The Physical Exchange of Factor VIII (FVIII) between von Willebrand Factor and Activated Platelets and the Effect of the FVIII B-Domain on Platelet Binding

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ABSTRACT: Normal hemostasis proceeds through the assembly of coagulant complexes on a lipid surface derived from activated platelets. The activation complex assembly is governed by multiple factors including the binding constants (K_d) of the coagulant factors for the lipid surface. The formation of the tenase complex requires delivery of factor VIII (FVIII) to the activated lipid surface by von Willebrand factor (vWF). Using electrophoretic quasi-elastic light scattering (ELS), we have examined the interaction of FVIII in the presence and absence of vWF with both resting and activated gel-filtered human platelets. Resting platelets do not bind FVIII. Platelets activated by thrombin, epinephrine, or SFLLRN, but not ADP or collagen, bind unactivated FVIII if vWF is not present. In the absence of vWF, unactivated FVIII binds to activated platelets with a K_d of 10.4 nM. B-domain deleted FVIII binds to activated platelets with a K_d of 5.1 nM. Thrombin -activated FVIII ($FVIII_a$) binds to activated platelets with a K_d of 1.7 nM. The activation of FVIII while bound to the platelet surface can be monitored as a function of time. In the presence of vWF, binding of unactivated FVIII to activated platelets was inhibited, but not the binding of $FVIII_a$. Displacement of bound unactivated FVIII from the platelet surface occurs when vWF is added to the FVIII−platelet complex. The binding of FVIII to activated platelets is affected by the B-domain, the state of FVIII activation, and the presence of soluble vWF and proceeds as a multistep process. FVIII binding by activated platelets is not affected by platelet gpIIb/IIIa or by platelet vWF.

Assembly of the tenase complex, which is composed of factor $VIII_a$ ($FVIII_a$),[1] factor IX_a (FIX_a), and factor X (FX), is fundamental to the establishment of normal hemostasis as evidenced by the severe hemorrhagic states caused by deficiencies of these factors. Factor VIII is a nonzymogen factor essential to the formation of the tenase complex and serves as a surface itself to concentrate and spatially organize the enzymatic interaction between FIX_a and FX (Hemker & Kahn, 1967). The tenase complex can be assembled on phospholipid derived from either activated platelets or lipid vesicles and serves to catalyze the conversion of FX to factor X_a (FX_a). Although FIX_a by itself is capable of this conversion, a platelet surface with $FVIII_a$ enhances the rate of conversion of FX to FX_a by 4−5-fold (van Dieijen et al., 1981; Ahmad et al., 1989). FVIII is present at a concentration of 0.2 μg/mL (0.7 nM) in normal human plasma and does not circulate freely, but as a noncovalently bound complex with von Willebrand factor (vWF), $K_d = 0.2$ nM (Lollar & Parker, 1987; Leyte et al., 1989). The plasma concentration of vWF is 20 nM (10 μg/mL) with respect to its monomer concentration, but vWF circulates as a polydisperse polymer with a multimer molecular weight range of 0.5×10^6 to 20×10^6. vWF protects FVIII from inactivation by protein C; it prevents activation of FVIII by FX_a (Neuenschwander & Jesty, 1988; Koedam et al., 1988; Fay et al., 1991), and it prevents FVIII from binding to phospholipid or to a platelet surface (Gilbert et al., 1990; Andersson & Brown, 1981; Nesheim et al., 1991).

In order to bind platelets, FVIII must be delivered to the platelet and then released from vWF. Cleavage of Arg^{1689} in the light chain of FVIII releases FVIII from vWF (Hill-Eubanks et al., 1989) and leads to a favorable K_d for binding of FVIII to the activated platelet surface, the initial event in the tenase complex assembly (Monroe et al., 1994). Cleavages of Arg^{372}, Arg^{740}, and Arg^{1689}, with the release of a highly charged activation peptide comprising the B-domain, are required to form the fully active FVIII heterotrimer (Eaton et al., 1986). Although vWF does not protect FVIII from activation by thrombin (Neuenschwander & Jesty, 1988; Hamer et al., 1987), studies suggest that vWF can promote thrombin-mediated cleavage of the FVIII light chain (Hill-Eubanks et al., 1990). $FVIII_a$ binds to activated platelets with a K_d of 1.6 nM, and approximately 500 molecules are bound per platelet (Nesheim et al., 1988; Li et al., 1994).

In this study, electrophoretic light scattering (ELS), which detects ligand binding (Gabriel et al., 1993), was used to show for the first time that in the absence of vWF the state of activation of FVIII affects its binding to platelets, that through its higher K_d, vWF regulates the FVIII distribution between activated platelets and vWF, and that the surface activation of FVIII can be detected and monitored in real time.

MATERIALS AND METHODS

Platelet Isolation. Fresh gel-filtered platelets were used in all experiments and prepared from 20 mL of venous blood

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[1] Abbreviations: FVIII, unactivated factor VIII; $FVIII_a$, activated factor VIII; vWF, von Willebrand factor; FIX_a, activated factor IX; ELS, electrophoretic quasi-elastic light scattering; K_d, binding constant; SFLLRN, thrombin receptor peptide; PPACK, phenylalanylprolylarginine chloromethyl ketone.

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drawn into 3 mL of acid−citrate−dextrose (ACD) with 5 μg/mL prostacyclin (PGI_2; Sigma Chemical Co., St. Louis, MO) (Fox, 1992). After thoroughly mixing, the sample was centrifuged at 200*g* for 5 min at room temperature, and the top two-thirds of the platelet-rich plasma (PRP) was collected. The PRP was then centrifuged at 650*g* for 25 min at room temperature. The platelet pellet was resuspended in 5 mL of CGS buffer (13 mM citrate, 123 mM NaCl, and 50 mM dextrose, pH 7.0) with 5 μg/mL PGI_2. The platelet pellet, which was washed twice, was then resuspended in less than 1 mL of albumin-free, calcium-free Tyrode's buffer containing 5 μg/mL PGI_2. Gel-filtered platelets were obtained from the application of washed platelets to a Sepharose CL-2B column equilibrated with albumin-free, calcium-free Tyrode's buffer. Platelets for light scattering experiments at a concentration of 10^7 were suspended in standard ELS buffer containing 50 mM NaCl, 225 mM sucrose, and 2 mM HEPES, pH 7.4 (Ware, 1974). The calcium concentration refers to calcium ion added to the system and does not include calcium contributed by the platelet itself. We estimate the calcium contributed by activated platelets to be <2 mM. Reopro (Centocor BV, Leiden, The Netherlands), a monoclonal antibody (c7E3) against human platelet glycoprotein IIb/IIIa (gpIIb/IIIa), was used to inhibit vWF binding to gpIIb/IIIa (Coller et al., 1991). The calcium concentration is reported as added calcium and does not include calcium contributed from the platelet. An unstirred system was used throughout to avoid platelet aggregation.

Platelet activation was carried out with one of the following agonists: 20 μM adenosine diphosphate (ADP) (Sigma Chemical Co.), 0.2 NIH unit/mL human α-thrombin (Sigma Chemical Co.), 10 μM epinephrine (Sigma Chemical Co.), or 10 μg/mL collagen (Chronolog, Havertown, PA).

Proteins. Purified recombinant factor VIII preparation containing no vWF was obtained from Bayer Laboratories Inc., Elkhart, IN. Purified recombinant factor VIII preparation containing about 2 ng of vWF/IU of FVIII was obtained from Baxter Healthcare Corp., Glendale, CA. FVIII−vWF complex purified from human plasma was obtained from Alpha Therapeutics, Los Angles, CA. Recombinant B-domainless FVIII was a gift from Novo Nordisk, Copenhagen, Denmark. vWF was prepared from plasma by gel filtration on Sepharose CL-6B in 20 mM HEPES, pH 7.4, 150 mM NaCl.

Electrophoretic Quasi-Elastic Light Scattering (ELS). ELS can measure the electrophoretic mobility and the diffusion coefficient and assess the heterogeneity of suspended platelets and the effect of ligand binding on the electrokinetics of platelets. ELS measurements were made on a multiangle quasi-elastic light scattering spectrometer (DELSA 440, Coulter Electronics, Inc., Hialeah, FL). Measurements were made simultaneously at four different scattering angles. The electrophoretic effect was obtained by superimposing a uniform electric field (usually 150−500 V/cm) across the sample. The field was pulsed and its polarity alternated to avoid mass accumulation. The scattered intensity (I_s) from a moving particle at a fixed angle (θ_s) is observed as an oscillating intensity described in the heterodyne experiment as a second-order field autocorrelation function (Ware, 1974; Johnson & Gabriel, 1981, 1994):

$$G^2_{\mathrm{Lhet}}(\tau) = I_L^2 + 2I_L\langle I_s\rangle \cos(K\nu_d)e^{-DK^2\tau} \quad (1)$$

where I_L is the intensity of the reference beam (local oscillator) and K is the scattering vector defined by

$$K = \frac{4\pi n}{\lambda}\sin\left(\frac{\Theta_s}{2}\right) \quad (2)$$

where n is the refractive index, λ is the wavelength of the incident light, ν_d is the velocity of the scattered particle, D is the diffusion coefficient, and τ is the time increment. The important quantity in eq 1 is $K\nu_d$, the Doppler shift of the signal resulting from particle motion. The Fourier transform of the measured autocorrelation function gives the power spectrum from which the particle electrophoretic mobilities are calculated (Johnson & Gabriel, 1981, 1994). Temperature, ionic strength, pH, and conductivity were carefully controlled and monitored by measuring the conductivity of each sample throughout the light scattering experiment. Joule heating was governed by regulation of the pulse duration and the frequency of the electric field. Thermal lensing was avoided by controlling the incident laser power. Electroosmosis was minimized by coating the scattering cell first with (γ-glycidoxypropyl)trimethoxysilane, drying at 70 °C, coating with methylcellulose, and drying again at 70 °C. Lack of an electroosmosis artifact was verified by a flat electrophoretic profile across the scattering volume. Snell's law correction was made for all scattering angles.

ELS Measurements Made in the Debye Limit. Charged particles in solution orient oppositely charged counterions about their surface so that the electrical potential of the particle's surface decreases with the distance from the particle surface (Figure 1). This layer of counterions, the electrical double layer, has a thickness predicted by the Debye−Hückel theory, and for platelets is estimated to be 8 Å (Jung et al., 1982; Pthica, 1961). The double layer, which is determined by the platelet surface potential, can be subdivided into smaller layers shown in Figure 1. The electrophoretic mobility for particles the size of blood cells, where the ratio of the particle diameter to the Debye screening length is greater than 30, assuming a platelet diameter of at least 10 000 Å, is governed by the magnitude of the surface charge density and not by frictional factors (Smoluchowski, 1921; Ware, 1974; Uzgiris et al., 1979)

Binding of Proteins to Platelets. Three different preparations of FVIII were used throughout these experiments and include the following: a plasma-derived FVIII in complex with vWF, a recombinant FVIII with 2 ng of vWF/IU of FVIII, and a recombinant FVIII free of vWF. In this manner, the effect of vWF as well as the effect of an intact Arg^{1689} bond on FVIII binding could be assessed. Optimal FVIII activation time was determined by monitoring FVIII activity for 20 min after 0.2 NIH unit/mL human α-thrombin was added. The maximal FVIII activity was reached within 5−10 min after the addition of thrombin, then decreased after 10 min, and finally became inactivated after 15 min. Activated platelets were prepared by incubation at 25 or 37 °C with 0.2 NIH unit/mL human α-thrombin for 5 min followed by the addition of 2.5 μM phenylalanylprolylarginine chloromethyl ketone (PPACK; Calbiochem-Novabiochem, La Jolla, CA) to inactivate thrombin. When agonists other than thrombin were used, activated platelets were prepared by incubation with 20 μM ADP, 10 μM epinephrine, 10 μg/

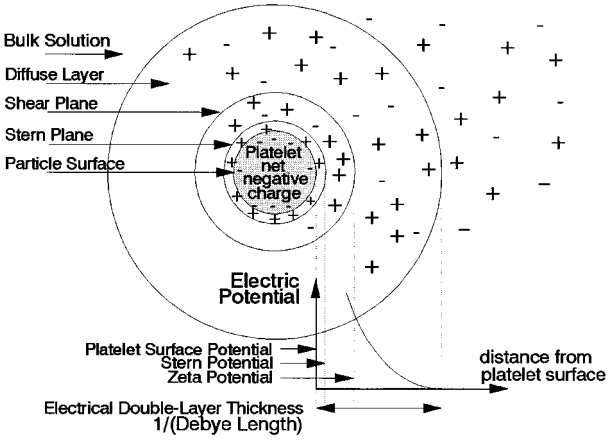

FIGURE 1: Definition of the surface potential layer for a charged particle. Counterions from the solvent are organized tightly in a layer surrounding a charged particle. The thickness of this tightly organized layer, the stern layer, depends on the surface charge density of the particle. The influence of the surface charge of the particle on the solution counterions decreases with the distance from the particle surface. At a distance defined by Debye−Huckle theory, the particle no longer has an influence on the solution counterions. The distance from this point to the particle surface is called the Debye−Huckle length or the electrical double layer. At some intermediate point between the Stern layer and the electrical double layer, the Coulombic attraction is insufficient to maintain the counterion cloud surrounding the charged particle for a mobile particle. The electrical potential at this shear plane is called the zeta potential. The magnitude of these electrical potentials is not to scale in this figure. As shown by Smoluchowski, Ware, and Uzgiris, the electrophoretic mobility of particles the size of blood cells depends on the surface charge density and not on frictional forces.

mL collagen, or 50 μg/mL SFLLRN peptide, which specifically activates the thrombin receptor (Coughlin et al., 1992; Kinlough-Rathbone et al., 1995), for 10 min followed by addition of FVIII containing no vWF. The final solution was incubated for 5 min before ELS measurement of mobility spectra. The binding of activated platelets with $FVIII_a$ was performed by activating both FVIII and platelets by the addition of 0.2 NIH unit/mL human α-thrombin for 5 min, followed by the addition of 2.5 μM PPACK just prior to ELS measurement. The FVIII−vWF complex was prepared by mixing the two proteins for 10 min before addition to platelets. Factor VIII and the FVIII−vWF complex and the solutions of $FVIII_a$ and vWF were then incubated with thrombin-activated platelets for 5 min before ELS measurement. Mechanical shear to the platelet preparations is minimized by using an unstirred system to avoid aggregation. Experiments were conducted at 25 and 37 °C. The binding constant (K_d) of proteins to platelets was determined by fitting the data from the binding experiments to

$$\mu = \mu_0 + \Delta\mu \frac{[\text{protein}]}{K_d + [\text{protein}]} \qquad (3)$$

where μ is the electrophoretic mobility, μ_0 is the mobility in the absence of added protein, and $\Delta\mu$ is the calculated maximal change in mobility at the saturating concentration of protein. This model assumes one class of platelet binding sites for the specific protein ligand.

RESULTS

Activation of the platelet results in a decrease in the surface charge density (Coller, 1978; Seamen &Vassar, 1966; Gabriel et al., 1993). When a ligand binds to the activated platelet surface, the platelet surface charge density further changes, which in turn changes the electrophoretic mobility of the platelet. The effect of ligand binding can cause either a decrease or an increase in the platelet mobility, depending on the ligand. Ligand binding can be prevented by inhibition of platelet activation by aspirin or prostacyclin (Gabriel et al., 1993; Hawiger et al., 1980). Accuracy is shown by the normal K_d's for fibrinogen binding, $K_d = 0.09$ μM (Figure 4C, filled circles), and for FIX_a binding, $K_d = 1.9$ nM (data not shown), and by agreement with the K_d published for $FVIII_a$ binding to platelets. In the typical experiment, the change in the electrophoretic mobility caused by ligand binding is shown in the ordinant, and the dependence of the change in platelet mobility on the ligand concentration is shown in the abscissa. The relationship between these variables permits calculation of binding constants. It is not the magnitude of the change in the electrophoretic mobility that determines the value of the binding constant but how the mobility depends on the ligand concentration. The presence of other ligands or proteins does not interfere with the assessment of a specific ligand unless there is competition for the same binding site.

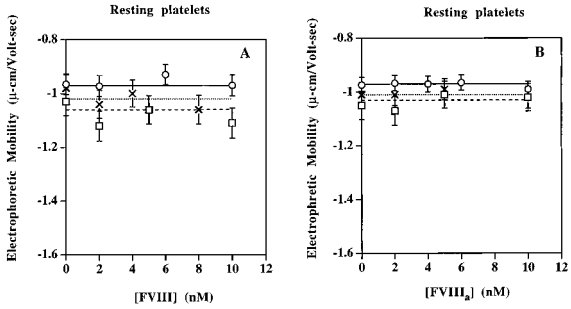

FIGURE 2: Interaction between FVIII and resting platelets. Panel A shows the interaction between resting platelets and FVIII derived from three different sources: plasma-derived FVIII with vWF (open squares), recombinant FVIII expressed with trace vWF (open circles), and recombinant FVIII expressed in the absence of vWF (×'s). Panel A shows no effect of unactivated FVIII on resting platelets. Panel B also shows that resting platelets do not bind thrombin-activated FVIII. Excess thrombin is inhibited by PPACK. Resting platelets do not bind either activated or unactivated FVIII.

The state of activation of the platelet on binding of FVIII was investigated in the next two figures. In Figure 2A, unactivated FVIII containing vWF purified from plasma (open squares), unactivated recombinant FVIII expressed in the presence of vWF (open circles), or unactivated recombinant FVIII expressed in the absence of vWF (×'s) are incubated with resting human platelets. No change in the platelet mobility is observed as the concentration of FVIII is increased from 0 to 10 nM, which indicates no binding of unactivated FVIII to resting platelets. In Figure 2B, the same experimental conditions are present, except that each FVIII preparation has been activated by 0.2 NIH unit/mL of human α-thrombin. Following FVIII activation, the thrombin activity was inhibited by an excess of PPACK prior to addition of $FVIII_a$ to the resting platelets. Again no change in the platelet mobility is observed as the concentration of $FVIII_a$ is increased form 0 to 10 nM, indicating no binding of activated FVIII to the resting platelets.

In Figure 3, the conditions are the same as in Figure 2 except that platelets were activated by 0.2 NIH unit/mL human α-thrombin. Excess thrombin was completely neu-

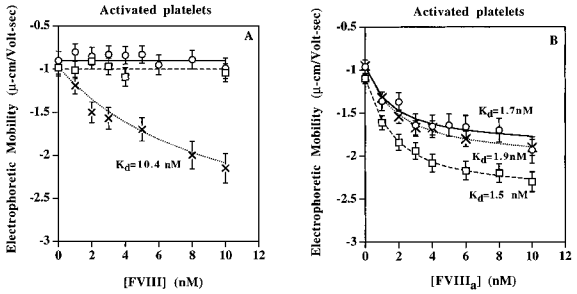

FIGURE 3: Interaction between FVIII and activated platelets. The same experimental conditions as Figure 2, except that the platelets were activated with 0.2 NIH unit/mL human α-thrombin. Excess thrombin used in the platelet activation is inhibited with PPACK. In panel A, unactivated FVIII expressed in the absence of vWF (×'s) is shown to bind to activated platelets as evidenced by the change in the platelet electrophoretic mobility. The change in mobility shows binding. The dependence of the change in mobility on the ligand concentration defines the binding constant. Plasma-derived FVIII containing vWF (open squares) and recombinant FVIII expressed with trace vWF (open circles) do not bind to thrombin-activated platelets. In panel B, thrombin-activated FVIII from all three sources is shown to bind to activated platelets and exhibit a similar K_d.

tralized by PPACK. In Figure 3A, the unactivated FVIII−vWF complex purified from plasma (open squares) and the unactivated recombinant FVIII expressed with vWF (open circles) show no binding to activated platelets. However, in experiments using unactivated recombinant FVIII lacking vWF (×'s), an increase in the platelet mobility from −1.0 (μ-cm)/(V-s) to −2.2 (μ-cm)/(V-s) is observed as the concentration of FVIII is increased from 0 to 10 nM, showing that unactivated FVIII in the absence of vWF is bound to activated platelets with a K_d of 10.4 nM.

In Figure 3B, each FVIII preparation was activated with thrombin and added to the thrombin-activated platelets. As the concentration of activated FVIII (FVIII$_a$) was increased from 0 to 10 nM, the platelet mobility increased, showing binding of FVIII$_a$ to activated platelets. The break in each binding curve occurs at the same concentration of FVIII, demonstrating that activated FVIII for each preparation binds to platelets with nearly the same K_d of 1.7 nM. The new information from these experiments is that in the absence of vWF unactivated FVIII binds to activated platelets with a K_d of 10.4 nM. When 10 μg/mL vWF is added to unactivated recombinant FVIII expressed in the absence of vWF, no FVIII binding occurs, but with activation of FVIII, binding does occur (data not shown).

In Figure 4A, the concentration range of FVIII lacking vWF has been expanded so that a full binding curve with saturation of binding sites could be demonstrated. Albumin was used to block nonspecific binding. As the concentration of unactivated FVIII containing no vWF is increased (Figure 4A, ×'s) from 0 to 50 nM, the platelet mobility increases from −1.0 (μ-cm)/(V-s) to −2.7 (μ-cm)/(V-s). In view of the fact that platelets may release vWF from their α-granules, the possible role of platelet vWF in FVIII binding was determined. Similar studies in which activated platelets were first saturated with a polyclonal anti-vWF antibody followed by addition of unactivated FVIII (filled diamonds) serve as an important control. Addition of the antibody to the platelet decreased the platelet mobility, but did not change the binding constant significantly, K_d = 9.4 nM. As second proof that platelet vWF does not influence our results, we

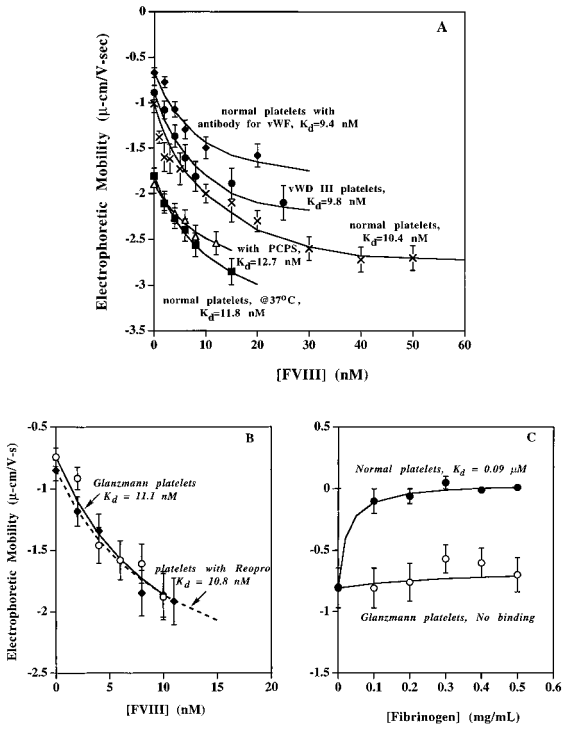

FIGURE 4: Complete binding curve for unactivated FVIII to activated platelets. Figure 4A shows the electrophoretic mobility of platelets obtained from four different donors. Error bars represent the small variation in platelets from different donors. A K_d of 10.4 nM is estimated for unactivated FVIII to activated platelet in the absence of vWF (×'s). No effect of inhibition of vWF was observed when platelets treated with a polyclonal antibody to vWF were exposed to FVIII lacking vWF (closed diamonds). Platelets from patients with type III von Willebrand disease (closed circles) show a similar binding curve for unactivated FVIII. FVIII binding curve at 37 °C is shown by closed squares. Phospholipid vesicles composed of phosphatidylcholine and phosphatidylserine (72:28, 10 μM) show no ability to remove FVIII from activated platelets (open triangles). Figure 4B shows that the absence of glycoprotein IIb/IIIa either in platelets from a patient with Glanzmann's Thrombastheina (open circles), K_d = 11.1 nM, or in activated platelets whose glycoprotein IIb/IIIa has been blocked by Reopro (filled diamonds), K_d = 10.8 nM, still bind FVIII with a K_d similar to normal platelets. Figure 4C provides an additional control and shows that the same Glanzmann's platelets (open circles) activated with 20 μM ADP do not bind fibrinogen, but normal platelets (filled circles) activated with 20 μM ADP do bind fibrinogen, K_d = 0.09 μM. Note that fibrinogen decreases the platelet mobility rather than increases the mobility as seen with FVIII.

repeated the binding experiments using type III von Willebrand disease (vWD) platelets lacking vWF (filled circles), which gave a K_d of 9.84 nM similar to the K_d for FVIII binding to normal activated platelets. The binding curve was repeated at 37 °C, which shows slightly weaker binding, K_d = 11.8 nM (filled squares). The conclusions from these experiments are that FVIII will bind to thrombin-activated platelets, that the binding is saturable, that the binding constant is temperature-dependent, and that platelet vWF does not mediate FVIII binding. The ability of phospholipid vesicles composed of phosphatidylcholine and phosphatidylserine (72:28) to remove unactivated FVIII from activated platelets at 37 °C was investigated. When a 10 μM aliquot of lipid vesicles is added with the addition of unactivated FVIII, no change in the binding curve is observed (K_d = 12.7 nM, open triangles).

The possible binding of the intact vWF−FVIII complex by the activated glycoprotein IIb/IIIa as an explanation of

Table 1: Differences in the Ability of Specific Platelet Agonists to Activate FVIII Binding

agonist	K_d (nM)
10 μM ADP	no binding
10 μg/mL collagen	no binding
10 μM epinephrine	11.6 ± 3.5
0.2 unit/mL thrombin	10.1 ± 2.8
50 μg/mL SFLLRN	7.1 ± 3.9

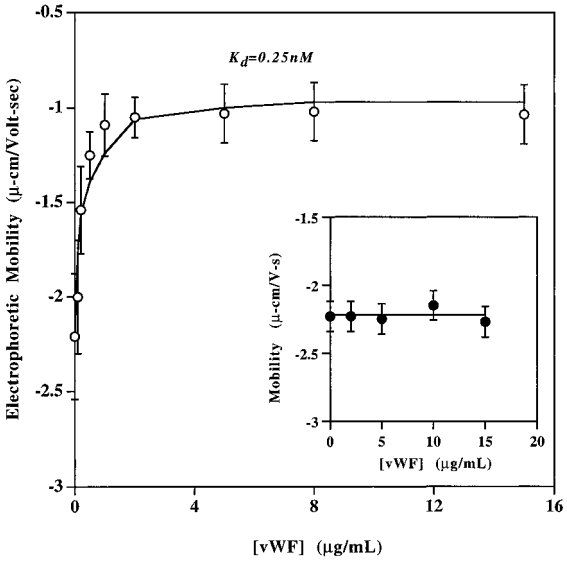

FIGURE 5: Determination of the distribution of unactivated FVIII between vWF and activated platelets. In this experiment, activated platelets are first loaded with 10 nM unactivated FVIII (open circles). As vWF is added to platelet-bound FVIII, the platelet electrophoretic mobility decreases, indicating displacement of FVIII from the platelet surface. In the control experiment, vWF is first treated with a polyclonal antibody to block FVIII binding to vWF, before addition of vWF to the FVIII-loaded platelets (filled circles). No change in platelet mobility is observed, indicating no FVIII is displaced from the activated platelet surface.

our results is shown in Figure 4B where two additional control experiments are shown. The first experiment (filled diamonds) shows binding of FVIII to activated platelets previously saturated with 8 μg/mL Reopro, a RGD peptide that blocks gpIIb/IIIa (Coller, 1985; Coller et al., 1991; The EPIC Investigators, 1994). Under these conditions, we obtain a K_d of 10.8 nM for FVIII binding, which is comparable to FVIII binding in the absence of the gpIIb/IIIa inhibitor. The second control (open circles) repeats the FVIII binding curve using platelets from a patient with Glanzmann's thrombstenia, which lacks gpIIb/IIIa (Nurden & Caen, 1974; Phillips & Poh Agin, 1977). The K_d of 11.1 nM for FVIII binding to Glanzmann's platelets is also close to that for normal platelets. In Figure 4C as a further proof of the validity of these measurements, we also show that fibrinogen does not bind to the Glanzmann's platelets (open circles), but does bind to normal platelets (filled circles) with the expected K_d of 0.09 μM (Bennett & Vilaire, 1979).

Table 1 shows that platelets activated by thrombin, epinephrine, or SFLLRN bind unactivated FVIII with no vWF. Platelets activated by ADP (at 25 or 37 °C) or type I collagen do not bind unactivated FVIII.

Figure 5 shows experiments in which unactivated FVIII was first added to saturate thrombin-activated platelets, followed by the addition of increasing amounts of vWF. Conditions were chosen that were unfavorable for vWF binding to platelets, i.e., 4 mM calcium ion (data not shown) (Moore et al., 1989). These experiments show that because of the higher affinity of unactivated FVIII for vWF (K_d = 0.2 nM) than for activated platelets (K_d = 10.4 nM), vWF can dissociate unactivated FVIII from the platelet surface. Displacement of unactivated FVIII from the platelet is shown as a decrease in the platelet electrophoretic mobility toward that for the unliganded, activated platelet. As the concentration of vWF is increased from 0 to 15 μg/mL, the platelet mobility is seen to decrease from −2.3 (μ-cm)/(V-s) to −1.0 (μ-cm)/(V-s), indicating displacement of FVIII from the platelet surface. In a control experiment, vWF was blocked with an anti-vWF polyclonal antibody prior to its addition to activated platelets saturated with FVIII (see insert to Figure 5). In this case, the antibody-blocked vWF does not modify the mobility of the platelet-bound FVIII, indicating that FVIII did not dissociate from the platelet surface. These experiments also permit the calculation of the binding constant of vWF for FVIII. A K_d of 0.25 nM for vWF binding to FVIII is observed and is consistent with previous reports (Lollar & Parker, 1987; Volt et al., 1995). In other control experiments, we demonstrate that vWF does not remove FIX from platelets as evidenced by no change in FIX−platelet complex mobility as the concentration of vWF is increased (data not shown).

Because of the large difference in charge between unactivated and activated FVIII, the mobility of FVIII-bound platelets is different, depending on the activation state of FVIII, as shown in Figure 3A; i.e., the mobility of unactivated FVIII−platelet complex is −2.2 (μ-cm)/(V-s) compared to −1.7 (μ-cm)/(V-s) for activated $FVIII_a$−platelet complex at 10 nM factor VIII (Figure 3B). This difference in mobility permits direct observation of the thrombin activation of platelet-bound FVIII as a change in the electrophoretic mobility of the platelet. Figure 6 shows the effect of the thrombin concentration on the activation of FVIII on the platelet surface. At time zero, 10 nM unactivated FVIII is added to platelets, which shows the base line electrophoretic mobility of −2.7 (μ-cm)/(V-s) for platelets saturated with FVIII. After addition of thrombin, activation of FVIII proceeds with loss of the highly charged B-domain, and the electrophoretic mobility of FVIII−platelet complex decreases to reach a terminal mobility of −1.7 (μ-cm)/(V-s). This indicates activation of FVIII on the platelet surface. The control experiment in which no FVIII is added, but thrombin is added (open triangles), shows no change in mobility for the normal mobility of activated platelets, indicating that the observed change in mobility was not from further platelet activation by the second addition of thrombin. In a second control experiment (open squares), the platelets are saturated with unactivated FVIII, but no additional thrombin is added, which shows that FVIII addition alone does not explain the observation. Figure 6 also shows that the rate of activation of FVIII on the platelet surface is dependent on the thrombin concentration. As the thrombin concentration is increased from 0.002 to 0.01 NIH unit/mL, the first-order rate constant increases from 0.006 to 0.04 s^{-1}.

To investigate the influence of FVIII's B-domain on binding, recombinant unactivated B-domainless FVIII was studied. As expected (Figure 7), activated platelets complexed to unactivated B-domainless FVIII (open circles) have a lower electrophoretic mobility reflecting the lower charge and tighter binding, K_d = 5.1 nM. When the B-domainless

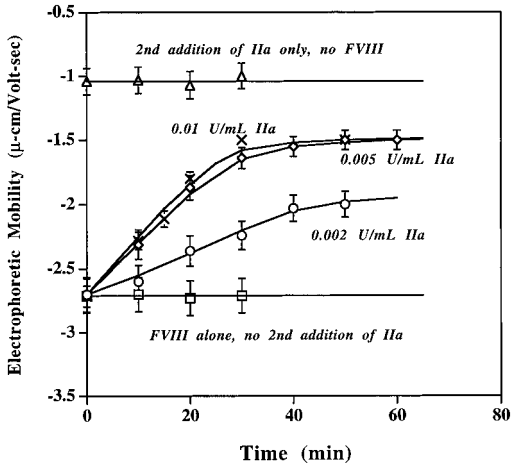

FIGURE 6: Activation of FVIII on the activated platelet surface as a function of time and thrombin concentration. Activated platelets were first saturated with unactivated FVIII at 10 nM, which causes the typical increase in the platelet mobility from −1 to −2.7 (μ-cm)/(V-s). Addition of thrombin (×, 0.01; open diamonds, 0.005; and open circles, 0.002 NIH unit/mL) cleaves the FVIII B-domain, resulting in a decrease in the charge contribution from FVIII, which results in a decrease in the platelet mobility. As the thrombin concentration is increased from 0.002 NIH unit/mL (open circles) to 0.01 NIH unit/mL (×'s), the first-order rate constant increases from 0.006 to 0.04 s^{-1}. Controls include the omission of thrombin to the FVIII-saturated platelets (open squares) which show that thrombin is required for activation of FVIII, and the second addition of thrombin to activated platelets lacking FVIII (open triangles), which shows that FVIII is required for the effect. The further decrease in platelet mobility is the result of FVIII activation, not further platelet activation.

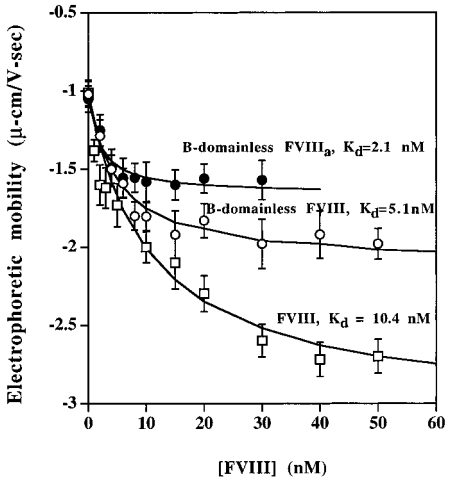

FIGURE 7: Effect of the B-domain on the binding constant of FVIII for activated platelets. Multiple peptide cleavages occur during FVIII activation, which produces tighter binding of FVIII to platelets. Unactivated FVIII has the highest charge and the lowest binding constant (open squares). Recombinant B-domainless FVIII has a lower charge and a 2-fold tighter binding (open circles). Thrombin-activated B-domainless FVIII cleaved at Arg^{372} and Arg^{741} further decreases the mobility and increases the binding affinity (filled circles). Thus, the B-domain of FVIII appears to affect FVIII binding affinity, which requires at least three different binding steps, each with a different binding constant.

FVIII is activated with thrombin and added to activated platelets, the mobility is lower yet and the binding even tighter, K_d = 2.1 nM, similar to activated native FVIII.

DISCUSSION

We have used ELS to study the effect of the state of activation of FVIII on the physical exchange of FVIII between vWF and activated platelets and the effect of the FVIII B-domain on platelet binding. ELS is highly useful for these types of experiments, since heterogeneity in the mobility and therefore in the binding affinity of a large population of platelets can be rapidly monitored. Our results show that neither the line width of the scattering envelope changes nor side bands develop as the mobility of the activated platelets increases during addition of FVIII, suggesting that the binding is homogeneous and distributed throughout the entire platelet population.

The first event of the tenase complex assembly (Hemker & Kahn, 1967; Monroe et al., 1994) is FVIII binding to platelets which requires delivery of FVIII to the platelet surface by vWF. Thus, details of the interaction between vWF, FVIII, and platelets are central to understanding the regulation of the tenase complex assembly. The hypothesis developed here is focused on how the state of activation of FVIII affects the partitioning of FVIII between its transport protein, vWF, and activated platelets. The distribution of FVIII is governed by the binding constant of FVIII for these two targets. In plasma-unactivated FVIII, binding favors vWF as seen by the difference in the K_d of binding to vWF (0.25 nM) compared to 10.4 nM for activated platelets. In the special case of FVIII concentrate infusion, and especially if high molecular weight multimers of vWF are absent, the bioavailability of FVIII may be altered. At this point, the weakly bound unactivated FVIII on the platelet surface may be dissociated from the platelet surface by circulating vWF (Figure 5). Neither unactivated FVIII nor activated FVIII binds to resting platelets. When vWF binds to the platelet, whether through gpIb/IX or gpIIb/IIIa, the FVIII is brought to the platelet surface. Trace concentrations of thrombin bound to the platelet surface, generated by tissue factor and FVIIa (Nemerson, 1988; Rapport & Rao, 1995), which are not present on the platelet, may then clip Arg^{1689} in FVIII (Ruggeri et al., 1983; Meyer & Girma, 1993; Fay, 1993). Arg^{1689} cleavage is critical because, as also shown in this report, the K_d of vWF for unactivated FVIII is approximately 50-fold tighter than for the activated platelet. Thus, the equilibrium of FVIII binding is immediately shifted in favor of the platelet. Since cleavage of Arg^{1689} abrogates FVIII binding to vWF, it would appear highly unlikely that vWF is the platelet receptor for FVIII as also shown in Figure 4. Loss of the FVIII B-domain by further FVIII activation reduces the binding constant for the FVIII−platelet interaction by half (from 10.4 nM to 5.1 nM, Figure 7), perhaps by decreasing the Coulombic repulsion between the negatively charged B-domain and the negatively charged platelet surface. Further activation of FVIII leads to tighter binding of FVIII as seen in the K_d of 2.1 nM (Figure 7). These results establish that $FVIII_a$ binding to activated platelets proceeds through a multistep process.

In this report using unactivated FVIII lacking vWF, unactivated FVIII is shown to bind to activated platelets with a K_d of 10.4 nM, approximately 5-fold higher than for $FVIII_a$. Saturation of activated platelets with albumin or fibrinogen has no effect on FVIII binding. Activated platelets coated with polyclonal anti-vWF antibodies bind FVIII normally with a K_d of 9.4 nM. Platelets from type III vWD, which lack platelet vWF, also bind FVIII with a K_d of 9.8 nM. Activated platelets from a patient with Glanzmann's thrombasthenia bound FVIII, but not fibrinogen, as expected.

Activated platelets inhibited with a monoclonal antibody against gpIIb/IIIa also bound FVIII. Thus, platelet vWF in normal platelets may be present in too low a concentration on the platelet surface (<1 ng of vWF/IU of FVIII under our experimental conditions) to be significant relative to the amount of FVIII bound by other sites on the platelet surface. Platelets saturated with $FVIII_a$ also bind unactivated FVIII with a $K_d = 9.8$ nM (data not shown). Thus, platelet vWF does not influence FVIII binding, $FVIII_a$ does not compete with FVIII for platelet binding, and gpIIb/IIIa does not influence FVIII binding.

Unactivated FVIII binds to vWF with a K_d of 0.2 nM (Lollar & Parker, 1987; Leyte et al., 1989; Volt et al., 1995), 50-fold higher than unactivated FVIII for activated platelets. Nesheim et al. have shown that activated FVIII binds to activated platelets with a K_d of 2.9 nM (Nesheim et al., 1988). In the absence of vWF, we show thrombin activation of FVIII changes the FVIII K_d for platelet binding from 10.4 nM to 1.7 nM. Complete activation of FVIII is required to optimally expose tight binding sites on FVIII. The use of a recombinant FVIII lacking the B-domain, but with the remainder of the molecule intact, binds tighter than native FVIII, K_d of 10.4 nM versus 5.1 nM. Further activation of the B-domainless FVIII with thrombin induces tighter binding ($K_d = 2.1$ nM), in good agreement with that obtained from native thrombin-activated FVIII, $K_d = 1.7$ nM. Because of the large difference in Coulombic charge between FVIII and $FVIII_a$, we can monitor the surface activation of FVIII by thrombin using ELS (Figure 3). The results of these experiments show that the binding of platelet-bound FVIII becomes tighter with each FVIII activation step largely because of the loss of the B-domain, and is consistent with the multistate binding of FVIII (Bardelle et al., 1993).

In addition, we have shown that the FVIII binding is different for different platelet agonists. For example, activation by 0.2 NIH unit/mL human α-thrombin, 10 μM epinephrine, and 50 μg/mL SFLLRN is associated with FVIII binding. However, no FVIII binding occurs when platelets are activated with adenosine diphosphate (ADP) at 20 μM at 25 or 37 °C or collagen at 10 μg/mL. The importance of the difference in the ability of a different agonist to activate the FVIII binding is not known, but may reflect the need for mechanical shear with some agonists.

We have confirmed that vWF will inhibit binding of FVIII to activated platelets, but have also made the new observation that vWF displaces unactivated FVIII from the platelet surface because of the large differences in the binding constants between the FVIII−vWF complex ($K_d = 0.25$ nM) and the FVIII−platelet complex ($K_d = 10.4$ nM, Figure 7). No effect is seen when vWF is added to platelets saturated with activated $FVIII_a$, which serves as an important control. vWF binds to activated platelets at a calcium ion concentration of 25 μM, but not when no additional calcium is added or at physiologic calcium (>2 mM) (Ruggeri et al., 1982, 1983; Moore et al., 1989). These experiments emphasize the importance of studying the tertiary system including vWF, FVIII, and activated platelets, so that the influence of each component can be assessed.

In summary, the physical exchange of FVIII between vWF and activated platelets and the effect of the FVIII B-domain on FVIII binding to activated platelets were studied. ELS provides a simple, rapid, and effective method to study interactions between vWF, FVIII, and the platelet surface in real time and under native conditions. Unactivated FVIII is shown to bind to activated platelets with a K_d of 10.4 nM, and vWF can displace unactivated FVIII from the platelet surface. The predominant binding site for FVIII does not appear to be platelet vWF or to be facilitated by activated gpIIb/IIIa. Thrombin-, epinephrine-, and SFLLRN-activated platelets will bind FVIII in contrast to ADP- or collagen-activated platelets that do not bind FVIII. The binding of FVIII to activated platelets is highly consistent with a multistep binding process that is influenced by the FVIII B-domain, the state of activation of FVIII, and the presence of vWF. Finally, we have shown that FVIII activation on the platelet surface can be monitored in real time using ELS and is dependent on thrombin concentration.

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