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Purification of Botrocetin from *Bothrops jararaca* Venom. Analysis of the Botrocetin-Mediated Interaction between von Willebrand Factor and the Human Platelet Membrane Glycoprotein Ib-IX Complex†

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ABSTRACT: Interaction of von Willebrand factor (vWF) with its platelet receptor only occurs in vitro in the presence of a modulator such as ristocetin. We have recently confirmed that the human platelet membrane glycoprotein (GP) Ib-IX complex is the receptor involved in the ristocetin-dependent binding of vWF by reconstitution with the purified components [Berndt, M. C., Du, X., & Booth, W. J. (1988) *Biochemistry 27*, 633-640]. We have now developed a similar solid-phase reconstitution assay using an alternate modulator, botrocetin, for the competitive analysis of functional domains in both vWF and the GP Ib-IX complex. Botrocetin was purified from *Bothrops jararaca* venom by ammonium sulfate fractionation and subsequent DEAE-cellulose and hydroxylapatite chromatography. The purified protein was a 25-kilodalton (kDa) disulfide-linked dimer with apparent subunit molecular weights of 14 000 and 14 500. Binding studies with immobilized botrocetin demonstrated that botrocetin bound to vWF and to a 52/48-kDa region of vWF that contains the GP Ib binding domain, but not to glycocalicin, a proteolytic fragment of GP Ib that contains the vWF binding site. Binding of ^{125}I-labeled vWF to GP Ib-IX complex coated beads and to platelets was strictly botrocetin-dependent with half-maximal binding at a botrocetin concentration of $\simeq$0.27 μM. Botrocetin-dependent binding of vWF was specific, saturable, and comparable to that observed with ristocetin. An anti-vWF monoclonal antibody, 3F8, inhibited ristocetin- but not botrocetin-dependent binding of vWF, suggesting the presence of distinct ristocetin and botrocetin modulator sites on vWF. The botrocetin reconstitution assay was at least an order of magnitude more sensitive than the corresponding ristocetin assay for the competitive analysis of functional domains on both vWF and the GP Ib-IX complex and has confirmed the localization of the vWF-binding domain to the 45-kDa N-terminal region of GP Ib.

One human platelet membrane receptor, the glycoprotein (GP)[1] Ib-IX complex, appears to play a key role in several aspects of the hemostatic process. Binding of the adhesive glycoprotein, von Willebrand factor (vWF), to the GP Ib-IX complex is crucial for the initial contact adhesion of platelets to the exposed subendothelium at high shear flow [for a review, see Girma et al. (1987)] and for the binding of platelets to fibrin (Loscalzo et al., 1986; Parker & Gralnick, 1987).

vWF has a subunit molecular weight of 275 000 consisting of 2050 amino acid residues (Titani et al., 1986) and circulates in plasma as a series of disulfide-linked multimers ranging in molecular weight from 1×10^6 to $>10 \times 10^6$ (Girma et al., 1987). The GP Ib-IX complex binding domain of vWF resides in a 52/48-kDa region, as revealed by the biological activity of a reduced and alkylated tryptic fragment extending

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[1] Abbreviations: Da, dalton; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; GP, glycoprotein; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; vWF, von Willebrand factor.

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from amino acid residue Val-449 to residue Lys-728 (Fujimura et al., 1986). Prior to reduction, the native fragment is a disulfide-linked homodimer with one major intrasubunit disulfide bond between Cys-509 and Cys-695 (Andrews et al., 1989).

GP Ib and GP IX exist in the intact platelet membrane as a native heterodimer complex (Du et al., 1987). GP Ib (170 kDa) consists of two disulfide-linked subunits, GP Ibα (135 kDa) and GP Ibβ (25 kDa). GP IX has a molecular weight of 22 000 (Berndt et al., 1985b). Both the α- and β-chains of GP Ib have been cloned (Lopez et al., 1987, 1988), and the α-chain of GP Ib has been partially protein-sequenced (Titani et al., 1987). GP Ibα consists of 610 amino acid residues, including a 29 amino acid transmembrane segment and an intracellular domain of $\simeq$100 amino acids. Trypsin cleaves the α-chain of GP Ib into three distinct domains: a 45-kDa N-terminal region, a highly glycosylated central macroglycopeptide core (85 kDa), and a $\simeq$25-kDa fragment that remains membrane-associated, disulfide-linked to the β-subunit, and complexed with GP IX (Berndt et al., 1985a, 1988; Handa et al., 1986; Fox et al., 1988). Glycocalicin, a water-soluble derivative of the α-chain of GP Ib, consists of both the macroglycopeptide and the N-terminal domains. Polyclonal and monoclonal antibodies directed against the 45-kDa N-terminal region of the GP Ib–IX complex inhibit the interaction with vWF, suggesting that this region contains the vWF-binding domain (Wicki & Clemetson, 1985; Handa et al., 1986; Berndt et al., 1988).

In normal circulation, vWF does not bind to its platelet receptor. This interaction in vivo requires the prior binding of vWF to the subendothelial matrix (Sakariassen et al., 1979) or the presence of fibrin monomer (Loscalzo et al., 1986; Parker & Gralnick, 1987). This interaction is more conveniently studied in vitro by using ristocetin, a glycopeptide antibiotic isolated from *Nocardia lurida*, that mimics the active constituent(s) of the exposed vessel wall and causes the binding of human vWF to human platelets [reviewed in Berndt and Caen (1984)]. We have recently confirmed that the human platelet GP Ib–IX complex is the receptor for the ristocetin-dependent binding of vWF by reconstitution with the purified components using a solid-phase bead assay (Berndt et al., 1988). It is presently unclear whether ristocetin modulates the binding of vWF to the GP Ib–IX complex by interacting with either ligand or receptor. This uncertainty coupled with the observation that soluble fragments of receptor competed poorly with immobilized GP Ib–IX complex for the binding of vWF led us to examine an alternative modulator of GP Ib–IX complex–vWF interaction. Botrocetin, a partially purified protein from the venom of the South American pit viper, *Bothrops jararaca*, has previously been shown to cause the vWF-dependent agglutination of platelets (Howard et al., 1984; Fujimura et al., 1987a). In this paper, we report the purification and preliminary characterization of botrocetin and demonstrate that it binds to the same 52/48-kDa fragment of vWF that also binds to the GP Ib–IX complex. We further show that the botrocetin-dependent binding of vWF to purified and immobilized GP Ib–IX complex provides a more sensitive competitive assay than ristocetin for the analysis of functional domains on vWF and on GP Ib. These studies provide additional evidence that the 45-kDa N-terminal region of GP Ib contains the binding domain for vWF.

Materials and Methods

Materials. Ristocetin sulfate was purchased from Lundbeck, Copenhagen, Denmark. Bovine serum albumin (fraction V) and *B. jararaca* venom were purchased from Sigma, St. Louis, MO; bovine pancreatic trypsin was purchased from Calbiochem, La Jolla, CA. *Serratia marcescens* metalloprotease was the gift of Dr. T. J. Kunicki, Milwaukee, WI. Sodium [^{125}I]iodide was obtained from New England Nuclear, Boston, MA; Matrex pel 102 beads from Amicon, Danvers, MA; and DEAE-cellulose (DE 52) from Whatman, Maidstone, England. Heparin–Sepharose CL-6B, wheat germ agglutinin–Sepharose CL-6B, and Sephadex G-100 (superfine), G-25, and G-10 were purchased from Pharmacia, Uppsala, Sweden, and hydroxylapatite was purchased from Bio-Rad, Richmond, CA. Human factor VIII concentrate was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia. Thrombospondin, fibrinogen, and fibronectin were purified as previously described (Booth et al., 1984a; Palascak & Martinez, 1977; Coller, 1980).

Bovine serum albumin, thrombospondin, fibronectin, glycocalicin, vWF, and the 52/48-kDa vWF dimer at 1 mg/mL in 0.01 M Tris, 0.15 M sodium chloride, 0.02% (w/v) sodium azide, pH 7.4, were iodinated with Iodobeads (Pierce, Rockford, IL) according to the manufacturer's instructions. Labeled protein was separated from unbound label by chromatography on Sephadex G-25 equilibrated with the same buffer.

Monoclonal Antibodies. All murine monoclonal antibodies were of the immunoglobulin G (IgG) class and were purified and characterized as previously described (Berndt et al., 1988). AK 1, AK 2, AK 3, HIP 1, SZ 1, WM 23, and FMC 25 are directed against the GP Ib–IX complex (Berndt et al., 1988). 2C9 and 3F8 are directed against distinct epitopes on the vWF molecule (Booth et al., 1984b). WM 15 is an irrelevant monoclonal antibody against a 165-kDa protein of myeloid cells and was employed as a control monoclonal antibody (Bradstock et al., 1985).

Purification of the GP Ib–IX Complex and Its Proteolytic Digestion Fragments. The human platelet GP Ib–IX complex was affinity-purified as described elsewhere (Berndt et al., 1985b). Glycocalicin was purified by affinity chromatography on wheat germ agglutinin–Sepharose CL-6B. Briefly, GP Ib–IX complex (0.5 mg/mL) in 0.01 M Tris, 0.15 M sodium chloride, and 0.1% (v/v) Triton X-100, pH 7.4, was equilibrated with *S. marcescens* metalloprotease (10 μg/mL) for 30 min at 25 °C. The digest was loaded at 20 mL/h onto a 1 × 15 cm column of wheat germ agglutinin–Sepharose CL-6B equilibrated with the same buffer. After a wash with 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4, glycocalicin was eluted with 0.5 M *N*-acetylglucosamine in the washing buffer. Glycocalicin was further proteolyzed to macroglycopeptide and N-terminal peptide by treatment with trypsin (0.1 mg/mg of glycocalicin) for 24 h at 22 °C, and the resulting fragments were isolated by gel filtration on Sephadex G-100. The glycocalicin, macroglycopeptide, and N-terminal peptide fractions were homogeneous as evaluated by SDS–polyacrylamide gel electrophoresis. Under reducing conditions, the 45-kDa N-terminal fragment electrophoresed at 35 kDa, indicating that a secondary cleavage at Lys-237 within an intramolecular disulfide loop had proceeded to completion (Handa et al., 1986).

Purification of von Willebrand Factor. vWF was purified from lyophilized human factor VIII concentrate essentially as described elsewhere (Booth et al., 1984b). The purified vWF used in these experiments consisted of high molecular weight multimers ($\simeq 1 \times 10^6$ to $>10 \times 10^6$) as judged by electrophoretic analysis (Ruggeri & Zimmerman, 1981). A weight-average molecular weight of 4.3×10^6 was determined by laser densitometry and integration of the SDS–agarose electrophoretic pattern.

Reduced and Alkylated vWF Dimer. For some experiments, multimeric native vWF in 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4, was reduced with 2-mercaptoethanol (10 mM, 30 min, 22 °C) and then alkylated with iodoacetamide (0.075 M, 30 min, 22 °C) to give a dimer of ≃500 kDa (Michelson et al., 1986).

52/48-kDa Dimeric Fragment of vWF. Since the 52/48-kDa tryptic fragment of vWF that contains the GP Ib–IX complex binding domain also contains a binding domain for heparin (Fujimura et al., 1987b), 52/48-kDa dimer[2] was purified by heparin affinity chromatography. vWF (1 mg/mL) in 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4, was treated with trypsin (0.2 mg/mg of vWF) for 2 h at 37 °C. The reaction was terminated by making the digest 0.1 mg/mL in soybean trypsin inhibitor, 0.2 mM in phenylmethanesulfonyl fluoride, and 0.1 M in benzamidine. The digest was loaded at 20 mL/h onto a 1 × 15 cm column of heparin–Sepharose CL-6B equilibrated at 22 °C with the same buffer. The 52/48-kDa dimer was eluted with a 200-mL linear 0.15–0.4 M sodium chloride gradient and dialyzed exhaustively against 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4, at 4 °C. The purified 52/48-kDa dimeric tryptic fragment was >90% homogeneous as evaluated by SDS–polyacrylamide gel electrophoresis. Analysis by SDS–polyacrylamide gel electrophoresis of ^{125}I-labeled 52/48-kDa dimer bound to GP Ib–IX complex coated beads in the presence of ristocetin indicated that the 52/48-kDa fragment was the only GP Ib–IX complex binding fragment present in the preparation.

Purification of Botrocetin. Botrocetin activity was monitored throughout the purification procedure by measuring the ability of individual fractions to support the binding of ^{125}I-labeled vWF to purified GP Ib–IX complex using the reconstitution assay (see below). Protein composition was visualized by SDS–polyacrylamide gel electrophoresis under both nonreducing and reducing conditions. Crude, lyophilized venom from *B. jararaca* (1 g) was taken up in 30 mL of 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4 (TSa buffer), and fractionated by 60–80% ammonium sulfate precipitation at 22 °C. The precipitate was resuspended in 30 mL of TSa buffer and dialyzed exhaustively against the same buffer at 4 °C. The dialyzed protein was loaded at 20 mL/h onto a 1.5 × 45 cm column of DEAE-cellulose equilibrated at 22 °C, and bound protein was eluted with a 250-mL linear 0.15–0.4 M sodium chloride gradient. Fractions with peak botrocetin activity were pooled and dialyzed exhaustively against 0.005 M sodium phosphate buffer, pH 6.8, at 4 °C. The dialyzed protein was loaded at 20 mL/h onto a 1 × 10 cm column of hydroxylapatite equilibrated at 22 °C, and bound protein was eluted with a 200-mL linear 0.005–0.2 M sodium phosphate, pH 6.8, gradient. Fractions with peak botrocetin activity were pooled, dialyzed exhaustively against TSa buffer at 4 °C, and concentrated where necessary by using an Amicon ultrafiltration cell fitted with a YM 10 membrane. A putative inactive analogue of botrocetin, which closely resembled botrocetin on SDS–polyacrylamide gel electrophoresis and which eluted after botrocetin on DEAE-cellulose chromatography, was also purified to homogeneity by hydroxylapatite chromatography under identical conditions.

Reconstitution Assay. The reconstitution assay for the binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads has been previously described in detail (Berndt et al., 1988). Briefly, FMC 25 coupled Immunobeads were prepared as previously described (Berndt et al., 1988) and coated with purified platelet membrane GP Ib–IX complex by incubating the monoclonal antibody coupled beads with ≃250 μg/mL of the complex in buffer A for 2 h. Buffer A contained 0.01 M Tris, 0.15 M sodium chloride, 0.001 M EDTA, 0.1% (v/v) Triton X-100, and 0.02% (w/v) sodium azide, pH 7.4. The GP Ib–IX complex coated beads were washed by centrifugation once with buffer A and once with TSa buffer. Test assays performed in duplicate at 22 °C consisted of GP Ib–IX complex coated beads (4–5 mg/mL final concentration), bovine serum albumin (1 mg/mL), ^{125}I-labeled vWF (0–200 μg/mL), vWF dimer (1 μg/mL) or 52/48-kDa dimer (2 μg/mL), and either ristocetin (1 mg/mL) or botrocetin (20 μg/mL) in a final volume of either 50 or 100 μL of TSa buffer. Nonspecific binding of ^{125}I-labeled vWF, vWF dimer, or 52/48-kDa dimer was evaluated as previously described (Berndt et al., 1988). In studies on the effect of monoclonal antibodies on the botrocetin-dependent binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads, 50 μg/mL final concentration of monoclonal IgG was included in the assay in a total volume of 100 μL. Similarly, for studies of the effects of vWF and GP Ib fragments on the ristocetin- and botrocetin-dependent binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads, 52/48-kDa dimer (10^{-9} to 2×10^{-6} M) or glycocalicin, macroglycopeptide, or N-terminal fragments (5×10^{-9} to 2×10^{-6} M) were included in the assay in a total volume of 100 μL. The assays were terminated by sedimenting the beads in a Beckman microfuge at 8750*g* for 2 min. The supernatant was carefully removed by use of a Terumo syringe fitted with a fine bore (26 gauge) needle. ^{125}I-Labeled vWF, vWF dimer, or 52/48-kDa dimer associated with the pelleted beads was then measured in a γ counter. Related experiments to those described above were performed to address the binding of ^{125}I-labeled albumin, fibrinogen, or fibronectin (10–100 μg/mL) to GP Ib–IX complex coated beads in the absence or presence of 30 μg/mL botrocetin.

Botrocetin-Dependent Binding of ^{125}I-Labeled vWF to Human Platelets. The botrocetin-dependent binding of ^{125}I-labeled vWF to human platelets was determined by using a minor modification of a previously published procedure (Michelson et al., 1986; Berndt et al., 1988). The assay incorporated 1.25×10^7 washed platelets, ^{125}I-labeled vWF (0–60 μg/mL), and botrocetin (0–40 μg/mL) in a final volume of 250 μL. Assay buffer was 0.01 M Tris and 0.15 M sodium chloride, pH 7.4. For experiments examining the effect of anti-GP Ib–IX complex and anti-vWF monoclonal antibodies on the botrocetin-dependent binding of ^{125}I-labeled vWF to platelets, monoclonal IgG was included in the assay at a final concentration of 50 μg/mL. After 50 min at 22 °C, 200-μL aliquots of each sample were sedimented for 2 min at 8750*g* through a 500-μL cushion of 17% (w/v) sucrose in the same buffer. Where appropriate, the sucrose cushion included botrocetin at the same concentration as present in the assay. The ^{125}I-labeled vWF associated with the pellet was measured in a γ counter after careful aspiration of the supernatant.

Coupling of Botrocetin to Matrex pel 102 Beads. Matrex pel 102 beads are impermeable beads (1–3 μm in diameter) with surface carboxylate and *N*-hydroxysuccinimidyl carboxylic acid ester groups. Botrocetin was covalently coupled to the beads according to the manufacturer's instructions. Control beads were prepared by coupling either ethanolamine or bovine serum albumin by the same method.

[2] In this paper, the term "52/48-kDa dimer" is used to describe the ≃100-kDa nonreduced disulfide-linked homodimer consisting of two 52/48-kDa tryptic fragments. Both the 52-kDa and 48-kDa fragments have an identical amino acid sequence, Val-449–Lys-728, but are variably glycosylated (Fujimura et al., 1986).

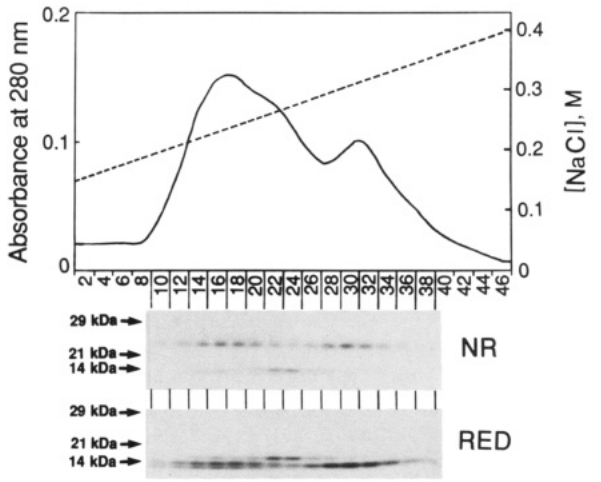

FIGURE 1: Elution profile of botrocetin on DEAE-cellulose chromatography showing the absorbance at 280 nm (solid line) and sections of the SDS–5–20% exponential gradient polyacrylamide electrophoresis gels run under nonreducing (NR) and reducing (RED) conditions. Botrocetin activity was measured in fractions 10–28 by using the reconstitution assay as described under Materials and Methods. The volume of each fraction was 5 mL.

Binding Assay with Botrocetin-Coated Beads. Assays consisted of botrocetin-coated beads (0.7 mg), bovine serum albumin (1 mg/mL), and ^{125}I-labeled protein (0–200 μg/mL) and were performed in a final volume of 140 μL of TSa buffer at 22 °C. The assay was terminated by the centrifugation (8750*g*, 2 min, 22 °C) of a 120-μL aliquot through a 500-μL cushion of 20% (w/v) sucrose in TSa buffer. Radiolabel in the pellet was measured in a γ counter after aspiration of the supernatant. Nonspecific binding of radiolabeled protein to the beads was measured either by using a 50-fold excess of unlabeled ligand in a parallel assay or by substituting the ethanolamine or bovine serum albumin coated control beads for the assay beads in an independent experiment.

Analytical Methods. Electrophoretic analysis on SDS–5–20% exponential gradient polyacrylamide gels was performed according to the method of Laemmli (1970). Protein staining was performed as previously described (Berndt et al., 1985a).

The botrocetin-dependent agglutination of platelets was examined by using citrated platelet-rich plasma (2×10^8 platelets/mL) stirred at 900 rpm at 37 °C by using a Payton dual-channel lumiaggregometer. Citrated platelet-rich plasma was prepared as previously described (Berndt et al., 1985a).

Samples were prepared for amino acid analysis by acid hydrolysis in sealed evacuated tubes containing 6 M hydrochloric acid, 0.3% (v/v) 2-mercaptoethanol, and 0.1% (v/v) phenol at 110 °C for 24, 48, and 72 h. Evaporated hydrolysates were reconstituted in 0.1 M hydrochloric acid and subjected to ion-exchange separation (Waters 80002 column) followed by ninhydrin postcolumn derivatization (Moore, 1968).

Automated protein sequence analysis (Edman & Begg, 1967) was performed with vapor-phase delivery of critical reagents (Hewick et al., 1981) in an automated sequenator (Model 470A protein sequencer, Applied Biosystems) equipped with on-line HPLC for determination of phenylthiohydantoin amino acids (Gorman et al., 1987). An octadecasilica cartridge column (22 cm × 2.1 mm, Applied Biosystems) was employed for chromatographic separation of these amino acid derivatives.

Results

Purification and Preliminary Physicochemical Characterization of Botrocetin. A partially purified protein, botrocetin, from the venom of the South American pit viper, *B. jararaca*, has been previously reported to modulate the binding of vWF to platelets in similar manner to ristocetin (Howard et al., 1984; Fujimura et al., 1987a). Botrocetin was purified to homogeneity from crude lyophilized venom by ammonium sulfate fractionation, followed by DEAE-cellulose and hydroxylapatite chromatography. The elution profile of botrocetin from the DEAE-cellulose column is shown in Figure 1. Peak botrocetin activity was detected in fractions 10–28 by using the reconstitution assay (Materials and Methods) and corresponded to the elution of a disulfide-linked dimer of apparent molecular weight of 25 000. Late fractions, 28–38, contained a protein with similar molecular weight characteristics but which was inactive. Both botrocetin and its putative analogue were purified to homogeneity by hydroxylapatite chromatography. Typical yields were $\simeq$7 and $\simeq$2 mg, respectively, from 1 g of lyophilized venom. By SDS–polyacrylamide gel electrophoresis, botrocetin had an apparent molecular weight of 25 000 under nonreducing conditions, while under reducing conditions the protein consisted of two closely spaced, equally stained subunits with apparent molecular weights of 14 000 and 14 500 (Figure 2, lane 1). The corresponding molecular weights for the putative inactive analogue were 25 000 (nonreduced) and 14 000 (reduced) (Figure 2, lane 2). Neither protein stained for carbohydrate with periodic acid Schiff reagent. Both proteins had a very similar amino acid composition (Table I), and identical N-terminal sequences were obtained for both proteins (Table II). This suggests the possibility that the analogue may be a precursor form of botrocetin that becomes active upon post-translational modification. The analogue protein was completely ineffective in supporting binding of vWF to the GP

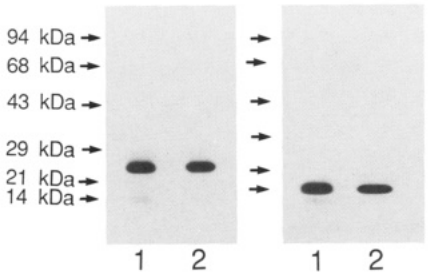

FIGURE 2: SDS–5–20% exponential gradient polyacrylamide gel electrophoresis of purified botrocetin (lane 1, 10-μg load) and its putative inactive analogue (lane 2, 10-μg load) run under nonreducing (left panel) and reducing conditions (right panel). The molecular weight standards (arrows) in order of decreasing molecular weight are phosphorylase B (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (21 000) and lysozyme (14 000).

Table I: Amino Acid Compositions of Botrocetin and Its Putative Inactive Analogue

amino acid	botrocetin (mol/100 mol)	analogue (mol/100 mol)	amino acid	botrocetin (mol/100 mol)	analogue (mol/100 mol)
Asx	10.0	10.6	Ile	2.7	2.0
Thr	2.9	4.2	Leu	5.4	4.4
Ser	11.5	11.4	Tyr	4.6	4.9
Glx	16.4	16.0	Phe	5.1	6.3
Pro	3.3	4.2	His	2.5	1.3
Gly	6.5	6.0	Lys	6.8	7.3
Ala	7.1	4.4	Arg	2.8	2.8
Val	4.9	6.4	Trp	ND[a]	ND
Met	1.7	1.1	Cys[b]	5.8	6.7

[a] ND, not determined. [b] Half-cystine plus cysteine determined as *S*-(carboxymethyl)-Cys.

Table II: Amino-Terminal Amino Acid Sequence Analysis of Botrocetin and Its Putative Inactive Analogue[a]

cycle	PTH amino acid	yield[b] (pmol) botrocetin	analogue
1	Asp	84	148
2	Xaa		
3	Pro	121	120
4	Ser	37	12
5	Asp	69	64
6	Trp	76	57
7	Ser	56	29
8	Pro	24	9
9	Tyr	33	46
10	Glu	46	20
11	Gly	57	23

[a] Supported by duplicate analyses. [b] Includes lag from subsequent cycle.

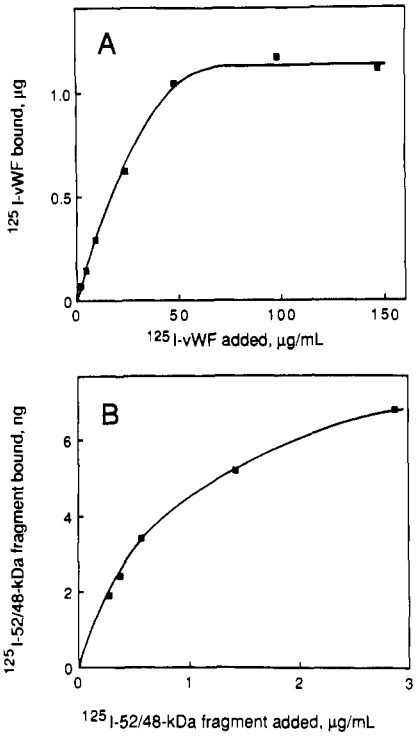

FIGURE 3: Specific binding of ^{125}I-labeled (A) vWF and (B) 52/48-kDa vWF fragment to botrocetin-coated beads (5 mg/mL) at 22 °C.

Ib–IX complex and at 10-fold higher concentration failed to inhibit the botrocetin-mediated binding of vWF to GP Ib–IX complex (data not shown).

Botrocetin Binds to the 52/48-kDa Dimeric Tryptic Fragment of vWF. Botrocetin covalently coupled to Matrex pel 102 beads (≃10 μg of botrocetin/mg of beads) was used to examine the potential interaction of botrocetin with vWF, with a 52/48-kDa dimeric trytpic fragment of vWF,[2] and with glycocalicin, a proteolytic fragment of GP Ibα that contains the vWF binding domain. Binding of ^{125}I-labeled proteins to the botrocetin-coupled beads was complete within 15 min at 22 °C and remained unchanged up to 60 min (data not shown). Specific binding of ^{125}I-labeled vWF and the 52/48-kDa dimer of vWF was saturable (Figure 3). Scatchard analysis indicated that there was a single class of binding sites on the botrocetin-coupled beads for vWF and the 52/48-kDa dimer (for the 52/48-kDa dimer, K_d ≃12 nM; data not shown). In contrast, there was no detectable specific binding of ^{125}I-labeled glycocalicin, fibronectin, thrombospondin, or bovine serum albumin (at 10 μg/mL) to the botrocetin-coupled

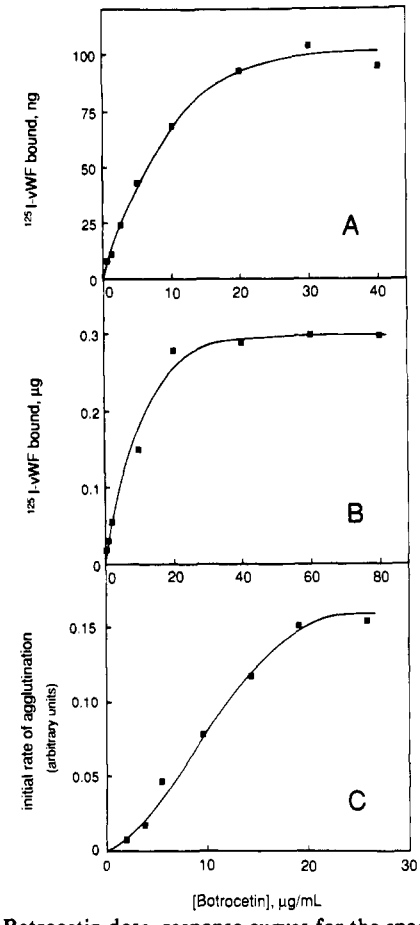

FIGURE 4: Botrocetin dose–response curves for the specific binding of ^{125}I-labeled vWF to (A) washed human platelets (10^8 per mL; [vWF] = 5 μg/mL) and to (B) GP Ib–IX complex coated beads (4 mg/mL; [vWF] = 10 μg/mL) at 22 °C. (C) is the corresponding botrocetin dose–response curve for the agglutination of human platelet rich plasma at 37 °C.

beads under identical conditions. These data strongly suggest that botrocetin induces the interaction of vWF with the GP Ib–IX complex (see below) by virtue of its specific binding to a site on the 52/48-kDa dimer of vWF which also contains the GP Ib–IX complex binding domain. Comparable experiments with ristocetin-coupled beads failed to discriminate the role of ristocetin in the vWF–GP Ib–IX complex interaction since both vWF and purified GP Ib–IX complex bound specifically and saturably to immobilized ristocetin (Andrews, Booth, and Berndt, unpublished observations).

Botrocetin-Dependent Binding of vWF to Purified GP Ib–IX Complex. The botrocetin-dependent binding of vWF to GP Ib–IX complex coated beads was evaluated by using our previously described reconstitution assay (Berndt et al., 1988) by the substitution as modulator of botrocetin for ristocetin. Botrocetin-dependent binding of ^{125}I-labeled vWF to the GP Ib–IX complex coated beads was complete within 10 min. In the absence of botrocetin, ^{125}I-labeled vWF bound nonspecifically (≃7% of total counts) to the GP Ib–IX complex coated beads (Berndt et al., 1988). With platelets and GP Ib–IX complex coated beads, the amount of ^{125}I-labeled vWF specifically associated with the platelets or beads increased with increasing concentrations of botrocetin and reached a plateau at ≃20 μg/mL of botrocetin (Figure 4A,B). Half-maximal binding occurred at a concentration of ≃6.5 μg/mL (0.27 μM) in both assays. Botrocetin-dependent agglutination of platelets in platelet-rich plasma had a similar dose–response profile (Figure 4C). Studies with washed platelets indicated that the agglutination of platelets by botrocetin was vWF

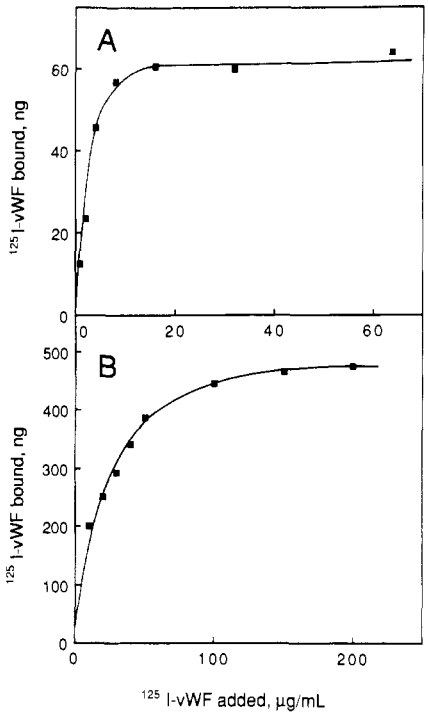

FIGURE 5: Specific binding of ^{125}I-labeled vWF to (A) washed human platelets (10^8 per mL) and (B) GP Ib–IX complex coated beads (4 mg/mL) in the presence of 20 μg/mL of botrocetin at 22 °C.

dependent (data not shown). In contrast, botrocetin did not augment the nonspecific binding of either ^{125}I-labeled fibrinogen, fibronectin, or bovine serum albumin to GP Ib–IX complex coated beads, suggesting that the modulation by botrocetin is specific to the interaction between vWF and the GP Ib–IX complex. The modulation by botrocetin of the binding of vWF to GP Ib–IX complex was EDTA insensitive but was abolished by reduction and alkylation of the botrocetin (data not shown).

In the presence of botrocetin (20 μg/mL), the specific binding of ^{125}I-labeled vWF was saturable both with platelets (Figure 5A) and with GP Ib–IX complex coated beads (Figure 5B). To confirm that botrocetin modulated the binding of vWF to GP Ib–IX complex by a mechanism similar to that of ristocetin, we examined the effect of a series of anti-GP Ib–IX complex and anti-vWF monoclonal antibodies on the botrocetin-dependent binding of vWF to platelets and GP Ib–IX complex coated beads (Figure 6). WM 15 is an irrelevant monoclonal antibody directed against a 165-kDa membrane protein on myeloid precursor cells (Bradstock et al., 1985) and served as a negative control. Two monoclonal antibodies directed against epitopes on the 45-kDa, N-terminal region of GP Ibα, HIP 1 and AK 2, strongly inhibited the botrocetin-dependent binding of vWF to platelets and GP Ib–IX complex coated beads. Monoclonal antibodies against other regions of the GP Ib–IX complex, AK 1 and SZ1 (membrane-associated region) and WM 23 and AK 3 (macroglycopeptide), were without effect, indicating that the botrocetin-dependent binding of vWF to purified GP Ib–IX complex occurred with similar specificity to the binding with intact platelets. In addition, since the antibody-inhibition profile is identical with that observed for ristocetin-dependent binding of vWF to GP Ib–IX complex (Berndt et al., 1988), the data further suggest that a common functional domain on GP Ib is probably involved in both botrocetin- and ristocetin-dependent binding of vWF. 2C9 and 3F8 are both anti-vWF monoclonal antibodies that map into the 52/48-kDa, GP Ib binding domain of vWF (data not shown). Neither antibody inhibited the botrocetin-dependent binding of vWF to either platelets or purified GP Ib–IX complex. This is in contrast to the data for ristocetin-dependent binding of vWF for which 3F8 was inhibitory (Berndt et al., 1988). This suggests that botrocetin and ristocetin modulate the binding of vWF to GP Ib–IX complex by different mechanisms and that 3F8 may inhibit ristocetin-dependent binding of vWF to receptor by interfering with a modulator site on the 52/48-kDa dimer distinct from that for botrocetin.

Evaluation of Functional Domains in vWF and GP Ib–IX Complex by Competitive Binding Analysis. Specific binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads in the presence of ristocetin or botrocetin was inhibited by increasing concentrations of unlabeled native vWF (Figure 7A) or unlabeled 52/48-kDa dimer (Figure 7B). The botrocetin-dependent reconstitution assay was approximately 5-fold more sensitive to inhibition by the 52/48-kDa dimer of vWF than was the corresponding reconstitution assay with ristocetin ($IC_{50} \simeq 0.2$ versus 1 μM, respectively). This differential sensitivity to inhibition is also observed for competition with cold vWF (Figure 7A).

The vWF-binding domain of the GP Ib–IX complex resides within glycocalicin, a 130-kDa water-soluble, proteolytic fragment of the α-subunit of GP Ib comprising both the macroglycopeptide and N-terminal domains. For example, glycocalicin has been reported to inhibit both ristocetin-dependent binding of ^{125}I-labeled vWF to platelets (Michelson et al., 1986) and ristocetin-dependent platelet agglutination (Okumura & Jamieson, 1986). The specific binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads in the presence of botrocetin was inhibited by increasing concentrations of unlabeled glycocalicin (Figure 8A, squares) with an $IC_{50} \simeq 0.2$ μM. Essentially identical inhibition curves for glycocalicin were obtained for the botrocetin-dependent binding of ^{125}I-labeled vWF dimer (Figure 8A, circles) and ^{125}I-labeled 52/48-kDa homodimer (Figure 8A, triangles), indicating that the degree of inhibition by glycocalicin was independent of the multimeric composition of vWF. In contrast, 2.1 μM glycocalicin only slightly inhibited the ristocetin-dependent binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads (Figure 8B). A reasonable explanation for this difference derives from the multimeric nature of vWF. Since the inhibition by glycocalicin in the botrocetin reconstitution assay is independent of the multimeric composition of vWF, this suggests that each vWF multimer interacts with a single receptor on the beads. In contrast, our previous studies suggest that ristocetin allows the interaction of a single vWF multimer with multiple receptors on the GP Ib–IX complex coated beads (Berndt et al., 1988). One would therefore predict that fluid-phase receptor (glycocalicin) should be a more effective inhibitor of botrocetin-dependent binding of vWF than of ristocetin-dependent binding of vWF. By extension of this argument, one would further predict that glycocalicin should be relatively more inhibitory of the ristocetin-dependent binding of vWF dimer and 52/48-kDa dimer than of native multimeric vWF, a conclusion supported by the data of Figure 8B.

To further characterize the vWF-binding domain on the GP Ib–IX complex, glycocalicin was treated with trypsin, and the resulting 85-kDa macroglycopeptide and 45-kDa N-terminal regions were purified by gel filtration. The inhibitory activity of glycocalicin for the botrocetin-dependent binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads (Figure 9, squares) resided completely within the 45-kDa N-terminal region (Figure 9, circles), which was equally inhibitory on a

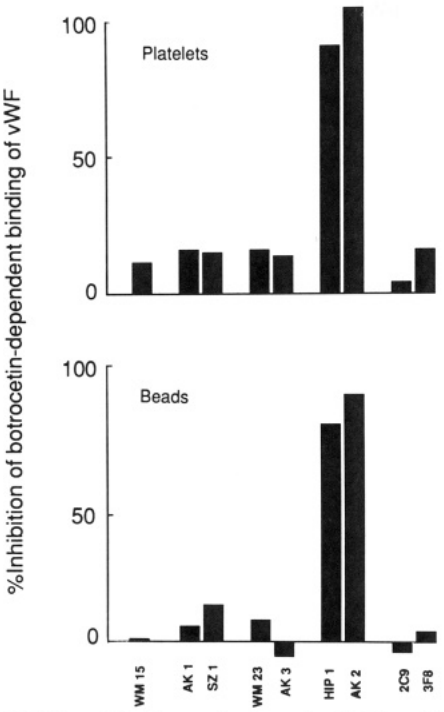

FIGURE 6: Inhibition of the botrocetin-dependent binding of ^{125}I-labeled vWF (1 μg/mL, platelets; 10 μg/mL, beads) to washed human platelets (upper panel; 10^8 platelets per mL) and to GP Ib–IX complex coated beads (lower panel; 4 mg/mL) by monoclonal antibodies at 22 °C. Platelets or beads were equilibrated with monoclonal antibody (50 μg/mL) for 10 min before the addition of ^{125}I-labeled vWF and botrocetin (20 μg/mL, final concentration).

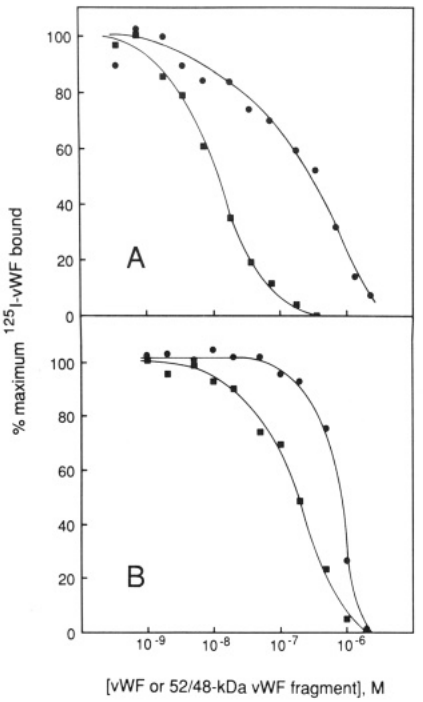

FIGURE 7: Inhibition of the (■) botrocetin-dependent and (●) ristocetin-dependent specific binding of ^{125}I-labeled vWF (1 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) by (A) intact vWF and by (B) 52/48-kDa dimer at 22 °C. The concentrations of botrocetin and ristocetin were 20 μg/mL and 1 mg/mL, respectively. The concentration of vWF has been calculated on the basis of a subunit molecular weight of 275 000 (Fujimura et al., 1986).

molar basis. Concentrations of macroglycopeptide up to 1 μM gave less than 10% inhibition under identical conditions (Figure 9, triangles).

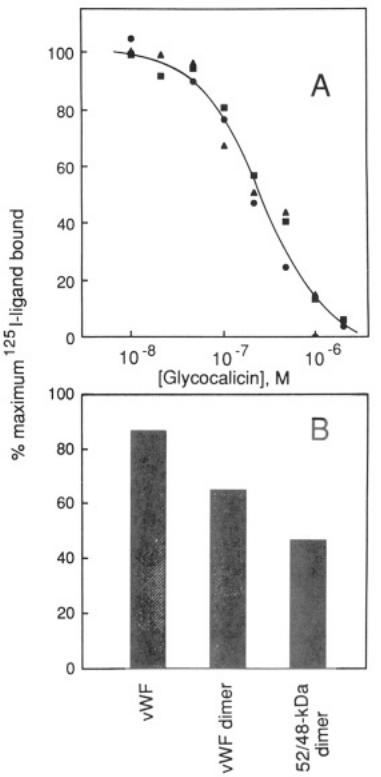

FIGURE 8: (A) Inhibition by glycocalicin of the botrocetin-dependent (20 μg/mL) specific binding of ^{125}I-labeled (■) vWF (1 μg/mL), (●) vwF dimer (2 μg/mL), and (▲) 52/48-kDa dimer (2 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) at 22 °C. (B) Effect of glycocalicin (2.1 μM) on the ristocetin-dependent (1 mg/mL) specific binding of ^{125}I-labeled vWF (1 μg/mL), vWF dimer (2 μg/mL), and 52/48-kDa vWF dimer (2 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) at 22 °C.

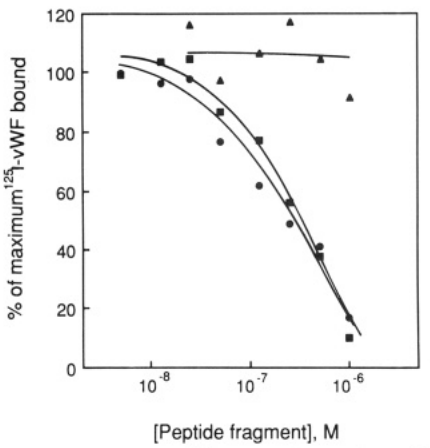

FIGURE 9: Specific binding of ^{125}I-labeled vWF (1 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) at 22 °C in the presence of 20 μg/mL botrocetin and unlabeled (■) glycocalicin, (●) 45-kDa peptide tail, or (▲) 85-kDa macroglycopeptide.

Discussion

The adhesion of blood platelets to exposed vascular subendothelium constitutes the initial event in hemostasis. At high shear flow, the adhesive interaction is dependent upon vWF incorporated in, or bound to, the subendothelial matrix and upon a specific platelet vWF receptor, the human platelet GP Ib–IX complex [for a review, see Girma et al. (1987)]. In a plasma milieu, the GP Ib–IX complex binding domain of vWF is cryptic and only becomes expressed by a poorly understood mechanism when vWF is associated with the subendothelial matrix or with fibrin (Sakariassen et al., 1979; Loscalzo et al., 1986; Parker & Gralnick, 1987). In vitro, ristocetin, a

glycopeptide antibiotic isolated from *N. lurida*, and botrocetin, a component of the venom of the South American pit viper, *B. jararaca*, have been found to mimic the subendothelial matrix in that they cause the binding of human vWF to human platelets (Kao et al., 1979; Schneider-Trip et al., 1979; Fujimura et al., 1987a). It is probable that the detailed understanding of the mechanism of how ristocetin and botrocetin modulate this interaction will help clarify the mechanism by which binding of vWF to subendothelium causes expression of the GP Ib–IX complex binding domain and platelet adhesion. We have previously confirmed that the human platelet membrane GP Ib–IX complex is the receptor involved in the ristocetin-dependent binding of vWF by reconstitution with the purified components using a solid-phase bead assay (Berndt et al., 1988). In this paper, we report the first purification of botrocetin and demonstrate that botrocetin also mediates the specific binding of vWF to purified GP Ib–IX complex by a similar overall mechanism to that of ristocetin. The botrocetin-dependent reconstitution assay, however, was $\simeq$5- to >10-fold more sensitive to inhibition by proteolytic fragments of vWF and GP Ib than was the corresponding ristocetin-dependent assay. These and other studies localize the vWF-binding domain of the GP Ib–IX complex to an N-terminal 45-kDa domain on the α-chain of GP (Wicki & Clemetson, 1985; Handa et al., 1986; Berndt et al., 1988; Vicente et al., 1988).

Botrocetin is a protein with an apparent molecular weight of $\simeq$25 000 consisting of two disulfide-linked subunits with apparent molecular weights of 14 000 and 14 500. Botrocetin mediated the specific binding of vWF both to platelets and to GP Ib–IX complex coated beads with maximal binding at botrocetin concentrations >20 μg/mL (0.83 μM). Half-maximal binding occurred at a botrocetin concentration of $\simeq$6.5 μg/mL. Botrocetin also caused the vWF-dependent agglutination of platelets with maximal effect at a concentration of 20 μg/mL. Reduction and alkylation abolished the functional activity of botrocetin. EDTA had no effect on the functional activity of botrocetin. The botrocetin-dependent specific binding of vWF to platelets and to GP Ib–IX complex coated beads was saturable and strictly dependent upon the presence of botrocetin. In contrast, botrocetin did not cause the specific binding to GP Ib–IX complex coated beads of bovine serum albumin or of other adhesive glycoproteins, such as fibrinogen or fibronectin, indicating that the effect of botrocetin was specific to the interaction between vWF and the GP Ib–IX complex. This conclusion was strongly supported by studies examining the effect of anti-GP Ib–IX complex monoclonal antibodies. Monoclonal antibodies directed against the 45-kDa N-terminal region of GP Ib that strongly inhibit the ristocetin-dependent binding of vWF to platelets and to GP Ib–IX complex coated beads (Berndt et al., 1988) also strongly inhibited the corresponding botrocetin-dependent binding of vWF in both assays. Conversely, monoclonal antibodies directed against either the macroglycopeptide or membrane-associated regions of the GP Ib–IX complex did not inhibit either the ristocetin- or botrocetin-dependent binding of vWF to platelets or to purified GP Ib–IX complex. These results are in complete accord with those of Fujimura et al. (1986, 1987a), who studied the effects of a smaller series of anti-GP Ib antibodies on ristocetin- and botrocetin-mediated platelet agglutination. The combined data indicate that botrocetin-mediated binding of vWF to platelets and, hence, platelet agglutination can be accounted for solely in terms of vWF binding to the GP Ib–IX complex on platelets. Further, the data suggest that a common domain on the GP Ib–IX complex is involved in both the ristocetin-dependent and botrocetin-dependent binding of vWF. In contrast, Howard et al., (1984) reported that platelet-rich plasma from patients with Bernard–Soulier syndrome, whose platelets genetically lack the GP Ib–IX complex (Berndt et al., 1983), showed partial agglutination with botrocetin but not with ristocetin. The significance of this observation, however, is uncertain since their stdies employed only partially purified botrocetin.

It is of particular interest that DEAE-cellulose chromatography separated botrocetin from a protein with similar molecular weight characteristics. This protein had an identical apparent molecular weight of $\simeq$25 000 and, like botrocetin, it consisted of two disulfide-linked subunits of molecular weight $\simeq$14 000. Both botrocetin and its putative analogue had very similar amino acid compositions and identical N-terminal amino acid sequences (Asp-Xaa-Pro-Ser-Asp-Trp-Ser-Pro-Tyr-Glu-Gly), confirming that they are closely related proteins. However, the analogue did not support the binding of vWF to purified GP Ib–IX complex and did not inhibit the effect of botrocetin. Future detailed structural analysis of both proteins should help to define the differences between these two proteins and those features necessary for the function of botrocetin as a modulator of vWF–GP Ib–IX complex interaction.

Since botrocetin and ristocetin both mediate the binding of purified vWF to purified GP Ib–IX complex, they must modulate this interaction by binding to either vWF or the GP Ib–IX complex (or both). Previous studies, however, suggest that botrocetin and ristocetin mediate vWF binding to the GP Ib–IX complex by different mechanisms. First, there are pronounced species differences in the ability of botrocetin and ristocetin to cause platelet agglutination (Read et al., 1978). Second, Howard et al., (1984) have described a patient with a variant form of von Willebrand's disease whose platelet-rich plasma shows normal botrocetin-dependent but absent ristocetin-dependent platelet agglutination. Finally, a structurally related antibiotic to ristocetin, vancomycin, inhibits ristocetin-induced but not botrocetin-induced platelet agglutination (Howard et al., 1984). In this study, we have investigated the mechanism by which botrocetin modulates vWF–GP Ib–IX complex interaction by evaluating the binding of vWF and its receptor to botrocetin covalently immobilized on impermeable Matrex pel 102 beads. Although glycocalicin, a proteolytic fragment of the α-chain of GP Ib that contains the vWF-binding domain, could compete with solid-phase GP Ib–IX complex for the botrocetin-dependent binding of vWF, it did not bind to immobilized botrocetin. In contrast, both vWF and a 52/48-kDa dimeric fragment of vWF were found to bind specifically and saturably to the botrocetin-coupled beads. The latter result is consistent with the report of Howard et al. (1984) that a mixture of botrocetin and vWF coeluted on Sepharose CL-4B chromatography. Previous studies have failed to discriminate between a mechanism that involves binding of ristocetin to vWF (Floyd et al., 1977; Moake et al., 1980) or binding of ristocetin to platelets (Coller & Gralnick, 1977; Coller, 1978; Jenkins et al., 1979; Moake et al., 1980). We have found that 3F8, an anti-vWF monoclonal antibody directed against an epitope on the 52/48-kDa tryptic fragment of vWF, inhibits ristocetin-mediated but not botrocetin-mediated binding of vWF to purified GP Ib–IX complex and to platelets (Berndt et al., 1988; this study). Since it is most likely that the same domains on vWF and on GP Ib are involved in binding mediated by both modulators (Fujimura et al., 1987a; Berndt et al., 1988; this study), the simplest explanation for this observation is that 3F8 inhibits ristocetin-dependent

binding of vWF to platelets by interfering with a ristocetin modulator site on vWF. The combined evidence is therefore consistent with the presence of distinct ristocetin and botrocetin modulator sites, both of which reside on the 52/48-kDa region of vWF that also binds to GP Ib (Fujimura et al., 1986, 1987a; this study).

The ristocetin-dependent binding domain for vWF has been localized within the glycocalicin region of the α-chain of GP Ib (Michelson et al., 1986; Handa et al., 1986). In this study, we have used both the 52/48-kDa fragment of vWF and glycocalicin to evaluate the sensitivity of the botrocetin- and ristocetin-dependent reconstitution assays for the competitive analysis of functional domains on vWF and the GP Ib–IX complex. We found that the botrocetin-dependent reconstitution assay was much more sensitive to inhibition both by the 52/48-kDa fragment of vWF and by glycocalicin. By use of the botrocetin-dependent reconstitution assay, the inhibitory activity of glycocalicin was found to reside totally within the 45-kDa N-terminal region, strongly suggesting that it is this region of the α-chain of GP Ib consisting of amino acid residues His-1–Lys-297 that contains the vWF-binding domain, a conclusion supporting previous studies (Wicki & Clemetson, 1985; Brower et al., 1985; Handa et al., 1986; Berndt et al., 1988; Vicente et al., 1988). In this regard, Vicente et al. (1988) have demonstrated direct ristocetin-dependent binding of the 45-kDa N-terminal peptide to immobilized vWF. However, these authors also found that both the 45-kDa peptide and macroglycopeptide fragments of glycocalicin inhibited ristocetin-dependent binding of vWF to platelets to a similar degree.

In conclusion, we believe that the botrocetin-dependent reconstitution of vWF binding to purified GP Ib–IX complex provides a sensitive and specific assay for the further analysis and definition of the functional binding domains on vWF and GP Ib that mediate their interaction. In this regard, in the following paper (Andrews et al., 1989) we further define the GP Ib–IX complex receptor-binding domain of vWF to a 39/34-kDa monomeric fragment extending from Leu-480/Val-481 to Gly-718 within the primary sequence of vWF and directly demonstrate botrocetin-dependent and -independent cross-linking of this fragment to the 45-kDa N-terminal region of GP Ib–IX complex on intact platelets.

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

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Cross-Linking of a Monomeric 39/34-kDa Dispase Fragment of von Willebrand Factor (Leu-480/Val-481-Gly-718) to the N-Terminal Region of the α-Chain of Membrane Glycoprotein Ib on Intact Platelets with Bis(sulfosuccinimidyl) Suberate†

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ABSTRACT: A 39/34-kilodalton (kDa) monomeric dispase fragment of von Willebrand factor (vWF) has been purified by heparin affinity chromatography. Detailed structural analysis of the individual 39- and 34-kDa fragments indicated that they had identical amino acid sequences extending from Leu-480/Val-481 to Gly-718 with an intramolecular disulfide bond between Cys-509 and Cys-695. In addition to the binding site for heparin, the 39/34-kDa fragment also contained binding sites for collagen and for platelet membrane glycoprotein (GP) Ib. Unlike native vWF, the 39/34-kDa fragment bound to GP Ib without the requirement for a modulator but showed increased binding in the presence of botrocetin. The 39/34-kDa vWF fragment was cross-linked to intact human platelets by using the membrane-impermeable, homobifunctional cross-linking reagent bis(sulfosuccinimidyl) suberate. Two distinct cross-linked species of similar molecular weight (220/200 kDa, nonreduced; 190/175 kDa, reduced) were identified by SDS-polyacrylamide gel electrophoresis and autoradiography, consistent with the cross-linking of the ^{125}I-labeled 39/34-kDa vWF fragment to GP Ib. The formation of these cross-linked species was enhanced 1.5-2.5-fold in the presence of the modulator botrocetin. The platelet membrane protein involved in cross-linking was shown unequivocally to be GP Ib since (i) neither cross-linked species was formed with Bernard-Soulier syndrome platelets, which genetically lack the GP Ib-IX complex, (ii) both cross-linked species were specifically immunoprecipitated by anti-GP Ib polyclonal and monoclonal antibodies, and (iii) the formation of the cross-linked species was completely inhibited only by those anti-GP Ib-IX complex monoclonal antibodies that inhibited vWF-GP Ib-IX complex interaction. Proteolysis of cross-linked platelets with endoproteinase Lys-C, which preferentially cleaves off the N-terminal peptide domain on the α-chain of GP Ib, indicated that the 39/34-kDa vWF fragment was cross-linked exclusively to this region of the GP Ib-IX complex.

Human von Willebrand factor (vWF)[1] is a multifunctional adhesive glycoprotein (GP) that circulates in plasma as disulfide-linked multimers ranging in molecular weight from 1×10^6 to $>10 \times 10^6$. vWF has a subunit molecular weight of ≈275 000 consisting of 2050 amino acid residues and containing 18.7% carbohydrate by weight. Distinct domains on the vWF subunit have been defined to interact with factor VIII, heparin, and collagen and with the platelet membrane glycoproteins, the GP Ib-IX complex, and the GP IIb-IIIa complex [for a review, see Girma et al. (1987)]. The GP Ib binding domain of vWF has been localized to reside within a 52/48-kDa region comprising amino acid residues Val-499-Lys-728 of the primary sequence of vWF (Fujimura et al., 1986). This fragment also contains distinct sites involved in the binding of vWF to collagen (Pareti et al., 1986) and to heparin (Fujimura et al., 1987). Studies with monoclonal antibodies indicate that the binding sites on vWF for GP Ib

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[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; Da, dalton; EDTA, ethylenediaminetetraacetic acid; 5-IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; FAB-MS, fast atom bombardment-mass spectrometry; GP, glycoprotein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; vWF, von Willebrand factor.

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