Divalent Cations Differentially Regulate Integrin α_{IIb} Cytoplasmic Tail Binding to β_3 and to Calcium- and Integrin-binding Protein*

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We have used recombinant or synthetic α_{IIb} and β_3 integrin cytoplasmic peptides to study their in vitro complexation and ligand binding capacity by surface plasmon resonance. αβ heterodimerization occurred in a 1:1 stoichiometry with a weak K_D in the micromolar range. Divalent cations were not required for this association but stabilized the αβ complex by decreasing the dissociation rate. αβ complexation was impaired by the R995A substitution or the KVGFFKR deletion in α_{IIb} but not by the β_3 S752P mutation. Recombinant calcium and integrin-binding protein (CIB), an α_{IIb}-specific ligand, bound to the α_{IIb} cytoplasmic peptide in a Ca^{2+}- or Mn^{2+}-independent, one-to-one reaction with a K_D value of 12 μM. In contrast, in vitro liquid phase binding of CIB to intact α_{IIb}β_3 occurred preferentially with Mn^{2+}-activated α_{IIb}β_3 conformers, as demonstrated by enhanced coimmunoprecipitation of CIB with PAC-1-captured Mn^{2+}-activated α_{IIb}β_3, suggesting that Mn^{2+} activation of intact α_{IIb}β_3 induces the exposure of a CIB-binding site, spontaneously exposed by the free α_{IIb} peptide. Since CIB did not stimulate PAC-1 binding to inactive α_{IIb}β_3 nor prevented activated α_{IIb}β_3 occupancy by PAC-1, we conclude that CIB does not regulate α_{IIb}β_3 inside-out signaling, but rather is involved in an α_{IIb}β_3 post-receptor occupancy event.

Integrins are αβ heterodimeric cell-surface receptors that promote not only adhesion to components present within the extracellular matrix or on the surface of opposite cells but also transfer information into and out of a cell (1). The adhesive functions of integrins can be regulated by intracellular processes referred to as “inside-out signaling.” Conversely, ligand binding to the extracellular domain of integrins initiates a cascade of intracellular events termed “outside-in signaling” that generate a large spectrum of cellular responses, such as cell migration, proliferation, differentiation, and gene expression (2). Integrin cytoplasmic tails appear to be key elements in these bidirectional signaling pathways, despite their short size as compared with other signaling receptors and the absence of any demonstrable catalytic activity (3, 4). Integrin α and β cytoplasmic domains are thought to mediate signaling events through modifications of their own structural and spatial organization and/or through interactions with specific cytoplasmic components. Various proteins have been identified that bind, at least in vitro, to the cytoplasmic tail of α and β subunits and are likely to play a role in regulating integrin signaling functions. These include cytoskeletal components such as talin and α-actinin, as well as several signaling or regulatory proteins such as integrin-linked kinase p59Flik, focal adhesion kinase p52FAK, Grb2, β3-endorphin, cytohesin-1, integrin cytoplasmic domain-associated protein ICAP-1, calreticulin and calcium- and integrin-binding protein CIB (reviewed in Refs. 5 and 6).

Recently used methods for studying protein-protein interactions, such as the two-hybrid system, have allowed the identification of integrin-specific intracellular ligands (7–12). These methods are based on the use of a unique linear amino acid sequence as a bait and consequently do not take into account the secondary and tertiary structural features of the interacting molecules. However, numerous studies tend to demonstrate that α and β cytoplasmic domains adopt a defined conformation and that the preservation of these structural constraints is crucial to maintain the functional properties of integrin receptors (13–20).

One of the best studied integrins is the platelet fibrinogen receptor, integrin α_{IIb}β_3, that undergoes conformational changes necessary for receptor function. In order to elucidate further the structural relationship of the cytoplasmic tails of α_{IIb} and β_3, we have used surface plasmon resonance biosensor technology to monitor real time assembly of the integrin α_{IIb} and β_3 cytoplasmic tails and to investigate their ligand binding capacity.

EXPERIMENTAL PROCEDURES

Antibodies and Synthetic Peptides—The anti-β_3 monoclonal antibody (mAb) 4D10G3, the anti-β_3 cytoplasmic domain mAb C3a.19.5, and the anti-α_{IIb} mAb SL1.3 were kindly provided by Dr. D. R. Phillips (Cor Therapeutics, South San Francisco, CA), the anti-β_3 mAb D3GP3 by Dr. L. K. Jennings (University of Tennessee, Memphis, TN), and the anti-α_{IIb}β_3 complex-specific mAb 10E5 by Dr. B. S. Coller (Mount Sinai School of Medicine, New York, NY). The anti-α_{IIb}β_3 mAb PAC-1 was 1

1 The abbreviations used are: CIB, calcium- and integrin-binding protein; CHO, Chinese hamster ovary; ConA, concanavalin A; GST, glutathione S-transferase; HEL, human erythroleukemia; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; RU, resonance unit; SPR, surface plasmon resonance; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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from Becton-Dickinson (San Jose, CA), and the anti-human α, mAb VNR 139 was from Life Technologies, Inc. (Merelbeke, Belgium). Polyclonal anti-α,β3 antibodies were raised in rabbits against purified human platelet α,β3. Synthetic peptides corresponding to either the wild type α, cytoplasmic domain (α, Lys99–Gln108), the α, cytoplasmic domain with a C-terminal sequence deleted of the 995KVGFFKR995 motif (α, ΔLys99–Gln108) were all purchased from NeoSystem (Strasbourg, France).

Platelets and Cell Lines—Outdated platelet concentrates were kindly provided by Dr. J.-C. C. Faber (Luxembourg Red Cross Blood Transfusion Center). The stable transfected CHO cell line A06, expressing high levels of human α, integrin (100 × 200 μm diameter) with a 0–40% linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The amino acid sequence of the recombinant peptide was checked by microsequencing on an Applied Biosystems Procise sequencer. The purified peptide was stored dessicated at 4 °C.

Construction of pGEX-4T-2 Expression Plasmids—The cDNA encoding the wild type or S762P mutant human β3 integrin cytoplasmic tail (Lys716–Thr726) was generated by the polymerase chain reaction (PCR) using full-length pBlJ-β3 plasmands as templates (21). The upstream (sense) primer was a 28-mer with a BamHI site (GATCC) corresponding to the β3 nucleotide sequence 2245–2266, 5′-GATCCAAACTCCCTACAGTCGAC-3′. The downstream (antisense) primer was a 30-mer corresponding to the β3 nucleotide sequence 2365–2388 and comprising an Smal restriction site (CCC GGG) followed by a stop codon 5′-CCCCGGGTATGCTCCTGACCTAATT-3′. PCR amplification was performed using the Takara PCR kit (Shiga, Japan). The full-length cDNA encoding human CIB was obtained by reverse transcriptase-PCR (RT-PCR) of HEL-5J20 cell mRNA. Briefly, total RNA was isolated from 5 × 109 cells according to the method of Chomczynski and Sacchi (23), and RT-PCR was performed using the RNA-seq cDNA synthesis kit from Promega (Madison, WI). The sense primer was a 36-mer corresponding to the α, cytoplasmic domain (α, Lys99–Gln108) motif (α, ΔLys99–Gln108) and was incubated for 30 min at room temperature.

purification, the thrombin hydrolsate was extensively dialyzed against PBS and then passed through a glutathione-Sepharose column in order to remove GST. The flow-through fraction containing CIB was kept frozen at −20 °C until use.

Preparation of α,β3 or α,β3-enriched Glycoprotein Concentrates—For preparation of α,β3-enriched glycoprotein concentrates, outdated platelets were washed and then lysed in 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM Tris-HCl (TBS), pH 7.4, containing 1% Triton X-100, 10 μM leupeptin, 500 μM PMSF, 2 mM N-ethylmaleimide, 0.2% Na3VO4, according to the procedure described by Fitzgerald et al. (24). The lysate was applied to a unconcanavalin A (ConA)-Sepharose 4B (Amersham Pharmacia Biotech) column (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.0, 0.1% Triton X-100 (TBS/ConA), and bound glycoproteins were eluted with 100 mM α-methyl-D-mannose in running buffer. For α,β3-enriched glycoprotein concentrates, α,β3-expressing CHO cells (cell clone A06) were detached with EDTA buffer, pH 7.4, for 10 min at 37 °C and then washed in cold PBS. Cells (5–10 × 106) were lysed for 30 min in 500 μl of ice-cold lysis buffer, pH 7.5 (150 mM NaCl, 50 μM aminothylenbenzenesulfonic fluoride, 1% Triton X-100, 10 mM Tris-HCl), and the lysate was precleared by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was incubated for 2 h at 4 °C with 1 volume of 50% slurry ConA-Sepharose 4B suspension. After extensive washes with cold TBS/ConA, bound glycoproteins were eluted as described above and kept on ice until use.

α,β3-binding to Immobilized Human Fibrogenin—α,β3 binding to fibrogenin was determined according to Kounis et al. (25) with some modifications. 96-well microtiter plates (Costar, Cambridge, MA) were coated overnight at 4 °C with 100 μg/well purified human fibrogenin (Sigma, Bornem, Belgium) at 5 μg/ml in TBS. The plates were then saturated with TBS containing 3.5% bovine serum albumin and 0.05% Na3VO4, (125 μg/ml) overnight at 4 °C. The ConA-enriched platelet glycoprotein fraction was serially diluted in TBS/ELISA alone (TBS containing 1% bovine serum albumin, 0.035% Triton X-100) or in TBS/ELISA containing either 2 μg/ml D3GP3 mAb or 10 μM MnCl2, 100-μl aliquots were added to the wells and incubated for 1 h at room temperature. After three washes with TBS, 1 μg/ml polyclonal rabbit anti-α,β3 antibodies in TBS/ELISA were added (100 μl/well) for 2 h at room temperature. The wells were washed three times with TBS, followed by 90 min incubation at room temperature with 100 μl/well donkey anti-rabbit Ig antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). After three washes with TBS, 100 μl of 0.1 mg/ml 3,3′,5′,5′-tetramethylbenzidine, 0.01% H2O2 in 140 mM sodium acetate-citrate buffer, pH 6.0, were dispensed into each well. The enzymatic reaction was stopped by addition of 25 μl of 2% H2SO4, and the absorbance was measured at a wavelength of 450 nm.

In Vitro Liquid Phase α,β3-CIB Binding Assays—ConA-purified platelet glycoproteins (250 μg) or α,β3-enriched CHO-A06 cell glycoproteins (1 mg) were incubated with 50 μg of purified GST-CIB or GST alone for 2 h at 4 °C under gentle stirring. Experiments were carried out in ice-cold 0.1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, containing either 2 μM CaCl2, 2.5 mM EGTA, or 1 mM CaCl2, 1 mM EGTA. Blood samples (1 ml) were added to the cell lysate, which was preincubated for 30 min at room temperature in 10 mM MnCl2 or in 2% -free buffer before incubation with GST fusion proteins. For α,β3 capture, the mixtures were incubated with 5 μg of the 10E5 mAb for 2 h at 4 °C or, alternatively, with 4 μg of the mAb PAC-1 for 2 h at room temperature followed by 5 μg of rabbit anti-mouse μ-chain-specific antibodies (Jackson Immunoresearch, West Grove, PA) for an additional 2 h at 4 °C. Protein A-Sepharose CL 4B beads (80 μl of a 50% slurry suspension) were added and incubated for 2 h at 4 °C. For GST fusion protein capture, the mixtures were incubated for 2 h at 4 °C with 80 μl of 50% slurry glutathione-Sepharose 4B suspension. Control experiments were performed using non-substituted Sepharose CL4B. The adsorbsents were washed three times with 500 μl of the respective ice-cold incubation buffer, and the captured proteins were recovered by boiling the beads in 30–50 μl of 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 25 μg/ml bromphenol blue in 15.625 mM Tris-HCl, pH 6.8. Each sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting as described below.

Protein Assay, Electrophoresis, and Western Blot Analysis—Protein concentration was determined using the Bio-Rad Protein assay reagent. SDS-PAGE was performed at room temperature on a Mini Protean II gel electrophoresis system (Bio-Rad), and Tris-Tricine SDS-PAGE was carried out according to the method described by Schagger and von Jagow (26). Electrophoresed samples were transferred onto Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech) using a semi-dry transblot apparatus (Amersham Pharmacia Biotech). The membranes were blocked overnight in blotting buffer (5% dry milk, 0.1% Tween 20, 150
mm NaCl, 20 mM Tris·HCl, pH 7.4), and incubated for 2 h with the anti-β3 4D10G3 mAb mixed with either the anti-αIIb S1.3 mAb or the anti-αV NVR 139 mAb diluted in blotting buffer. After three washes in blotting buffer, membranes were incubated for 1 h with diluted sheep anti-mouse Ig conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Membranes were again washed three times in blotting buffer and then in 137 mM NaCl, 20 mM Tris·HCl, pH 7.4 (TBS/WB), and developed using the chemiluminescence ECL kit (Pierce) according to the manufacturer’s instructions. The membranes were then stripped by successive washes in TBS/WB containing 100 mM β-mercaptoethanol, 2% SDS, 32.5 mM Tris·HCl, pH 6.7, for 30 min at 50 °C, and again in TBS/WB. After an overnight incubation in blotting buffer, the membranes were reprobed for 2 h with polyclonal goat anti-GST antibodies (Amersham Pharmacia Biotech), and antibody binding was detected as described above with horseradish peroxidase-conjugated rabbit anti-goat IgG (Jackson Immunoresearch).

**Surface Plasmon Resonance Binding Studies**—Real-time biomolecular interaction analysis was performed using the Biaevaluate™ or Biacore XT™ instruments (Biacore, Uppsala, Sweden). Purified proteins were covalently attached to carboxymethyl dextran (CM5) chips (Biacore) running buffers either Biacore HBS (150 mM NaCl, 3.4 mM EDTA, turer’s instructions. Experiments were performed at 25 °C using as ethyl-

For the transformation of crude data, the preparation of overlay plots, and the determination of kinetic parameters of the binding reactions were performed using the Biaevaluation 3.0 software. The association rate constant (kₐ) and the dissociation rate constant (kₐ₋ₐ) were determined separately from individual association and dissociation phases, respectively, assuming a one-to-one interaction. The affinity constant (Kₐ) was calculated as kₐ/kₐ₋ₐ. Experimental values from the first 20 s at the beginning of each phase were not considered in the fitting to avoid distortions due to injection and mixing.

**RESULTS**

**Generation of Recombinant Wild Type or Mutant β3 Integrin Cytoplasmic Peptides and of CIB**—In order to perform in vitro studies of αIIb and β3 cytoplasmic tail association, we generated recombinant GST fusion proteins containing a thrombin cleavage site and corresponding to the entire wild type or S752P mutant cytoplasmic domain of the β3 integrin (residues 716–762) and to the αIIb-binding protein CIB (residues 1–191). The fusion proteins were isolated from bacterial cell lysates by glutathione affinity chromatography, and the GST-β3 proteins were further immunopurified using the anti-β3 monoclonal antibody (mAb) C3a.19.5. Thrombin-released wild type β3 cytoplasmic tail peptide and CIB were purified free of GST using reverse-phase HPLC and glutathione affinity chromatography, respectively. The accurate amino acid sequence of the β3 peptides was confirmed by microsequencing. SDS-PAGE analysis of isolated proteins revealed a purity greater than 98%, as evaluated by densitometric scanning of the gel (Fig. 1). The apparent molecular masses of both GST-β3 and GST-β3(S752P) (94 kDa), GST-CIB (48 kDa), CIB (25 kDa), and GST (29 kDa) were in good agreement with the predicted mass deduced from the amino acid composition. When analyzed with higher resolution SDS-PAGE and on a C18 HPLC column, the 5.6-kDa β3 peptide appeared homogeneous with only slight impurities (Fig. 2).

**In Vitro Complex Formation of αIIb and β3 Integrin Cytoplasmic Domains as Monitored by SPR Analysis**—In vitro complex formation of wild type αIIb and β3 integrin cytoplasmic tails was investigated by surface plasmon resonance (SPR) in order to monitor real-time biomolecular interactions. We first examined whether the synthetic αIIb peptide was able to bind to the β3 cytoplasmic tail fused to GST. Purified anti-GST polyclonal antibodies were immobilized on a sensor chip through amine coupling and were allowed to stably capture native GST or the GST-β3 fusion protein before injection of the αIIb peptide. As shown in Fig. 3A, a characteristic binding signal was monitored when the peptide was brought into contact with captured GST-β3, but not with either an uncoated surface, immobilized antibodies alone, or GST-antibody complexes. In these latter control experiments, the rapid change in the resonance signal was due to a dilution buffer-induced, nonspecific change in the bulk refractive index. The maximum response monitored at the end of the peptide injection phase was about 60–70 RU for 1100 RU of initially captured GST-β3 protein. The corresponding molar ratio was estimated at ~0.8 mol/mol and was consistent with a 1:1 interaction. Further studies showed that αIIb binding was dose-dependent and could be almost completely inhibited by soluble GST-β3 protein but not by GST alone (Fig. 3, B and C). Taken together, these data demonstrate that αIIb specifi-
Divalent Cations—

In order to determine the role of divalent ions in integrin αIIbβ3 cytoplasmic tails, the experimental sensorgrams were recorded on a BiaLite apparatus using TBS/Bia as running buffer and a flow rate of 5 μl/min (except for B which is 50 μl/min). Data are given as absolute responses. A, response curves recorded during and after injection of 41.7 μM αIIb peptide on an uncoated surface (curve 1) or on a chip with immobilized polyclonal anti-GST antibodies before (curve 4) and after capture of ~1100 RU of GST (curve 3) or GST-β3 (curve 2). GST/antibody interaction was very stable over time with a decrease in signal of only 1–2 RU/min. B, overlaid dose-response binding curves obtained with various concentrations of αIIb peptide injected over a GST-β3-coated chip surface (~12,500 RU). C, interaction of covalently immobilized GST-β3 (~12,500 RU) with 41.7 μM of αIIb peptide preincubated with 1.5 mg/ml free purified GST (curve 2) or GST-β3 (curve 1). The binding curve obtained in the absence of the competitor is shown as a reference (curve 3).

The αIIbβ3 Cytoplasmic Domain Complex Is Stabilized by Divalent Cations—In order to determine the role of divalent cations in integrin αIIbβ3 cytoplasmic domain association, we investigated the binding of αIIb to immobilized GST-β3 in the presence of 2 mM CaCl2 or MgCl2. The influence of Ca2+ on the interaction was apparent from the slopes of the sensorgrams in Fig. 4. Interestingly, the rates of association of αIIb with GST-β3 were similar, independent of the presence or absence of Ca2+. In contrast, the dissociation of αIIb was more rapid in the absence of Ca2+, as demonstrated by a greater slope in the curve. Similar results were obtained with a low cation concentration (50 μM) or with a Mg2+-containing buffer (data not shown). To characterize further the dynamic parameters of the interaction, we determined binding isotherms in the presence or absence of Ca2+ by injecting αIIb peptide solutions ranging from 4.2 to 83.3 μM over a GST-β3 fusion protein-coated chip (Fig. 3B). From these curves, the association and dissociation rates and the apparent Kd of the binding were determined as indicated under “Experimental Procedures.” As shown in Table I, the on rates with or without Ca2+ were very similar. In contrast, the peptide dissociation was slower when Ca2+ ions were present in the flow as compared with Ca2+-free buffer. This resulted in an increased affinity, although the Kd value was still in the range of weak interactions. Taken together, these data suggest that divalent ions have a different effect on the association and dissociation of αIIb and β3 cytoplasmic tails.

In Vitro α/β Heterodimerization Is Impaired by the R995A Substitution or the KVGFKR Deletion within the αIIb Cytoplasmic Tail but Not by the S752P Substitution in the β3 Cytoplasmic Domain—Several mutations or deletions within the αIIb and β3 cytoplasmic tails have been shown to disturb αIIb-β3-mediated signaling, such as the αIIb (R995A) substitution, the αIIb membrane-proximal GFFKR truncation, or the β3 (S752P) point mutation (15, 28, 29). To investigate the influence of these mutations on the in vitro αβ complexation, a chip coated with GST-β3 was used. The binding curves obtained following the injection of equimolar solutions of αIIb (R995A) or αIIb (A389G390G1008) peptides were strongly reduced as compared with the curve monitored with wild type αIIb (Fig. 5A). Residual binding calculated from these curves 20 s after the end of the sample injection, using wild type αIIb peptide binding as a 100% reference, were only ~15% for the substituted mutant and ~20% for the deleted mutant. In contrast, sensorgrams obtained when wild type αIIb peptide was injected over GST-β3 or GST-β3 (S752P) were superimposable (Fig. 5B). The presence of 2 mM Ca2+ in the running buffer only slightly improved...
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Experiments were performed on a Biacore instrument using a flow rate of 50 μl/min and TBS/Bia buffer ± 2 mM CaCl2 as running and dilution buffer. The association (k_{on}) and dissociation (k_{off}) rate constants were determined using the Biacore evaluation analysis software, from data recorded for a range of synthetic αIIb peptide concentrations (4.2–83.3 μM) injected over a GST-β3 fusion protein-coated surface (~12,500 RU). The affinity constants (K_{D}) were calculated as k_{on}/k_{off} from curve-fitting analysis as described under “Experimental Procedures.” The kinetic data presented are the mean values ± S.D. of at least two separate experiments.

| Buffer                    | k_{on} ± σ  | k_{off} ± σ | K_{D} ± σ |
|---------------------------|-------------|-------------|----------|
| Without CaCl2 (n=3)       | 4.5 ± 1.0   | 2.2 ± 1.0   | 50.0 ± 21.0 |
| 2 mM CaCl2 (n=2)          | 5.5 (±1.3)  | 0.41 (±0.05) | 7.7 (±0.9) |

The affinity constants were generated using the Biaevaluation analysis software, from fitting analysis as described under “Experimental Procedures.” The interaction in the absence or presence of 2 mM CaCl2 were essentially the same, independent of the presence or absence of Ca^{2+}, and revealed a weak affinity of 12 μM.

αβ dimerization and stabilization, suggesting that the β3 C-terminal part is not involved in these processes.

Calcium-independent Interaction of CIB with the αIIb Cytoplasmic Tail—We have used purified recombinant CIB as a control reporter protein to monitor αIIb cytoplasmic tail ligand binding functions. As shown in Fig. 6A, a binding signal was recorded when the αIIb peptide was passed over sensorchips with antibody-captured GST-CIB but not with anti-GST antibodies alone, GST, or ethanolamine-substituted dextran, indicating that the interaction occurred specifically between αIIb and CIB. The molar ratio calculated from these curves was ~0.7 mol/mol and was consistent with a 1:1 stoichiometry. Binding experiments performed in the presence or absence of 2 mM CaCl2 in the running buffer showed that CaCl2 did not significantly modify either the association or the dissociation phase (Fig. 6B), demonstrating that the αIIb interaction with CIB was Ca^{2+}-independent, despite the fact that CIB is a Ca^{2+}-binding protein (12). The on and off rates, and the apparent K_{D}, calculated as described previously from overlaid sensograms, are summarized in Table II. As predicted from the sensograms in Fig. 6B, all the dynamic parameters were essentially the same, independent of the presence or absence of Ca^{2+}, and revealed a weak affinity of 12 μM.

The αIIb Cytoplasmic Peptide Can Bind Simultaneously to the β3 Cytoplasmic Tail and to CIB—We further examined whether the binding of the αIIb peptide to antibody-captured GST-CIB was influenced by the β3 cytoplasmic tail. Samples of αIIb mutant binding to GST-β3 (22% residual binding for both). Calcium stabilized the interaction of the wild type αIIb peptide with GST-β3 (S752P) in the same way as with GST-β3 and kinetic parameters determined for the αIIb/GST-β3 (S752P) interaction in the absence or presence of 2 mM CaCl2 were essentially the same as those obtained with wild type β3 (data not shown). These results demonstrate that the αIIb membrane-proximal KVGGFKR sequence and the αIIb residue Arg995 are crucial for the formation of αβ complexes, as well as their subsequent stabilization by divalent cations. In contrast, the S752P mutation in the β3 cytoplasmic tail does not affect

**Fig. 5.** SPR analysis of the binding properties of αIIb and β3 cytoplasmic tail mutants. Sensorgrams were monitored on a Biacore X instrument using Ca^{2+}-free TBS as running and dilution buffer and a flow rate of 20 μl/min. Data are expressed as relative responses after subtraction of the background signal recorded on a reference surface made up of ethanolamine-substituted dextran matrix. A, 100 μM peptide solutions of either wild type αIIb (curve 1), αIIb (R995A) (curve 2), or αIIb (K98m–Gln1008) (curve 3) were passed over a chip with immobilized GST-β3 (~7300 RU). B, 50 μM wild type αIIb peptide solution was injected over a chip with ~2000 RU of antibody-captured GST-β3 (curve 1) or GST-β3 (S752P) (curve 2).

**Fig. 6.** Characterization by SPR analysis of the interaction between αIIb cytoplasmic domain and CIB. The experiments were performed on a Biacore X instrument using TBS/Bia as running and dilution buffer and a flow rate of 20 μl/min. Data are given as relative responses. A, αIIb peptide (41.7 μM) was passed over a chip with immobilized polyclonal anti-GST antibodies before (curve 2) or after capture of ~1300 RU of GST (curve 3) or GST-CIB (curve 1). B, sensograms recorded during the interaction of 41.7 μM of αIIb peptide with GST-CIB (~1200 RU) previously captured by immobilized anti-GST antibodies. The experiments were performed with TBS/Bia either alone or complemented with 2 mM CaCl2.
TABLE II

Kinetics of the interaction of αIIb peptide with GST-CIB fusion protein as a function of [Ca^{2+}]

Sensogram and curves collected for a range of αIIb peptide concentrations (4.2–83.3 μM) interacting with antibody-captured GST-CIB fusion protein (1200–1400 RU). Measurements were carried out on a Biacore X instrument at a flow rate of 50 μl/min using TBS/Bia ± 2 mM CaCl₂ as running and dilution buffer. On and off rates (with $K_0 = k_{on}k_{off}$) were evaluated using Bioevaluation software as described under "Experimental Procedures." Kinetic data presented are the mean values ± S.D. of three separate experiments.

| Buffer | $k_{on} ± σ$ | $k_{off} ± σ$ | $K_0 ± σ$ |
|--------|-------------|-------------|-----------|
| Without CaCl₂ ($n = 3$) | 1.0 ± 0.2 | 1.3 ± 0.2 | 12.0 ± 1.0 |
| 2 mM CaCl₂ ($n = 3$) | 1.2 ± 0.2 | 1.4 ± 0.2 | 12.0 ± 3.0 |

FIG. 7. Interaction of the αIIb β₃ cytoplasmic tail complex with GST-CIB fusion protein. Experiments were carried out on a Biacore X apparatus at a flow rate of 20 μl/min. TBS/Bia without CaCl₂ was used as running and dilution buffer. Data are expressed as relative responses. Both αIIb and β₃ peptides were injected at a concentration of 40 μM. Sensograms were obtained using an antibody-captured GST-CIB chip surface (1700–1800 RU) after sequential injection (A) of each of the peptides (curve 1, αIIb, followed by β₃; curve 2, β₃ followed by αIIb) or after a single injection (B) of the two peptides previously incubated together for at least 15 min at room temperature with (curve 3) or without 2 mM CaCl₂ (curve 4). Sample injections are indicated by arrows.

whereas the α/β mixture preincubated in the presence of Ca²⁺ supported an enhanced resonance signal (Fig. 7B). Comparable results were obtained when the experiments were carried out with running buffer containing 2 mM CaCl₂ (data not shown). considering that the SPR response is directly related to the mass of the analyte adsorbed on the chip surface, we conclude from these results that binding of preformed αIIb/β₃ complexes was responsible for the difference observed with the reference signal recorded with the αIIb peptide alone. These data indicate that αIIb and β₃ cytoplasmic tails can form a multimolecular complex together with CIB, suggesting that the regions in the αIIb amino acid sequence implicated in the interaction with β₃ and CIB are distinct. Since the αIIb membrane-proximal region has been shown to contain the key contact sites for β₃ binding, CIB interaction is likely to involve the αIIb C terminus.

The Membrane-proximal KVGFFKR Domain and the Arg⁹⁹⁵ Residue of αIIb Cytoplasmic Tail Are Also Required for Optimal αIIb Binding to CIB—To delineate further the contact site of CIB within the αIIb cytoplasmic tail, we investigated the binding of αIIb(R995A) and αIIb(Asn⁹⁸⁶–Gln¹⁰⁰⁸) peptides to GST-CIB. Interestingly, the sensograms obtained with both mutants were markedly reduced, as compared with the binding curve monitored with the wild type αIIb peptide (Fig. 8), although residual binding of ~30% persisted for the two mutants independent of the presence or absence of calcium. These results indicate that CIB binding to αIIb does not rely exclusively on the C-terminal part of αIIb and that the KVGFFKR sequence or the Arg⁹⁹⁵ residue within αIIb are required for optimal binding.

Calcium-independent Binding of CIB to Intact Platelet αIIbβ₃—To confirm further the calcium-independent interaction of CIB with the αIIb cytoplasmic domain, we performed in vitro liquid phase binding assays using intact αIIbβ₃. For this purpose, an αIIbβ₃-rich glycoprotein fraction was prepared from platelets by ConA affinity chromatography. Similarly, an αIIbβ₃-enriched glycoprotein concentrate was prepared from CHO cells expressing human αIIbβ₃ for control experiments. SDS-PAGE and Western blot analysis demonstrated that these samples contained appreciable levels of β₃ integrins (Fig. 9A, lanes 2 and 3). Platelet or CHO-A06 cell glycoproteins were incubated with GST-CIB or GST alone in the presence of 2 mM CaCl₂ or 2.5 mM EGTA. GST fusion proteins were recovered using glutathione-Sepharose beads, and bound proteins were analyzed by SDS-PAGE and Western blotting using anti-αIIb,
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with crude sample than with fractions incubated either with the activating anti-β3 mAb D3GP3 or with 10 mM Mn2+, demonstrating that the ConA-purified platelet extract contained essentially inactive αIIbβ3 and minor amounts of activated αIIbβ3.

To investigate further whether Mn2+-induced activation of platelet αIIbβ3 was able to influence αIIbβ3/CIB interaction, we performed in vitro binding studies in the presence or absence of MnCl2. Interestingly, the binding of αIIbβ3 to GST-CIB was more pronounced in the presence of 10 mM MnCl2 than in Mn2+-free buffer (Fig. 10B). Also, αIIbβ3 heterodimers were undetectable in competitive assays using an excess of αIIb cytoplasmic peptide or in control experiments performed with purified GST alone, demonstrating that the specificity of the interaction was not modified by Mn2+. To confirm further these results, we studied the binding of CIB to platelet αIIbβ3 as a function of Mn2+ by immunoprecipitation experiments. Crude or Mn2+-treated platelet glycoproteins were first incubated with purified GST-CIB and then with the anti-αIIbβ3 complex-specific mAb 10E5 to retain total αIIbβ3, or with the anti-β3 fibronogen-mimetic mAb PAC-1 to capture activated αIIbβ3. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting using anti-αIIb, anti-β3, and anti-GST antibodies. As shown in Fig. 11, αIIbβ3 binding to PAC-1 was weak in the absence of Mn2+ (B, lane 1) and was significantly enhanced in 10 mM MnCl2 buffer (B, lane 2), demonstrating that the activated αIIbβ3 conformers were increased in the Mn2+-treated platelet glycoprotein concentrate. In Mn2+-free buffer, few GST-CIB immunoprecipitated with 10E5-captured αIIbβ3 (A, lane 3), whereas GST-CIB was not detectable with PAC-1-bound αIIbβ3, probably on account of the small recovery in αIIbβ3 obtained with this sample, containing a weak proportion of active heterodimers (B, lane 3). Conversely, Mn2+ treatment of the platelet glycoproteins led to a marked increase in GST-CIB coprecipitation in experiments performed with either 10E5 or PAC-1 mAb (A and B, lane 4). This effect was particularly apparent from the level of GST-CIB coprecipitated in 10E5 assays, which was significantly greater in the presence of 10 mM Mn2+ than in Mn2+-free buffer, although the amount of captured αIIbβ3 remained unchanged (A, lanes 3 and 4). As expected from our previous assays, addition of αIIb cytoplasmic peptides to the incubation mixture almost completely inhibited GST-CIB binding to platelet αIIbβ3 (A and B, lane 5), and no GST was coprecipitated with αIIbβ3 (A and B, lane 6). Taken together, these results demonstrate that CIB binds preferentially to the Mn2+-treated, activated form of αIIbβ3 integrin. These data further suggest that CIB is unlikely to have a regulatory effect on αIIbβ3 ligand binding function, since its interaction with αIIbβ3 does not stimulate PAC-1 binding to inactive αIIbβ3 nor inhibit Mn2+-activated αIIbβ3 occupancy by PAC-1.

DISCUSSION

SPR technology has been successfully used by several authors to analyze the interaction of structural domains of receptor cytoplasmic tails with their intracellular targets, such as the interaction of cytoplasmic tail sorting sequences with the adaptor proteins AP-1, AP-2, and AP-3 (31–33) or of growth factor receptors with the Src homology 2 (SH2) or the phosphotyrosine-binding (PTB) domains of tyrosine kinases (34, 35). SPR has also been used to characterize the interaction of various integrin receptors with extracellular matrix proteins (36–38), ligand-mimetic antibodies (39, 40), or with integrin counter-receptors (41). In this report, we have used SPR technology to study in vitro complexation of the α- and β-cytoplasmic tails of integrin αIIbβ3 as data from mutagenesis, spectroscopic, and computer modeling studies indicate this heterodimerization.

anti-αIIb, anti-β3, and anti-GST antibodies. As shown in Fig. 9B (panel 1), GST-CIB was able to retain αIIbβ3 independent of the presence or absence of Ca2+, whereas GST alone was not. In contrast, no αIIbβ3 integrin was detected in either GST-CIB- or GST-containing samples incubated with the αIIbβ3-enriched fraction (Fig. 9B, panel 2). In competitive inhibition experiments, an excess of free αIIb cytoplasmic peptide abrogated αIIbβ3/GST-CIB coprecipitation (Fig. 9B, panel 3). Taken together, these results demonstrate that CIB binds specifically to intact platelet αIIbβ3 in a Ca2+-independent manner.

CIB Binds Preferentially to Mn2+-activated αIIbβ3—Since both inactive and active αIIbβ3 conformers are isolated from platelet lysate by ConA affinity chromatography (30), we determined the activation state of the αIIbβ3 heterodimers present in our ConA-purified platelet extract by examining the binding capacity of αIIbβ3 to immobilized fibrinogen. Fig. 10A shows that specific αIIbβ3 binding was significantly weaker
concentrations of crude ConA-purified platelet glycoproteins were incubated for 4 h at room temperature with 2 µg/ml D3GP3 mAb (A), 10 mM MnCl₂, or buffer alone (B) in 96-well microtiter plates coated with 5 µg/ml human fibrinogen. After washing, bound α₁bβ₃ was detected by ELISA using rabbit anti-α₁bβ₃ antibodies as stated under “Experimental Procedures.” The figure is representative of two independent experiments, each data point corresponding to the mean of duplicates. B, ConA-purified platelet glycoproteins (250 µg) were incubated with 50 µg of purified GST-CIB or GST in the presence (+) or absence (−) of 10 mM MnCl₂. Binding inhibition experiment was performed using 25 nmol of α₁bβ₃ cytoplasmic peptide. Proteins were recovered using glutathione-Sepharose beads and were analyzed by 7.8% reducing SDS-PAGE and by Western blot. The membrane was first probed with anti-β₃ (4D10G3) and anti-α₁b (S1.3) mAbs (upper panel), stripped and reprobed with anti-GST antibodies (lower panel). Two nonspecific bands of high electrophoretic mobility were observed with the GST-CIB-containing samples.

The affinity of the interaction was determined in both the presence and absence of MnCl₂. In contrast to previous studies, in which the binding partners were immobilized on surfaces (A), our results demonstrate that the affinity of the interaction is low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65).

In vitro GST-CIB binding to platelet α₁bβ₃ as a function of MnCl₂. ConA-purified platelet glycoproteins (250 µg) were incubated with GST-CIB (50 µg) and, α₁bβ₃ complexes were immunoprecipitated using either the anti-α₁bβ₃ mAb 10E5 (A) or the fibrinogen-mimetic mAb PAC-1 (B) and analyzed by SDS-PAGE and Western blot as described in the legend to Fig. 10. Samples were analyzed as follows: platelet glycoproteins in Mn²⁺ free buffer (lane 1) or in 10 mM MnCl₂ buffer (lane 2); platelet glycoproteins incubated with GST-CIB in Mn²⁺ free buffer (lane 3), 10 mM MnCl₂ buffer (lane 4), 10 mM MnCl₂ buffer containing 25 nmol of α₁bβ₃ cytoplasmic peptide (lane 5); platelet glycoproteins incubated with purified GST in 10 mM MnCl₂ buffer (lane 6). The affinity of the interaction was determined in both the presence and absence of MnCl₂. In contrast to previous studies, in which the binding partners were immobilized on surfaces (A), our results demonstrate that the affinity of the interaction is low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65). These affinities are low when compared with affinities determined using intact platelets (64, 65).

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are not required for αIIbβ3 complexation but rather stabilize the heterodimeric structure by reducing the dissociation rate. Since αβ association proceeded with the same extent, independent of the presence of cations, the cation coordination sites within αIIb and β3 cytoplasmic tails are likely to be distinct from those involved in intersubunit binding, namely the membrane-proximal region of each integrin subunit. Interestingly, our study indicates that the αIIb membrane-proximal region is involved in divalent cation-induced αβ complex stabilization, since an alteration in the KVGFFKR sequence impaired the stability of the heterodimers. In support of these results, a functional cation binding domain has previously been mapped to the negatively charged acidic C-terminal region of αIIb (residues 999–1008) and was found to bind divalent cations in coordination with sites located in the αIIb 985–998 sequence (16, 19). Based on the structural model of αIIbβ3, Haas and Plow (19) speculated that a cation coordination site rearrangement (19) increased in active αIIbβ3 complexes that mimic the cytoplasmic tails. Since Mn²⁺ activation or ligand occupancy (49, 50). Alternatively, an increase in αIIbβ3 avidity for CIB cannot be excluded, since the fibrinogen-mimetic mAb PAC-1 used to capture active αIIbβ3 is a multimeric IgM antibody and is thus likely to trigger oligomerization of αIIbβ3 complexes that mimic integrin clustering (51). Finally, our data provide evidence that CIB is unlikely to have a regulatory effect on αIIbβ3 ligand binding function, since its interaction with αIIb does not trigger ligand binding to inactive αIIbβ3 nor inhibit activated αIIbβ3 occupancy by a ligand, suggesting that CIB is most likely involved in αIIbβ3 post-receptor occupancy events.

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