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The PI^{A2} polymorphism of integrin β_3 enhances outside-in signaling and adhesive functions

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Genetic factors are believed to influence the development of arterial thromboses. Because integrin $\alpha_{IIb}\beta_3$ plays a crucial role in thrombus formation, we analyzed receptor adhesive properties using Chinese hamster ovary and human kidney embryonal 293 cells overexpressing the PI^{A1} or PI^{A2} polymorphic forms of $\alpha_{IIb}\beta_3$. Soluble fibrinogen binding was no different between PI^{A1} and PI^{A2} cells, either in a resting state or when $\alpha_{IIb}\beta_3$ was activated with anti-LIBS6. PI^{A1} and PI^{A2} cells bound equivalently to immobilized fibronectin. In contrast, significantly more PI^{A2} cells bound to immobilized fibrinogen in an $\alpha_{IIb}\beta_3$ -dependent manner than did PI^{A1} cells. Disruption of the actin cytoskeleton by cytochalasin D abolished the increased binding of PI^{A2} cells. Compared with PI^{A1} cells, PI^{A2} cells exhibited a greater extent of polymerized actin and cell spreading, enhanced tyrosine phosphorylation of pp125^{FAK}, and greater fibrin clot retraction. These adhesion differences appear to depend on a signaling mechanism sensitive to receptor occupancy. Thus, the PI^{A2} polymorphism altered integrin-mediated functions of adhesion, spreading, actin cytoskeleton rearrangement, and clot retraction.

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Introduction

With few exceptions, defects in single genes do not cause ischemic coronary disease, and it is likely that contributions of multiple inherited and environmental influences culminate in the event recognized as a myocardial infarction. Platelet aggregation mediated by integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) plays a key role in unstable ischemic coronary syndromes (1), and the possibility that the PI^{A2} polymorphism of integrin β_3 might contribute to the genetic component of ischemic vascular disease has been given credence by some (2–5) but not other (6, 7) clinical epidemiology studies (for review, see ref. 8). Platelet studies on donors of known PI^A status have suggested a possible effect on platelet function. For example, most anti- PI^{A1} antibodies completely inhibit the aggregation of $PI^{A1/A1}$ platelets and only retard or partially inhibit aggregation of $PI^{A1/A2}$ platelets (9, 10). Feng et al. found that the PI^{A2} polymorphism was associated with a lower threshold of platelet aggregation (11), and we have observed that compared with PI^{A2} -negative individuals, PI^{A2} -positive platelets have a lower threshold for $\alpha_{IIb}\beta_3$ activation and α granule release (12).

Using platelets in formal studies of the consequences of the PI^{A2} polymorphism on $\alpha_{IIb}\beta_3$ function presents several challenges. First, the marked heterogeneity in in vitro functional assays among ostensibly normal donors presents difficulties in the detection of small differences. An example of this heterogeneity comes from estimates of the K_d for fibrinogen binding to $\alpha_{IIb}\beta_3$, which have var-

ied by 2 orders of magnitude (13–15). Secondly, age, medications, gender, cigarette smoking, co-morbid conditions, or other unknown variables affect platelet function and limit both the interpretation of the data obtained and the pool of potential donors. Lastly, PI^{A2} -homozygous individuals represent less than 2% of the population (16), making these platelets difficult to obtain. Stable cell lines overexpressing the $\alpha_{IIb}\beta_3$ have been used to elucidate many of the kinetic and signaling properties of $\alpha_{IIb}\beta_3$ receptor function (17, 18). The aim of the current study was to determine whether the PI^{A2} polymorphism could influence functions relevant to the process of thrombosis by using Chinese hamster ovary (CHO) and 293 cell lines overexpressing the 2 PI^A forms of $\alpha_{IIb}\beta_3$. We found that PI^{A2} -expressing cells demonstrated increased adhesive functions compared with their PI^{A1} -expressing counterparts, and these differences appear to be the result of a differential dependency on outside-in signaling pathways. These functional differences provide physiologic support for the epidemiologic association between the genetic and platelet components of coronary artery disease.

Methods

Materials. The CHO cell line CHO-K1 (CCL-61) and human embryonal kidney cell line 293 (CRL 1573) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). All reagents that do not specify the name of the manufacturers were from Sigma Chemical Co. (St. Louis, Missouri, USA).

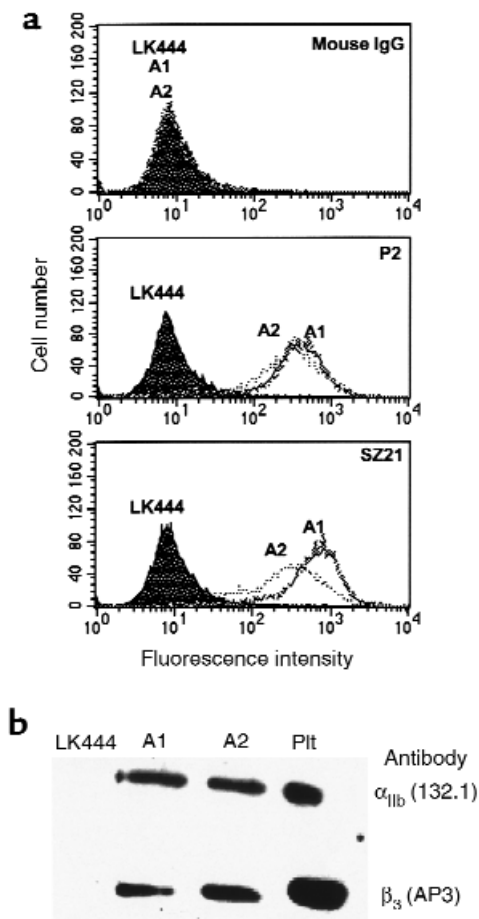


Figure 1 Characterization of stable $\alpha_{IIb}\beta_3$ -expressing CHO cell lines. (a) Flow cytometric analysis. LK444, A1, and A2 cell lines were labeled with 3 $\mu\text{g}/\text{mL}$ isotype-matched control IgG (upper panel), 3 $\mu\text{g}/\text{mL}$ $\alpha_{IIb}\beta_3$ complex-specific mAb P2 (middle panel), or 2 $\mu\text{g}/\text{mL}$ SZ21 mAb that distinguishes PI^{A1} from PI^{A2} (lower panel). LK444, solid line; A1, heavy dotted line; A2, fine dotted line. (b) Western immunoblotting. Fifteen micrograms of CHO cell lysates or 2 μg of platelet lysates were separated by nonreduced SDS-PAGE, transferred to nitrocellulose, and probed with either 132.1 (α_{IIb} -specific) or AP3 (β_3 -specific) mAbs.

Generation of cell lines. The cDNA for β_3 (19) corresponding to the PI^{A1} polymorphism was mutated to code for the PI^{A2} polymorphism using the Altered Sites II in vitro Mutagenesis Kit (Promega Corp., Madison, Wisconsin, USA) as described previously (20) and sequenced to confirm authenticity. The cDNAs for α_{IIb} (21) and for β_3 (either PI^{A1} or PI^{A2}) were engineered into the LK444 vector (22) and used to transfect CHO cells. In addition, the cDNA for α_{IIb} was engineered into the pZeoSV2 vector (Invitrogen Corp., Carlsbad, California, USA) and the cDNA for β_3 (either PI^{A1} or PI^{A2}) engineered into the pcDNA3.1 vector (Invitrogen Corp.) and used to transfect 293 cells. Both CHO and 293 cells were transfected using lipofectin (Life Technologies Inc., Gaithersburg, Maryland, USA) (23) with the respective plasmids for both α_{IIb} and β_3 subunits. Control CHO cells (designated LK444) were transfected

with the LK444 parental vector whereas the control 293 cells (designated Pc/Z) were transfected with empty pcDNA3.1 and pZeoSV2 vectors. Stable CHO cell lines expressing the neomycin resistance gene were selected in the presence of 250 $\mu\text{g}/\text{mL}$ G418 (Life Technologies) and cultured in αMEM (Life Technologies) containing 10% FBS (Gemini Bio-Products, Calabasas, California, USA), and antibiotics (penicillin, streptomycin, amphotericin; Life Technologies). Stable 293 cell lines expressing the neomycin and zeocin resistance genes were selected in the presence of 300 $\mu\text{g}/\text{mL}$ G418 and 100 $\mu\text{g}/\text{mL}$ zeocin (Invitrogen Corp.) and cultured in DMEM (Life Technologies) containing 10% FBS containing the same antibiotics as above. $\alpha_{IIb}\beta_3$ -expressing cells were sorted by flow cytometry using the $\alpha_{IIb}\beta_3$ complex-specific mAb, P2 (Immunotech, Marseilles, France) to select for high and equivalent expression. These cells were pooled and used for the study.

Flow cytometry. CHO and 293 cells were grown to 70–80% confluency and detached using 0.05% trypsin (Life Technologies). After neutralization with complete media, the cells were suspended in PBS (pH = 7.4; 0.137 M NaCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 2.7 mM KCl) with 2% BSA and incubated with either 3 $\mu\text{g}/\text{mL}$ P2, 2 $\mu\text{g}/\text{mL}$ anti- β_3 mAb (clone SZ21; Immunotech), or 3 $\mu\text{g}/\text{mL}$ control mouse IgG (Pierce Chemical Co., Rockford, Illinois, USA) for 1 hour on ice. The cells were washed, incubated with a secondary goat anti-mouse FITC-labeled antibody, and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, California, USA), with fluorescence data acquired in the logarithmic mode and light scattering data acquired in the linear mode (24). The mean channel

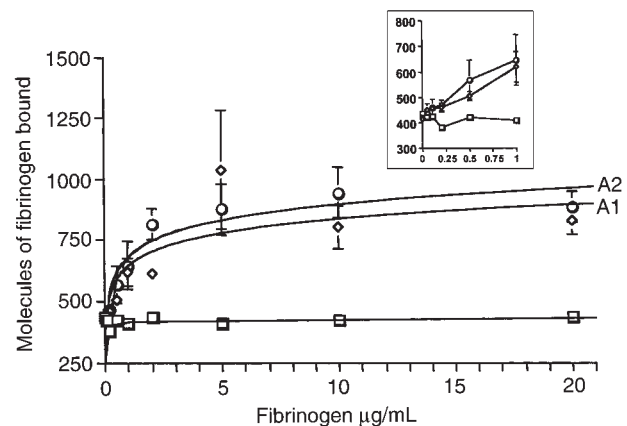


Figure 2 Binding to soluble fibrinogen. LK444 (open squares), A1 (open diamonds), and A2 (open circles) cells were incubated with 5 μM LIBS-6 to activate $\alpha_{IIb}\beta_3$ and varying concentrations of FITC-labeled fibrinogen, then analyzed by flow cytometry. The graph is expressed as SEM of 4 experiments. Best-fit curves for LK444, A1, and A2 cells were generated using the logarithmic mode in CA Cricket Graph III version 1.5.2. The inset shows an expanded view of the fibrinogen concentrations between 0 and 1 $\mu\text{g}/\text{mL}$.

number, which corresponded to cell-fluorescence intensity, was used as the measure of surface-expressed $\alpha_{IIb}\beta_3$ for the whole population.

Western blot analysis. Cells were lysed in 15 mM HEPES, pH 7.0, 145 mM NaCl, 0.1 mM $MgCl_2$, 10 mM EGTA, 1% Triton X-100, 1 mM $NaVO_4$, 250 $\mu g/mL$ 4-2-aminoethyl-benzene sulfonylfluoride, 15 $\mu g/mL$ of protease inhibitors chymostatin, antipain, and pepstatin, and 55 $\mu g/mL$ of the protease inhibitor leupeptin. Fifteen micrograms of cell lysate or 2 μg platelet lysate were separated in 7% SDS polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose, and then subjected to Western blotting using a 1:500 dilution of monoclonal anti- α_{IIb} (132.1) and monoclonal anti- β_3 (AP-3) (gifts from Peter Newman, Blood Research Institute, Milwaukee, Wisconsin, USA) or 1:1000 dilution of SZ21 antibody. After the addition of horseradish peroxidase-labeled goat anti-mouse (1:3000 dilution), blots were developed using enhanced chemiluminescence (ECL; Amersham Life Science, Piscataway, New Jersey, USA) (25).

Binding to soluble fibrinogen. CHO cells were trypsinized and rinsed as above, and 5×10^5 cells were resuspended in 50 μL Tyrode's buffer (138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.36 mM Na_2HPO_4 , 5.5 mM glucose, pH 7.4) containing 1 mg/mL BSA, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. The cells were incubated with varying concentrations of FITC-conjugated fibrinogen (0–100 $\mu g/mL$) in the presence or absence of 0.5 μM $\alpha_{IIb}\beta_3$ -activating mAb LIBS-6 (gift of Mark Ginsberg, The Scripps Research Institute, La Jolla, California, USA) for 30 minutes at 22°C. Cells were analyzed for FITC fluorescence by flow cytometry. Specific fibrinogen binding was defined as the difference in mean fluorescence between LIBS-6-activated and nonactivated cells.

Adhesion to immobilized ligands. Cells were trypsinized and rinsed as above, suspended in 0.01% BSA in Tyrode's buffer for adhesion studies to fibrinogen (Enzyme Research Laboratories Inc., South Bend, Indiana, USA) or in 0.01% BSA in HBSS (136 mM NaCl, 5.3 mM KCl, 0.33 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 5.5 mM glucose, pH 7.4) for adhesion studies to fibronectin (Life Technologies). Cells were further labeled with ^{51}Cr (Amersham) by incubating in 25 $\mu Ci/200 \mu L$ at 37°C for 1 hour with gentle shaking. Cells were washed 3 times with Tyrode's buffer or HBSS and resuspended at a concentration of 3.5×10^5 cells/mL in Tyrode's buffer or HBSS with 1.8 mM $CaCl_2$ and 0.49 mM $MgCl_2$. Adhesion to immobilized fibrinogen and fibronectin were performed as described previously (26). Twenty-four-well tissue culture plates (Falcon, Lincoln Park, New Jersey, USA) were coated with varying concentrations (2.5, 5, 12.5, and 20 $\mu g/mL$) of fibrinogen or fibronectin in Tyrode's buffer or HBSS, respectively, for 2 hours at 22°C. Wells were washed twice with Tyrode's buffer or HBSS and incubated with 500 μL of 5 mg/mL of heat-inactivated BSA for 2 hours at 22°C, with constant shaking on a platform shaker to block nonspecific binding to the plates. Control wells were coated only with 500 μL of 5 mg/mL heat-inactivated BSA. Before the adhesion assay the wells were washed twice with Tyrode's buffer or HBSS. For adhesion studies 0.7×10^5 ^{51}Cr -labeled cells in 0.2 mL were added to each well and incubated at 37°C for 5 minutes. Unbound cells were removed by inverting the plates. Each well was washed 3 times with 200 μL of Tyrode's buffer or HBSS by briskly tilting the plate 10 times at an angle of 90° along an axis parallel to that of the length of the plate, followed by 10 times along an axis perpendicular to that of the length of the plate. The buffer was removed by inverting the plate, and the excess liquid was dabbed on a filter paper. Note that each of the cell lines

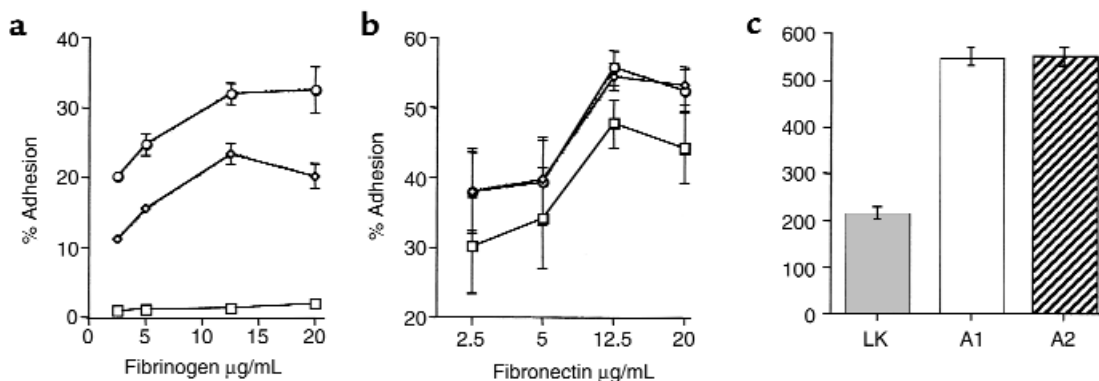


Figure 3

Adhesion of CHO cell lines to immobilized ligands. The ^{51}Cr -labeled LK444 (open squares), A1 (open diamonds), and A2 (open circles) cells were allowed to adhere to immobilized fibrinogen (a) or fibronectin (b) for 5 minutes. The results are expressed as \pm SEM for 5–21 observations. Compared with A1 cells, A2 cells demonstrated 80% greater adhesion at 2.5 $\mu g/mL$ ($P < 0.001$), 60% greater adhesion at 5 $\mu g/mL$ ($P < 0.001$), 31% greater adhesion at 12.5 $\mu g/mL$ ($P < 0.001$), and 61% greater adhesion at 20 $\mu g/mL$ ($P = 0.001$). There was no difference in adhesion between A1 and A2 cells at any concentration of fibronectin ($P \geq 0.22$). (c) Mean fluorescence intensity of P2 binding (α_{IIb} -specific) to the 3 CHO cell lines determined within 24 hours of the adhesion assay.

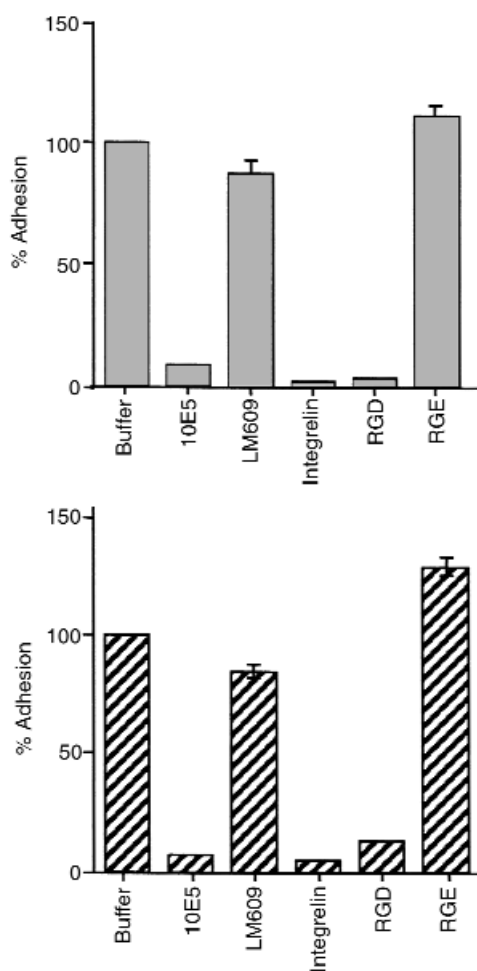


Figure 4

Adhesion of A1 and A2 CHO cells is $\alpha_{IIb}\beta_3$ specific. Adhesion was as in Figure 3, except that A1 and A2 cells were first incubated for 10 minutes with buffer or 2.1 $\mu\text{g}/\text{mL}$ 10E5, 10 $\mu\text{g}/\text{mL}$ LM609, 5 μM integrilin, 200 μM RGD peptide, or 200 μM RGE peptide. Cells were allowed to bind for 5 minutes before washing. Binding without inhibitors is displayed as 100%. The experiment was performed 3 times; error bars were too narrow to be seen with the 10E5, integrilin, and RGD inhibition. Binding of A1 and A2 cells after inhibition with 10E5, integrilin, and RGD was equivalent to basal binding of the LK444 control line.

under comparison was present on the same plate and hence exposed to identical adhesion and washing conditions. The bound cells were lysed with 0.1% SDS, and the lysate was counted on a gamma counter. The specific percent of cells bound to fibrinogen in each well was calculated as: (cpm from fibrinogen/fibronectin-coated wells) – (cpm from BSA-coated wells) \times 100/total cpm added to each well.

Specificity of adhesion to fibrinogen was investigated by incubating the ^{51}Cr -labeled cells for 10 minutes with 1 of the following inhibitors: 2.1 $\mu\text{g}/\text{mL}$ 10E5, a blocking mAb against $\alpha_{IIb}\beta_3$ (gift from Barry Coller, Mt. Sinai Hospital, New York, New York, USA); 5 μM integrilin, a blocking cyclic peptide specific for $\alpha_{IIb}\beta_3$ (gift from COR Therapeutics Inc. South San Francisco, California,

USA); 200 μM RGD peptide; 10 $\mu\text{g}/\text{mL}$ anti- $\alpha_v\beta_3$ mAb LM609 (Chemicon International Inc., Temecula, California, USA); 200 μM RGE peptide; or 10 $\mu\text{g}/\text{mL}$ mouse IgG. In certain experiments ^{51}Cr -labeled cells were incubated for 30 minutes with either 10 μM cytochalasin D, 12 μM bisindolylmaleimide, or control DMSO. A 5-minute adhesion assay was then performed as described above. In certain experiments 10- or 15-minute adhesion assays were also performed.

Confocal microscopy. Glass coverslips were coated with 12.5 $\mu\text{g}/\text{mL}$ fibrinogen in Tyrode's buffer for 2 hours at 22°C. Fibrinogen-coated coverslips were incubated with 10^5 CHO cells in 100 μL for 5 minutes at 37°C. Unbound cells were removed and cells fixed with 3.5% paraformaldehyde for 8 minutes, permeabilized with 0.1% NP-40 for 15 minutes, and transferred to PBS containing 10% FCS for 20 minutes at 22°C. Cells were stained for actin with 0.6 μM rhodamine-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at 22°C. The coverslips were washed twice and mounted in permafluor (Lipshaw Immunon, Pittsburgh, Pennsylvania, USA). Fluorescent images were obtained with Zeiss LSM 410 confocal imaging system attached to a Zeiss C-apo $\times 40$ water immersion lens microscope (Carl Zeiss Inc., Thornwood, New York, USA). The degree of fluorescence and the area of cell spreading were quantitated by scoring for individual cells using the Metamorph image analysis system (Universal Imaging Corp., Westchester, Pennsylvania, USA).

Clot retraction assay. CHO cells grown to confluence were harvested as described above and resuspended in αMEM containing 28 mM CaCl_2 and 25 mM HEPES, pH 7.4. A total of 4×10^6 cells in 300 μL were mixed with 200 μL of fibronectin-depleted plasma, 250 μg of fibrinogen, 5 μg of aprotinin in a 75 \times 100-mm glass tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Clot formation was initiated by the addition of 2.5 U thrombin (Sigma). After incubation at 37°C for varying time periods, clot retraction was quantitated by measuring the volume of liquid not incorporated into the clot, as described (27). The percentage of clot retraction was calculated as: (amount of liquid collected)/(the total amount of liquid before the clot) \times 100. Percentage of clot retraction was normalized for CHO cell $\alpha_{IIb}\beta_3$ -receptor density by dividing by the mean fluorescence intensity (clot retraction units). In certain experiments, cells were pretreated for 15 minutes with 10 $\mu\text{g}/\text{mL}$ of LM609 or 5 μM of integrilin.

Tyrosine phosphorylation of pp125^{FAK}. Twenty-four-well tissue culture plates were coated with either 12.5 $\mu\text{g}/\text{mL}$ of fibrinogen or 2.5 mg/mL heat-treated BSA. Two hundred microliters of 3.5×10^5 CHO cells/mL were added to each well and incubated for 5 minutes at 37°C in 5% CO_2 . The adherent cells were lysed with ice-cold lysis buffer (described above), and the lysate was incubated for 30 minutes on ice, clarified by centrifugation at 14,000 g for 20 minutes, and the protein content determined using then Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, California, USA).

One hundred fifty microliters of proteins from each lysate were precleared with protein A-Sepharose (Pharmacia Biotech, Piscataway, New Jersey, USA) and incubated with 2 μg of anti-pp125^{FAK} antibody (Upstate Biotechnology Inc., Lake Placid, New York, USA) for 16 hours at 4°C and precipitated using protein A-Sepharose beads. After washing the beads 3 times with ice-cold lysis buffer, proteins were eluted in boiling Laemmli sample buffer containing 1 mM orthovanadate. Proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, probed with antiphosphotyrosine 4G10 antibody (Upstate Biotechnology) or anti-pp125^{FAK} antibody, and developed as described above. The signals were scanned using PhotoShop 5 software and the densitometric quantitation performed using NIH Image software [developed at the Research Services Branch of the National Institute of Mental Health in Bethesda, Maryland, USA (<http://rsb.info.aov/nih-image/default.html>)].

Results

Overexpression of the PI^{A1} and PI^{A2} isoforms of $\alpha_{IIb}\beta_3$ in CHO cells. Wild-type CHO cells were stably transfected with expression plasmids containing the cDNAs for α_{IIb} and β_3 (either the PI^{A1} or PI^{A2} forms) or the empty parental LK444 vector. Flow cytometric analysis with a mAb specific for the $\alpha_{IIb}\beta_3$ complex revealed equivalent levels of receptor expression on the cell lines generated with the PI^{A1} and PI^{A2} isoforms, designated as lines A1 and A2, respectively (Figure 1a). The neomycin-resistant control CHO cells, designated LK444, did not express detectable $\alpha_{IIb}\beta_3$. The mAb SZ21 was used to distinguish the 2 forms of β_3 because it binds the PI^{A2} isoform of β_3 with a much lower affinity than the PI^{A1} isoform (25). In addition, Western immunoblot analysis demonstrated the presence of α_{IIb} and β_3 in cell lysates of A1 and A2 cells, but not LK444 cells, (Figure 1b). As with the flow cytometry, the PI^{A1} form, but not PI^{A2} form, of β_3 could be immunodetected using SZ21 antibody (not shown).

Binding to soluble fibrinogen. We assessed initially whether the PI^{A2} isoform was in a conformation favoring soluble ligand binding. Similar to previous studies where $\alpha_{IIb}\beta_3$ -expressing CHO cells did not bind soluble fibrinogen in response to physiologic agonists (18), we observed no binding of soluble fibrinogen or the mAb PAC-1 (specific for the activated conformation of $\alpha_{IIb}\beta_3$) to the “resting” states of either the A1 and A2 cell lines (data not shown). In addition, neither the thrombin receptor-activating peptide (100 μM) nor epinephrine (20 μM) caused fibrinogen binding to either cell line. However, the $\alpha_{IIb}\beta_3$ -activating mAb LIBS-6 induced equivalent fibrinogen binding to both the PI^{A1} and PI^{A2} CHO cell lines, both at subsaturating (Figure 2) and saturating (20–100 $\mu\text{g}/\text{mL}$; data not shown) fibrinogen concentrations. The concentration of fibrinogen producing half-maximal binding appeared to be no different for A1 cells and A2 cells. Thus, although both isoforms were functional in CHO

cells, neither the resting nor activated conformation of PI^{A2} resulted in greater soluble fibrinogen binding compared with PI^{A1} , and we conclude that there is no significant difference in the binding kinetics of soluble fibrinogen to the 2 isoforms of β_3 .

Adhesion to immobilized ligands. Because in vivo thrombus growth requires platelet adhesion to fibrinogen, we next examined the ability of $\alpha_{IIb}\beta_3$ -expressing CHO cells to bind immobilized ligands. Both A1- and A2-expressing cells bound in significantly greater numbers than did the LK444 to immobilized fibrinogen, suggesting $\alpha_{IIb}\beta_3$ mediated adhesion (Figure 3a). Whereas there was a slight trend toward greater binding to soluble fibrinogen in A2 cells compared with A1 (Figure 2), significantly greater A2 cell adhesion was observed over a range of immobilized fibrinogen concentrations ($P \leq 0.001$). In contrast, there was equivalent adhesion of A1 and A2 cells to fibronectin (a ligand for endogenous CHO cell $\alpha_5\beta_1$) (Figure 3b), suggesting that the differential adhesion due to the PI^A polymorphism was

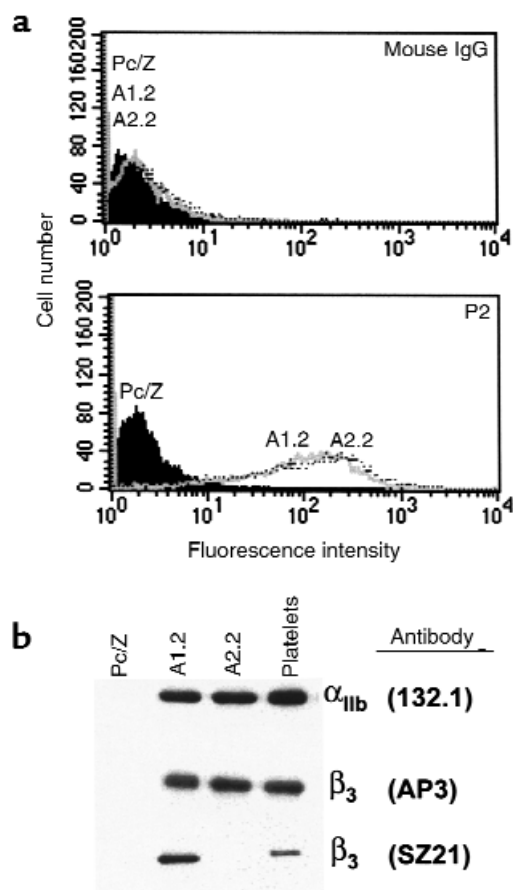


Figure 5 Characterization of stable $\alpha_{IIb}\beta_3$ -expressing 293 cell lines. (a) Flow cytometric analysis. Pc/Z, A1.2, and A2.2 cell lines were labeled with 3 $\mu\text{g}/\text{mL}$ isotype-matched control IgG (upper panel), 3 $\mu\text{g}/\text{mL}$ $\alpha_{IIb}\beta_3$ complex-specific mAb P2 (lower panel). Pc/Z, solid line; A1.2, heavy dotted line; A2.2, fine dotted line. (b) Western immunoblotting of the 293 cell lines. Fifteen micrograms of 293 cell lysates or 2 μg of platelet lysates were separated by nonreduced SDS-PAGE, transferred to nitrocellulose, and probed with 132.1 (α_{IIb} -specific), AP3 (β_3 -specific), or SZ21 (PI^{A1} -specific) mAbs.

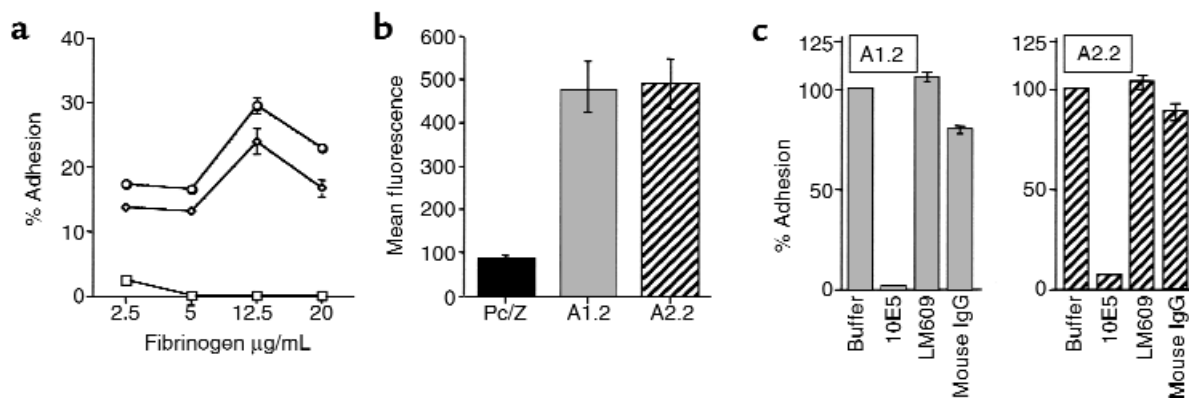


Figure 6

Adhesion of 293 cell lines to immobilized fibrinogen. (a) Pc/Z (open squares), A1.2 (open diamonds), and A2.2 (open circles) cell adhesion was performed as in Figure 3. The results are expressed as \pm SEM of 9 observations for all concentrations. Compared to A1 cells, A2 cells demonstrated 26% greater adhesion for 2.5 μ g/mL, 25% greater adhesion for 5 μ g/mL, 23% greater adhesion for 12.5 μ g/mL, and 37% greater adhesion for 20 μ g/mL. The increased adhesion of A2 over A1 was significant at $P = 0.005$ for 2.5 μ g/mL, $P = 0.05$ for 5 μ g/mL, $P = 0.006$ for 12.5 μ g/mL. (b) Mean fluorescence intensity of P2 binding to the three 293 cell lines, determined within 24 hours of the adhesion assay. (c) Inhibition of A1.2 and A2.2 cell lines. A1.2 and A2.2 cells were first incubated for 10 minutes with buffer or 2.1 μ g/mL 10E5, 10 μ g/mL LM609, 10 μ g/mL mouse IgG. Cells were allowed to bind for 5 minutes before washing. Binding without inhibitors is displayed as 100%. Results are expressed as \pm SEM of 3 observations. Error bars were too narrow to be seen with the 10E5 inhibition.

ligand specific. Similarly, no difference in adhesion to an immobilized anti- β_3 antibody (AP3) was observed between A1 and A2 cells (data not shown). Surface expression of $\alpha_{IIb}\beta_3$ was not detectably different between the A1 and A2 cell lines and could not account for the observed difference in adhesion (Figure 3c). Specificity of binding to fibrinogen by both the A1 and A2 cell lines was further demonstrated by more than 90% inhibition with $\alpha_{IIb}\beta_3$ -specific blocking antibody 10E5, integrilin, and RGD peptides (Figure 4). RGE peptides showed no inhibition, and the LM609 mAb, which recognizes the chimeric hamster-human $\alpha_v\beta_3$ receptor, inhibited little of the A1 and A2 cell adhesion to fibrinogen (Figure 4), indicating adhesion was mediated primarily through $\alpha_{IIb}\beta_3$. In addition, these assays were performed in the presence of 1.8 mM calcium and no manganese, conditions not permissive for the $\alpha_v\beta_3$ -mediated adhesion to fibrinogen (28).

Studies in 293 cells. Although CHO cell lines were generated by cell sorting and should not have been subject to clonal variation, we generated a second set of cell lines to confirm the functional difference associated with the PI^{A2} polymorphism. Wild-type 293 cells were stably transfected as described in Methods. Equivalent levels of receptor expression on the cell lines generated with the PI^{A1} (called A1.2) and PI^{A2} (called A2.2) isoforms were seen by flow cytometry (Figure 5a). Both A1.2 and A2.2 cells, but not the vector-only control (called Pc/Z) cells, showed α_{IIb} and β_3 with Western blot analysis. SZ21 antibody was used to distinguish the 2 forms of β_3 (Figure 5b).

Both the A1.2 and A2.2 cells bound in significantly greater numbers than the Pc/Z to immobilized fibrinogen (Figure 6). A2.2 cells exhibited greater binding over A1.2 cells after 5 minutes over a range of immobilized fibrinogen concentrations. (Figure 6a). The

observed difference in adhesion between A1.2 and A2.2 cells was not because of difference in their surface expression of $\alpha_{IIb}\beta_3$ (Figure 6b). Furthermore, this adhesion was abolished by the 10E5 antibody but not by LM609 antibody or mouse IgG (Figure 6c). Thus, 2 independent sets of cell lines from 2 different parental strains yielded similar data regarding greater adhesion of PI^{A2} -expressing cells compared with PI^{A1} -expressing cells. This makes clonal variation a highly unlikely explanation for these data and greatly strengthens the conclusion that the different adhesive properties are intrinsic to the PI^A polymorphism.

Role of actin cytoskeleton in the differential adhesion to fibrinogen. During the course of these studies, it appeared that the bound A2 cells exhibited a different morphology than the bound A1 cells. The actin cytoskeleton plays an important role in a large number of cellular functions, including cell adhesion, shape, and motility (29) and is rapidly remodeled in response to external stimuli. We used confocal microscopy of rhodamine-phalloidin-stained actin and observed more F actin in the periphery of A2 cells compared with A1 cells (Figure 7a, upper row). With additional time for adhesion, substantially greater cell spreading was observed in the A2 cells compared with the A1 cells (Figure 7a, lower row). Quantitative image analysis revealed a greater actin staining ($P < 0.001$; Figure 7b) and greater surface area ($P = 0.01$; Figure 7b) for A2 cells adhered to fibrinogen compared with A1 cells. Figure 8a shows how cytochalasin D abolishes the difference in adhesion between the A1 and A2 cell lines over a range of fibrinogen concentrations at the 5-minute time point. Cytochalasin D inhibited both A1 and A2 cell adhesion, but the overall percent of inhibition was greater in the A2 cells. The inhibitory effects of cytochalasin D on both cell lines was time dependent and largely lost after

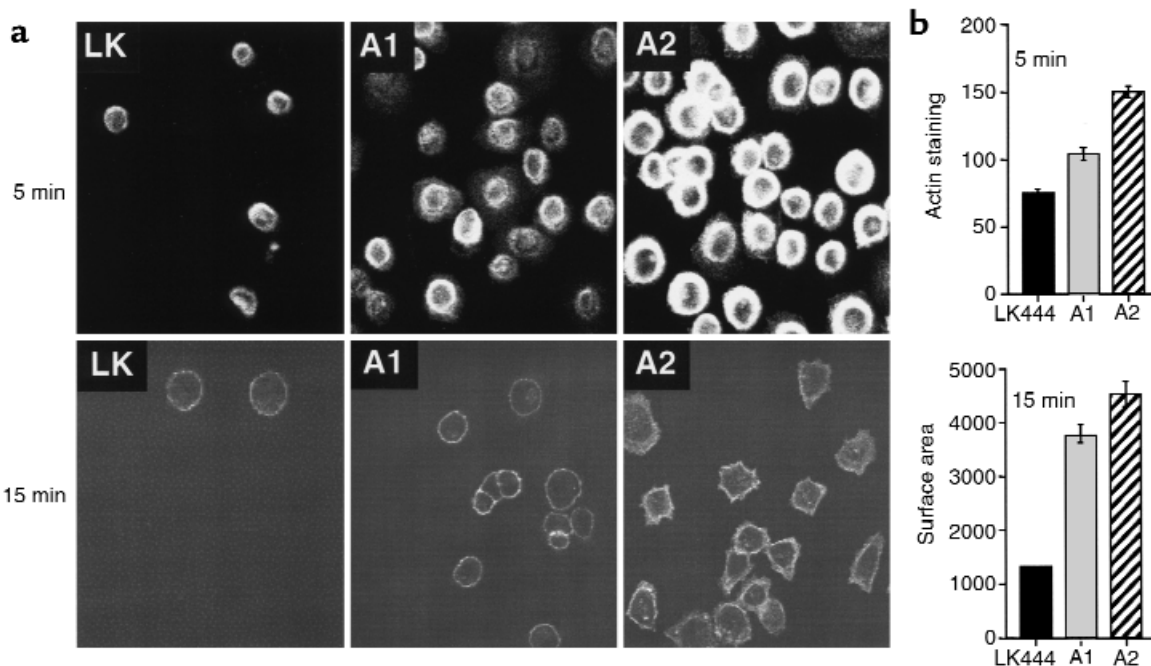


Figure 7
 (a) Confocal microscopy of the actin cytoskeleton in CHO cells adhered to fibrinogen. Cells adherent to coverslips coated with fibrinogen were stained with rhodamine phalloidin to detect F-actin. A greater actin polymerization was seen for A2 cells bound to fibrinogen for 5 minutes compared with A1 cells (upper row). After 15 minutes of incubation, greater spreading was seen for A2 compared with A1 cells (lower row). (b) The morphometric analysis of actin staining and cell spreading is shown to the right in the upper and the lower graphs, respectively. At 5 minutes F-actin staining of A2 compared with A1 cells was significantly greater ($P < 0.001$). Actin staining is shown as arbitrary units of fluorescence defined by Metamorph software. Results are expressed as \pm SEM of values obtained from 50 cells. At 15 minutes the greater surface area for A2 cells compared with A1 cells adhered to fibrinogen was significant at $P = 0.01$. Surface area is shown as arbitrary units of cell area. Results are expressed as \pm SEM of values obtained from 35 cells.

the 5-minute time point (Figure 8b), perhaps because of the recruitment of other noncytoskeletal mechanisms that stabilize cell adhesion. However, the ability of cytochalasin D to eliminate the adhesion difference between A1 and A2 cells persisted at these later time points. Surface expression of $\alpha_{IIb}\beta_3$ was not detectably different between the A1 and A2 cell lines in these experiments (Figure 8c). These findings indicate that

an intact cytoskeletal architecture is required for the rapid stable adhesion of both A1 and A2 cells to fibrinogen, but the PI^{A2} isoform of β_3 demonstrates a more robust and brisk actin reorganization.

Analysis of signaling pathways that influence adhesion. We investigated postreceptor occupancy signaling pathways that might affect reorganization of the actin cytoskeleton and hence, cell adhesion. Focal adhesion

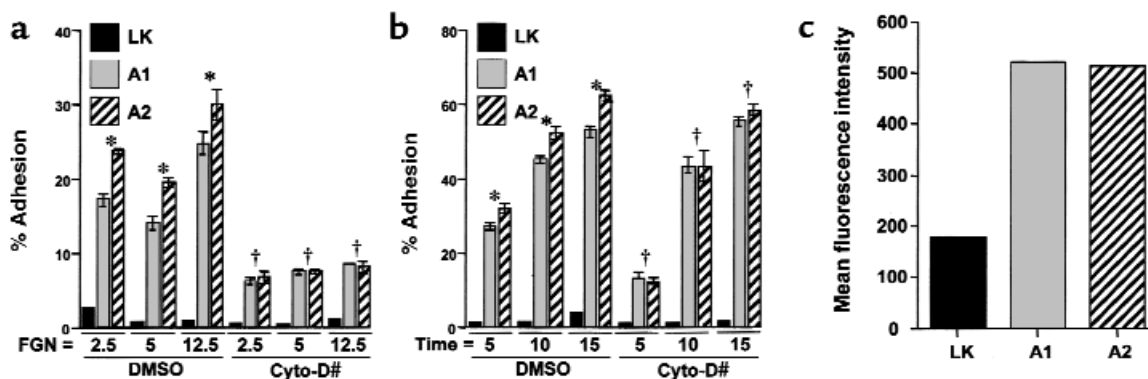


Figure 8
 Effect of cytochalasin D on CHO cell adhesion. Cells were treated with either DMSO or 10 μ g/mL of cytochalasin D for 30 minutes, washed twice, and adhesion assays performed as above. (a) Adhesion for 5 minutes to different concentrations of fibrinogen. $*P \leq 0.008$ for A1 vs. A2; $\dagger P > 0.46$ for A1 vs. A2; $\#P < 0.03$ for A1 (DMSO vs. cyto-D), and $P < 0.001$ for A2 (DMSO vs. cyto-D). The results are expressed as \pm SEM of 6 observations. (b) Adhesion to 12.5 μ g/mL fibrinogen for different time points. $*P \leq 0.007$ for A1 vs. A2; $\dagger P \leq 0.08$ for A1 vs. A2; $\#P \leq 0.0008$ for A1 and A2 (DMSO vs. cyto-D at 5 minutes), and $P > 0.19$ for A1 (DMSO vs. cyto-D at 10 and 15 minutes), and $P = 0.06$ for A2 (DMSO vs. cyto-D at 10 and 15 minutes). (c) Mean fluorescence intensity of P2 binding to the 3 CHO cell lines. Error bars are too narrow to be seen.

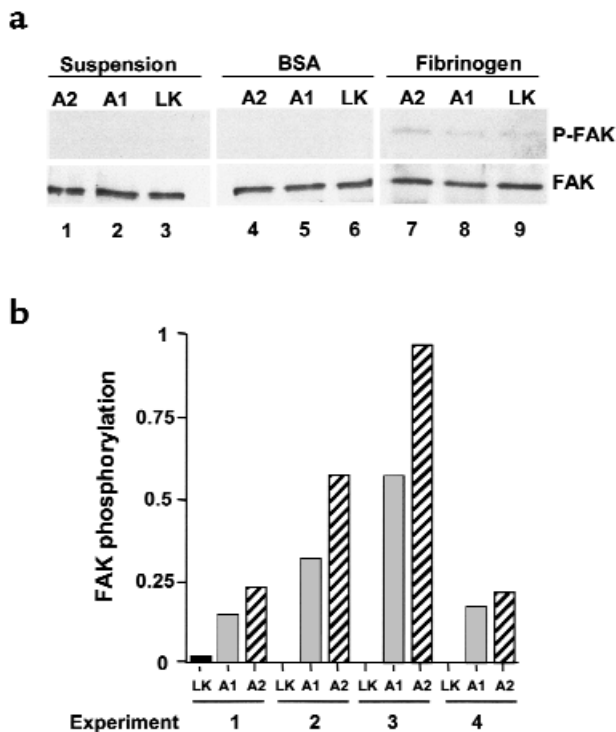


Figure 9 pp125^{FAK} tyrosine phosphorylation of CHO cells. (a) Immunoblot showing tyrosine phosphorylation of pp125^{FAK} (P-FAK). LK444, A1, and A2 cells either in suspension (lanes 1–3), or adhered to BSA (lanes 4–6) or fibrinogen (lanes 7–9) were solubilized. P-FAK was immunoprecipitated, separated by SDS-PAGE, and blotted with an antiphosphotyrosine antibody (upper panel). The lower panel shows the same filter stripped and reprobbed with an anti-P-FAK antibody. (b) Densitometric quantitation of the P-FAK phosphorylation (ratio of tyrosine phosphorylation P-FAK to total P-FAK immunoprecipitated in arbitrary units) in 4 different experiments. FAK phosphorylation in panel a is experiment 1 displayed in b.

kinase, pp125^{FAK}, a cytoplasmic tyrosine kinase, is an important regulator of signaling in focal contacts. Cell spreading and focal contact is dictated partly by the tyrosine phosphorylation status of this protein, which in turn is regulated by protein kinase C (PKC) (30). Incubation of the A1 and A2 cells with the PKC inhibitor, bisindolylmaleimide, caused a greater inhibition in adhesion of A2 cells (83.3%; $P = 0.02$) than A1 cells (32.7%; $P = 0.173$) (data not shown), so we assessed pp125^{FAK} phosphorylation in the CHO cell lines (Figure 9a). A small but consistent increase in pp125^{FAK} phosphorylation was observed in the A2 cells compared with the A1 cells that had adhered to fibrinogen (Figure 9a, lane 7 versus lane 8). This difference depended upon adhesion to fibrinogen because it was not observed in suspension cells (Figure 9a, lanes 1–3) or in cells incubated on albumin (Figure 9a, lanes 4–6). In most experiments LK444 cells showed little pp125^{FAK} phosphorylation. Figure 9b shows the ratio of phosphorylated pp125^{FAK} to immunoprecipitated pp125^{FAK} in 4 experiments. Although the difference in signals between A1

and A2 cells was modest, this data raised the possibility that downstream $\alpha_{IIb}\beta_3$ signaling might be different in A2 cells compared with A1 cells.

Fibrin clot retraction represents another outside-in signaling (downstream) cellular function involving $\alpha_{IIb}\beta_3$ that also depends on actin cytoskeletal rearrangement. We found that both A1 and A2 cells, but not LK444 cells, could retract a fibrin clot (Figure 10a). Compared with A1 cells, A2 cells exhibited a small but significant increase in fibrin clot retraction at all time points. This clot retraction could be inhibited by an $\alpha_{IIb}\beta_3$ -specific peptide, but not by a blocking anti- $\alpha_v\beta_3$ antibody (Figure 10, b and c). These clot retraction studies provided additional evidence for functional differences in A1 and A2 cells that was downstream of receptor occupancy.

Discussion

In this work we have studied the adhesive and signaling properties of cell lines expressing the PI^{A1} or PI^{A2} polymorphism of integrin β_3 . Compared with PI^{A1}-expressing cells, the PI^{A2}-expressing cells exhibited increased adhesion to immobilized fibrinogen with greater cell spreading and F-actin content and increased clot retraction, all of which suggest differences in outside-in signaling events. Should similar properties hold true in platelets, this genetic alteration of integrin β_3 might account for some component of the reported associations between PI^{A2} and acute coronary thrombotic syndromes.

We used a CHO and 293 cell system to address functional differences between the PI^{A1} and PI^{A2} forms of $\alpha_{IIb}\beta_3$ to overcome some of the limitations of platelets, and several issues related to our experimental conditions should be mentioned. First, because cell lines derived from single-cell clones can acquire properties that might affect function beyond that induced by the exogenously expressed cDNAs, we generated stable populations of overexpressing CHO and 293 cells by cell sorting. These “pools” of clones make genetic drift a much less likely explanation for the differences we observed. Through cell sorting, we maintained the equivalent expression of $\alpha_{IIb}\beta_3$ receptors and did not perform studies if there was a greater than 10% difference in surface density of $\alpha_{IIb}\beta_3$ between the A1 and A2 cell lines. Importantly, we always observed greater A2 cell binding even when the surface density of $\alpha_{IIb}\beta_3$ was greater on A1 compared with A2 cells (data not shown), further substantiating a qualitative rather than quantitative difference in the 2 β_3 isoforms. Second, the low levels of chimeric hamster-human $\alpha_v\beta_3$, expressed in our cell lines had little contribution to cell adhesion, as demonstrated by the minor degree of inhibition by LM609. The use of a calcium-containing buffer, which does not support $\alpha_v\beta_3$ -fibrinogen binding (28), may have contributed to the $\alpha_{IIb}\beta_3$ specificity of our studies. Lastly, although these studies were performed under static conditions, shear was applied during the washing procedure. Indeed, in complementary studies (31),

shear was found to augment the difference in A2 cell binding to fibrinogen relative to A1 cells.

The Leu→Pro substitution at amino acid 33 of integrin β_3 results in a conformational change in the extracellular domain (32). With respect to soluble fibrinogen at subsaturating concentrations, we found this change in conformation had no effect on binding in either the resting or activated state of the receptor (Figure 2). This argues against any difference in the apparent K_d between the 2 forms of β_3 for fibrinogen. This was consistent with findings by Bennett et al., who found no difference in soluble fibrinogen-binding kinetics to platelets from donors of $PI^{A1/A1}$ and $PI^{A1/A2}$ genotype (33). Because thrombus formation involves platelet binding to both soluble and immobilized fibrinogen, we studied this latter process and found significantly greater $\alpha_{IIb}\beta_3$ -mediated binding of A2 cells compared with A1 cells, regardless of the concentration of immobilized fibrinogen used or the time of incubation. Adhesion data (Figure 6a) from a second set of cell lines generated in a different parental strain further confirmed the functional differences between the 2 forms of β_3 . In contrast, adhesion of CHO cells to fibronectin (a ligand for endogenous $\alpha_5\beta_1$) showed no difference by PI^A genotype. Immobilization of fibrinogen at the densities used in these experiments is known to result in a conformation that differs significantly from that of soluble fibrinogen (34), and this might explain the difference we observed in binding between the soluble and immobilized forms (Figures 2 and 3).

Our findings indicated that the increased adhesion of A2 cells was due to a greater reorganization of the actin cytoskeleton. This conclusion is based on: (a) greater F-actin content seen in the periphery of A2 cells adhered to fibrinogen for 5 minutes compared with A1 cells (Figure 7); (b) greater spreading of A2 cells compared with A1 cells after 15 minutes (Figure 7); and (c) the difference in adhesion between A1 and A2 cells was

abolished in the presence of cytochalasin D (Figure 8). Rapid remodeling of cytoskeletal machinery in A2 cells compared with A1 could also explain the greater clot retraction seen in A2 cells. Certain lines of evidence suggested the greater reorganization of the actin cytoskeleton seen in the A2 cell line was because of differences in postreceptor occupancy signaling. A small increase in pp125^{FAK} phosphorylation was consistently observed in A2 cells adhered to fibrinogen compared with A1 cells (Figure 9, a and b). This difference occurred only after ligand binding and required integrin $\alpha_{IIb}\beta_3$. Tyrosine phosphorylation of pp125^{FAK} represents one of the early-recognized events during outside-in signaling, and our findings are consistent with evidence that tyrosine phosphorylation of pp125^{FAK} correlates with cell spreading, focal adhesion plaque formation, and stress fiber assembly (35). In addition, A2 cells demonstrated greater reduction in adhesion after PKC inhibition with bisindolylmaleimide. Because PKC regulates tyrosine phosphorylation of pp125^{FAK} and spreading in platelets (30), a theoretical mechanism to explain these data would be as follows: compared with A1 cells, adhesion of A2 cells to fibrinogen induces greater activation of PKC with subsequent activation of MAPK, which influences rapid cytoskeletal changes that favor stronger and sustained adhesion of A2 cells compared with A1 cells. Further studies are underway to address this hypothesis.

In summary, cells overexpressing the PI^{A2} form of the $\alpha_{IIb}\beta_3$ exhibited several functional differences compared with its PI^{A1} counterpart: greater adhesion to immobilized fibrinogen, greater spreading and actin reorganization, and greater clot retraction. It remains unclear how these pathways are linked to the Leu → Pro substitution at amino acid 33 of β_3 . Perhaps the conformational change in the β_3 extracellular domain favors the interaction with an associated activation molecule, such as one containing an immunoreceptor tyrosine-based

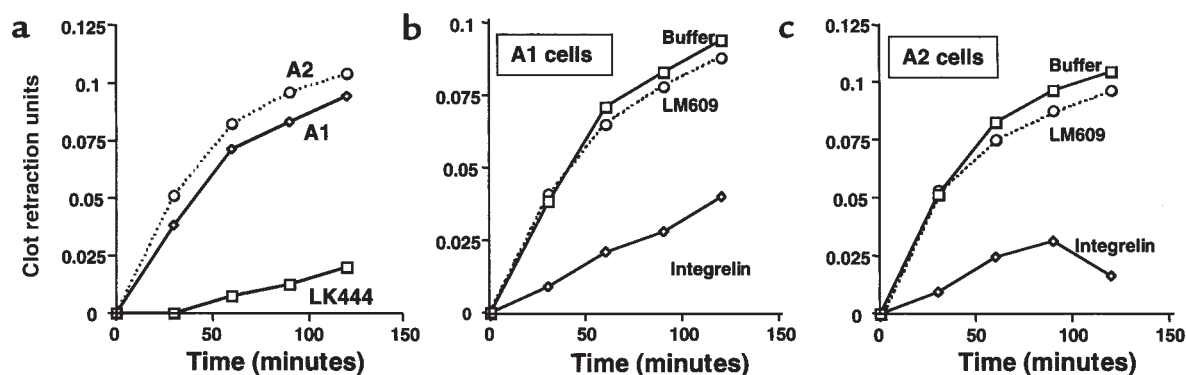


Figure 10

Fibrin clot retraction. (a) Clot retraction of LK444 (open squares), A1 (open diamonds), or A2 (open circles) cells was performed as described in Methods. The increased fibrin clot retraction of A2 over A1 was significant at $P = 0.002$, $P = 0.003$, $P = 0.004$, $P = 0.03$ for 30, 60, 90, and 120 minutes, respectively. The results are \pm SEM of 6 experiments performed. Error bars were too small to be displayed. (b) A1 cells and (c) A2 cells demonstrate that clot retraction is mediated through $\alpha_{IIb}\beta_3$. Cells were incubated with buffer (open squares), 10 μ g/mL anti $\alpha_{IIb}\beta_3$ LM609 (open circles), or 5 μ M integrin (open diamonds), and clot retraction was performed as above. The results are \pm SEM of 3 experiments performed. Error bars are too small to be displayed.

activation motif. Our findings support a prothrombotic feature of the PI^{A2} isoform of β_3 that provides biological plausibility to the clinical associations that have been described. Given the prevalence of PI^{A2} in the population, it should not be too surprising that the functional differences between the PI^{A1} and PI^{A2} forms of β_3 are modest. More substantial changes may have produced a phenotype that would not have survived negative evolutionary pressure. Considering both the central role of $\alpha_{IIb}\beta_3$ in thrombus formation and its abundance on platelets, even a modest functional alteration could have profound effects over an individual's lifetime.

Acknowledgments

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