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Abstract

The ability of platelets to tether to and translocate on injured vascular endothelium relies on the interaction between the platelet glycoprotein receptor Ib α (GPIb α) and the A1 domain of von Willebrand factor (vWF-A1). To date, limited information exists on the kinetics that govern platelet interactions with vWF in hemodynamic flow. We now report that the GPIb α -vWF-A1 tether bond displays similar kinetic attributes as the selectins including: 1) the requirement for a critical level of hydrodynamic flow to initiate adhesion, 2) short-lived tethering events at sites of vascular injury in vivo, and 3) a fast intrinsic dissociation rate constant, k_{off}^0 ($3.45 \pm 0.37 \text{ s}^{-1}$). Values for k_{off} , as determined by pause time analysis of transient capture/release events, were also found to vary exponentially ($4.2 \pm 0.8 \text{ s}^{-1}$ to $7.3 \pm 0.4 \text{ s}^{-1}$) as a function of the force applied to the bond (from 36 to 217 pN). The biological importance of rapid bond dissociation in platelet adhesion is demonstrated by kinetic characterization of the A1 domain mutation, I546V that is associated with type 2B von Willebrand disease (vWD), a bleeding disorder that is due to the spontaneous binding of plasma vWF to circulating platelets. This mutation resulted in a loss of the shear threshold phenomenon, a approximately sixfold reduction in k_{off} , but no significant alteration in the ability of the tether bond to resist shear-induced forces. Thus, flow dependent adhesion and rapid and force-dependent kinetic properties are the predominant features of the GPIb α -vWF-A1 tether bond that in part may explain the preferential binding of platelets to vWF at sites of vascular injury, the lack of spontaneous platelet aggregation in circulating blood, and a mechanism to limit thrombus formation.

Comments

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Selectin-Like Kinetics and Biomechanics Promote Rapid Platelet Adhesion in Flow: The GPIIb α -vWF Tether Bond

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ABSTRACT The ability of platelets to tether to and translocate on injured vascular endothelium relies on the interaction between the platelet glycoprotein receptor Ib α (GPIIb α) and the A1 domain of von Willebrand factor (vWF-A1). To date, limited information exists on the kinetics that govern platelet interactions with vWF in hemodynamic flow. We now report that the GPIIb α -vWF-A1 tether bond displays similar kinetic attributes as the selectins including: 1) the requirement for a critical level of hydrodynamic flow to initiate adhesion, 2) short-lived tethering events at sites of vascular injury in vivo, and 3) a fast intrinsic dissociation rate constant, k_{off}^0 ($3.45 \pm 0.37 \text{ s}^{-1}$). Values for k_{off} , as determined by pause time analysis of transient capture/release events, were also found to vary exponentially ($4.2 \pm 0.8 \text{ s}^{-1}$ to $7.3 \pm 0.4 \text{ s}^{-1}$) as a function of the force applied to the bond (from 36 to 217 pN). The biological importance of rapid bond dissociation in platelet adhesion is demonstrated by kinetic characterization of the A1 domain mutation, I546V that is associated with type 2B von Willebrand disease (vWD), a bleeding disorder that is due to the spontaneous binding of plasma vWF to circulating platelets. This mutation resulted in a loss of the shear threshold phenomenon, a approximately sixfold reduction in k_{off} , but no significant alteration in the ability of the tether bond to resist shear-induced forces. Thus, flow dependent adhesion and rapid and force-dependent kinetic properties are the predominant features of the GPIIb α -vWF-A1 tether bond that in part may explain the preferential binding of platelets to vWF at sites of vascular injury, the lack of spontaneous platelet aggregation in circulating blood, and a mechanism to limit thrombus formation.

INTRODUCTION

Rapid localization of leukocytes and platelets at sites of inflammation or vascular injury, respectively, relies on the unique binding properties of two distinct groups of adhesion receptors. For leukocytes, this interaction is primarily mediated by the selectin (CD62P, E, and L) family of adhesion molecules, whereas platelets utilize a receptor that is a member of the leucine-rich motif family, GPIIb. Classification of these receptors into two distinct groups has been largely based on homologies in structure. For instance, each selectin molecule has an N-terminal carbohydrate-recognition domain characteristic of Ca²⁺-dependent (C-type) lectins, followed by an epidermal growth factor-like motif, a series of short consensus repeats, a transmembrane domain, and a short cytoplasmic tail (Lasky, 1992). In contrast, glycoprotein receptor Ib α (GPIIb α) consists of a globular domain at the amino terminus that contains the seven leucine-rich tandem repeats, a mucin-like segment (macro-glycopeptide) that separates the ligand binding domain from the plasma membrane, a transmembrane segment, and a cytoplasmic domain (Lopez, 1994). The amino-terminal globular domain contains the major binding site for the A1

domain of von Willebrand factor (vWF-A1). vWF is a multimeric plasma glycoprotein that supports platelet adhesion at sites of vascular injury by virtue of its ability to form a bridge between GPIIb α and exposed components of the extracellular matrix (Coller et al., 1983; Sakariassen et al., 1979; Turitto et al., 1980). Although apparent differences in structure and ligand binding requirements exist between the selectins and GPIIb α , the ability of both adhesion families to promote and sustain cell adhesion in flow suggests similarities in the kinetic properties of their receptor-ligand bond.

It is known that selectin-dependent rolling of leukocytes in response to a hydrodynamic force is a consequence of the rapid formation and breakage of adhesive bonds formed between selectin molecules and their respective glycoprotein ligands. The kinetic properties of selectin-ligand bonds are critical for controlling leukocyte adhesion in vivo, as rolling is a prerequisite for integrin-mediated firm adhesion and subsequent transmigration of cells. Estimation of the dissociation rate constants for these interactions as determined by either measurement of the duration of adhesion of leukocytes that transiently interact with surface-immobilized selectin substrates in flow (Alon et al., 1995, 1997; Smith et al., 1999; Ramachandran et al., 2001; Kaplanski et al., 1993) or by surface plasmon resonance (SPR) (Mehta et al., 1998; Nicholson et al., 1998), range from 0.7 s^{-1} to $> 10 \text{ s}^{-1}$. It is reasonable to assume that the rate constants for GPIIb α binding to vWF-A1 would be correspondingly fast as effective hemostasis requires rapid platelet deposition at sites of vascular injury. This is supported by previous in

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vitro studies demonstrating that platelets rapidly tether to and translocate on surface-immobilized vWF (Savage et al., 1996; Cruz et al., 2000). Yet, slow intrinsic binding kinetics have been reported to mediate rapid platelet adhesion to vWF (Miura et al., 2000). In fact, the dissociation rate constant for the GPIb α -wild type (WT) vWF-A1 bond as determined by equilibrium binding and Scatchard analysis was estimated to be 0.0038 s^{-1} , a value 10-fold lower in magnitude than that reported for integrin-ligand interactions (Labadia et al., 1998). Based on these results, it has been predicted that effective platelet adhesion does not require rapid intrinsic binding kinetics as does selectin-dependent adhesion of leukocytes. The proposed paradigm of slow kinetics and fast adhesion would be unique among adhesion receptors that promote the rapid attachment and translocation of hematogenous cells. This study, however, does not provide insight into whether the mechanical properties of GPIb α -vWF-A1 bond are also distinct from selectin-ligand interactions. This includes the ability to resist an applied force (a measure of the reactive compliance of the bond) and whether the adhesive behavior of this receptor-ligand pair in flow also fits to the Bell model (Alon et al., 1995, 1997; Smith et al., 1999; Ramachandran et al., 2001).

We have performed a detailed kinetic analysis of the GPIb α -vWF-A1 tether bond in flow to determine the impact of hydrodynamic forces on this adhesive interaction and to permit for direct comparison with the biomechanical properties of the bonds that govern selectin-dependent adhesion of leukocytes. By studying the kinetics of transient adhesive events between platelets and vWF in flow, we observed that the cellular dissociation rate constant for this receptor-ligand pair was not only similar in magnitude to those reported for selectin-dependent interactions but varied as a function of the force applied to the bond. Analysis of platelet behavior at sites of injured vascular endothelium in vivo confirmed that the duration of tether bond lifetimes for transiently interacting cells was consistent with our in vitro observations of rapid bond dissociation. Demonstration that alterations in the dynamic properties of the receptor-ligand can have a profound impact on cell adhesion is provided by a detailed kinetic characterization of GPIb α interactions with the naturally occurring type 2B-vWF mutation, I546V (Federici et al., 1997). Patients with this gain-of-function mutation in vWF have a bleeding disorder due to spontaneous binding of plasma vWF to circulating platelets and subsequent clearance of both of these hemostatic elements from the blood, an interaction that normally only occurs at sites of vascular injury. Importantly, our results indicate that evaluation of receptor-ligand interactions under physiological relevant conditions is paramount to understanding how biomechanical properties of tether bonds ultimately control the process of cell adhesion.

MATERIALS AND METHODS

Antibodies and constructs

Antibodies 6D1, a monoclonal antibody (mAb) to the vWF-A1 binding region of GPIb, and 7E3, anti-GPIIb/IIIa, were generous gifts of Dr. B. Coller (Mount Sinai Medical Center, New York, NY). Mouse anti-6-HIS mAb was purchased from Research Diagnostics, Inc. (Flanders, NJ). Anti-vWF-A1 mAb AMD-1 (mouse anti-IgG1) was generated to human vWF-A1 protein using standard techniques for the production of hybridomas (Langone and Van Vunakis, 1986). Fab fragments were prepared using ImmunoPure Fab preparation kit (Pierce Chemical Co., Rockford, IL). Mutations were introduced into vWF-A1 cDNA with a polymerase chain reaction-based mutagenesis strategy and the resulting polymerase chain reaction product subsequently inserted into pQE9 vector (Cruz et al., 2000). Recombinant vWF-A1 proteins, containing residues 475 to 709 of the mature human vWF, was expressed and purified as previously described (Cruz et al., 2000).

Platelet tethering, accumulation, and velocity measurements in flow

Platelet adhesion was assessed in a parallel-plate flow chamber apparatus as previously described (Cruz et al., 2000). Briefly, platelets purified from citrated whole blood (5×10^7 per mL) were perfused over adsorbed vWF-A1 proteins (1 to 100 $\mu\text{g}/\text{mL}$ coating concentrations) or plasma vWF (25 $\mu\text{g}/\text{mL}$) at shear stresses ranging from 0.25 to 4 dyn cm^{-2} . Wall shear stress was calculated from the momentum balance on a Newtonian fluid, assuming a viscosity of 1.0 cP (Lawrence and Springer, 1991). An enzyme-linked immunosorbent assay was used to ensure that equivalent concentrations of recombinant proteins were adsorbed to polystyrene plates (Cruz et al., 2000). Platelet attachment and their subsequent motion were recorded on Hi-8 videotape using a Nikon microscope with a plan 10 \times or 20 \times objective, respectively. Inhibition studies were performed by preincubation of platelets with mAb 6D1 for 15 min at a final concentration of 20 $\mu\text{g}/\text{mL}$ (Karpatkin et al., 1988).

Preparation of vWF-A1-coated microspheres

Recombinant vWF-A1 proteins were bound to polystyrene microspheres (goat anti-mouse IgG (FC); Bangs Lab, Inc., Fishers, IN) of 7 μm in diameter that were initially coated with mouse anti-6-HIS mAb (100 $\mu\text{g}/\text{mL}$). Estimation of the amount of vWF-A1 coupled to beads was determined using mAb AMD-1 and a calibrated microbead system (Quantum Simply Cellular; Flow Cytometry Standards Corp., San Juan, PR) following the manufacturer's instructions. The site density of vWF-A1 on beads coated with 5 $\mu\text{g}/\text{mL}$ of protein was estimated to be ≥ 30 sites per μm^2 . In flow assays involving protein-coated microspheres, purified platelets were incubated with 10 mM sodium azide (NaN_3), 50 ng/mL prostaglandin E_1 , and 10 μm indomethacin (Sigma, St. Louis, MO). Platelets were subsequently allowed to settle in stasis on Fab 7E3 fragment-coated glass plates to form a reactive substrate. Platelet coverage of $>90\%$ of the glass surface area was used in determining the tethering frequency and resistance to detachment forces of vWF-A1 coated beads in flow while a total platelet coverage of $<10\%$ was used for kinetic assays to ensure bead interactions with only individual platelets. Confirmation that platelets immobilized in this manner were not activated was documented by the lack of P-selectin expression as assayed by immunofluorescence microscopy.

Tethering frequency and detachment assays for microspheres

The frequency of tethering for microspheres coated with various amounts of vWF-A1 proteins (per 10 \times field of view) was measured by determining

the percentage of beads that paused, but did not translocate, on antibody-immobilized platelet substrates (>90% total platelet coverage). Tethers per minute were divided by the flux of beads near the wall per minute to obtain the frequency of this adhesive interaction (Finger et al., 1996). To ensure that the beads were in close proximity to the substrate at all shear stresses tested and thus have a similar probability of interacting, tethering frequency was determined only after the first bead was noted to bind (~1 min of flow). Only one tethering event per bead was counted during the observation period and coating concentrations of beads were chosen that only supported transient adhesive events. For detachment assays, beads ($1 \times 10^9/\text{mL}$) were infused into the parallel-plate flow chamber at 0.85 dyn cm^{-2} and allowed to accumulate for 5 min. Subsequently, the wall shear stress was increased every 10 s to a maximum 36 dyn cm^{-2} . The number of beads remaining bound at the end of each incremental increase in wall shear stress was determined and expressed as the percentage of the total number of beads originally bound.

Pause time analysis

The interaction times between platelets and vWF-A1 adsorbed surfaces per field of view (i.e., pause time or duration of a transient tether) were quantitated by high temporal resolution videomicroscopy as previously described (Schmidtke and Diamond, 2000). A transient tether event was defined as a flowing platelet that abruptly halted forward motion for a defined period of time and subsequently released, without evidence of translocation, to resume a velocity equivalent to that of a noninteracting cell. The vast majority of transient tethers were $>0.02 \text{ s}$ at all wall shear stresses tested. Dissociation rate constants were determined by plotting the natural log of the number of platelets that interacted as a function of time after the initiation of tethering (Alon et al., 1995, 1997; Smith et al., 1999; Ramachandran et al., 2001). The slope of the line is $-k_{\text{off}}$.

Estimation of k_{off} values for vWF-A1 coated microspheres transiently interacting with surface-immobilized platelets was determined by recording images from a Nikon X60 DIC objective (oil immersion) viewed at a frame rate of 235 fps (Speed Vision Technologies, San Diego, CA).

In vivo studies

The surgical preparation of animals for all in vivo studies were performed using standard techniques (Coxon et al., 1996). The cremaster muscle of anesthetized adult male mice (C57Bl/6, Jackson Laboratory) was surgically exposed and positioned over a circular glass coverslip (25 mm) on a custom-built plexiglass board for viewing. Carboxyfluorescein-labeled platelets (Diacovo et al., 1996) were videotaped during their passage through the arterial microcirculation under fluorescent stroboscopic epillumination via observation through a $60\times$ Olympus objective (LUMPlanFl, numerical aperture 0.9∞). The arterial shear rates of pre- and postvessel injury were 1224 ± 285 (mean \pm SD) and 1324 ± 186 as determined from optical doppler velocimeter measurements of centerline erythrocyte velocity. Platelet-vessel wall interactions were classified as either rolling or firmly adherent. Rolling flux was determined by counting the number of rolling platelets that cross an imaginary perpendicular line through the vessel per unit time. A MacIntosh-based interactive image analysis system was used to determine vessel diameter and rolling flux.

Vascular trauma was generated as follows: the segment of arteriole initially identified and recorded as preinjury was visualized under a dissecting scope. Subsequently, an electrically induced vascular lesion was created by a brief application of a current using a fine tip epilator (Bourgain et al., 1985).

Monte Carlo simulation

Simulations of the formation and dissociation of vWF-A1 (wt or type 2B) and GPIIb α was based upon Gillespie's algorithm for stochastic reactions

(Gillespie, 1976). Monte Carlo (MC) began with a single bond between the platelet and bead. Subsequently, three types of events were permitted: 1) dissociation of a bond; 2) creation of a new bond; and 3) departure of the unbound bead from the platelet. According to stochastic theory these events have the following probabilities: 1) $a_1 dt = k_{\text{off}}(F)ndt$; 2) $a_2 dt = k_{\text{on}}X_{\text{A1}}X_{\text{GPIIb}}dt$; and 3) $a_3 dt = \gamma_w \delta_{n,0} dt$, in which $X_{\text{A1}} = 5$ and $X_{\text{GPIIb}} = 390$ are the numbers of A1 domains and GPIIb α receptors in the contact area ($0.78 \mu\text{m}^2$) for 25,000 GPIIb α per platelet and 30 vWF sites per μm^2 as determined by flow cytometry (Rènyi, 1953; McQuarrie, 1963). n is the number of bonds, k_{on} and $k_{\text{off}}(F)$ are the association and force-dependent dissociation rate constants, γ_w is the wall shear rate, and $\delta_{i,j}$ is the Kronecker delta-function. This approach extends the method of Tees by rigorous selection of the time between events, calculation of the escape probability, and inclusion of bond formation (Tees and Goldsmith, 1996). The definition of the escape probability relies upon the use of the escape velocity of the bead, which is detectable within 1 frame (<4 ms) of the experiments. It is possible that the surface repulsion and bead diffusion are also involved as mechanisms in the escape process, however these give equally small departure times relative to $1/k_{\text{off}}$ and thus should not dramatically alter the results of the MC. This was in fact the case as the MC was relatively insensitive to the magnitude of the escape probability. The use of the escape probability allows a definitive conclusion of the MC when the adhesion event ends.

Using MC, the rate constants k_{on} and k_{off}^0 were determined by fitting sets of simulated pause times to the sets of experimental pause times at each experimental condition. The Bell model was used for the off-rate, $k_{\text{off}}(F) = k_{\text{off}}^0 \exp(\sigma F/nk_B T)$ in which k_{off}^0 is the zero-force dissociation constant and σ is the reactive compliance (Bell, 1978). Moreover, F is the hydrodynamic force pulling on the bond as determined from force balance equations (Chen and Springer, 1999), T is the temperature and k_B is Boltzmann's constant. The force on the bead was related to the wall shear stress using Goldman's equation (Goldman et al., 1967). Simulations were conducted over a wide range of values of k_{on} , k_{off}^0 , and σ . Each parameter was systematically varied in all combinations, resulting in 2.0×10^6 simulations altogether. Because the pause time is a random variable, the average pause time $\langle t_{\text{pause}}(\tau_w) \rangle$ is an unbiased estimator. Consequently, the optimal fit between our model and experiment was specified by the global minimum of the quantity $\epsilon(\tau_w) = \langle t_{\text{pause,model}}(\tau_w) \rangle - \langle t_{\text{pause,exp}}(\tau_w) \rangle / \langle t_{\text{pause,exp}}(\tau_w) \rangle$ over all shear stresses. Therefore, the optimal fit between simulation and experiment was selected by minimization of the largest value of the quantity:

$$\epsilon(\tau_w) = \frac{|\bar{t}_{\text{pause,exp}}(\tau_w) - \bar{t}_{\text{pause,MC}}(\tau_w)|}{\bar{t}_{\text{pause,exp}}(\tau_w)}$$

over all experimental wall shear stresses, in which $\bar{t}_{\text{pause,exp}}(\tau_w)$ and $\bar{t}_{\text{pause,MC}}(\tau_w)$ are the means of the experimental and simulated pause time distributions at τ_w . The parameters k_{on} , k_{off}^0 , and σ were systematically varied in all combinations during $\epsilon(\tau_w)$ minimization for a total of 2.0×10^6 simulations for each vWF-A1 species.

RESULTS

Injured arterial endothelium supports rapid platelet tethering and translocation in vivo

To determine whether the dynamics of platelet adhesion in vitro truly reflect the physiological properties of platelet adhesion at sites of arterial injury, an event initiated by GPIIb α -vWF interactions, we initially examined and classified platelet interactions in vivo using a murine vascular injury model. Circulating, fluorescently labeled platelets were observed to rapidly tether to and release from or

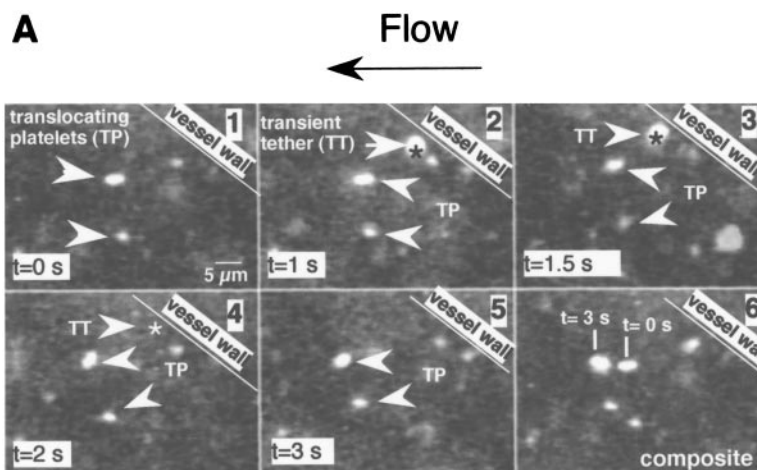
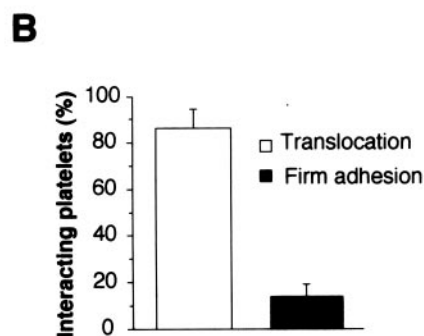


FIGURE 1 In vivo characterization of platelet interactions with injured arteriolar vascular endothelium. (A) Representative intravital photomicrographs depict the range of platelet interactions that occur at a site of vascular injury (60 \times magnification). Platelets were observed to either transiently pause (*) or rapidly tether to and translocate (TP) on damaged arterial endothelium. A composite image demonstrates translocation of two platelets over a 3-s interval of time (panel 6). (B) Dynamics of platelet adhesion in vivo. Interacting platelets at the site of arterial injury were classified as either undergoing translocation or firm adhesion (sticking) during an observation period of 1 min. The nature of these interactions was determined for 50 to 60 platelets in five separate experiments. Data represent the mean \pm SD.



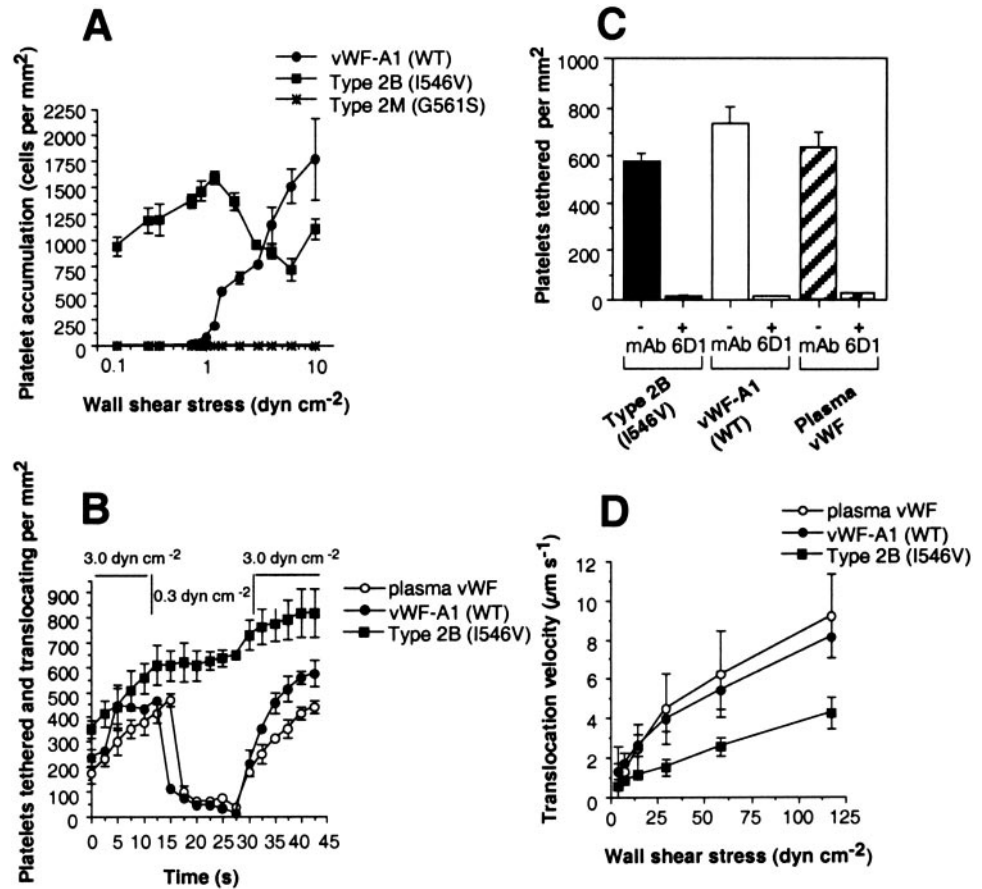
subsequently translocate on injured arterial endothelium in a manner reminiscent of selectin-dependent adhesion of leukocytes at sites of venular inflammation (Fig. 1 A). Evidence to support the concept that rapid formation and breakage of adhesive bonds are characteristic of the receptor-ligand pair(s) involved in mediating platelet attachment to sites of vascular damage is further suggested by platelet-vessel wall interaction times of <1.0 s (panels 2–4). Thrombus formation was not observed in our system, as $<15\%$ of translocating platelets eventually became firmly adherent suggesting low levels of ligands for platelet integrin receptors or lack of an activating stimulus (Fig. 1 B). A role for the A1 domain of vWF in mediating this rapidly reversible interaction is supported by the ability of murine, but not human recombinant A1 protein, to inhibit platelet adhesion in vivo (Doggett and Diacovo, unpublished observation).

Effect of hydrodynamic flow on platelet-vWF interactions

After establishing that the adhesive behavior of platelets in vitro are in deed representative of those observed in vivo, we next evaluated the impact of shear flow on the kinetics

that govern the interactions between GPIIb/IIIa and vWF. This was accomplished by assessing platelet adhesion to vWF-A1 domain proteins in vitro under various wall shear stresses. Recombinant monomeric A1 has been shown to mediate platelet tethering and translocation to a similar extent as observed for multimeric plasma vWF (Cruz et al., 2000; Miyata and Ruggeri, 1999). Flow rates that support both transient tethers and rolling adhesions of leukocytes on purified selectin molecules were initially chosen to study this interaction so to enable comparisons with the kinetics of tether bonds established for selectin-ligand pairs. Platelets were observed to transiently interact with saturating concentrations of the WT substrate or plasma vWF only after achieving a shear stress 0.73 dyn cm^{-2} (Fig. 2 A). The specificity of the interaction was demonstrated by the inability of platelets to adhere to a vWF-A1 substrate into which the type 2M mutation was incorporated (G561S). This naturally occurring mutation has been shown to impair interactions between vWF and GPIIb/IIIa on platelets in flow (Cruz et al., 2000). Evidence that direct surface-immobilization does not alter the affinity of vWF-A1 for GPIIb/IIIa was demonstrated by the requirement for the identical level of shear stress to support platelet adhesion to vWF-A1 bound by an immobilized antibody that specifically recognizes the

FIGURE 2 Role of hydrodynamic flow in promoting platelet tethering and translocation. **(A)** Purified platelets were perfused over surface-immobilized recombinant A1 proteins (100 $\mu\text{g}/\text{mL}$) or plasma vWF (25 $\mu\text{g}/\text{mL}$) at wall shear stress ranging from 0.25 to 4 dyn cm^{-2} for 5 min. The number of platelets that tethered to and/or translocated (>1 -platelet diameter) on the indicated substrates as a function of wall shear stresses was determined. **(B)** Reversibility of GPIIb α -vWF-A1 interactions was evaluated by rapidly altering shear flow. Platelets were infused for 1 min at 3.0 dyn cm^{-2} before taking measurements ($t = 0$). At $t = 15$ s, the flow was reduced to 0.3 dyn cm^{-2} , and the number of platelets remaining bound was quantified per second. At $t = 30$ s, flow was increased again to 3.0 dyn cm^{-2} and platelet reattachment measured as a function of time. Values represent the mean \pm SD. **(C and D)** Comparison of platelet attachment and translocation velocities on WT and mutant vWF-A1 substrates at high shear stresses. Platelet accumulation on the indicated substrates was evaluated at a wall shear stress of 16 dyn cm^{-2} for a duration of 5 min. Mean translocation velocities ($\mu\text{m s}^{-1}$) for individual interacting platelets ($n = 30$ –40) were determined at the indicated wall shear stresses for three independent experiments performed in duplicate. The specificity of the interactions was demonstrated by the ability of GPIIb α function blocking antibody, mAb 6D1, to inhibit platelet adhesion to all vWF-A1 substrates.



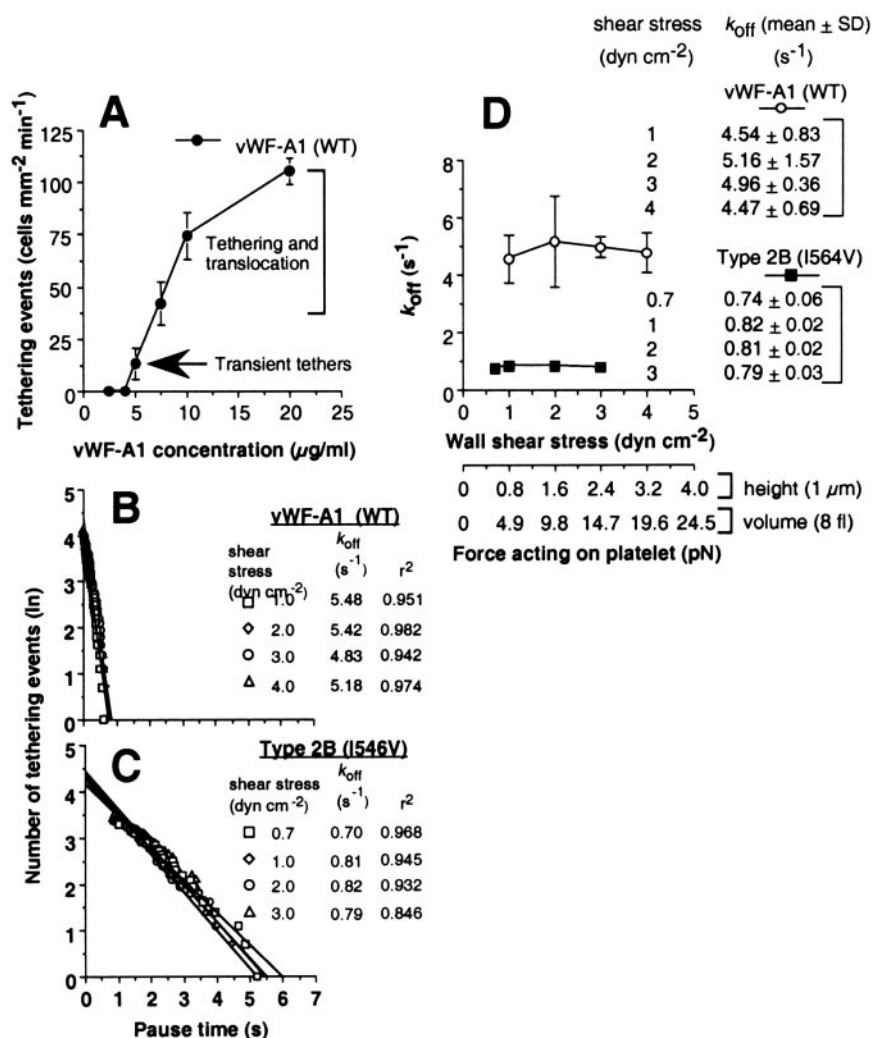
amino terminus His-tag of the recombinant protein (data not shown). Incorporation of the type 2B mutation, I546V, into the A1 domain abolished the requirement for a critical level of shear stress as flowing platelets readily accumulated and translocated on the mutant substrate under identical flow conditions. Similar results were obtained with type 2B mutants R543Q and R543W (data not shown).

To demonstrate that the shear stress-dependent interaction between GPIIb α and vWF-A1 was a rapidly reversible phenomenon, a kinetic attribute associated with selectin-ligand interactions, platelet accumulation on either plasma vWF or recombinant proteins was evaluated as wall shear stress was reduced below and reinstated above the critical threshold value. Platelets that attached and translocated on WT vWF-A1 at a wall shear stress of 3.0 dyn cm^{-2} quickly released (<1 s) from the substrate as flow was lowered to 0.3 dyn cm^{-2} (Fig. 2 B). This rapid reduction in flow,

however, did not result in the detachment of platelets from the type 2B vWF-A1, demonstrating the consequences of altered bond kinetics. In fact, platelets continued to accumulate on the mutant substrate despite the 10-fold reduction in wall shear stress. Platelet adhesion to WT vWF-A1 could also be rapidly reestablished (<1 s) by an increase in wall shear stress, indicating that fluid shear does not irreversibly modulate GPIIb α -vWF-A1 binding interaction.

Clinically, individuals with vWF type 2B vWD appear to have a mild bleeding disorders suggesting that the remaining mutant vWF multimers can support platelet adhesion at sites of arterial injury. Yet, such afflicted individuals are not prone to thrombotic events as would be anticipated if type 2B mutant vWF significantly enhanced platelet deposition in damaged arterial beds as suggested by its ~ 10 -fold higher affinity ($K_d \sim 0.44 \pm 0.07$) for GPIIb α than WT vWF-A1. To determine whether a type 2B mutation would

FIGURE 3 Estimation of the k_{off} values for the GPIIb α -vWF-A1 tether bond based on the duration of transient tether events. (A) Platelets were perfused over WT vWF-A1 substrate at a wall shear stress of 3 dyn cm $^{-2}$ for 5 min and the number and behavior of interacting platelets determined as a function of the bulk concentration of vWF-A1 adsorbed to polystyrene dishes. Values shown are the mean \pm SD of three experiments at each protein concentration tested. (B and C) Kinetics of dissociation of transiently tethered platelets on WT and type 2B mutant vWF-A1 substrates. Dissociation rate constants were estimated at various wall shear stresses using a 5 μ g/mL coating concentration of recombinant proteins. Representative experiments for each protein substrate are shown for comparison. Error bars indicate SD, $n = 2$ to 4. (D) Effect of shear stress on k_{off} for WT and mutant substrates. The force (F_s) acting on a platelet in shear flow was estimated based on equations of Goldman ($F_s = 6\pi R h C_F \tau$; $T_s = 4\pi R^3 C_F \tau$). As the geometry of a resting platelet is discoid and not spherical, a low and high estimate of the shear force and torque were determined for spheres with either a diameter (1 μ m) or a volume (8 fL) equal to that of a platelet.



enhance platelet adhesion at wall shear stresses encountered in the arterial circulation, the ability of platelets to accumulate on a mutant vWF-A1 substrate under high flow rates was evaluated. Incorporation of the type 2B mutation, I546V, into the vWF-A1 domain did not dramatically alter platelet accumulation as compared with substrates containing WT vWF-A1 or plasma vWF at saturating concentrations of protein (Fig. 2 C). Platelet translocation velocities on either substrate were also comparable, demonstrating that the isolated A1-domain reflects the biological activities of the mature plasma glycoprotein. In contrast, a twofold reduction in platelet translocation velocity was observed for the mutant substrate, suggesting an alteration in dissociation rate constant (Fig. 2 D).

Kinetics of dissociation of transient tethers in response to hydrodynamic force

Fast dissociation rate constants, k_{off} , are characteristic of receptor-ligand pairs that mediate rolling adhesive interac-

tions of hematogenous cells in biological systems. To date, values meeting this criterion have only been determined for the selectin family of adhesion receptors. Estimations of intrinsic k_{off} values for these adhesion molecules as determined by measuring lifetimes of tether bonds in flow (Alon et al., 1995, 1997; Smith et al., 1999; Ramachandran et al., 2001; Kaplanski et al., 1993) or by SPR (Mehta et al., 1998; Nicholson et al., 1998) yielded similar results. However, the ability of platelets to tether to and translocate on vWF in vitro and at sites of vascular injury in vivo is suggestive of a very rapid rate of dissociation for the bond formed between GPIIb α and the A1 domain of this multimeric plasma protein. To estimate k_{off} for both WT and mutant substrates and to better evaluate the effect of flow-induced forces on tether bond lifetimes, the lowest concentration of recombinant protein (5 μ g/mL) was chosen that supported transient interactions of platelets at all shear stresses tested (Fig. 3 A). Transient tethers, the smallest unit of adhesive interaction observable in shear flow, have a distribution of bond lifetimes that obey first order dissociation kinetics (Alon et al.,

1995, 1997; Smith et al., 1999; Ramachandran et al., 2001). Pause time analysis of such adhesive events indicates that the majority of transient tethers that dissociated rapidly (>90% of all interactions) fit a straight line for both WT and mutant vWF-A1 proteins (Fig. 3, B and C). Dissociation rate constants for platelets interacting with WT vWF-A1 were approximately sixfold greater in magnitude than those observed for the mutant protein, I546V, at the identical coating concentration and shear stresses. Moreover, k_{off} values for both proteins were essentially unchanged as a function of wall shear stress, unlike previous reports for selectin-ligand interactions. This suggests that the forces acting on the GPIIb α -vWF-A1 tether bond may not be sufficient to alter the rate of dissociation (Fig. 3 D). Evidence to support this hypothesis is provided by estimation of the forces acting on a platelet under wall shear stresses ranging from 1 to 4 dyn cm $^{-2}$ (minimum of 0.8 pN to a maximum of 19.6 pN, respectively). These values are significantly lower than those calculated for leukocytes under identical flow conditions (57.9 to 231.6, respectively assuming a diameter of 8.5 μm).

Effect of increased hydrodynamic force on the kinetics of the GPIIb α -vWF-A1 bond

To gain insight into the strength of the GPIIb α -vWF-A1 bond and to permit direct comparison with selectin-ligand interactions, we examined transient tethering events between vWF-A1-coated microspheres and surface-immobilized platelets in flow. By using microspheres of 7 μm in diameter, the hydrodynamic force acting on the bond formed between this receptor-ligand pair would then be comparable with that experienced by leukocytes interacting with adherent selectin molecules under identical flow conditions. Transient adhesive events were the predominant interactions for coating concentrations of <10 $\mu\text{g}/\text{mL}$ for both WT and mutant forms of the recombinant protein at wall shear stresses ranging from 0.5 to 4.0 dyn cm $^{-2}$ (Fig. 4 A). Interestingly, adhesion of protein-coated beads was limited to a shear stress of <4 dyn cm $^{-2}$, the maximal flow conditions that support selectin-dependent adhesion of leukocytes. The distinct transitions in motion that occur as a vWF-A1 coated bead forms a tether bond with GPIIb α on the surface of an immobilized platelet are depicted in Fig. 4, B and C. From measurements of the escape velocity relative to the approach velocity, it was apparent that the bead had rotated after capture to a position that was in extremely close proximity to the surface. A gap separation distance of <100 nm was determined from the measured escape velocity of $288 \pm 90.4 \mu\text{m}/\text{s}$ ($n = 9$) at a wall shear rate of 150 s^{-1} using the solution of the Stokes equation (Goldman et al., 1967). This is consistent with previous determinations of gap separation distance for beads or for human neutrophils released from spread platelets (Schmidtke and Diamond, 2000; Pierres et al., 1995).

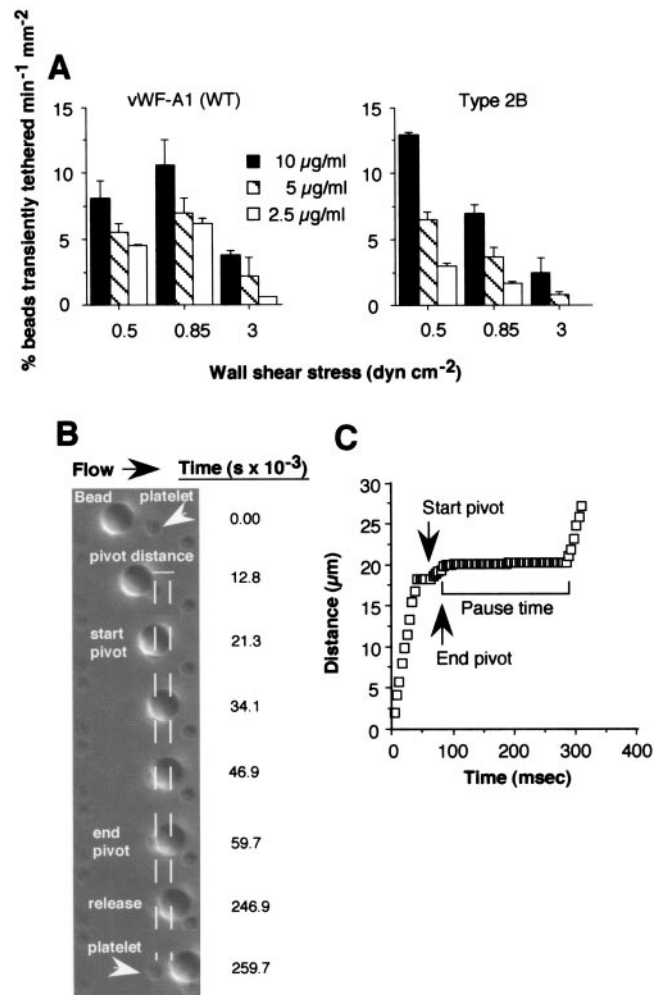


FIGURE 4 Characterization of transient tethers of vWF-A1 coated microspheres. (A) Microspheres were perfused over a confluent monolayer of surface-immobilized platelets at wall shear stresses ranging from 0.5 to 3 dyn cm $^{-2}$. The number of beads that transiently tethered to the substrate for 1 min was quantified and normalized by dividing by the flux of beads near the wall. Data represent the mean \pm SD of three independent experiments. (B and C) Direct visualization of bead-platelet interaction under flow (60 \times DIC microscopy). An approaching bead moving at a velocity of $609 \pm 97 \mu\text{m}/\text{s}$ (wall shear stress of 1.5 dyn cm $^{-2}$) is captured by a surface-immobilized platelet at ($t = 12.8$ ms), pivots a distance of less than 3 μm in under 40 ms, and is then released after a pause time of $t_p = 228.2$ ms into the flow stream (escape velocity = $288 \pm 90.4 \mu\text{m}/\text{s}$).

After establishing the location of the bead relative to the platelet and glass surfaces, the relationship between wall shear stress and force on the tether bond (F_b) was determined. Thus, the measured k_{off} values for transient tether events could be plotted as a function of wall shear stress and the estimated force on the tether bond. The lifetimes of the GPIIb α -vWF-A1 tether bond were measured for beads coated using concentrations of recombinant protein (<5 $\mu\text{g}/\text{mL}$) that only supported transient interactions at all wall shear stresses tested. Pause time analysis of such adhesive events indicates that the majority of transient tethers that

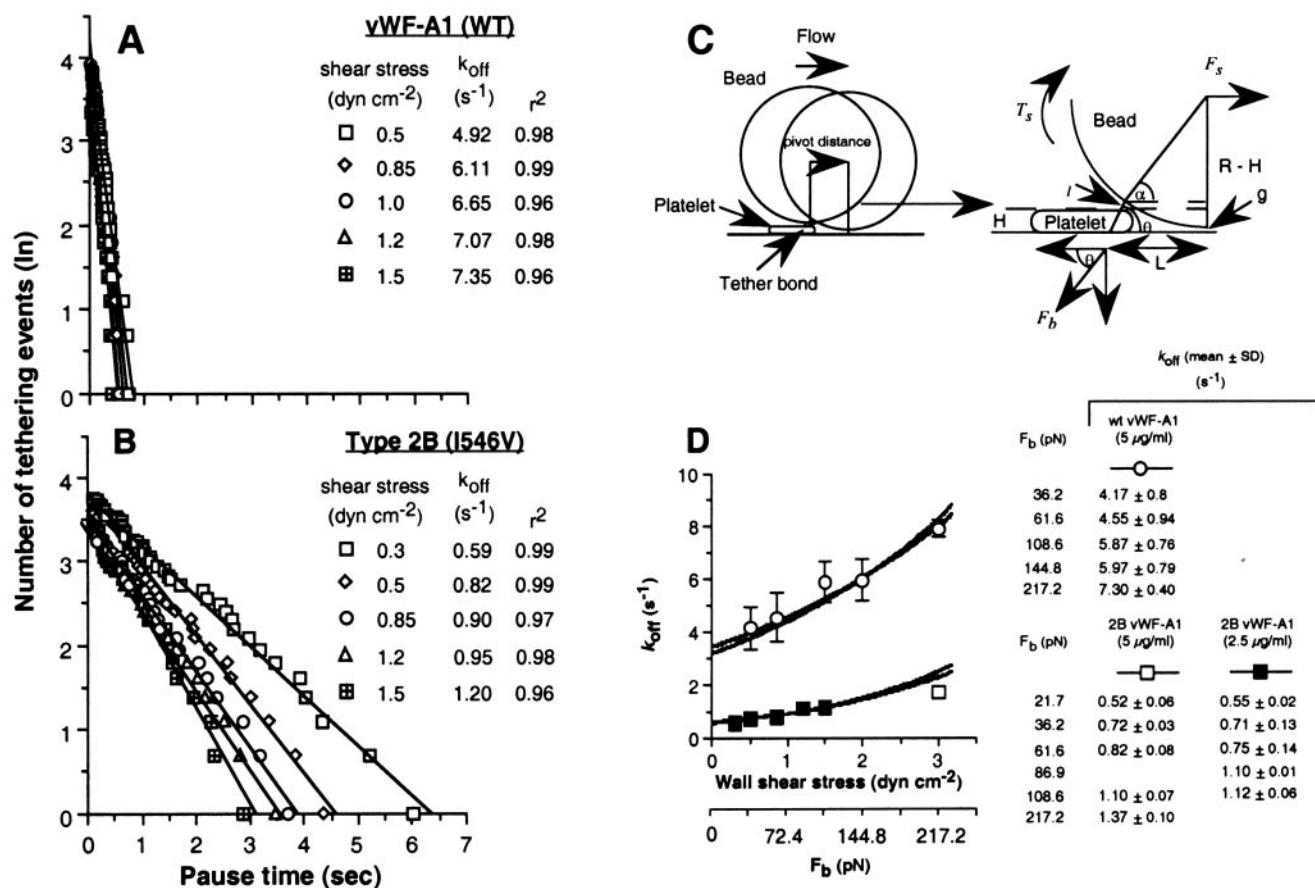


FIGURE 5 Kinetics of dissociation and estimation of the forces acting on the tether bond for vWF-A1 coated microspheres. k_{off} values for microspheres coated with WT (A) or type 2B mutant proteins (B) that transiently tethered to surface-immobilized platelets based on a distribution of pause times. Dissociation rate constants were estimated at various wall shear stresses using a 2.5- $\mu\text{g}/\text{mL}$ coating concentration of recombinant proteins, the lowest concentration capable of supporting adhesion. Representative experiments for each protein substrate are shown for comparison. Error bars indicate SD, $n = 5$. (C) Estimation of the force on the GPIIb α -vWF-A1 tether bond. The force on the bond was calculated based on a lever arm (L) determination for the height of the platelet ($H = 1 \mu\text{m}$) and the known bead radius ($R = 3.5 \mu\text{m}$). The angle $\theta = 57.16^\circ$ satisfied the force balances $F_b \cos \theta = F_s$ and $(L)F_b \sin \theta = RF_s + T_s$ in which the force on the bond F_b is related to the lever arm $L = H/\tan\theta + R\cos(\sin^{-1}(1 - H/R))$, the shear force F_s and the torque T_s (Shao et al., 1998). Thus, F_b (in pN) = $(72.41) \tau_w$ in which the wall shear stress τ_w (in dyn cm⁻²) is related to the wall shear rate $\dot{\gamma}_w$ (in s⁻¹) by $\tau_w = (0.01 \text{ Poise}) \dot{\gamma}_w$. (D) Effect of force on the GPIIb α -vWF-A1 tether bond as a function of k_{off} . F_b was calculated as described above. Similar values for dissociation rate constants were obtained using two different coating concentrations of vWF-A1. Error bars indicate SD, $n = 3$ to 5. Data were fit to the Bell equation.

dissociated rapidly (>90% of all interactions) fit a straight line for both WT and mutant vWF-A1 proteins (Fig. 5, A and B). Dissociation rate constants for platelets interacting with WT vWF-A1 were approximately sevenfold greater in magnitude than those observed for the mutant protein, I546V, at the identical coating concentration and shear stresses. This is in agreement with our estimates for k_{off} obtained for platelets transiently interacting with surface-bound vWF-A1 proteins at a similar level of hydrodynamic force (i.e., <40 pN). Moreover, k_{off} values for both proteins varied as a function of shear stress suggesting that the increased force experienced by the beads versus platelets is now sufficient to impact on the biomechanical properties of the tether bond (Fig. 5 C). All data were fit to Bell's

equation to quantitate the reactive compliance (σ), a measure of the mechanical stability of a tether, and to yield values for k_{off} in the absence of force (Table 1). The kinetics of dissociation for these transient tether events varied exponentially as a function of the force in accordance with Bell's equation (Bell, 1978). Strikingly, both the reactive compliance and k_{off}^0 determined for GPIIb α interactions with WT vWF-A1 were within the range reported for selectin-ligand interactions (Mehta et al., 1998; Nicholson et al., 1998; Alon et al., 1995, 1997; Smith et al., 1999; Ramachandran et al., 2001; Kaplanski et al., 1993). In comparison with the WT protein, the GPIIb α -type 2B vWF-A1 tether bond varied significantly with respect to the intrinsic k_{off} (approximate sixfold reduction). The differences in val-

TABLE 1 Dissociation rates and reactive compliance values for the GPIIb α – vWF-A1 tether bond

	k_{off}^0 (s^{-1})	σ (nm)	R^2
vWF-A1 (WT)			
SPE	3.21 ± 0.15	0.018 ± 0.002	0.97
MCR	3.45 ± 0.37	0.016 ± 0.002	
Type 2B			
SPE	0.56 ± 0.02	0.026 ± 0.001	0.99
MCR	0.55 ± 0.03	0.029 ± 0.004	

Comparison between MC and experimental results were conducted for WT and mutant proteins at coating concentrations of 5 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respectively. The data represent the mean \pm SD for five regressions for the MC (MCR).

ues for σ were statistically significant (p value of 0.029) for WT and mutant proteins suggesting an alteration in the mechanical properties of the bond as well.

MC simulation and an additional independent statistical analyses of the experimental data using a single tether bond model of the pause time distribution were used to evaluate kinetic parameters of the GPIIb α -WT vWF-A1 and GPIIb α -type 2B vWF-A1 bonds. In the second model, statistical point estimates of k_{off} (F) were obtained from the roughly exponentially distributed pause times (Montgomery and Runger, 1994). Subsequently, both sets of estimates of k_{off} (F) were fit to the Bell model (Eq. 1) by standard linear regression to obtain the zero force off-rate k_{off} (F) and reactive compliance σ (Fig. 5 D). The results of the two analyses are given in Table 1. Strong agreement was observed between the methods. For all sets of kinetic parameters best fitting the experimental results at all shear stresses, the optimal values of the association rate constant k_{on} were effectively zero (10^{-5} to 10^{-7} s^{-1}). Standard deviations for the data were on the order of or exceeding the mean, indicating that tether bond formation beyond the first bond was experimentally insignificant. The insensitivity of both the MC regression and experimental design to the surface concentration of wt or type 2B mutant vWF-A1 on the beads supports this conclusion.

Resistant of protein-coated microspheres to shear-induced detachment forces and comparison of rolling velocities

The impact of the vWF type 2B mutation, I564V, on the strength of GPIIb α -mediated rolling was also determined by measuring the resistance of protein-coated beads to detachment on a platelet substrate as a function of increasing wall shear stress. Incorporation of this mutation into the vWF-A1 domain did not dramatically alter the ability of beads to resist shear stress-induced detachment forces as compared with WT vWF-A1, even at the lowest coating concentration of protein capable of supporting rolling interactions (Fig. 6 A). This is most evident at concentrations ≤ 20 $\mu\text{g}/\text{mL}$ in which similar quantities of beads remained bound at all

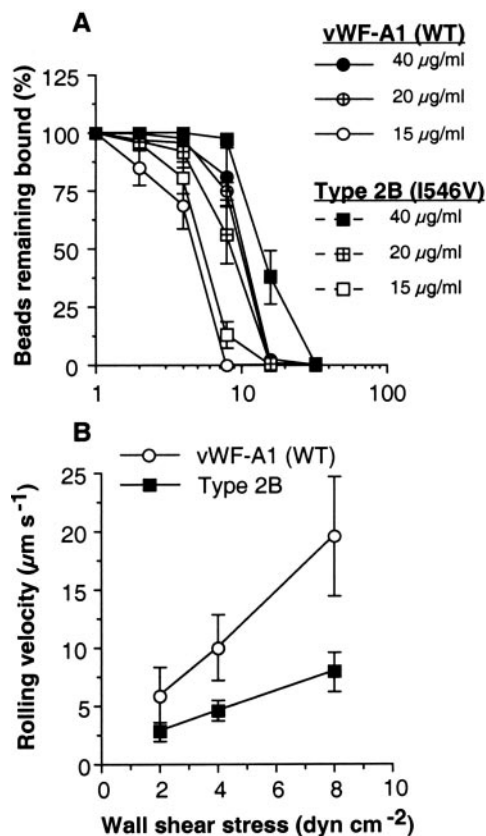


FIGURE 6 Effect of the type 2B mutation, I564V on microspheres detachment by shear forces and rolling velocities. (A) Detachment profile of beads coated with either WT or type 2B vWF-A1 at concentrations of 15, 20, and 40 $\mu\text{g}/\text{mL}$ as a function of increasing shear stress. (B) Rolling velocities of vWF-A1 coated beads (100 $\mu\text{g}/\text{mL}$). Beads that had accumulated on surface-immobilized platelets were subjected to incremental increases in wall shear stress and rolling velocities calculated for a minimum of 50 beads over a 10-s intervals. Data represent the mean \pm SD of three independent experiments performed in triplicate.

shear stresses tested. In contrast, rolling velocities were on the order of two to threefold lower for the type 2 B mutation than for the WT substrate, results are consistent with platelet translocation velocities on vWF-A1 protein coated plates (Fig. 6 B).

DISCUSSION

It has been well established that GPIIb α mediates the attachment and translocation of platelets on surface immobilized vWF in vitro. We confirm this observation in vivo and further demonstrate that rapid attachment and release of platelets at sites of arterial injury is a characteristic feature of this interaction. However, previous results evaluating GPIIb α interactions with the vWF-A1 domain in equilibrium binding assays suggest that slow intrinsic binding kinetics are responsible for rapid platelet adhesion (Miura et al., 2000). Estimated dissociation rate constant values reported

in this previous study were 0.0038 s^{-1} and 0.0036 s^{-1} , which correspond to bond lifetimes of $>4 \text{ min}$ for platelet interactions with wt or type 2B recombinant vWF-A1 domains, respectively. These findings suggest that the kinetic properties of this receptor ligand interaction are distinct from those reported for the selectin family of adhesion molecules, an adhesive event that relies on a high value of kinetic constants to promote rolling adhesion of leukocytes (Chang et al., 2000).

By examining the interactions between platelets and vWF under physiologically relevant conditions, hydrodynamic flow, we report that the GPIb α -vWF-A1 tether bond possess all the biomechanical properties associated with the selectin family of adhesion receptors. This includes flow dependent adhesion and rapid and force-dependent kinetics, properties that cannot be ascertained by techniques such as SPR or biochemical analysis of the bond in stasis. Our results were based on the lifetimes of transient tether events, which followed first-order kinetics and appeared to be independent of ligand density above the shear threshold required to promote platelet or microsphere translocation. Although such properties do not prove that transient tethers are indicative of the formation and dissociation of single bonds, these events are of physiological relevance as they represent the smallest functional unit of adhesion that permits cell interactions in flow. Thus, in contrast to measuring the kinetics of binding between purified receptor-ligand pairs by biochemical analysis, determination of the dynamics of cellular interactions in flow will, by its very nature, also be affected by parameters such as membrane deformability and cytoskeletal interactions with the receptor. Despite these contributions, measurements of bond lifetimes in flow have proven to be useful in estimating the intrinsic as well as the mechanical properties of a receptor-ligand pair. For instance, k_{off}^0 values obtained for P-selectin interactions with its ligand P-selectin glycoprotein-1 were $\sim 1.0 \text{ s}^{-1}$ versus 1.4 s^{-1} as determined by transient tethers versus SPR, respectively (Mehta et al., 1998; Alon et al., 1995). Based on the similarities in values obtained by these two different techniques, we believe that our results will also prove to be a reasonable representation of the kinetic properties of the bond that governs the interactions between the GPIb α and vWF. Interestingly, k_{off} values estimated for GPIb α interactions with WT and mutant vWF-A1 proteins under zero force conditions in our system were observed to be significantly higher than those previously reported (~ 900 and ~ 120 -fold greater for WT and mutant vWF-A1, respectively). Thus, even accounting for the possibility that our measurements are in fact representative of multiple homogenous bonds, which would lower estimations of k_{off}^0 , our values still do not approach those previously reported for this receptor-ligand pair (Miura et al., 2000). This discrepancy may be due to the effect of hydrodynamic forces on the receptor-ligand bonds as solution phase determinant of affinities are unable to quantify the impact of mechanical

forces on bond lifetimes. Further evidence to support our claim that the GPIb α -vWF tether bond exhibits characteristics attributed to selectins is demonstrated by its fit to the relationship proposed by Bell, $k_{\text{off}} = k_{\text{off}}^0 \exp(\sigma F/kT)$, a model demonstrated to best represent how force affects the dissociation of selectin-ligand bonds (Chen and Springer, 2001). This was confirmed by two-independent statistical analyses based on a tether bond model of pause time distribution. Importantly, our values for k_{off}^0 and reactive compliance are consistent with theoretical parameters established for adhesion receptors that support cell translocation in flow, including the selectins (Chang et al., 2000.).

Biologically, receptor-ligand bonds have evolved kinetic properties that are critical for their specific function(s). In the case of GPIb α and the selectins, both families of adhesion receptors have adopted unique dynamic properties well suited for their roles in initiating the cell adhesion cascade. To date, only mutations in GPIb α and the vWF-A1 domain have been described in man that result in perturbation in the cell adhesion process leading to dire consequences for the afflicted individual. In particular, specific gain-of-function mutations contained within the A1 domain of vWF, classified as type 2B vWD, result in spontaneous binding of circulating platelets to mutant vWF in plasma, clearance of both of these hemostatic elements from the blood, and ultimately a predilection to hemorrhage (Ruggeri et al., 1980; Cooney and Ginsburg, 1996). The majority of these mutations are localized within the disulfide loop of the A1 domain, between amino acid residues 463 and 716, in a region distinct from the putative GPIb α binding site (Ginsburg and Sadler, 1993; Meyer et al., 1997). Thus, the vWF type 2B mutation, I546V, provides a unique opportunity to determine the specific alterations in kinetic properties of the bond that are responsible for the observed phenotype. It is anticipated that if the mechanical properties of the tether bond are altered, this will manifest as a change in the dissociation rate constant as a function of force on the bond. In contrast, modifications in the intrinsic properties of the tether bond will result in a change in k_{off} in the absence of flow. Our results indicate that type 2B mutation, I546V, appears to have a major affect on the intrinsic kinetic properties of the bond formed with GPIb α . Incorporation of the type 2B mutation, I546V, resulted in sixfold reduction in the estimated dissociation under zero flow conditions and only a modest increase in the Bell's parameter σ . A larger σ correlates with a higher reactive compliance and thus a bond's increased susceptibility to force-driven dissociation. However, the magnitude of increase in bond dissociation for both wt and mutant proteins was similar (approximately twofold) as the force on the bond increased from 36.2 to 217.2 pN, suggesting that both sets of bond are of comparable mechanical strength. This is also supported by the similarity in detachment profiles for beads coated

with either wt or mutant vWF-A1 as a function of shear stress. These findings are unique from a previous report that evaluated the effects of artificial chemical modification of L-selectin ligands. In contrast to type 2B mutations, periodate treatment of ligands for L-selectin has been shown to modify only the mechanical properties, that is, the effect of force on the rate of bond dissociation but not on the intrinsic kinetics. This manifested as an approximate twofold decrease in reactive compliance but no significant change in k_{off}^0 (Puri et al., 1998). It is interesting to speculate that a prolongation of bond lifetime for type 2B vWF may permit multiple bond formation and subsequent vWF-induced platelet aggregation in flowing blood where platelets would experience relatively small forces due to their unique geometry. The relatively rapid intrinsic k_{off} for native vWF may preclude such an event from occurring. Thus, mutations associated with type 2B vWD provide a unique insight into the specific properties of a receptor-ligand bond that when altered dramatically impact on cell adhesion.

Based on our results, it would not be surprising if kinetic evaluation of mutations associated with platelet-type vWD, which result in a gain-of-function of GPIb α will yield similar values in rates of dissociation as the vWF mutation, I546V. Platelets from affected individuals also spontaneously bind to plasma vWF (Miller, 1996; Miller and Castell, 1982). Interestingly, transfected CHO cells expressing these mutant forms of GPIb α have significantly slower rolling velocities on saturating concentrations of surface-immobilized plasma vWF than cells expressing the WT receptor (Dong et al., 2000). This suggests the possibility of an alteration in the rate of bond dissociation as this kinetic parameter is an important determinant of this adhesive event. These studies, however, were performed under conditions that support multiple bond formation and as such provide limited insight into the biomechanical properties of the GPIb α -vWF-A1 bond. Thus, a detailed kinetic analysis of these mutations under flow conditions is warranted before direct comparisons can be made between platelet and vWF type 2B mutations.

Overall, our data suggest that rapid bond kinetics are essential for the ability of platelets to attach to and translocate on damaged vascular endothelium under hydrodynamic flow conditions. Moreover, this dynamic behavior may serve as a mechanism to allow surveillance of injured vascular endothelium without promoting thrombus formation unless an appropriate exogenous signal(s) are present. This is evident in a recent study demonstrating diminished arterial thrombosis in mice lacking the protease-activated receptor, PAR-4, a platelet receptor critical for thrombin-induced platelet activation (Sambrano et al., 2001). Our results also emphasize the importance of kinetic properties of receptor-ligand interactions for regulating platelet adhesion in shear flow and the clinical consequences that occur when these parameters are altered.

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