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Expression and Function of Calcium Binding Domain Chimeras of the Integrins α_{IIb} and α_5*

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To further identify amino acid domains involved in the ligand binding specificity of $\alpha_{IIb}\beta_3$, chimeras of the conserved calcium binding domains of α_{IIb} and the α subunit of the fibronectin receptor $\alpha_5\beta_1$ were constructed. Chimeras that replaced all four calcium binding domains, replaced all but the second calcium binding domain of α_{IIb} with those of α_5, or deleted all four calcium binding domains were synthesized but not expressed on the cell surface. Additional chimeras exchanged subsets or all of the variant amino acids in the second calcium binding domain, a region implicated in ligand binding. Cell surface expression of each second calcium binding domain mutant complexed with β_3 was observed. Each second calcium binding domain mutant was able to 1) bind to immobilized fibrinogen, 2) form fibrinogen-dependent aggregates after treatment with dithiothreitol, and 3) bind the activation-dependent antibody PAC1 after LIBS 6 treatment. Soluble fibrinogen binding studies suggested that there were only small changes in either the K_d or B_{max} of any mutant. We conclude that chimeras of α_{IIb} containing the second calcium binding domain sequences of α_5 are capable of complexing with β_3, that the complexes are expressed on the cell surface, and that mutant complexes are capable of binding both immobilized and soluble fibrinogen, suggesting that the second calcium binding domain does not determine ligand binding specificity.

Integrins function as adhesion receptors that mediate cell-extracellular matrix, cell-cell, and cell-soluble ligand interactions (1). The platelet integrin $\alpha_{IIb}\beta_3$ (membrane glycoprotein IIb-IIIa) functions to mediate platelet-platelet and platelet-subendothelial matrix interactions through the binding of its ligands: fibrinogen, von Willebrand factor, thrombospondin, fibronectin, and vitronectin (2, 3). Although progress has been made in elucidating ligand structure and potential ligand binding sites in integrins, questions regarding areas that determine both ligand binding and ligand binding specificity in $\alpha_{IIb}\beta_3$ remain. While integrins bind diverse ligands, three themes are apparent in ligand binding. First, many ligands have an acidic amino acid, usually aspartic acid, in the recognition sequence (4, 5). Certain integrins, including $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$, bind ligands that contain the tripeptide sequence arginine-glycine-aspartic acid (RGD) (6), while the recognition motif of others is more varied (7). Second, the ligand recognition sequence is usually a short peptide presented on an extended loop containing a β-turn (5). For example, the crystal and NMR structures of the ligand recognition sequence of fibronectin have been determined (8, 9), revealing the RGD sequence to be present on an extended loop with a β-turn. Although not as well defined, NMR studies have shown that the fibrinogen C-terminal γ-chain peptide assumes a helical conformation in solution with a β-turn centered at residues 408 and 409 (10, 11). Third, ligand binding specificity is varied. For example, while $\alpha_5\beta_1$ binds primarily to fibronectin, $\alpha_{IIb}\beta_3$ is a more promiscuous receptor and binds to multiple ligands.

Much work has been done to determine areas of $\alpha_{IIb}\beta_3$ involved in ligand binding. Previous studies have implicated multiple ligand interaction areas on both subunits, leading to models in which the ligand binding pocket is proposed to be formed by amino acids contributed by both subunits (12, 13). The $\alpha_{IIb}\beta_3$ complex must undergo a conformational change before it is capable of high affinity binding of fibrinogen, but the resting complex is still capable of binding small peptide ligands. Site-directed mutagenesis (14), peptide and antibody inhibition (15–18), chemical cross-linking studies (19), and analysis of thrombasthenic mutations (20–23) have implicated two areas of β_3, amino acids 109–133 and 212–222, in ligand binding. Recently, a third area, amino acids 274–368, has been reported to bind fibrinogen (24).

Multiple areas of α_{IIb} appear to be important in ligand binding. A recombinant truncated fragment, amino acids 171–464, has been shown to bind fibrinogen in a calcium-dependent manner (25), while ligand binding specificity has been localized to the first 334 amino acids of α_{IIb} using $\alpha_{IIb}\alpha_v$ chimeras (26). Site-directed mutagenesis of residues in a predicted β-turn in the third N-terminal repeat of α_{IIb}, α_4, and α_5 (amino acids 184–193 of α_{IIb}) has shown the region to be critical for ligand binding (27, 28). A synthetic peptide corresponding to α_{IIb}-(656–667) has been demonstrated to bind to soluble fibrinogen, and both the peptide and antibodies to the peptide inhibit platelet aggregation (29). Only one area of the $\alpha_{IIb}\beta_3$ has been shown to bind the fibrinogen γ-chain HHLGGAKQAGDV (H12) peptide in a calcium-dependent manner. Chemical cross-linking of the H12 peptide followed by proteolysis identified the second calcium binding domain of α_{IIb}, amino acids 296–314, as a critical region for binding the fibrinogen carboxyl-terminal γ-chain peptide (30). High affinity binding of the H12 peptide requires calcium and $\alpha_{IIb}\beta_3$ to be in the activated conformation (31). Peptides corresponding to amino acids 296–306 or 300–312 blocked platelet aggregation and bound directly to fibrinogen. Antibodies against α_{IIb}-(296–306) blocked binding of fibrinogen to either the peptide or $\alpha_{IIb}\beta_3$ (32, 33).

Amino acids 296–312 in α_{IIb} define one of four calcium binding domains of α_{IIb}. The integrin calcium binding domains are homologous with the "EF-hand" helix-loop-helix motif found in calmodulin and other proteins (34, 35). However, integrin cat-

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ion binding domains are missing the flanking helices and one of six divalent cation coordination sites. These coordination sites are presented as a linear array of amino acids having precisely spaced oxygenated side chains at positions 1, 3, 5, 9, and (in true EF-hands) 12. Position 7 contributes a coordination site through its main chain carbonyl. The divalent cation binding domains in integrins are missing the oxygenated, nearly invariant, glutamic acid residue at position 12, having a small hydrophobic residue instead. It has been postulated that the acidic residue in the ligand contributes the final coordination site (4) and that the metal ion, the ligand, and the receptor form a transient ternary complex before the metal ion dissociates (36). The second calcium binding domain of α_{IIb} and α_5 occur in a region of high homology, having 80% sequence identity over 35 amino acids.

In this paper, we examine the expression, complex formation, and function of a series of chimeric $\alpha_{IIb}\alpha_5\beta_3$ molecules in which we substituted clusters of the divergent α_5 amino acids into the second calcium binding domain of α_{IIb}. The mutations were made as chimeras in order to preserve both calcium and RGD binding, while probing ligand binding specificity. The results indicate that these mutants are expressed on the cell surface, complex with β_3 but not β_1, and function in a manner indistinguishable from wild type $\alpha_{IIb}\beta_3$, indicating that the second calcium binding domain of $\alpha_{IIb}\beta_3$ does not determine ligand binding specificity.

EXPERIMENTAL PROCEDURES

Materials—EA.hy 926, an endothelial-adenocarcinoma hybrid cell, λ-gt11 cDNA library was provided by C.-J. Edgell (University of North Carolina, Chapel Hill, NC) (37). A partial-length α_{IIb} was the gift of M. Poncz (University of Pennsylvania, Philadelphia, PA). A λ-gt11 liver cDNA library was provided by L. Brass (University of Pennsylvania). Cloned α_5 cDNA was provided by R. Juliano (University of North Carolina). Bluescript KS was from Stratagene (San Diego, CA). The plasmids pcDNAI/AMP and pRc/CMV were from Invitrogen (San Diego, CA). Oligonucleotides were obtained from the University of North Carolina Department of Pathology Oligonucleotide Synthesis Facility. The T7-GEN *In Vitro* Mutagenesis Kit was from U. S. Biochemical Corp. (Cleveland, OH). Horseradish peroxidase-conjugated streptavidin, LipofectAMINE, and tissue culture media and supplements, except for fetal bovine serum (FBS),[1] were from Life Technologies, Inc. FBS was from either HyClone (Logan, UT) or Irvine Scientific (Santa Clara, CA). Pefabloc SC was obtained from Roche Molecular Biochemicals. Protein A-Sepharose, GammaBind Plus Sepharose, and gelatin Sepharose were from Amersham Pharmacia Biotech. NHS-LC-biotin was obtained from Pierce. Biotinylated molecular weight markers, gelatin-agarose, and fluorescein isothiocyanate (FITC)-Celite were from Sigma. The ECL chemiluminescence detection system was from Amersham Pharmacia Biotech. Peptides were from the University of North Carolina Protein Chemistry Laboratory. Purified fibrinogen and fibronectin were the gift of L. Parise (University of North Carolina). Human fibrinogen, plasminogen- and von Willebrand factor-depleted, for soluble fibrinogen binding studies, was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Chelex 100 was from Bio-Rad. Other reagents were from standard sources.

Antibodies—The $\alpha_{IIb}\beta_3$ complex-specific monoclonal antibody (mAb) AP2 (38) was supplied by T. Kunicki (Scripps Research Institute, La Jolla, CA). Anti-β_3 mAb AP3 (39) and rabbit polyclonal anti-α_{IIb} SEW 8 were provided by P. Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI). The mAb Tab (40), specific for α_{IIb}, was provided by R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The mAbs A2A9 (41), recognizing the $\alpha_{IIb}\beta_3$ complex, and B1B5 (42), recognizing an epitope on α_{IIb}, were provided by J. Bennett (University of Pennsylvania, Philadelphia, PA). The $\alpha_{IIb}\beta_3$ complex-specific mAb 10E5 (43) was provided by B. Coller (Albert Einstein University, New York, NY). The anti-hamster β_1 mAb 7E2 (44) was provided by R. Juliano (University of North Carolina). The mAb LIBS 6, specific for β_3 (45), was provided by M. Ginsberg (Scripps Research Institute). PAC1 murine IgM (46) was from University of Pennsylvania Cell Center (Philadelphia, PA). Monoclonal antibodies against synthetic peptides containing the N- and C-terminal fibrinogen Aα RGD motifs (Aα 87–100 and Aα 566–580, respectively) were provided by Z. Ruggeri (Scripps Research Institute). Monoclonal antibody 4A5 (47) against the fibrinogen γ-chain H12 sequence was provided by Gary Matsueda (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). FITC-conjugated goat anti-mouse IgG $F(ab')_2$ was from Fisher. FITC-conjugated rabbit anti-mouse IgM was from Zymed Laboratories Inc. (South San Francisco, CA). FITC-conjugated chicken anti-human fibrinogen was from Biopool International (Ventura, CA). Normal mouse serum, goat anti-mouse IgG, goat anti-rabbit IgG, horseradish peroxidase-goat anti-mouse IgG, horseradish peroxidase-goat anti-rabbit IgG, and alkaline phosphatase-conjugated goat anti-rabbit IgG were from Sigma. Primary antibodies were used as either ascites fluid or as the IgG fraction after purification on either Protein A-Sepharose or GammaBind Plus Sepharose.

Isolation of α_{IIb} and β_3 cDNA Clones—A full-length α_{IIb} cDNA clone was isolated from a λ-gt11 liver cDNA library using restriction fragments of a partial-length α_{IIb} clone as probes. Partial-length cDNA clones of β_3 were isolated from both the liver cDNA library and an EA.hy 926, endothelial-adenocarcinoma hybrid cell, λ-gt11 cDNA library. All cDNA isolates were subcloned into Bluescript KS. Full-length β_3 was constructed from two partial clones that overlapped at the internal *Eco*RI site. The β_3 clone isolated had a G2069A mutation encoding a C655Y substitution. The mutation was repaired by subcloning a *Sap*I–*Mlu*I fragment containing the correct sequence from an EA.hy926 partial clone.

Construction of Mutant α_{IIb} and β_3 cDNA Clones—All clones were constructed using standard mutagenic and subcloning techniques. A cDNA encoding β_3 with a silent mutation destroying the internal *Eco*RI site, $\beta_3\Delta E$, was prepared using the T7-GEN *In Vitro* Mutagenesis Kit. Silent mutations in α_{IIb} creating *Bst*BI and *Nde*I sites at nucleotides 771 and 1411, respectively, were made simultaneously using the *dut-*, *ung-* method (48). This clone, named BN, was used to make IIbα5, a chimeric cDNA where all four calcium binding domains of α_{IIb} were replaced with the calcium binding domains of α_5 (amino acids 230–436), and ΔCa, an in-frame deletion of all four calcium binding domains of α_{IIb} (amino acids 234–438) (Fig. 1*A*). To construct IIbα5, the calcium binding domains of α_5 were amplified by PCR using oligonucleotides that contained *Bst*BI and *Nde*I sites, respectively, and that reconstructed the α_{IIb} sequence from the restriction site up to the start of the calcium binding domains. ΔCa was made by annealing oligonucleotides CGAACCCAGAGTACTTCGACGGCGCA and TATGCGCCGTCGAAGTACTCTGGGTT and ligating into BN digested with *Bst*BI and *Nde*I. The mutant D2 was made by subcloning the second calcium binding domain of α_{IIb} into IIbα5 using a subcloning and PCR strategy that precisely transferred α_{IIb}-(286–315) into IIbα5.

Second calcium binding domain mutants YAVAA, LD, and LMD were made using the *dut-*, *ung-* method after subcloning the cDNA into M13mp18. YAVAA-LD, YAVAA-LMD, and LD-LMD were made by using the appropriate second mutagenic oligonucleotide after isolating the first mutant. YAVAA-LD-LMD was made by using the YAVAA and LMD oligonucleotides simultaneously with the LD cDNA. The sequence of the second calcium binding domain of the mutant constructs is shown in Fig. 1*B*. The cDNAs were isolated from double-stranded M13mp18 by digestion with *Eco*RI and subcloned into Bluescript KS. α_{IIb} was subcloned into the expression vector pcDNAI/AMP by digestion with *Eco*RV and *Xba*I. α_{IIb} was also transferred into pRc/CMV, an expression vector containing the *neo* gene, by digesting the vector with *Hin*dIII, blunting with Klenow, and digesting with *Xba*I. The vector was then ligated with the *Eco*RV–*Xba*I fragment of α_{IIb}. Mutant constructs were transferred to the expression vectors by subcloning the *Not*I–*Nru*I fragment of each mutant into similarly digested α_{IIb}. $\beta3\Delta E$ and $\beta3\Delta E_{G2069A}$ were subcloned into pcDNAI/Amp and pRc/CMV by digestion with *Eco*RV and *Xba*I. The sequence of all clones was confirmed using dideoxy nucleotide sequencing (49).

Cell Culture and Transfections—Chinese hamster ovary K1 (CHO) cells were maintained and transfected as described previously (50). COS-7 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Wild type or mutant constructs were co-transfected with $\beta_3\Delta E$ into COS-7 cells using LipofectAMINE. Cells were plated at 1.3×10^5 cells/cm^2 in 25-cm^2 flasks. One day after plating, cells were washed with serum-free DMEM and incubated with 2.75 ml of serum-free DMEM and 0.5 ml of Opti-MEM I containing a total of 2.5 μg of $\beta_3\Delta E$ and α_{IIb} construct, each in pcDNAI/Amp, and 24

[1] The abbreviations used are: FBS, fetal bovine serum; CHO, Chinese hamster ovary K1, DMEM, Dulbecco's modified Eagle medium, high glucose formula; PBS, phosphate-buffered saline; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; DTT, dithiothreitol.

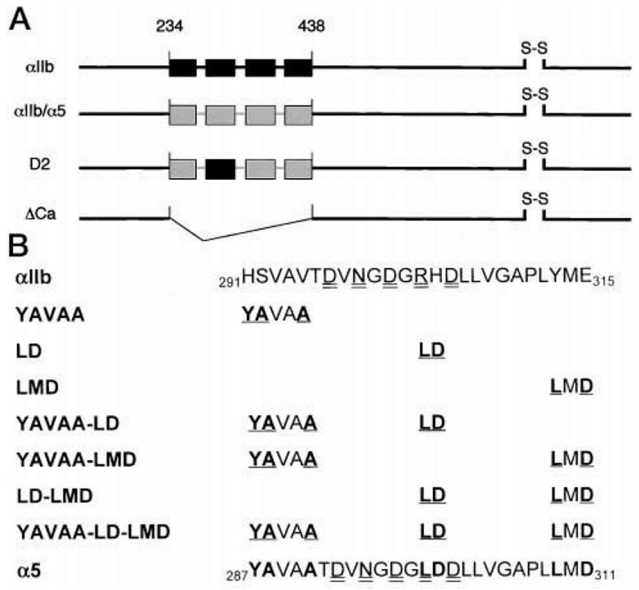

FIG. 1. *A*, schematic representation of IIbα5, D2, and ΔCa mutants. The position of the start and stop of the calcium binding domains of α_{IIb} is indicated. *Boxes* show calcium binding domains. *Black*, α_{IIb} sequence; *gray*, α_5 sequence. *B*, sequence of second calcium binding domain mutants. *Underlined*, *boldface* amino acids were mutated from α_{IIb} to α_5 sequence; *double underlined* amino acids indicate calcium liganding sites.

μl of LipofectAMINE. After 5 h at 37 °C, 2.5 ml of DMEM containing 20% FBS was added. Twenty-four h later, the medium was replaced with DMEM containing 10% FBS. Cells were assayed 72 h after the start of transfection.

Flow Cytometry—Flow cytometry was performed as described previously (50) except that for PAC1 studies, cells were incubated with a 1:100 dilution of LIBS 6 ascites with or without 1 mM GRGDSP peptide for 15 min at 37 °C. Cells were washed and stained with FITC-conjugated rabbit anti-mouse IgM. For studies of subunit dissociation, cells were harvested and washed and then resuspended in PBS containing 2% BSA. Immediately before the start of the experiment, EDTA was added to a final concentration of 5 mM. Cells were incubated at the indicated temperature for the indicated times and then immediately diluted 10-fold with PBS, centrifuged, and resuspended in PBC (PBS containing 2% BSA, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$) containing the $\alpha_{IIb}\beta_3$ complex-specific mAb AP2 and processed as above.

Immunoprecipitation—Surface labeling and immunoprecipitation were performed essentially as described previously (50).

Antibody Binding—One mg of mAb AP2 (IgG fraction) was iodinated with 2 mCi of $Na^{125}I$ using 2 mM chloramine T for 5 min at 22 °C, quenched with 2.3 mM $Na_2S_2O_5$, and separated from free $Na^{125}I$ by gel filtration. Cells were harvested with EDTA and trypsin as described above for flow cytometry and resuspended in Tris-buffered saline plus 0.5% BSA. Typically, 5×10^5 cells were incubated with varying concentrations of [^{125}I]AP2 for 30 min at 22 °C in a total volume of 0.5 ml. Triplicate 50-μl aliquots were layered over 0.4 ml of 20% sucrose, 0.5% BSA in Tris-buffered saline and centrifuged at 12,000 × *g* for 2 min. The samples were aspirated to dryness, and the radioactivity associated with the cell pellets was measured in a γ-spectrometer. Total radioactivity in the sample was determined by counting triplicate 50-μl aliquots of the reaction mixture. Nonspecific binding was defined as the radioactivity associated with the cell pellets of mock-transfected cells and was subtracted from that of $\alpha_{IIb}\beta_3$-expressing cells. The data were fit to equilibrium binding models (51).

Adhesion—Cells were harvested as above for flow cytometry and resuspended in PBC. Flat bottomed immunoassay 96-well polystyrene plates were incubated with either 2.5 μg/ml fibronectin or 4 μg/ml fibrinogen in PBS for 16 h at 4 °C. Plates were then incubated with PBS with 2% BSA for 2 h at 37 °C to block nonspecific binding sites on the plates. Typically, 2×10^6 cells/ml were incubated with PBC or PBC plus 1 mM GRGDSP for 30 min at 22 °C prior to plating 1×10^5 cells/well. Cells were allowed to adhere to the plates for 2 h at 37 °C. Plates were washed three times with PBS to remove nonadherent cells. Adherent cells were stained with 0.5% crystal violet in PBS containing 20% methanol for 30 min at 22 °C. Excess dye was removed by three washes with water, and cells were solubilized in 1% SDS for 16 h at 22 °C. Adhesion was quantified by measuring the absorbance at 540 nm in a BIO-TEK EL 340 microplate reader (BIO-TEK Instruments, Winooski, VT). Nonspecific binding was defined as the absorbance of cells binding to BSA-coated wells and was subtracted from the absorbance of cells binding to ligand-coated wells. All assays were performed in triplicate.

Aggregation—All operations were performed at 22 °C. Cells were harvested as above for flow cytometry and resuspended in HEPES-Tyrode's buffer, pH 7.5, at 2×10^7 cells/ml. Cells were incubated with 10 mM DTT for 20 min, pelleted, and resuspended at the same concentration in HEPES-Tyrode's buffer with or without inhibitors. Cells were incubated for 30 min, and then $CaCl_2$ was added to 0.4 mM. One hundred μl of cells was placed in wells of a 48-well tissue culture plate and incubated with or without 0.25 mg/ml fibrinogen for 30 min at 75 rpm on a gyrotory shaker. Cells were analyzed for aggregation by bright field microscopy.

Fibrinogen Binding—Fibrinogen was passed over a gelatin-agarose column two times to deplete the fibrinogen of fibronectin. Fibrinogen and AP2 were labeled using FITC-Celite essentially as described by Xia *et al.* (52). The fluorescein:protein ratio for fibrinogen ranged from 3 to 5, while the AP2 had a fluorescein:protein ratio of 3. Cells were harvested as above for flow cytometry and resuspended at a final concentration of 3×10^6 cells/ml in DMEM containing 20 mM HEPES, pH 7.5, LIBS 6 ascites at a 1:100 dilution, and either 0.25 mM ligand-blocking peptide RGDW or buffer. Cells were incubated at room temperature for 15 min, and then FITC-fibrinogen was added at the indicated final concentration. Cells were incubated for 30 min at room temperature, washed with PBS, fixed with 1% paraformaldehyde in PBS at 4 °C, and analyzed by flow cytometry. For both fibrinogen and AP2 binding, cytometry was scrupulously gated to analyze only single cells. In an effort to exclude cells lying outside the normal range, only the central 98% of the fluorescent signal (as determined by minimizing the coefficient of variance) was averaged to determine mean fluorescent intensity. Specific binding was defined as the difference in mean fluorescent intensity between cells incubated in the presence and absence of RGDW. Data was fitted to the one-site ligand binding equation $y = a_0 * x/(a_1 + x)$ using the iterative nonlinear curve fitting function of SlideWrite® Plus (Advanced Graphics Software, Inc., Carlsbad, CA).

To test the effect of the anti-N-terminal and anti-C-terminal fibrinogen Aα RGD motif and anti-γ-chain antibodies, cells were resuspended in HEPES-Tyrode's buffer without calcium or magnesium that had been treated with Chelex 100. Cells were incubated with 2 mM Ca^{2+} and LIBS 6 or with 1 mM Mn^{2+} in the presence or absence of 1 mM GRGDSP for 15 min at 22 °C. Fibrinogen and control or anti-fibrinogen peptide IgG were added to final concentrations of 50 nM and 10 μg/ml, respectively. Cells were incubated at 22 °C for 30 min, washed with HEPES-Tyrode's buffer, and then incubated at 22 °C for 30 min with a 1:10 dilution of FITC-chicken anti-human fibrinogen in HEPES-Tyrode's buffer. Cells were washed, fixed, and analyzed by flow cytometry as above.

RESULTS

Expression of Mutant Constructs—To determine if the various α_{IIb} calcium binding domain mutants were expressed on the cell surface, CHO cells co-transfected with the mutants and wild type β_3 were analyzed by flow cytometry using Tab, a murine monoclonal antibody that binds α_{IIb}. As shown in Fig. 2, all of the constructs with point mutations in the second calcium binding domain, including YAVAA-LD-LMD in which the entire second calcium binding domain of α_{IIb} was replaced by the equivalent sequence in α_5, were recognized by Tab, which indicated cell surface expression of the mutant subunit. Similar studies also showed that these mutants were recognized by complex-specific antibodies 10E5, AP2, and A2A9, suggesting complex formation with β_3 (data not shown). Immunoprecipitation with anti-β_1 and anti-β_3 mAbs confirmed that each of the α_{IIb} chimeras complexed with β_3 and not β_1 (data not shown). Radiolabeled antibody binding studies determined that each cell line expressed approximately 150,000 AP2 sites/cell (range 71,000–182,000) with the same dissociation constant, K_d, as reported for AP2 binding to platelets (38) (data not shown).

In contrast, more extended chimeras such as IIbα5, a chimera in which the entire calcium binding domain of α_{IIb} was

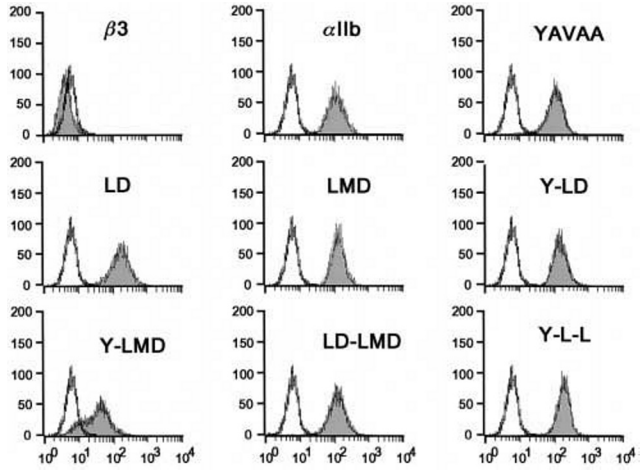

FIG. 2. **Detection of second calcium binding domain recombinant heterodimers on the cell surface of stably transfected CHO cells.** Cells were incubated with Tab, a mAb against α_{IIb}, washed, and stained with FITC-labeled goat anti-mouse IgG. Cells were analyzed by flow cytometry. *Open curve*, mock-transfected cells; *filled curves*, named cells. β_3, cells transfected with β_3 only. All other cell lines were co-transfected with the indicated α_{IIb} construct and β_3. α_{IIb}, wild type $\alpha_{IIb}\beta_3$; *Y-LD*, YAVAA-LD; *Y-LMD*, YAVAA-LMD; *Y-L-L*, YAVAA-LD-LMD.

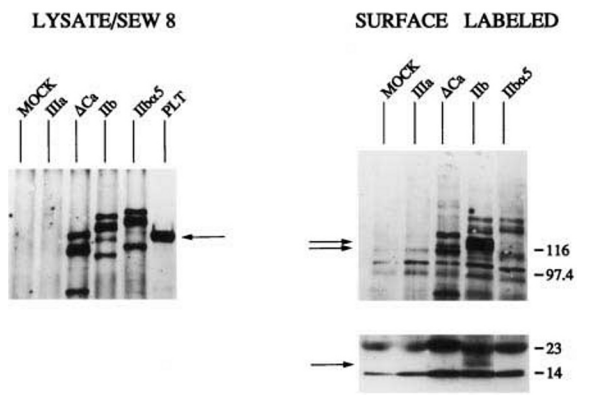

FIG. 3. **IIbα5, D2, and ΔCa are synthesized but not expressed on the cell surface of transiently transfected COS cells.** Cell lysates were immunoprecipitated with B1B5, a mAb to α_{IIb} that binds distal to the mutated region. The proteins were separated on 7.5% gels under reducing conditions and transferred to nitrocellulose. *Left*, proteins immunodetected with SEW 8, a polyclonal antibody to α_{IIb}. *Right*, lysates from cells that had been surface-labeled with biotin detected with streptavidin-horseradish peroxidase chemiluminescence. *PLT*, platelet lysate. In both *panels* the *top arrow* indicates $\alpha_{IIb\alpha}$. In the *right panel*, the *middle arrow* indicates β_3, and the lower arrow indicates $\alpha_{IIb\beta}$. *IIIa*, cells transfected with β_3 only; ΔCa, cells transfected with $\alpha_{IIb}\Delta Ca\beta_3$; *IIb*, cells transfected with wild type $\alpha_{IIb}\beta_3$; *IIbα5*, cells transfected with $\alpha_{IIb}\alpha_5\beta_3$.

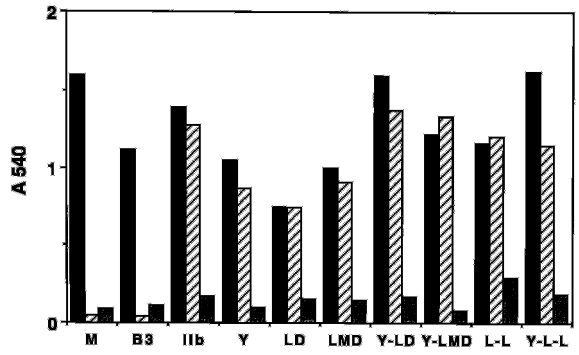

FIG. 4. **CHO cells expressing chimeric second calcium binding domain heterodimers adhere to ligand-coated plates.** Microtiter plates were coated overnight with fibronectin or fibrinogen. Nonspecific binding sites were blocked with BSA. Cells were allowed to attach to the plate for 2 h and washed, and the number of cells that bound were quantified by staining with methylene blue and subtracting nonspecific binding (binding to BSA). *Black bars*, adherence to fibronectin; *cross-hatched bars*, adherence to fibrinogen; *gray bars*, cells were incubated with 1 mM GRGDSP for 30 min before plating onto fibrinogen-coated wells. *M*, mock; *β3*, cells transfected with β_3 only. All other cells were transfected with β_3 and the indicated α-subunit. *IIb*, cells transfected with wild type α_{IIb}; *Y*, YAVAA; *Y-LD*, YAVAA-LD; *Y-LMD*, YAVAA-LD-LMD; *L-L*, LD-LMD; *Y-L-L*, YAVAA-LD-LMD.

replaced with the corresponding sequence of α_5, and D2, a chimera in which the first, third, and fourth calcium binding domains of α_{IIb} were replaced by the corresponding sequences of α_5, were not recognized by flow cytometric analysis with any anti-α_{IIb} or complex-specific antibody tested (data not shown), indicating lack of expression on the cell surface. Likewise, ΔCa, an in-frame deletion mutant in which the entire calcium binding domain was removed, was not expressed on the cell surface.

To examine further the synthetic defect in IIbα5, D2, and ΔCa, the mutant proteins were transiently expressed in COS-7 cells with β_3 and analyzed by immunoprecipitation of cell lysates using the mAb B1B5. B1B5 recognizes an epitope near the transmembrane region of α_{IIb} (42), an area that should not be disturbed by the mutations. When cell surface proteins were labeled with biotin prior to cell lysis and immunoprecipitation, cells transfected with the mutant constructs had very faint bands corresponding to mature α_{IIb} and β_3, confirming the flow cytometry data suggesting little or no surface expression of the chimeric complexes (Fig. 3 and data not shown). Analysis of immunoprecipitates of unlabeled cell lysates revealed that cells transfected with the mutants synthesized single chain precursors and a small amount of mature α_{IIb}-like proteins, but the molecular weight of each of the mutant α_{IIb} proteins was different from wild type α_{IIb} (Fig. 3). ΔCa was smaller than α_{IIb}, as expected from a protein with a 204-amino acid deletion, while IIbα5 and D2 were both slightly larger than α_{IIb}. Both of these chimeras contain α_5 sequence from the start of the calcium binding domains up to the second calcium binding domain and include the amino acid sequences NLT, NGS, and NFS, all potential sites for *N*-linked glycosylation. Thus, the increased molecular weight of IIbα5 and D2 compared with wild type α_{IIb} might be attributed to increased glycosylation.

Adhesion Studies—The adherence of mutant $\alpha_{IIb}\beta_3$-expressing CHO cells to fibronectin or fibrinogen immobilized on the wells of microtiter plates was examined. Resting platelets can bind to fibrinogen-coated surfaces; the $\alpha_{IIb}\beta_3$ complex does not have to assume an activated conformation to bind to solid phase fibrinogen (53). As shown in Fig. 4, all of the cell lines bound to fibronectin-coated wells as the result of constitutive expression of the $\alpha_5\beta_1$ fibronectin receptor. Cells expressing wild type $\alpha_{IIb}\beta_3$ also bound to fibrinogen, and the binding could be blocked by preincubation with the RGD peptide GRGDSP, but not by GRGESP. When we examined the ligand binding capabilities of the mutants, all of the second calcium binding domain chimeras bound to both fibronectin and fibrinogen, and fibrinogen binding was blocked by GRGDSP or by 250 μg/ml AP2 (data not shown) but not by GRGESP (data not shown). For each cell line, there was day to day variation in binding to the ligands, but the ratio of cells binding to fibrinogen *versus* fibronectin remained constant. Recently, Suehiro and co-workers reported that immobilized fibrinogen is a ligand for $\alpha_5\beta_1$ in the presence of calcium (54); yet in our studies, cell lines that did not express $\alpha_{IIb}\beta_3$ did not bind to fibrinogen, and binding to fibrinogen was blocked by $\alpha_{IIb}\beta_3$-specific mAbs, indicating that all fibrinogen binding was to $\alpha_{IIb}\beta_3$, not $\alpha_5\beta_1$.

Activation Studies—CHO cells expressing wild type $\alpha_{IIb}\beta_3$ do not respond to normal platelet agonists, including thrombin,

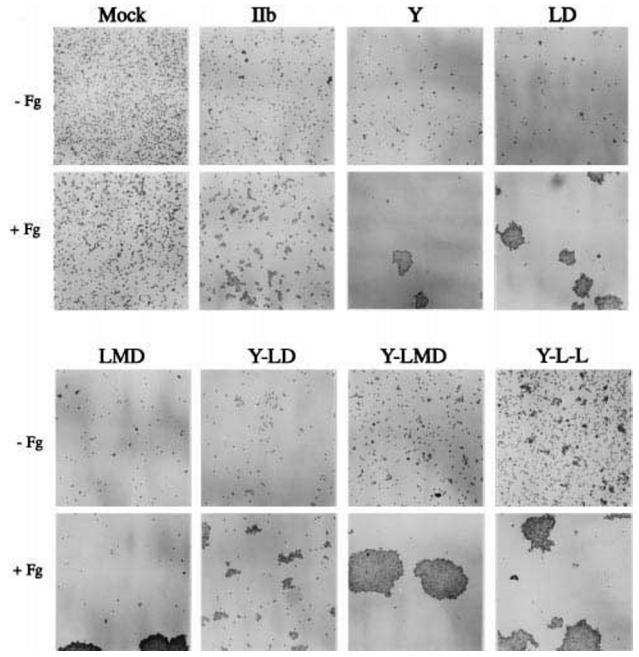

FIG. 5. **Aggregation of CHO cells expressing chimeric second calcium binding domain heterodimers.** Cells were incubated with 10 mM DTT for 20 min, washed, and incubated with buffer (−Fg) or 750 nM fibrinogen (+Fg) with agitation at 80 rpm, fixed with paraformaldehyde, and analyzed using bright field microscopy. *IIb*, wild type αIIb; *Y*, YAVAA; *Y-LD*, YAVAA-LD; *Y-LMD*, YAVAA-LMD; *Y-L-L*, YAVAA-LD-LMD.

phorbol myristate acetate, or epinephrine, to yield an active $\alpha_{IIb}\beta_3$ complex, presumably because the cells do not possess the necessary functional signaling pathways. However, $\alpha_{IIb}\beta_3$ expressed on the surface of platelets can be induced to assume an active conformation by treatment with the disulfide bond reducing agent DTT (55, 56). Cells were incubated with DTT for 20 min at room temperature, washed, and then incubated with soluble fibrinogen for 30 min at room temperature with stirring. Under these conditions, cells expressing $\alpha_{IIb}\beta_3$ formed large aggregates (Fig. 5). Aggregation was dependent on DTT treatment, since untreated cells expressing $\alpha_{IIb}\beta_3$ did not aggregate upon incubation with fibrinogen (data not shown). Aggregation could be inhibited by preincubation of the DTT-treated cells with the peptide inhibitor GRGDSP, H12, or 80 μg/ml $\alpha_{IIb}\beta_3$ complex-specific mAb A2A9 but not by preincubation with GRGESP or irrelevant antibody (data not shown). Cells expressing β_3 only did not aggregate when treated with DTT prior to incubation with fibrinogen (data not shown). As shown in Fig. 5, each of the second calcium binding domain mutants aggregated in the same manner as did cells expressing wild type $\alpha_{IIb}\beta_3$, indicating that each of the mutants maintained the ability to bind fibrinogen, despite the introduction of α_5 sequence.

Similar results were obtained when the mutant second calcium binding domain chimeras were activated using the anti-β_3 mAb LIBS 6, and activation was detected using PAC1, a ligand mimetic antibody that recognizes only the active conformation of $\alpha_{IIb}\beta_3$. Mock-transfected cells or those transfected with β_3 alone showed a minimal amount of PAC1 binding. Cells expressing wild type or each of the mutant $\alpha_{IIb}\beta_3$ heterodimers showed significant PAC1 binding after activation with LIBS 6 (Fig. 6). For each cell line, PAC1 binding was inhibited by incubation with GRGDSP (data not shown).

Since the aggregation studies used high concentrations of fibrinogen (750 nM), well above the reported K_d of 60 and 110 nM for $\alpha_{IIb}\beta_3$ expressed in tissue culture cells (22, 57), we examined the possibility that more subtle changes in fibrinogen binding were caused by the various chimeras. Soluble fibrinogen binding was carried out for each chimeric cell line at two fibrinogen concentrations, one above and one below the reported range for the K_d. Cells were activated with LIBS 6 antibody and incubated with FITC-fibrinogen in the presence or absence of RGDW blocking peptide and analyzed by flow cytometry (data not shown). Fibrinogen binding was dependent on activation of $\alpha_{IIb}\beta_3$, since cells incubated with FITC-fibrinogen but without LIBS 6 did not exhibit any specific binding. Mock-transfected cells and cells expressing only β_3 did not bind FITC-fibrinogen either before or after incubation with LIBS 6, indicating that measurable soluble fibrinogen binding was occurring only through binding to $\alpha_{IIb}\beta_3$. Each of the cell lines bound soluble fibrinogen at both ligand concentrations. However, when normalized for cell receptor number, some differences in FITC-fibrinogen binding were apparent. To determine if the differences were indicative of changes in either K_d or maximal binding (B_{max}), full equilibrium binding isotherms were performed on wild type cells and YAVAA and LD, the mutants with the most widely divergent binding at the two concentrations tested. Full binding isotherms revealed no difference in the K_d of YAVAA compared with wild type $\alpha_{IIb}\beta_3$ (300 ± 50 nM for wild type $\alpha_{IIb}\beta_3$ *versus* 280 ± 82 nM for YAVAA (Fig. 7). The maximal fibrinogen binding of YAVAA, however, was only 44 ± 5% that of wild type $\alpha_{IIb}\beta_3$ ($p < 0.001$). LD, on the other hand, demonstrated a somewhat reduced K_d of 110 ± 34 nM ($p < 0.005$) with a slightly increased B_{max} of 120 ± 11% of wild type $\alpha_{IIb}\beta_3$. The observed differences in binding parameters were small but reproducible over multiple experiments using several lots of FITC-fibrinogen.

Suehiro *et al.* (54) have reported that soluble fibrinogen can bind to $\alpha_5\beta_1$ in the presence of manganese but not calcium. Binding was shown to be mediated by the C-terminal Aα RGD sequence of fibrinogen by inhibition of fibrinogen binding with an antibody to this region but not with antibodies to either the N-terminal Aα RGD sequence or the γ-chain H12 sequence. To look for a potential difference in fibrinogen binding mechanism by the chimeric mutants, fibrinogen binding to wild type or YAVAA-LD-LMD, which substitutes the complete second calcium binding domain of α_5 into α_{IIb}, was examined in the presence of calcium or manganese, and inhibition of this binding with antibodies against the fibrinogen N- or C-terminal Aα RGD sequences or the γ-chain H12 sequence was determined. As shown in Fig. 8, neither the N- nor C-terminal Aα RGD sequence antibodies inhibited fibrinogen binding in the presence of either calcium or manganese, while the anti-γ-chain H12 antibody significantly inhibited soluble fibrinogen binding to both wild type (71% inhibition in calcium and 77% in manganese) and YAVAA-LD-LMD (81% in calcium and 80% in manganese), demonstrating that even when the second calcium binding domain of α_{IIb} is replaced with the second calcium binding domain of α_5, binding of soluble fibrinogen is mediated by the γ-chain H12 sequence.

Dissociation Studies—When platelets are incubated at 37° C with EDTA at alkaline pH, the $\alpha_{IIb}\beta_3$ complex irreversibly dissociates, and this subunit dissociation can be monitored by loss of binding of the complex-specific antibody AP2 (58). We looked for changes in the stability of the mutant $\alpha_{IIb}\beta_3$ complexes by incubating CHO cells expressing the complexes with 5 mM EDTA at pH 7.4 at varying temperatures. No loss of AP2 binding to wild type $\alpha_{IIb}\beta_3$ or any of the mutants occurred when the cells were incubated at 22° C for 1 h (data not shown). When wild type and mutant cells were incubated at 37° C, the complexes dissociated at different rates, as seen in Fig. 9. Wild type $\alpha_{IIb}\beta_3$ complex dissociation occurred with a half-time of

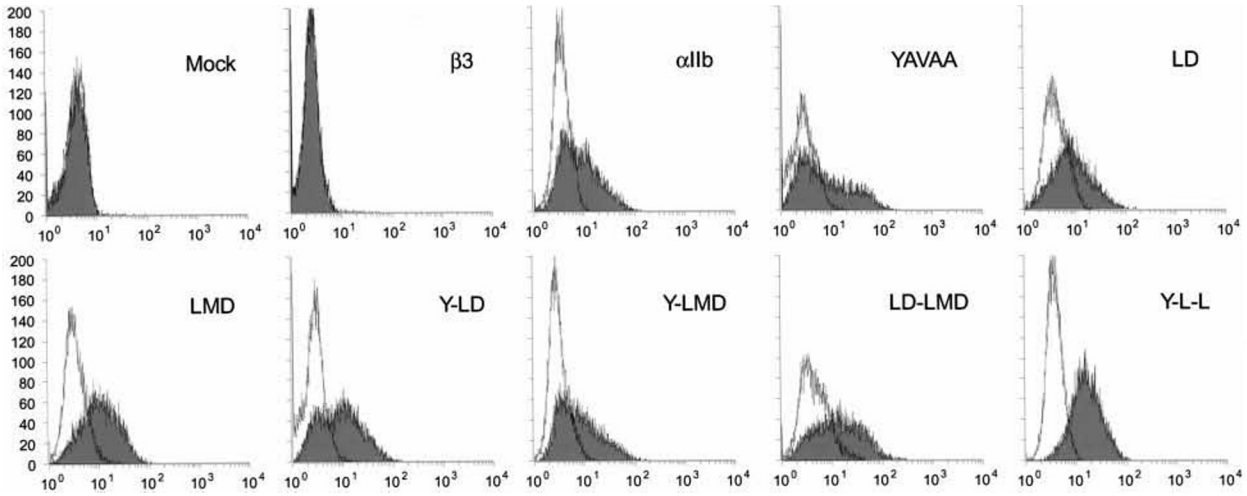

FIG. 6. **Binding of PAC1 to second calcium binding domain recombinant heterodimers on the cell surface of stably transfected CHO cells.** Cells were incubated with buffer (*open curves*) or activating anti-β_3 mAb LIBS 6 (*filled curves*). Cells were then incubated with PAC1 followed by FITC-rabbit anti-mouse IgM. Cells were analyzed by flow cytometry. *β3*, cells transfected with β_3 only. All other cell lines were transfected with β_3 and the indicated α-subunit. *αIIb*, wild type α_{IIb}; *Y-LD*, YAVAA-LD; *Y-LMD*, YAVAA-LMD; *Y-L-L*, YAVAA-LD-LMD.

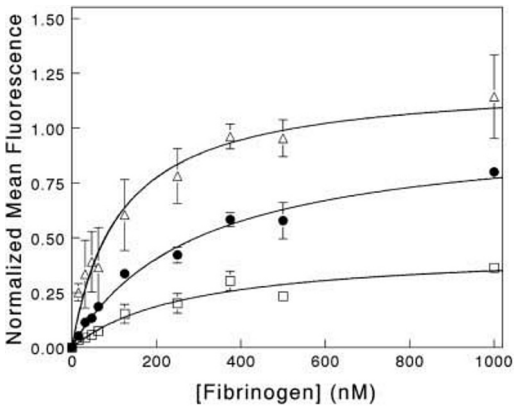

FIG. 7. **Mutant cell lines YAVAA and LD bind fibrinogen with properties similar to CHO cell expressing wild type $\alpha_{IIb}\beta_3$.** Specific binding of fibrinogen was measured by incubating cells with LIBS 6-activating antibody either in the presence or absence of 0.25 mM ligand-blocking peptide RGDW. FITC-fibrinogen was added at the indicated final concentration, and cells were incubated for 30 min at 22 °C, washed, fixed, and analyzed by flow cytometry. Specific binding was defined as FITC-fibrinogen binding minus FITC-fibrinogen binding in the presence of RGDW. Binding was normalized for differences in receptor number by determining the relative receptor number in the different mutants as compared with wild type by binding of the complex specific mAb AP2 that had been directly labeled with FITC. Maximum binding to wild type was set at 1. The results are the average of three (LD) or four (wild type and YAVAA) determinations. ●, wild type; □, YAVAA; △, LD.

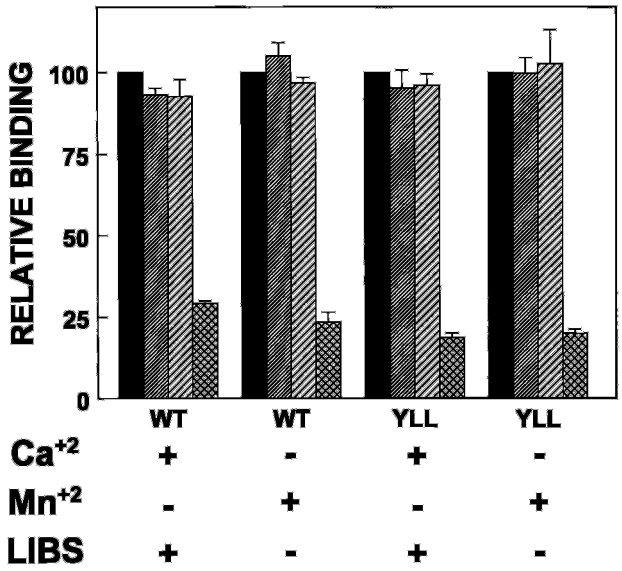

FIG. 8. **Inhibition of fibrinogen binding by anti-fibrinogen antibodies.** Cells were incubated with 2 mM Ca^{2+} and LIBS 6 or with 1 mM Mn^{2+}, as indicated, in the presence or absence of 1 mM GRGDSP for 15 min at 22° C. Fibrinogen and control or anti-fibrinogen peptide IgG were added to a final concentrations of 50 nM and 10 μg/ml, respectively, and cells were incubated for 30 min at 22° C, washed, and then incubated with FITC-chicken anti-human fibrinogen IgG for 30 min at 22° C, washed, fixed, and analyzed by flow cytometry. Specific binding was defined as fibrinogen binding minus fibrinogen binding in the presence of GRGDSP. Specific fibrinogen binding in the presence of control IgG was set at 100. The results are expressed as the mean ± S.E. of three independent experiments. *WT*, wild type; *YLL*, YAVAA-LD-LMD. *Black bar*, control IgG; *narrow diagonal bar*, anti-Aα chain N-terminal RGD IgG; *wide diagonal bar*, anti-Aα chain C-terminal RGD IgG; *cross-hatched bar*, anti-γ chain H12 IgG. Inhibition by the anti-γ-chain H12 IgG was significantly different from control IgG ($p < 0.001$) in all cases.

2.56 ± 0.07 min. YAVAA dissociated much faster, with a half-time of 0.82 ± 0.04 min, while LD was more stable, having a dissociation half-time of 3.73 ± 0.09 min. YAVAA-LD-LMD, which contains the complete α_5 second calcium binding domain, had a dissociation half-time of 2.19 ± 0.06 min, which is not statistically different from that of wild type $\alpha_{IIb}\beta_3$. When the cells were incubated with EDTA at 41° C, wild type and mutant $\alpha_{IIb}\beta_3$ complexes dissociated more rapidly than at 37° C but in the same rank order: YAVAA less stable than YAVAA-LD-LMD and wild type, which were less stable than LD (data not shown). When the cells were incubated with EDTA at temperatures below 37° C, only YAVAA showed a significant loss of AP2 binding (data not shown). Thus, there appears to be a correlation between complex stability and fibrinogen binding. For example, YAVAA was less stable than wild type $\alpha_{IIb}\beta_3$ and YAVAA-LD-LMD when incubated with EDTA and exhibited decreased maximal fibrinogen binding, while LD was more stable than wild type $\alpha_{IIb}\beta_3$ and YAVAA-LD-LMD and had an increased affinity for fibrinogen.

DISCUSSION

The major findings of this study are as follows. 1) Chimeric α_{IIb} containing the four calcium binding domains of α_5; containing domains 1, 3, and 4 of α_5; or deleted of calcium binding domains is synthesized but is neither processed nor expressed

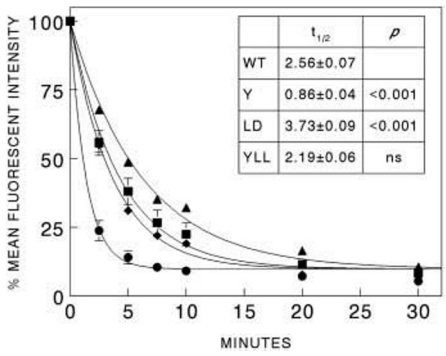

FIG. 9. **Loss of complex-specific antibody binding after incubation with EDTA at 37° C.** Cells were incubated with 5 mM EDTA at 37° C for the indicated time, washed, incubated with AP2, washed, incubated with FITC-goat anti-mouse IgG, washed, fixed, and then analyzed by flow cytometry. Each *curve* is the average of four separate experiments. ■, wild type (*WT*); ●, YAVAA (*Y*); ▲, LD; ♦, YAVAA-LD-LMD (*YLL*); *ns*, not significant.

on the cell surface. 2) Chimeras containing subsets or all of the variant amino acids of the second calcium binding domain of α_5 are synthesized, complexed with β_3, processed, and expressed on the cell surface, and they retain epitopes of α_{IIb} and $\alpha_{IIb}\beta_3$. 3) Second calcium binding domain chimeras retain the functions of adhesion to fibrinogen, binding of the ligand mimetic antibody PAC 1, binding soluble fibrinogen, and fibrinogen-dependent aggregation. 4) Second calcium binding domain chimeras bind fibrinogen utilizing the γ-chain H12 sequence as opposed to the C-terminal Aα RGD sequence that is used in fibrinogen binding to $\alpha_5\beta_1$. 5) Changes in the amino acid sequence of the second calcium domain have varying effects on subunit stability.

The fundamental observation of this study, that the chimeric second binding domain $\alpha_{IIb}\alpha_5\beta_3$ proteins were capable of binding fibrinogen as evidenced by soluble fibrinogen binding studies and aggregation of CHO cells expressing these proteins after activation with either LIBS 6 activating antibody or DTT and incubation with fibrinogen, conflicts with a number of previously published works, which indicate that amino acids 291–314 are important for ligand binding. D'Souza and co-workers (30) demonstrated that α_{IIb}-(296–306) inhibited ^{125}I-labeled fibrinogen binding to platelets, but a peptide with the conservative mutation D301E was a much less potent inhibitor. They also found that neither the α_5 nor α_v homologs of α_{IIb}-(296–306) inhibited binding of fibrinogen to purified $\alpha_{IIb}\beta_3$, while α_{IIb}-(296–306) did (36). Taylor and Gartner (33) reported that α_{IIb}-(300–312) blocked adhesion of platelets to fibrinogen, fibronectin, and vitronectin, while the α_v homolog would partially block platelet adhesion to vitronectin but not fibrinogen or fibronectin. Furthermore, the results of our experiments are surprising in light of recent NMR studies by Yao and Mayo (59), who suggested that both hydrophobic amino acids and the positively charged arginine and histidine residues play a significant role in binding of α_{IIb}-(300–314) to fibrinogen. They concluded that the RHDLL and PLYM sequences in that peptide have the strongest interaction with fibrinogen, yet the mutations that we made in these sequences, RHDLL → LDDLL and PLYM → PLLM, did not significantly change the fibrinogen binding functionality, either by themselves or together in the chimeric protein complexes.

Our data demonstrate that all of the chimeric mutants bound soluble fibrinogen at concentrations as low as 25 nM; however, some small, but statistically significant alterations in ligand binding appear to be present, especially for YAVAA and LD. YAVAA exhibited a B_{max} value less than half that of wild type $\alpha_{IIb}\beta_3$, possibly caused by a molecular structure that may have been more resistant to activation by LIBS 6. LD, with mutation of one of the calcium liganding amino acids and a −2 change in ionic charge, exhibited only a modest, 3-fold increase in affinity for fibrinogen. Overall, the data suggest that the chimeras with mutations in the second calcium binding domain may have some structural differences affecting $\alpha_{IIb}\beta_3$ complex stability, as evidenced by the altered EDTA dissociation profiles, but the chimeras do not exhibit major changes in either the specificity of fibrinogen binding or the amino acid sequence in fibrinogen required for binding.

A possible interpretation of the results is that the second calcium binding domain is important for ligand binding, but only in the sense that it must be present in a conformation that supports binding of divalent cation. Calcium (or magnesium) binding is required for not only subunit association, but for allowing the receptor to be in a conformation that supports ligand binding (60). Substituting the second calcium binding domain of α_5 into α_{IIb} should still allow formation of a divalent cation binding loop, since the oxygenated residues that provide calcium coordination sites were not mutated. Interestingly, one coordination site was changed, an Arg in α_{IIb} to a Leu in α_5. However, calcium coordination at this site is predicted to be through the main-chain carbonyl, based on the model of the calmodulin EF-hand structure (34). Thus, the amino acid differences between α_{IIb} and α_5 may produce subtle differences in the second calcium binding domain but differences that still support calcium and, therefore, ligand binding. Less conservative mutations to residues involved in calcium binding demonstrate the importance of the integrity of the loop structure for ligand binding. Masumoto and Hemler (61) performed alanine-scanning mutagenesis on the metal ion binding residues of the first divalent cation binding domain of α_4 and found that mutant protein complexes were not expressed on the cell surface. Only the conservative change of Glu for Asn or Asp at the third coordination site of any one of the divalent cation binding domains resulted in surface expression, and the mutant proteins exhibited decreased ligand binding. We have performed alanine-scanning mutagenesis and made more conservative mutations to the calcium-liganding residues in the second calcium binding domain of α_{IIb} and found that although the mutated α_{IIb} subunits were synthesized and were able to complex with β_3, they were not properly processed, and the complexes were not expressed on the cell surface.[2]

Another interpretation of the results is that the second calcium binding domain is required for ligand binding (*i.e.* it interacts with RGD, but the specificity of ligand binding resides in sequences that flank the second calcium binding domain). While the calcium binding domains, D*X*D*X*DG*XX*D, are highly conserved, occurring near the middle of an FG . . . (D*X*D*X*-DG*XX*D) . . . GAP . . . G*XX*Y conserved N-terminal repeat, the sequence outside the FG . . . G*XX*Y sequence is very poorly conserved among integrins (35). Even the sequence between the GAP and G*XX*Y sequences of the second calcium binding domains of α_{IIb} and α_5 is poorly conserved, differing at 9 of 14 amino acids. Several groups have demonstrated the importance of sequence flanking the RGD, RYD, or KGD recognition sequence of disintegrins in determining binding specificity (62–64). Kunicki and co-workers (65) have demonstrated the importance of flanking amino acids in experiments where the amino acids surrounding the RGD tripeptide in the Fab ligands PAC1.1 and AP7, not the RGD binding sequence itself, determined whether ligand binding was activation-sensitive. Flanking sequences have been shown to be important in determining ligand binding specificity for many receptors and enzymes in-

[2] S. Gidwitz and G. C. White II, manuscript in preparation.

cluding Src homology 2 and 3 domains, T-cell receptors, Kit, and α-glucan phosphorylase (66–70).

Recently, a new model for the N-terminal 440 amino acids of α-integrins was proposed (71). The seven FG . . . GAP repeats were predicted to form a seven-membered β-propeller similar to that formed by the β-subunit of heterotrimeric G proteins. Ligand binding was suggested to occur in a pocket on the upper surface of the β-propeller. In contrast, the calcium binding loops are modeled on the lower surface of the propeller, potentially in contact with the β-subunit. Thus, mutation of RH → LD at amino acids 302–303 in the second calcium binding loop would be predicted to be on the lower surface of the propeller and away from the ligand binding pocket. In contrast, His^{291} is suggested to be one of the residues lining the ligand binding pocket, as modeled on Gβ, while Tyr^{313} and Glu^{315} would be predicted to be in a loop that lies on the upper surface of the protein. If this model is correct, mutation of these residues to the corresponding α_5 sequence might be expected to change ligand binding specificity. Mutations at any of these residues, however, did not change ligand binding specificity for fibrinogen and had only small effects the K_d or B_{max}, suggesting that either these residues are not critical for ligand binding or that the mutations were conservative enough to allow ligand binding.

A potential explanation for the differences between the peptide studies and our results is that the second calcium binding domain of the chimeric constructs is constrained into a conformation that allows ligand binding, while the more highly mobile free peptides adopt conformations that may or may not block ligand binding. The wild type α_{IIb} peptide may reside in a conformation that blocks ligand binding, while a blocking conformation for the mutant α_{IIb} or wild type α_5 or α_v peptides may be thermodynamically unfavored. The effect of constraining a peptide structure has been demonstrated in studies with cyclic *versus* linear RGD peptides, where cyclization increases the affinity and specificity of the peptide for individual integrins (72, 73).

Three areas of $\alpha_{IIb}\beta_3$ have been implicated in binding of the C terminus of the γ-chain of fibrinogen. Two are in α_{IIb}, amino acids 294–312 and 656–667 (29–33). Alemany (24) and coworkers have presented evidence that β_3-(274–368) may also be involved. The properties of the implicated sites have some similarities and some striking differences. α_{IIb}-(294–312) has been demonstrated to be the cross-linking site for the H12 peptide. It binds fibrinogen in a calcium-dependent manner, and RGD peptides can compete for H12 binding (30). The site is not exposed on the resting $\alpha_{IIb}\beta_3$ complex. These properties mimic the way fibrinogen interacts with $\alpha_{IIb}\beta_3$ *in vivo*. The second calcium binding domain chimeras interact with fibrinogen in a similar fashion. α_{IIb}-(656–667) also binds fibrinogen with high affinity, but in a calcium-independent manner (29). Binding can be partially blocked by H12 peptide, but not by RGD, indicating a specificity for the γ-chain. This site, too, is cryptic on resting $\alpha_{IIb}\beta_3$. The C-terminal γ-chain-specific β_3 site at amino acids 274–368 is somewhat enigmatic in that soluble fibrinogen binding is calcium-independent and the site is exposed on both resting and activated $\alpha_{IIb}\beta_3$ (24). Considering that fibrinogen does not bind to resting platelets or to activated platelets in the absence of calcium, it is unclear how this site might function *in vivo*. Our data indicate that the fibrinogen binding site is not exposed on the resting $\alpha_{IIb}\beta_3$ or chimeric complex and that RGD can inhibit the fibrinogen interactions, suggesting that these later two sites are not involved in soluble fibrinogen binding.

In conclusion, our data indicate that chimeras in which the second calcium binding domain of α_{IIb} is replaced by the corresponding sequences of α_5 retain the capacity to bind fibrinogen. While one interpretation of this data is that the second calcium binding domain is not involved in ligand binding, we speculate that this region is important for ligation of the binding sequence(s) but that sequences outside the second calcium binding domain mediate ligand binding specificity.

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