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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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and for heparin are in close proximity (Fujimura et al., 1987).

A major interest of our laboratory has been the analysis of the interaction between vWF and the GP Ib–IX complex since this process mediates the initial contact adhesion of platelets to exposed vascular subendothelium, the initial event in hemostasis. In an attempt to further define the sequence of vWF involved in binding to GP Ib, we have digested vWF with a large number of proteinases and isolated the potential GP Ib binding fragment(s) by heparin affinity chromatography. In this paper, we report the purification and characterization of a monomeric 39/34-kDa dispase fragment of vWF that extends from Leu-480/Val-481 to Gly-718 of the primary sequence of vWF. This fragment retains the binding sites for GP Ib, collagen, and heparin. Unlike intact human vWF, which has an absolute requirement for the presence of a modulator such as ristocetin or botrocetin for binding to the GP Ib–IX complex (Berndt et al., 1988; Andrews et al., 1989), the 39/34-kDa vWF fragment bound to the GP Ib–IX complex in the absence of modulator, but showed increased binding in the presence of botrocetin. Further, we demonstrate that the 39/34-kDa vWF fragment could be covalently cross-linked to GP Ib–IX complex on intact platelets using the membrane-impermeable cross-linking reagent bis(sulfosuccinimidyl) suberate (BS^3). Selective proteolysis with endoproteinase Lys-C indicates that the domain of the GP Ib–IX complex involved in cross-linking with the 39/34-kDa vWF fragment resides within the N-terminal peptide region of the α-chain of GP Ib.

Materials and Methods

Materials. Dispase (neutral protease, grade I) and endoproteinase Lys-C were purchased from Boehringer-Mannheim, West Germany. *Vibrio cholerae* neuraminidase was purchased from Behring, West Germany, and bis(sulfosuccinimidyl) suberate (BS^3) from Pierce, Rockford, IL. All other reagents were of analytical grade quality or the best available commercial grade and were obtained as previously described (Andews et al., 1989). Botrocetin was purified from *Bothrops jararaca* venom as described in the preceding paper (Andrews et al., 1989).

Monoclonal and Polyclonal Antibodies. All murine monoclonal antibodies were of the immunoglobulin G (IgG) class and were purified and characterized as previously described (Ruan et al., 1987). 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., 1984). WM 15, an irrelevant monoclonal antibody against a 165-kDa protein of myeloid cells (Bradstock et al., 1985) and WM 18, directed against a complex-specific epitope on the human platelet membrane GP IIb–IIIa complex (Berndt et al., 1988), were employed as control monoclonal antibodies. Monoclonal IgG was purified by using protein A–Sepharose chromatography (Ruan et al., 1987). The affinity-purified rabbit anti-GP Ibα antibody was prepared as previously described (Du et al., 1987). Normal rabbit IgG was purified from rabbit serum by 0–40% ammonium sulfate fractionation and chromatography on DEAE-Affi–Gel blue.

Purification and Enzymatic Digestion of the Human Platelet Membrane GP Ib–IX Complex. GP Ib–IX complex and tritium-labeled GP Ib–IX complex were prepared and purified as previously described (Berndt et al., 1985b; Du et al., 1987). Purified, tritium-labeled GP Ib–IX complex (200 μg/mL) in 0.01 M Tris buffer, 0.15 M sodium chloride, and 0.1% (v/v) Triton X-100, pH 7.4, was proteolytically digested with endoproteinase Lys-C (20 μg/mL) at 22 °C. At various time intervals, the reaction was terminated by making the digest 0.2 mM in phenylmethanesulfonyl fluoride and 0.5 mM in diisopropyl fluorophosphate prior to analysis by SDS–polyacrylamide gel electrophoresis.

Purification of vWF. Native human vWF was purified from lyophilized factor VIII concentrate as previously described (Booth et al., 1984; Andrews et al., 1989).

Preparation of Asialo-vWF. Human asialo-vWF was prepared essentially as described by De Marco and Shapiro (1981). vWF (1 mg/mL) in 0.05 M sodium acetate, 0.15 M sodium chloride, and 0.008 M calcium chloride, pH 6.0, was equilibrated with *V. cholerae* neuraminidase (0.1 unit/mg of vWF) for 3 h at 37 °C. Asialo-vWF was purified by gel filtration chromatography on Sephadex G-100 and dialyzed exhaustively against 0.01 M Tris and 0.15 M sodium chloride, pH 7.4.

Preparation of the 39/34-kDa vWF Fragment. A 39/34-kDa dispase digestion fragment of human vWF was purified by heparin affinity chromatography. vWF (1 mg/mL) in 0.01 M Tris, 0.15 M sodium chloride, 0.2 mM calcium chloride, and 0.02% (w/v) sodium azide, pH 7.4, was equilibrated with dispase (0.3 mg/mg of vWF) for 6 h at 22 °C. The reaction was terminated by the addition of EDTA (0.01 M final concentration). The digest was loaded at 20 mL/h onto a 1.5 × 5 cm column of heparin–Sepharose CL-6B equilibrated at 22 °C in 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4 (TSa buffer). After extensive washing, the 39/34-kDa vWF fragment was eluted with 0.01 M Tris, 0.5 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4, and dialyzed exhaustively against TSa buffer at 4 °C. Where appropriate, the 39/34-kDa vWF fragment was ^{125}I-labeled by using Iodobeads (Pierce, Rockford, IL) according to the manufacturer's instructions.

Preparation of Reduced and Alkylated 39/34-kDa vWF Fragment. The 39/34-kDa vWF fragment (0.4 mg/mL) in 0.01 M Tris, 0.15 M sodium chloride, 6 M guanidinium chloride, and 0.02% (w/v) sodium azide, pH 7.4, was reduced with 2-mercaptoethanol (0.01 M, 60 min, 22 °C) and then alkylated with iodoacetamide (0.075 M, 15 min, 22 °C) and dialyzed exhaustively against TSa buffer at 4 °C. Reduction was confirmed by SDS–polyacrylamide gel electrophoresis under nonreducing and reducing conditions.

Amino-Terminal Sequence Analysis of the 39/34-kDa vWF Fragment. Automated amino-terminal 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 (2.1 mm × 22 cm, Applied Biosystems) was employed for chromatographic separation of these amino acid derivatives.

Isolation of Individual 39- and 34-kDa vWF Fragments. Procedures employed to label the 39/34-kDa fragments with 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (5-IAEDANS) after reduction with dithiothreitol have been described previously (Gorman, 1987; Gorman et al., 1987, 1988). After the Cys residues of the 39/34-kDa fragments were fluorescently labeled, the individual 39- and 34-kDa fragments were separated on 3-mm-thick 10–20% (w/v) slab gels (Laemmli, 1970) and isolated individually by electroelution according to published methods (Gorman, 1987; Gorman et al., 1987, 1988).

Isolation and Analysis of Peptides Derived by Tryptic Digestion of the 39- and 34-kDa vWF Fragments. Mixtures

of or individual 39- and 34-kDa fragments were digested by addition of two aliquots [1% (w/v) each] of TPCK-treated trypsin (Worthington, Freehold, NJ) and equilibrated at 37 °C. These additions were spaced by 2 h, and the total reaction time was 4 h. Tryptic peptides were either isolated by HPLC or analyzed directly by FAB–MS. Individual tryptic peptides were isolated by reverse-phase HPLC employing a Waters Associates gradient system with two Model 510 pumps, a Model 440 dual-channel fixed-wavelength detector, a Model 481 variable-wavelength detector, a Hitachi F100 fluorescence detector, a U6K injector, and a Model 840 gradient control and data acquisition system. Chromatography was performed at 1 mL/min on an octadecasilica-based column (Vydac C18, 4.6 mm × 25 cm, Separations Group, Hysperia, CA) using linear gradients of increasing concentration of acetonitrile relative to water while maintaining a constant concentration of 0.1% (v/v) trifluoroacetic acid. Absorbance of the eluates was monitored simultaneously at 210, 254, and 280 nm in addition to the fluorescence emission at 500 nm due to excitation at 340 nm. Individual peaks were collected manually into 1.5-mL polypropylene tubes (Sarstedt), and the solvent was removed by using a Savant centrifugal concentrator. 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). FAB–MS (Barber et al., 1982) of unfractionated digests or individual tryptic peptides was performed by using a VG 70/70 EHF forward geometry double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) equipped with an Ion Tech saddle-field fast-atom gun. Samples were applied in 2 μL of 0.1% (v/v) aqueous trifluoroacetic acid to the sample stage containing 1 μL of a preapplied mixture of dithiothreitol/dithioerythritol (5:1). Xenon atoms at a potential of 8 keV and a discharge potential of 1 mA were used for sample bombardment. Scans were performed at 40 s/decade at a resolution of 1500. Positive-ion spectra were acquired and reprocessed in a multichannel analysis mode using a VG 11/250J data system.

Purification of Bovine vWF. Bovine blood was collected at the slaughterhouse and was mixed immediately with a $^1/_{10}$ volume of 0.1 M trisodium citrate. Bovine plasma was obtained by differential centrifugation and stored at −20 °C in plastic bags. The plasma ice was crushed and made 3% (v/v) in ethanol by the slow addition, with mixing, of ice-cold 50% (v/v) ethanol. An ethanol cryoprecipitate was formed following slow thawing of the ice. The cryoprecipitate from 500 mL of plasma was pelleted by centrifugation and resuspended to a final volume of 150 mL in TSa buffer. All centrifugation steps were performed at 4000*g* for 10 min at 20 °C. The resolubilized material was maintained at 4 °C for 16 h, during which time a fibrinogen gel formed. The gel was broken up, pelleted, and discarded. The supernatant was made 4% (w/v) in polyethylene glycol 8000 by the slow addition, with constant mixing, of a 40% (w/v) stock solution. After the solution was mixed for 30 min at 22 °C, the precipitate was pelleted and the pellet resuspended to a volume of 40 mL in TSa buffer. The resolubilized material was made 1 M in β-alanine by the slow addition, with constant mixing, of a 4 M stock solution. The precipitate formed after 30 min of mixing at 4 °C was pelleted and discarded. The supernatant was dialyzed against TSa buffer and fractionated by Sepharose 4B gel chromatography, employing a 5 × 90 cm column, preequilibrated with TSa buffer, with a flow rate of 30 mL/h. The void volume fractions were pooled and loaded onto a 1 × 15 cm column of heparin–Sepharose CL-6B at 30 mL/h. The flow-through was discarded and the bound vWF eluted with 0.01 M Tris buffer, 0.5 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4. The eluted material was dialyzed against TSa buffer and further concentrated by using an Amicon ultrafiltration cell fitted with a YM 30 membrane. The final product was essentially pure vWF (>95%) as indicated by the major Coomassie Brilliant Blue stained band at 240 000 molecular weight when analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions. Analysis of ^{125}I-labeled bovine von Willebrand factor protein by SDS–agarose gel electrophoresis under nonreducing conditions (Ruggeri & Zimmerman, 1981) showed multimer bands with molecular weights predominantly in the range 1–5 × 10^6. The protein was radioiodinated by the Chloramine T method.

Ristocetin-Dependent and Botrocetin-Dependent Binding of the 39/34-kDa vWF Fragment to Purified GP Ib–IX Complex. The binding of ^{125}I-labeled 39/34-kDa vWF fragment (0–20 μg/mL) to GP Ib–IX complex coated beads was measured in duplicate at 22 °C in the presence of either ristocetin (1 mg/mL) or botrocetin (20 μg/mL) according to our previously described solid-phase bead reconstitution assay (Berndt et al., 1988; Andrews et al., 1989). Total binding was corrected for nonspecific binding as previously described (Berndt et al., 1988). For studies on the effect of anti-GP Ib–IX complex monoclonal antibodies on the botrocetin-dependent binding of ^{125}I-labeled 39/34-kDa vWF fragment to GP Ib–IX complex coated beads, 50 μg/mL final concentration of monoclonal IgG was included in the assay for 10 min prior to the addition of labeled fragment and botrocetin.

Inhibition of Binding of vWF to Purified GP Ib–IX Complex by the 39/34-kDa vWF Fragment. These experiments investigated the ability of the 39/34-kDa vWF fragment (0–7 μM) or the reduced and alkylated 39/34-kDa vWF fragment (0–8 μM) to inhibit the binding of ^{125}I-labeled human or bovine vWF (1 μg/mL) to purified GP Ib–IX complex. Studies with ^{125}I-labeled human vWF were performed in the presence of either ristocetin (1 mg/mL) or botrocetin (20 μg/mL). The reconstitution assays for these inhibition studies were performed as previously described in detail (Berndt et al., 1988; Andrews et al., 1989).

Inhibition of Binding of vWF to Collagen by the 39/34-kDa vWF Fragment. The inhibition of binding of ^{125}I-labeled vWF (1 μg/mL) to equine achilles tendon collagen (Horm, Munich) (167 μg/mL) by unlabeled 39/34-kDa vWF fragment (0–5.7 μM) was examined at 22 °C by using the binding assay of Pareti et al. (1986).

Preparation of Normal and Bernard–Soulier Syndrome Platelets. The patient with Bernard–Soulier syndrome, LS, has been characterized in detail elsewhere (Berndt et al., 1983). Normal and Bernard–Soulier syndrome platelets were isolated from venous blood as previously described (Chong et al., 1983; Berndt et al., 1985a). Platelets were washed by centrifugation three times in 0.033 M trisodium citrate, 0.123 M sodium chloride, and 0.013 M glucose, pH 7.0, and finally suspended at >10^9/mL in divalent-cation-free Tyrode's solution. Divalent-cation-free Tyrode's solution contained 0.0036 M sodium phosphate, 0.012 M sodium bicarbonate, 0.138 M sodium chloride, 0.0029 M potassium chloride, 0.0055 M glucose, and 0.14% (w/v) bovine serum albumin, pH 7.4.

Cross-Linking of the 39/34-kDa vWF Fragment to Platelets. Normal or Bernard–Soulier syndrome platelets

(10^9/mL final concentration) in divalent-cation-free Tyrode's solution were equilibrated in the presence of ^{125}I-labeled 39/34-kDa vWF fragment (10 μg/mL) and botrocetin (0 or 20 μg/mL) for 30 min at 22 °C prior to the addition of BS^3 (0.2 mM final concentration). After a further 10 min at 22 °C, the reaction was terminated by making the samples 0.01 M in Tris buffer, pH 7.4. The platelets were washed once with divalent-cation-free Tyrode's solution by centrifugation at 8730*g* for 30 s. For immunoprecipitation analysis, the platelets were resuspended in 0.01 M Hepes buffer, 0.15 M sodium chloride, and 0.001 M EDTA, pH 7.4, and solubilized with Triton X-100 in the presence of protease inhibitors as previously described (Berndt et al., 1985a). Alternatively, for electrophoretic analysis, the platelets were resuspended at 10^9/mL in 0.01 M Tris and 0.15 M sodium chloride, pH 7.4. Aliquots of each sample were solubilized by the addition of an equal volume of nonreducing or reducing electrophoresis sample buffer (Laemmli, 1970) either immediately or after treatment for 1 h at 22 °C with 10 μg/mL endoproteinase Lys-C.

The effect of monoclonal antibodies on the cross-linking of ^{125}I-labeled 39/34-kDa vWF fragment to normal platelets was studied in parallel experiments by equilibrating the platelets with monoclonal antibody (50 μg/mL) for 30 min at 22 °C prior to the addition of the labeled vWF fragment and botrocetin. Cross-linking of the ^{125}I-labeled 39/34-kDa vWF fragment to the platelets with BS^3 was then performed as described above.

General Methods. Electrophoretic analysis on SDS–polyacrylamide gels (Laemmli, 1970), immunoprecipitation, protein staining, and autoradiography were performed as previously described (Berndt et al., 1985a). Asialo-vWF-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 use of a Payton dual-channel lumiaggregometer. Citrated platelet-rich plasma was prepared as previously described (Berndt et al., 1985a).

Results

Isolation and Characterization of a 39/34-kDa vWF Fragment. Previous studies have localized the binding sites for platelet membrane GP Ib, collagen, and heparin to a 52/48-kDa region of vWF that extends from Val-449 to Lys-728 (Fujimura et al., 1986, 1987; Pareti et al., 1986). We have taken advantage of the observation that the binding sites for GP Ib and heparin are in close proximity as the basis of a general strategy for further defining the GP Ib binding site on vWF. Purified vWF was digested with a range of different proteinases, and the potential GP Ib binding fragments were purified by heparin affinity chromatography. One proteinase, dispase, generated a heparin-binding fragment of lower molecular weight than the 52/48-kDa tryptic fragment, and this dispase fragment has been the subject of further detailed analysis.

Treatment of vWF (1 mg/mL) with dispase (0.3 mg/mL) for 6 h at 22 °C yielded a discrete number of vWF fragments (Figure 1, lanes 1 and 2). Two fragments of similar molecular weight could specifically be purified by heparin affinity chromatography (Figure 1, lanes 3 and 4). Approximately 1.5 mg of the two fragments could be reproducibly isolated from 20 mg of purified vWF. The heparin-binding dispase fragments had molecular weights of 35 000 and 31 000 under nonreducing conditions. Both fragments increased in apparent molecular weight upon reduction to 39 and 34 kDa, respectively (Figure 1, lane 4 versus lane 3), indicating the presence of an intramolecular disulfide bond(s). Further treatment of the 39/34-kDa fragment with dispase was without additional effect, indicating that the 34-kDa fragment was not proteolytically derived from the 39-kDa fragment.

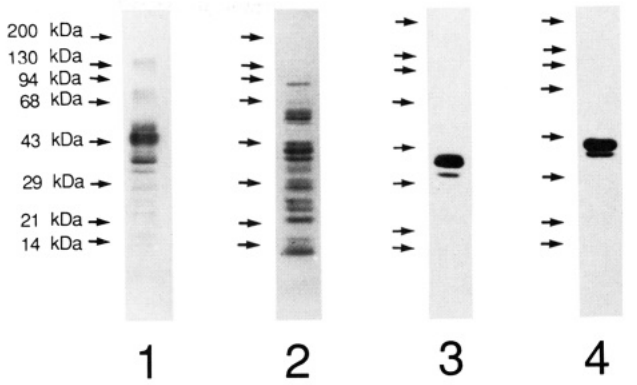

FIGURE 1: SDS–5–20% exponential gradient polyacrylamide gel electrophoresis of a 6-h dispase digest of vWF (lanes 1 and 2, 50-μg load) and of purified 39/34-kDa vWF fragment (lanes 3 and 4, 20-μg load) under nonreducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions. The molecular weight standards (arrows) in order of decreasing molecular weight are myosin (200 000), β-galactosidase (130 000), 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-Terminal Analysis of the 39/34-kDa vWF Fragment

	PTH amino acid[a]		yield[b] (pmol)	
cycle	sequence 1	sequence 2	sequence 1	sequence 2
1	Leu	Val	249	138
2	Val		323	
3	Val	Pro	222	146
4	Pro		276	
5	Pro	Xaa	160	
6	Xaa	Asp	64	
7	Asp	Ala	139	77
8	Ala	Pro	198	28
9	Pro	Val	75	33
10	Val	Xaa	97	
11	Xaa	Pro	32	
12	Pro	Xaa	17	

[a] Unidentified amino acid residues (Xaa) correspond to O-glycosylated threonine or serine residues in the vWF primary sequence (Titani et al., 1986). [b] Sequence 1 will unavoidably include the lag of the preceding cycle; however, no lag values have been included in the yields for sequence 2.

Detailed structural analysis of the separated 39- and 34-kDa fragments established that both fragments had identical amino acid sequences. Amino-terminal sequence analysis of the unfractionated 39/34-kDa fragments revealed two sequences corresponding to dispase-mediated cleavages preceding adjacent residues Leu-480 and Val-481 of vWF (Table I). FAB–MS of unfractionated tryptic digests of the 39/34-kDa fragment produced molecular ions corresponding to tryptic peptides spanning the region from residue Leu-512 to residue Arg-687 of vWF (data not shown). No ions corresponding to tryptic peptides outside this region were observed. Furthermore, identical data were found when separate FAB–MS maps were obtained with tryptic digests of individual 39- and 34-kDa fragments isolated from SDS–polyacrylamide gels. The separation and isolation protocol was preceded by reduction of the 39/34-kDa mixture and alkylation with 5-IAEDANS. This labeling was performed for the purpose of facilitating isolation of individual fragments by SDS–polyacrylamide gel electrophoresis and electroelution in a form suitable for extensive structural analysis (Gorman, 1987; Gorman et al., 1987) and to specifically visualize peptides

Table II: Analysis of Tryptic Digestion Peptides Derived from Unfractionated 39/34-kDa vWF Fragment[a]

fraction	sequence location[b]	
	FAB-MS[c]	amino acid analysis
1	668–673 (686)	668–673
2	nil	546–549
3	630–632 (393)	630–632
4	661–663 (416)	661–663
5[d]	nil	nil
6[d]	nil	nil
7	580–858 (645)	580–585
8	664–667 (502)	664–667
9	609–616 (943)	609–616
10[e]	573–578 (757) + 579–585 (801)	
11	550–552 (460)	550–552
12	572–578 (885)	572–578
13	633–636 (535)	633–636
14	637–643 (835)	637–643
15	637–642 (707)	637–642
16	586–599 (1439)	586–599
17	nil	480/481–511
18	553–569 (1858)	553–569
19	nil	480/481–511 + 553–569
20	525–534 (1165)	525–534
21[f]	617–629 (1776)	617–629
22	535–543 (1097)	535–543
23	645–660 (1655)	645–660
24	674–687 (1621)	674–687
25	646–660 (1527)	646–660
26	617–629 (1470)	617–629
27	nil	688–718
28	600–608 (1147)	600–608
29	nil	674–718
30	nil	674–718
31	512–524 (1448)	512–524

[a] Identical FAB-MS data were obtained for the separated 39- and 34-kDa vWF fragments. [b] Based on the primary amino acid sequence of human vWF (Titani et al., 1986). [c] Values in parentheses represent masses of protonated parent ions found in isolated fractions (cf. Figure 2) which correspond to the amino acid sequences of vWF spanned by the indicated residue numbers. [d] May be degraded fluorescent label released from protein during handling since no amino acids were detected in these fluorescent fractions. [e] Mixed fraction that gave no clear result on amino acid analysis. [f] Contains the S-alkylated methionine derivative of the peptide present in fraction 26.

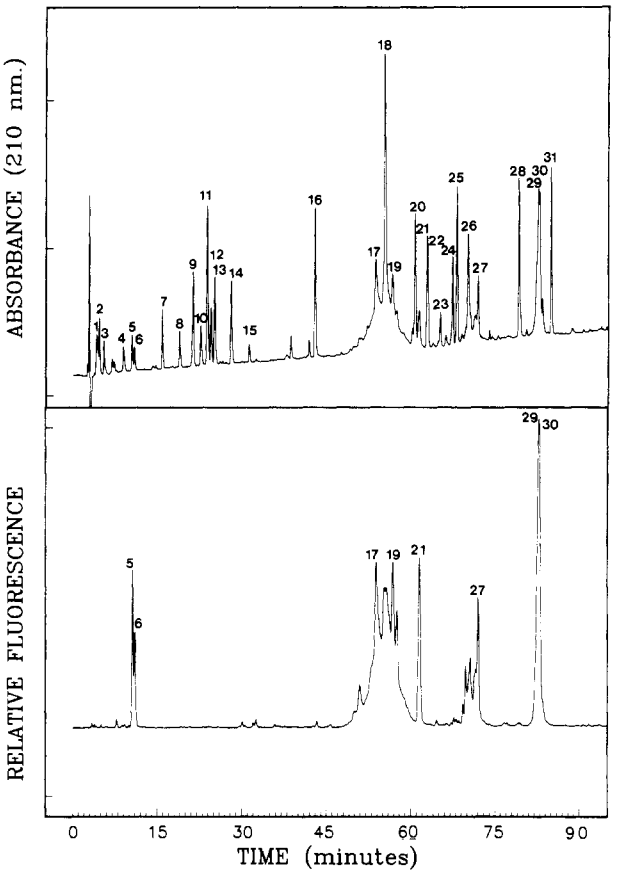

FIGURE 2: HPLC separation of peptides derived from tryptic digestion of the isolated 39-kDa dispase fragment of vWF. Numbered fractions produced analytical data corresponding to tryptic peptides within the primary sequence of vWF spanned by residues Leu-480/Val-481–Gly-718 (Tables II and III). The HPLC profiles of the tryptic digests for the unfractionated 39/34-kDa vWF fragment and for the separated 34-kDa fragment were essentially identical except for minor differences in detail in the midregion of fluorescence (fractions 17–19). The acetonitrile content of the eluant (volume/volume) was increased from 4% to 24% over 60 min, then to 40% over 25 min, and finally to 80% over 15 min.

containing the two Cys residues putatively situated at the amino- and carboxyl-terminal extremities of the fragments. FAB-MS analysis of individual tryptic peptides isolated by HPLC for the unfractionated 39/34-kDa fragment (Table II) and for the separated 39-kDa fragment (Figure 2) and 34-kDa fragment confirmed the data obtained with total tryptic digests. These analyses were substantiated by amino acid analysis of the isolated tryptic peptides for the unfractionated 39/34-kDa fragment (Table II).

Additional tryptic peptides were apparent by HPLC which did not produce interpretable data by FAB-MS (Figure 2; Table II). However, data were forthcoming on these peptides by amino acid analysis and stepwise sequence analysis. These peptides all exhibited fluorescent properties indicative of reactivity with 5-IAEDANS (Figure 2). One of the peptides (fraction 21) was found to have been labeled on Met-622. This peptide eluted as a fairly broad but homogeneous peak during HPLC. Other complex areas of fluorescence were apparent in the midregion and toward the end of the chromatogram. These data are presented in Figure 2 for the 39-kDa fragment. The pattern of nonfluorescent peptides was identical for the 34-kDa fragment, and only minor differences predominantly around fractions 17 and 19 were discerned for the complex fluorescent regions (not shown).

Amino acid analysis of fractions 17 and 19 (midregion fluorescence) derived from the unfractionated 39/34-kDa fragment and the separated 39-kDa fragment produced data indicating that they were both derivatives of the amino-terminal tryptic peptide of the fragments (Tables II and III). In particular, amino-terminal sequence analysis data from fraction 17 were identical with that for the mixture of intact 39/34-kDa fragments; that is, overlapping sequences starting at residues Leu-480 and Val-481 were apparent (not shown). Amino acid and sequence analysis of the later eluting fluorescent peptides served to identify the carboxyl termini of the unfractionated 39/34-kDa fragment and the separated 39-kDa fragment (Tables II and III). These peptides were derived from tryptic cleavage between residues Lys-673 and Ala-674 and dispase cleavage at residue Gly-718 and contain the Arg-687–Asp-688 peptide bond that was relatively insensitive to trypsin. This Arg-687–Asp-688 peptide bond was found to cleave only after protracted tryptic digestion. Data on fraction 27 indicated that it was derived by sluggish cleavage between Arg-687 and Asp-688. Amino-terminal sequence analysis of a mixture of the later eluting fluorescent peptides produced data for 31 cycles of degradation corresponding to the sequence from residue Ala-674 of vWF. Unfortunately, insufficient material was available to extend the analysis to the putative carboxyl termini (i.e., Gly-718), although the insensitive Arg-687–Asp-688 peptide bond was traversed during the sequence analysis. While there was insufficient material for the detailed analysis of fractions 17/19 and 29/30

Table III: Amino Acid Analysis of Tryptic Peptides Derived from the Amino and Carboxyl Termini of the 39-kDa vWF Fragment

amino acid	N-terminal peptide[a] (Leu-480/Val-481–Arg-511)	C-terminal peptide[b] (Ala-674–Gly-718)
Asx	3.01 (3)	4.08 (4)
Thr	2.76 (3)	1.55 (2)
Ser	2.95 (3)	3.36 (3)
Glx	2.00 (2)	7.73 (7)
Pro[c]	– (6)	– (7)
Gly	0.43 (0)	2.60 (2)
Ala	1.26 (1)	4.55 (4)
Val[d]	2.54 (4)	3.90 (5)
Met	0 (0)	0.73 (1)
Ile[d]	0.90 (1)	0.42 (1)
Leu	2.20[e] (2–3)	4.86 (5)
Tyr	1.65 (2)	1.02 (1)
Phe	1.04 (1)	1.06 (1)
His	0.97 (1)	0 (0)
Lys	0 (0)	0 (0)
Arg	0.93 (1)	1.22 (1)
Trp[f]	– (0)	– (0)
Cys[g]	0.79 (1)	0.95 (1)

[a] Data for fraction 17. Comparable data were obtained for fraction 19. [b] Data for fractions 30/31. [c] Detection of amino acids was via postcolumn derivatization with *o*-phthalaldehyde without oxidation; therefore, Pro was not determined. [d] Values may be low as only a 24-h hydrolysis was used. [e] Leu values influenced by ragged cleavage at Leu-480 and Val-481. [f] Not determined. [g] Determined as *S*-(carboxymethyl)-Cys.

for the 34-kDa fragment, the identity of the determined N- and C-terminal sequences for the unfractionated 39/34-kDa fragment and isolated 39-kDa fragment (Tables II and III) and the virtual identity of tryptic digest HPLC profiles for the unfractionated and separated 39- and 34-kDa fragments strongly suggest that the 39- and 34-kDa fragments have the same amino acid sequences. These data would therefore indicate that the Cys-containing amino- and carboxyl-terminal tryptic peptides are heterogeneously O-glycosylated. The more complex region of fluorescence was that associated with the N-terminal tryptic peptide (Figure 2B, fractions 17 and 19). Ragged dispase cleavage at residues Leu-480 and Val-481 does not entirely account for this complexity as overlapping sequences starting with these residues were detected in the same HPLC fraction (fraction 17). Moreover, this would indicate that ragged cleavage does not account for the differences in apparent size of the 39- and 34-kDa fragments. It is apparent that the differences in the 39- and 34-kDa fragments arose from O-glycosylation variability of five threonines and one serine in the sequences (Titani et al., 1986). Comparison of tryptic digests by HPLC (data not shown) supported this conclusion. It is noteworthy that the general elution positions of the complex regions of fluorescence were the same for the 39- and 34-kDa fragments, although these regions varied in detail.

Binding of the 39/34-kDa vWF Fragment to the Glycoprotein Ib–IX Complex and to Collagen. Like the 52/48-kDa vWF fragment (Fujimura et al., 1987; Pareti et al., 1986) the 39/34-kDa fragment contained not only a binding site for heparin but also a binding site for collagen since the purified 39/34-kDa fragment completely inhibited the binding of intact vWF to collagen with an IC_{50} of 1.2 μM (data not shown). This value is comparable with the IC_{50} reported for inhibition of vWF binding to collagen by the reduced and alkylated 52/48-kDa vWF fragment (0.3–0.5 μM) (Pareti et al., 1986).

The 39/34-kDa vWF fragment retained the capacity of intact vWF to bind to the human platelet membrane GP Ib–IX complex. However, unlike native multimeric vWF, the monomeric 39/34-kDa vWF fragment directly bound to GP Ib without the absolute requirement for the presence of a modulator such as ristocetin or botrocetin. Although this binding could not be directly established relative to the level of nonspecific binding in the reconstitution assay (≃6%), the spontaneous binding of the 39/34-kDa vWF fragment to GP Ib was indirectly demonstrated since the fragment strongly inhibited the asialo-vWF-dependent agglutination of platelets (data not shown) and the direct binding of bovine vWF to purified GP Ib–IX complex (Figure 3A), (IC_{50} = 10 μM).[2] Since asialo-vWF and bovine vWF both bind to GP Ib without the requirement for a modulator (Girma et al., 1987; Kirby & Mills, 1975), the inhibition by the 39/34-kDa vWF fragment is most simply explained by the competitive binding of this fragment to the same binding domain on the receptor. The 39/34-kDa fragment also completely inhibited the botrocetin-dependent (Figure 3B, squares) and ristocetin-dependent (Figure 3B, circles) binding of ^{125}I-labeled human vWF to purified GP Ib–IX complex with IC_{50} values of 15 nM and 5.5 μM, respectively. As for the competition by unlabeled vWF and the 52/48-kDa dimer (Andrews et al., 1989), the

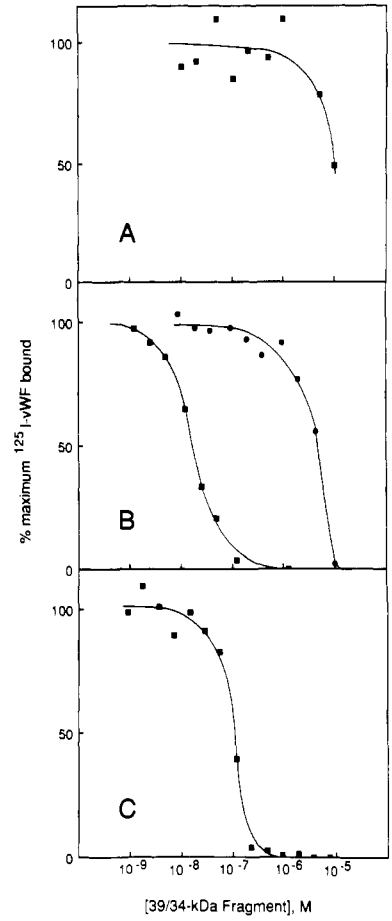

FIGURE 3: (A) Inhibition of the specific binding of ^{125}I-labeled bovine vWF (1 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) by the 39/34-kDa vWF fragment. (B) Inhibition of the (■) botrocetin-dependent or (●) ristocetin-dependent binding of ^{125}I-labeled human vWF (1 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) by the 39/34-kDa fragment. (C) Inhibition of the botrocetin-dependent binding of ^{125}I-labeled vWF (1 μg/mL) to GP Ib–IX complex coated beads (4 mg/mL) by reduced and alkylated 39/34-kDa vWF fragment. The final concentrations of botrocetin and ristocetin in the assays were 20 μg/mL and 1 mg/mL, respectively.

[2] Bovine vWF binds specifically and saturably to GP Ib–IX complex coated beads without the requirement for a modulator. The binding of bovine vWF to the GP Ib–IX complex coated beads is blocked by the same anti-GP Ib–IX complex monoclonal antibodies as for ristocetin-dependent binding of human vWF (Berndt et al., 1988) (Booth, Andrews, and Berndt, unpublished observations).

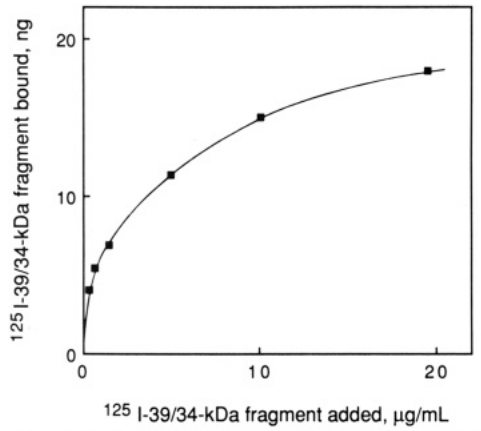

FIGURE 4: Specific binding of ^{125}I-labeled 39/34-kDa vWF fragment to GP Ib–IX complex coated beads (4 mg/mL) in the presence of 20 μg/mL of botrocetin at 22 °C.

botrocetin-dependent reconstitution assay again proved markedly more sensitive than the corresponding ristocetin assay for the analysis of the GP Ib binding domain(s) on vWF. The dispase digest of human vWF depleted of the 39/34-kDa vWF fragment (0.5 mg/mL) (the flow-through of the heparin affinity column, see Materials and Methods) had no inhibitory effect in either reconstitution assay. Reduction and alkylation of the disulfide bond between Cys-509 and Cys-695 in the 39/34-kDa fragment had little effect on the ability of the fragment to inhibit the botrocetin-dependent binding of vWF to GP Ib (part C versus part B of Figure 3, squares).

Specific binding of the ^{125}I-labeled 39/34-kDa vWF fragment to GP Ib–IX complex coated beads could be directly demonstrated in the presence of botrocetin (Figure 4), indicating that the 39/34-kDa vWF fragment also still contains the modulator site for botrocetin. We were unable to evaluate whether ristocetin also modulated binding of the 39/34-kDa fragment to purified receptor since ristocetin caused high levels of nonspecific binding ($\simeq$30%) in the reconstitution assay. For the botrocetin assay, preliminary time course experiments (not shown) indicated that binding of the 39/34-kDa vWF fragment was complete within 30 min. The botrocetin-dependent binding of the 39/34-kDa vWF fragment to GP Ib–IX complex coated beads was specific and saturable (Figure 4). This binding was strongly inhibited by anti-GP Ib–IX complex monoclonal antibodies directed against the 45-kDa N-terminal region of the α-chain of GP Ib, AK 2 and HIP 1, but was unaffected by monoclonal antibodies directed against other regions of the GP Ib–IX complex (data not shown). This inhibitory pattern is identical with that previously observed for the botrocetin-dependent and ristocetin-dependent binding of vWF to purified GP Ib–IX complex and to platelets (Berndt et al., 1988; Andrews et al., 1989) and confirms that the same region of GP Ib is involved in both the binding of vWF and the 39/34-kDa vWF fragment. SDS–polyacrylamide gel electrophoresis of the ^{125}I-labeled 39/34-kDa fragment used in the binding studies and of the labeled fragment bound to purified GP Ib–IX complex in the presence of botrocetin demonstrated that both the 39- and 34-kDa vWF fragments bound equally well to GP Ib (not shown).

Cross-Linking of the 39/34-kDa vWF Fragment to Platelets. Addition of bis(sulfosuccinimidyl) suberate (BS^3) to a preincubated mixture of ^{125}I-labeled 39/34-kDa vWF fragment and platelets led to the formation of a major cross-linked species of apparent molecular weight 220 000, nonreduced, and 190 000, reduced (Figure 5, lane 1). A second minor cross-linked species was of slightly lower apparent molecular weight: 200 000, nonreduced; 175 000, reduced.

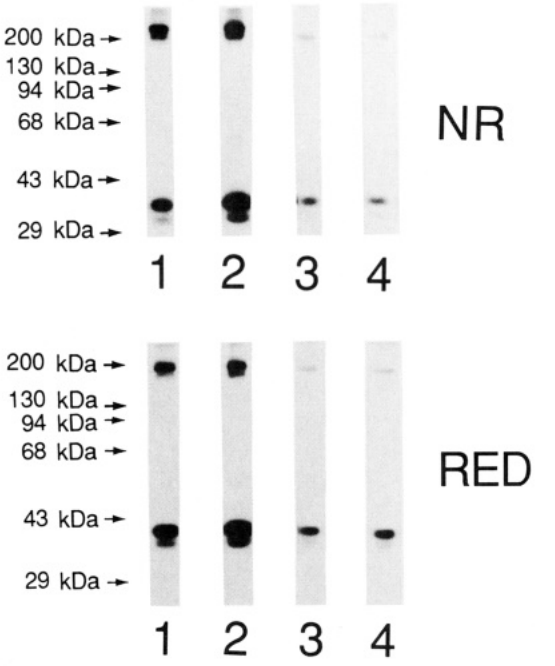

FIGURE 5: Autoradiographs of SDS–7.5% polyacrylamide gels run under nonreducing (NR, upper panel) or reducing (RED, lower panel) conditions. ^{125}I-labeled 39/34-kDa vWF fragment (20 μg/mL) was cross-linked with BS^3 (0.2 mM final concentration) for 10 min at 22 °C to normal (lanes 1 and 2) or Bernard–Soulier (lanes 3 and 4) platelets (10^9/mL) in the absence (lanes 1 and 3) or in the presence of 20 μg/mL botrocetin (lanes 2 and 4). Platelets were preequilibrated with the 39/34-kDa vWF fragment for 30 min at 22 °C prior to the addition of BS^3. Molecular weight standards are as described in the legend to Figure 1.

The relative intensities of the bands corresponding to both cross-linked species were similar to those for the platelet-associated, non-cross-linked 39-kDa fragment relative to the 34-kDa fragment of vWF (Figure 5, lane 1). This suggests that both of the cross-linked species derive from the interaction of the 39- and 34-kDa vWF fragments with a single platelet membrane protein. The following lines of evidence confirm that both cross-linked species derive from the association of the 39/34-kDa vWF fragment with the α-chain of GP Ib. First, the molecular weights of the cross-linked species were consistent with their derivation from the combination of the 39/34-kDa fragment of vWF with GP Ib. Under nonreducing conditions, GP Ib consists of two disulfide-linked subunits, GP Ibα and GP Ibβ, with a combined apparent molecular weight of 170 000 (Berndt et al., 1985b). On reduction, the α-chain of GP Ib has an apparent molecular weight of 135 000 (Berndt et al., 1985b). This decrease in apparent molecular weight on reduction is similar to that observed on reduction of the cross-linked species. Second, inclusion of botrocetin in the preincubation mixture of ^{125}I-labeled 39/34-kDa vWF fragment and platelets prior to the addition of BS^3 increased both the amount of platelet-associated, non-cross-linked 39/34-kDa fragment and the amount of the cross-linked species (Figure 5, lane 2 versus lane 1). In three separate experiments, botrocetin (20 μg/mL) increased the degree of cross-linking by 1.5–2.5-fold. This result is in accord with the evidence that botrocetin enhances the binding of the 39/34-kDa vWF fragment to purified GP Ib–IX complex (above). Third, Bernard–Soulier platelets, which genetically lack the GP Ib–IX complex (Berndt et al., 1983), failed to form both cross-linked species either in the absence or in the presence of botrocetin (Figure 5, lanes 3 and 4, respectively). A very minor cross-linked species of apparent molecular weight 190 000, nonre-

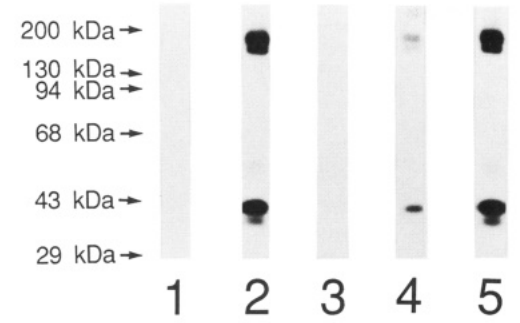

FIGURE 6: Autoradiograph of SDS–7.5% polyacrylamide gel run under reducing conditions of immunoprecipitates derived from solubilized, cross-linked platelets. Normal platelets (10^9/mL) were cross-linked with BS^3 (0.2 mM final concentration) for 10 min at 22 °C to ^{125}I-labeled 39/34-kDa vWF fragment (20 μg/mL) in the presence of 20 μg/mL botrocetin. (Lane, antibody) 1, control rabbit IgG; 2, rabbit anti-GP Ibα IgG; 3, WM 18 (negative control); 4, AK 1; 5, AK 3. Molecular weight standards are as described in the legend to Figure 1.

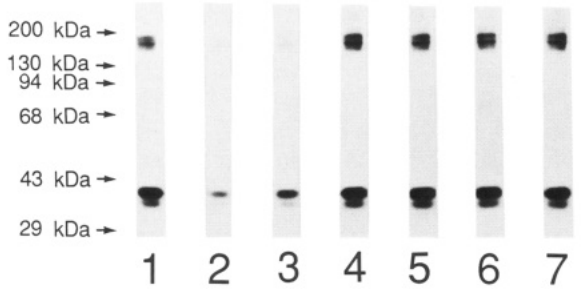

FIGURE 7: Autoradiograph of SDS–7.5% polyacrylamide gel run under reducing conditions showing the effect of anti-GP Ib–IX complex monoclonal antibodies on the BS^3-dependent cross-linking of ^{125}I-labeled 39/34-kDa vWF fragment (20 μg/mL) to normal platelets (10^9/mL). Cross-linking was performed in the presence of 20 μg/mL botrocetin for 10 min at 22 °C. Platelets were preequilibrated with monoclonal antibody (50 μg/mL final concentration) for 30 min at 22 °C prior to the addition of the 39/34-kDa fragment and botrocetin. BS^3 was added after a further 30 min at 22 °C. (Lane, monoclonal antibody) 1, WM 18 (negative control); 2, AK 2; 3, HIP 1; 4, AK 3; 5, WM 23; 6, AK 1; 7, SZ 1. Molecular weight standards are as described in the legend to Figure 1.

duced and reduced, could, however, be seen on prolonged autoradiography with the Bernard–Soulier platelets. While Bernard–Soulier platelets also lack GP V (88 kDa, reduced and nonreduced) (Clemetson et al., 1982; Berndt et al., 1983), no bands equivalent to GP V cross-linked with 39/34-kDa vWF fragment (ca. 127/122 kDa) appear even at long exposure (see Figure 5, lanes 1 and 2). Fourth, both cross-linked species were specifically immunoprecipitated by an affinity-purified rabbit anti-GP Ibα polyclonal antibody and by an anti-GP Ib–IX complex monoclonal antibody (Figure 6). The 39/34-kDa vWF fragment was coimmunoprecipitated under these conditions presumably because of its specific binding to solubilized GP Ib–IX complex (above). Finally, monoclonal antibodies directed against the 45-kDa N-terminal region of the α-chain of GP Ib, AK 2 and HIP 1, which inhibit the binding of the 39/34-kDa vWF fragment to purified GP Ib–IX complex, also inhibited the formation of both cross-linked species (Figure 7, lanes 2 and 3 relative to lane 1). Conversely, monoclonal antibodies directed against other regions of the GP Ib–IX complex neither inhibited the binding of the 39/34-kDa fragment to purified GP Ib–IX complex nor affected the formation of both cross-linked species (Figure 7, lanes 4–7 relative to lane 1).

Identification of the Region of the GP Ib–IX Complex Involved in Cross-Linking with the 39/34-kDa vWF Fragment.

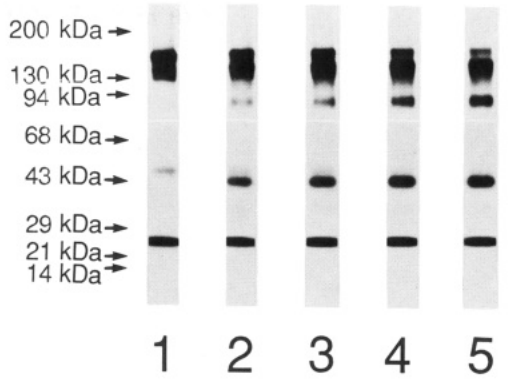

FIGURE 8: Autoradiograph of SDS–5–15% exponential gradient polyacrylamide gel run under nonreducing conditions showing the digestion of ^{3}H-labeled GP Ib–IX complex (lane 1, 200 μg/mL) with endoproteinase Lys-C (20 μg/mL final concentration) for 5 min (lane 2), 10 min (lane 3), 30 min (lane 4), or 1 h (lane 5) at 22 °C. For clarity, different exposure times are shown for the high molecular weight (>68 000) and low molecular weight (<68 000) regions. Molecular weight standards are as described in the legend to Figure 1.

In the course of other studies, we have investigated a large number of proteinases for their effect on the GP Ib–IX complex and on the 39/34-kDa vWF fragment. To further characterize the region of the GP Ib–IX complex involved in cross-linking to the 39/34-kDa vWF fragment, we chose to digest the cross-linked species with endoproteinase Lys-C since the 39/34-kDa vWF fragment is not degraded by this proteinase and since this proteinase preferentially cleaves off the N-terminal region of the α-chain of GP Ib. The time course of hydrolysis of tritium-labeled GP Ib–IX complex (200 μg/mL) by endoproteinase Lys-C is shown in Figure 8. Assignments of proteolytic fragments are based on the apparent molecular weights of the fragments under nonreduced conditions (Figure 8) and reduced conditions (not shown) and the relative labeling intensity of each of the bands (Okumura et al., 1976) and by the results of immunoprecipitation with previously defined, epitope-mapped anti-GP Ib–IX complex monoclonal antibodies (Berndt et al., 1988) (data not shown). GP Ib (170 kDa) is initially cleaved to yield an N-terminal fragment of apparent molecular weight 40 000, nonreduced and reduced, and a fragment of apparent molecular weight 150 000, nonreduced, which consists of an 100-kDa remnant of the α-chain disulfide-linked to the 25-kDa β-chain of GP Ib (and complexed with GP IX). Within 1 h, a second slower cleavage converts a small fraction of the 150-kDa fragment into macroglycopeptide (90 kDa) and a ≃30-kDa remnant of the α-chain disulfide-linked to the 25-kDa β-chain (and complexed with GP IX; membrane-associated region). Immunoprecipitation analysis indicated that identical GP Ib derived fragments were generated by endoproteinase Lys-C treatment of intact platelets (data not shown).

Treatment of 39/34-kDa vWF fragment cross-linked platelets with 10 μg/mL endoproteinase Lys-C for 1 h gave almost complete degradation of both cross-linked species and yielded two closely spaced, cross-linked fragments of apparent molecular weight ≈80 000, nonreduced, and ≈89 000, reduced (Figure 9). The proteolyzed cross-linked fragments were not platelet-associated as they were only detectable in the supernatant of endoproteinase Lys-C treated platelets (data not shown). Identical results were obtained with the platelet cross-linked species formed in the absence and presence of botrocetin (Figure 9, lanes 2 and 4, respectively). As discussed above, there are only two potential water-soluble fragments, the 40-kDa N-terminal peptide and the 90-kDa macroglyco-

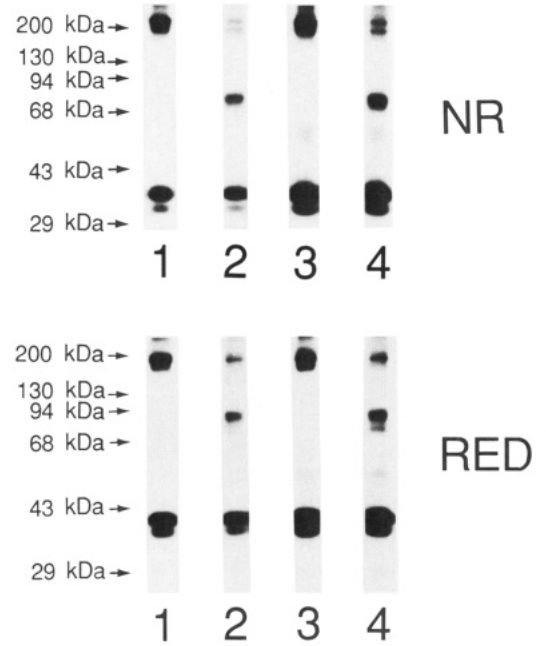

FIGURE 9: Autoradiographs of SDS–7.5% polyacrylamide gels run under nonreducing (NR, upper panel) or reducing (RED, lower panel) conditions. ^{125}I-Labeled 39/34-kDa vWF fragment (20 μg/mL) was cross-linked with BS^3 (0.2 mM final concentration) for 10 min at 22 °C to normal platelets (10^9/mL) in the absence (lanes 1 and 2) or in the presence of 20 μg/mL botrocetin (lanes 3 and 4). Platelets were preequilibrated with the 39/34-kDa vWF fragment for 30 min at 22 °C prior to the addition of BS^3. Cross-linked platelets shown in lanes 2 and 4 were treated with endoproteinase Lys-C (10 μg/mL final concentration) for 1 h at 22 °C prior to direct solubilization of the whole digest by addition of electrophoresis sample buffer. Molecular weight standards are as described in the legend to Figure 1.

peptide, that can be formed by treatment of the GP Ib–IX complex on intact platelets with endoproteinase Lys-C. Of these two possibilities, two lines of evidence demonstrate that the proteolyzed cross-linked fragments shown in Figure 9 (lanes 2 and 4) must be derived from the cross-linking of the ^{125}I-labeled 39/34-kDa vWF fragment to the 40-kDa N-terminal domain of GP Ib. First, under the experimental conditions employed for proteolysis, the N-terminal peptide fragment is the predominant hydrolytic species with only a small degree of formation of macroglycopeptide (cf. Figure 8 and data not shown). Second, the molecular size of the proteolyzed cross-linked fragments (80 kDa, nonreduced, and 89 kDa, reduced) is fully consistent with the cross-linking of the vWF fragment (35/31 kDa, nonreduced, 39/34 kDa, reduced) to the N-terminal region of GP Ib (40 kDa, nonreduced, and reduced), but is not consistent with cross-linking involving the 90-kDa macroglycopeptide.

Discussion

In this paper, we have identified and purified a GP Ib–IX complex binding, monomeric 39/34-kDa dispase fragment of vWF extending from Leu-480/Val-481 to Gly-718 and have provided direct evidence that the binding domain for vWF on the GP Ib–IX complex resides within the N-terminal region of the α-chain of GP Ib. A comparison of the dispase cleavage sites on vWF that generate the 39/34-kDa fragment indicates that they are remarkably similar (Table IV). Both the N-terminal and C-terminal cleavages occur within hydrophobic sequences rich in glycine, leucine, proline, and valine. Two of the three cleavages involve the scission of a Gly–Leu peptide bond, and all three cleavages occur four to five amino acid residues downstream from an O-linked glycosylation site

Table IV: Dispase Cleavage Sites on vWF Giving Rise to the 39/34-kDa vWF Fragment

cleavage site	amino acid sequence[a]
N-terminus:	-Gln$_{475}$-Glu-Pro-Gly-Gly-Leu-Val-Val-Pro-Pro-Thr$_{485}$-
	-Gln$_{475}$-Glu-Pro-Gly-Gly-Leu-Val-Val-Pro-Pro-Thr$_{485}$-
C-terminus:	-Thr$_{714}$-Val-Gly-Pro-Gly-Leu-Leu-Gly-Val-Ser-Thr$_{724}$-

[a] Based on the primary amino acid sequence of human vWF, where underlined residues are sites of O-glycosylation (Titani et al., 1986).

(Titani et al., 1986). Detailed structural analysis indicated that both the 39- and 34-kDa fragments had identical amino acid sequences, from Leu-480/Val-481 to Gly-718. The ragged cleavage at the N-terminus of both fragments therefore cannot account for the weight difference between the predominant 39-kDa fragment and the minor 34-kDa fragment, suggesting that the molecular difference must be due to heterogeneity in glycosylation. The 39/34-kDa fragment contains six O-linked glycosylation sites at Thr-485, Thr-492, Thr-493, Ser-500, Thr-705, and Thr-714 (Titani et al., 1986). Since the peptide backbone, Leu-480/Val-481–Gly-718, contributes $\simeq$27 kDa to the fragment, this suggests that one or more of these sites are not glycosylated in the minor 34-kDa fragment. In contrast, Fujimura et al. (1986) reported that the heterogeneity in the 52/48-kDa tryptic fragment of vWF, Val-449–Lys-728, was resolved by treatment with endoglycosidase F, suggesting that the heterogeneity was due to the N-linked glycosylation site at Asn-468 (Titani et al., 1986). The reason for the discrepancy between our study and that of Fujimura et al. (1986) is unclear.

The sequence Leu-480/Val-481–Gly-718 of the vWF subunit contains several interesting structural features. In the present paper, detailed chemical analysis of the purified 39/34-kDa vWF fragment has provided secure evidence for an intramolecular disulfide bond between Cys-509 and Cys-695. Marti et al. (1987) have previously provided evidence for the presence of a disulfide bond between Cys-509 and Cys-695, but could not distinguish whether this bond was inter- or intramolecular. The definition of this disulfide bond within the structural repeat domain A1 of vWF potentially indicates the presence of an intramolecular disulfide bond involving the conserved residues Cys-923 and Cys-1109 within the homologous A3 domain, while the A2 domain lacks a conserved Cys residue corresponding to Cys-509 (Shelton-Inloes et al., 1986). In the A1 domain, this disulfide bond brings into proximity the sequences Leu-480–Cys-509 and Cys-695–Gly-718, which between them contain six O-linked glycosylation sites. There are no positively charged amino acids in these two sequences. Since the O-linked carbohydrate is probably sialated (Federici et al., 1984), this portion of the 39/34-kDa vWF fragment is strongly negatively charged. In contrast, the sequence within the disulfide loop, Ser-510–Leu-694, contains predominantly positively charged amino acid residues (30 Arg, Lys; 22 Glu, Asp). The 39/34-kDa fragment is also unusual in that it contains three distinct binding domains within a linear sequence of 239 amino acids (or less). Previous studies have shown that a reduced and alkylated 52/48-kDa vWF fragment extending from Val-449 to Lys-728 contains binding sites for heparin (Fujimura et al., 1987), collagen (Pareti et al., 1986), and GP Ib (Fujimura et al., 1986). Inhibition studies with monoclonal antibodies directed against epitopes within this sequence have indicated that each of these

binding sites is distinct (Fujimura et al., 1986; Pareti et al., 1986). In the present study, we have refined the sequence that contains these binding sites to a 39/34-kDa vWF fragment that extends from Leu-480/Val-481 to Gly-718 of the primary amino acid sequence of vWF (Titani et al., 1986). The 39/34-kDa vWF fragment retained the heparin-binding site since it could be specifically isolated from a dispase digest of native vWF by heparin affinity chromatography. Since heparin typically binds to small, highly positively charged peptide sequences (Hirose et al., 1987), the heparin site on the 39/34-kDa vWF fragment must reside within the intramolecular disulfide loop. The 39/34-kDa fragment also contained a collagen-binding site since it inhibited the specific binding of intact vWF to equine achilles tendon collagen fibrils. A collagen-binding site has been shown to reside on a cyanogen bromide peptide, Glu-542–Met-622, within the intramolecular disulfide loop (Roth et al., 1986). Finally, the 39/34-kDa sequence still bound to GP Ib since this fragment inhibited the botrocetin-dependent and ristocetin-dependent binding of intact vWF to purified GP Ib–IX complex.

The similarity of the IC_{50} values for the inhibition of the botrocetin-dependent binding of ^{125}I-labeled vWF to purified GP Ib–IX complex by vWF (IC_{50} = 12 nM, calculation based on the concentration of 275-kDa subunit; Andrews et al., 1989) and by the 39/34-kDa fragment (IC_{50} = 15 nM) suggests that the 39/34-kDa fragment encompasses the entire GP Ib recognition site. In support of this, the dispase digest of native vWF specifically depleted of the 39/34-kDa fragment has no inhibitory effect on the botrocetin-dependent binding of vWF to purified GP Ib–IX complex. These results are consistent with the direct demonstration of botrocetin-dependent binding of the 39/34-kDa vWF fragment to purified GP Ib–IX complex. This also confirms that the botrocetin-binding site on the 52/48-kDa vWF fragment (Andrews et al., 1989) also resides within the sequence Leu-480/Val-481–Gly-718. Mohri et al. (1988) have recently demonstrated that two synthetic peptides corresponding to the sequence Cys-474–Pro-488 and Leu-694–Pro-708 inhibit both ristocetin-dependent vWF binding and asialo-vWF binding to platelets. These two peptide sequences would be brought into proximity by the Cys-509–Cys-695 disulfide bond. Our data suggest that the sequence Cys-474–Gly-479, however, is not critical for GP Ib binding.

Unlike native vWF, which has an absolute requirement for the presence of a modulator such as botrocetin or ristocetin for binding to GP Ib (Berndt et al., 1988; Andrews et al., 1989), the 39/34-kDa fragment could spontaneously bind to GP Ib since this fragment inhibited asialo-vWF-induced platelet agglutination and the binding of bovine vWF to purified GP Ib–IX complex. We have directly confirmed the spontaneous and botrocetin-mediated binding of the 39/34-kDa vWF fragment to the N-terminal domain of the α-chain of GP Ib on intact platelets by covalent cross-linking analysis. Cross-linking studies employed the monomeric 39/34-kDa fragment of vWF that includes the GP Ib binding domain. Radioiodinated fragment was incubated with washed platelets in the absence or presence of botrocetin prior to the addition of the membrane-impermeable cross-linking agent bis(sulfosuccinimidyl) suberate (BS^3). SDS–polyacrylamide gel electrophoresis demonstrated the presence of two cross-linked bands, a major species (220 kDa, nonreduced; 190 kDa reduced) and a minor species (200 kDa, nonreduced; 175 kDa reduced). As expected from our reconstitution studies (Berndt et al., 1988; Andrews et al., 1989; this study), both these species were found to represent cross-linked products derived from combination of the 39/34-kDa vWF fragment with the α-chain of GP Ib. This was established by analysis of the molecular weights of the cross-linked species relative to the molecular weight characteristics of GP Ib, by the botrocetin-dependent enhancement of cross-link formation, by the absence of these cross-linked products in corresponding experiments with Bernard–Soulier syndrome platelets that genetically lack the GP Ib–IX complex (Berndt et al., 1983), by the specific immunoprecipitation of both cross-linked species by polyclonal and monoclonal anti-GP Ib antibodies, and by the inhibition of cross-link formation by some anti-GP Ib monoclonal antibodies.

In an extension of these experiments, cross-linked platelets were digested with endoproteinase Lys-C to define the region of GP Ib involved in binding of the 39/34-kDa vWF fragment. Under the conditions used for this experiment, endoproteinase Lys-C cleaves GP Ib predominantly into two fragments, a 40-kDa N-terminal fragment and a 150-kDa fragment (nonreduced) consisting of a 100-kDa α-chain remnant disulfide-linked to the 25-kDa β-chain of GP Ib. The average molecular weight of the cross-linked species after endoproteinase Lys-C digestion was 80 000, nonreduced, and 89 000, reduced, indicating that the 39/34-kDa vWF fragment was exclusively associated with the N-terminal region of the α-chain of GP Ib. This evidence directly confirms previous evidence that it is this region of the GP Ib molecule that is involved in vWF binding. First, GP Ib–vWF interaction is only inhibited by antibodies directed against the N-terminal region of the GP Ib–IX complex (Wicki & Clemetson, 1985; Handa et al., 1986; Berndt et al., 1988; Andrews et al., 1989). In this study, a similar correlation was found in that only monoclonal antibodies directed against the N-terminal domain of GP Ib inhibited cross-link formation. Second, selective proteolytic removal of this region of GP Ib on intact platelets by human neutrophil elastase abolishes the ability of the receptor to bind vWF (Brower et al., 1985; Wicki & Clemetson, 1985). Third, we have shown that the purified 45-kDa N-terminal tryptic fragment of GP Ib completely inhibits the botrocetin-dependent binding of vWF to purified GP Ib–IX complex (Andrews et al., 1989). Finally, Vicente et al. (1988) have shown the ristocetin-dependent binding of purified 45-kDa N-terminal fragment of GP Ib to immobilized vWF, although they also found that both the 45-kDa N-terminal fragment and the macroglycopeptide fragment of GP Ib inhibited the ristocetin-dependent binding of vWF to platelets.

Rotary shadowing electron microscopy of purified GP Ib–IX complex indicates that the N-terminal region of GP Ib potentially extends up to 60 nm from the surface of the platelet membrane, further than any other known platelet membrane protein (Fox et al., 1988). When platelets in the circulation contact exposed vascular subendothelium, it is therefore this region of the GP Ib–IX complex that is initially exposed to immobilized vWF in the subendothelial matrix. That this region of the GP Ib–IX complex contains the vWF-binding domain may therefore be of major physiological importance with respect to the mechanism of platelet adhesion. The combined data of these two papers (Andrews et al., 1989; this study) localize vWF–GP Ib interaction to the sequences Leu-480–Gly-718 of vWF and His-1–Lys-297 of GP Ib. Current studies are under way to further define the sequences of vWF and GP Ib involved in their interaction.

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Registry No. vWF, 109319-16-6; botrocetin, 85537-36-6; ristocetin, 1404-55-3.

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