Functional Modulation of the Isolated Glycoprotein Ib Binding Domain of von Willebrand Factor Expressed in *Escherichia coli*†

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ABSTRACT: We have expressed in *Escherichia coli* the domain of von Willebrand factor (vWF) containing the binding site for platelet glycoprotein (GP) Ib and used it to study the regulation of vWF–platelet interaction. The recombinant fragment, comprising residues 445–733 of the mature vWF subunit and designated rvWF$^{445-733}$, did not have the native conformation of the corresponding domain in the intact molecule because, in order to prevent formation of random aggregates, the seven cysteine residues in the sequence were reduced and alkylated. Unlike native vWF, rvWF$^{445-733}$ bound to GP Ib in the absence of any modulator, suggesting that the lack of disulfide bonds and/or carbohydrate side chains within this domain may expose platelet interaction sites. In the presence of two modulators, the glycopeptide ristocetin and the snake protein botrocetin, rvWF$^{445-733}$ inhibited native vWF binding to GP Ib as well as platelet aggregation mediated by vWF, suggesting that both the fragment and the native molecule interact with the same site on platelets. This conclusion was also supported by the observation that the recombinant fragment competed with the binding to platelets of an anti-GP Ib monoclonal antibody known to inhibit vWF binding. Botrocetin formed a complex with rvWF$^{445-733}$, but the affinity of this interaction was approximately 25-fold lower than with native vWF. However, the complexes of botrocetin with either rvWF$^{445-733}$ or multimeric native vWF bound to GP Ib with similar dissociation constant. Therefore, conformational attributes of vWF regulate its affinity for botrocetin, but once the complex is formed, interaction with GP Ib is independent of native vWF conformation. These findings provide insights into the regulation of vWF–platelet interaction.

The interaction of von Willebrand factor (vWF)[1] with platelets is part of the physiologic mechanism leading to the arrest of bleeding from damaged vessels (Ruggeri & Zimmerman, 1987). Moreover, in pathological conditions, vWF may contribute to the processes that cause thrombotic vascular occlusion (Fuster et al., 1978; Nichols et al., 1986; Bellinger et al., 1987; Weiss et al., 1989). Several structural and functional characteristics of the vWF molecule have been partly clarified, including the location of the domain responsible for interacting with the platelet receptor, glycoprotein (GP) Ib–IX complex (Fujimura et al., 1986; Mohri et al., 1988, 1989). The binding of vWF to GP Ib is considered essential for normal platelet adhesion to damaged vascular walls (Weiss et al., 1978, 1986; Stel et al., 1985) and for platelet aggregation under flow conditions of high shear stress (Peterson et al., 1987).

Understanding the mechanisms that regulate vWF interaction with GP Ib is of crucial biological importance but has been hampered by the fact that measurable platelet binding of soluble vWF does not occur under normal experimental conditions unless nonphysiologic modulators such as ristocetin (Howard & Firkin, 1971; Kao et al., 1979; Ruggeri et al., 1983) or botrocetin (Read et al., 1978, 1989; Andrews et al., 1989a) are used. In order to gain insights into these problems, we have expressed in *Escherichia coli* a single-chain polypeptide corresponding to the region of vWF containing the GP Ib binding domain (Fujimura et al., 1986), and we have analyzed the characteristics of its interaction with platelets. The results obtained demonstrate that this recombinant fragment interacts with platelet GP Ib at the same site involved in binding of the native molecule. In contrast to native vWF, however, the binding of rvWF$^{445-733}$ occurs even in the absence of exogenous modulators. Nevertheless, the fragment forms a complex with botrocetin that then binds specifically to GP Ib with affinity similar to that of the corresponding complex of native vWF, and greater than that observed with the fragment in the absence of botrocetin. These findings suggest that conformational transitions possibly induced by interaction with specific molecules may regulate vWF affinity for its platelet receptor.

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[1] Abbreviations: vWF, von Willebrand factor; GP, platelet membrane glycoprotein; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; TEAP, triethylammonium phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepes buffer, buffer composed of 20 mM HEPES and 150 mM NaCl, pH 7.4; BSA, bovine serum albumin; PBS/NP-40 buffer, buffer composed of 40 mM phosphate buffer, pH 7.4, 150 mM NaCl, and 0.1% Nonidet P-40; IC_{50}, concentration of a substance necessary to inhibit the specific binding of a given ligand by 50%.

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Experimental Procedures

Expression and Purification of the Recombinant vWF Fragment. A cDNA clone encoding a portion of the amino acid sequence of vWF, cloned in the vector pGEM2, was provided by Dr. Dennis Lynch (Dana-Farber Cancer Institute, Boston, MA). The expression plasmid pET-8C (Km^R) and the *E. coli* host BL21 (DE3) were provided by Dr. F. William Studier, Brookhaven National Laboratory, Upton, NY. The nucleotide sequence of the plasmid demonstrated that an 857 bp *Mst*II/*Nco*I fragment encoded amino acid residues 445–733 of the mature vWF subunit, essentially corresponding to the tryptic 52/48-kDa fragment comprising amino acid residues 449–728 (Fujimura et al., 1986, 1987) and containing the GP Ib binding domain. This fragment was modified at both the 5′ and 3′ ends to provide for translation termination and vector insertion and then inserted into the *Nco*I site of the expression plasmid pET-8c (Km^R) containing the bacteriophage T7 promoter and the kanamycin resistance gene. The resulting vector encoded an open reading frame consisting of the amino-terminal methionine followed by residues 445–733 of the mature vWF subunit. The expression plasmid was used to transform *E. coli* BL21 (DE3) cells, a λ lysogen of BL21 in which the prophage carries a copy of the gene for T7 RNA polymerase under the control of the lac UV5 promoter (Studier & Moffatt, 1986). A single isolate from this transformation was then used for expression of the vWF fragment.

To direct expression of the recombinant fragment, overnight cultures were diluted 1:100 and grown to an optical density of 0.4 (absorbance at 595 nm). Cultures were adjusted to 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubated further for various times at 37 °C. Cells were collected, mechanically disrupted in the presence of 0.5 g/L sodium deoxycholate and 0.025% Tween 80, and briefly centrifuged to yield an insoluble fraction enriched for vWF fragment. This fraction was solubilized by addition of 6 M guanidine hydrochloride in 50 mM Tris, pH 8.8, and the vWF fragment was reduced and alkylated by the addition of 10 mM dithiothreitol (DTT) at 37 °C for 1 h under nitrogen, followed by 50 mM iodoacetamide for an additional hour. The sample was supplemented with 10 mM DTT and then dialyzed against a buffer composed of 25 mM Tris, pH 8.0, containing 0.1 mM disodium ethylenediaminetetraacetate (EDTA), 0.1 mM DTT, 20 mM KCl, and 6 M urea (buffer A). The reduced and alkylated fragment was purified by high-performance liquid chromatography (HPLC; Perkin-Elmer) employing the anion-exchanger Q Sepharose Fast Flow (Pharmacia) followed by the cation-exchanger Protein-Pack SP 8HR (Waters). Fractions containing the vWF fragment were pooled, concentrated by ultrafiltration, dialyzed against citrate buffer, and stored at −70 °C at low pH. Purity of the fragment was assessed by polyacrylamide gel electrophoresis (PAGE) in a 4–20% gradient gel in the presence of sodium dodecyl sulfate (SDS), performed according to Laemmli (1970), and by immunoblotting (Burnette, 1981) using two anti-vWF monoclonal antibodies (see below). The fragment was designated $rvWF^{445-733}$.

Monoclonal Antibodies. The monoclonal antibodies used in this study were as follows: anti-vWF LJ-RG46 and LJ-52K2, which react with vWF residues 474–488 and 694–708, respectively, representing the two discontinuous regions of the GP Ib binding domain (Mohri et al., 1988); anti-vWF LJ-2.2.9, which binds to the carboxyl terminus of native vWF and does not affect its binding to platelets; anti-GP Ib LJ-Ib1 and LJ-P3 (Handa et al., 1986); anti-GP IIb-IIIa LJ-CP8 (Niiya et al., 1987). These antibodies were prepared as previously described (Liu et al., 1980; Trapani-Lombardo et al., 1985; Handa et al., 1986; Berliner et al., 1988). IgGs were purified on protein A–Sepharose (Sigma) according to a published procedure (Ey et al., 1978).

Purification of Botrocetin. Crude, lyophilized venom (2 g) from *Bothrops jararaca* (Sigma, lot 119F0599) was dissolved in 50 mL of 50 mM Tris buffer, pH 7.4, and loaded onto a diethylaminoethyl (DEAE)-HPLC column (1 × 25 cm; Perkin Elmer) with a flow rate of 4 mL/min. After the column was extensively washed with Tris buffer, bound proteins were eluted with a linear gradient of 0–1 M NaCl (120-mL total volume). Two major protein peaks were resolved at 0.15 and 0.3 M NaCl, respectively, and botrocetin cofactor activity, i.e., the ability to induce ^{125}I-vWF binding to platelet GP Ib, was detected in the second of the two peaks. Fractions enriched in the activity were pooled, and $(NH_4)_2SO_4$ was added at a final concentration of 1.6 M. The sample was then applied onto a polypropylaspartamide hydrophobic interaction HPLC column (0.46 × 15 cm; The Nest Group) equilibrated with 0.2% triethylammonium phosphate (TEAP), pH 6.8, containing 1.6 M $(NH_4)_2SO_4$. Most of the activity was recovered in the flow-through fraction, whereas most of the fibrinogen clotting activity was bound to the column. The concentration of $(NH_4)_2SO_4$ in the flow-through fraction was increased to 4 M, and the solution was loaded onto a hydroxypropyl hydrophobic interaction HPLC column (0.46 × 15 cm; SynChrom) equilibrated with the same 0.2% TEAP buffer, pH 6.8, containing 4 M $(NH_4)_2SO_4$. The column was washed extensively with the same buffer but containing 2 M $(NH_4)_2SO_4$ and then eluted with a gradient of 2–0 M $(NH_4)_2SO_4$. Two major protein peaks were detected, one corresponding to 1.8 M and the other to 0.7 M $(NH_4)_2SO_4$; neither peak showed fibrinogen clotting activity, but only the second one contained botrocetin cofactor activity. The corresponding fractions were pooled and dialyzed overnight against a buffer composed of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 150 mM NaCl, pH 7.4 (Hepes buffer). Typical yield of the final product was approximately 4 mg. By SDS–PAGE analysis (4–20% gradient), the purified protein expressing botrocetin cofactor activity migrated as a single band of 27 kDa under nonreducing conditions and as a closely spaced doublet of 15–14.8 kDa following reduction of disulfide bonds.

Biotinylation of Botrocetin. Purified botrocetin (100 μg) was dialyzed against 0.1 M $NaHCO_3$ for 18 h at 4 °C. An equal amount (w/w) of NHS-LC-Biotin (Pierce), dissolved in dimethyl sulfoxide, was added to the dialyzed botrocetin solution and the mixture incubated for 2 h in the dark at 22–25 °C. The resulting biotinylated botrocetin was dialyzed against Hepes buffer for 18 h at 4 °C and stored at −70 °C until used.

Radioiodination of Proteins. vWF was purified from cryoprecipitate and characterized as described (Ruggeri et al., 1983). Botrocetin, vWF, and IgG were radiolabeled with $Na^{125}I$ (Amersham) using Iodo-Gen (Pierce) according to the method of Fraker and Speck (1978). The specific activity of the radiolabeled ligands was between 1.2×10^9 and 7.5×10^9 cpm/mg.

Inhibition of vWF and Anti-GP Ib Monoclonal Antibody Binding to Platelets by $rvWF^{445-733}$. To evaluate the effect of $rvWF^{445-733}$ on the ristocetin- or botrocetin-mediated binding of ^{125}I-vWF to GP Ib, washed platelets (Walsh et al., 1977) at a final count of 1×10^8/mL were incubated for 30 min at 22–25 °C with ^{125}I-vWF (2 μg/mL), Hepes buffer or $rvWF^{445-733}$ at various concentrations, and ristocetin (Sigma;

1 mg/mL) or purified botrocetin (0.4 μg/mL); the concentrations of ristocetin and botrocetin used were such as to give maximal binding of vWF to GP Ib. In some experiments, citrated plasma from a patient with severe von Willebrand diseases (vWF antigen levels >0.1% of normal average) was added to the experimental mixtures in a volume corresponding to 52% of the total. To evaluate the effect of the recombinant fragment on the binding of anti-GP Ib monoclonal antibodies to platelets, rvWF$^{445-733}$ at various concentrations was incubated with washed platelets (1×10^8/mL) and ^{125}I-LJ-Ib1 (20 μg/mL) or ^{125}I-LJ-P3 (1 μg/mL) in the presence or absence of botrocetin (5 μg/mL). The concentrations of the two antibodies were chosen to give approximately half-maximal saturation of the corresponding epitopes on the platelet surface, while the concentration of botrocetin was such as to give maximal interaction with the recombinant vWF fragment. In parallel experiments, the effect of purified vWF was compared to that of the recombinant fragment. After incubation, platelets were centrifuged through 20% sucrose to separate bound from free ligand (Ruggeri et al., 1983) and platelet-associated radioactivity was determined in a γ-scintillation counter. Nonspecific binding was estimated in mixtures containing the same reagents as test mixtures, with the addition of a 50-fold excess of unlabeled ligand; the corresponding bound counts (<5% of total bound) were subtracted from all data points. Binding was expressed as a percentage of that measured in the control mixtures containing Hepes buffer instead of rvWF$^{445-733}$.

Platelet Agglutination Studies. The inhibitory effect of rvWF$^{445-733}$ on platelet agglutination induced by ristocetin (1 mg/mL) or botrocetin (1.0 μg/mL) was evaluated in a dual-channel aggregometer (ChronoLog Corp.). Various concentrations of recombinant fragment (or the appropriate volume of buffer in control mixtures) was added to citrated platelet-rich plasma containing 5 mM EDTA and maintained at 37 °C with constant stirring, as described previously (De Marco et al., 1985), followed by either ristocetin or botrocetin. EDTA was used to prevent any aggregation dependent on the function of the GP IIb–IIIa receptor. Platelet agglutination was recorded as an increase in light transmittance.

Measurement of Complex Formation between Botrocetin and vWF. Two different methods were used for this assay. In the first, wells of polystyrene microtiter plates were each coated with 100 μL of a 10 μg/mL IgG solution of the anti-vWF antibody LJ-2.2.9 in 50 mM bicarbonate buffer (pH 9.6) for 2 h at 22–25 °C. This concentration of antibody gave maximum vWF binding to the wells as determined in preliminary experiments. This antibody was chosen because it has no reactivity with the vWF region comprised between residues Val^{449} and Lys^{728} (not shown) and, thus, was assumed to capture vWF complexed to botrocetin as well as free vWF. The coating solution was removed, and the plastic surface was saturated with 200 μL of 1% bovine serum albumin (BSA) for 1 h at 22–25 °C. Wells were either used immediately or stored overnight at 4 °C. The BSA was aspirated off, and wells were washed 3 times with 200 μL of 40 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.1% Nonidet P-40 (PBS/NP-40). For the assay, purified vWF (1 or 5 μg/mL), or Hepes buffer in control mixtures, biotinylated botrocetin (0–10 μg/mL), and BSA (5 mg/mL) were incubated in a 120-μL incubation volume for 30 min at 22–25 °C, after which two 50-μL aliquots of each mixture were placed into the antibody-coated wells. After an additional 30-min incubation at 22–25 °C, wells were washed 3 times with 200 μL of PBS/NP-40 and incubated for 20 min at 22–25 °C with 50 μL of peroxidase-conjugated streptavidin (Zymed Lab.) diluted to a final concentration of 0.25 μg/mL with PBS/NP-40 containing 1% BSA. After removal of this solution, wells were washed 3 times with PBS/NP-40, and the peroxidase substrate o-phenylenediamine was added in 50-μL aliquots at a final concentration of 1 mg/mL. After 10–20 min, the reaction was blocked with 2 M H_2SO_4. The intensity of the color developed, measured at 490 nm, was taken to represent the amount of biotinylated botrocetin complexed to vWF. For competitive inhibition studies, biotinylated botrocetin and rvWF$^{445-733}$ were preincubated for 30 min at 22–25 °C and then further incubated in the presence of vWF at fixed concentrations for an additional 30 min at 22–25 °C.

In the second method, polystyrene microtiter plates were coated with 100 μL of a solution of purified vWF (10 μg/mL) as described above for IgG. In control experiments, microtiter wells were coated with either fibrinogen, fibronectin, or vitronectin [purified as referenced in Berliner et al. (1988)] instead of vWF. Wells were washed and saturated with BSA as described above; then 50-μL aliquots of various concentrations of biotinylated botrocetin (0–10 μg/mL) were added and incubated for 30 min at 22–25 °C. Wells were washed and assayed for bound biotinylated botrocetin as described above. For competitive inhibition studies, a fixed amount of biotinylated botrocetin was mixed with varying concentrations of rvWF$^{445-733}$ for 30 min at 22–25 °C before addition to the vWF-coated wells.

Binding of ^{125}I-Labeled Botrocetin to Platelets in the Presence of Native vWF or rvWF$^{445-733}$. The effect of vWF on ^{125}I-botrocetin binding to platelets was demonstrated by incubating a fixed concentration of botrocetin (1 μg/mL) with increasing concentrations of native vWF or rvWF$^{445-733}$ and washed platelets (1×10^8/mL) for 30 min at 22–25 °C. Platelet-associated radioactivity was then measured as described above. The affinity of the interaction between GP Ib and the complex of botrocetin with either native vWF or rvWF$^{445-733}$ was calculated from binding isotherms obtained as follows. Complex formation was allowed to occur by incubating at 22–25 °C for 30 min a saturating amount of ^{125}I-labeled botrocetin (6 μg/mL) with native vWF (0.0125 μM) or rvWF$^{445-733}$ (0.5 μM) or Hepes buffer in control mixtures. Then, varying dilutions of the mixtures were prepared in Hepes buffer and added to washed platelets to give a final platelet count of 1×10^8/mL and varying concentrations of total botrocetin and vWF or recombinant fragment. After an additional 30 min, platelet-bound radioactivity was determined as described above. Scatchard analysis of the binding data was performed by using the computer-assisted program LIGAND (Munson & Rodbard, 1980; Munson, 1983).

RESULTS

Expression and Characterization of the Recombinant vWF Fragment. The vWF cDNA corresponding to residues 445–733 of the mature vWF subunit was inserted into a vector that contains a promoter selectively recognized by T7 RNA polymerase. Addition of IPTG to a growing culture of cells induced expression of a chromosomal copy of the gene for T7 RNA polymerase under the control of the lac UV5 promoter; the T7 RNA polymerase, in turn, transcribed the target DNA in the plasmid. Analysis by SDS–PAGE of samples obtained at time points between 0.5 and 3 h after induction of expression revealed the presence of a new, quantitatively predominant protein band with an apparent molecular mass of 36 kDa (Figure 1). By 3-h postinduction, this fragment constituted more than 25% of the total cellular protein, a level of expression that was an order of magnitude higher than observed

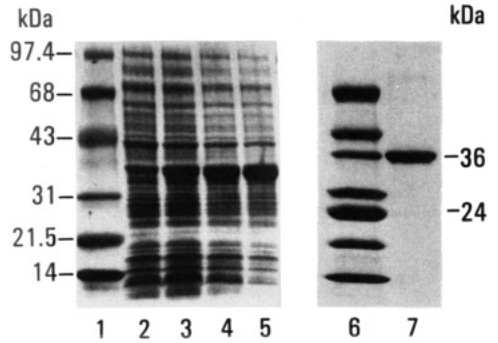

FIGURE 1: SDS–polyacrylamide gel electrophoretic analysis of the expressed rvWF$^{445-733}$. Lanes 1 and 6 contain marker proteins; lanes 2–5 contain samples from cells at 0 min, 30 min, 1 h, and 3 h after induction with IPTG; lane 7 contains the purified recombinant vWF fragment, rvWF$^{445-733}$. Lanes 1 through 5 and lanes 6 and 7 are from two different gels, respectively, thus explaining the different mobility of the marker proteins and vWF fragment. Molecular masses are indicated on the left and right sides of the gels in kilodaltons (kDa).

by using similar constructs with lac, tac, or P_R promoter vectors (not shown). Amino acid sequence analysis of the reduced and alkylated purified 36-kDa fragment identified 15 amino-terminal residues that were identical with those predicted from the DNA sequence in the expression vector, except that the initiating methionine was absent in the peptide. Sequence analysis of proteolytic fragments comprising greater than 70% of the 36-kDa peptide provided results in complete agreement with those predicted from the DNA sequence. Finally, immunoblotting analysis using the anti-vWF monoclonal antibodies LJ-RG46 and LJ-52K2 confirmed the identity of the recombinant fragment (not shown).

Effect of rvWF$^{445-733}$ on Native vWF Binding to Platelets and Agglutination Mediated by Ristocetin and Botrocetin. The recombinant vWF fragment failed to inhibit completely the ristocetin-mediated binding of intact vWF to platelets when the assay was performed with washed platelets resuspended in buffer; rather, the apparent amount of vWF associated with the platelet pellet was paradoxically increased at the higher fragment concentrations added (Figure 2, upper panel). In contrast, when plasma proteins were added to the experimental mixtures (in the form of severe von Willebrand disease plasma devoid of vWF), the inhibition of ristocetin-induced vWF binding to platelets by rvWF$^{445-733}$ was complete and dose-dependent. In two separate experiments, the corresponding IC_{50} (the concentration necessary to inhibit 50% of binding) was 66 and 72 nM, respectively. With botrocetin as a modulator of the interaction, the platelet binding of intact vWF was completely inhibited by rvWF$^{445-733}$ in a dose-dependent manner, regardless of the presence of plasm proteins in the mixture (Figure 2, lower panel). The corresponding IC_{50} varied between 12 and 16 nM in three distinct experiments. In agreement with the inhibition of vWF binding to GP Ib, rvWF$^{445-733}$ inhibited in a dose-dependent fashion the agglutination induced by addition of ristocetin or botrocetin to normal platelet-rich plasma (Figure 3). In these experiments, aggregation mediated by the GP IIb–IIIa receptor was blocked by EDTA.

Interaction of rvWF$^{445-733}$ with GP Ib. This was evaluated by testing the ability of the recombinant fragment to interfere with the platelet binding of anti-GP Ib monoclonal antibodies directed at different epitopes in the 45-kDa amino-terminal region of GP Ibα known to contain the vWF binding domain (Vicente et al., 1988, 1990). The two monoclonal antibodies used were LJ-Ib1, which markedly inhibits botrocetin-mediated binding of vWF to GP Ib, and LJ-P3, which has a negligible

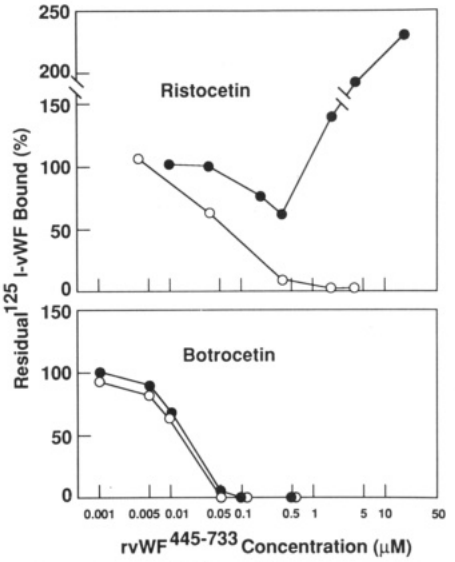

FIGURE 2: Effect of rvWF$^{445-733}$ on ^{125}I-vWF binding to platelets mediated by ristocetin or botrocetin. Washed platelets (1×10^8/mL), resuspended in either Hepes buffer (filled circles) or in plasma from a patient with severe von Willebrand disease (vWF antigen level <0.1% of average normal) in the proportion of 52% of the final volume (open circles), were incubated with ^{125}I-vWF (2 μg/mL) without (control) or with the addition of rvWF$^{445-733}$ at the concentrations indicated on the abscissa, followed by either ristocetin (1 mg/mL; upper panel) or botrocetin (0.4 μg/mL; lower panel). All indicated concentrations are final. Platelet-associated radioactivity was measured after a 30-min incubation at 22–25 °C. Binding in the mixtures containing rvWF$^{445-733}$ is expressed as percentage of that measured in the control mixtures, after subtraction of nonspecific binding from all data points.

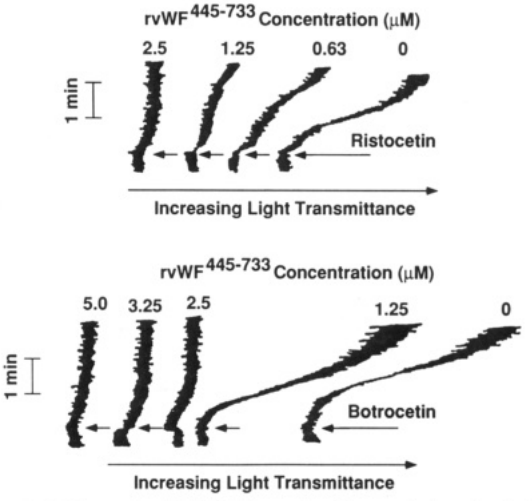

FIGURE 3: Effect of rvWF$^{445-733}$ on platelet agglutination induced by ristocetin or botrocetin. Platelet-rich plasma (2×10^8 platelets/mL), containing 5 mM EDTA to inhibit aggregation, was stirred (1200 rpm) in an aggregometer cuvette at 37 °C and mixed with rvWF$^{445-733}$ at the indicated concentrations followed by either ristocetin (1 mg/mL) or botrocetin (1 μg/mL). All indicated concentrations are final. Platelet agglutination was measured as the increase in light transmittance as a function of time.

inhibitory effect. In the presence of botrocetin, both intact vWF and the recombinant fragment inhibited platelet binding of LJ-Ib1 in a dose-dependent manner but did not affect binding of LJ-P3 (Figure 4). The IC_{50} values observed with native vWF were 6 and 8 nM in two separate experiments; with rvWF, between 30 and 50 μM in three separate experiments. The recombinant fragment, therefore, was approximately 8-fold less effective than native vWF in inhibiting LJ-Ib1 binding. At the concentrations tested, the inhibition of LJ-Ib1 binding by vWF was strictly dependent on the

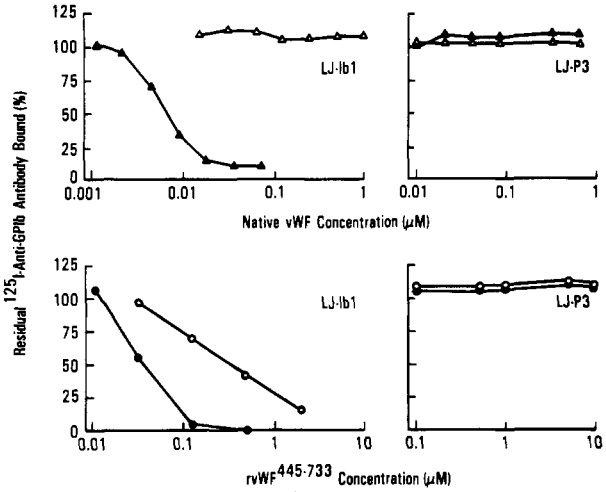

FIGURE 4: Inhibition of ^{125}I-labeled anti-GP Ib monoclonal antibody binding to platelets by native vWF or rvWF$^{445-733}$. (Upper panels) Washed platelets (1×10^8/mL) were incubated with ^{125}I-labeled IgG of monoclonal antibody LJ-Ib1 (20 μg/mL; left) or LJ-P3 (1 μg/mL; right) and native vWF at the various concentrations indicated on the abscissa, or the same volume of Hepes buffer in the control mixture, with (filled symbols) or without (open symbols) the addition of botrocetin (5 μg/mL). (Lower panels) Same as above, except that rvWF$^{445-733}$ was used instead of native vWF. After a 30-min incubation at 22–25 °C, platelet-bound radioactivity was measured as indicated under Experimental Procedures. Binding is expressed as a percentage of that measured in the control mixture containing Hepes buffer instead of vWF or rvWF$^{445-733}$, after subtraction of nonspecific binding.

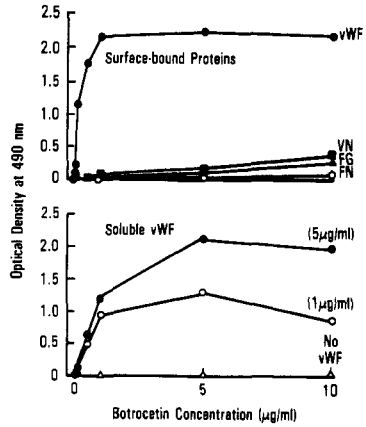

FIGURE 5: Complex formation between botrocetin and vWF. Biotinylated botrocetin was added to microtiter plates coated with purified solutions (10 μg/mL) of either vWF, fibrinogen (FG), fibronectin (FN), or vitronectin (VN) (upper panel) or (lower panel) incubated with vWF in solution (1 or 5 μg/mL, as indicated by open or filled circles, respectively) or Hepes buffer in control mixtures (open triangles) before addition into microtiter wells previously coated with the anti-vWF monoclonal antibody LJ-2.2.9. The amount of botrocetin bound to the wells was then measured as described under Experimental Procedures. The values reported on the ordinate represent the optical density at 490 nm of the color developed in the assay.

presence of botrocetin, whereas rvWF$^{445-733}$ inhibited also in the absence of botrocetin (Figure 4). In this case, the IC_{50} varied between 0.4 and 0.45 μM in three separate experiments, demonstrating approximately an 8–10-fold lower affinity than in the presence of botrocetin. Neither the fragment nor vWF had any effect on the platelet binding of an anti-GP IIb–IIIa monoclonal antibody, LJ-CP8; moreover, the same anti-GP IIb–IIIa antibody did not affect the ability of the fragment or native vWF to inhibit the platelet binding of the anti-GP Ib monoclonal antibody (results not shown).

Interaction of Botrocetin with Native vWF and rvWF$^{445-733}$. Botrocetin formed a dose-dependent and saturable complex

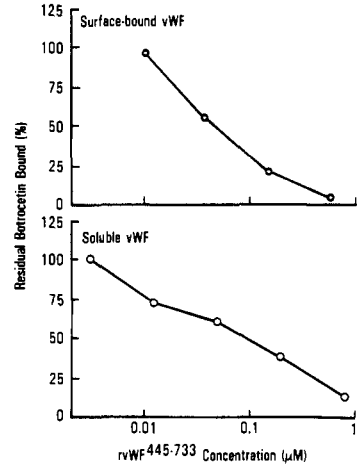

FIGURE 6: Inhibition of botrocetin–vWF complex formation by rvWF$^{445-733}$. These experiments were performed as indicated in the legend to Figure 5, except that rvWF$^{445-733}$ at the concentrations indicated on the abscissa was incubated with biotinylated botrocetin (0.2 μg/mL) for 30 min at 22–25 °C before addition of the latter to either vWF-coated microtiter wells or vWF in solution (5 μg/mL). Residual binding measured in the presence of rvWF$^{445-733}$ is expressed as a percentage of that measured in the control mixture containing Hepes buffer instead of the fragment.

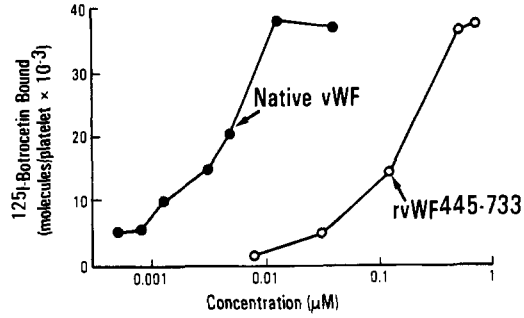

FIGURE 7: Dose-dependent ability of native vWF or rvWF$^{445-733}$ to support ^{125}I-botrocetin binding to platelets. Binding was measured by using washed platelets (1×10^8/mL) and ^{125}I-botrocetin at a fixed concentration (1 μg/mL) in the presence of the indicated concentrations of native vWF or rvWF$^{445-733}$. After a 30-min incubation at 22–25 °C, platelet-associated radioactivity was measured as described under Experimental Procedures.

with both surface-bound and soluble vWF (Figure 5). The concentration necessary to occupy half of the vWF binding sites was between 0.27 and 0.81 μg/mL (10–30 nM, assuming a botrocetin molecular mass of 27 kDa). In both experimental assays, formation of the complex was inhibited by rvWF$^{445-733}$ (Figure 6). The corresponding IC_{50} values were 85 and 95 nM in two separate experiments with soluble vWF, and between 40 and 52 nM in three separate experiments with surface-bound vWF.

Platelet Binding of Botrocetin in Complex with Native vWF or rvWF$^{445-733}$. Both native vWF and rvWF$^{445-733}$ supported the interaction of ^{125}I-labeled botrocetin with platelets in a dose-dependent manner (Figure 7). In two separate experiments, half-maximal binding of botrocetin was observed with 4.5–6 nM native vWF and 100–150 nM rvWF$^{445-733}$. Equivalent amounts of botrocetin were bound to platelets when saturating concentrations of vWF or recombinant fragment were present (Figure 7). The anti-GP IIb–IIIa monoclonal antibody, LJ-CP8, had no effect on the platelet binding of botrocetin mediated by either native vWF or recombinant vWF fragment; accordingly, the same antibody had no effect on the botrocetin-mediated binding of vWF to platelets (results not shown). The binding of ^{125}I-labeled botrocetin to platelets in the presence of native vWF or rvWF$^{445-733}$ was saturable; no

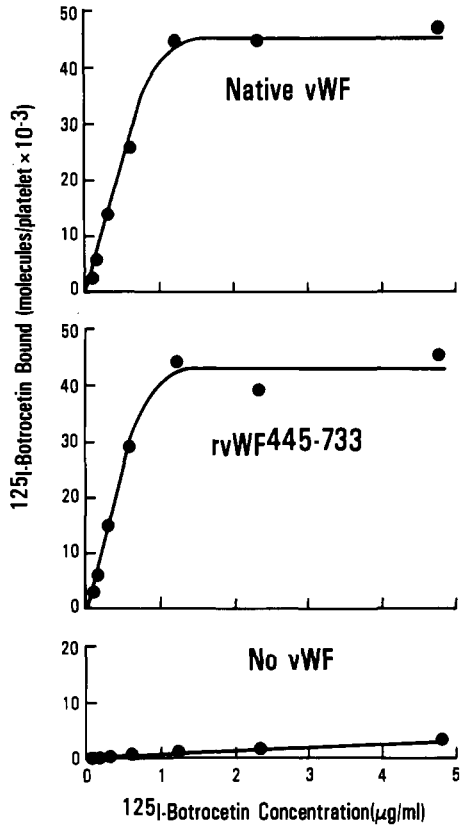

FIGURE 8: Isotherms of ^{125}I-botrocetin binding to platelets in the presence of native multimeric vWF or rvWF$^{445-733}$. (Upper panel) ^{125}I-Botrocetin (6.0 μg/mL) was incubated with native vWF (0.0125 μM) for 30 min at 22–25 °C to allow complex formation; the mixture was then diluted with Hepes buffer containing 5 mg/mL BSA to give the botrocetin concentrations indicated in the figure. Washed platelets (1×10^8/mL) were added to each sample mixture and incubated for an additional 30 min at 22–25 °C. The amount of ^{125}I-botrocetin bound to platelets was then measured as described under Experimental Procedures, and is represented in the graph as total binding. (Middle panel) Same as above, except that rvWF$^{445-733}$ (0.5 μM) was used instead of native vWF. (Bottom panel) Same as above, except that Hepes buffer was used instead of vWF or recombinant fragment. Scatchard-type analysis of binding was performed with the computer-assisted program LIGAND (Munson, 1983); nonsaturable binding was calculated as a fitted parameter from the total binding curve and was subtracted from all experimental points. B_{max} values were 66 253 botrocetin molecules bound per platelet when in complex with native vWF and 54 060 molecules per platlet when in complex with rvWF$^{445-733}$; the corresponding K_d values were 4.3×10^{-8} and 2.37×10^{-8} M, respectively.

appreciable binding occurred with botrocetin alone (Figure 8). In these experiments, vWF or rvWF$^{445-733}$ were used at a concentration (0.0125 and 0.5 μM, respectively) that gave maximal binding of ^{125}I-botrocetin (see Figure 7). Scatchard analysis of these binding data demonstrated similar dissociation constants (K_d) whether botrocetin was in complex with native vWF or recombinant fragment, as well as an essentially identical number of molecules bound at saturation (B_{max}) (Figure 8).

DISCUSSION

The GP Ib binding domain of vWF contains seven cysteine residues (Titani et al., 1986); four are involved in two intrachain disulfide bonds between residues 471–474 (Marti et al., 1987) and 509–695 (Mohri et al., 1988; Andrews et al., 1989b), while at least one of the others (residues 459, 462, and 464) is involved in an interchain bond, as shown by the fact that the domain comprising residues 449–728 is isolated as a disulfide-linked homodimer following tryptic digestion of native vWF (Mohri et al., 1989). However, the conformation in solution of the recombinant bacterial fragment characterized here differs from that of the corresponding domain in native vWF, due to the fact that cysteine residues were alkylated before renaturation to prevent random disulfide bond formation during purification. Moreover, because of the bacterial expression system used, the carbohydrate side chains normally present in this domain of vWF (Titani et al., 1986) are not represented in the recombinant fragment. Both factors may account for some of the functional characteristics that distinguish the recombinant fragment from native vWF.

Perhaps the most important difference is that, unlike native multimeric vWF, the recombinant fragment interacts directly with GP Ib in the absence of any exogenous modulator. This could not be demonstrated with direct binding isotherms using radioiodinated rvWF$^{445-733}$, since preliminary experiments suggested that radioiodination decreased markedly the GP Ib binding function of the fragment, but was proven by the ability of the latter to inhibit platelet binding of an anti-GP Ib monoclonal antibody. The choice of this methodological approach was justified by the need of using a ligand capable of interacting with GP Ib in the absence of any exogenous substance, a property of the monoclonal antibody but not of native vWF. The contention that the epitope recognized by this antibody must be very close if not coincident with the vWF binding site on GP Ib is supported by two lines of evidence. First, the antibody inhibits vWF binding to GP Ib (Handa et al., 1986); second, a variant GP Ibα molecule in a patient with Bernard-Soulier syndrome was found to lack vWF binding function and, concomitantly, lacked expression of this antibody epitope (De Marco et al., 1990). Therefore, it seems reasonable to assume that the inhibition of monoclonal antibody binding by the recombinant fragment is the consequence of its ability to interact with the vWF binding site of GP Ibα in the absence of any modulator. The structural characteristics responsible for the property of rvWF$^{445-733}$ to bind directly to GP Ib remain presently unexplained. A possibly relevant factor in this regard is the lack of inter- and intramolecular disulfide bonds and/or carbohydrate side chains that may result in the exposure of the limited sequences indicated as possible GP Ib interaction sites, residues 474–488 and 694–708 (Mohri et al., 1988). These sites may be functionally hidden in the native conformation of fully glycosylated vWF in solution. The absence of carbohydrate chains in the recombinant fragment may be particularly important, in view of the fact that desialylated multimeric vWF has been shown to interact with GP Ib in the absence of any exogenous substance (De Marco et al., 1985).

The binding of native vWF to GP Ib, whether mediated by ristocetin or botrocetin, was inhibited by rvWF$^{445-733}$. With ristocetin, however, the recombinant fragment failed to inhibit binding completely in experiments performed with washed platelets in the absence of plasma proteins; rather, a paradoxical increase in binding was observed at higher concentrations of fragment, possibly due to the formation of aggregates upon addition of ristocetin to the solution. This problem could be eliminated by the addition of vWF-deficient plasma to the assay mixtures, suggesting that some molecule in plasma either increases the solubility of the fragment and/or prevents the precipitating effect of ristocetin. Thus, in spite of differences in the experimental approach, our findings are in agreement with those reported previously by other investigators (Pietu et al., 1989), who, using a similar recombinant fragment, concluded that it binds to platelets in the presence of ristocetin. It should be noted, however, that the previous work

was performed with a nonpurified bacterial extract and no experimental evidence was provided that the fragment used inhibited the ristocetin-mediated binding of vWF to platelets.

These studies also demonstrate that botrocetin modulates the binding of rvWF$^{445-733}$ to GP Ib and that the mechanisms involved are identical with those responsible for modulation of intact vWF binding. Indeed, that rvWF$^{445-733}$ contains the interaction site, or sites, for botrocetin was shown by its ability to inhibit botrocetin complex formation with multimeric vWF. Moreover, both intact vWF and the isolated recombinant fragment supported the binding of botrocetin to platelets, converse of the property of botrocetin to support vWF binding. These results are in agreement with the concept that botrocetin and vWF form a bimolecular complex that, in turn, binds to platelets (Read et al., 1989). While the affinity of interaction with botrocetin is markedly lower for the isolated recombinant fragment than for intact vWF, the complexes of botrocetin with either native vWF or rvWF$^{445-733}$ exhibit identical binding affinity for platelets. Thus, although the botrocetin binding function of the recombinant fragment may be negatively affected by the lack of native conformation and/or normal glycosylation, formation of the complex regardless of the inherent affinity determines the same functional modulation of the interaction of intact vWF or recombinant fragment with GP Ib.

The structural correlates of this activity of botrocetin remain unknown at present. One possibility is that botrocetin bound to vWF contributes directly to the interaction with GP Ib by creating a new functional site existing only on the complex of the two molecules. Alternatively, without participating in the interaction, botrocetin may impart a conformation to the GP Ib binding site of vWF resulting in higher affinity for the platelet receptor. In any case, the effect of modulation by botrocetin is not dependent on the intrinsic conformation of the GP Ib binding site itself nor on the presence of normal glycosylation, a conclusion supported by the fact that native vWF and bacterial recombinant fragment had similar behavior once bound to botrocetin. Even though the recombinant bacterial fragment has an inherent ability to interact with GP Ib, botrocetin can increase the affinity of this interaction by approximately 10-fold as judged by the relative concentrations of recombinant fragment necessary to inhibit anti-GP Ib antibody binding in the absence or presence of botrocetin. Moreover, the present result supports the concept that no other domain of vWF is essential for achieving high-affinity interaction with platelet GP Ib. In this regard, functional modulation by botrocetin may mimic the effect of subendothelial components such as heparin-like proteoglycans and collagen that may also form a complex with the GP Ib binding domain of vWF (Pareti et al., 1986, 1987; Fujimura et al., 1987; Mohri et al., 1989) and may influence its function in supporting platelet adhesion. Confirmation of this hypothesis, however, will require rigorous experimental evidence.

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Registry No. vWF, 109319-16-6; botrocetin, 85537-36-6.

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Ion-Binding Properties of Calbindin D_{9k}: A Monte Carlo Simulation Study

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ABSTRACT: Monte Carlo simulations are used to calculate the binding constant of two Ca^{2+} ions to the protein bovine calbindin D_{9k}. The change in binding constant with respect to mutation of charged amino acids, presence of various electrolytes, protein concentration, solution pH, and competitive binding of monovalent ions is investigated. Each of these factors may have a large influence on the binding constant. The simulations are performed in a dielectric continuum model, the so-called primitive model of electrolyte theory, with a fixed protein structure and a uniform dielectric permittivity. The calculated binding constants are in excellent agreement with experimental data and describe changes in the binding constant over six orders of magnitude.

During the last five years or so breakthroughs in genetic engineering techniques have made possible the production of synthetic biomolecules. In particular, specifically designed proteins are now able to be manufactured in large quantities, which will have a large impact on the field of pharmacology. A need to understand changes in the function and efficacy of proteins, due to specific mutations and changes in ambient conditions (e.g., electrolyte concentration), has stimulated interest in theoretical models, which may hopefully provide some predictive power. Indeed, in the near future, one can expect that theory will become an increasingly important contributor to the advancement of the predominantly experimental field of molecular biology.

The theoretical approach to these many-atomic systems is based on statistical mechanics. However, while approximate statistical mechanical theories easily deal with systems of say spherical or cylindrical symmetry, awkward numerical procedures are generally needed to solve for more complex geometries, such as that of a protein molecule. It is easy to appreciate the possibilities offered by simulation techniques such as Monte Carlo (MC) and molecular dynamics. Both methods have already proven their utility for simple liquids (Hansen & McDonald, 1976), and their ease of applicability is not greatly affected by the symmetry of the system under study. To date the most detailed calculations utilizing these methods have included an all-atom description of the protein together with a number of surrounding solvent and solute molecules. However, simulations of these "ab initio" models (Ahlström et al., 1987) suffer from the enormous computer time needed, not to mention the problem of choosing realistic atomic interactions.

When an attempt is made to calculate equilibrium properties of proteins in solution, the formidable computational problems, alluded to above, force one to turn to simpler models. For example, when focussing on electrostatic interactions, it is possible to avoid an explicit treatment of solvent molecules by instead invoking a dielectric continuum. In this case solvent molecules are replaced by a uniform continuum with a suitable dielectric constant. A further, commonly used, simplification is to treat the protein as a rigid body. Sometimes simple shapes are also assumed, e.g., hard spheres (Tanford & Kirkwood 1957; Bratko et al., 1988). Other descriptions have used lattice models to better reproduce the irregular shape of proteins (Gilson et al., 1985).

As the protein will exclude solvent molecules, the question arises as to what one should choose for the dielectric permittivity of the interior region. A seemingly viable choice is the electronic permittivity (Harvey, 1989). However, this may lead to an underestimation as the assumption of a fixed protein structure precludes a dielectric response due to nuclear motions. Furthermore, it is not apparent where one should locate the dielectric boundary between the protein and solvent. A common choice is the so-called "solvent-accessable surface" (SAS) (Harvey, 1989), which is the surface traced out by a solvent molecule as it rolls over the bare protein. A model invoking a Stern layer (Stern, 1924) gives a discontinuity that is shifted outward with respect to the SAS. On the other hand, one may expect that, in reality, water is able to penetrate the

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