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Arginine-Glycine-Aspartic Acid Mimics Can Identify a Transitional Activation State of Recombinant αIIbβ3 in Human Embryonic Kidney 293 Cells

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SUMMARY

The platelet-specific integrin αIIbβ3 achieves a high affinity binding state in response to extracellular agonists such as thrombin, ADP, or collagen. During this activation, the receptor undergoes a number of conformational changes. To characterize the different conformations of αIIbβ3, we expressed recombinant αIIbβ3 in human embryonic kidney (HEK) 293 cells. Antigenic and peptide recognition specificities of the full-length recombinant receptor resembled those of the native receptor in platelets. We used an array of peptidic and nonpeptidic arginine-glycine-aspartic acid (RGD) mimics that specifically bind to human platelet αIIbβ3 to determine the affinity state of the receptor. Some of these RGD mimics were previously shown to clearly discriminate between resting and activated αIIbβ3. Solution-phase binding of these RGD mimics to the recombinant cells suggested that in HEK 293 cells the full-length αIIbβ3 is expressed in a "transitional" activation state. This observation was confirmed by the binding of the activation-specific, monoclonal anti-αIIbβ3 antibody PAC1 to cells expressing the full-length recombinant αIIbβ3. Deletion of the entire cytoplasmic domain of the β subunit was sufficient to convert the receptor in HEK 293 cells to a fully active form, as found in activated platelets. In addition, the full-length receptor was capable of mediating agonist-independent aggregation of cells in the presence of fibrinogen. Thus, by using RGD mimics, we have identified a functional transitional activation state of αIIbβ3 that is capable of mediating fibrinogen-dependent cell aggregation.

The integrin family of receptors mediate many of the cell-cell and cell-substratum interactions that are central to cell adhesion, migration, growth, and differentiation. Integrins are noncovalent α/β heterodimers. Each subunit contains a large extracellular region, a transmembrane domain, and a short cytoplasmic tail (1). Integrins bind to a wide variety of ligands, including extracellular matrix proteins, counter-receptors on other cells, and circulating plasma proteins (2). The affinity and specificity of an integrin binding site are defined by the specific pairing of the α and β subunits (3). In addition, previous studies support a model in which amino acid sequences in both subunits coordinate ligand and cations in close proximity to form a "reactive" center for ligand binding (4). The cytoplasmic tails of integrins interact with intracellular proteins, including cytoskeletal proteins such as talin and α-actinin (5) and a number of regulatory proteins such as focal adhesion kinase (6), integrin-linked kinase (7), endonexin (8), and cytohesin-1 (9). Upon ligand binding, integrin-mediated signaling events, which include rearrangement of the cytoskeleton, gene regulation, and cellular differentiation, are induced by a process called "outside-in" signaling (10). Alternatively, intracellular signaling events can modulate the affinities of integrins for extracellular ligands (11). These pathways involve phospholipids, protein kinases (5, 6), intracellular calcium fluxes, and low-molecular weight G proteins (12, 13). The process whereby cytoplasmic signals result in changes in receptor conformation and ligand binding affinity is termed "inside-out" signaling. The modulation of outside-in and inside-out signals is important for the regulation of integrin function (2).

Platelet adhesive interactions are of primary importance in normal hemostasis as well as thrombotic disorders. αIIbβ3 is the major integrin involved in attachment, spreading, and aggregation of platelets. On resting platelets, αIIbβ3 is in a "latent" or basal state that does not bind fibrinogen, one ligand present in abundance in the circulation (14). A wide variety of agonists, such as thrombin, ADP, or collagen, can stimulate platelets, which results in the "activation" of αIIbβ3 and the binding of soluble fibrinogen or other ligands (including von Willebrand factor, vitronectin, and throm-

ABBREVIATIONS: RGD, arginine-glycine-aspartic acid; CHO, Chinese hamster ovary; DPBS, Dulbecco's phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; AI, activation index; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; HEK, human embryonic kidney; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

bospondin) that are important for thrombus formation (15). This inside-out signaling is mediated by conformational changes in αIIbβ3 and is modulated by intracellular events (2, 5). In addition to these agonists, synthetic peptides containing the RGD sequence, which is present within the fibrinogen molecule and serves as a recognition site for binding to αIIbβ3, activate αIIbβ3 (16). RGD peptides bind to resting αIIbβ3, leading to conformational changes in its extracellular domain that enable it to bind soluble fibrinogen, after the removal of the RGD peptide. αIIbβ3 activated by RGD peptides expresses novel sites on its extracellular domain (termed ligand-induced binding sites), whereas αIIbβ3 activated by agonist does not express ligand-induced binding sites unless it binds fibrinogen (17, 18). These findings indicate that αIIbβ3 has at least two distinct conformational states that can bind soluble fibrinogen, i.e., agonist- and RGD-activated states (19).

To analyze the conformational states of αIIbβ3, the ligand-binding properties of recombinant human platelet αIIbβ3 expressed in HEK 293 cells were examined by using synthetic peptidic and nonpeptidic RGD mimics. These molecules have differential specificity for agonist-activated and resting platelets and thus can be used as markers to identify activation states of αIIbβ3. Here, we identify αIIbβ3 in HEK 293 cells to be in a transitional activation state that is distinct from the fully activated receptor in platelets. This is confirmed by using the activation-specific, anti-αIIbβ3 mAb PAC1. Furthermore, Glanzmann's thrombasthenic mutations, which disrupt either ligand binding [β3(D119Y)] or receptor signaling [β3(S752P)] (20), do not permit binding to PAC1 and abrogate the physiological ligand-binding functions of αIIbβ3. However, deletion of the cytoplasmic domain of the β subunit is sufficient to convert αIIbβ3 in HEK 293 cells to a fully competent receptor, as in activated platelets. The full-length receptor, which is in a transitional activation state, is also shown to mediate agonist-independent aggregation of cells in the presence of fibrinogen. This fibrinogen-mediated aggregation requires an intact ligand binding domain in αIIbβ3 and is dependent on cytoskeletal rearrangement. These results suggest that αIIbβ3 can exist in multiple transitional conformational states, which may modulate specific physiological functions of the integrin in platelets.

Experimental Procedures

Antibodies. Mouse mAbs to αIIbβ3 (mAb CA3) and α5β1 (mAb JBS5) were purchased from Chemicon (Temecula, CA). mAb PAC1 (21), against the activated form of αIIbβ3, was purchased from the Cell Center, University of Pennsylvania (Philadelphia, PA). Polyclonal antibodies against purified human platelet αIIbβ3 were generated in rabbits and affinity-purified. These antibodies specifically bind to αIIbβ3 in platelets and human erythroleukemia cells.[1] FITC-conjugated donkey anti-mouse IgM and FITC-conjugated goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Synthetic ligands. The αIIbβ3 antagonists MK-852, L-692,884, and L-734,217 were synthesized by the Medicinal Chemistry Department, Merck Research Laboratories (West Point, PA). L-692,884 [cyclo-4-iodo-benzoyl-(Cys-Asn-Pro-Arg-Gly-Asp-Cys)-OH], a synthetic cyclic RGD peptide, binds preferentially to activated αIIbβ3 (22). MK-852 [cyclo-*N*-acetyl-[Cys-Asn-(5′,5-dimethyl-4-thiazolidinecarbonyl)-(4-aminomethyl-Phe)-Gly-Asp-Cys]-OH] and L-734,217 [*N*-[3(*R*)-(2-(piperidin-4-yl)ethyl)-2-piperidon-1-yl]acetyl-3(*R*)-methyl-β-Ala] bind specifically to activated αIIbβ3 in platelets (23, 24). αIIbβ3-specific antagonists Ro 43–5054 and Ro 44–9883 were also synthesized by the Medicinal Chemistry Department and characterized as previously described (25). The detailed characterization and binding properties of these fibrinogen receptor antagonists with purified human platelet αIIbβ3 are described elsewhere[2] and are summarized in Table 1.

TABLE 1

Dissociation binding constants of RGD mimics with purified human αIIbβ3

The ratio of differentiation is defined as the ratio of the K_d values for the unactivated and activated forms. It is a qualitative measure of the ability of the compounds to distinguish between the two forms of the receptor.

Compound	K_d		Ratio of differentiation
	Activated form	Unactivated form	
	nM		
Echistatin	4.6	4.9	1.1
RO 44-9883	0.96	6.3	6.5
L-692,884	1.4	75	54
MK-852	42	2900	69
RO 43-5054	6	580	97
L-734,217	4.5	650	140

cDNA constructs. The full-length human cDNA encoding αIIb in the expression vector pM2ADA was a kind gift from Dr. Joel Bennett (University of Pennsylvania, Philadelphia, PA) (26). The cDNA insert was subcloned into the eukaryotic expression vector pR135, which was under the transcriptional control of the cytomegalovirus promoter and contained the hygromycin-selectable marker. The isolation and construction of cDNA encoding full-length human platelet β3, β3(Δ717), β3(D119Y), and β3(S752P) have been previously described (27). The mutation β3(Δ693) was introduced by polymerase chain reaction using a 5′ primer (CAGCTCGAGCTATTAGTCAGGGCCCTTAGGGACACTCTGG) that contained a *Pst*I restriction site and the 3′ oligonucleotide (TGCCATTGGGCCTCATA) that contained an *Xho*I site. The resulting polymerase chain reaction fragment was digested with *Pst*I and *Xho*I. The full-length β3 cDNA was digested with *Hin*dIII and *Pst*I. The expression vector pCDNA3 (Invitrogen, CA) was digested with *Hin*dIII and *Xho*I. Ligation of the three resulting fragments generated a stop codon before the transmembrane domain of the β3 cDNA. The β3 cDNA constructs were subcloned into the expression vector pCDNA3, containing the neomycin-selectable marker. All constructs were characterized by restriction digestion, purified by CsCl centrifugation, and verified by DNA sequence analysis before transfection.

Cell culture and transfection. HEK 293 cells were obtained from the American Type Culture Collection (Rockville, MD). HEK 293 cells were grown in minimal essential medium with Earle's salt supplemented with 10% fetal calf serum, 1% kanamycin, and 2 mM glutamine (GIBCO-BRL Life Technologies, Gaithersburg, MD).

Stable transfection of cells (1×10^6) was by electroporation in the presence of 10 μg of DNA, using a Gene Pulser ElectroCell manipulator (Bio-Rad Laboratories, Richmond, CA) at 960 μF and 200 V. Twenty-four hours after electroporation, cells were resuspended in selection medium containing 800 μg/ml G418 (GIBCO-BRL) and 100 μg/ml hygromycin B (Calbiochem, La Jolla, CA), and resistant clones were isolated after 2 weeks in culture. Positive clones were sorted (exclusion mode) by flow cytometry in a FACSCalibur (Becton Dickinson, San Jose, CA), using anti-αIIb subunit-specific antibodies

[1] D. G. Abraham and B. Bednar, unpublished observations.

[2] R. A. Bednar, S. L. Gaul, T. G. Hamill, M. S. Egbertson, J. A. Shafer, G. D. Hartman, R. J. Gould, and B. Bednar, Identification of low molecular weight GP11b/111a antagonists that bind preferentially to activated platelets, manuscript in preparation.

(mAb SZ0.22). After sorting, the cells were maintained in selection medium containing 400 μg/ml G418 and 50 μg/ml hygromycin B. Surface expression of αIIbβ3 was not significantly altered between passage 5 and passage 25.

Flow cytometry. Surface expression levels of integrins were analyzed by single-color flow cytometry. Cells (2×10^5) were harvested with trypsin/EDTA (GIBCO-BRL), washed once with 5 volumes of minimal essential medium with Earle's salt containing 10% fetal calf serum and twice with DPBS, and incubated with either 20 μg/ml mAb CA3 (anti-αIIbβ3) or 15 μg/ml mAb JBS5 (anti-α5β1), in DPBS containing 1 mM $CaCl_2$ and 1% BSA, for 45 min at 4°, in a total volume of 100 μl. The cells were pelleted, washed once with DPBS, and incubated with FITC-conjugated goat anti-mouse IgG. After a 45-min incubation at 4°, the cells were washed once with DPBS, resuspended in 350 μl of flow cytometric buffer (100 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$), and analyzed by flow cytometry. The light scatter and fluorescence intensity of 10,000 cells were collected using logarithmic gain.

The affinity state of the receptor was assessed by PAC1 binding. Cells (2×10^5) were harvested, washed as described above, and incubated with PAC1 (20 μg/ml) in the presence or absence of RGD peptide or RGD mimics for 1 hr at 4°. The cells were then washed once in DPBS and incubated with FITC-conjugated goat anti-mouse IgM for 30 min at 4°. The cells were resuspended in 350 μl of flow buffer immediately before analysis by flow cytometry. Because the activating anti-αIIbβ3 antibodies were not available for this study, the receptor was activated by preincubating the cells with 2 mM DTT for 5 min at room temperature before the addition of PAC1. Although receptor activation by DTT has not been thoroughly characterized, treatment of platelets with DTT has been shown to promote αIIbβ3-dependent platelet aggregation (28).

To define activity state, histograms depicting PAC1 staining in the absence or presence of the competitive inhibitor were compared. As a quantitative measure of affinity state, an AI for the PAC1 binding data was calculated for each construct. The AI was essentially that described by O'Toole *et al.* (29), $AI = 100 \times (F_o - F_r)/(F_oDTT - F_rDTT)$, where F_o is the median fluorescence intensity of PAC1 binding, F_r is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor, F_oDTT is the median fluorescence intensity of PAC1 binding in the presence of 2 mM DTT, and F_rDTT is the median fluorescence intensity of PAC1 binding in the presence of 2 mM DTT and competitive inhibitor. For example, when αIIbβ3 is inactive, as in resting platelets, PAC1 cannot bind and F_o is low. After DTT treatment, PAC1 binds and F_o is high. Thus, the AI for PAC1 alone on unactivated platelets is low. If αIIbβ3 is already fully activated, DTT cannot further activate and AI approaches 100, because $F_o = F_oDTT$.

Platelets were isolated from whole blood as previously described (14). Platelets (1×10^7/ml) were treated with 10 nM thrombin for 5 min at ambient temperature before the addition of PAC1 (20 μg/ml) and were incubated at room temperature for 30 min, in a total volume of 100 μl. FITC-conjugated goat anti-mouse IgM was then added in a total volume of 50 μl. After a 30-min incubation, 200 μl of flow cytometric buffer were added and the samples were subjected to flow cytometric analysis as described above. In some cases, specific inhibitors of αIIbβ3 were added at the time of PAC1 incubation.

Cell attachment. The cell attachment assay was performed as previously described (27). Essentially, 96-well plates were coated with fibrinogen (5 μg/ml), vitronectin (1.5 μg/ml), or fibronectin (4 μg/ml) in DPBS. Cells were harvested, washed three times with serum-free minimal essential medium with Earle's salt, and then resuspended in attachment solution (calcium- and magnesium-free Hanks' balanced salt solution, 20 mM HEPES, 1 mg/ml heat-inactivated BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Cells (1×10^4) were added to each well and allowed to attach for 1–2 hr at 37° in a humidified 5% CO_2 incubator. Unattached cells were washed with Hanks' balanced salt solution. Attached cells were determined by colorimetric development of glucosaminidase activity. The number of attached cells was quantitated spectrophotometrically at 405 nm in triplicate, according to a standard curve.

Surface labeling and immunoprecipitation. Transfectants (2×10^6 cells) were surface-labeled with 2 mM Immunopure Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) and then solubilized in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 100 μg/ml leupeptin. Cell extracts were immunoprecipitated overnight with rabbit polyclonal anti-αIIbβ3 antibodies, followed by protein A-Sepharose for 2 hr at 4°. The protein A-Sepharose beads were pelleted, washed in RIPA buffer, resuspended in sample buffer (50 mM Tris·HCl, pH 6.8, 2% SDS, 0.002% bromphenol blue, 10% glycerol), and boiled for 5 min. After centrifugation, immunoreactive proteins were resolved by reducing 8% SDS-polyacrylamide gel electrophoresis (Novex, San Diego, CA). The proteins were transferred to nitrocellulose, stained with horseradish peroxidase-conjugated streptavidin (Amersham, Arlington Heights, IL), and developed with the enhanced chemiluminescence system (NEN-DuPont, Boston, MA).

Ligand binding assays. Saturation binding studies were performed using 1×10^4 cells/tube and increasing concentrations of ^{125}I-L-692,884 in the presence or absence of unlabeled L-692,884 (1 μM), in a total volume of 200 μl, as described below. Using the LIGAND program, αIIbβ3 cells were shown to express, on average, 4×10^5 receptors/cell ($K_d = 12 \times 10^{-9}$ M), whereas αIIbβ3(Δ717) cells express 7.5×10^5 receptors/cell ($K_d = 2 \times 10^{-9}$ M) (data not shown).

Ligand binding assays were performed using the recombinant cells expressing the various constructs. Cells (2.5×10^5/ml) were incubated with 20 pM ^{125}I-L-692,884 (2200 Ci/mmol) in binding buffer (DPBS containing 1 mM $CaCl_2$ and 1%, w/v, BSA), in a total volume of 200 μl. Competitor ligand (antagonist) or control buffer was added as indicated. Binding reactions were incubated for 1 hr at 4°. Cells were collected using a Skatron cell harvester, with phosphate-buffered saline. The bound ^{125}I-ligand was determined with a γ counter (Packard, Downers Grove, IL). Each point represents the average of triplicate determinations, and each experiment was repeated at least three times with similar results. The concentration of the competitor ligand that inhibited binding by 50% (IC_{50}) was determined by a four-parameter nonlinear analysis of bound radioactivity versus the concentration of the ligand. The concentration of the radiolabeled ligand (20 pM) used was much lower than its dissociating binding constant ($K_d = 12$ nM). Thus, under these experimental conditions, the IC_{50} is equal to the dissociation binding constant.

Aggregation of recombinant cells. Agonist-independent aggregation of cells was performed as previously described (18). Typically, 100 μl of cells (1×10^7/ml in DPBS containing 1 mM $CaCl_2$) were added to wells of a 24-well tissue culture plate in the presence or absence of inhibitor (1 μM Ro 43–4054 or L-734,217), in a total volume of 200 μl, and were allowed to remain at room temperature for 30 min. Fibrinogen (1 μM; Sigma Chemical Co., St. Louis, MO) was added, the contents of the wells were mixed by hand-swirling, and the plates were then subjected to gyrorotation at 100 rpm for 30 min. Aggregation was stopped after 20 min by addition of 2% paraformaldehyde (150 μl). The plate was allowed to remain at room temperature for 15 min before analysis by light microscopy. Aggregation was monitored as the formation of aggregates during the rotary agitation. In some cases, cells were pretreated with 0.5 μM cytochalasin D (Sigma) or 0.1% dimethylsulfoxide for 30 min at 4° before addition of fibrinogen. All experiments were performed in triplicate.

Results

Surface expression of αIIbβ3 and mutants in HEK 293 cells. Full-length αIIbβ3 and its mutants were expressed in HEK 293 cells (Fig. 1). Parental HEK 293 cells do not express αIIb or β3, as shown in Fig. 1A. To obtain high

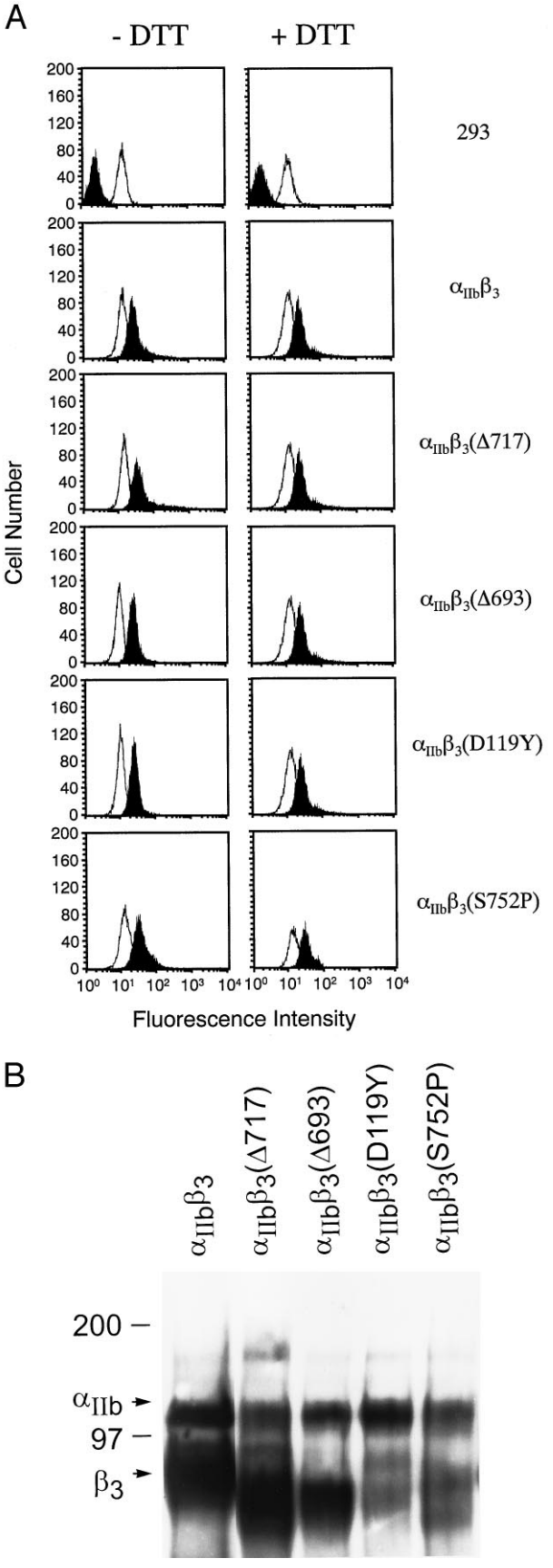

Fig. 1. Surface expression of recombinant αIIbβ3 in HEK 293 cells. A, The expression pattern of αIIbβ3 and $\alpha5\beta1$ in the presence or absence of 2 mM DTT was determined by flow cytometry. Cells (2×10^4) transfected with cDNAs coding for the α and β subunits noted were stained with either anti-αIIbβ3 (mAb CA3) or anti-$\alpha5\beta1$ (mAb JBS5) and then incubated with FITC-conjugated goat anti-mouse IgG. The cells were then subjected to flow cytometry as described in Experimental Procedures. *Filled histograms,* cells labeled with anti-αIIbβ3; *open histograms*, cells labeled with anti-$\alpha5\beta1$. Note that all αIIbβ3 heterodimers were expressed at similar levels. B, Cell extracts of the biotin-surface-labeled HEK 293 cells were immunoprecipitated with polyclonal anti-αIIbβ3 antibody. The immunoprecipitated proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred, and visualized as described in Experimental Procedures. Deletion of the cytoplasmic domain of the β3 subunit leads to a shift in the mobility of the β3 subunit bands on the gels. Compared with the level of receptor expression determined by flow cytometry, the decrease in the level of β3 subunit in the point mutants (D119Y and S752P) may be the result of the decrease in the efficiency of biotinylation, rather than the level of expression.

levels of integrin expression, we first transfected αIIb cDNA into HEK 293 cells and selected clones that expressed high αIIb mRNA levels (data not shown). The full-length β3 subunit and its mutants were then transfected into these cell lines expressing αIIb. The β3 mutants included those with deletion of the cytoplasmic domain [$\beta3(\Delta717)$] or deletion of the transmembrane and cytoplasmic domains [$\beta3(\Delta693)$] and the two variants of Glanzmann's mutants [$\beta3$(D119Y) and $\beta3$(S752P)].

To determine whether the mutations in the β3 subunit affected integrin surface expression of αIIbβ3, transfectants were analyzed by flow cytometry using an αIIbβ3-specific mAb (CA3). Constructs encoding the wild-type subunit and all β3 variants demonstrated comparable levels of surface expression (Fig. 1A). Heterodimer formation and surface expression were also confirmed by surface labeling and immunoprecipitation (Fig. 1B); thus, the β subunit mutations do not disrupt normal subunit association or cell surface expression. In addition, the exogenous expression of these constructs did not alter the level of the endogenous fibronectin receptor $\alpha5\beta1$ in HEK 293 cells (Fig. 1A).

The ability of these stably expressed αIIbβ3 receptors to promote cell adhesion to fibrinogen or vitronectin was investigated using the attachment assay, as described in Experimental Procedures. Parental HEK 293 cells attached readily to fibronectin but poorly to vitronectin or fibrinogen (Fig. 2). Expression of αIIbβ3, αIIb$\beta3(\Delta717)$, or αIIb$\beta3(\Delta693)$ specifically promoted cell attachment to both fibrinogen and vitronectin; however, disruption of ligand binding [$\beta3$(D119Y)] or inside-out signaling [$\beta3$(S752P)] abolished cell attachment to fibrinogen but not that to fibronectin, which suggests that the full-length αIIbβ3 expressed in these cells is a functional receptor.

Discrimination, by binding affinities, of the transitional state of αIIbβ3 in HEK 293 cells. The cyclic RGD peptide L-692,884, cyclo-4-iodo-benzoyl-(Cys-Asn-Pro-Arg-Gly-Asp-Cys)-OH, binds differentially to activated (K_d = 1.4 nM) and unactivated (K_d = 75 nM) αIIbβ3 in human platelets (22).[2] To evaluate the affinity state of the receptor, we used a number of αIIbβ3 antagonists (RGD mimics) (Table 1) to displace the binding of radioiodinated L-692,884 from recombinant cells. These antagonists fall into two categories, i.e., ones that selectively bind to the activated αIIbβ3 (MK-852, L-734,217, L-692,884, and Ro 43–5054) and others that do not discriminate between the activated and unactivated αIIbβ3 (echistatin and Ro 44–9883). Echistatin and

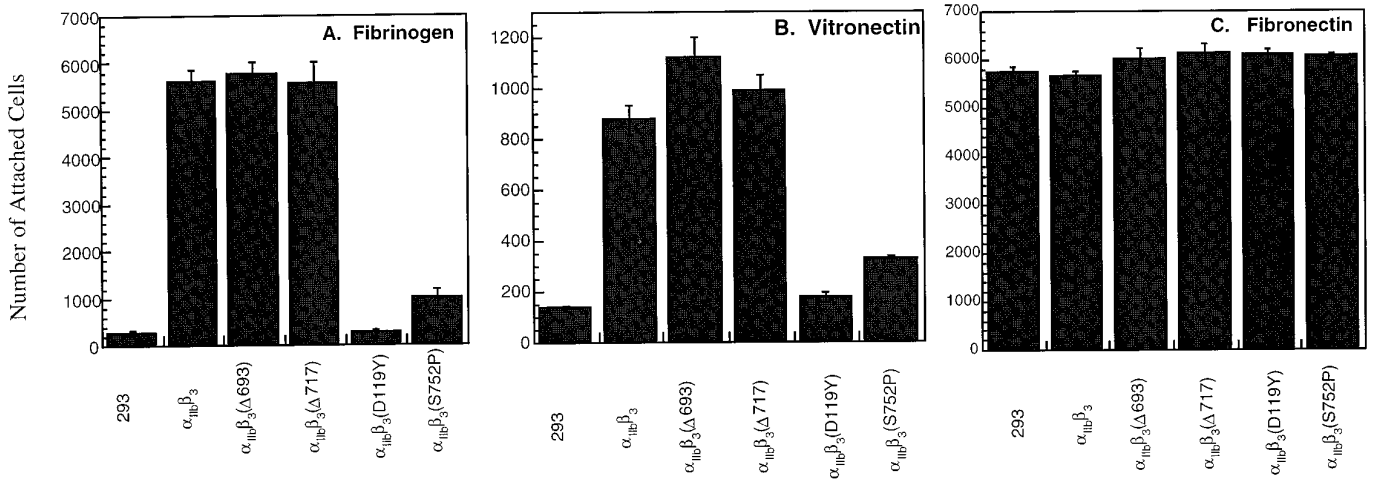

Fig. 2. Cell attachment assay of cells expressing αIIbβ3 constructs. Cells (1×10^4/well) were plated on 96-well plates that had been precoated with fibrinogen (A), vitronectin (B), or fibronectin (C), as described in Experimental Procedures. The numbers of adherent cells after a 2-hr incubation at 37° are reported as the means of triplicate determinations.

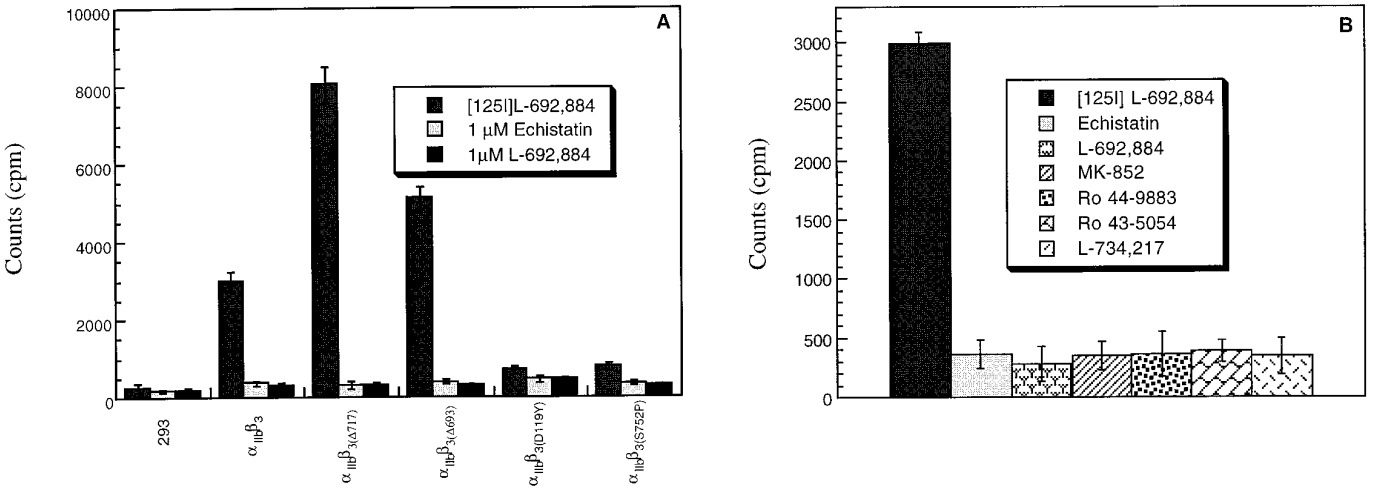

Fig. 3. Binding of ^{125}I-L-692,884 to αIIbβ3 transfectants. A, Specific binding of ^{125}I-L-692,884 to cell lines, determined as described in Experimental Procedures. Cells (5×10^4) were incubated with 20 pM ^{125}I-L-692,884 in the absence or presence (1 μM) of inhibitor (echistatin or L-692,884). The radiolabeled ligand bound specifically only to cells expressing αIIbβ3, αIIbβ3(Δ717), or αIIbβ3(Δ693). B, Displacement of ^{125}I-L-692,884, in HEK 293 cells expressing αIIbβ3, by echistatin, L-692,884, MK-852, Ro 44–9883, Ro 43–5054, or L-734,217.

L-692,884 bind to both αIIbβ3 and αVβ3[1]; all of the other compounds listed in Table 1 are specific for αIIbβ3.[2]

As shown in Fig. 3A, ^{125}I-L-692,884 does not bind to nontransfected HEK 293 cells but selectively binds to cells expressing αIIbβ3, αIIbβ3(Δ717), or αIIbβ3(Δ693). The binding specificity of ^{125}I-L-692,884 for these cells was further demonstrated by displacement using an excess (1 μM) of unlabeled L-692,884 or echistatin (Fig. 3A). The radiolabeled ligand did not bind to cells expressing the Glanzmann's mutant αIIbβ3(D119Y) or αIIbβ3(S752P). This demonstrates that these mutations effectively alter the binding of low-molecular weight ligands to the receptor.

To further characterize the affinity state of αIIbβ3, we analyzed the L-692,884 displacement binding properties of these various RGD mimics with cells expressing wild-type αIIbβ3. As shown in Fig. 3B, the RGD mimics inhibited ^{125}I-L-692,884 binding to HEK 293 cells expressing full-length αIIbβ3. Furthermore, in detailed analysis, the antagonist L-734,217 displaced ^{125}I-L-692,884 binding to the receptor with an IC_{50} of 150 nM. This affinity is intermediate between that for activated αIIbβ3 (4.5 nM) and that for unactivated αIIbβ3 (650 nM) in platelets. However, in the case of αIIbβ3(Δ717) in HEK 293 cells, displacement of L-692,884 binding by L-734,217 was achieved with an IC_{50} of 5.1 nM, which is similar to the value for activated αIIbβ3 in platelets. In fact, all of the RGD mimics examined in this study displaced the radiolabeled L-692,884 from HEK 293 cells expressing wild-type αIIbβ3 with an IC_{50} intermediate between values for the activated and unactivated forms of αIIbβ3 determined in platelets (Table 2). In contrast, the displacement affinity for αIIbβ3(Δ717) in HEK 293 cells was similar to that for the activated form of αIIbβ3 in platelets. These data suggest that the full-length αIIbβ3 expressed in HEK 293 cells may be in a transitional activated state and deletion of the cytoplasmic domain of the β subunit is sufficient to convert it to a fully activated receptor.

PAC1 binding to αIIbβ3 in HEK 293 cells. PAC1 is a murine IgMκ antibody specific for the high affinity conformation of αIIbβ3 (21). PAC1 mimics the ligand binding characteristics of the natural ligand fibrinogen (30) and fails to

TABLE 2

Competitive binding of RGD mimics to HEK 293 cells expressing αIIbβ3 and αIIbβ3(Δ717)

Inhibition of ^{125}I L-692,884 binding to HEK 293 cells expressing αIIbβ3 or αIIbβ3(Δ717) was determined at various concentrations of the RGD mimics. Cells (2.5×10^4) were incubated with 20 pM ^{125}I L-692,884 in the presence of increasing concentrations of RGD mimics, as described in Experimental Procedures. The concentrations of the compounds inhibiting binding of ^{125}I L-692,884 by 50% (IC_{50}) were determined by a four-parameter nonlinear analysis and are represented as mean ± standard error. Under the experimental conditions, IC_{50} is equal to the dissociation binding constant (K_d). The ratio of differentiation is defined as the ratio of the IC_{50} (K_d) values for αIIbβ3 and αIIbβ3(Δ717).

Compound	IC_{50}		Ratio of differentiation
	αIIbβ3(Δ717)	αIIbβ3	
	nM		
Echistatin	3.3 ± 0.2	4.6 ± 0.3	1.4
RO 44-9883	0.26 ± 0.02	2.3 ± 0.2	8.9
L-692,884	0.4 ± 0.2	5.3 ± 0.4	13
MK-852	5.1 ± 0.4	118 ± 16	23
RO 43-5054	0.5 ± 0.02	10.3 ± 2.1	21
L-734,217	5.1 ± 0.3	150 ± 20	30

bind to ligand binding-defective mutants of αIIbβ3 (20). Consequently, PAC1 binding to the receptor mimics the binding of the physiological soluble ligand fibrinogen and serves as a marker for the activated state of αIIbβ3.

Binding of PAC1 to platelets was performed as described in Experimental Procedures. As shown in Fig. 4A, PAC1 did not bind to unactivated platelets but bound to activated platelets upon stimulation with thrombin or pretreatment with 2 mM DTT. In addition, PAC1 binding to platelets was specific and blocked by inhibitors of platelet aggregation such as echistatin, L-692,884, L-734,217, MK-852 (data not shown), or Ro 43–5054 (Fig. 4A).

To assess ligand binding affinity of transfected αIIbβ3 in HEK 293 cells, we examined PAC1 binding to αIIbβ3 transfectants (Fig. 4B). Cells transfected with the full-length αIIbβ3 bound PAC1, albeit weakly. Enhancement of PAC1 binding was seen when the cells were pretreated with 2 mM DTT (Fig. 4B) but not thrombin (data not shown). Pretreatment of cells with 2 mM DTT did not alter the expression levels of the integrins (αIIbβ3 and α5β1), as shown in Fig. 1. This binding of PAC1 to αIIbβ3 was abrogated in the presence of 2 mM EDTA (data not shown). Cells transfected with β subunits with deletions [αIIbβ3(Δ717) and αIIbβ3(Δ693)] bound PAC1 with greater fluorescence intensities. Pretreatment of these cells with 2 mM DTT did not further increase PAC1 binding. The binding of PAC1 to the recombinant cells was also inhibited by the presence of Ro 43–5054 (Fig. 4B). In addition, L-692,884, L-734,217, and MK-852 were also able to block PAC1 binding to cells (data not shown). Interestingly, PAC1 did not bind to cells expressing either of the Glanzmann's thrombasthenic β3 mutants [αIIbβ3(D119Y) or αIIbβ3(S752P)], which indicates that PAC1 is sensitive to an activated conformation surrounding the ligand binding site.

In this study, the PAC1 binding and affinity state for the various constructs are expressed numerically as the AI (29) (Fig. 5). PAC1 bound to HEK 293 cells expressing the full-length receptor with an AI (AI = 61.2 ± 5.6) intermediate between those of resting (AI = 8.3 ± 1.1) and activated (AI = 96.3 ± 4.5) platelets. However, upon deletion of the cytoplasmic domain of the β subunit, the AI of PAC1 binding to these cells was similar to that in activated platelets (Fig. 5), which suggests that, in HEK 293 cells, deletion of the cytoplasmic

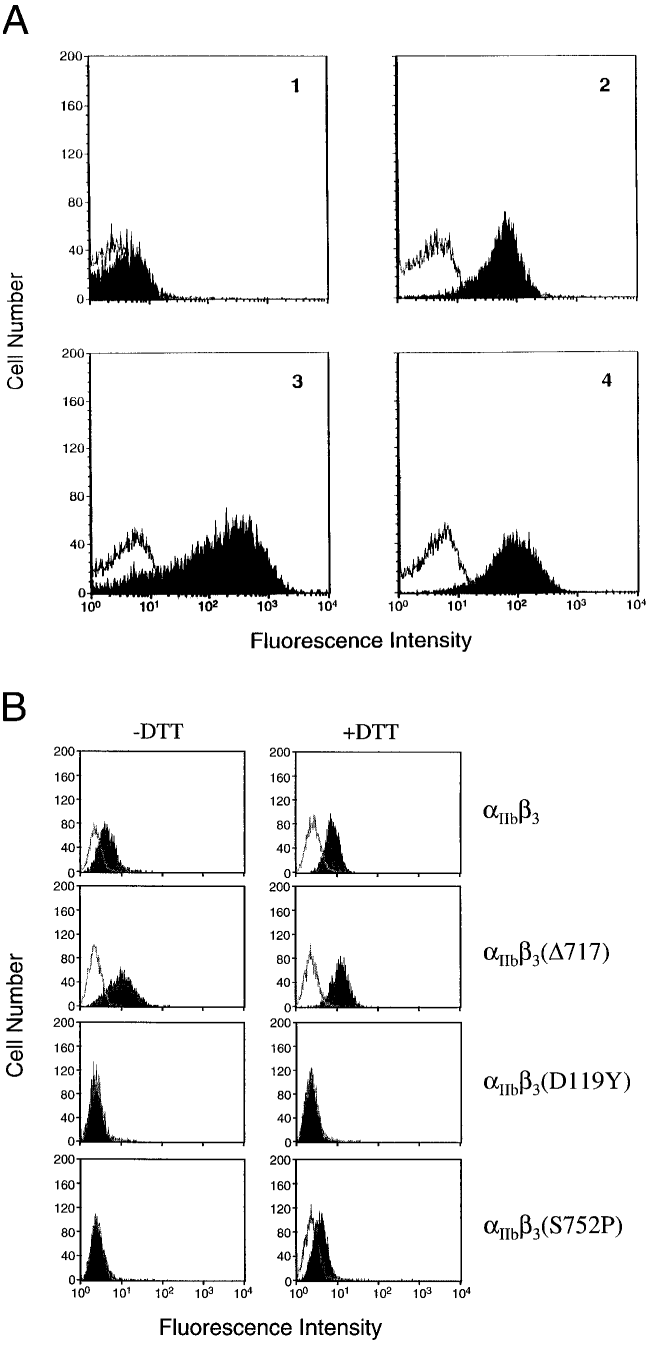

Fig. 4. PAC1 binding to activated platelets and HEK 293 cells expressing αIIbβ3. A, PAC1 binding to platelets in the absence (*filled histograms*) or presence (*open histograms*) of Ro 43–5054 (1 μM). Resting (*1* and *2*) or thrombin-activated (*3* and *4*) platelets were treated with 2 mM DTT (*2* and *4*) or not treated (*1* and *3*) before incubation with PAC1. PAC1 does not bind to resting platelets and binds specifically to activated platelets or DTT-treated resting platelets. B, Flow cytometric histograms illustrating PAC1 binding to HEK 293 cells expressing wild-type αIIbβ3 and the mutants, in the presence (*open histograms*) or absence (*filled histograms*) of the competitive inhibitor Ro 43–5054.

domain of the β subunit results in a fully activated αIIbβ3 receptor. This finding agrees with the data from CHO cells, where deletion of the entire cytoplasmic domain of the β subunit was required for the activation of the αIIbβ3 (5).

Aggregation of cells. Aggregation of platelets requires the presence of αIIbβ3 in the activation state capable of

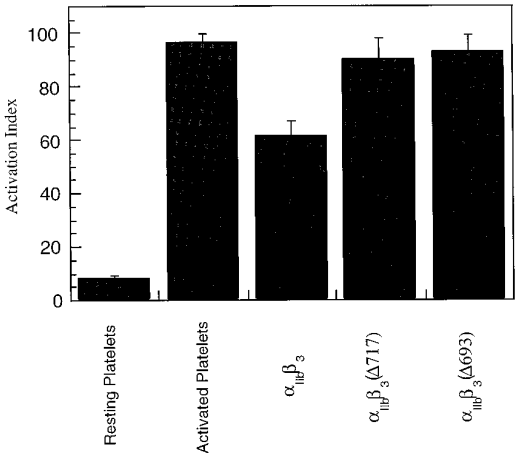

Fig. 5. AI values of αIIbβ3. To obtain a numerical estimate of integrin activation, an AI was calculated for each of the αIIbβ3 mutants, as described in Experimental Procedures. PAC1 binding was measured in HEK 293 cells expressing wild-type αIIbβ3, αIIbβ3(Δ717), or αIIbβ3(Δ693). Depicted are the mean AI ± standard error of three independent experiments.

binding to fibrinogen or other adhesive macromolecules with high affinity (14). In CHO cells, recombinant αIIbβ3 exhibits fibrinogen-dependent aggregation only after the addition of activating antibodies. This fibrinogen-dependent aggregation has also been observed for recombinant cells transfected with an αIIbβ3 construct lacking the entire cytoplasmic domain of the α subunit (19). In the present study, HEK 293 cells expressing the full-length αIIbβ3 were capable of promoting fibrinogen-dependent aggregation in the absence of activating antibodies (Fig. 6A). This αIIbβ3-mediated, fibrinogen-dependent aggregation of HEK 293 cells was completely blocked by L-734,217, an αIIbβ3-specific inhibitor (Fig. 6B). Furthermore, an intact ligand binding site was required for this fibrinogen-dependent aggregation, because cells expressing the Glanzmann's mutation αIIbβ3(D119Y) failed to aggregate (Fig. 6A). Aggregation of cells was completely inhibited by the presence of cytochalasin D (0.5 μM) (Fig. 6B), which indicates that cytoskeletal reorganization is required for aggregation. These results suggest that in HEK 293 cells the full-length αIIbβ3 is capable of mediating agonist-independent cell aggregation.

Discussion

Adhesion is controlled by the kinetics of integrin binding to extracellular matrix. Cells can rapidly change integrin function by altering the binding affinity as well as the avidity of integrins for ligands (3). This modulation of the binding characteristics of integrins is cell type specific and depends on the cytoplasmic domains of widely divergent structures (5).

In this study, we have examined the expression of recombinant αIIbβ3 and its mutants in HEK 293 cells. Cells expressing αIIbβ3, αIIbβ3(Δ717), or αIIbβ3(Δ693) preferentially adhered to immobilized fibrinogen. In contrast, parental HEK 293 cells and cells expressing the Glanzmann's mutants, with mutations in either the ligand binding site [αIIbβ3(D119Y)] or the receptor signaling region [αIIbβ3(S752P)], did not adhere to fibrinogen. As a control, the adhesion to fibronectin, which is presumably mediated by the endogenous integrin α5β1, was similar in parental HEK 293 cells and all recombinant cell lines.

The activation state of the full-length αIIbβ3 receptor in HEK 293 cells was examined by the binding of an activation-dependent antibody (PAC1), peptidic RGD mimics (echistatin, L-692,884, and MK-852), and nonpeptidic RGD mimics (L-734,217, Ro 43–5054, and Ro 44–9883). These RGD mimics include those that discriminate between resting and activated αIIbβ3, as well as those that do not. For example, echistatin, an RGD-containing peptide initially isolated from snake venom, shows no selectivity in binding to unactivated or activated αIIbβ3 (31). Similarly, Ro 44–9883 does not discriminate in its binding to activated or unactivated αIIbβ3. In contrast, L-692,884, MK-852, L-734,217, and Ro 43–5054 bind preferentially to activated αIIbβ3 (Table 1) (22–25).[2]

The cyclic RGD mimic L-692,884 was shown to bind specifically to αIIbβ3 expressed in HEK 293 cells. This binding was sensitive to the presence of inhibitors of αIIbβ3. As summarized in Table 2, the binding specificities of the compounds for the full-length αIIbβ3 in HEK 293 cells are intermediate between those observed for unactivated and activated platelets. However, all of these compounds bind to cells expressing the β-cytoplasmic deletion mutant αIIbβ3(Δ717) with affinities that resemble those in activated platelets. These results indicate that the full-length αIIbβ3 expressed in HEK 293 cells is indeed in a conformational state different from those in resting and activated platelets. We suggest that the receptor may exist in a transitional state.

To further explore the aforementioned hypothesis, the activation state of the recombinant αIIbβ3 expressed in HEK 293 cells was evaluated using PAC1, an antibody specific for activated αIIbβ3. PAC1 bound to cells expressing αIIbβ3, αIIbβ3(Δ717), and αIIbβ3(Δ693). Binding of PAC1 to the full-length αIIbβ3 receptor, although weak, was sensitive to DTT. Pretreatment of the cells with DTT before the addition of the antibody PAC1 increased PAC1 binding to the full-length receptor. In contrast, DTT treatment of cells expressing αIIbβ3(Δ717) and αIIbβ3(Δ693) did not alter PAC1 binding. An AI was defined as a measure of the activation state of the receptor. The full-length receptor, with an AI of 52, was identified to be in an activation state intermediate between that of resting platelets (AI = 8) and that of activated platelets (AI = 93). The AIs for the β3 subunit cytoplasmic and transmembrane deletion mutants αIIbβ3(Δ717) (AI = 88) and αIIbβ3(Δ693) (AI = 86) were indicative of fully activated receptors. These results suggest that the full-length αIIbβ3 expressed in HEK 293 cells is in a "transitional" activation state, which is converted to a fully activated form by pretreatment with DTT. Recent studies[1] have shown that FITC-labeled fibrinogen binds to HEK 293 cells expressing αIIbβ3 and αIIbβ3(Δ717) but does not bind to parental cells or the cells expressing the Glanzmann's mutants. The binding of FITC-labeled fibrinogen to αIIbβ3 was energy dependent and could be abolished by the presence of sodium azide.[1]

The functional state of the full-length αIIbβ3 receptor expressed in HEK 293 cells was assessed by its ability to mediate cell aggregation in the presence of fibrinogen. This aggregation required an intact ligand binding site on the receptor; thus, the transitional activation state of αIIbβ3 in HEK 293 cells was sufficient to support aggregation.

Ginsberg *et al.* (5) examined αIIbβ3 expression in CHO

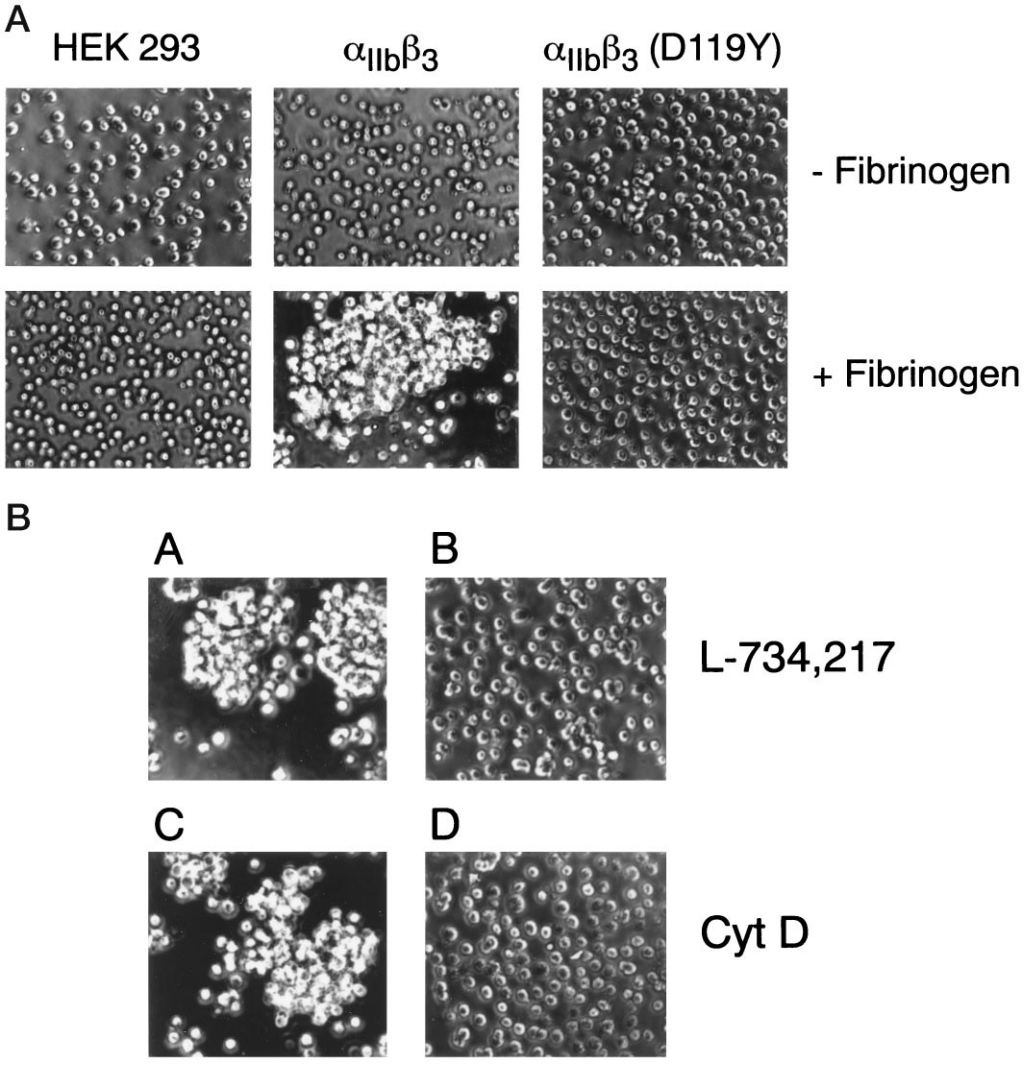

Fig. 6. Agonist-independent aggregation of cells expressing αIIbβ3. A, Cells expressing αIIbβ3 and parental HEK 293 cells were subjected to aggregation in the presence or absence of 1 mM fibrinogen. The cells were subjected to gyrorotation for 30 min at room temperature, and the aggregation was stopped by the addition of (2%) paraformaldehyde. B, Aggregation of recombinant cells was also performed in the absence (*A*) or presence (*B*) of 1 μM L-734,217 or in the absence (*C*) or presence (*D*) of cytochalasin D (*Cyt D*), as described in Experimental Procedures.

cells and found that in this cell system the receptor exists in an unactivated state. Deletion of the membrane-proximal region of either of the cytoplasmic domains of the integrin subunit is sufficient to convert the receptor into an activated form (5, 19). Recently, those authors showed that a charge-reversal mutation in the membrane-proximal region can activate the receptor, presumably through the disruption of a potential salt bridge between the membrane-proximal portions of the α and β subunit cytoplasmic domains (32). Our results are consistent with those studies, in that deletion of the cytoplasmic domain of the β subunit leads to the activation of the receptor. These cytoplasmic deletions render the receptor competent to bind PAC1 and RGD mimics with affinities that resemble those of activated αIIbβ3 in platelets. In contrast to αIIbβ3 expressed in CHO or K562 cells, αIIbβ3 expressed in HEK 293 cells appears to be in a transitional activation state, as determined by the intermediate affinities for RGD peptide and RGD mimics and the ability to bind to PAC1 and mediate fibrinogen-dependent, cell-cell aggregation. We have not been able to isolate a form of αIIbβ3 in HEK 293 cells equivalent to that found in resting platelets.

It is conceivable that several conformations of αIIbβ3 can exist in platelets. In fact, comparative binding studies of soluble fibrinogen and fibronectin suggest that in platelets conformational changes in αIIbβ3 are not "all or none." Intermediate states in the conformational range of the receptor may further modulate the selectivity of soluble versus surface-bound conformations of any one ligand (33). Nakatani *et al.* (34) have established that, when platelets are stimulated with different agonists (and RGD-containing peptides), they bind to fibrinogen with different affinities. Thus, varied conformational states of activated αIIbβ3 may exist in platelets. Kunicki *et al.* (35) have suggested the existence of subpopulations of αIIbβ3 by direct binding of Fab fragments of the antibodies AP7 and PAC1 to αIIbβ3 purified from human platelets, and it has been postulated that these subpopulations of αIIbβ3 result in distinctive phenotypes of human platelets (36). These observations support the notion that recombinant αIIbβ3 may exist in multiple conformational states in HEK 293 cells, with the most predominant being the "transitional" activation state, which we have identified here.

Although the reason for recombinant αIIbβ3 being in a unique conformation in HEK 293 cells is unclear, previous studies have demonstrated that other integrins have cell-specific differences in ligand binding activity. For example, $\alpha 2\beta 1$ purified from platelets does not bind to laminin; however, that purified from endothelial cells binds readily to

laminin, which suggests a role for cell-specific factors in the modulation of integrin affinity (37). Cell-specific intracellular factors that are unique to HEK 293 cells could also account for this activation state of αIIbβ3. Recently, a novel cytosolic regulatory molecule, cytohesin-1, was shown to interact with the cytoplasmic domain of β2 and to increase the strength of αLβ2 interactions with its ligand, intercellular adhesion molecule-1 (9). Alternatively, proteins associated with the β3 integrin, such as integrin-associated protein (38), cell adhesion regulator (39), and endonexin (8), do not appear to influence the affinity state of αIIbβ3 in CHO cells. The regulatory influence of these proteins in modulating αIIbβ3 function in HEK 293 cells requires further investigation.

In conclusion, our results demonstrate that, with the use of novel small peptidic and nonpeptidic RGD mimics, the activation state of αIIbβ3 can be precisely determined. We show that, in HEK 293 cells, αIIbβ3 exists in a transitional activation state that is functionally competent to mediate aggregation of cells in the presence of fibrinogen. Cell-specific conformations of αIIbβ3 *in vivo* could indicate an alternative ligand binding specificity for αIIbβ3 in platelets or megakaryocytes. Such subpopulations of transitionally active αIIbβ3 may serve a physiological role in such events as attachment to specific extracellular matrices or transportation of these proteins into the cell for storage. To understand the conformation-specific integrin-matrix interactions that may be found in different cells, the functional role of the transitionally active αIIbβ3 receptor must be further explored.

Acknowledgments

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