The Platelet Glycoprotein Ib–von Willebrand Factor Interaction activates the Collagen Receptor α2β1 to bind Collagen: activation-dependent conformational change of the α2-I domain.

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ABSTRACT

Integrin α2β1 (GP Ia/IIa) is a major platelet receptor for collagen, containing its collagen binding site within the α2 I domain. α2β1 changes conformation upon platelet activation, increasing its affinity for collagen. We observed that two antibodies known to bind within the α2 I domain, 12F1 and 6F1, bound preferentially to ADP-activated platelets. Interestingly, when whole blood was perfused over a surface coated with either 12F1 or 6F1, only 6F1 supported the adhesion of unstimulated platelets. To test whether the interaction of GP Ib with VWF directly activates α2β1, we used 12F1 as a probe of integrin activation. We perfused blood over a surface coated with a mixture of VWF A1 domain (a GP Ib ligand) and 12F1 or VWF A1 and mouse IgG. Platelets rolled and did not attach stably on the A1/IgG surface, but they firmly bound and covered the A1/12F1 surface. We corroborated that 12F1 binds an active conformation of the I domain by showing that it binds with higher affinity to a gain-of-function mutant than to either wild-type I domain or a loss-of-function mutant. These results strongly suggest that the interaction of platelet GP Ib with VWF mediates the activation of α2β1, increasing its affinity for collagen.
Introduction

The integrin α2β1, also called glycoprotein (GP) Ia-IIa, is a major collagen receptor in platelets. This receptor plays a vital role in mediating the firm adhesion of platelets from flowing blood as they act to seal vascular defects. When the integrity of the lining endothelium is disrupted, the circulating platelets make their first contact with the exposed subendothelial von Willebrand factor (VWF) bound to collagen. This initial contact decelerates the platelets, allowing α2β1 to bind collagen. The interaction arrests the platelets and contributes to their activation and aggregation.

It appears that high-affinity binding of collagen by α2β1 requires the integrin to assume an active conformation. In platelets, α2β1 activation can be detected by an increase in the binding of soluble collagen to platelets stimulated with agonists such as ADP, thrombin, and the GP VI-binding collagen related peptide (CRP). In fact, very recently Schoolmeester et al. reported that the activated conformation of platelet α2β1 could be detected by an antibody against the α2-I domain. Although it has been proposed that the GP Ib-IX-V complex and α2β1 play synergistic roles in platelet adhesion, there is no direct evidence to demonstrate that the initial interaction of the GP Ib-IX-V complex with VWF activates α2β1.

Within the α2 subunit, the I domain (amino acids 140–349) contains the principal binding site for collagen. Like other I domains, the α2 I domain contains a cation-binding site within a motif known as the metal ion-dependent adhesion site (MIDAS), which explains why collagen-induced platelet adhesion and activation require divalent cations. A recombinant α2 I domain has been reported that inhibits the interaction of platelets with collagen and extracellular matrix (ECM) under flow. By crystallography and mutagenesis, two conformations were demonstrated for the α2 I domain, which correlated with low and high affinity states. Based on this observation, we
hypothesize that a conformational shift switches the I domain from a low affinity to a high affinity state as the result of the activation of α2β1.

Support for this hypothesis came also from results obtained with the anti-human α2 integrin monoclonal antibodies 12F1 and 6F1. Both antibodies were capable of blocking the inhibitory effect of the isolated α2 I domain on collagen-induced platelet aggregation (CIPA) but themselves failed to inhibit CIPA in platelet-rich plasma (PRP). A likely explanation is that the epitopes recognized by the two antibodies are exposed in the isolated I domain while being masked in the intact α2β1 on platelets. A similar activation-dependent epitope has been described within αIIbβ3, which binds the antibody PAC-1 only when the platelets have been activated.

In this study, we demonstrate that 12F1 and 6F1 each has a higher affinity on activated platelets than on unstimulated platelets. We took advantage of this finding to demonstrate that the interaction of the GP Ib-IX-V complex with its binding site on VWF, the A1 domain, is sufficient to activate α2β1 and enable the firm adhesion of platelets in flowing blood to a mixed matrix of VWF A1 and 12F1. Finally, by mutagenesis of the α2 I domain, we demonstrate that 12F1 binds preferably to the high-affinity conformation of the domain.
Materials and Methods

Reagents and Antibodies—We used three monoclonal antibodies in these studies, 12F1, 6F1, and P1E6, the first two recognizing the α2 I domain and P1E6 binding the α2 subunit. 12F1 was obtained from Pharmingen and from Dr. Virgil L. Woods (University of California, San Diego, CA), 6F1 from Dr. Barry Coller (The Rockefeller University, New York, NY), and P1E6 and mouse IgG were purchased from Calbiochem (San Diego, CA). Recombinant VWF A1 and wild-type and Y285F mutant α2 I domains were expressed in bacteria and purified as described earlier. All of the recombinant I domains were monomeric, as previously determined by either SDS-PAGE or gel-filtration chromatography.

Construction, expression and purification of the Y285F/E318W mutant—The gain-of-function α2 I domain mutant, Y285F/E318W, was constructed by introducing the E318W mutation into the vector containing the Y285F mutation (pQE9-α2-I/Y285F) using the QuikChange II XL site-directed mutagenesis kit (Stratagene, LA Jolla, CA). As per instructions supplied by the manufacturer, we introduced the mutation by polymerase chain reaction using two complementary oligonucleotide primers containing the mutation (5´ primer = GTGTCTGATTGGGCAGCTC and the 3´ primer = GAGCTGCCCAATCAGACAC). The mutant insert was sequenced to confirm the presence of the mutation. The recombinant Y285F/E318W mutant was expressed in E. coli as a fusion protein containing the 12 residue His tag (MRGSHHHHHHGS) at the N terminus and purified as described previously for the Y285F mutant.

Surface Plasmon Resonance Collagen Binding Assay—Surface plasmon resonance binding studies were performed using a BIAcore 2000 system (BIACore, Piscataway, NJ) as previously described, with minor modifications. Either acid-soluble collagen type I from human placenta (250 µg/ml in 50 mM sodium acetate, pH 4.8) or bovine serum albumin (BSA, as control) was
covalently coupled via amine coupling to a sensor chip (CM5) as directed by the supplier. The immobilization of the collagen resulted in 3,000 resonance units, corresponding to a surface density of approximately 3 ng/mm². The binding assays were performed in 10 mM Hepes, 150 mM NaCl, 0.005% Tween-20, pH 7.4 at 25°C at a flow rate of 10 µl/min. The binding of the α2 I domain variants to collagen-coated channel was corrected for non-specific binding to the control (BSA) channel (between 5% and 10%). Collagen binding at equilibrium was determined at several concentrations of wild-type, Y285F and Y285F/E318W I domains (0.5, 1.0, 5.0, 10 and 20 µM). Kinetic rate constants were calculated from the binding and the dissociation curves by BIAevaluation software (version 3.0) supplied by the manufacturer.

**12F1 binding assay in ELISA**—Monoclonal antibody 12F1¹⁹ was diluted to 10 µg/ml in 50 mM sodium carbonate, pH 9.6 and coated onto the wells of a microtiter plate. Coating was carried out overnight at 4°C. The wells were washed with 25 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween-20, pH 7.4 (TBS-T) and blocked with 3% BSA in TBS-T for 60 min at 37°C. The binding of wild-type and mutant α2 I domains was assessed by adding each to the wells at increasing concentrations, and incubating for 60 min at 37°C. After the incubation, the solution was removed and the wells were washed with TBS-T. The remaining bound α2-I protein was detected by enzyme-linked immunosorbent assay (ELISA) by first adding a 1:20,000 dilution of peroxidase-conjugated monoclonal anti-polyhistidine antibody (Sigma, St. Louis, MO) for 60 min at 37°C, washing, then adding the peroxidase substrate o-phenylenediamine (Sigma). After 30 min of substrate conversion, the reactions were stopped with 0.025 ml of 2N H₂SO₄, and the plates were read at 490 nm. Specific binding was determined by subtracting the OD values from wells coated only with BSA from the total binding values obtained.
**Flow cytometry**—Blood was collected from healthy donors into 3.8% sodium citrate anticoagulant. Approval was obtained from the Baylor College of Medicine institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki. Unstimulated or ADP (20 µM)-stimulated platelets in whole blood or platelet-rich plasma (PRP) were incubated with monoclonal antibodies (5 µg/mL) for 40 min at 22°C, washed twice, then incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse antibody for 40 min, washed, and resuspended in phosphate buffered saline-BSA buffer. The samples were then analyzed in an EPICS XL Coulter Flow Cytometer (Coulter). Background binding was determined from parallel samples incubated with mouse IgG and the FITC-conjugated secondary antibody. In some experiments, ADP was substituted with ristocetin (1 mg/ml final concentration).

**Platelet Adhesion under Flow**—Blood from healthy donors was drawn into D-phenylalanyl-prolyl-arginine chloromethyl ketone anticoagulant (final concentration 75 µM). The flow assays were performed as previously described. The antibodies and purified recombinant VWF A1 were immobilized onto glass coverslips using coating solution concentrations of 200 µg/ml and 150 µg/ml, respectively. The coverslips were incubated with antibody only or with the mixture of antibody and VWF A1 for either 1 h at 37°C or overnight at 4°C. Thus, we produced coverslips coated with 6F1, 12F1, A1 and 12F1, A1 and 6F1, or A1 and mouse IgG. The coverslips were then washed to remove the unbound protein, then blocked with 3% BSA in TBS for 1 hr at 37°C before use. A coated coverslip formed the bottom of the parallel-plate flow chamber (Glycotech, Rockville, MD) and a silicone rubber gasket determined the flow path height of 254 µm. A syringe pump (Harvard Apparatus Inc., Holliston, MA) was used to aspirate blood through the flow chamber. A flow rate of 0.6 ml/min produced a wall shear rate of 1500s⁻¹. To assess platelet adhesion, we perfused anticoagulated whole blood through the chamber for 2 min or the flow was stopped for 1
min, then washed the coverslip with TBS and visualized the adherent platelets by phase-contrast microscopy. In other experiments, we studied the platelet interactions with these substrates using platelets labeled with the fluorescent dye mepacrine (quinacrine dihydrochloride; Sigma) at a final concentration of 200 µg/ml. These experiments were recorded in real time on videotapes and 10 images were captured from tapes to quantify the number of adherent platelets. In some experiments, whole blood was incubated with 10 U/ml apyrase (grade III, Sigma), 10 µg/ml aspirin, or 10 µM prostacyclin (PGE$_1$) for 30 min before perfusion. To analyze the interaction of activated platelets with immobilized 12F1, we treated the blood with either 20 or 10 µM ADP in the presence of the α$_{III}$β$_3$ antibody 10E5 (20 µg/ml) (to prevent aggregation) before perfusing them over the 12F1 surface. The flow experiments were performed in duplicate on different days using different donors.

**Preparation of plasma-free blood**—Plasma-free blood was prepared as described elsewhere with some modifications.$^{20}$ Blood was collected from normal donors into plastic syringes containing one part of acid-citrate-dextrose (pH 4.5) (ACD). The blood was supplemented with 2 U/ml of the ADP scavenger apyrase (grade III or VII, Sigma), 10 µM of PGE$_1$ and 5 mM EDTA to block platelet activation, and was centrifuged at 2100 x g for 13 min at room temperature. The supernatant plasma was discarded and the cells were resuspended to their original volume with Hepes-Tyrode buffer (10 mM Hepes, 138 mM NaCl, 2.7 mM KCl, 0.4 mM Na$_2$HPO$_4$, 10 mM NaHCO$_3$, and 5 mM dextrose), pH 6.5 containing 1 U/ml apyrase and 5 mM EDTA. The suspended cells were centrifuged again and the supernatant was removed. The process was repeated twice more with no apyrase in the third wash cycle. After the last centrifugation, the cells were resuspended in Hepes-Tyrode buffer pH 7.4 containing 20 mg/ml BSA.

**Preparation of A1/collagen coated dishes**—Acid-soluble human placental collagen type III in 50 mM acetic acid was diluted to 100 µg/ml in 65 mM sodium phosphate buffer, pH 6.5, and added to
35 mm culture dishes and incubated for 60 min at 37°C as described elsewhere. The dishes were then washed twice with phosphate buffered saline, pH 7.4 (PBS) and blocked with 3% BSA in TBS for one hour at 37°C. After one washed the monomeric recombinant VWF-A1 (2 µM) was then added to the dishes and incubated for 30-45 min at 37°C. The dishes were then used immediately in blood perfusion studies as described below.

**Flow Assays**—The flow assays were performed as described above and elsewhere. Briefly, the collagen-coated dish formed the lower surface of the parallel-chamber. Plasma-free blood was perfused through the chamber for 2 min, followed by TBS. For antibody inhibition studies, plasma-free blood was incubated with 15 µg/ml of either 6F1 or the function-blocking GPIbα monoclonal antibody 6D1 for 15 min at room temperature before the attachment assay. Tethered platelets were observed with phase-contrast objectives and the images recorded by videomicroscopy. The number of platelets tethered to the surface was determined by overlaying a 15 square grid on 6 frames and counting and averaging the number of platelets in 6 randomly selected squares. For the control and the inhibitory experiments, the whole frame was counted. All experiments were performed in duplicate and on different days.

**Protein Quantitation**—Protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, IL). Coomassie Blue staining of SDS-PAGE gels was used to assess the purity of the recombinant proteins.

**Results**

*Antibody 12F1 recognizes an active conformation of integrin α2β1*—We evaluated whether an active conformation of integrin α2β1 can be detected through increased binding of monoclonal antibodies, much as the active form of αIIbβ3 is recognized by the antibody PAC-1.
We initially evaluated two antibodies, 6F1 and 12F1, both known to bind within the I domain of the \( \alpha 2 \) subunit. Both antibodies bound unactivated platelets above the background level set by an irrelevant antibody, and the binding of both increased with ADP activation of the platelets, 12F1 displaying the most marked increase (Figure 1). Increased binding did not result from increased surface expression of the integrin, as another \( \alpha 2 \) antibody, P1E6, did not display differential binding depending on the activation state of the platelets.

We then evaluated whether the two antibodies could detect the active conformation of the integrin under conditions of flow. For this, we perfused whole blood at a high shear rate (1500 s\(^{-1}\)) over a surface coated with either 6F1 or 12F1. Unactivated platelets rapidly adhered to the 6F1 surface (Figures 2A and 2B) but, unexpectedly, not to the 12F1 surface. Identical results were obtained when flow was interrupted for one minute to allow the platelets to interact with the surface-bound antibody and then resumed (Figure 2C). This indicates that the binding of immobilized 12F1–resting \( \alpha 2 \beta 1 \) is not strong enough to support platelet firm adhesion under a higher shear stress. Nevertheless, the immobilized 12F1 is capable of recognizing the active conformation of \( \alpha 2 \beta 1 \). When ADP-treated blood (in the presence of the \( \alpha IIb\beta 3 \) blocker, 10E5, to prevent platelet aggregation) was incubated with the 12F1-coated surface for 1 min before flow was initiated, the number of firmly adherent platelets was 9 times greater than in untreated blood (data not shown). However, the \( \alpha 2 \beta 1–12F1 \) bond was unable to capture the platelets from the flowing blood because without the brief incubation period only a very small number of ADP-activated platelets adhered to the 12F1 surface (data not shown).

We took advantage of the observation that \( \alpha 2 \beta 1 \)-mediated attachment to a 12F1 surface requires both platelet tethering and integrin activation to evaluate whether the first molecular interaction in the sequence of platelet adhesion from flowing blood—the binding of the GP Ib-IX-
V complex to VWF—is able to directly signal to activate α2β1 and enable firm adhesion to collagen. 12F1 is able to act as a surrogate for the α2β1-binding site of collagen, obviating the need to use collagen, which has several receptors on the platelet surface,21 and is difficult to obtain in pure form. We therefore perfused whole blood over a surface coated with a mixture of the isolated VWF A1 domain—containing the binding site for GP Ib—and 12F1, reasoning that if the binding of the platelets to A1 activated α2β1, they would become firmly adherent to the surface. Surfaces coated with mixtures of A1 and 6F1 or A1 and mouse IgG served as positive and negative controls, respectively. We observed extensive platelet deposition on coverslips coated with either A1/12F1 or A1/6F1, and virtually no adherent platelets on the surface coated with A1/mouse IgG (Figure 3). When analyzed in real time, the platelets perfused over the A1/12F1 surface were seen to arrest on the surface very soon after they first attached, whereas those on the A1/mouse IgG surface rolled continuously across the surface and failed to attach firmly.

These results provide compelling evidence that the GP Ib-IX-V–VWF interaction sends signals that activate α2β1, exposing the 12F1 epitope. The results were identical when performed in the presence of apyrase, an ADP scavenger, aspirin, or PGE1 (not shown), indicating that the signaling pathway was direct, and not a consequence of either stimulated release of granules and secondary ADP-mediated integrin activation or of thromboxane A2 generation.

The above results were consistent with those obtained by substituting 12F1 with collagen as a α2β1 ligand. We perfused reconstituted blood—in which washed platelets and erythrocytes are suspended in buffer—at high shear stress over a surface coated with collagen or with a mixture of A1 and collagen (Figure 4A and 4B). The firmly adherent platelets were quantified. The platelet number on the collagen surface was only 7% of that on the A1/collagen surface. The binding required both the GP Ib-IX-V complex and α2β1 complexes, as blocking GP Ibα with antibody
6D1 completely prevented platelet attachment (Figure 4D) and blocking α2β1 with 6F1 decreased platelet binding by eight-nine percent (Figure 4C).

VWF in suspension was also able to induce α2β1 activation. The binding of 12F1 increased by an average of 26% when platelets were incubated with soluble VWF in the presence of ristocetin (data not shown).

*12F1 binding is favored by the open conformation of the α2 I domain*—Enhanced binding of 12F1 within the α2 I domain would be seen if platelet activation either unmasks the epitope or induces a high-affinity conformation of the I domain. To distinguish between the two, we compared the binding of the antibody to the wild-type, recombinant I domain with its binding to two mutants, one with lower affinity for collagen, one with higher affinity. As described earlier, the Y285F mutant α2 I domain binds collagen with lower affinity than does the wild-type α2 I domain. In fact, based on the comparison of the crystal structure of the I domain alone with that of the I domain in complex with a collagen peptide, we proposed that the Y285F mutant adopts a closed low-affinity conformation (Figure 5A). To construct an I domain with a high-affinity conformation, we introduced a second mutation into the Y285F mutant, E318W (Figure 5A). Aquilina et al. showed that this mutation, by itself, resulted in a gain of collagen-binding function. In our hands, the double mutant, Y285F/E318W, also displayed gain-of-function, binding collagen with higher affinity than either Y285F or wild-type α2 I domain, as demonstrated using surface plasmon resonance (Figure 5B). Similarly, the affinity of the double mutant for 12F1 was greater than the affinity of the Y285F mutant and slightly greater than the affinity of the wild-type α2 I domain (Figure 6), proving that the Y285F mutation altered antibody binding allosterically.
Discussion

Platelet adhesion to sites of vessel injury, particularly in regions of rapidly flowing blood, has been modeled as involving sequential adhesive interactions that initially attach the platelets from bulk flow, allow them to decelerate by rolling, and then allow them to firmly attach to the exposed collagen. Firm attachment involves integrins, most prominently \( \alpha 2 \beta 1 \), which generally must be activated to bind their ligands with high affinity. Recent studies have demonstrated that \( \alpha 2 \beta 1 \), like other integrins, can indeed assume an active conformation capable of binding collagen with high affinity.\(^6\)\(^{23,24}\) This active conformation is induced when platelets are stimulated by a number of soluble agonists, including ADP, thrombin and the GP VI–binding peptide, CRP. Of these, only CRP activates a receptor that would be able to activate the integrin early in the adhesive cascade. In fact, it has been proposed that collagen engagement of GP VI during the adhesive process is a prerequisite to integrin activation and firm platelet adhesion.\(^6\)\(^{23,25}\) However, the potential for signals from the GP Ib-IX-V complex to activate \( \alpha 2 \beta 1 \) has never been addressed directly, largely for technical reasons, among them being the fact that both VWF and collagen have more than one receptor on the platelet surface. For this reason, we investigated whether a monoclonal antibody could be used as a surrogate for collagen to recognize the active state of the integrin, much as has been described for the \( \alpha I I b \beta 3 \) antibody PAC-1, which recognizes only the active, ligand-competent form of \( \alpha I I b \beta 3 \).\(^12\)

Two antibodies tested, 12F1 and 6F1 in solution, bound activated platelets at higher levels than they bound unstimulated platelets. When immobilized on the surface, only one of these antibodies, 6F1, was able to support the adhesion of unstimulated platelets under flow. Immobilized 12F1 only supports the adhesion of activated platelets under static conditions.
However, once activated platelets bound immobilized 12F1, they remained firmly adhered, even at elevated shear stresses.

Further evidence that 12F1 recognizes an active form of α2β1 comes from studies with the isolated I domains. The antibody bound best to a gain-of-function form of the I domain with a structure predicted to be the one assumed when the domain binds collagen,11 and least well to a form with low collagen-binding activity (Figure 5A). These data are in agreement with those of Jung et al.,6 who reported that α2β1 on agonist-stimulated platelets bound significantly more soluble collagen than did the integrin on resting platelets. These investigators also noted that the affinity for collagen was different between platelets activated with thrombin and those activated with ADP. Thus, α2β1 on platelets can assume at least two different conformational states of activation, and possibly more. In the intact integrin, the antibody 12F1 recognizes an active conformation.

This property of the 12F1 made it possible for us to design a system to test GP Ib–mediated activation of α2β1 using ligands relatively restricted in their reactivity with the two receptors: the A1 domain of VWF, containing the GP Ib-IX-V binding site, and 12F1, a specific probe of α2β1 activation. The ability of a surface coated with a mixture of these two proteins to support the rapid and firm adhesion of platelets under flow argues strongly that signals emanating from GP Ib after it binds A1 activate the integrin to a high-affinity conformation that can bind 12F1. These signals are transmitted rapidly, as we observed the platelets to arrest immediately after their initial contact with the matrix.

These signals are not only rapid, they may also be local or specifically induce only a small number of changes normally associated with platelet activation. For example, our failure to observe the formation of microthrombi on the A1/12F1-coated surface suggests that the signals were insufficient to activate receptors on the side of the platelet not in contact with the matrix. That the
signals may act locally is made more likely by the known proximity of the GP Ib-IX-V complex and α2β1 on the platelet membrane. 2,15,26 This proximity should allow the approximately 20:1 ratio of GP Ib to α2β1 complexes to concentrate the signals at the integrin. A second possibility is suggested by the fact that both receptors are attached through their cytoplasmic domains to the cytoskeletal protein filamin A. 27-29 Thus, it is possible that a direct mechanical signal is transmitted to activate α2β1 when torque is applied to ligand-engaged GP Ib complex. This possibility is particularly attractive when one considers that the GP Ib–VWF bond is subjected to considerable torque as the platelet attaches from flowing blood to the immobile vessel wall. 30,31 Such stress would be directly transferred to cytoskeletal structures attached to the platelet receptor.

Another implication of this work is that firm adhesion of platelets at sites of vessel injury does not require GP VI–generated activation signals. However, such signals may be important for platelets to build thrombi, a possibility that we are currently examining.

In summary, we demonstrate that integrin α2β1 in platelets can be directly activated by the interaction of GP Ib with VWF. This activation is reflected in a conformational change of the I domain in the α2 subunit recognized by specific antibodies. Activation of α2β1 allows the integrin to mediate firm platelet attachment to collagen, which is then followed by platelet activation and aggregation.
References


(5) Saelman EU, Nieuwenhuis HK, Hese KM et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (α2β1-integrin). Blood. 1994;83:1244-1250.


Figure Legends

Figure 1. Analysis of the binding of monoclonal antibodies 12F1 and 6F1 to ADP-stimulated platelets. **Unstimulated**: PRP was incubated with each MoAbs 12F1, 6F1 or P1E6 and examined by flow cytometry (**dashed lines**). **ADP Stimulated**: PRP containing each of the antibodies and ADP (20 µM) is represented by the **dotted lines**. Mouse IgG was used as control and is represented by **solid lines**. ADP stimulation increases 12F1 binding by an average of 38%, based on mean fluorescence intensities. The data were obtained from 8 different donors. There was no difference of P1E6 binding between unstimulated and ADP-stimulated platelets.

Figure 2. Interaction of platelets with immobilized monoclonal antibodies 6F1 and 12F1. (A) Whole blood containing 75 µM PPACK as anticoagulant, 200 ug/ml mepacrine to make platelets fluorescent and unstimulated platelets was perfused over glass surface coated with antibodies as indicated at a flow shear rate of 1500s⁻¹. (B) The graph represents the number of platelets adhered on each surface during the time of perfusion. (C) Conditions as described (A), but the flow was interrupted for one minute to allow platelets to interact with the antibody-coated surface. The adhered platelets are visualized after resumed the flow at 1500s⁻¹. The images represent four separate experiments using blood from different donors.

Figure 3. Interaction of flowing platelets with immobilized VWF-A1/12F1 and VWF-A1/6F1. Whole blood was perfused over surface coated with the VWF-A1 (150 µg/ml) mixed with 6F1, 12F1 or mouse IgG (200 µg/ml). After 2-min perfusion, the coverslips were washed with TBS and several frames of attached platelets were recorded. The photomicrographs represent three separated assays.
Figure 4. Interaction of flowing platelets with a collagen or A1/collagen surface. Plasma-free blood was perfused at a shear rate of 1,500 s\(^{-1}\) over different coating substrates in the absence or presence of inhibitors. (A) a collagen surface, (B) a A1/collagen surface, (C) on a A1/collagen surface, in the presence of antibody 6F1 (against the \(\alpha_2\)-I domain), (D) on a A1/collagen surface, in the presence of antibody 6D1 (against GPIb\(\alpha\)). The photomicrographs depict the platelets tethered to the surface after 2 min of perfusion and represent three separate experiments with blood from different donors.

Figure 5. Collagen-binding analysis of the mutant Y285F/E318W-I domain. (A) The coordinates for the isolated \(\alpha_2\)-I domain (1DZI) and \(\alpha_2\)-I-domain in complex with the collagen peptide (1AOX) were obtained from the Protein Data Bank (PDB). The closed or “low affinity” conformation adopted by the isolated I domain and the open or “high affinity” conformation found in the I domain:collagen complex (the collagen triple helix is shown in dark gray) were constructed using the program Rasmol. Black spheres indicate the position of the two residues mutated in the recombinant Y285F/E318W-I domain. (B) Analysis of the binding of recombinant \(\alpha_2\)-I variants to collagen type I from human placenta by Surface Plasmon Resonance (SPR). Binding curves of the WT or Y285F/E318W mutant were obtained perfusing different concentrations of each purified I domain protein over the collagen surface as described in the methods. The figures depicted overlay plots of sensograms observed for the interaction at different concentrations. RU stands for resonance units. Dissociation constant (\(K_D\)) obtained for each \(\alpha_2\)-I variant was 1.0±0.2 \(\mu\)M for WT; 2.0±0.15 \(\mu\)M for Y285F, and 0.02±0.09 \(\mu\)M for Y285F/E318W.

Figure 6. Binding of \(\alpha_2\)-I variants to monoclonal antibody 12F1. The \(\alpha_2\) I proteins were incubated with immobilized monoclonal antibody 12F1. Bound protein was determined by ELISA
as described in the methods. The figure shows the mutant proteins that had a markedly different binding curve than the wild type. Each point represents the mean ± S.D. of values obtained in two independent sets of triplicate assays.
Figure 1
Figure 2
Figure 3
**Figure 5**

Diagram A shows a molecular model with labeled points Y285 and E318. The model is depicted in two states: Closed (Low Affinity) and Open (High Affinity). The model is annotated with "Collagen peptide".

Graph B compares two sets of data: WT and Y285F/E318W. The graphs show changes over time in a quantitative measure, with time on the x-axis and the measure on the y-axis.
Figure 6
The platelet glycoprotein Ib - von Willebrand Factor interaction activates the collagen receptor $\alpha_2\beta_1$ to bind collagen: activation-dependent conformational change of the $\alpha_2$-I domain

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