

# Integrin $\alpha_{\text{IIb}}\beta_3$ Inside-out Activation

## AN IN SITU CONFORMATIONAL ANALYSIS REVEALS A NEW MECHANISM\*

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**Background:** The transmembrane domain of integrins plays a critical role in mediating receptor inside-out activation.

**Results:** Inside-out activation triggers the repartitioning of the intracellular border of  $\alpha_{\text{IIb}}$  but not the  $\beta_3$  transmembrane domain into the lipid bilayer in living cells.

**Conclusion:** Complex conformational changes occur in the transmembrane domain upon integrin  $\alpha_{\text{IIb}}\beta_3$  inside-out activation.

**Significance:** Our findings represent a new mechanism for integrin inside-out activation.

Integrins are a family of heterodimeric adhesion receptors that transmit signals bi-directionally across the plasma membranes. The transmembrane domain (TM) of integrin plays a critical role in mediating transition of the receptor from the default inactive to the active state on the cell surfaces. In this study, we successfully applied the substituted cysteine scanning accessibility method to determine the intracellular border of the integrin  $\alpha_{\text{IIb}}\beta_3$  TM in the inactive and active states in living cells. We examined the aqueous accessibility of 75 substituted cysteines comprising the C terminus of both  $\alpha_{\text{IIb}}$  and  $\beta_3$  TMs, the intracellular membrane-proximal regions, and the whole cytoplasmic tails, to the labeling of a membrane-permeable, cysteine-specific chemical biotin maleimide (BM). The active state of integrin  $\alpha_{\text{IIb}}\beta_3$  heterodimer was generated by co-expression of activating partners with the cysteine-substituted constructs. Our data revealed that, in the inactive state, the intracellular lipid/aqueous border of  $\alpha_{\text{IIb}}$  TM was at Lys<sup>994</sup> and  $\beta_3$  TM was at Phe<sup>727</sup> respectively; in the active state, the border of  $\alpha_{\text{IIb}}$  TM shifted to Pro<sup>998</sup>, whereas the border of  $\beta_3$  TM remained unchanged, suggesting that complex conformational changes occurred in the TMs upon  $\alpha_{\text{IIb}}\beta_3$  inside-out activation. On the basis of the results, we propose a new inside-out activation mechanism for integrin  $\alpha_{\text{IIb}}\beta_3$  and by inference, all of the integrins in their native cellular environment.

Integrins are a large family of adhesion receptors on the cell surfaces that mediate cell adhesion, migration, and extracellular matrix assembly (1, 2). 18 integrin  $\alpha$  subunits and 8  $\beta$  subunits form 24 heterodimers, of which each subunit consists of a large extracellular domain, a single transmembrane domain (TM),<sup>3</sup> and a short unstructured cytoplasmic tail. Integrins exist in a default low affinity state on the cell surfaces. Upon stimulation by intracellular signals, integrins convert to the active state that permits extracellular ligands binding (inside-

out activation), which in turn promotes interactions of intracellular proteins with the cytoplasmic tails (outside-in signaling) (3, 4). In this way, integrins transmit signals bi-directionally across plasma membranes.

Biochemical analyses (5, 6), electron microscopy (7, 8), and fluorescent energy transfer studies (9) have established that a constraint is present in the intracellular membrane-proximal (MP) region that restrains integrin  $\alpha_{\text{IIb}}\beta_3$  in the inactive state. Intracellular signals interact with the  $\beta_3$  cytoplasmic tail that breaks the constraint to initiate a conformational change that traverses the TMs, which ultimately triggers activation of the extracellular domain. Two intracellular proteins, talin and kindlin, are reported to interact with the  $\beta_3$  cytoplasmic tail and work synergistically to activate  $\alpha_{\text{IIb}}\beta_3$  (10–12). The extracellular domain of integrin  $\alpha_{\text{IIb}}\beta_3$  was crystallized (13), and a low resolution electron cryomicroscopy structure (8) of the full heterodimer was also reported; however, attempts to obtain a crystal structure of the full-length receptor were not successful.

Two stretches of highly conserved amino acids in the intracellular MP region of integrin  $\alpha_{\text{IIb}}\beta_3$  play pivotal roles in controlling the receptor inside-out activation (<sup>991</sup>GFFKR<sup>995</sup> in  $\alpha_{\text{IIb}}$ ; <sup>717</sup>LLITIH<sup>723</sup> in  $\beta_3$ ) (5, 6, 14) (Fig. 1A). A salt bridge between  $\alpha_{\text{IIb}}$  Arg<sup>995</sup> and  $\beta_3$  Asp<sup>723</sup> was proposed to form a clasp constraining the receptor in the inactive state (15). Intense efforts including site mutagenesis scanning (15, 16), transmission electron microscopy (7), nuclear magnetic resonance (NMR) (17–20), chimeric swapping (21), and computational modeling (16, 22) have been invested to determine how the intracellular clasp is formed, which gave conflicting results. Recent studies using NMR on the structure of associated  $\alpha_{\text{IIb}}\beta_3$  TM peptides in the lipid bicelles (20),  $\alpha_{\text{IIb}}\beta_3$  TM and cytoplasmic tail complex in CD<sub>3</sub>CN/H<sub>2</sub>O (1:1) mixture (17), and by using disulfide constraints coupled with structural modeling (16) have yielded three different views on the intracellular clasp of  $\alpha_{\text{IIb}}\beta_3$ : the first proposed that the clasp is formed by the interaction between residues  $\alpha_{\text{IIb}}$  Phe<sup>992</sup>/Phe<sup>993</sup> and  $\beta_3$  Tyr<sup>715</sup>, the second proposed that the clasp is formed by an inhibitory ligand binding in the MP region, and the third proposed that the clasp is formed between  $\beta_3$  Lys<sup>716</sup> side chain and the peptide backbone of  $\alpha_{\text{IIb}}$  <sup>991</sup>GFFKR<sup>995</sup> motif.

All of the aforementioned studies have greatly improved our knowledge on the conformation of the TMs and the intracellu-

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<sup>3</sup> The abbreviations used are: TM, transmembrane domain; BM, biotin maleimide (3-(N-maleimidylpropionyl) biocytin); MP, membrane-proximal; SCSAM, substituted cysteine scanning accessibility method.

## Conformational Analysis of Integrin $\alpha_{IIb}\beta_3$ in Living Cells

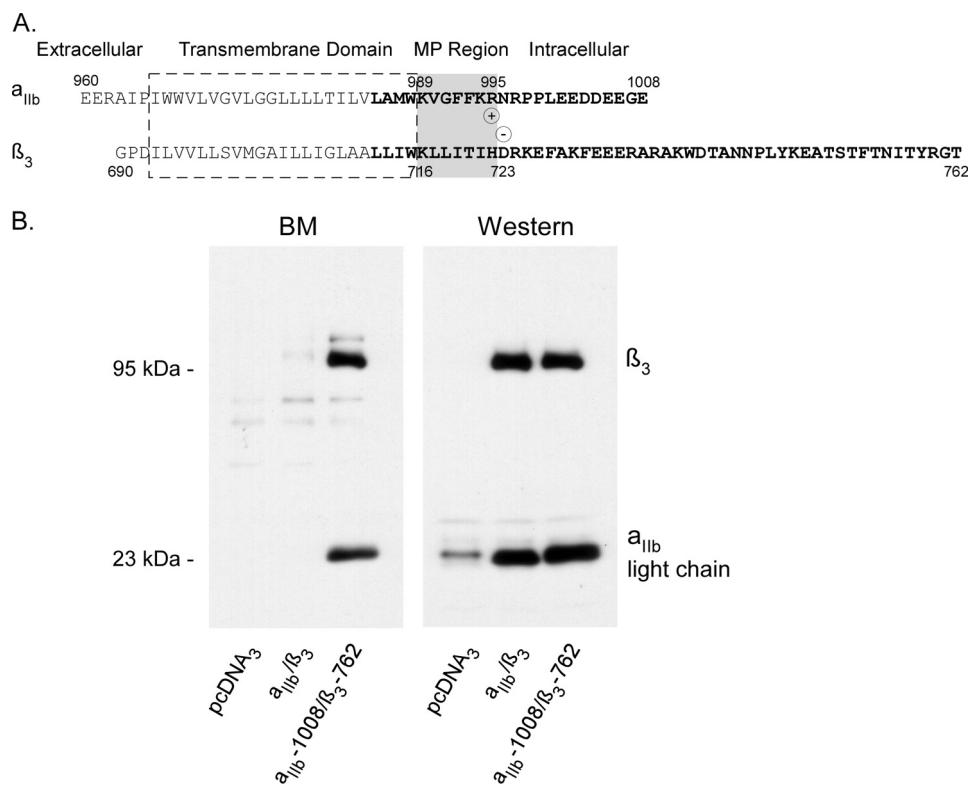


FIGURE 1. A, amino acid sequences of the TMs and the cytoplasmic tails of integrin  $\alpha_{IIb}\beta_3$ . The proposed TMs are depicted in a box with broken lines, MP regions are colored in gray, and cysteine-substituted residues are highlighted in bold. The potential ionic interaction between  $\alpha_{IIb}$  Arg<sup>995</sup> and  $\beta_3$  Asp<sup>723</sup> is indicated as circled positive and negative symbols. B, BM labeling of wild-type and cysteine-substituted integrin  $\alpha_{IIb}\beta_3$ . HEK 293 cells transfected with cloning vector pcDNA3, wild-type  $\alpha_{IIb}\beta_3$ , and mutant  $\alpha_{IIb}$  E1008C/ $\beta_3$  T762C were collected and labeled with BM at room temperature for 20 min. Cells were lysed, and integrin  $\alpha_{IIb}\beta_3$  heterodimer was immunoprecipitated, resolved on 10% SDS-PAGE, and transferred to a PVDF membrane. Incorporated biotin was detected by HRP-streptavidin and ECL. The blot was stripped and probed with an anti- $\alpha_{IIb}$  light chain and an anti- $\beta_3$  antibody simultaneously to detect the amount of protein in each sample.

lar clasp in the MP region of integrin  $\alpha_{IIb}\beta_3$ ; however, questions remain. It is known that the regulated association of  $\alpha_{IIb}\beta_3$  TMs is driven by domains outside of the plasma membranes, whether the NMR structures of fragmented  $\alpha_{IIb}\beta_3$  TM peptides correspond to any physiological states of the receptor is obscure. Questions such as how the TMs of integrin  $\alpha_{IIb}\beta_3$  undergo spatial and conformational changes upon activation and how intracellular proteins interact with the cytoplasmic tails of  $\alpha_{IIb}\beta_3$  to initiate inside-out activation can only be genuinely answered by the analyses of intact integrin protein in the cellular environment.

Here, we present a successful analysis of the cellular location of integrin  $\alpha_{IIb}\beta_3$  TM intracellular borders in the receptor inactive and active states in the living cells by using the substituted cysteine scanning accessibility method (SCSAM). Our data revealed a new mechanism for integrin  $\alpha_{IIb}\beta_3$  inside-out activation in cell membranes.

### EXPERIMENTAL PROCEDURES

**Materials**—Site-directed mutagenesis kits were from Stratagene. Biotin maleimide, DMEM, and all cell culture reagents were from Invitrogen. Anti-integrin  $\alpha_{IIb}$  or  $\beta_3$  rabbit or mouse antibodies were from Santa Cruz Biotechnology. Protein A- and G-Sepharose, streptavidin/biotinylated-horseradish peroxidase complex (streptavidin-HRP), and goat anti-rabbit IgG-conjugated horseradish peroxidase were from GE Healthcare. Igepal was from Sigma. PVDF membrane was from Millipore.

**Site-directed Mutagenesis**—A wild-type human  $\alpha_{IIb}$  and a  $\beta_3$  cDNA were used as the templates for site-directed mutagenesis. Amino acids at the position of Leu<sup>985</sup> to Glu<sup>1008</sup> in  $\alpha_{IIb}$  and Leu<sup>712</sup> to Thr<sup>762</sup> in  $\beta_3$  were individually replaced with cysteines. Mutagenesis was performed using the Stratagene site-directed mutagenesis kit following the manufacturer's instructions. The complete cDNA sequence of each mutant was verified by DNA sequencing.

**Protein Expression**—Cysteine-substituted  $\alpha_{IIb}\beta_3$  was transiently expressed in the human embryonic kidney 293 cells (HEK 293) by using Lipofectamine 2000 (from Invitrogen) transfection following the manufacturer's instructions. Chinese hamster ovary (CHO) cells were also used in some experiments. Cells were grown at 37 °C in a 5% CO<sub>2</sub> atmosphere and harvested 48 h after transfection.

**Flow Cytometry**—Flow cytometry assays for assessing the effects of cysteine substitutions on  $\alpha_{IIb}\beta_3$  activation were performed as described previously (23). In brief, cysteine-substituted  $\alpha_{IIb}$  or  $\beta_3$  constructs were co-expressed with the wild-type partners in the CHO cells. 24 h after transfection, cells were stained with antibody D57, which measures receptor surface expression, and PAC1, which detects the active state of  $\alpha_{IIb}\beta_3$ , in the presence and absence of Ro43-5054 or anti-LIBS6, and then subjected to FACS scan. Ro43-5054 is a competitive antagonist of  $\alpha_{IIb}\beta_3$ , which was used to estimate nonspecific PAC1 binding ( $F_0$ ), and anti-LIBS6 is an  $\alpha_{IIb}\beta_3$ -activating anti-

body that was used to estimate maximal PAC1 binding ( $F_{max}$ ). The activation index was calculated as  $100 \times (F - F_0)/(F_{max} - F_0)$ , where  $F$  = mean fluorescence intensity of PAC1 staining under the test condition. For detailed methods, see Ref. 23.

**Biotin Maleimide Labeling and Immunoprecipitation**—Whole cell labeling with BM was performed as described previously (24). Briefly, transfected HEK 293 cells were collected and resuspended in PBSCM (PBS containing 0.1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ , pH 7.0) solution and subsequently labeled with BM (0.2 mM final) at room temperature for 20 min. Reactions were stopped by adding 5-fold glutathione in molar ratio. Cells were then washed with PBSCM and lysed in IPB buffer (150 mM NaCl, 1% (v/v) Igepal, 0.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) containing 0.2% (w/v) BSA and protease inhibitors (from Roche Applied Science) on ice for 10 min.  $\alpha_{IIB}\beta_3$  proteins were immunoprecipitated by a mouse anti-human  $\alpha_{IIB}\beta_3$  monoclonal antibody (sc-21783 from Santa Cruz Biotechnology) and protein G beads for 4 h at 4 °C.

**SDS-PAGE and Immunoblotting**—Protein samples were resolved on 7.5% (for  $\beta_3$  subunit) and 10% (for  $\alpha_{IIB}$  light chain) SDS-polyacrylamide gels, respectively, and transferred to PVDF membranes. Biotinylated proteins were detected by incubation of blots with 1:10,000 diluted streptavidin-biotinylated horseradish peroxidase (GE Healthcare) in TBSTB buffer (TBST buffer (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 0.5% (w/v) BSA). Protein expression levels were determined by probing the blot with rabbit anti- $\alpha_{IIB}$  or  $\beta_3$  polyclonal antibodies at 1:3,000 dilutions in TBSTM buffer (TBST buffer containing 5% (w/v) nonfat milk).

**Membrane Isolation and  $\text{Na}_2\text{CO}_3$  Treatment**—Membrane treatment with  $\text{Na}_2\text{CO}_3$  was performed as described previously (25). Briefly, transfected cells (10-cm plate) were collected, washed (TBS buffer: 140 mM NaCl, 10 mM Tris, pH 7.4), and incubated with the homogenization buffer (10 mM Tris, pH 7.4, with Roche protease inhibitors) for 30 min on ice. Cells were then lysed by Dounce homogenization. Cell debris were removed by low speed centrifugation ( $4,000 \times g$ , 5 min, 4 °C), and the membrane fractions were collected by high speed centrifugation ( $35,000 \times g$ , 30 min, 4 °C). Membrane pellets were first resuspended in 100  $\mu\text{l}$  of 0.3 M sucrose and then mixed with 2 ml of ice-cold 0.1 M or 3 M  $\text{Na}_2\text{CO}_3$ , pH 11.5, and incubated for 30 min on a rotating shaker at 4 °C. Membranes were then collected by centrifugation, washed with PBSCM, and resuspended in 1.0 ml of PBSCM followed by BM labeling. In some experiments, the protein levels of the stripped and unstripped membranes were determined by the BCA method (from Thermo Scientific). Equal amounts of protein samples were resolved on SDS-PAGE and stained with Coomassie Blue.

**Image and Data Analysis**—Films from immunoblots and biotinylation blots were scanned with a Hewlett-Packard Scanjet 5590. Scanned images were quantified with UN-SCAN-IT gel<sup>TM</sup> version 6.1 software. Biotinylation levels were calculated according to Ref. 24.

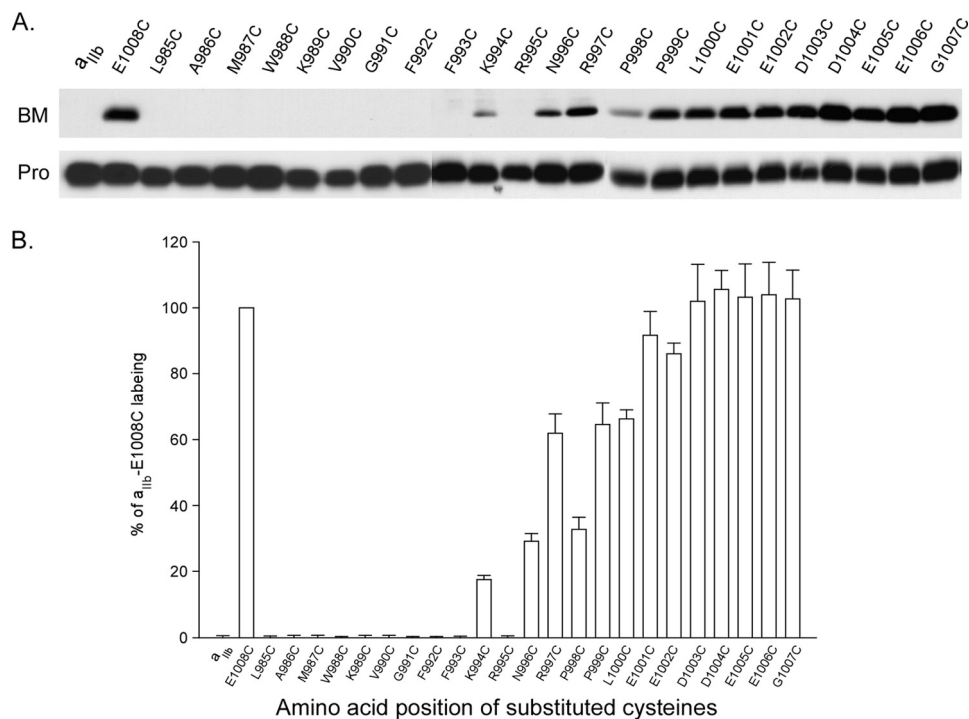
**Statistical Analysis**—Means  $\pm$  S.E. were calculated with SigmaPlot 10 software. Statistical analysis was performed using SigmaPlot 10 software.

## RESULTS

**Substituted Cysteine Scanning Accessibility Method for Integrin  $\alpha_{IIB}\beta_3$  Conformational Analysis**—SCSAM uses sulfhydryl reactive chemical probes to determine the location of the introduced cysteines in a target membrane protein that is free of endogenous reactive cysteines (26, 27). BM, a membrane-permeable sulfhydryl-specific reagent, has been used successfully for the topological analysis of transmembrane proteins (24, 28). SCSAM is based on the observation that the chemical reaction only occurs in the aqueous environment. Cysteines residing in the aqueous medium can react with BM, whereas cysteines residing in the lipid bilayer, the protein interior, or forming disulfide bonds cannot. BM minimally labels the endoplasmic reticulum-retained membrane proteins (24), which is probably due to the high content of glutathione in the cytosol. Integrin  $\alpha_{IIB}\beta_3$  is a cysteine-rich membrane receptor that contains 20 endogenous cysteines in  $\alpha_{IIB}$  and 56 in the  $\beta_3$  subunit. The recent crystal structure of the extracellular domain of  $\alpha_{IIB}\beta_3$  showed that all of the cysteines in  $\alpha_{IIB}\beta_3$  form disulfide bonds (13); however, conflicting reports also suggested that the receptor may have endogenous thiol isomerase activity that exposes free cysteines (29). To determine whether free reactive cysteines are present in the wild-type  $\alpha_{IIB}\beta_3$ , we tested wild-type integrin  $\alpha_{IIB}\beta_3$  for labeling with BM. Wild-type  $\alpha_{IIB}\beta_3$  and mutant constructs  $\alpha_{IIB}$ -E1008C/ $\beta_3$ -T762C were expressed in the HEK 293 cells and subjected to the whole cell labeling. Fig. 1B shows that the wild-type  $\alpha_{IIB}$  or  $\beta_3$  has no detectable BM labeling, whereas the light chain of  $\alpha_{IIB}$  with a single cysteine substitution (E1008C) at the C terminus or  $\beta_3$  with a cysteine substitution at Thr<sup>762</sup> (residue at the C terminus of  $\beta_3$ ) was strongly labeled, suggesting that no free endogenous cysteines are present in wild-type  $\alpha_{IIB}$  or  $\beta_3$  that are available for BM labeling. Western blots verified that  $\alpha_{IIB}$  (sc-6602, from Santa Cruz Biotechnology) and  $\beta_3$  (sc-6627, from Santa Cruz Biotechnology) are both well expressed. Based on the results, we individually substituted single amino acids (between Leu<sup>985</sup> and Glu<sup>1008</sup>) with cysteines in the C-terminal region of  $\alpha_{IIB}$  that covers the proposed C terminus of the TM, the intracellular MP region, and the whole intracellular tail, and the positions between Leu<sup>712</sup> and Phe<sup>730</sup> in  $\beta_3$  covering the proposed MP region of  $\beta_3$  TM (Fig. 1A).

**Whole Cell Labeling of Cysteine-substituted Integrin  $\alpha_{IIB}\beta_3$  with BM**—Integrin  $\alpha_{IIB}$  carrying substituted cysteines was co-expressed with the wild-type  $\beta_3$  in the HEK 293 cells using Lipofectamine 2000 transfection. 48 h after transfection, cells were collected and subjected to BM labeling. Fig. 2 shows that the positive control,  $\alpha_{IIB}$  E1008C was strongly labeled, whereas the negative control, wild-type  $\alpha_{IIB}$ , had no labeling. Amino acids between Leu<sup>985</sup> and Phe<sup>993</sup> were not labeled, suggesting that they are embedded in the lipid bilayer; amino acids between Asn<sup>996</sup> and Glu<sup>1008</sup> were increasingly labeled, indicating the C-terminal tail is free of any cytosolic protein interactions. Interestingly, K994C was weakly labeled, whereas the adjacent residue, R995C was unlabeled compared with the controls. These data suggest that in the intact  $\alpha_{IIB}\beta_3$  complex in living cells, the intracellular border of  $\alpha_{IIB}$  TM is at Lys<sup>994</sup>, which leaves the proposed salt bridge  $\alpha_{IIB}$  Arg<sup>995</sup>/ $\beta_3$  Asp<sup>723</sup>

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**FIGURE 2. BM labeling of cysteine-substituted  $\alpha_{IIb}$  co-expressed with wild-type  $\beta_3$ .** *A*, representative results of BM labeling on integrin  $\alpha_{IIb}$ -substituted cysteines. 24 amino acids in the C terminus of  $\alpha_{IIb}$  were individually substituted with cysteines and labeled with BM as described under "Experimental Procedures." *B*, summary of BM labeling of integrin  $\alpha_{IIb}$ -substituted cysteines. The level of biotin incorporation into each sample was quantified by densitometry, and the signal was normalized to the amount of integrin  $\alpha_{IIb}$  light chain present in the sample. In each experiment, the level of biotinylation was compared with that of the  $\alpha_{IIb}$  E1008C, whose labeling was set to 100%. Data represent mean of 3–5 experiments  $\pm$  S.E. (error bars).

outside of the plasma membrane at the lipid/aqueous interface in the cytosol.

We then performed whole cell labeling on the cysteine-substituted integrin  $\beta_3$  constructs co-expressed with wild-type  $\alpha_{IIb}$  in HEK 293 cells. Fig. 3, *A* and *C*, shows that none of the substituted cysteines in the region between Leu<sup>712</sup> and Glu<sup>726</sup> was labeled with BM, and the first BM labeled residue was at Phe<sup>727</sup> marking the intracellular border of  $\beta_3$  TM. The intensity of BM labeling on the cysteine substitutions from F727C to F730C was similar suggesting the peptide after Phe<sup>727</sup> enters the aqueous medium abruptly.

**Functional Analysis of Cysteine-substituted Integrin  $\alpha_{IIb}\beta_3$** —Functional effects of cysteine substitutions in the TM and the MP regions of integrin  $\alpha_{IIb}\beta_3$  have recently been reported (16), in that cysteine substitution of any of the residues in the <sup>991</sup>GFFKRN<sup>996</sup> region of  $\alpha_{IIb}$  activated the receptor to various degrees, whereas substitution in the remaining portion of the  $\alpha_{IIb}$  intracellular tail had no functional effect. Surprisingly, in the MP region of  $\beta_3$ , only one cysteine substitution at amino acid Lys<sup>716</sup> substantially activated integrin  $\alpha_{IIb}\beta_3$ . Because most of the functional studies of integrin  $\alpha_{IIb}\beta_3$  were performed in the CHO cells, we expressed the cysteine-substituted constructs in the CHO cells and subjected them to FACS analysis with PAC1 antibody that recognizes the active state of integrin  $\alpha_{IIb}\beta_3$ . Fig. 4 shows that, indeed, our findings were in agreement with the previous report.

**Intracellular Border of Integrin  $\alpha_{IIb}$  TM in Fully Active State**—Functional results indicated that the intracellular border of integrin  $\alpha_{IIb}$  TM represented mixed states of active/inactive receptors. Substitution of  $\beta_3$  Lys<sup>716</sup> with Cys or Pro was

shown to activate integrin  $\alpha_{IIb}\beta_3$  close to the full activation level (16) in the HEK 293 cells. To obtain the intracellular border of  $\alpha_{IIb}$  TM in the active state, we co-expressed  $\alpha_{IIb}$  cysteine-substituted constructs with  $\beta_3$  K716P and subjected them to whole cell labeling with BM. Fig. 5 shows that activation of integrin  $\alpha_{IIb}\beta_3$  had no effect on the labeling in the region of Pro<sup>998</sup> to Glu<sup>1008</sup>; however, the region of Lys<sup>994</sup>-Arg<sup>997</sup> was no longer labeled with BM, and the first labeled residues started with Pro<sup>998</sup>, which differs significantly from the previous results obtained in the mixed active/inactive states of the receptor. We further tested BM labeling on the  $\alpha_{IIb}$  cysteine-substituted constructs co-expressed with a truncated form of  $\beta_3$  at Lys<sup>716</sup>, which also produces a highly active state of  $\alpha_{IIb}\beta_3$  (14). The same results were observed (data not shown). To test whether breaking of the predicted outer membrane clasp (highly activates integrin  $\alpha_{IIb}\beta_3$ ) affects conformation of the region, we whole cell labeled the  $\alpha_{IIb}$  cysteine-substituted constructs co-expressed with  $\beta_3$  G708I (20). Again, we observed a similar pattern of labeling (data not shown), indicating that this conformational change in  $\alpha_{IIb}$  is irrespective to the breaking of either the inner or the outer membrane clasp.

It was previously reported that the GFFKR region of  $\alpha_{IIb}$  may associate with intracellular peripheral proteins such as calcium- and integrin-binding protein (30) which potentially shield the substituted cysteines from being labeled. To test this, we isolated the plasma membranes from cells expressing the  $\alpha_{IIb}$  cysteine-substituted constructs (Phe<sup>993</sup>-Phe<sup>998</sup>) with  $\beta_3$  K716P and subjected to chemical stripping with 0.1 M Na<sub>2</sub>CO<sub>3</sub> prior to BM labeling. Compared with the unstripped plasma membranes, Na<sub>2</sub>CO<sub>3</sub> treatment effectively removed multiple

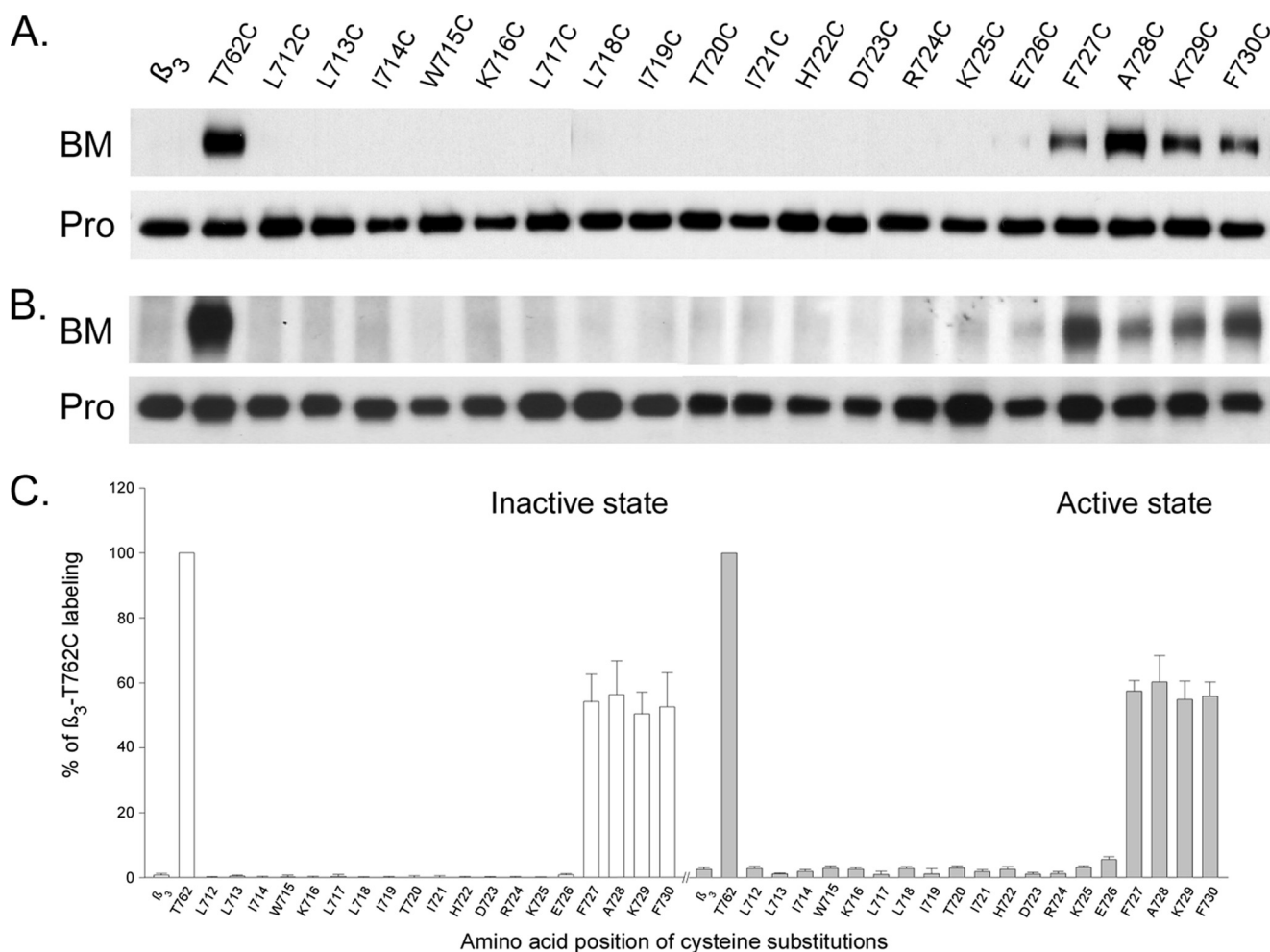


FIGURE 3. **BM labeling on integrin  $\beta_3$ -substituted cysteines.** Amino acids between Leu<sup>712</sup> and Phe<sup>730</sup> in the  $\beta_3$  subunit were individually substituted with cysteines and labeled with BM. *A*, BM labeling of cysteine-substituted  $\beta_3$  co-expressed with wild-type  $\alpha_{IIb}$ . *B*, BM labeling of cysteine-substituted  $\beta_3$  co-expressed with mutant  $\alpha_{IIb}$  F992A/F993A. Similar results were obtained with co-expression of  $\alpha_{IIb}$  Gly<sup>991</sup> truncation. *C*, summary of BM labeling of cysteine-substituted  $\beta_3$ . Biotin incorporation into each sample was quantified as described in Fig. 2 and was compared with  $\beta_3$ -T762C, whose labeling was set to 100%. Data represent mean of 3–6 experiments  $\pm$  S.E. (error bars).

protein bands from the membrane samples as shown on the Coomassie Blue-stained SDS-PAGE (Fig. 6A). Fig. 6, *B* and *C*, shows no differences in BM labeling were detected compared with the fully active state of  $\alpha_{IIb}\beta_3$ . We then tested BM labeling on the membranes stripped with 3 M Na<sub>2</sub>CO<sub>3</sub>, a stringent condition inducing both counter-ion and ionic strength that could effectively expose a deeply buried residue in the sodium bicarbonate co-transporter 1 (25). Again, we did not observe any labeling on the substituted cysteines in Phe<sup>993</sup>-Arg<sup>997</sup> (data not shown). Interestingly, the Na<sub>2</sub>CO<sub>3</sub>-treated  $\alpha_{IIb}\beta_3$  protein was no longer immunoprecipitated by the antibody recognizing the protein complex, but instead, by an antibody that recognizes  $\alpha_{IIb}$ , suggesting that a conformational change in the extracellular domain of the receptor must have been induced by the Na<sub>2</sub>CO<sub>3</sub> treatment. Our data indicated that activation of  $\alpha_{IIb}\beta_3$  triggers repartition of the  $\alpha_{IIb}$  MP region from aqueous into the lipid bilayer.

**Intracellular Border of Integrin  $\beta_3$ -TM in Fully Active State—**The flow cytometry assay showed that none of the cysteine substitutions except K716C in the  $\beta_3$  TM/MP region activated  $\alpha_{IIb}\beta_3$ . Therefore, the first labeled cysteine-substituted residue,

F727C, marks the intracellular border of  $\beta_3$  TM in  $\alpha_{IIb}\beta_3$  inactive state in living cells. To determine the intracellular border of  $\beta_3$  TM in activated  $\alpha_{IIb}\beta_3$ , we co-expressed  $\beta_3$  cysteine-substituted constructs with  $\alpha_{IIb}$  F992A/F993A, a construct that fully activates the receptor (16). Fig. 3, *B* and *C*, shows that activation of  $\alpha_{IIb}\beta_3$  did not expose any  $\beta_3$  endogenous cysteines to aqueous. Interestingly, F727C again remained the first residue labeled with BM, indicating its aqueous accessibility. We were somewhat surprised that no differences were observed in the  $\beta_3$  TM BM labeling between the active and the inactive state of  $\alpha_{IIb}\beta_3$ , and therefore we performed additional BM labeling assays on the co-expressed  $\beta_3$  cysteine-substituted constructs with a truncated form of  $\alpha_{IIb}$  at Gly<sup>991</sup>, which generates the constitutively active state of  $\alpha_{IIb}\beta_3$  (6). Indeed, the same results were obtained (data not shown). These results suggested that  $\beta_3$  TM/MP regions do not repartition between lipid bilayer and aqueous medium upon the receptor activation as seen in  $\alpha_{IIb}$  subunit.

**Aqueous Accessibility of the Predicted Membrane-anchored Integrin  $\beta_3$  Intracellular Tail—**A recent NMR analysis on the complex of fragmented  $\alpha_{IIb}$  and  $\beta_3$  intracellular tails has sug-

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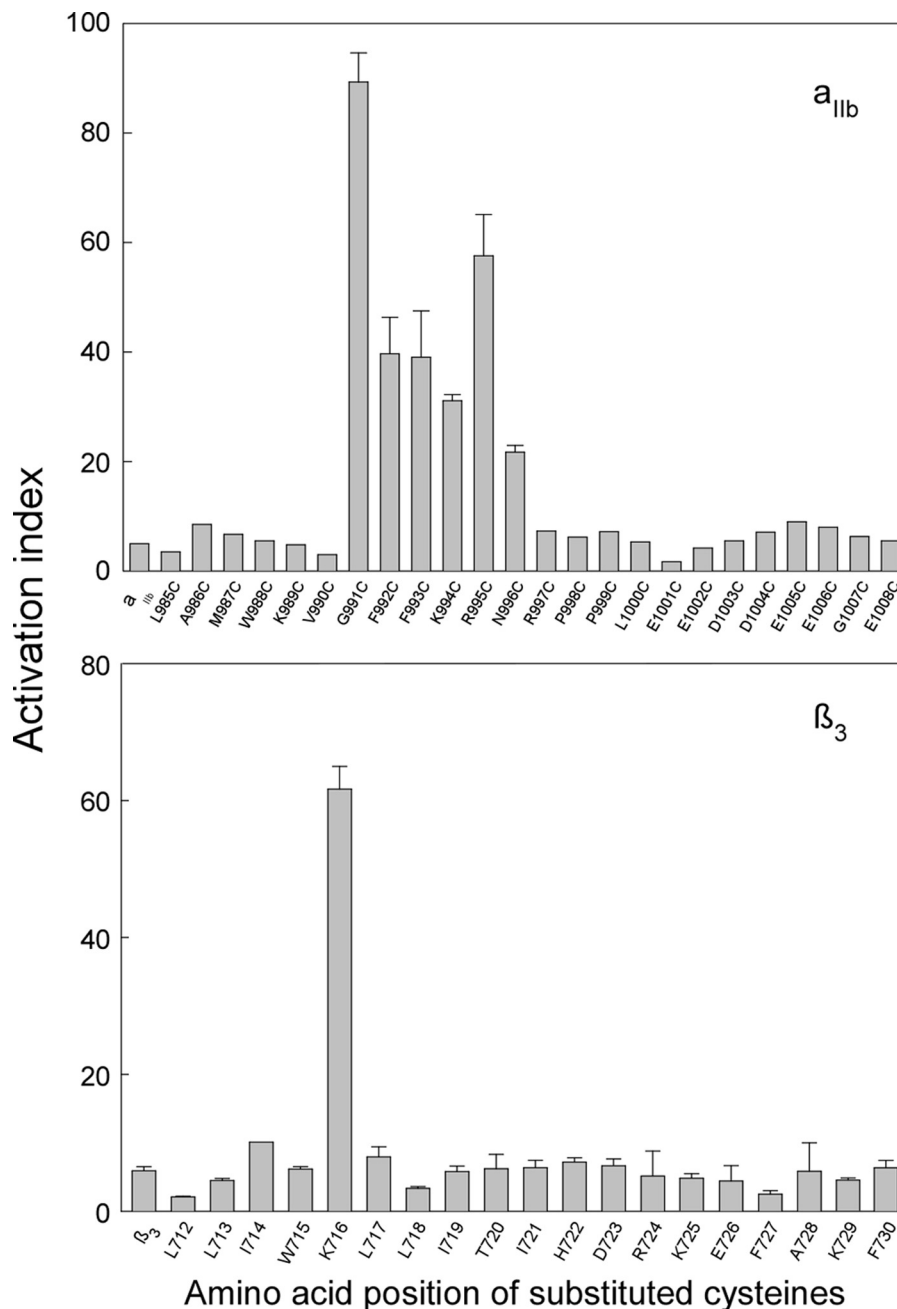


FIGURE 4. **Effect of cysteine substitutions in  $\alpha_{IIb}$  or  $\beta_3$  subunit on integrin  $\alpha_{IIb}\beta_3$  activation.** Upper, activation index of cysteine-substituted  $\alpha_{IIb}$  co-expressed with wild-type  $\beta_3$ . Lower, activation index of cysteine-substituted  $\beta_3$  co-expressed with wild-type  $\alpha_{IIb}$ . Cysteine-substituted  $\alpha_{IIb}$  or  $\beta_3$  were co-expressed with the respective wild-type partners in the CHO cells. 24 h after transfection, cells were stained with PAC1 antibody to measure activation and with D57 antibody to measure surface expression. Data represent mean of 1–3 experiments  $\pm$  S.E. (error bars). Details are described under “Experimental Procedures.”

gested that regions of Phe<sup>727</sup>-Trp<sup>739</sup> and Tyr<sup>747</sup>-Tyr<sup>759</sup> in the  $\beta_3$  tail form membrane-anchored  $\alpha$ -helices in loose contact with the lipid bilayer (31). To determine whether these predicted membrane-anchored helices are accessible to BM labeling, we individually substituted amino acids from Glu<sup>731</sup> to Thr<sup>762</sup> (in wild-type  $\beta_3$ ) with cysteines that cover the whole intracellular tail of  $\beta_3$ . The cysteine-substituted constructs were co-expressed with wild-type  $\alpha_{IIb}$  in HEK 293 cells and subjected to whole cell BM labeling. Fig. 7 shows that all of the substituted cysteines are increasingly strongly labeled. Therefore, these predicted membrane-anchored helices are accessi-

ble to aqueous that differs from Leu<sup>712</sup>-Glu<sup>726</sup> region that is embedded in the lipid bilayer.

### DISCUSSION

*Intracellular Borders of Integrin  $\alpha_{IIb}$  and  $\beta_3$  TMs in Living Cells*—Here, we present for the first time the application of SCSAM in analyzing the conformational changes of integrin  $\alpha_{IIb}\beta_3$ , a membrane adhesion receptor in living cells. The intracellular MP region of integrin  $\alpha_{IIb}\beta_3$  has been the target of intensive study because of its critical role in controlling the receptor inside-out activation. In this study, we have unambig-

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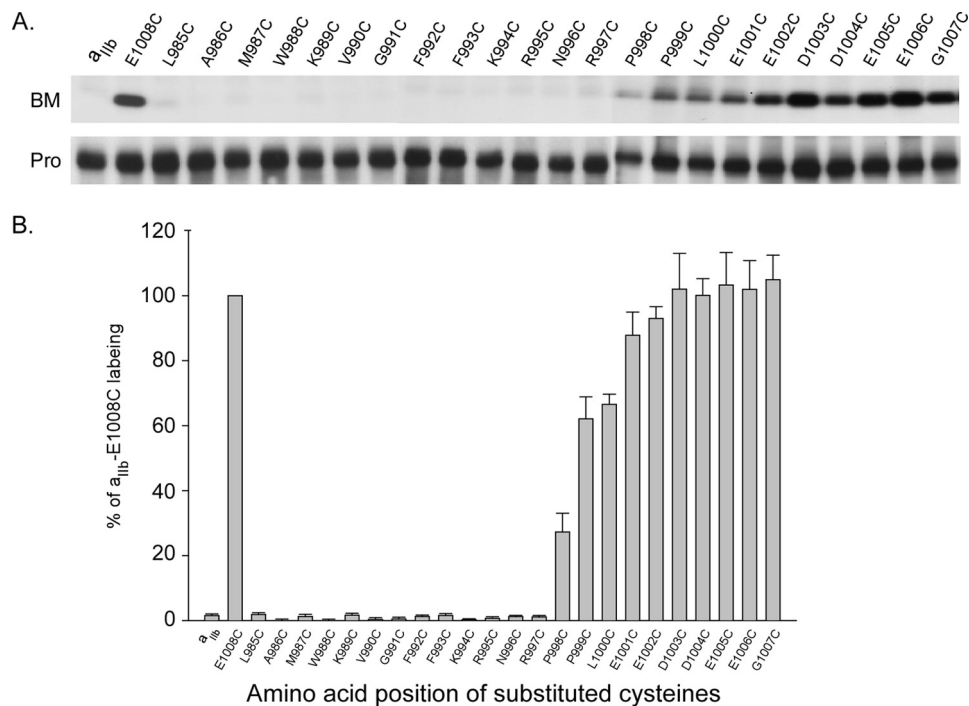


FIGURE 5. **BM labeling of cysteine-substituted  $\alpha_{IIb}$  co-expressed with  $\beta_3$  K716P.** *A*, representative results of BM labeling on integrin  $\alpha_{IIb}$ -substituted cysteines. The experiments were performed as described in the Fig. 3. *B*, summary of BM labeling of integrin  $\alpha_{IIb}$ -substituted cysteines. Results were analyzed same as described in Fig. 3. Data represent mean of 3–5 experiments  $\pm$  S.E. (error bars). Similar labeling results were obtained on cysteine-substituted  $\alpha_{IIb}$  co-expressed with  $\beta_3$  Lys<sup>716</sup> truncation or  $\beta_3$  G708I mutation.

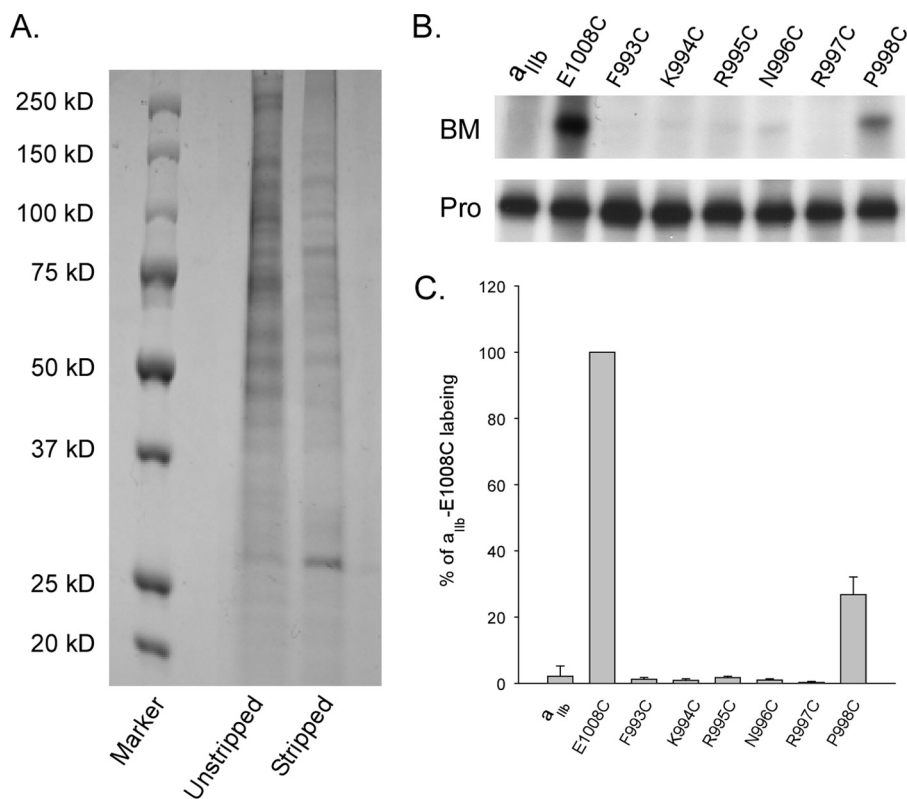


FIGURE 6. **BM labeling of cysteine-substituted  $\alpha_{IIb}$  co-expressed with  $\beta_3$ -K716P after  $\text{Na}_2\text{CO}_3$  stripping.** *A*, comparison of protein samples from membranes without and with  $\text{Na}_2\text{CO}_3$  stripping. Half-fraction of the isolated cell membranes was treated with 0.1 M  $\text{Na}_2\text{CO}_3$  for 30 min on ice. Membranes were then pelleted by centrifugation and washed once with PBS. Membrane pellets were lysed in IPB buffer, and protein levels were determined by the BCA method. Equal amounts of protein samples were loaded on the 4–20% SDS-polyacrylamide gel. *B*, representative BM labeling of cysteine-substituted  $\alpha_{IIb}$  Phe<sup>993</sup>-Pro<sup>998</sup> co-expressed with  $\beta_3$  K716P after 0.1 M  $\text{Na}_2\text{CO}_3$  treatment. Similar labeling results were obtained after 3 M  $\text{Na}_2\text{CO}_3$  treatment. *C*, summary of BM labeling. Results were analyzed the same as described in Fig. 3. Data represent mean of 3–6 experiments  $\pm$  S.E. (error bars).

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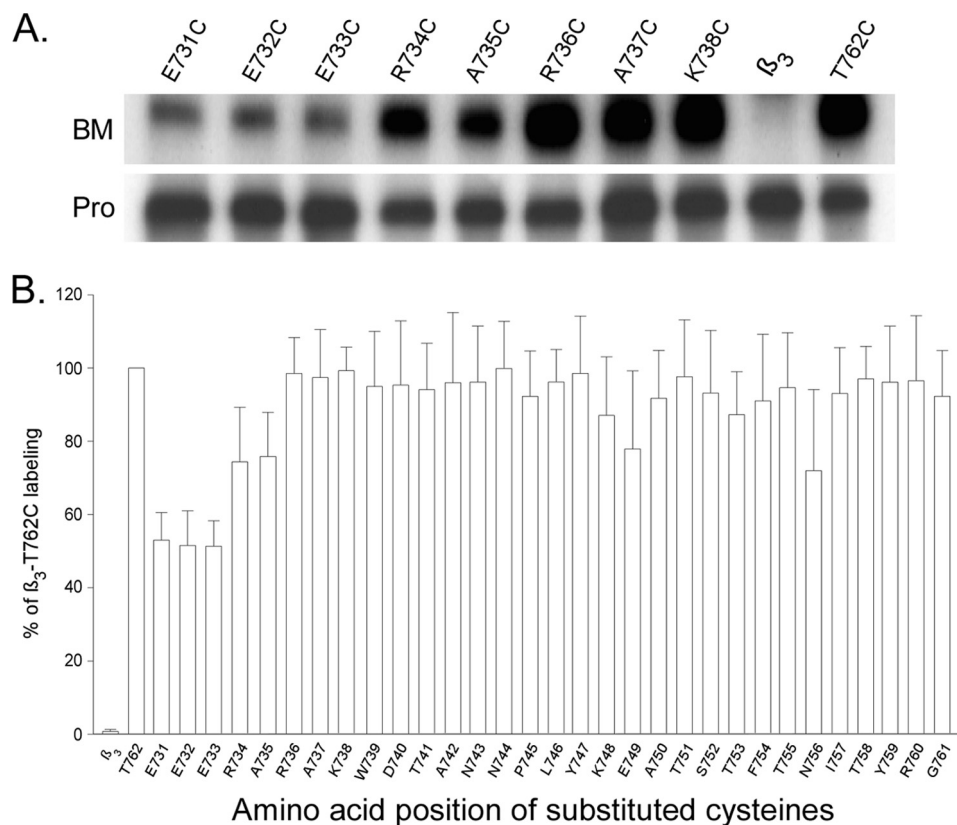


FIGURE 7. **BM labeling of cysteine-substituted integrin  $\beta_3$  intracellular tail.** Amino acids between Glu<sup>731</sup> and Thr<sup>762</sup> in the  $\beta_3$  subunit were individually substituted with cysteines and labeled with BM. A, representative results of BM labeling on substituted cysteines in integrin  $\beta_3$  intracellular tail co-expressed with wild-type  $\alpha_{IIb}$ . B, summary of BM labeling of cysteine-substituted  $\beta_3$  intracellular tail. Results were analyzed the same as described in Fig. 3. Data represent mean of 3 experiments  $\pm$  S.E. (error bars).

ously resolved the intracellular borders of integrin  $\alpha_{IIb}$  and  $\beta_3$  TM domain in the receptor active and inactive states on the surface of living cells. Our data revealed that, unlike the results from *in vitro* analysis, the intracellular borders of the active state integrin  $\alpha_{IIb}$  and  $\beta_3$  TMs reside at amino acids Pro<sup>998</sup> and Phe<sup>727</sup>, respectively. This located the proposed MP regions of both subunits in the lipid bilayer in the active state. The conclusion is supported by two sets of BM labeling experiments by using highly active mutation (K716P and G708I) or truncation (at Lys<sup>716</sup>) in the  $\beta_3$  subunit, and highly active mutation (F992A/F993A) or truncation (at Gly<sup>991</sup>) in the  $\alpha_{IIb}$  subunit that generates constitutive active states of integrin  $\alpha_{IIb}\beta_3$ .

A puzzling pattern of BM labeling was observed on the determination of  $\alpha_{IIb}$  TM border in the receptor inactive state. When co-expressed with wild-type  $\beta_3$  subunit, weak labeling was detected on  $\alpha_{IIb}$  K994C; strong labeling on N996C, R997C, and P998C; however, no labeling on F992C, F993C, and R995C. This result is readily explained by the functional data from the current and the previous studies (16), which showed cysteine substitution of Arg<sup>995</sup> activated the receptor close to the maximum level in the HEK 293 cells, whereas Phe<sup>992</sup>, Phe<sup>993</sup>, Lys<sup>994</sup>, Asn<sup>996</sup>, Arg<sup>997</sup>, and Pro<sup>998</sup> were ~0–40% activation, indicating that >60% of the population of the mutant receptors on the cell surface were not active. Additionally, the recent Rosetta model on the  $\alpha_{IIb}$  MP region showed that the side chain of Lys<sup>994</sup> points away from the dimer interface in the receptor inactive

state, suggesting its accessibility to the aqueous medium (16). Therefore, labeled K994C, N996C, R997C, and P998C represent the major population of inactive receptors. Consistent with this, no BM labeling was detected on  $\alpha_{IIb}$  R995C co-expressed either with wild-type or activating  $\beta_3$  subunit because both conditions activated the receptor at a high level. Based on these observations, we concluded that the labeled K994C defines the intracellular border of  $\alpha_{IIb}$  TM in the inactive state. One possibility is that in the active state, intracellular proteins may bind to the unclamped  $\alpha_{IIb}$  tail that shields the substituted cysteines to BM labeling. We ruled out this possibility by stripping the isolated membranes with 0.1 M or 3 M Na<sub>2</sub>CO<sub>3</sub> that removes potential bound peripheral proteins prior to BM labeling and showed no detectable differences compared with the samples without treatment.

In contrast to  $\alpha_{IIb}$ , determination of the  $\beta_3$  TM intracellular border was clear and compelling because none of the cysteine substitutions in the proposed MP region except Lys<sup>716</sup> activated the receptor. Our data showed that none of the cysteine substitutions prior to Phe<sup>727</sup>, including the highly charged <sup>722</sup>HDRKE<sup>726</sup> stretch, was accessible to BM labeling, suggesting their lipid embedment. Residues starting at Phe<sup>727</sup> became strongly labeled, indicating their aqueous exposure. Therefore, we assigned the intracellular border of  $\beta_3$  TM in the inactive state to Phe<sup>727</sup>. Indeed, the strong labeling of the cysteine-substituted  $\beta_3$  tail membrane-anchored  $\alpha$ -helices further supports



that Phe<sup>727</sup> marks the intracellular lipid/aqueous interface of  $\beta_3$  TM in the living cells.

**Comparison with the *in Vitro* Determined TM Borders**—Our findings differ significantly from the results obtained in the *in vitro* studies, including glycosylation mapping in the cell-free system (32, 33) and the recent NMR structures of  $\alpha_{IIB}$  (34) and  $\beta_3$  TM (35) peptides in the lipid bicelles, which both placed the intracellular borders of  $\alpha_{IIB}$  TM at Lys<sup>994</sup> and  $\beta_3$  TM at Asp<sup>723</sup>. Because integrin TMs are fully separated in the active state (36), these *in vitro* findings may represent the TM borders of the integrin active state. Because the isolated TM fragments do not contain the intracellular clasp in the MP region and more importantly do not carry the extracellular domains, the physiologic significance is questionable. It is known that the extracellular domain of integrin  $\alpha_{IIB}$  and  $\beta_3$  forms a dimer independent of the TM domains (13), and further, dimerization of the extracellular domains precedes the dimerization of the TM domains during biosynthesis. It is conceivable that the  $\alpha_{IIB}\beta_3$  TM domain dimerization and lipid embedding are influenced or guided by the dimerized extracellular domain. In support of this, mutations that separated the dimerized  $\alpha_{IIB}\beta_3$  TM fragment in the lipid bicelles failed to activate the intact receptor in the cells (20), confirming that the extracellular domain is involved in stabilizing the  $\alpha_{IIB}\beta_3$  TM dimer and that the previous *in vitro* observations may not be physiological.

Our study has considerable advantages over the *in vitro* studies because the analyses were performed on intact integrin  $\alpha_{IIB}\beta_3$  protein in living cells. Our data showed, in contrast to the *in vitro* findings, that Lys<sup>994</sup> marks the intracellular border of inactive state  $\alpha_{IIB}$  TM. When the receptor is active, the border shifts to Pro<sup>998</sup>, indicating a significant peptide repartitioning from aqueous medium into the lipid bilayer. The extracellular domain of integrin was shown to switch from a bent to an extended conformation in the active state (36). We suggest that the peptide repartitioning is caused by the pulling of the extended extracellular domain rather than by a spontaneous repartitioning of the peptide itself. Indeed, upon close examination of the lipid-embedded extracellular end of  $\alpha_{IIB}$  TM, two amphiphilic residues (Trp<sup>997</sup>, Trp<sup>998</sup>) immediately follow the first lipid embedded residue Ile<sup>996</sup>, making it possible for them to repartition into the aqueous medium as a result of the stretching forces induced by the extracellular domain extension. In support of this, when the extracellular domain was missing, the <sup>994</sup>KRNRP<sup>998</sup> stretch of  $\alpha_{IIB}$  TM was not embedded in the lipid bilayer, indicating that spontaneous lipid repartitioning of the peptide is not favorable (32–34). This phenomenon can only be observed in intact integrin in a cellular environment.

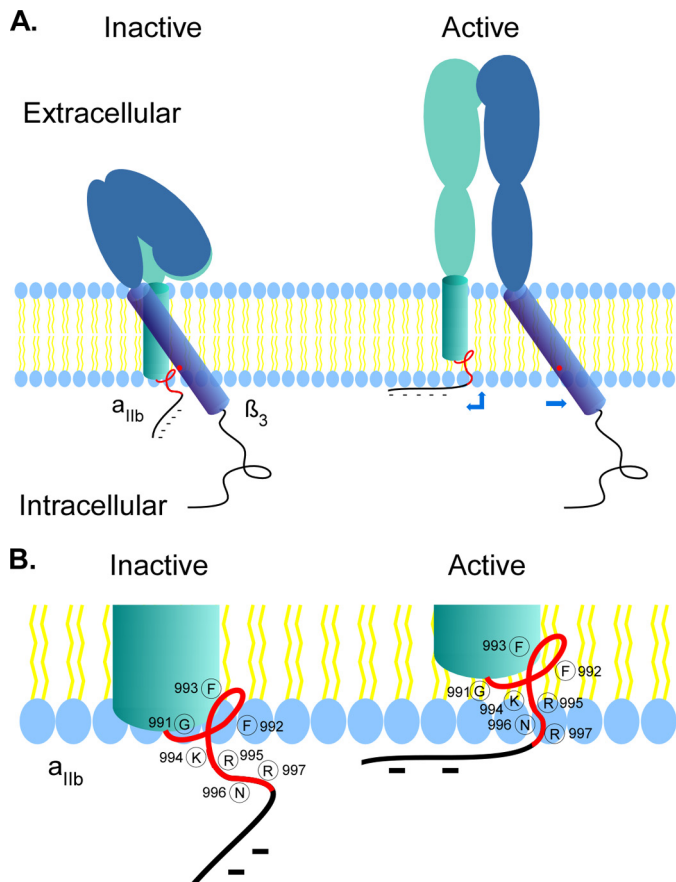
Compared with the reported *in vitro* determined  $\beta_3$  TM intracellular border, our data are more complex: exposure to the aqueous starts at amino acid Phe<sup>727</sup>, leaving the stretch of charged residues <sup>722</sup>HDRKE<sup>726</sup> in the membrane. In the *in vitro* system, to place such a highly charged peptide in the lipid environment is energetically unfavorable. The  $\beta_3$  TM NMR structure showed that the TM helix terminates at Asp<sup>723</sup> (20, 35), whereas cysteine cross-linking analyses in the intact protein suggested that the helical structure extended to Phe<sup>730</sup> (16).

Following the latter observation, our findings suggest that there is an additional helix turn at the C terminus of  $\beta_3$  TM in the lipid bilayer,  $\sim 5.4$  Å distant from the aqueous medium. Residue Asp<sup>723</sup> has been shown to face the dimer interface (16, 20); therefore Arg<sup>724</sup>, Lys<sup>725</sup>, and Glu<sup>726</sup> would be 100 degrees apart, which potentially interacts with the negatively charged phosphate groups and the positively charged choline head of membrane phospholipids, which forms ionic interactions to stabilize the end of TM in the lipid bilayer. In agreement with this, we observed that activation of the receptor had no effect on membrane embedding of this region, suggesting that it is unlikely that the C terminus of  $\beta_3$  TM could move out of the lipid bilayer upon activation. Lipid embedding of heavily charged peptides has been shown in the crystallized voltage sensor of the voltage-gated potassium channel (37), and potentially, these charged residues are hydrated (38).

A recent analysis of the integrin  $\beta_3$  TM intracellular border using a  $\beta_3$  TM fragment suggested that  $\beta_3$  Lys<sup>716</sup> helps determine the integrin  $\beta_3$  TM topography (39). Removal of the positive charge at this position (active state) induced aqueous exposure of the residues in the proposed  $\beta_3$  MP region. Because our experiments analyzed the intact receptor in a cellular environment, the difference between our findings and this observation is potentially due to differences in the experimental approaches used.

**Implications for Integrin Inside-out Activation**—The TMs of integrin have been proposed to undergo “separation” (9), “piston” (40), or “scissors” (40, 41) movement upon inside-out activation. We found that, upon activation of integrin  $\alpha_{IIB}\beta_3$  in the living cells, the intracellular border of  $\alpha_{IIB}$  TM undergoes an upward shift that repartitions into the lipid bilayer, whereas the position of  $\beta_3$  TM border remains unchanged. It has been shown that the  $\alpha_{IIB}$  TM is perpendicular in the lipid bilayer whereas  $\beta_3$  TM tilts at a significant angle when dimerized (16, 20). Our finding that the membrane embedded region of  $\beta_3$  TM extends to Glu<sup>726</sup> suggests that the TM tilts at more extended angles. Taking our *in vivo* findings together with the reported *in vitro* TM structures (16, 20, 23), we propose a new mechanism for integrin inside-out activation (Fig. 8). In the resting state, the MP region of  $\alpha_{IIB}$  <sup>991</sup>GFFKR<sup>995</sup> interacts with  $\beta_3$  Lys<sup>716</sup> forming a clasp that keeps the receptor inactive. Upon inside-out activation, the breakage of the intracellular clasp leads to extensive conformational changes in the extracellular domain from a bent to an extended conformation. Because the two TMs are dissociated, the extended  $\alpha_{IIB}$  extracellular domain pulls its TM upward causing four consecutive residues in the MP region repartition into the lipid bilayer. The NMR structure shows the region after  $\alpha_{IIB}$  Val<sup>990</sup> has a left reverse turn rather than a helical structure; this would minimize the energy required for the peptide repartition into the lipid bilayer. Additionally, the up shift of  $\alpha_{IIB}$  TM would draw the negatively charged <sup>1001</sup>EED-DEEGE<sup>1008</sup> stretch close to the surface of lipid bilayer, which may form ionic interactions with the positively charged choline head of membrane phospholipids to serve as a “break” for the prevention of “whiplash” of  $\alpha_{IIB}$  extracellular domain, to keep integrin in the correct active state conformation on the surface of cells. Indeed, the unique reverse folding structure prior to the acidic stretch introduced by two prolines

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**FIGURE 8. Proposed mechanism of integrin inside-out activation.** A, schemes showing that in the inactive state, the  $\alpha_{IIb}$  TM (aqua) associates with  $\beta_3$  TM (blue) in the lipid bilayer with a clasp formed by GFFKRNRR (red line) and Lys<sup>716</sup> (red dot) at the intracellular lipid/aqueous interface. Upon inside-out activation, extension of the  $\alpha_{IIb}$  extracellular domain applies stretching forces on the  $\alpha_{IIb}$  TM, causing its upward shift that results in repartitioning of four amino acids in the MP region (red line) into the lipid bilayer and drawing the negatively charged tail (dashed black lines) close to the inner leaflet of the plasma membrane that may form ionic interactions with the positive head groups of membrane phospholipids. B, detailed positions of the residues in the  $\alpha_{IIb}$  MP region involving lipid bilayer repartitioning upon inside-out activation. The structure of the  $\alpha_{IIb}$  MP region was adapted from Refs. 16 and 34.

(998 and 999) in the  $\alpha_{IIb}$  tail makes the break hypothesis plausible (42).

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### REFERENCES

- Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673–687
- Anthis, N. J., and Campbell, I. D. (2011) The tail of integrin activation. *Trends Biochem. Sci.* **36**, 191–198
- Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010) The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell. Biol.* **11**, 288–300
- Wegener, K. L., and Campbell, I. D. (2008) Transmembrane and cytoplasmic domains in integrin activation and protein-protein interactions. *Mol. Membr. Biol.* **25**, 376–387
- 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
- O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F., and

- Ginsberg, M. H. (1991) Modulation of the affinity of integrin  $\alpha_{IIb}\beta_3$  (GPIIb-IIIa) by the cytoplasmic domain of  $\alpha_{IIb}$ . *Science* **254**, 845–847
- Takagi, J., Petre, B. M., Walz, T., and Springer, T. A. (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**, 599–611
- Adair, B. D., and Yeager, M. (2002) Three-dimensional model of the human platelet integrin  $\alpha_{IIb}\beta_3$  based on electron cryomicroscopy and x-ray crystallography. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 14059–14064
- Kim, M., Carman, C. V., and Springer, T. A. (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* **301**, 1720–1725
- Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M., and Fässler, R. (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat. Med.* **14**, 325–330
- Moser, M., Legate, K. R., Zent, R., and Fässler, R. (2009) The tail of integrins, talin, and kindlins. *Science* **324**, 895–899
- Ma, Y. Q., Qin, J., Wu, C., and Plow, E. F. (2008) Kindlin-2 (Mig-2): a co-activator of  $\beta_3$  integrins. *J. Cell Biol.* **181**, 439–446
- Zhu, J., Luo, B. H., Xiao, T., Zhang, C., Nishida, N., and Springer, T. A. (2008) Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Mol. Cell* **32**, 849–861
- Hughes, P. E., O'Toole, T. E., Ylänne, J., Shattil, S. J., and Ginsberg, M. H. (1995) The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J. Biol. Chem.* **270**, 12411–12417
- Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., 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
- Zhu, J., Luo, B. H., Barth, P., Schonbrun, J., Baker, D., and Springer, T. A. (2009) The structure of a receptor with two associating transmembrane domains on the cell surface: integrin  $\alpha_{IIb}\beta_3$ . *Mol. Cell* **34**, 234–249
- Yang, J., Ma, Y. Q., Page, R. C., Misra, S., Plow, E. F., and Qin, J. (2009) Structure of an integrin  $\alpha_{IIb}\beta_3$  transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17729–17734
- Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) A structural mechanism of integrin  $\alpha_{IIb}\beta_3$  “inside-out” activation as regulated by its cytoplasmic face. *Cell* **110**, 587–597
- Vinogradova, O., Vaynberg, J., Kong, X., Haas, T. A., Plow, E. F., and Qin, J. (2004) Membrane-mediated structural transitions at the cytoplasmic face during integrin activation. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 4094–4099
- Lau, T. L., Kim, C., Ginsberg, M. H., and Ulmer, T. S. (2009) The structure of the integrin  $\alpha_{IIb}\beta_3$  transmembrane complex explains integrin transmembrane signalling. *EMBO J.* **28**, 1351–1361
- Kim, C., Lau, T. L., Ulmer, T. S., and Ginsberg, M. H. (2009) Interactions of platelet integrin  $\alpha_{IIb}$  and  $\beta_3$  transmembrane domains in mammalian cell membranes and their role in integrin activation. *Blood* **113**, 4747–4753
- Gottschalk, K. E. (2005) A coiled-coil structure of the  $\alpha_{IIb}\beta_3$  integrin transmembrane and cytoplasmic domains in its resting state. *Structure* **13**, 703–712
- Wegener, K. L., Partridge, A. W., Han, J., Pickford, A. R., Liddington, R. C., Ginsberg, M. H., and Campbell, I. D. (2007) Structural basis of integrin activation by talin. *Cell* **128**, 171–182
- Zhu, Q., Lee, D. W., and Casey, J. R. (2003) Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1. *J. Biol. Chem.* **278**, 3112–3120
- Zhu, Q., Kao, L., Azimov, R., Newman, D., Liu, W., Pushkin, A., Abuladze, N., and Kurtz, I. (2010) Topological location and structural importance of the NBCe1-A residues mutated in proximal renal tubular acidosis. *J. Biol. Chem.* **285**, 13416–13426
- Karlin, A., and Akabas, M. H. (1998) Substituted-cysteine accessibility method. *Methods Enzymol.* **293**, 123–145
- Zhu, Q., and Casey, J. R. (2007) Topology of transmembrane proteins by scanning cysteine accessibility mutagenesis methodology. *Methods* **41**, 439–450

28. Zhu, Q., Kao, L., Azimov, R., Abuladze, N., Newman, D., Pushkin, A., Liu, W., Chang, C., and Kurtz, I. (2010) Structural and functional characterization of the C-terminal transmembrane region of NBCe1-A. *J. Biol. Chem.* **285**, 37178–37187
29. Yan, B., and Smith, J. W. (2000) A redox site involved in integrin activation. *J. Biol. Chem.* **275**, 39964–39972
30. 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  $\alpha_{11b}$  cytoplasmic domain. *J. Biol. Chem.* **277**, 28877–28883
31. Metcalf, D. G., Moore, D. T., Wu, Y., Kielec, J. M., Molnar, K., Valentine, K. G., Wand, A. J., Bennett, J. S., and DeGrado, W. F. (2010) NMR analysis of the  $\alpha_{11b}\beta_3$  cytoplasmic interaction suggests a mechanism for integrin regulation. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 22481–22486
32. Armulik, A., Nilsson, I., von Heijne, G., and Johansson, S. (1999) Determination of the border between the transmembrane and cytoplasmic domains of human integrin subunits. *J. Biol. Chem.* **274**, 37030–37034
33. Stefansson, A., Armulik, A., Nilsson, I., von Heijne, G., and Johansson, S. (2004) Determination of N- and C-terminal borders of the transmembrane domain of integrin subunits. *J. Biol. Chem.* **279**, 21200–21205
34. Lau, T. L., Dua, V., and Ulmer, T. S. (2008) Structure of the integrin  $\alpha_{11b}$  transmembrane segment. *J. Biol. Chem.* **283**, 16162–16168
35. Lau, T. L., Partridge, A. W., Ginsberg, M. H., and Ulmer, T. S. (2008) Structure of the integrin  $\beta_3$  transmembrane segment in phospholipid bilayers and detergent micelles. *Biochemistry* **47**, 4008–4016
36. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* **25**, 619–647
37. Long, S. B., Tao, X., Campbell, E. B., and MacKinnon, R. (2007) Atomic structure of a voltage-dependent  $K^+$  channel in a lipid membrane-like environment. *Nature* **450**, 376–382
38. Krepiak, D., Mihailescu, M., Freitas, J. A., Schow, E. V., Worcester, D. L., Gawrisch, K., Tobias, D. J., White, S. H., and Swartz, K. J. (2009) Structure and hydration of membranes embedded with voltage-sensing domains. *Nature* **462**, 473–479
39. Kim, C., Schmidt, T., Cho, E. G., Ye, F., Ulmer, T. S., and Ginsberg, M. H. (2012) Basic amino acid side chains regulate transmembrane integrin signalling. *Nature* **481**, 209–213
40. Williams, M. J., Hughes, P. E., O'Toole, T. E., and Ginsberg, M. H. (1994) The inner world of cell adhesion: integrin cytoplasmic domains. *Trends Cell Biol.* **4**, 109–112
41. Kalli, A. C., Campbell, I. D., and Sansom, M. S. (2011) Multiscale simulations suggest a mechanism for integrin inside-out activation. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 11890–11895
42. Vinogradova, O., Haas, T., Plow, E. F., and Qin, J. (2000) A structural basis for integrin activation by the cytoplasmic tail of the  $\alpha_{11b}$  subunit. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1450–1455