mg/ml Fn7–10 in the presence of 1 mM Ca<sup>2+</sup>/1 mM Mg<sup>2+</sup> or 0.2 mM Ca<sup>2+</sup>/0.2 mM Mn<sup>2+</sup> at room temperature for 30 min and then with anti-LIBS mAb on ice for 30 min followed by FITC-conjugated anti-mouse IgG and flow cytometry. LIBS antibody binding is presented as the MFI of anti-LIBS antibody staining as a percentage of the MFI of conformation-independent mAb AP3 staining.

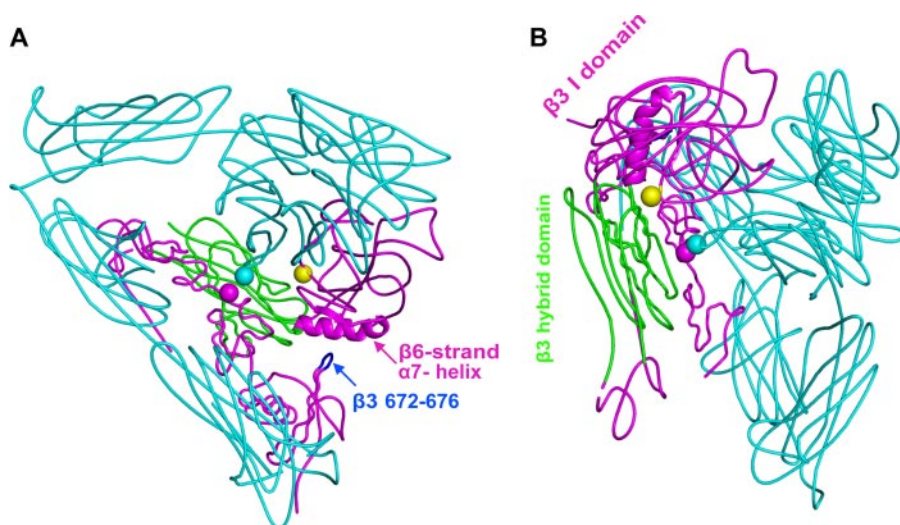
**Establishment of CHO Stable Cells**—The QuikChange mutagenesis kit (Stratagene; La Jolla, CA) was used to introduce a 15-nucleotide deletion into human integrin  $\beta$ 3 cDNA to generate cDNA encoding  $\Delta$ Asp-672–Lys-676. CHO cells were transfected with human integrin  $\alpha$ IIb (in pcDNA3.1 Zeo; Invitrogen) and one of two forms of human integrin  $\beta$ 3 (wild type or  $\Delta$ Asp-672–Lys-676 in pcDNA3.1 Neo; Invitrogen) using a calcium phosphate transfection kit (Invitrogen). 48 h after transfection, the growth medium (F12K; Mediatech, Inc, Herndon, VA) was supplemented with 0.6 mg/ml G418 and 0.35 mg/ml Zeocin (Invitrogen).

**Evaluation of  $\alpha$ IIb $\beta$ 3 Activation State on CHO Cells**—CHO cells that had been stably transfected with cDNAs encoding human  $\alpha$ IIb and either human  $\beta$ 3 or human  $\beta$ 3 $\Delta$ 672–676 were incubated with 5  $\mu$ g/ml anti-integrin  $\beta$ 3 monoclonal antibody, AP3, conjugated to Alexa Fluor 647 (Invitrogen) and either 120  $\mu$ g/ml FITC-fibrinogen or 2.5  $\mu$ g/ml PAC-1 (BD Biosciences) in the presence of 1 mM Ca<sup>2+</sup> or 1 mM Mn<sup>2+</sup>. Purified fibrinogen was kindly provided by Dr. Michael Moseson (Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI) and was labeled with FITC according to previously described methods (34).

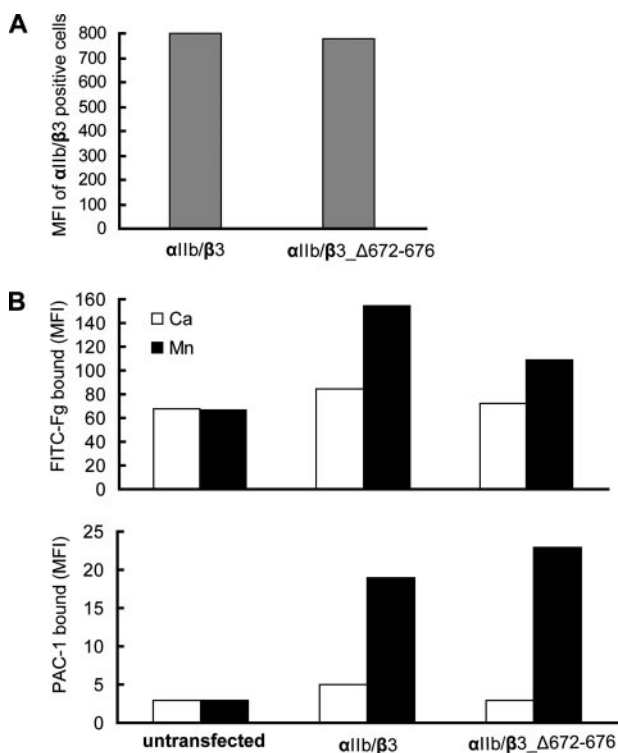
## RESULTS

**Deleting or Mutating Residues of the  $\beta$ -Tail Domain CD Loop Has No Effect on Ligand Binding**—According to the deadbolt model, a hairpin loop (between  $\beta$ -strands C and D) of the  $\beta$ 3 tail domain (the CD loop) contacts the  $\beta$ 3 I domain and restrains integrin activation (Fig. 1). Two groups of investigators independently tested the deadbolt model and have combined their data in this paper. One group produced and assayed ligand binding to CHO cell lines stably expressing either wild-type or mutant  $\alpha$ IIb $\beta$ 3 with five residues (Asp-672–Lys-676) deleted from the  $\beta$ -tail CD loop. The activation state of these integrins was evaluated by measuring the ability of CHO cell transfectants to bind macromolecular ligands. Wild-type and mutant  $\alpha$ IIb $\beta$ 3 were expressed similarly in CHO cells (Fig. 2A). Basal binding of fibrinogen and the ligand-mimetic, activation-dependent antibody PAC-1 (28, 29) in the presence of Ca<sup>2+</sup> was low for both wild-type  $\alpha$ IIb $\beta$ 3 and the  $\alpha$ IIb $\beta$ 3 $\Delta$ 672–676 mutant (Fig. 2B), *i.e.* it was similar to that in mock transfectants. In contrast, Mn<sup>2+</sup> induced similar binding of fibrinogen and PAC-1 mAb to the wild-type and  $\alpha$ IIb $\beta$ 3 $\Delta$ 672–676 CHO cell transfectants (Fig. 2B). These data show that deletion of the deadbolt does not constitutively activate  $\alpha$ IIb $\beta$ 3 integrin.

The other group deleted three residues (Asp-672–Ser-674) of the CD loop or mutated them to Ala individually. These mutations had no effect on the expression of  $\alpha$ IIb $\beta$ 3 or  $\alpha$ V $\beta$ 3 on the cell surface (data not shown). The activation state of these mutant integrins was evaluated by measuring soluble



**FIGURE 1. Locations of the mutations in the  $\alpha$ V $\beta$ 3 crystal structure (3).** The  $\alpha$ V subunit is in cyan. The  $\beta$ 3 hybrid domain is in green, and other  $\beta$ 3 domains are in magenta. The  $\beta$ 3 tail domain CD loop (residues Asp-672–Lys-676) is in blue. Residues mutated to cysteine are shown with spheres at the positions of  $\alpha$ V G307 C $\alpha$  (cyan) and  $\beta$ 3 R563 C $\alpha$  (magenta). The position of the N-glycan wedge introduced by the  $\beta$ 3 NIN305T mutation is shown with a yellow sphere at Asn-303 C $\alpha$ . *A*, this view emphasizes the  $\beta$ -I domain  $\beta$ 6-strand and  $\alpha$ 7-helix, the only elements shown as a ribbon, and their proximity to the  $\beta$ -tail domain CD loop. *B*, this view is rotated relative to *A* and emphasizes the glycan wedge introduced into a crevice between the hybrid and  $\beta$ -I domain that widens in the open headpiece conformation.



**FIGURE 2. Effect of  $\beta$ -tail domain CD loop mutation on ligand binding by  $\alpha$ IIb $\beta$ 3 expressed on CHO cells.** *A*, expression of wild-type and mutant  $\alpha$ IIb/ $\beta$ 3 integrins in CHO cells is shown as MFI of  $\alpha$ IIb/ $\beta$ 3-positive cells stained with AP3-conjugated with Alexa Fluor 647. *B*, mock transfectants or  $\alpha$ IIb/ $\beta$ 3-positive cells gated for equivalent  $\beta$ 3 expression using AP3 mAb were quantitated for binding of FITC-Fg or the fibrinogen mimetic antibody, PAC-1, in the presence of 1 mM  $\text{Ca}^{2+}$  or 1 mM  $\text{Mn}^{2+}$ .

ligand binding to 293T cells transiently transfected with the mutant or wild type receptors (Fig. 3). None of the CD loop mutants showed constitutively high affinity for human fibrino-

gen, fibronectin, or the ligand mimetic antibody PAC-1 under physiological conditions (Fig. 3, A–C). Furthermore, CD loop mutants all were activated by  $\text{Mn}^{2+}$  and activating mAbs to the same level as wild-type integrins. In addition, CD loop mutations had no effect on the binding of fibronectin fragments Fn7–10 or Fn9–10 (Fig. 3D). As a positive control, the cytoplasmic GFFKR to GAAKR mutation ( $\alpha$ V\_GAAKR), which mimics inside out activation (7), constitutively activated binding of the fibronectin fragments (Fig. 3D). These data demonstrate that the  $\beta$ -tail domain CD loop does not restrain activation of either integrin  $\alpha$ IIb $\beta$ 3 or  $\alpha$ V $\beta$ 3.

*Integrins Locked in the Bent Conformation Are Resistant to Inside-out Activation*—Fluorescence resonance energy transfer

and mutagenesis studies revealed that integrin inside-out activation requires unclamping or separation of the cytoplasmic and transmembrane domains (7, 8, 35, 36). However, it has been questioned whether integrin extension is required for inside-out activation (37). Previously, we showed that introducing a disulfide bond between residues 307 of the  $\alpha$ V  $\beta$ -propeller domain and 563 of the  $\beta$ 3 I-EGF domain 4, with the  $\alpha$ V G307C and  $\beta$ 3 R563C mutations, locked the  $\alpha$ V $\beta$ 3 integrin in the low affinity, bent conformation (4). Residues  $\alpha$ V-307 and  $\beta$ 3-563 are in close proximity in the bent  $\alpha$ V $\beta$ 3 conformation (3) (Fig. 1). To test the requirement for integrin extension during inside-out activation, we combined the disulfide bond-forming  $\alpha$ V G307C and  $\beta$ 3 R563C mutations with the  $\alpha$ V\_GFFKR/GAAKR cytoplasmic domain mutation, which induces inside-out activation. The disulfide bond was formed in high efficiency in this  $\alpha$ V\_GAAKR\_G307C/ $\beta$ 3\_R563C mutant (Fig. 4A). Consistent with previous results (7), the GAAKR mutant ( $\alpha$ V\_GAAKR/ $\beta$ 3) bound anti-LIBS antibodies D3 and LIBS1 to the  $\beta$ 3 leg (31) constitutively, indicating that the integrin was extended (Fig. 4B). However, binding of LIBS antibodies by  $\alpha$ V\_GAAKR/ $\beta$ 3 was reversed when the intersubunit disulfide bond was introduced in the  $\alpha$ V\_GAAKR\_G307C/ $\beta$ 3\_R563C mutant (Fig. 4B). The high affinity ( $\text{IC}_{50} = 2.5$  nM) (38) cyclo-RGDfV peptide induced LIBS binding by wild-type  $\alpha$ V $\beta$ 3 and further enhanced LIBS binding by  $\alpha$ V\_GAAKR/ $\beta$ 3 (Fig. 4B). By contrast, the mutants  $\alpha$ V\_GAAKR\_G307C/ $\beta$ 3\_R563C and  $\alpha$ V\_G307C/ $\beta$ 3\_R563C did not bind LIBS antibodies even in the presence of cyclo-RGDfV (Fig. 4B), showing that these mutants were locked in the bent conformation. Ligand binding assays showed that mutant integrin  $\alpha$ V\_GAAKR/ $\beta$ 3 bound fibrinogen and fibronectin constitutively in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  (Fig. 4C). By contrast, the mutants  $\alpha$ V\_GAAKR\_G307C/ $\beta$ 3\_R563C and  $\alpha$ V\_G307C/ $\beta$ 3\_R563C did not bind fibrinogen and fibronectin even in the presence of  $\text{Mn}^{2+}$  and activating mAb (Fig.

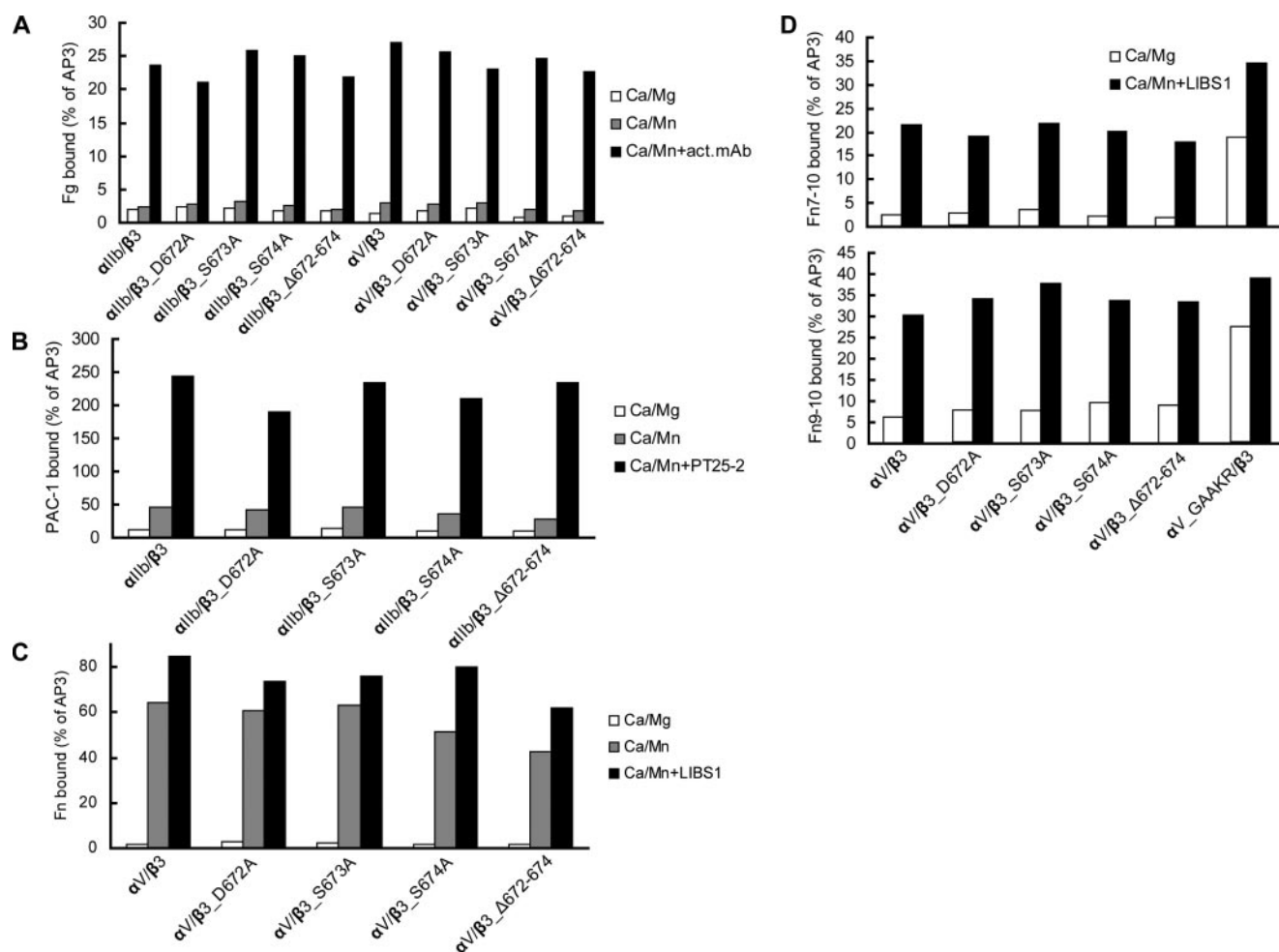


FIGURE 3. Effect of  $\beta$ -tail domain CD loop mutation on ligand binding by  $\alpha IIb\beta 3$  and  $\alpha V\beta 3$  expressed in 293T cells. A, human Fg binding to  $\alpha IIb\beta 3$  and  $\alpha V\beta 3$ . B, ligand-mimetic PAC-1 antibody binding to  $\alpha IIb\beta 3$ . C, human Fn binding to  $\alpha V\beta 3$ . D, binding of fibronectin fragments Fn9–10 and Fn7–10 to  $\alpha V\beta 3$ . 293T cell transfectants were treated with the indicated conditions and incubated with 50  $\mu\text{g}/\text{ml}$  fluorescently labeled ligands or 10  $\mu\text{g}/\text{ml}$  PAC-1 IgM as described under “Experimental Procedures.” Binding is expressed as MFI of ligand staining as a percentage of MFI of Cy3-AP3 antibody staining.

4C). These data demonstrate that integrins locked in the bent conformation are resistant to inside-out activation, *i.e.* integrin extension is required for inside-out activation as measured by both LIBS epitope exposure and soluble ligand binding.

**Ligand Accessibility Is Important for Integrin Ligand Binding**—A negative stain EM study suggested that the soluble integrin  $\alpha V\beta 3$  in the bent conformation could bind a fragment of its ligand, fibronectin (21). These data lead to the suggestion that integrin extension may not be required for ligand binding. We designed experiments to investigate whether bent integrins are accessible to ligands on the cell surface. Introduction of a glycan wedge into the hybrid domain at its interface with the  $\beta$ -I domain by introducing an NIT305 sequon with the  $\beta 3_{N305T}$  mutation has been shown to stabilize integrins in the high affinity state by favoring swing-out of the hybrid domain (Fig. 1) (23). We combined the  $\alpha V_{G307C}/\beta 3_{R563C}$  mutations that form the intersubunit disulfide bond with the glycan wedge mutation. The disulfide bond was formed with high efficiency in this  $\alpha V_{G307C}/\beta 3_{N305T_{R563C}}$  mutant (Fig. 4A). For wild-type  $\alpha V\beta 3$  integrin, LIBS epitope exposure was partially

induced by  $\text{Mn}^{2+}$  treatment and fully induced by cyclo-RGDfV peptide or Fn7–10 in the presence of  $\text{Mn}^{2+}$  (Fig. 5A). The wedge mutant ( $\alpha V/\beta 3_{N305T}$ ) bound LIBS antibodies constitutively, irrespective of the conditions used. By contrast, the  $\alpha V_{G307C}/\beta 3_{N305T_{R563C}}$  and  $\alpha V_{G307C}/\beta 3_{R563C}$  mutants did not bind LIBS antibodies even in the presence of cyclo-RGDfV peptides or Fn7–10 (Fig. 5A), showing that these mutant integrins were locked in the bent conformation. In contrast to wild-type  $\alpha V\beta 3$ , the disulfide-bonded wedge mutant  $\alpha V_{G307C}/\beta 3_{N305T_{R563C}}$  did not bind full-length human fibronectin, even when activated (Fig. 5B).

Distinctive results were obtained with small fibronectin fragments. The disulfide-bonded wedge mutant  $\alpha V_{G307C}/\beta 3_{N305T_{R563C}}$  bound the Fn7–10 and Fn9–10 fragments constitutively (Fig. 5, C and D). However, the amount of binding was lesser than that for the other mutants and wild-type  $\alpha V\beta 3$  (Fig. 5, C and D). Consistent with previous results, the disulfide-bonded mutant  $\alpha V_{G307C}/\beta 3_{R563C}$  did not bind fibronectin, Fn7–10, and Fn9–10, even in activating conditions. As controls, the wedge mutant ( $\alpha V/\beta 3_{N305T}$ ) or the wedge mutation combined with single cysteine muta-

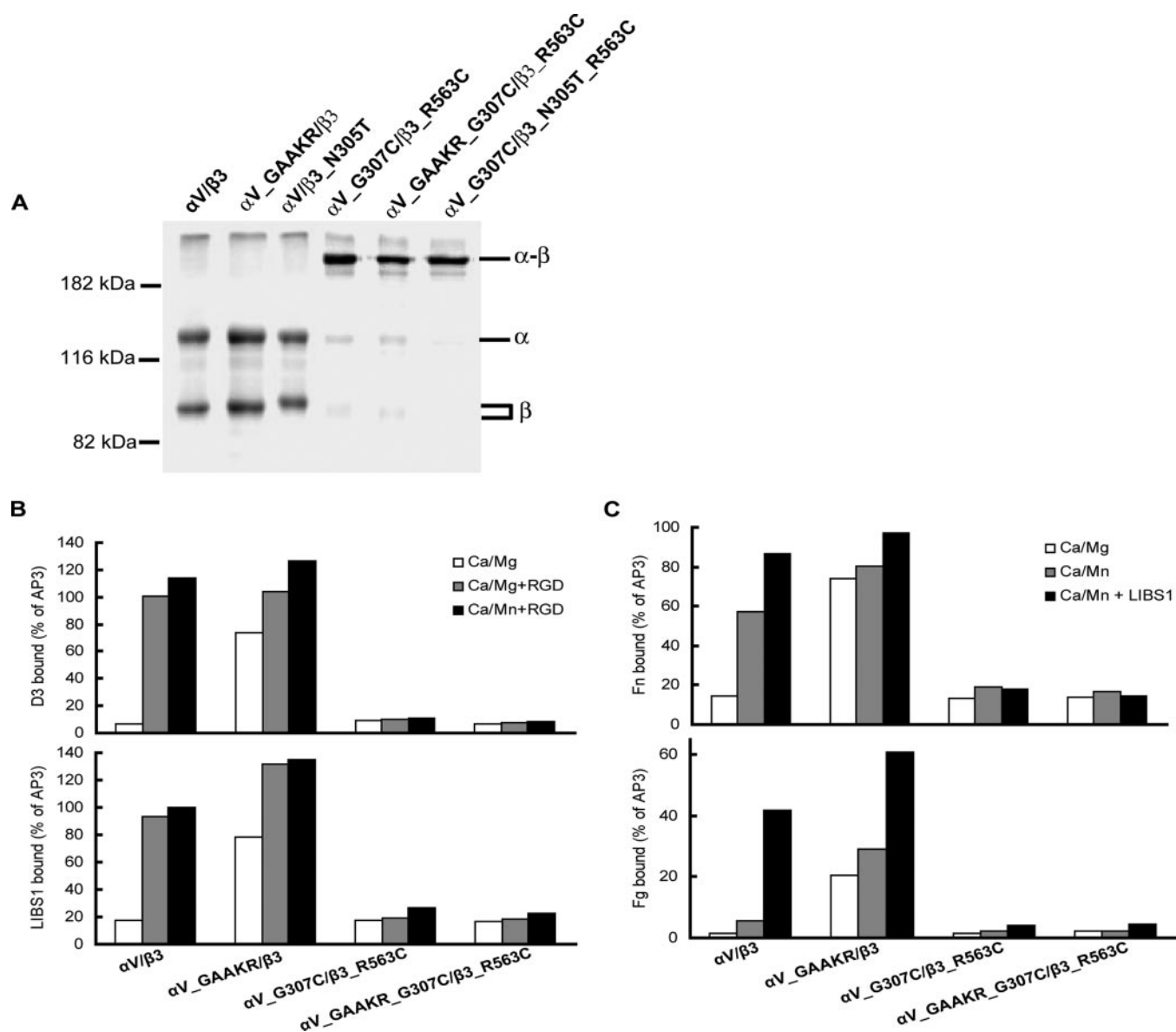


FIGURE 4. Intersubunit disulfide bond formation and effect on LIBS exposure and ligand binding. *A*, disulfide bond formation by cysteine mutants. Lysates from  $^{35}\text{S}$ -labeled 293T cells that had been transiently transfected with wild-type or mutant integrins as indicated were immunoprecipitated with mAb AP3 (anti- $\beta 3$ ) and subjected to non-reducing SDS-7.5% PAGE. Bands of  $\alpha V$  ( $\alpha$ ),  $\beta 3$  ( $\beta$ ), and  $\alpha V\beta 3$  heterodimer ( $\alpha$ - $\beta$ ) are indicated. Positions of protein molecular size markers are shown on the left. *B*, LIBS exposure. 293T cell transfectants were stained with anti-LIBS mAb D3 or LIBS1 in the presence of 1 mM  $\text{Ca}^{2+}$ /1 mM  $\text{Mg}^{2+}$  or 1 mM  $\text{Ca}^{2+}$ /1 mM  $\text{Mn}^{2+}$ , with or without 25  $\mu\text{M}$  cyclo-RGDV (RGD). LIBS epitope expression is expressed as MFI of the D3 or LIBS1 staining as a percentage of MFI of AP3 mAb staining. *C*, soluble Fn or Fg binding to 293T cell transfectants in the presence of 1 mM  $\text{Ca}^{2+}$ /1 mM  $\text{Mg}^{2+}$  or 1 mM  $\text{Ca}^{2+}$ /1 mM  $\text{Mn}^{2+}$  or 1 mM  $\text{Ca}^{2+}$ /1 mM  $\text{Mn}^{2+}$  plus 10  $\mu\text{g}/\text{ml}$  activating mAb, LIBS1. Binding is expressed as MFI of ligand staining as a percentage of MFI of staining with Cy3-AP3 antibody.

tions ( $\alpha V\_G307C/\beta 3\_N305T$  and  $\alpha V/\beta 3\_N305T\_R563C$ ) bound fibronectin and the Fn7–10 or Fn9–10 fragments constitutively (Fig. 5, *B–D*). These data demonstrate that even when activated, integrins in the bent conformation are not accessible to large soluble ligands, although they are accessible to small soluble ligand fragments.

**Fn Fragments Induce Extension of Cell Surface  $\alpha V\beta 3$** —As mentioned above, a negative stain EM study reported that soluble  $\alpha V\beta 3$  remained in the bent conformation when exposed to 1 mg/ml Fn fragment in 0.2 mM  $\text{Ca}^{2+}$ /0.2 mM  $\text{Mn}^{2+}$  (21). Notably, under the same conditions, we found that Fn 7–10 efficiently induced extension of cell surface  $\alpha V\beta 3$ , as reported both by the LIBS1 and D3 antibodies (Fig. 5*A*).

## DISCUSSION

In the  $\alpha V\beta 3$  structure (3), residue Ser-674 at the tip of the CD loop of the  $\beta$ -tail domain contacts the  $\beta$ -I domain. The buried surface area is quite small at 60  $\text{\AA}^2$ . According to the deadbolt model, the residues at the tip of the CD loop, by contacting the  $\beta 6$ -strand near the  $\beta 6$ - $\alpha 7$  loop of the  $\beta$ -I domain, restrain the integrin in the resting, low affinity state (22, 37). Activating mutations in the  $\beta 6$ - $\alpha 7$  loop of the  $\beta 2$  I domain (39) have been interpreted as supporting the deadbolt model (37). However, rearrangement of this region is directly coupled to rearrangement of the ligand binding site and thus to the affinity change (12). Introduction of disulfide bonds (40) or other mutations

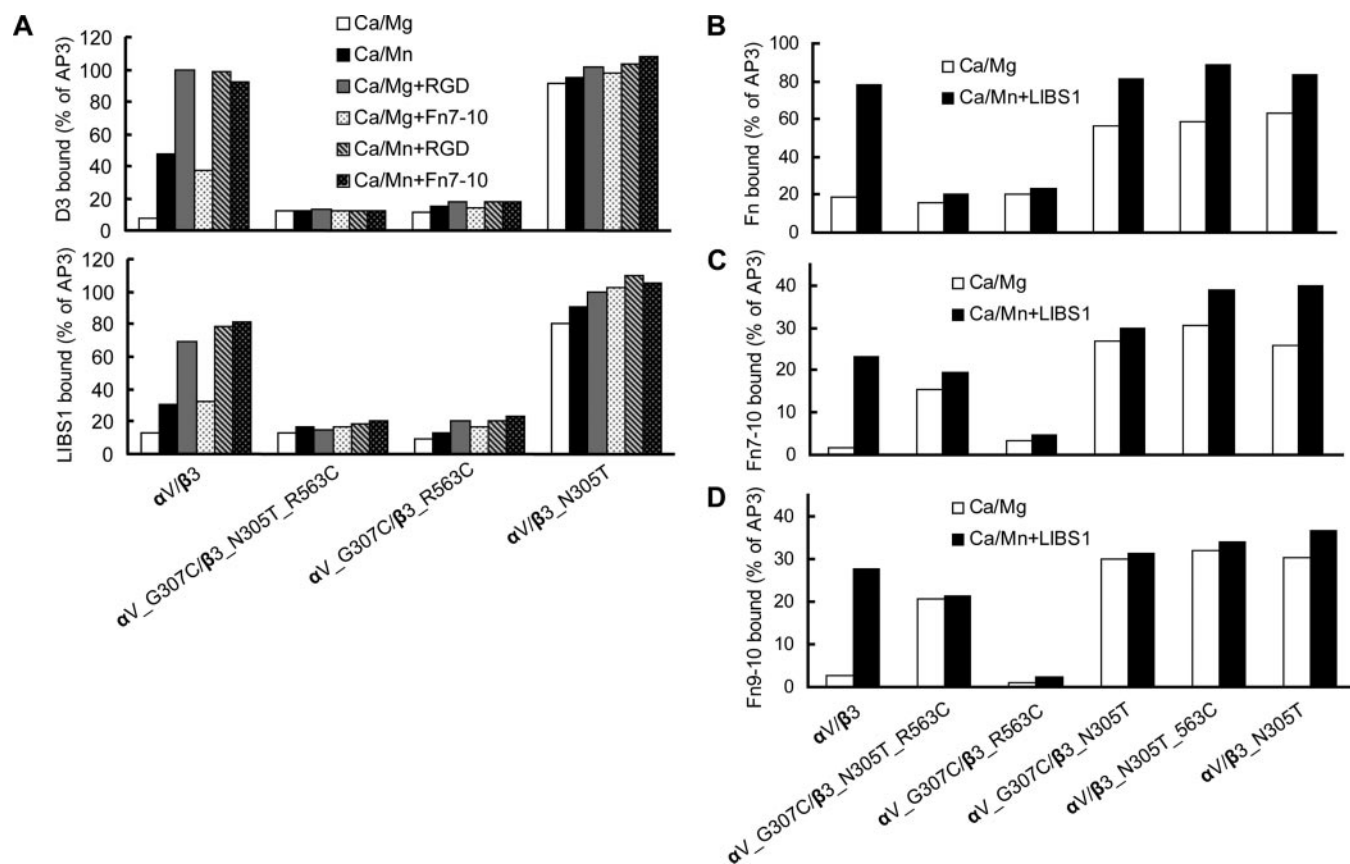


FIGURE 5. **LIBS exposure and ligand binding of disulfide-bonded and glycan wedge mutants.** *A*, LIBS exposure. 293T cell transfectants were stained with anti-LIBS antibodies, D3 or LIBS1, in the presence of 1 mM  $Ca^{2+}$ /1 mM  $Mg^{2+}$  or 0.2 mM  $Ca^{2+}$ /0.2 mM  $Mn^{2+}$ , with or without 25  $\mu M$  cyclo-RGDfV or 1 mg/ml Fn7-10. LIBS epitope expression is expressed as MFI of the D3 or LIBS1 staining as a percentage of MFI of AP3 mAb staining. *B*, Fn binding. *C*, fibronectin fragment Fn7-10 binding. *D*, fibronectin fragment Fn9-10 binding. In *B–D*, 293T cell transfectants were stained with 50  $\mu g/ml$  fluorescently labeled ligands in the presence of 1 mM  $Ca^{2+}$ /1 mM  $Mg^{2+}$  or 1 mM  $Ca^{2+}$ /1 mM  $Mn^{2+}$  plus 10  $\mu g/ml$  activating mAb, LIBS1, and subjected to flow cytometry. Binding is expressed as MFI of ligand staining as a percentage of MFI of Cy3-AP3 antibody staining.

(41, 42) into the  $\beta 6$ - $\alpha 7$  loop and  $\alpha 7$ -helix induced high affinity by causing the rearrangement of the ligand binding site of the  $\beta$ -I domain, and there is no evidence that these mutations disrupt interaction with the  $\beta$ -tail domain CD loop. Therefore, mutation of the  $\beta$ -tail domain CD loop is required to test the deadbolt model. This model was independently tested using different mutations and cell lines in two different laboratories, with similar results. We found that mutations to Ala of three residues in this loop, or deletions of residues 672–674 or 672–676, did not activate ligand binding by integrins  $\alpha V\beta 3$  or  $\alpha IIb\beta 3$  and had no effect on the ability of these integrins to be activated by  $Mn^{2+}$  or antibodies. If local rearrangement of the  $\beta 3$  tail domain were sufficient for integrin affinity regulation, as proposed in the deadbolt model, we should at least detect the binding of small fibronectin fragments to CD loop mutants. However, we found that the CD loop mutations had no effect on the binding of the Fn7-10 and Fn9-10 fragments. Therefore, we conclude that the  $\beta$ -tail domain CD loop does not regulate ligand binding and does not act as a deadbolt. Recently, it was reported that exchanging D658GMD in the  $\beta$ -tail domain CD loop of the  $\beta 2$  subunit with D672SSG of the  $\beta 3$  subunit or with N658GTD, which introduces a glycan wedge sequence, activated the  $\alpha M\beta 2$  integrin (43). A crystal structure of  $\alpha M\beta 2$  is not available. Whether the  $\beta$ -tail domain CD loop of  $\alpha M\beta 2$  contacts the  $\beta$ -I domain is unknown, and the overall orienta-

tion of the  $\beta$ -tail domain relative to the  $\beta$ -I domain in  $\beta 2$  integrins is unknown, making the structural consequences of mutations in the CD loop difficult to predict. However, it is known that multiple substitutions in the  $\beta 2$  subunit, including those in I-EGF domain 3, are activating, consistent with disruption of interactions in the bent conformation (15, 44). Integrin activation is observed after many types of mutations. We believe that our finding of a lack of effect of deletion or mutation of the  $\beta$ -tail domain CD loop, in the context of the structurally characterized  $\alpha V\beta 3$  integrin, which served as the basis for the deadbolt proposal, is the more telling test of this model.

Our data further demonstrate that transition from the bent to extended conformation (*i.e.* integrin extension) is not only required for integrin affinity regulation but also required for accessibility to biological ligands. Integrin  $\alpha V\beta 3$  with the  $\alpha V\_GFFKR/GAAKR$  cytoplasmic mutation, which induces inside-out activation, bound ligands with high affinity in non-activating conditions and was in the extended conformation, as shown by constitutive LIBS epitope exposure. However, this constitutive LIBS exposure and ligand binding activity was reversed when a disulfide bond was introduced between the  $\alpha V$   $\beta$ -propeller domain and the  $\beta 3$  I-EGF 4 domain, which locked the integrin in the bent conformation. It has been demonstrated that activating mutations in the transmembrane and cytoplasmic regions of  $\alpha IIb\beta 3$  induce constitutive LIBS epitope

exposure (7, 35), showing that integrin extension can occur in the absence of ligand binding. These results are consistent with a wide body of evidence that suggests that integrin affinity regulation is concomitant with extension during inside-out activation (11). Integrin  $\alpha V\beta 3$  with a disulfide bond between the  $\alpha V\beta$ -propeller domain and the  $\beta 3$  I-EGF 4 domain and activated with a hybrid domain glycan wedge appeared to remain in the bent conformation as intended because the LIBS1 and D3 epitopes were not exposed. Although the glycan wedge mutant activated binding to soluble fibronectin, this binding was completely reversed by the introduced disulfide bond. However, the double mutant with the glycan wedge and the disulfide to restrain the bent conformation bound Fn7–10 and Fn9–10 fragments, although binding was lower than in the absence of the disulfide. These results demonstrate that the introduced disulfide did not prevent activation and that integrin extension is required for access to the large macromolecular ligands to which integrins bind physiologically.

In addition, we demonstrate that  $Mn^{2+}$  and binding of Fn7–10 induce LIBS epitope exposure in integrin  $\alpha V\beta 3$ , suggesting that these agents induced  $\alpha V\beta 3$  integrin extension on the cell surface. Notably, we used the same  $Mn^{2+}$  concentration (0.2 mM) and fibronectin fragment concentration (1 mg/ml) as used in a recent EM study that suggested that after binding of these agents,  $\alpha V\beta 3$  remained in a bent conformation (21). However, aggregation was present in the EM preparations, which may have resulted in the absence of sampling of extended integrins (11, 21). Our results on cell surface  $\alpha V\beta 3$  are consistent with other EM studies on soluble  $\alpha V\beta 3$  bound to cyclo-RGDfV peptide (4), soluble  $\alpha IIb\beta 3$  bound to RGD peptide (13), and  $\alpha 5\beta 1$  headpiece fragments bound to Fn7–10 (14), which showed that integrins were extended and/or had the open headpiece conformation with the hybrid domain swung out after ligand binding. In conclusion, our results fail to show any role for a deadbolt in the integrin in which this model was proposed,  $\alpha V\beta 3$ , or in integrin  $\alpha IIb\beta 3$ , suggest that integrins extend on the cell surface under conditions in which extension was not found in one EM study, and show that integrin extension is important for both affinity regulation and ligand accessibility.

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