ACTIVATION OF PLATELET αIIbβ3 BY AN EXOGENOUS PEPTIDE CORRESPONDING TO THE TRANSMEMBRANE DOMAIN OF αIIb*

Hang Yin†, Rustem I. Litvinov‡, Gaston Vilaire†, Hua Zhu†, Gregory A. Caputo‡, David T. Moore‡, James D. Lear‡, John W. Weisel†, William F. DeGrado‡§, Joel S. Bennett†**

From ‡Department of Biochemistry and Biophysics, School of Medicine; ¥Department of Cell and Developmental Biology, School of Medicine; †Hematology-Oncology Division, Department of Medicine, School of Medicine; §Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

**Address correspondence to: Joel S. Bennett, Hematology-Oncology Division, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, Tel. 215-573-3280; Fax. 215-573-7039; E-Mail: bennetts@mail.med.upenn.edu

Running Title: Integrin αIIb Transmembrane Peptide

A transmembrane domain heterodimer, acting in concert with a membrane-proximal cytoplasmic domain clasp, is thought to maintain integrins in a low-affinity state. To test whether helix-helix interactions between the αIIb and β3 transmembrane domains regulate the activity of integrin αIIbβ3, we synthesized a soluble peptide corresponding to the αIIb transmembrane domain, designated αIIb-TM, and studied its ability to affect αIIbβ3 activity in human platelets. αIIb-TM was α-helical in detergent micelles and phospholipid vesicles, readily inserted into membrane bilayers, bound to intact purified αIIbβ3, and specifically associated with the transmembrane domain of αIIb, rather than the transmembrane domains of β3, α2, and β1, other integrin subunits present in platelets. When added to suspensions of gel-filtered platelets, αIIb-TM rapidly induced platelet aggregation that was not inhibited by pre-incubating platelets with the prostaglandin PGE1 or the ADP scavenger apyrase, but was prevented by the divalent cation chelator EDTA. Further, αIIb-TM induced fibrinogen binding to platelets, but not the binding of osteopontin, a specific ligand for platelet αvβ3. The peptide also induced fibrinogen binding to recombinant αIIbβ3 expressed by Chinese hamster ovary cells, confirming that its effect was independent of platelet signal transduction. Lastly, transmission electron microscopy of purified αIIbβ3 revealed that αIIb-TM shifted the integrin from a closed configuration with its stalks touching to an open configuration with separated stalks. These observations demonstrate that transmembrane domain interactions regulate integrin function in situ and that it is possible to target intra-membranous protein-protein interactions in a way that can have functional consequences.

The affinity of integrins for ligands appears to be regulated by interactions between the transmembrane (TM) and/or cytoplasmic domains of their α and β subunits (1). Thus, it is likely that a TM domain heterodimer, acting in concert with a membrane-proximal cytoplasmic domain clasp, maintains integrins in a low-affinity state. The prototypic example of integrin regulation is platelet αIIbβ3. αIIbβ3, a receptor for the plasma proteins fibrinogen, von Willebrand factor, fibronectin, and vitronectin, is maintained in an inactive state on circulating platelets, but following vascular trauma, it shifts allosterically to an active conformation, a prelude to the formation of hemostatic platelet aggregates (1).
Data supporting the heteromeric association of the αIIb and β3 TM domains in unstimulated platelets are largely indirect (2,3). Moreover, proteins containing these domains also associate homomERICALLY in micelles and bacterial membranes (4,5). It is noteworthy that mutations that either enhance or disrupt homomeric αIIb and β3 TM domain interactions in vitro can activate the intact integrin expressed in Chinese hamster ovary (CHO) cells. These observations suggest a “push-pull” mechanism for αIIbβ3 regulation in which processes that destabilize heteromeric αIIb and β3 TM domain interactions push αIIbβ3 to its activated state, whereas processes that favor their homomeric association pull αIIbβ3 toward its active conformation (6).

In the absence of the extracellular portion of the protein, αIIb TM domain peptides tend to form homodimers in micelles and E. coli membranes, rather than associating with the β3 TM domain (5). Furthermore, the residues involved in αIIb homo-dimerization are also implicated in binding of the αIIb TM domain to the β3 TM domain, implying that the two interaction sites overlap. Accordingly, we would expect that an αIIb TM domain peptide might be able to bind to the TM region of αIIb in αIIbβ3-expressing cells, thereby disrupting heteromeric αIIb/β3 TM domain helix-helix interactions. If helix-helix interactions between the αIIb and β3 TM domains are indeed critical for αIIbβ3 activation, disruption of these interactions should cause activation of the integrin. We show here that a synthetic peptide, designated αIIb-TM, corresponding to the wild type αIIb TM domain, undergoes specific association with the αIIb TM domain in micelles and bacterial membranes and induces platelet aggregation by interacting directly with αIIbβ3. These results provide strong support for the hypothesis that separation of the αIIb and β3 TM helices is required for αIIbβ3 activation. Further, they demonstrate that it is possible to alter the function of an integrin in situ by targeting its TM domains, providing a new approach for the development of novel therapeutic agents. Finally, these results demonstrate a way to perturb TM-TM interactions in intact integrins, allowing determination of the role of these interactions in integrin activation and clustering.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification - Peptides were synthesized using an Applied Biosystems 430A peptide synthesizer at 0.25 mmole scales and on a Rink Amide AM resin (200-400 mesh) (Nova Biochem) with a substitution level of 0.71 mmole/g. Activation of the free amino acids was achieved with N,N,N',N'-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) as a 0.40 M solution in N,N'-dimethylformamide (DMF) and using a reaction solvent containing 25% dimethylsulfoxide (DMSO) and 75% 1-methyl-2-pyrrolidinone (NMP). Side chain deprotection and simultaneous cleavage from the resin was performed with a mixture of trifluoroacetic acid (TFA)/H2O/1,2-ethanediethyl/anirole (94:2.5:2.5:1 v/v) at room temperature under N2 flow for 2 hours. Crude peptides collected after precipitation with cold diethyl ether were dissolved in a mixture of 2-propanol:acetonitrile:water (6:3:1) and then lyophilized. Peptides were then purified on a preparative reverse phase HPLC system (Varian ProStar 210) with a C-8 semi-preparative column (Vydac) using a linear gradient of buffer A (0.1% TFA in Millipore water) and buffer B, 2-propanol/acetonitrile/water (6:3:1 v/v) containing 0.1% TFA. The N-terminus of nascent peptides was labeled with fluorescein isothiocyanate (FITC) using a standard protocol (7). To direct the modification to only the N-terminal amine, the reaction was conducted at the end of the automated peptide synthesis while the peptide was still attached to the resin and its lysine side chains protected by Boc-groups.

Circular Dichroism (CD) Spectrometry - CD spectrometry was performed using a J-810 spectropolarimeter (JASCO). Samples were
prepared at 20 µM concentrations in micelles (2.5 mM 1,2-dihexanoyl-sn-glycero-3-phosphocholine, critical micellar concentration (CMC) 1.0 mM, Sigma) or unilamellar vesicles (500 µM total phospholipid composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) (Avanti Polar Lipids Inc.) at an 8:2 ratio. Measurements were conducted at 25°C in step scanning mode with a response time of 4 seconds.

**Analytical Ultracentrifugation** - Equilibrium sedimentation was performed in a Beckman XL-I analytical ultracentrifuge (Beckman Coulter) at 25°C using six-channel carbon-epoxy composite centerpieces. Peptides, prepared at concentrations of 50 µM, were dissolved in 20 mM HEPES buffer (pH 7.4) containing 5 mM C14-betaine (3-(N,N-dimethylmyristyl-ammonio)propanesulfonate, Sigma) and 29% D2O previously determined to match the density of the detergent component (8). Partial specific volumes of volumes of peptides were calculated using a previously described method (9) and residue molecular weights corrected for the 29% D2O exchange expected for the density-matched buffer. The solvent density (1.0245 g.ml⁻¹) was measured using a Paar densitometer. Aqueous solution molar extinction coefficients were calculated using the program Sednterp and multiplied by the molar detergent concentration to provide mole fraction units. Data fitting and analysis was accomplished as previously described (10).

**Tryptophan Fluorescence** - Peptide insertion into lipid vesicles was assayed by monitoring changes in the emission spectrum of tryptophan as previously described (11). An aliquot of peptide from a concentrated stock in DMSO was added to 10 mM HEPES buffer containing 150 mM NaCl, pH 7.0, to a final concentration of 2 µM in 1.7 ml and was allowed to equilibrate for 5 minutes at which point the Trp emission spectra was recorded. The sample was then titrated with aliquots of vesicles (POPC:POPG, 8:2). After incubation for 10 min with constant mixing, the Trp emission spectra were re-recorded.

**Attenuated Total Reflection Infrared (ATR-IR) Spectroscopy** – ATR-IR spectroscopy was performed as previously described (12). Briefly, αIIb-TM, solubilized in a 1:1 (v:v) mixture of 2-propanol:H2O, was mixed with lipid and dried under a stream of N2. The dried film was reconstituted in 5 mM HEPES buffered D2O at pH 7.1 by vigorous vortexing. The peptide-lipid suspension was then extruded 17 times using an Avestin liposofast mini extruder (Avestin Inc.) equipped with 2 stacked poly carbonate membranes with average pore diameter of 200 nm. The peptide-containing vesicles were deposited on the ATR crystal, gently spread with a Teflon bar to form a film, and dried under a gentle stream of N2. Infrared spectra were recorded on a Nicolet 4700 infrared spectrophotometer (Thermo-Electron Corp.) equipped with a DTGS detector and a ZnSe wire-grid polarizer. The internal reflection element was a zinc-selenide ATR crystal (80 × 20 × 3 mm) with an angle of 45° yielding 25 internal reflections. A total of 512 scans at polarizations of 0° and 90° were collected for each sample. Spectra were recorded at 2 cm⁻¹ resolution and analyzed using the OMNIC software package for peak deconvolution and area analysis. Helix orientation angle was calculated from the spectra as previously described (13) with the exception that the value used in this study for the crystal refractive index was 2.42 (ZnSe).

**Size Exclusion Chromatography** - Size exclusion chromatography was performed using an AKTA FPLC system (Amersham Biosciences). FITC-labeled peptide (1 µM) and purified αIIbβ3 protein (10 mg/ml) were mixed and incubated overnight. The mixture was loaded onto a Superdex 200 HR 10/30 FPLC column (Amersham Pharmacia) equilibrated with the elution buffer (10 mM HEPES, 60 mM N-octyl-β-D-glucopyranoside, 0.5 mM CaCl₂, 0.02% NaN₃, and 200 mM NaCl, pH 7.4.) at a flow rate of 0.5 ml/min. 0.5 ml fractions were
collected and fluorescence was measured using a SpectraMax Gemini EM microplate fluorometer (Molecular Devices) with excitation and emission wavelengths at 485 nm and 515 nm, respectively.

**Fluorescence Anisotropy** - Full-length αIIbβ3 in 10 mM HEPES buffer containing 60 mM N-octyl-β-D-glucopyranoside, 0.5 mM CaCl₂, 0.02% NaN, pH 7.5, was prepared as previously reported (14). Fluorescence polarization experiments were conducted on an ATF105 spectrofluorometer (Aviv Instrument, Inc) using a 0.3 cm path length cuvette. Spectra were measured at 25 °C using 1.0 nm slit widths. Excitation at 485 nm was used for the FITC-labeled peptide and the emission maximum at 515 nm was monitored. Anisotropy measurements were recorded by titrating a 64 nM FITC-αIIb-TM peptide solution with increasing concentrations of purified αIIbβ3 protein. A dissociation constant and baseline parameters were derived from the fluorescence anisotropy signal/concentration isotherm using previously described methods (15).

**SDS-PAGE and Immunoblotting** - The contents of the elution fractions from the size exclusion chromatography were identified using precast SDS polyacrylamide gels (4-12% NuPAGE Bis-Tris gels, Invitrogen). Before electrophoresis, each sample was incubated at 90°C for 7 min. Electrophoresis was carried out at room temperature with NuPAGE MOPS (3-[N-morpholino]propanesulfonic acid) SDS running buffer (Invitrogen). Immunoblotting was performed after electrophoretically transferring proteins to nitrocellulose paper (0.45 µm, Schleicher & Schuell). The paper was then incubated with the anti-β3 monoclonal antibody SSA6. Primary antibody binding was detected using ECL Western blotting detection reagents after 6000-fold dilution of the stock solution, followed by fluorography using Kodak Biomax MS film.

**TOXCAT** – TM peptide dimerization in membrane bilayers was measured by the TOXCAT assay using the expression vector pccKAN kindly provided by Donald M. Engelman (Yale University, New Haven, CT) (16). After changing the EcoRV restriction site between the TM region and the maB gene in pccKAN to a BamHI site, the vector was digested with NheI-BamHI, and cDNA encoding integrin TM helices were ligated into the vector in-frame. The resulting plasmids were transformed into E. coli MM39 cells. Chloramphenicol acetyltransferase (CAT) synthesis was assayed by using a CAT-ELISA kit (Roche Applied Sciences, Indianapolis), as described (4). Chimeric protein expression was quantified from immunoblots using a Personal Densitometer SI (Molecular Dynamics) and was used to compare CAT expression by the various constructs.

**Platelet Function Assays** - Turbidometric measurements of platelet aggregation were performed in a Chrono-Log Lumi-Dual Aggregometer as described previously (17). Briefly, platelet-rich plasma, prepared from human blood anti-coagulated with 0.1 volume 0.13 M sodium citrate, was gel-filtered on Sepharose 2B (Amersham Biosciences) using an elution buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 0.35 mg/ml bovine serum albumin, 3.3 mM NaH₂PO₄, and 4 mM HEPES, pH 7.4. 400 µl aliquots of the gel-filtered platelet suspension were stirred at 900 rpm in siliconized aggregometer cuvettes and were supplemented with human fibrinogen (Enzyme Research Laboratories) and CaCl₂ to final concentrations of 200 µg/ml and 1 mM, respectively, prior to adding platelet agonists.

Binding of FITC-conjugated fibrinogen to gel-filtered platelets was measured by fluorescence activated cell sorting (FACS) as previously described (18). Following the addition of ADP or the αIIb-TM peptide to platelet suspensions, the platelets were fixed with 0.37% formalin in PBS buffer for 10 minutes, washed, and examined by FACS analysis.
Peptide-Induced Cell Lysis – Three methods were used to assess peptide-induced cell lysis. First, peptide-induced lysis of unilamellar vesicles containing the dye Tb(DPA)_3 was measured as previously described (19). Melittin and buffer alone were positive and negative controls, respectively. Second, peptide-induced lysis of erythrocytes was measured as previously described (20). Briefly, 1% (v/v) suspensions of washed human erythrocytes were incubated with peptide in 4 mM HEPES buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 0.35 mg/ml bovine serum albumin, and 3.3 mM NaH₂PO₄, pH 7.4, at 37°C for 3 min. After centrifuging the samples at 14,000 rpm, the optical density of the supernatant was measured at 405 nm. Third, peptide-induced release of ¹⁴C-serotonin from non-aggregating platelets was measured as described previously (17). Platelets were loaded with ¹⁴C-serotonin, gel-filtered, and incubated with peptide for 30 min in the absence of stirring. The ¹⁴C-serotonin content of 100 µl aliquots of platelet-free supernatant were then measured and compared to the ¹⁴C-serotonin content of a 100 µl aliquot of the original platelet suspension.

Rupture Force Spectroscopy (Laser Tweezers) - A custom-built laser tweezers setup was used to measure the strength of fibrinogen and osteopontin binding to CHO cells or platelets (21,22). Purified human fibrinogen and recombinant osteopontin (OPN) were covalently bound to 1.87 µm carboxylate-modified latex beads. An individual platelet was manually attached to a 5 µm diameter silica pedestal coated with polylsine, whereas CHO cells were permitted to settle and attach spontaneously to the polylsine-coated bottom of the chamber. A ligand-coated bead, trapped by the laser light, was brought into proximity of an immobilized cell, oscillated at 10 Hz, and then brought into repeated intermittent contact with the cell by micromanipulation. Data collection was initiated at the first contact. Rupture forces following repeated contacts were collected into 5 pN- or 10 pN-wide bins. The percentage of events in a particular bin represented the probability of rupture events at that tension. Optical artifacts observed with or without trapped latex beads produced signals that appear as forces below 10 pN; rupture forces in this range were not considered when data were analyzed.

Transmission Electron Microscopy (TEM) – Electron microscopy of purified αIIbβ3 heterodimers was performed as previously described (23). Rotary-shadowed samples were prepared using a modification of standard procedures (24) by spraying a dilute solution of molecules in a volatile buffer (0.05 M ammonium formate) and glycerol (30-50%) onto freshly-cleaved mica and shadowing with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ). All specimens were examined in a FEI/Philips 400 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ), operating at 80 kV and at a magnification of 60,000.

RESULTS

Synthesis and Characterization of an αIIb TM peptide - We prepared a 22-residue peptide, designated αIIb-TM, encompassing residues Trp968-Lys989 of the αIIb TM domain, a length we previously determined to be optimal for αIIb TM domain homodimerization (4). Two lysine residues were then added at each terminus of the peptide to enhance its solubility, inhibit the formation of peptide aggregates, and facilitate its insertion into membranes (25) (Table 1). We also prepared mutant peptides in which one or both of the Gly residues of the critical αIIb GX₃G motif were changed to Leu, as well as the unrelated peptide MS-1, a model membrane peptide that forms dimers and trimers in membranes (26).

Circular dichroism (CD) spectroscopy of αIIb-TM (Fig. 1a, b) and LX₃L (not shown) in micelles (2.5 mM DPC, CMC 1.0 mM) and phospholipid vesicles (500 µM phospholipid, POPC:POPG 7:3) revealed minima at 208 and
222 nm, confirming that both peptides assume an α-helical secondary structure in micelles and phospholipid bilayers. Further, analytical ultracentrifugation demonstrated that both αIIb-TM and FITC-labeled αIIb-TM assemble into homodimers in zwitterionic C14-betaine and DPC micelles, as we had shown previously for a peptide that encompassed the αIIb TM and cytoplasmic domains (5). The pKdiss values for αIIb-TM and FITC-αIIb-TM in betaine micelles (expressed in mole fraction units, the concentration of peptide in the detergent micellar phase) were 1.8±0.3 and 3.0±1.0, respectively (Supplement Fig. 1).

Next, the propensity of αIIb-TM to insert into phospholipid vesicles was examined using fluorescence spectroscopy. Measurement of the maximum fluorescence emission (λmax) of the Trp residues in αIIb-TM revealed a shift from 351 nm to 340 nm as the peptide inserted into the less polar environment of the lipid vesicle (Fig. 1c). A λmax value of 340 nm is consistent with emission from a Trp located in the polar headgroup region of a bilayer (12). Further, at a peptide concentration of 2.0 μM, the shape of the binding curve exhibited near linear titration behavior at low lipid concentrations, indicating that the peptide binds very tightly to the vesicles with an estimated Kdiss in the low micromolar range.

While the Trp fluorescence experiments indicated that αIIb-TM binds to POPC/POPG vesicles, polarized attenuated total reflection infrared (ATR-IR) spectroscopy was used to confirm that αIIb-TM is capable of forming a TM α-helix in this environment. ATR-IR spectroscopy exploits the fact that in an ordered sample, a given bond will absorb infrared radiation differentially depending on the polarization of the light and the angle at which the bond is oriented relative to the polarized light. If the secondary structure of a peptide is known, the dichroic ratio (RATR) of the amide-I absorbance when the incident light is polarized at 0° to the amide absorbance when the light is polarized at 90° can be used to calculate the angle between the helical axis of the peptide and the bilayer normal. The amide-I vibration observed at 1656 cm⁻¹ indicated that αIIb-TM adopted primarily an α-helical conformation in the POPC/POPG bilayers, consistent with the CD data (Fig. 1d). The RATR of 4.7 corresponds to an angle of ~15°, indicating that the peptide was inserted nearly perpendicular to the plane of the bilayer.

The αIIb TM Peptide Binds to αIIbβ3 in Micelles and Membranes - Two approaches were taken to determine whether αIIb-TM can associate with full-length αIIbβ3. First, FITC-αIIb-TM, either alone or mixed with purified αIIbβ3, was loaded onto a Superdex 200 HR 10/30 size exclusion column and the amount of fluorescently-labeled peptide in the eluted fractions was measured. As shown in Fig. 2a, the fluorescence intensity of eluted fractions decreased progressively when increasing amounts of αIIbβ3 were present and a new peak of early eluting fluorescence appeared. SDS-PAGE and immunoblotting of the early peak revealed that it contained αIIbβ3, confirming that the elution of FITC-αIIb-TM was affected by its association with αIIbβ3. By contrast, when the gel-filtration was performed using LX3L instead of αIIb-TM, there was no decrease in the LX3L peak in the presence of increasing amounts of αIIbβ3 (data not shown), implying that the GX3G motif in αIIb-TM plays a critical role in its interaction with αIIbβ3.

Second, the physical association of FITC-αIIb-TM with αIIbβ3 was detected by measuring the fluorescence anisotropy of peptide/αIIbβ3 mixtures. As shown in Fig. 2b, measurement of FITC-αIIb-TM anisotropy as a function of αIIbβ3 concentration resulted in a binding isotherm with an apparent Kdiss of 2.6±1.1 ×10⁻⁵ in mole fraction units, consistent with high affinity binding of αIIb-TM to full-length αIIbβ3.

The experiments described above demonstrated that αIIb-TM interacts with full-length αIIbβ3 in detergent micelles. The TOXCAT assay was then used to address
whether the peptide can bind to the TM domain of αIIb in a membrane bilayer (16). In TOXCAT, a chimeric protein consisting of an N-terminal ToxR' DNA binding domain, a C-terminal maltose-binding protein domain, and an interposed TM domain is expressed in the inner membrane of E. coli (16). TM domain-mediated dimerization of the chimeric protein drives the transcriptional activation of a chloramphenicol acetyl transferase (CAT) reporter gene. The ability of exogenously-added αIIb-TM to inhibit CAT synthesis was used as a measure of its association with the TM domain of the chimeric protein. αIIb-TM inhibited CAT synthesis driven by a chimeric protein containing the wild type αIIb TM domain by 44±3% (Fig. 3a). Replacing the first glycine in αIIb-TM’s GXαG motif with Leu (LXαL) significantly impaired the ability of the peptide to inhibit CAT synthesis (30±1%, p>0.02) and replacing both glycines with Leu (LXαG) reduced inhibition to that of the solvent control (4.8±0.1% vs. 4.7±0.1%, respectively). Thus, these data demonstrate that not only can αIIb-TM associate the αIIb TM domain in a lipid bilayer, but that the peptide’s GXαG motif plays an essential role in this interaction.

TOXCAT was also used to confirm that the interaction of αIIb-TM with the αIIb TM domain was specific. TM domains from the integrin subunits β3, α2, and β1 were substituted for the αIIb domain in the TOXCAT chimera and the ability of the exogenous αIIb-TM peptide to inhibit CAT synthesis was measured. As shown in Fig. 3b, αIIb-TM again significantly inhibited CAT synthesis induced by the αIIb TM domain-containing chimera, but there was no change in CAT synthesis when the chimera contained the β1 TM domain and there were non-significant increases in CAT synthesis when chimeras contained either the β3 or the α2 TM domains (145% and 118%, p<0.11 and p<0.16, respectively).

The αIIb-TM Peptide Induces Platelet Aggregation by Directly Activating αIIbβ3 - The ability of αIIb-TM to interact with the αIIb TM domain in situ in platelets was assessed by its ability to induce platelet aggregation. Platelets undergo fibrinogen-dependent, αIIbβ3-mediated aggregation when stimulated by agonists such as ADP, a process preceded by a change in platelet shape from a disc to a spiny sphere (17). Adding micromolar concentrations of αIIb-TM to suspensions of gel-filtered human platelets rapidly induced platelet aggregation (Fig. 4a). Like ADP-stimulated aggregation, αIIb-TM-induced aggregation was inhibited by EDTA. But unlike ADP, peptide-induced aggregation was unaffected by the adenylyl cyclase agonist PGE1 or the ADP scavenger apyrase and was not accompanied by platelet shape change (Fig. 4a-c). This suggests that αIIb-TM induced platelet aggregation independent of platelet signal transduction or secreted ADP by interacting directly with αIIb.

To verify that αIIb-TM interacts specifically with αIIb, the ability of αIIb-TM LXαL and the unrelated peptide MS1 to induce platelet aggregation was tested. As shown in Fig. 4d, neither 3 μM LXαL nor MS1 induced platelet aggregation, although increasing the LXαL concentration nearly 7-fold to 20 μM had a partial effect. Thus, these results indicate that the interaction of αIIb-TM with αIIb in platelets is specific and requires the presence of a GXαG motif.

Because fibrinogen binding to αIIbβ3 is a prerequisite for platelet aggregation (1), αIIb-TM-induced binding of FITC-labeled fibrinogen to platelets was assessed using flow cytometry (18). As shown by the histograms in Fig. 5, both 20 μM ADP and 2.0 μM αIIb-TM-induced FITC-fibrinogen binding to gel-filtered platelets. In both instances, fibrinogen binding was prevented by EDTA, consistent with fibrinogen binding to αIIbβ3.

Platelets express a second β3-containing integrin, αvβ3, that mediates agonist-stimulated platelet adhesion to surfaces coated with the matrix protein OPN (27). Because agonist-induced binding of OPN to αvβ3 on platelets can be specifically measured using laser
tweezers (22), this methodology was used to address whether αIlb-TM can also activate αvβ3. However in contrast to ADP, αIlb-TM did not induce a peak of specific rupture forces between platelets and beads coated with OPN (Supplement Fig. 2). Thus, these results demonstrate that αIlb-TM specifically recognizes the TM domain of αIlb despite the presence of the closely related integrin α subunit αv.

Although αIlb-TM induced platelet aggregation was not inhibited by pre-incubating platelets with PGE1 or apyrase, it is still conceivable that the aggregation tracings resulted from peptide-induced platelet lysis, rather than platelet aggregation. This possibility was addressed in three ways (Supplement Fig. 3). First, αIlb-TM did not induce leakage of the small fluorescent molecule Tb(DPA)3 from phospholipid vesicle at peptide/lipid ratios up to 1:10. Second, the peptide did not cause lysis of erythrocytes at concentrations as high as 10 µM. Third, the peptide did not cause the leakage of 14C-serotonin from platelet dense granules in the absence of platelet aggregation.

**αIlb-TM Activates Recombinant αIlbβ3 Expressed by CHO Cells** - The ability of αIlb-TM to activate αIlbβ3 independent of signal transduction was corroborated by measuring fibrinogen binding to recombinant αIlbβ3 expressed by CHO cells using laser tweezers-based force spectroscopy (28). The results of these experiments are shown in Fig. 6. CHO cells readily express recombinant αIlbβ3 that cannot be activated by cellular agonists. Thus, in the absence of αIlb-TM, rupture forces between fibrinogen-coated beads and αIlbβ3-expressing CHO cells were < 20 pN and resulted from non-specific protein-protein interactions (28). By contrast, when the cells were incubated in Mn2+-containing media, up to 10% of rupture forces were > 20 pN, with a characteristic peak at 70 pN. The effect of adding αIlb-TM was similar to Mn2+: in this case ≈ 7% of rupture forces were > 20 pN with a rupture force maximum at 70 pN. Further, there was a substantial reduction in the rupture force peak when αIlb-TM was added along with either the αIlbβ3 antagonist abciximab (Fig. 6d) or the divalent cation chelator EDTA, confirming that the peak resulted from the rupture force of fibrinogen bound to activated αIlbβ3.

**TEM of αIlbβ3 in the Presence or Absence of αIlb-TM** - Integrins are inactive when their TM-containing stalks are in proximity and active when the stalks separate (23,29). Thus, as we showed previously, TEM of inactive purified αIlbβ3 in buffer containing octyl glucoside and 1 mM CaCl2 revealed that the majority of the αIlbβ3 molecules had a closed configuration with their stalks touching at the their tips (Fig. 7, CaCl2; Table 2) (23). By contrast, when αIlb-TM was present, most of the αIlbβ3 molecules had an open configuration with separated stalks (Fig. 7, CaCl2+αIlb-TM). These observations are consistent with the notion that αIlb-TM activates αIlbβ3 by disrupting the TM heterodimer maintaining the integrin in its inactive state. It is noteworthy that when αIlbβ3 is activated by Mn2+ (23), nearly 40% of the open molecules are present as dimers and higher order oligomers. However, when αIlbβ3 was shifted to an open configuration by αIlb-TM, only 0.8% of the molecules were present as dimers and no higher order oligomers were observed. This result suggests that although αIlb-TM is able to induce an active αIlbβ3 conformation, oligomerization of the active molecules cannot occur because the required oligomerization site on αIlb is occupied by the peptide.

**DISCUSSION**

The ability of integrins to bind to ligands with high affinity is regulated by cell metabolism (1). This is most apparent for integrins on circulating blood cells where integrin function is desirable under only specific circumstances; it is less apparent for adherent cells, but is equally important, since cycles of integrin activity and inactivity are necessary for
cell movement (30). Integrins reside on cell membranes in an equilibrium between low affinity (inactive) and high affinity (active) conformations (31). The agonist-mediated shift between these conformations appears to involve the disruption of intra-molecular interactions involving subunit TM and/or cytoplasmic domains (1). The most extensively studied intra-molecular interaction involves conserved membrane-proximal cytoplasmic domain sequences (32). These sequences are thought to form a low affinity activation-constraining "clasp", a notable feature of which is a salt-bridge between a conserved α subunit Arg and a conserved β subunit Asp (33). There is also mounting evidence that TM domain sequences participate in integrin regulation. These domains, including those of αIβ and β3, can undergo both heteromeric (2,34,35) and homomeric (5) interactions, but it is likely that heteromeric interactions are paramount in constraining integrin activity. In the case of αIββ3, for example, single disruptive amino acid replacements in the TM domain of either αIβ or β3 are sufficient to induce constitutive αIββ3 activity when the mutants are expressed in tissue culture cells (36).

Despite the mutational data cited above, proteins corresponding to the wild-type αIβ and β3 TM and cytoplasmic domains undergo homomeric, not heteromeric, association in micelles (5). This raises the possibility that an exogenous αIβ TM peptide would bind in a homomeric manner to the αIβ TM domain and cause platelet aggregation by disrupting the constraining αIβ/β3 TM domain heterodimer. However, TM segments are inherently hydrophobic and would not be expected to be soluble in aqueous buffers. Nonetheless, peptides corresponding to such segments can be solubilized in aqueous buffers by appending polar residues (e.g. lysine) to their amino- and carboxyl-termini (25,37,38). Such peptides insert spontaneously into lipid membranes where they fold into α-helices and assume their native monomeric or oligomeric states (25,38). Based on these observations, we synthesized a 22-residue αIβ TM peptide and added two lysine residues to each end to enhance its solubility. As expected, the peptide was soluble in aqueous buffer and rapidly inserted into small unilamellar phospholipid vesicles where it assumed an α-helical conformation. Further, the peptide formed homodimers in detergent micelles, bound with high affinity to purified αIββ3, and as shown by the TOXCAT assay, interacted specifically with the αIβ TM domain rather than the TM domains of β3, β1, or α2.

The plasma membrane of platelets is a mosaic of at least 83 different proteins (39), including five different integrins (1). Nonetheless, αIββ3 is the only platelet protein known to support platelet aggregation. Thus, the ability of αIβ-TM to cause platelet aggregation implies that the peptide can recognize αIβ in this complex environment. Normally, αIββ3 activity is regulated by “inside-out” signals initiated by specific platelet agonists, several of which, notably ADP and thromboxane A2, are either released or generated by the platelets themselves (40). Thus, it is essential to be certain that αIβ-TM did not activate αIββ3 by causing inside-out signaling. Agonist-stimulated platelet function can be prevented when signal transduction is interrupted by increases in the platelet content of cAMP. This is usually accomplished by exposing platelets to the prostaglandins PGI2 or PGE1. However, pre-incubating platelets with PGE1 had only a minimal effect on αIβ-TM-induced aggregation, strong evidence that the effect of the peptide was independent of signal transduction. Further, as would be predicted from the lack of a PGE1 effect, the ADP-ase apyrase did not inhibit αIβ-TM-induced aggregation, nor was αIβ-TM-induced aggregation accompanied by platelet shape change, an agonist-induced phenomenon.

Turbidometric platelet aggregation measures the increase in light transmission that occurs when platelets in suspension aggregate. However, platelet lysis, by increasing light
transmission, could be mistaken for aggregation and could be an alternative explanation for the aggregometry results discussed above. But we found that αIIb-TM did not cause the release of serotonin from non-aggregating platelets, as would occur if the peptide had disrupted the integrity of the platelet plasma membrane. Nor did it disrupt the integrity of unilamellar liposomes or cause red cell lysis. Thus, platelet lysis is not a tenable explanation for aggregation tracings shown in Fig. 4.

Mutating the GX₃,G motif in αIIb-TM impaired its ability to bind to the αIIb TM domain and to activate αIIbβ3. First recognized as a framework for the homomeric association of the glycoporphin A (GpA) TM domain, GX₃,G has been consistently identified as the most over-represented sequence motif in TM domain databases (41). In GpA, GX₃,G permits extensive backbone-backbone contacts at the homodimer interface because a groove created by the glycines in one monomer packs tightly against a ridge created by the side chains of the residues that succeed each glycine in the other. Previously, we expressed an extensive series of single-site mutations of the αIIb TM domain in TOXCAT and found that its GX₃,G motif was essential for dimerization (4). The mutagenesis data were then used to construct an atomic model for an αIIb TM domain dimer that placed GX₃,G in the dimerization interface, although in this model, residues preceding the glycines formed the ridge that packed against the glycine-created groove (42). The GX₃,G mutations in αIIb also resulted in constitutive αIIbβ3 activation when αIIbβ3 was expressed in CHO cells, implying that GX₃,G is not only involved in homomeric αIIb TM domain interactions, but in heteromeric αIIb/β3 TM domain interactions as well (6). That being the case, the ability of αIIb TM to activate αIIbβ3 indicates that the tendency of the αIIb TM domain to interact homomERICally is substantially greater than its tendency to interact heteromERICally with β3, a suggestion consistent with the behavior of small proteins corresponding to the αIIb and β3 TM and cytoplasmIC domains. It also suggests that sequestration of the αIIb GX₃,G motif in an αIIb/β3 heterodimer is important factor for maintaining αIIbβ3 in an inactive state.

There is a substantial body of experimental evidence that integrins are inactive when their TM and/or cytoplasmIC domains are in proximity. Thus, replacing the cytoplasmIC domains of αLβ2 and α5β1 with complementary acidic and basic peptides maintains the integrins in a low affinity state (43, 44). Likewise, using TEM, we found that the tips of the stalks of most αIIbβ3 molecules purified from unstimulated platelets appeared to be touching in the absence of αIIb-TM. But when the peptide was present at a concentration sufficient to activate αIIbβ3 in platelets and CHO cells, the majority of molecules had separated stalks. These observations are entirely consistent with the “push-pull” hypothesis for integrin regulation and imply that TM domain interactions alone are at least sufficient to regulate integrin activity.

Clusters of αIIbβ3 molecules have been detected on thrombin-stimulated platelets (45) and we found αIIbβ3 present in patches on the surface of CHO cells expressing constitutively active αIIbβ3 due to mutations in the αIIb or β3 TM domains (6, 36). TEM images of Mn²⁺-activated αIIbβ3 revealed that nearly 40% of the molecules formed dimers, trimers, and higher order oligomers via interactions involving the distal ends of αIIb and β3 stalks (23). It is noteworthy then that αIIbβ3 oligomers were rarely seen when purified αIIbβ3 was incubated with αIIb-TM, even though most of the molecules were in an open active conformation. Because small proteins corresponding to the αIIb and β3 TM and cytoplasmIC domains tend to undergo homomERIC, rather than heteromERIC, association (5), it is likely that the clustering of Mn²⁺-activated αIIbβ3 resulted from the homomERIC interaction of these domains in the intact protein. The paucity of αIIbβ3 oligomers when αIIbβ3 is incubated with αIIb-TM is consistent with this conclusion because in the
presence of the peptide, there would be no free αIIb TM domains to support polymerization. Whether homomeric αIIb and β3 TM domain association participates in αIIbβ3 clustering on agonist-stimulated platelets is not known. Nonetheless, these observations suggest that such interactions can occur and could be responsible for the formation of the signaling complexes that mediate signal transduction following ligand binding to integrins.

In summary, we report that a lysine-flanked peptide corresponding to the αIIb TM domain not only binds to αIIbβ3 when the purified integrin is present in detergent micelles and in bacterial membranes, but also induces platelet aggregation by interacting directly with αIIbβ3 in the platelet membranes. Thus, these observations demonstrate that TM domain interactions regulate integrin function in situ and that it is possible to target intra-membranous protein-protein interactions in a way that can have functional consequences.

REFERENCES

1. Bennett, J. S. (2005) J. Clin. Invest. 115(12), 3363-3369
2. Luo, B. H., Springer, T. A., and Takagi, J. (2004) Plos Biology 2(6), 776-786
3. Partridge, A. W., Melnyk, R. A., Yang, D., Bowie, J. U., and Deber, C. M. (2003) J. Biol. Chem. 278(24), 22056-22060
4. Li, R., Gorelik, R., Nanda, V., Law, P. B., Lear, J. D., DeGrado, W. F., and Bennett, J. S. (2004) J. Biol. Chem. 279(25), 26666-26673
5. Li, R. H., Babu, C. R., Lear, J. D., Wand, A. J., Bennett, J. S., and DeGrado, W. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98(22), 12462-12467
6. Li, W., Metcalf, D. G., Gorelik, R., Li, R. H., Mitra, N., Nanda, V., Law, P. B., Lear, J. D., DeGrado, W. F., and Bennett, J. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102(5), 1424-1429
7. http://www.anaspec.com
8. Tanford, C., and Reynolds, J. A. (1976) Biochim. Biophys. Acta 457(2), 133-170
9. Kharakoz, D. P. (1997) Biochemistry 36(33), 10276-10285
10. Kochendoerfer, G. G., Salom, D., Lear, J. D., Wilk-Orescan, R., Kent, S. B., and DeGrado, W. F. (1999) Biochemistry 38(37), 11905-11913
11. Hammond, K., Caputo, G. A., and London, E. (2002) Biochemistry 41(9), 3243-3253
12. Caputo, G. A., and London, E. (2003) Biochemistry 42(11), 3275-3285
13. Arkin, I. T., MacKenzie, K. R., and Brunger, A. T. (1997) J. Am. Chem. Soc. 119(38), 8973-8980
14. Weisel, J. W., Nagaswami, C., Vilaire, G., and Bennett, J. S. (1992) J. Biol. Chem. 267(23), 16637-16643
15. Yin, H., Lee, G. I., Sedey, K. A., Rodriguez, J. M., Wang, H. G., Sebti, S. M., and Hamilton, A. D. (2005) J. Am. Chem. Soc. 127(15), 5463-5468
16. Russ, W. P., and Engelman, D. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96(3), 863-868
17. Bennett, J. S., and Vilaire, G. (1979) J. Clin. Invest. 64(5), 1393-1401
18. Basani, R. B., D’Andrea, G., Mitra, N., Vilaire, G., Richberg, M., Kowalska, M. A., Bennett, J. S., and Poncz, M. (2001) J. Biol. Chem. 276(17), 13975-13981
19. Heuck, A. P., Tweten, R. K., and Johnson, A. E. (2003) J. Biol. Chem. 278(33), 31218-31225
20. Liu, D., and DeGrado, W. F. (2001) J. Am. Chem. Soc. 123(31), 7553-7559
21. Litvinov, R. I., Shuman, H., Bennett, J. S., and Weisel, J. W. (2002) Proc. Natl. Acad. Sci. U. S. A. 99(11), 7426-7431
22. Litvinov, R. I., Vilaire, G., Shuman, H., Bennett, J. S., and Weisel, J. W. (2003) J. Biol. Chem. 278(51), 51285-51290
23. Litvinov, R. I., Nagaswami, C., Vilaire, G., Shuman, H., Bennett, J. S., and Weisel, J. W. (2004) Blood 104(13), 3979-3985
24. Weisel, J. W., Stauffacher, C. V., Bullitt, E., and Cohen, C. (1985) Science 230(4732), 1388-1391
25. Gerber, D., Sal-Man, N., and Shai, Y. (2004) J. Biol. Chem. 279(20), 21177-21182
26. Bilgicer, B., and Kumar, K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101(43), 15324-15329
27. Bennett, J. S., Chan, C., Vilaire, G., Mousa, S. A., and DeGrado, W. F. (1997) J. Biol. Chem. 272(13), 8137-8140
28. Litvinov, R. I., Vilaire, G., Li, W., DeGrado, W. F., Weisel, J. W., and Bennett, J. S. (2006) Biochemistry 45(15), 4957-4964
29. Kim, M., Carman, C. V., and Springer, T. A. (2003) Science 301(5640), 1720-1725
30. Hynes, R. O. (2002) Cell 110(6), 673-687
31. Takagi, J., and Springer, T. A. (2002) Immunol. Rev. 186, 141-163
32. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) Biochemistry 45(15), 4957-4964
33. Kim, M., Carman, C. V., and Springer, T. A. (2003) Science 301(5640), 1720-1725
34. Hynes, R. O. (2002) Cell 110(6), 673-687
35. Takagi, J., and Springer, T. A. (2002) Immunol. Rev. 186, 141-163
36. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) Biochemistry 45(15), 4957-4964
37. Hessa, T., Kim, H., Bihlmaier, K., Lundin, C., Boekel, J., Andersson, H., Nilsson, I., White, S. H., and von Heijne, G. (2005) Nature 433(7024), 377-381
38. White, S. H., and von Heijne, G. (2005) Curr. Opin. Struct. Biol. 15(4), 378-386
39. Moebius, J., Zahedi, R. P., Lewandrowski, U., Berger, C., Walter, U., and Sickmann, A. (2005) Mol Cell Proteomics 4(11), 1754-1761
40. Shattil, S. J., Kashiwagi, H., and Pampori, N. (1998) Blood 91(8), 2645-2657
41. Senes, A., Gerstein, M., and Engelman, D. M. (2000) J. Mol. Biol. 296(3), 921-936
42. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) Science 276(5309), 131-133
43. Takagi, J., Erickson, H. P., and Springer, T. A. (2001) Nat. Struct. Biol. 8(5), 412-416
44. Lu, C., Takagi, J., and Springer, T. A. (2001) J. Biol. Chem. 276(18), 14642-14648
45. Fox, J. E., Shattil, S. J., Kinlough-Rathbone, R. L., Richardson, M., Packham, M. A., and Sanan, D. A. (1996) J. Biol. Chem. 271(12), 7004-7011

FOOTNOTES
1 Abbreviations used: ATR-IR, attenuated total reflection infrared; CAT, chloramphenicol acetyltransferase; CD, circular dichroism; CHO, Chinese hamster ovary; CMC, critical micellar concentration; DMSO, dimethylsulfoxide; DMF, N,N'-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; DPA, dipicolinic acid; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; HATU, N,N',N',N'-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate; NMP, 1-methyl-2-pyrrolidinone; OPN, osteopontin; PGE1, prostaglandin E1; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TM, transmembrane.

ACKNOWLEDGEMENTS
We thank the NIH (HL40387, HL54500, GM60610 and GM54616) for support of this work.

FIGURE LEGENDS
Fig. 1. Biophysical characterization of αIIb-TM. Circular dichroism spectra of αIIb-TM in: a, 2.5 mM 1,2-dihexanoyl-sn-glycerol-3-phosphocholine (DPC, CMC 1.0 mM) and b, 500 µM unilamellar vesicles (POPC:POPG 8:2). c, Maximum Trp fluorescence emission (λmax) shifts along increasing concentrations of phospholipid vesicles (POPC:POPG 8:2). d, Polarized ATR-IR spectra of αIIb-TM reconstituted into vesicles (POPC:POPG 7:3). Shown are representative spectra from the same sample irradiated with light polarized at 0° (⊥) and 90° (∥). The amide-I vibration is centered at 1656 cm⁻¹ and exhibits an average dichroic ratio of 4.7.

Fig. 2. FITC-αIIb-TM binds to purified full-length αIIbβ3. a, Size exclusion gel filtration tracing of 1.0 µM αIIb-TM in the presence of 0 (1), 4.0 (2), and 8.0 (3) µM full-length αIIbβ3. Tracing (4) is that of 4 µM αIIbβ3 alone in 10 mM HEPES buffer, pH 7.5, containing 60 mM N-octyl-β-D-glucopyranoside, 0.5 mM CaCl₂, and 0.02% NaN₃. b, Anisotropic titrations of FITC-αIIb-TM with full-length αIIbβ3 in 10 mM HEPES buffer, pH 7.5, containing 60 mM N-octyl-β-D-glucopyranoside, 0.5 mM CaCl₂, and 0.02% NaN₃ at 25°C. Data are mean values ± S.D. of a set of 15 individual data points.

Fig. 3. αIIb-TM binds specifically to the TM domain of the integrin subunit αIIb. The ability of αIIb-TM to associate with the TM domains of αIIb and other platelet integrin subunits was examined using the TOXCAT assay as described in the “Experimental Procedures”. a, Effect of exogenous αIIb-TM and the mutant αIIb-TM peptides LX₃G and LX₃L on CAT synthesis. b, CAT synthesis induced by the TM domains of β3, α2, and β1 in the presence (+) and absence (-) of 3 µM αIIb-TM. Data shown are mean ± S.D. of four experiments.

Fig. 4. αIIb-TM causes the aggregation of gel-filtered human platelets. a, The extent of platelet aggregation as a function of αIIb-TM concentration; b, e, Platelet aggregation induced by 3 µM αIIb-TM (b) or 10 µM ADP (e) in the presence of 2.0 µM PGE₁, 10 units/ml apyrase, or 2.5 mM EDTA; d, Platelet aggregation induced by 3.0 µM αIIb-TM, by the mutant αIIb-TM peptide LX₃L at 3.0 µM and 20 µM, and by the unrelated TM helix MS1.

Fig. 5. Measurement of FITC-fibrinogen to gel-filtered human platelets by flow cytometry. a, Fibrinogen binding stimulated by 20 µM ADP. b, Fibrinogen binding stimulated by 20 µM ADP in the presence of 5 mM EDTA. c, Fibrinogen binding induced by 2 µM αIIb-TM. d, Fibrinogen binding induced by 2 µM αIIb-TM in the presence of 5 mM EDTA.

Fig. 6. Rupture force histograms of αIIb-TM induced-fibrinogen binding to recombinant αIIbβ3 expressed by Chinese hamster ovary (CHO) cells. The distribution of rupture forces ≥ 20 pN between CHO cells and beads coated with fibrinogen was measured using laser tweezers. a, Rupture forces measured between transfected CHO cells and fibrinogen-coated beads in the absence of αIIb-TM; b, Rupture forces measured between transfected CHO cells and fibrinogen-coated beads in the presence of 1 mM Mn²⁺; c, Rupture forces measured between transfected CHO cells and fibrinogen-coated beads in the presence of 3 µM αIIb-TM; d, Effect of the αIIbβ3 antagonist abciximab on the αIIb-TM induced interaction of fibrinogen-coated beads with transfected CHO cells; e, Effect of 1 mM EDTA on the αIIb-TM induced interaction of fibrinogen-coated beads with transfected CHO cells.

Fig. 7. Transmission electron microscopy of purified αIIbβ3 in CaCl₂-containing buffer alone and in CaCl₂-containing buffer + 3 µM αIIb-TM. Individual αIIbβ3 molecules were visualized using transmission electron microscopy after rotary shadowing with tungsten. The images of αIIbβ3 could be classified into 4 groups: (1) Closed structures with the tips of the αIIb and β3 stalks...
touching; (2) Open structures with the αIIb and β3 stalks separated; (3) Globular headpieces with no visible stalks; (4) Open αIIbβ3 dimers. Bar = 30 nm.
Table 1: Amino acid sequences of the αIIb TM and control peptides

|    | αIIb-TM          | LX3G           | LX3L           | MS1                |
|----|-----------------|----------------|----------------|--------------------|
|    | KKWVLVGVLGGLLLLTILVLAMWKKK | KKWVLVLVLGLLLLLTILVLAMWKKK | KKWVLVLVLGLLLLLTILVLAMWKKK | BQLLIAVLLLIATNLILLIAVARLRYLG (B = β-Ala) |

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Table 2: Analysis of TEM images of purified αIIbβ3 molecules dissolved in CaCl$_2$-containing buffer in the absence and presence of the αIIb-TM peptide

|                  | n   | Group 1: closed structures | Group 2: open structures | Group 3: globular structures |
|------------------|-----|----------------------------|--------------------------|-----------------------------|
| CaCl$_2$ alone   | 1106| 61±4%                      | 16±2%                    | 23±3%                       |
| CaCl$_2$+αIIb-TM * | 764 | 14±2%                      | 68±6%                    | 18±3%                       |

*The αIIb TM peptide and purified αIIbβ3 were incubated at a molar ratio of 10:1 for 30 min at 37°C. Differences within and between Groups 1 and 2 are statistically significant at p<0.001.
Fig. 2

a. 

Fluorescence (arbitrary units)

Elution volume (ml)

b. 

Anisotropy

αββ3/detergent
Fig. 4

(a) Graph showing the percentage aggregation of platelets with varying concentrations of 3.0 μM, 1.5 μM, 1.0 μM, 0.75 μM, 0.5 μM, and 0.25 μM. The graph includes the following treatments: +Apyrase +αIIb-TM +PGE₁.

(b) Graph showing the percentage aggregation of platelets with +EDTA treatment.

(c) Graph showing the percentage aggregation of platelets with ADP treatment.

(d) Graph showing the percentage aggregation of platelets with +PGE₁ +Apyrase +EDTA treatment.

Platelet shape change is indicated by the arrow.
Fig. 6

a. 

b. 

c. 

d. 

e. 

Rupture forces, pN 

Rupture forces, pN 

Rupture forces, pN 

Probability 

Probability 

Probability 

 Probability 

Rupture forces, pN 

Rupture forces, pN 

Rupture forces, pN 

Probability
Activation of platelet αIIbβ3 by an exogenous peptide corresponding to the transmembrane domain of αIIb

Hang Yin, Rustem I. Litvinov, Gaston Vilaire, Hua Zhu, Wei Li, Gregory A. Caputo, David T. Moore, James D. Lear, John W. Weisel, William F. DeGrado and Joel S. Bennett

J. Biol. Chem. published online October 10, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605877200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/10/11/M605877200.DC1