Mocarhagin, a Novel Cobra Venom Metalloproteinase, Cleaves the Platelet von Willebrand Factor Receptor Glycoprotein Ibα. Identification of the Sulfated Tyrosine/Anionic Sequence Tyr-276−Glu-282 of Glycoprotein Ibα as a Binding Site for von Willebrand Factor and α-Thrombin†

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ABSTRACT: Platelet adhesion to the subendothelium is the initiating event in hemostasis and thrombosis and involves the binding of von Willebrand factor (vWF) by the platelet membrane glycoprotein (GP) Ib−IX complex. The α-chain of GP Ib contains binding sites for both vWF and α-thrombin within a 45-kDa N-terminal tryptic fragment. In the present study, we have further delineated these sites using smaller proteolytic fragments and functional antibodies. Mocarhagin, a cobra venom metalloproteinase, generates the fragment His-1−Glu-282, while cathepsin G, a neutrophil granule serine protease, generates a slightly smaller fragment, His-1−Leu-275. His-1−Glu-282 was as effective as intact soluble GP Ibα (glycocalicin) in inhibiting botrocetin-dependent binding of vWF to washed platelets (IC_{50} ∼0.3 μM), whereas His-1−Leu-275 was an order of magnitude less effective (IC_{50} ∼3 μM). Residues Tyr-276−Glu-282 (YDYYPEE) are part of an anionic region homologous to thrombin-binding molecules such as hirudin. In ligand blot analysis, thrombin blotted the His-1−Glu-282 fragment, but not His-1−Leu-275. The three tyrosine residues within Tyr-276−Glu-282 meet the consensus criteria for O-sulfation. A method was developed to distinguish O-sulfated from nonsulfated tyrosine residues based on differences in the UV absorbance spectra. Residues Tyr-276−Glu-282 were isolated from glycocalicin by proteolysis with mocarhagin and cathepsin G. Ion spray mass spectrometry confirmed that Tyr-278 and Tyr-279 were O-sulfated to at least 90%, whereas Tyr-276 was only ∼50% O-sulfated. Four anti-GP Ibα monoclonal antibodies (SZ2, ES85, C34, and VM16d) were found to be modulator-specific, strongly inhibiting botrocetin-dependent binding of vWF, but having less or no effect on ristocetin-dependent vWF binding. These antibodies also inhibited the binding of thrombin to fixed platelets. Immunoprecipitation with GP Ibα fragments defined the epitopes for these antibodies as SZ2 (Tyr-276−Glu-282), ES85 (Asp-283−Arg-293), C34 (His-1−Glu-282), and VM16d (His-1−Leu-275). An antibody which inhibited ristocetin-dependent, as well as botrocetin-dependent, vWF binding but had no effect on thrombin binding (AK2) had an epitope within His-1−Leu-275. These findings indicate that the sulfated tyrosine/anionic GP Ibα residues Tyr-276−Glu-282 are important for the binding of thrombin and the botrocetin-dependent binding of vWF, but that vWF also interacts with residues within His-1−Leu-275.

The platelet membrane glycoprotein (GP)[1] Ib−IX complex mediates platelet adhesion to damaged blood vessels at high shear, the first step in hemostasis and thrombosis, by recognition of the multimeric adhesive glycoprotein von Willebrand factor (vWF) bound to the subendothelial matrix (Booth et al., 1990). The interaction of vWF with the GP Ib−IX complex is dependent upon an activation event or conformational change in vWF structure when bound to matrix and/or exposure to shear. This process is mimicked *in vitro* by specific modulators such as ristocetin and botrocetin that bind to vWF (Andrews et al., 1989a; Berndt et al., 1992) or by desialation of vWF (De Marco & Shapiro, 1981). Ristocetin is a bacterial glycopeptide that binds to anionic proline-rich amino acid sequences flanking the Cys-509−Cys-695 disulfide loop of the GP Ib−IX-binding domain of vWF, whereas botrocetins are snake venom proteins that bind to predominantly positively-charged sequences within the disulfide loop (Sugimoto et al., 1991; Berndt et al., 1992). We have previously identified a sequence of vWF, Asp-514−Glu-542, that binds GP Ib−IX and proposed that ristocetin and botrocetin modulate vWF by exposing this sequence by altering electrostatic interactions within the GP Ib−IX-binding domain (Berndt et al., 1992). A heparin-binding sequence within this same domain (Tyr-565−Ala-587) (Sobel et al., 1992) may be involved in this modulation of vWF (Andrews et al., 1995). The GP Ib−IX complex also contains a high-affinity binding site for α-thrombin that facilitates platelet activation (Coughlin et

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[1] Abbreviations: BSA, bovine serum albumin; Da, dalton; DFP, diisopropyl fluorophosphate; GP, glycoprotein; IC_{50}, concentration of inhibitor giving 50% inhibition; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; vWF, von Willebrand factor.

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al., 1992). Binding of vWF to GP Ib−IX also induces platelet activation (Kroll et al., 1991), which may involve the interaction of the cytoplasmic domain of the GP Ib−IX complex with the platelet cytoskeleton or phospholipase A_2 (Fox, 1993; Du et al., 1994). While a discrete linear sequence of vWF has been identified that mediates binding to the GP Ib−IX complex (Berndt et al., 1992), structure−function relationships of the GP Ib−IX complex remain to be fully defined. Our ultimate aim in the present study was to identify specific amino acid sequences involved in vWF and α-thrombin binding.

GP Ib−IX is a heterotrimeric transmembrane complex comprised of the disulfide-linked ∼130-kDa α-chain and ∼25-kDa *β*-chain of GP Ib and noncovalently associated GP IX (∼22 kDa). GP Ibα, GP Ib*β*, and GP IX are all members of the leucine-rich protein family and contain conserved ∼24 residue leucine-rich sequences numbering 7, 1, and 1, respectively, as well as conserved N- and C-terminal flanking sequences (Booth et al., 1990; López, 1994). GP V, a transmembrane protein associated with the GP Ib−IX complex in the platelet membrane and cleaved during thrombin stimulation of platelets, contains 15 leucine-rich repeats (Hickey et al., 1993; Lanza et al., 1993). The α-chain of GP Ib consists of three distinct structural domains: an ∼40-kDa globular N-terminal peptide domain containing the seven leucine-rich repeats and Cys-bonded flanking sequences; a highly glycosylated mucin-like macroglycopeptide domain; and a membrane-associated C-terminal region that contains the disulfide bridge to GP Ib*β* and the transmembrane and cytoplasmic sequences (Fox et al., 1988; López, 1994). The vWF-binding site has been clearly defined to reside within the N-terminal 293 amino acids of GP Ibα (Handa et al., 1986; Berndt et al., 1988; Vicente et al., 1988; Andrews et al., 1989b). Additional studies with synthetic peptides have identified sequences Ser-251−Glu-285 (Vicente et al., 1990) and Asp-235−Lys-262 (Katagiri et al., 1990) that may participate in vWF binding. However, these peptides (IC_{50} ∼0.5 mM) are 2−4 orders of magnitude less effective at inhibiting vWF binding to platelets than the ∼45-kDa tryptic fragment derived from native GP Ibα (His-1−Arg-293).

A striking feature of this region of GP Ibα is the presence of a cluster of negatively charged amino acids between Asp-269 and Asp-287, DEGDTDLYDYYPEEDTEGD (López et al., 1987; Titani et al., 1987). This sequence contains three tyrosine residues, Tyr-276, Tyr-278, and Tyr-279, that meet the consensus for O-sulfation by the golgi processing enzyme tyrosylprotein sulfotransferase (Nierhs & Huttner, 1990), and recent reports confirm sulfation of these residues, at least when the GP Ib−IX complex is expressed in CHO cells (Dong et al., 1994; Marchese et al., 1995). While these studies suggest that sulfation is important for vWF (Dong et al., 1994; Marchese et al., 1995) and α-thrombin recognition (Marchese et al., 1995), functional data involving point mutations of these tyrosine and adjacent residues are less easy to interpret since monoclonal antibody-binding results suggest that site-directed mutagenesis in this region may be affecting conformation (Marchese et al., 1995).

In this study, we have used an alternate approach to address the function of the negative charge/sulfated tyrosine cluster in GP Ibα, an approach which has also allowed assessment of the sulfation status of these tyrosine residues in the native receptor isolated from circulating platelets. Fundamental to these studies has been our identification of a novel cobra venom metalloproteinase, termed mocarhagin, which selectively cleaves between Glu-282 and Asp-283 on GP Ibα as the sole detectable event on the human platelet surface. Our approach was to examine the functional effect of N-terminal GP Ibα fragments that encompass the entire negative charge/sulfated tyrosine cluster (trypsin: His-1−Arg-293; Titani et al., 1987), two-thirds (mocarhagin: His-1−Glu-282) and one-third of the cluster (cathepsin G: His-1−Leu-275; Pidard et al., 1994), and, in addition, to evaluate the functional effect of monoclonal antibodies defined to interact with this region by analysis of immunoreactivity with these fragments. These studies suggest that the human platelet GP Ibα sequence, Tyr-276 to Glu-282, YDYYPEE, containing either two or three sulfated tyrosine residues is an important determinant for both vWF and α-thrombin binding.

MATERIALS AND METHODS

Materials. *Naja mocambique mocambique* and *Bothrops jararaca* venom, human thrombin, bovine serum albumin (BSA), and diisopropyl fluorophosphate (DFP) were purchased from Sigma, St. Louis, MO. Heparin−Sepharose CL-6B, Sepharose CL-6B, and Sephadex G200SF were bought from Pharmacia, Uppsala, Sweden; trypsin (bovine pancreas) from Calbiochem, San Diego, CA; and chloramine T from Riedel-de Häen, Selze, Germany. Ristocetin was purchased from Boehringer-Mannheim, West Germany, and botrocetin was purified as previously described (Andrews et al., 1989a). Sodium [^{125}I]iodide was purchased from Australian Radioisotopes, Lucas Heights, Australia. Lyophilized human factor VIII concentrate was a kind gift of the Commonwealth Serum Laboratories, Melbourne, Australia. Purified cathepsin G was a kind gift from Dr. P. Hogg, Sydney, Australia. Nonsulfated cholecystokinin 26−29 (CCK: H-Asp-Tyr-Met-Gly-OH), sulfated cholecystokinin 26−29 [CCK-S: H-Asp-Tyr(SO_3H)-Met-Gly-OH], and trifluoroacetic acid (TFA) were obtained from Auspep, Parkville, Australia. Acetonitrile was purchased from Waters, Lane Cove, Australia, and HPLC grade water from Mallinckrodt, Paris, NY.

Purification and Labeling of vWF and Asialo-vWF. vWF was prepared from lyophilized human factor VIII concentrates as previously described (Andrews et al., 1989a), and iodinated with Na^{125}I using Iodobeads (Pierce, Rockford, IL) according to the manufacturer's instructions. Asialo-vWF was prepared after the method of DeMarco and Shapiro (1981) with minor modification (Andrews et al., 1989a).

Purification of Mocarhagin. The purification of mocarhagin from *Naja mocambique mocambique* venom based on the heparin-binding properties of cobra lectins (Ogilvie & Gartner, 1984) has been previously reported (De Luca et al., 1995). DFP-treated mocarhagin was prepared by incubating 250 *μ*g of mocarhagin in 1 mL of 0.01 M Tris, 0.15 M sodium chloride, pH 7.4 (TS buffer), with 8 mM (final concentration) DFP for 1 h at 22 °C, followed by dialysis against TS buffer.

Purification and Proteolytic Digestion of Glycocalicin. Glycocalicin, a soluble ∼135-kDa proteolytic fragment comprising the extracytoplasmic portion of GP Ibα, was prepared from human platelet lysates as described (Andrews et al., 1989a) and iodinated where necessary using Na^{125}I and Iodobeads. For trypsin digestion, glycocalicin in TS

buffer was incubated with 5 μg/mL trypsin (final concentration) for 4 h at 22 °C, and the reaction was stopped by addition of an equal volume of TS buffer containing 100 μg/mL soybean trypsin inhibitor, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 200 μg/mL leupeptin. For mocarhagin digestion, glycocalicin in TS buffer containing 1 mM calcium chloride was incubated with 10 μg/mL mocarhagin (final concentration) for 1 h at 22 °C, and digestion was stopped by adding EDTA to 0.01 M. For cathepsin G digestion, glycocalicin in TS buffer containing 1 mM calcium chloride was incubated with 5 μg/mL cathepsin G (final concentration) for 4 h at 22 °C, and digestion was stopped by making the sample 1 mM in PMSF. For some experiments, glycocalicin fragments were purified by gel filtration on Sephadex G200, and their purity was confirmed by SDS−polyacrylamide gel electrophoresis. For determining mocarhagin and cathepsin G cleavage sites in synthetic peptides based on the GP Ibα sequence, 0.5 mg/mL peptide (Chiron Mimotopes, Clayton, Australia) in 0.05 M sodium phosphate, 1 mM calcium chloride, pH 7.6, was digested with 10 μg/mL protease for 4 h at 22 °C. The sample was diluted 1/5 by 0.1% (v/v) TFA in methanol and centrifuged at 8750*g* to remove precipitated protein, and peptide fragments in the supernatant were separated by reverse-phase HPLC on a Waters 510 system equipped with a NovaPak C18 column eluted at 1 mL/min with a linear 2.1−70% (v/v) acetonitrile gradient in 0.08% (v/v) TFA.

Cleavage of Glycocalicin by Mocarhagin. The proteolytic activity of mocarhagin was characterized by equilibrating the protease with purified glycocalicin (350 μg/mL) in TS buffer containing 1 mM calcium chloride at 22 °C, and analyzing the digests by SDS−polyacrylamide gel electrophoresis. The metal ion dependence of proteolysis was determined in an assay including either 10 mM EDTA without added divalent cations, or 1 mM EDTA plus 10 mM calcium chloride, magnesium chloride, or zinc chloride. The effect of protease inhibitors on glycocalicin proteolysis was assessed in the presence of 1 mM calcium chloride by including either 0.2 mM phenylmethanesulfonyl fluoride, 8 mM DFP, 1 mM benzamidine, 1 μM pepstatin, 0.1 mM dichloroisocoumarin, 0.1 mg/mL leupeptin, 0.1 mM iodoacetamide, or 0.2 mg/mL aprotinin (all final concentrations). Inhibition was also measured in the presence of heparin at a final concentration of 100 μg/mL.

Monoclonal Antibodies. All murine monoclonal antibodies were of the immunoglobulin G (IgG) class and were purified and characterized as previously described (Ruan et al., 1987). Several of the antibodies were generous gifts from Dr. C. Ruan, Suzhou, China (SZ2), Dr. J. Miller, Syracuse, NY (C34), and Dr. Q. Sun, Beijing, China (ES85). AK2 (Berndt et al., 1988), SZ2 (Ruan et al., 1987), and VM16d (Mazurov et al., 1991) are all directed against the N-terminal region of the α-chain of GP Ib. SZ1 (Du et al., 1987) recognizes a complex-specific epitope on the GP Ib−IX complex. AK7 is an irrelevant control antibody directed against the human integrin α2 subunit (Gamble et al., 1993). FMC25 recognizes an epitope on GP IX (Berndt et al., 1988). Monoclonal antibodies were radiolabeled with $Na^{125}I$ using the chloramine T method (Berndt et al., 1985).

N-Terminal Sequencing. Protein samples for N-terminal sequencing were dialyzed against 0.02% SDS and concentrated by vacuum centrifugation (Speedvac concentrator, Savant). N-Terminal sequence was determined using an Applied Biosystems Model 470A protein sequencer equipped with an on-line Model 120A phenylthiohydantoin analyzer. Polybrene was used as a carrier.

Platelet Preparation, Surface Labeling, and Aggregation. Citrated platelet-rich plasma and washed platelets were prepared as previously described (Booth et al., 1984). Platelet surface membrane radiolabeling was performed with ^{125}I using a lactoperoxidase method (Berndt et al., 1985) and with ^{3}H using a periodate/sodium borohydride method (Berndt & Phillips, 1981). For platelet aggregation studies, citrated platelet-rich plasma was stirred at 900 rpm at 37 °C in a Whole Blood Lumiaggregometer (Chronolog, Havertown, PA).

Ristocetin- and Botrocetin-Dependent Binding of ^{125}I-Labeled vWF to Platelets. The effect of mocarhagin and DFP-treated mocarhagin on ristocetin- or botrocetin-dependent binding of ^{125}I-labeled vWF to washed platelets was studied essentially as previously described (Berndt et al., 1988). The assay incorporated 1×10^8 platelets, 0.1% (w/v) BSA, either 1.5 mg/mL ristocetin or 50 μg/mL botrocetin, and 1 μg/mL ^{125}I-labeled vWF in a final volume of 200 μL of TS buffer containing 1 mM calcium chloride. Platelets were pretreated with 5 μg/mL (final concentration) mocarhagin or DFP-treated mocarhagin for 10 min at 22 °C prior to their addition to the assay. After a 30 min incubation at 22 °C, two 80 μL aliquots were centrifuged at 8750*g* for 3 min, the supernatants were carefully aspirated, and the ^{125}I-labeled vWF associated with the pellets was measured in a γ counter. The effect of monoclonal antibodies on the ristocetin- and botrocetin-dependent binding of ^{125}I-labeled vWF to platelets was examined using the same method except that platelets were preincubated with monoclonal antibody at a final concentration of 50 μg/mL for 5 min at 22 °C. Similarly, for studies on the effect of GP Ibα fragments on the binding of ^{125}I-labeled vWF to platelets, glycocalicin and the trypsin-, mocarhagin-, and cathepsin G-derived N-terminal GP Ibα fragments (2×10^{-10} to 4×10^{-6} M) were included in the routine assay.

Binding of ^{125}I-Labeled Anti-GP Ib−IX Monoclonal Antibodies to Platelets. The effect of mocarhagin on antibody binding was assessed using the method of Berndt et al. (1985), with minor modifications. Platelet-rich plasma was pretreated with 5 μg/mL mocarhagin for 10 min at 22 °C; then 100 μL was added to various concentrations of ^{125}I-labeled antibodies in TS buffer in a final volume of 150 μL. Some assays also contained 10 mM EDTA to assess the divalent cation-dependence, or a 50-fold excess of unlabeled monoclonal antibody to determine nonspecific binding. After 30 min at 22 °C, two 50 μL aliquots were overlaid onto 0.5 mL of 25% (w/v) sucrose in TS buffer and centrifuged at 8750*g* for 3 min. ^{125}I-Labeled antibody associated with the pellet was measured in a γ counter.

Binding of ^{125}I-Labeled Thrombin to Platelets. Binding of ^{125}I-labeled thrombin to platelets was studied with platelets that were fixed using 1% (w/v) paraformaldehyde in 0.01 M phosphate, 0.15 M sodium chloride, and 0.001 M EDTA, pH 7.4, for 30 min at 22 °C and washed 3 times in TS buffer. α-Thrombin was radiolabeled with $Na^{125}I$ (1 mCi of $Na^{125}I$/100 units of thrombin) using chloramine T (Berndt et al., 1985) and separated from free iodine by gel filtration on Sephadex G25.

Ligand Blotting with ^{125}I-Labeled Thrombin. Nitrocellulose strips with electrotransferred proteins were blocked

with 5% (w/v) BSA in blotting buffer (0.05 M Tris, 0.2 M sodium chloride, pH 7.4) for 3 h at 22 °C, and incubated with ^{125}I-labeled thrombin in blotting buffer containing both 5% (w/v) BSA and 8.8 mg/mL poly(ethylene glycol) 8000 for 16 h at 4 °C. Blots were washed 3 times in TS buffer and air-dried for autoradiography.

Analysis of Tyrosine Sulfation. HPLC samples were run on a Hewlett Packard HP 1090 liquid chromatograph with data analysis performed on a HP 79994A HPLC workstation. Diode array detection allowed spectral monitoring at 215, 260, and 280 nm. Buffer A comprised 0.1% (v/v) TFA in HPLC grade water, and buffer B, 0.08% (v/v) TFA, 70% (v/v) acetonitrile; the standard gradient used was a linear 30 min gradient from 97% A and 3% B to 100% B. Unless otherwise specified, a C18 Hypersil ODS 4 × 540 mm column was used for HPLC runs, at a flow rate of 0.5 mL/min. Spectroscopic analysis of CCK and CCK-S was also performed on a Hitachi 220A double-beam spectrophotometer, using 1 cm quartz cuvettes. The analysis of residues Tyr-276−Glu-282 was performed by sequentially digesting purified glycocalicin (1 mL at 720 μg/mL in TS buffer containing 0.001 M calcium chloride) with 5 μg/mL cathepsin G for 2 h at 22 °C and then adding 10 μg/mL mocarhagin for a further 2 h at 22 °C. The fragment Asp-277−Glu-282 was generated by simultaneous digestion with cathepsin G and mocarhagin for 2 h under equivalent conditions. The peptide digest was separated from intact glycocalicin and proteases by centrifugation in a Centrifree micropartition system (Amicon, Danvers, MA) at 1000*g* for 7 min. The ultrafiltrate containing the peptide digest was loaded directly onto HPLC, and fractions were collected for N-terminal amino acid sequencing and ion spray mass spectrometry (performed by Chiron Mimotopes Peptide Systems using an API III Sciex instrument, run in the negative ion mode).

Analytical Methods. SDS−polyacrylamide gel electrophoresis, Western blotting, immunoprecipitation, and autoradiography were performed as previously described (Laemmli, 1970; Berndt et al., 1985).

RESULTS

Purification of Mocarhagin. One of the striking features of the primary structure of GP Ibα is the presence immediately N-terminal to the sialomucin core of a cluster of negatively charged aspartate and glutamate residues containing three tyrosine residues (López et al., 1987; Titani et al., 1987) that meet the consensus for sulfation by the golgi tyrosylprotein sulfotransferase (Niehrs & Huttner, 1990). Our approach to analysis of the function of this negative charge/sulfated tyrosine cluster was to analyze N-terminal fragments of GP Ibα generated by proteases which selectively cleaved through this region. A key protease in our analysis was a metalloproteinase, termed mocarhagin, which we identified in the venom of the Mozambiquan spitting cobra, *Naja mocambique mocambique*, by its ability in crude venom to abolish vWF-dependent platelet agglutination. Mocarhagin had an apparent molecular mass by SDS−polyacrylamide gel electrophoresis of ~55 kDa under nonreducing and reducing conditions (Figure 1). Typically, 1−2 mg of purified mocarhagin was obtained from 0.5 g of lyophilized venom. N-Terminal sequencing of purified mocarhagin was achieved up to 24 residues (Figure 2). The early cycles showed several peaks that are suggestive of a ragged N-terminus. The N-terminal sequence showed significant homology to members of the *Viperidae* snake venom metalloproteinase−disintegrin family (Figure 2) (Gould et al., 1990), suggesting that mocarhagin, although from the *Elapidae* family, was also a metalloproteinase.

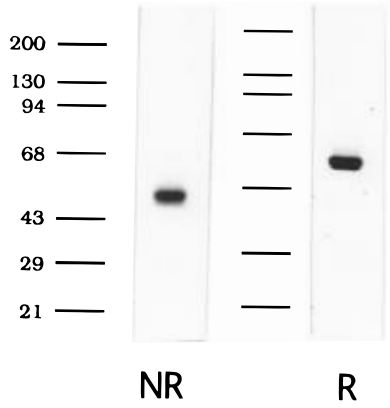

FIGURE 1: SDS−5−20% exponential gradient polyacrylamide gel electrophoresis of purified mocarhagin under nonreducing (NR) and reducing (R) conditions stained with Coomassie blue. Molecular mass standards are myosin (200 kDa), β-galactosidase (130 kDa), phosphorylase B (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (21 kDa).

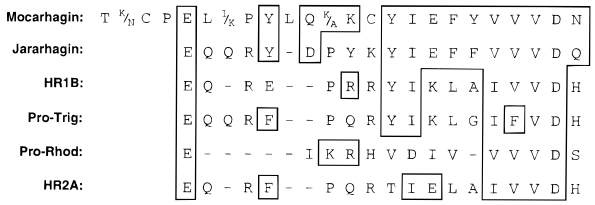

FIGURE 2: Comparison of the N-terminal amino acid sequence determined for cobra venom mocarhagin with metalloprotease−disintegrins from viper venoms. Jararhagin is a 52-kDa protease purified from *Bothrops jararaca* (Paine et al., 1992); HR1B and HR2A are hemorrhagins derived from *Trimeresurus flavoviridis* venom (Takeya et al., 1989, 1990); the sequences for pro-trigramin (Pro-Trig) and pro-rhodostomin (Pro-Rhod) are inferred from the cDNA sequences of the disintegrins trigramin (from *T. gramineus* venom; Neeper & Jacobson, 1990) and rhodostomin (from Malayan pit viper *Calloselasma rhodostoma* venom; Au et al., 1991). Identical amino acid residues or conservative substitutions are boxed.

Effect of Mocarhagin on vWF-Dependent Agglutination of Platelet-Rich Plasma. Purified mocarhagin did not induce platelet aggregation in platelet-rich plasma. Treatment of platelet-rich plasma with 10 μg/mL mocarhagin for 6 min at 37 °C had no effect on aggregation induced by 5 μM ADP or 5 μg/mL collagen, and the response to 0.1 unit/mL α-thrombin was normal after an increased lag time (data not shown). It was, however, a potent inhibitor of vWF-dependent platelet agglutination in response to either ristocetin, botrocetin, or bovine vWF. Mocarhagin at a final concentration of 10 μg/mL completely inhibited ristocetin-induced platelet agglutination when platelets were preincubated with mocarhagin for 6 min at 37 °C, whereas mocarhagin concentrations of 5 μg/mL or less only partially inhibited platelet agglutination. This inhibitory activity was time-dependent. There was no inhibition of agglutination if ristocetin was added to the assay immediately after mocarhagin (10 μg/mL final concentration), ~10% inhibition after 1 min incubation with mocarhagin, and complete

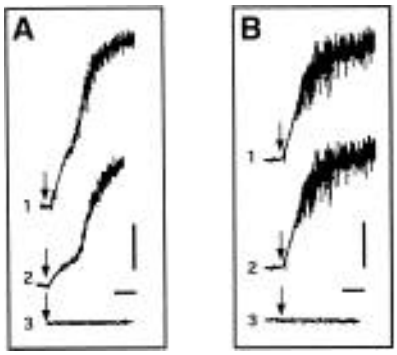

FIGURE 3: Inhibition by mocarhagin of ristocetin-induced agglutination in citrated platelet-rich plasma (2×10^8 platelets/mL) at 37 °C. Ristocetin was added where indicated by the arrow at a final concentration of 1 mg/mL. Panel A: trace 1, platelet-rich plasma with no additions; trace 2, platelet-rich plasma pretreated (6 min, 37 °C) with 10 μg/mL DFP-treated mocarhagin; trace 3, platelet-rich plasma pretreated with 10 μg/mL mocarhagin. Panel B: trace 1, control platelet-rich plasma plus 10 mM EDTA, final concentration; trace 2, 10 mM EDTA added immediately before pretreatment of platelets with 10 μg/mL mocarhagin for 6 min at 37 °C; trace 3, 10 mM EDTA added after pretreatment of platelets with 10 μg/mL mocarhagin. The horizontal bar represents 1 min; the vertical bar represents 20% maximal transmittance.

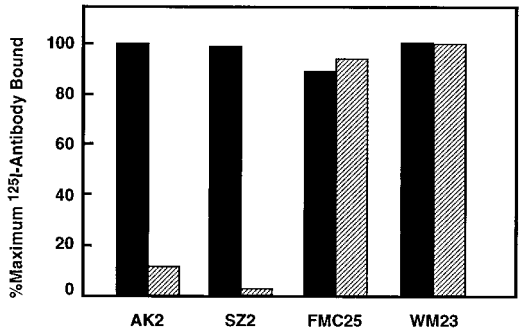

FIGURE 4: Inhibition of binding of ^{125}I-labeled anti-GP Ibα monoclonal antibodies (1 μg/mL) to platelets pretreated for 10 min at 22 °C with 5 μg/mL mocarhagin in the absence (hatched bars) or presence (solid bars) of 10 mM EDTA. Nonspecific binding was assessed for each antibody in the presence of a 50-fold excess of unlabeled antibody.

inhibition after 3 min incubation with mocarhagin. Mocarhagin at 10 μg/mL also completely inhibited vWF-dependent platelet agglutination in platelet-rich plasma induced by 50 μg/mL botrocetin and direct agglutination by bovine vWF. EDTA (10 mM, final concentration) completely blocked the inhibitory effect of mocarhagin on ristocetin-induced platelet agglutination, and mocarhagin treated with 8 mM DFP also lacked inhibitory activity (Figure 3), results consistent with proteolysis of the vWF receptor on platelets. Three additional results confirm this conclusion. First, treatment of platelets with mocarhagin under identical conditions to those inhibiting ristocetin-dependent platelet agglutination fully inhibited binding of two monoclonal antibodies, AK2 and SZ2, with epitopes within the N-terminal peptide domain of the α-chain of GP Ib (Figure 4). Both AK2 and SZ2 inhibit ristocetin-dependent binding of vWF to GP Ibα (Ruan et al., 1987; Berndt et al., 1988). In contrast, mocarhagin treatment had no effect on the binding of monoclonal antibodies against either the macroglycopeptide region of GP Ibα (WM23) or GP IX (FMC25). This suggests that mocarhagin treatment was not causing activation-dependent down-regulation of the surface-expressed GP Ib−IX complex

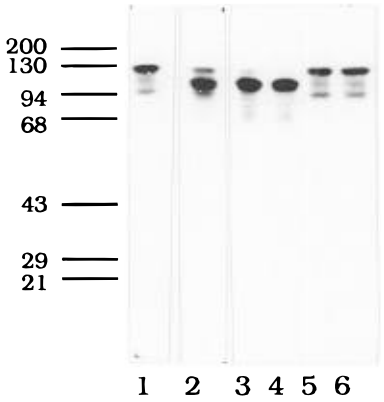

FIGURE 5: Cleavage of platelets in platelet-rich plasma by 10 μg/mL mocarhagin for 30 min at 22 °C analyzed by SDS−5−20% exponential gradient polyacrylamide gel electrophoresis, Western blotting with ^{125}I-labeled anti-GP Ibα monoclonal antibody, WM23, and autoradiography. Lane 1, untreated platelets; lane 2, platelets treated with 10 μg/mL mocarhagin for 6 min at 22 °C; lane 3, 15 min; lane 4, 30 min; lane 5, platelets treated with 10 μg/mL mocarhagin for 30 min in the presence of 10 mM EDTA; lane 6, platelets treated with 10 μg/mL DFP-treated mocarhagin for 30 min.

into the surface-connected canalicular system of the platelet (Hourdille et al., 1990; Michelson et al., 1991). When 10 mM EDTA was added prior to mocarhagin, there was no effect on binding of any of the antibodies to platelets (Figure 4). Second, in the presence of calcium ion, glycocalicin, a soluble ~135-kDa extracytoplasmic fragment of GP Ibα, was digested in a time-dependent manner, giving rise to two stable products of ~40 and ~100 kDa, respectively. The ~40-kDa fragment was detectable on SDS−polyacrylamide gels by Coomassie blue staining whereas the ~100-kDa highly glycosylated macroglycopeptide fragment was only detectable by staining with periodic acid−Schiff reagent (data not shown). Mocarhagin cleavage of glycocalicin was inhibited by EDTA, and this inhibition could be overcome by an excess of calcium ion or zinc ion, but not magnesium ion. Of the protease inhibitors tested (see Materials and Methods), only DFP completely and irreversibly inhibited the protease activity of mocarhagin. In addition, heparin at a final concentration of 100 μg/mL strongly inhibited the mocarhagin-dependent proteolysis of glycocalicin. Further, analysis of mocarhagin-treated platelets in platelet-rich plasma by Western blot analysis with the anti-GP Ibα monoclonal antibody WM23, directed against the macroglycopeptide region, was consistent with similar cleavage of GP Ibα (Figure 5). Finally, mocarhagin treatment of lactoperoxidase-radioiodinated platelets gave similar results to those observed with glycocalicin, showing the loss of a band corresponding to GP Ib (~170 kDa nonreduced) and the appearance of an ~150-kDa band (nonreduced) consisting of the remnant of the GP Ibα-chain disulfide-linked to GP Ibβ, and an ~40-kDa cleavage product (nonreduced and reduced) in the supernatant (data not shown). [^{3}H]Borohydride/periodate-labeled platelets treated with mocarhagin and analyzed on two-dimensional SDS−polyacrylamide gels showed only loss of GP Ib (Figure 6A, arrow, *vs* Figure 6B, arrow) with the remainder of the GP Ibα chain remaining platelet-associated (Figure 6B, arrowhead). Isolation by gel filtration of the ~100-kDa fragment after mocarhagin cleavage of glycocalicin yielded an N-terminal sequence of DTEGDKVR, consistent with mocarhagin cleaving GP Ibα at a single site between residues Glu-282 and Asp-283 in

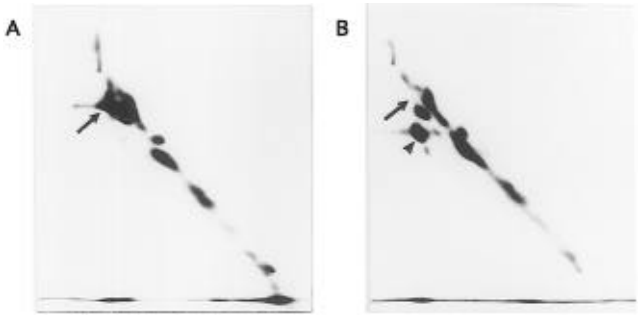

FIGURE 6: Two-dimensional nonreduced/reduced gel electrophoresis of periodate ^{3}H-surface-labeled platelets. Panel A: control platelets. Panel B: platelets treated with 10 μg/mL mocarhagin for 30 min at 22 °C. The position of GP Ibα is shown by an arrow and the position of the cleaved membrane-associated fragment of GP Ibα by an arrowhead.

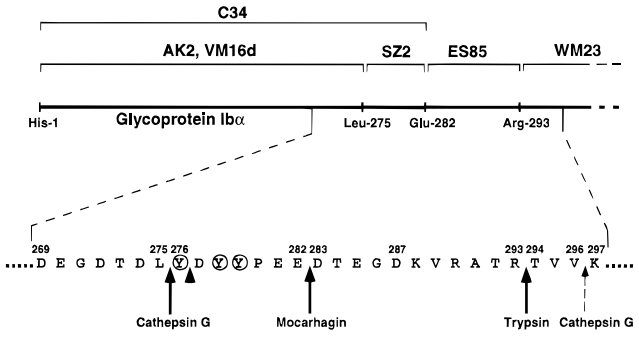

FIGURE 7: Diagram of the N-terminal peptide domain of GP Ibα (His-1−Arg-293) showing the epitopes for anti-GP Ibα monoclonal antibodies and the amino acid sequence of the anionic/sulfated tyrosine region (Asp-269 to Asp-287), tyrosine sulfation sites (circled), and the cleavage sites (solid arrows) for cathepsin G (Leu-275/Tyr-276; Pidard et al., 1994), mocarhagin (Glu-282/Asp-283; this study), and trypsin (Arg-293/Thr-294; Titani et al., 1987). A secondary cleavage site for cathepsin G (Val-296/Lys-297) is indicated by the broken arrow (Pidard et al., 1994). The arrowhead represents the cathepsin G cleavage site determined for a 15-mer synthetic peptide spanning this region, but not observed in the full-length protein.

the negative charge/sulfated tyrosine cluster (Figure 7). Analysis of the effect of mocarhagin on synthetic peptides spanning this region was also consistent with cleavage only occurring between these two residues (data not shown).

Effect of Glycocalicin Fragments on Botrocetin-Dependent vWF Binding to Platelets. As an initial approach to characterization of the functional role of the negative charge/sulfated tyrosine cluster in GP Ibα, proteolytic fragments of glycocalicin corresponding to the N-terminal segment of GP Ibα containing all of the negative charge/sulfated tyrosine cluster (trypsin: His-1−Arg-293; Titani et al., 1987) and two-thirds (mocarhagin: His-1−Glu-282) and one-third of the cluster (cathepsin G: His-1−Leu-275; Pidard et al., 1994) were evaluated for their effect on botrocetin-dependent binding of ^{125}I-vWF to platelets. On a molar basis, glycocalicin, the tryptic fragment His-1−Arg-293, and the mocarhagin fragment His-1−Glu-282 all showed equivalent inhibition of binding (IC_{50} ~0.3 μM), indicating that the botrocetin-dependent vWF-binding site on the platelet GP Ib−IX complex was located within the N-terminal 282 amino acids (Figure 8). In contrast, the cathepsin G fragment, His-1−Leu-275, was an order of magnitude less effective as an inhibitor (Figure 8), indicating that the sequence Tyr-276−Glu-282, YDYYPEE, containing the three consensus tyrosine

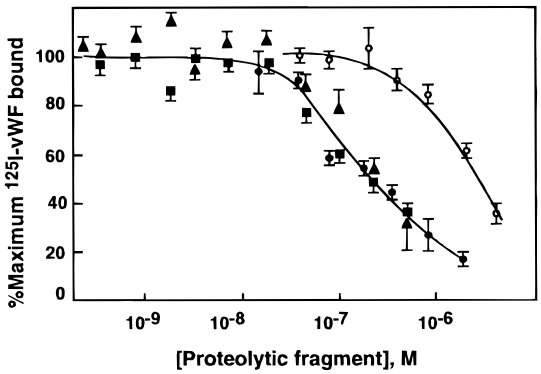

FIGURE 8: Inhibition of botrocetin-dependent binding of ^{125}I-labeled vWF (1 μg/mL) to washed platelets (5 × 10^7/mL) in TS buffer, 0.1% (w/v) BSA by intact glycocalicin (triangles), the tryptic His-1−Arg-293 fragment (squares), the mocarhagin His-1−Glu-282 fragment (solid circles), and the cathepsin G His-1−Leu-275 fragment (open circles).

Table 1: Immunoprecipitation by Monoclonal Antibodies of ^{125}I-Labeled Glycocalicin and Its Proteolytic Fragments Derived by Digestion with Mocarhagin and Cathepsin G

GP Ibα fragment	AK7	AK2	VM16d	SZ2	C34	ES85
glycocalicin	−	+	+	+	+	+
trypsin fragment (His-1−Arg-293)	−	+	+	+	+	+
mocarhagin fragment (His-1−Glu-282)	−	+	+	+	+	−
cathepsin G fragment (His-1−Leu-275)	−	+	+	−	ND[a]	ND

[a] ND, not determined.

sulfation sites, is an important but not absolute determinant for vWF recognition. Similar experiments evaluating the effect of glycocalicin and fragments on ristocetin-dependent binding of vWF to platelets were not performed since glycocalicin is a much less effective inhibitor in this system (IC_{50} >2 μM; Andrews et al., 1989a).

Effect of Monoclonal Antibodies Mapping to the Negative Charge/Sulfated Tyrosine Cluster on vWF Binding to Platelets. As an alternative to evaluating the functional role of the negative charge/sulfated tyrosine cluster on vWF binding to the GP Ib−IX complex, anti-GP Ib antibodies submitted to the Vth Leukocyte Workshop were mapped by analysis of their immunoreactivity toward the tryptic His-1−Arg-293, mocarhagin His-1−Glu-282, and cathepsin G His-1−Leu-275 fragments and analyzed for their effects on vWF−platelet interaction. The immunoreactivity profile for the anti-GP Ibα antibodies, AK2, VM16d, SZ2, C34, and ES35, relative to the anti-VLA-2 control antibody, AK7, is shown in Table 1. The epitopes of the monoclonal antibodies were initially mapped by immunoprecipitation of intact or proteolytic fragments of ^{125}I-labeled glycocalicin and analysis by SDS−polyacrylamide gel electrophoresis and autoradiography (Table 1). A control monoclonal antibody, AK7, directed against the human integrin (VLA-2) α2 subunit failed to precipitate any of the glycocalicin fragments. The N-terminal tryptic fragment of glycocalicin was immunoprecipitated by the anti-GP Ibα monoclonal antibodies AK2, SZ2, and VM16d (Figure 7), consistent with previously published results (Ruan et al., 1987; Berndt et al., 1988; Mazurov et al., 1991). The His-1−Glu-282 mocarhagin fragment was precipitated by AK2, VM16d, and weakly by SZ2, whereas the cathepsin G His-1−Leu-275 fragment was

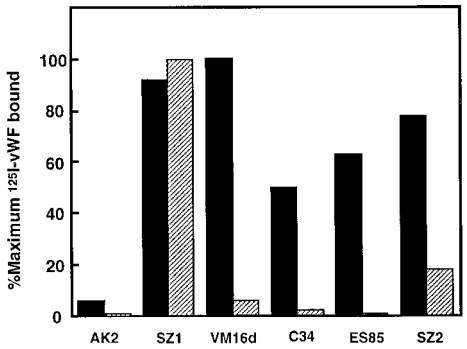

FIGURE 9: Inhibition of binding of ^{125}I-labeled vWF (1 μg/mL) to washed platelets (5×10^7/mL) by anti-GP Ibα monoclonal antibodies (50 μg/mL) in the presence of 1.5 mg/mL ristocetin (solid bars) or 50 μg/mL botrocetin (hatched bars).

precipitated by AK2 and VM16d, but not by SZ2. This suggests that the epitopes for AK2 and VM16d are contained within the sequence His-1−Leu-275 and that the epitope for SZ2 is lost by cleavage between residues Leu-275 and Tyr-276. Two other anti-GP Ibα monoclonal antibodies, C34 and ES85, both immunoprecipitated the N-terminal tryptic fragment, while only C34, but not ES85, recognized the N-terminal mocarhagin fragment, suggesting that the epitope for C34 was within His-1−Glu-282 and that the epitope for ES85 was sensitive to cleavage between residues Glu-282 and Asp-283 (Figure 7). Of these antibodies, only SZ2 and ES85 recognized glycocalicin or its proteolytic fragments on Western blots. SZ2 blotted the tryptic fragment His-1−Arg-293 and the mocarhagin fragment His-1−Glu-282, but not the His-1−Leu-275 cathepsin G fragment. ES85 only blotted the His-1−Arg-293 fragment, and not the smaller fragments (data not shown). The combined data suggest that ES85 recognizes an epitope on GP Ibα between residues Asp-283 and Arg-293, and that the epitope for SZ2 lies between residues Tyr-276 and Glu-282 and is lost following cleavage between residues Leu-275 and Tyr-276.

Monoclonal antibodies against GP Ibα (at a final concentration of 50 μg/mL) were tested for their ability to inhibit the ristocetin- and botrocetin-dependent binding of ^{125}I-labeled vWF to GP Ibα on washed platelets (Figure 9). AK2 completely inhibited vWF binding with either modulator as reported previously (Berndt et al., 1988; Andrews et al., 1989a). SZ1, directed against the membrane-associated region of the GP Ib−IX complex, was used as a control and had no effect on vWF binding. The monoclonal antibodies C34, ES85, and SZ2, which map to the negative charge cluster, and VM16d, all showed modulator-specific inhibition, with complete inhibition of botrocetin-dependent binding, but only a partial (0−50%) inhibition of ristocetin-dependent binding. In contrast to the vWF-binding studies, SZ2, ES85, and C34 completely inhibited vWF-dependent agglutination in platelet-rich plasma induced by 1.5 mg/mL ristocetin (data not shown), suggesting that this assay is more sensitive to inhibition by anti-GP Ibα monoclonal antibodies. AK2 completely blocked ristocetin-dependent agglutination. In order to test the interaction of vWF with platelets in the absence of modulators, asialo-vWF was added to platelet-rich plasma at a final concentration of 20 μg/mL. The resulting aggregation could be completely inhibited by SZ2, ES85, and C34 as well as by AK2 (Berndt et al., 1992; this study, data not shown). SZ2 and AK2 also inhibited

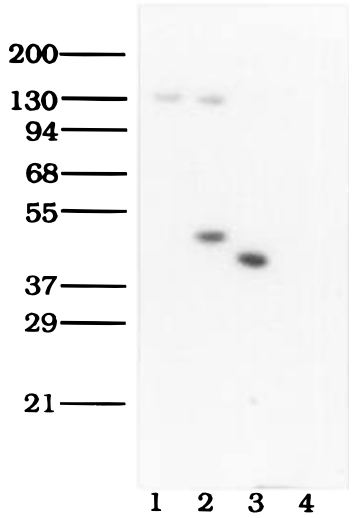

FIGURE 10: ^{125}I-Labeled thrombin ligand blot of intact glycocalicin and protease-digested glycocalicin electrophoresed on SDS−5−20% exponential gradient polyacrylamide gels and transferred to nitrocellulose. Lane 1, intact glycocalicin; lane 2, trypsin-treated glycocalicin; lane 3, mocarhagin-treated glycocalicin; lane 4, cathepsin G-treated glycocalicin. Molecular mass markers are as described in the legend of Figure 1.

agglutination of platelets induced by bovine vWF (data not shown).

Role of the Negative Charge/Sulfated Tyrosine Cluster in Binding of α-Thrombin to GP Ibα. A ligand blot assay was developed to investigate the thrombin-binding domain on GP Ibα. Glycocalicin and glycocalicin fragments were electrophoresed on SDS−polyacrylamide gels and transferred to nitrocellulose. ^{125}I-Labeled thrombin bound to intact glycocalicin, to the tryptic ~45-kDa fragment (His-1−Arg-293), and to the mocarhagin ~40-kDa fragment (His-1−Glu-282), but not to the cathepsin G fragment (His-1−Leu-275) (Figure 10), indicating that the sequence Tyr-276−Glu-282, YDYYPEE, implicated as important for vWF recognition (see above) is also important for binding of α-thrombin. Consistent with this, monoclonal antibodies with epitopes mapping to this region of GP Ibα inhibited specific binding of ^{125}I-labeled thrombin to fixed platelets. C34 and ES85 completely inhibited binding, while SZ2 inhibited by 91%. Other monoclonal antibodies had less effect on binding, with AK2 and WM23 inhibiting by 37% and 15%, respectively. SZ2 produced a dose-dependent inhibition of platelet aggregation induced by low-dose thrombin (0.05 unit/mL) (data not shown). VM16d has previously been demonstrated to inhibit both platelet aggregation induced by low-dose thrombin and the binding of ^{125}I-labeled thrombin to paraformaldehyde-fixed platelets (Mazurov et al., 1991).

Analysis of Tyrosine Sulfation. Since the sequence YDYYPEE appears important for the binding of both vWF and α-thrombin and contains three tyrosine residues that meet the consensus for O-sulfation, it was important to establish the degree of tyrosine sulfation within this sequence in the native protein on platelets. Our strategy was to employ glycocalicin isolated from washed platelets and to splice out the tyrosine-containing sequence by proteolysis with cathepsin G and mocarhagin. We have previously reported that cathepsin G cleaves glycocalicin between Leu-275 and Tyr-276 (Pidard et al., 1994). In contrast, cathepsin G cleaves synthetic peptides spanning this region between Tyr-276 and Asp-277 (Ward and Berndt, unpublished observations),

Table 2: Analysis by Mass Spectroscopy of Peptides Derived from Glycocalicin by Digestion with Mocarhagin and Cathepsin G

peptide	relative intensity observed (%)	molecular weight	molecular weight expected
DY(SO_3H)Y(SO_3H)PEE	100.0	974.1	973.0
DYYPEE	19.7	786.4	786.8
YDY(SO_3H)Y(SO_3H)PEE	100.0	1135.2	1136.2
Y(SO_3H)DY(SO_3H)Y (SO_3H)PEE	86.0	1215.6	1215.3

suggesting that the cathepsin G cleavage site is dependent on the extent of sequence C-terminal to the potential cleavage site. We were able to use this property of cathepsin G to isolate the GP Ibα sequences, YDYYPEE and DYYPEE, depending on the time of addition to glycocalicin of cathepsin G relative to mocarhagin.

An experimental system for detecting the presence of sulfated tyrosine residues was developed using HPLC and UV absorbance spectroscopy. Comparison of the synthetic peptides CCK (H-Asp-Tyr-Met-Gly-OH) and CCK-S [H-Asp-Tyr(SO_3H)-Met-Gly-OH] showed that the sulfated form, CCK-S, eluted earlier from a C18 HPLC column (retention time of 11.2 min) than did CCK (12.0 min). The UV absorbance spectra were distinctive for the two tyrosine-containing peptides with a difference in λ_{max} (CCK: 276 nm; CCK-S: 262 nm) and the $A_{280/260}$ ratio (CCK, 2.09; CCK-S, 0.24). From these data, UV spectra were calculated for a theoretical peptide containing three tyrosine residues with variable degrees of sulfation and compared with the measured UV spectrum of isolated peptide fragments of the native protein. Purified glycocalicin was first digested simultaneously with mocarhagin and cathepsin G. Digested peptides were separated from the proteases and larger fragments by ultrafiltration through a YMT Centricon membrane. The filtrate was analyzed by HPLC and showed a major absorbance peak eluting at 11.5 min, with the amino acid sequence Asp-Tyr-Tyr-Pro-Glu-Glu (DYYPEE), corresponding to residues Asp-277−Glu-282. UV spectral analysis of DYYPEE gave a spectrum approximating the theoretical spectrum for two sulfated tyrosines (measured $A_{280/260}$ 0.42; theoretical $A_{280/260}$ 0.24). By mass spectrometry, the fragment had the expected molecular weight for DYYPEE with both tyrosines sulfated, with a smaller component (∼10% of the relative signal intensity) consistent with nonsulfation of both residues (Table 2). These findings indicate that residues Tyr-278 and Tyr-279 are both sulfated in ∼85% of GP Ibα molecules. In order to assess sulfation of Tyr-276, glycocalicin was sequentially digested for 2 h with cathepsin G followed for 2 h with mocarhagin. The resulting filtrate gave a later peak (12.4 min) on HPLC, confirmed as YDYYPEE by amino acid sequencing. The UV spectrum of YDYYPEE differed from that of the smaller fragment, and was closer to the theoretical spectrum for a peptide with 2.4 sulfated tyrosines and 0.6 nonsulfated tyrosine (measured $A_{280/260}$ 0.86; theoretical $A_{280/260}$ 0.86) than that for three sulfated residues ($A_{280/260}$ 0.24). Mass spectrometry of the YDYYPEE fragment showed two signals of equal intensity, corresponding in molecular weight to either doubly or triply sulfated peptide (Table 2). This provides evidence for variable sulfation (∼50%) of Tyr-276, suggesting heterogeneity of posttranslational processing at this residue.

DISCUSSION

The human platelet GP Ib−IX complex is a key membrane receptor mediating both platelet function and reactivity (López, 1994). Recognition of subendothelial-bound vWF by the GP Ib−IX complex allows platelets to adhere to damaged blood vessels and signals subsequent events in the hemostatic process. Further, by an as yet poorly defined mechanism, binding of α-thrombin to GP Ib facilitates platelet agonist responses to α-thrombin. In this paper, we have identified a heptapeptide sequence, Tyr-276−Glu-282, YDYYPEE, as an important recognition site for both the botrocetin-dependent binding of vWF and α-thrombin. In addition, we have confirmed that either two or three of the tyrosine residues within this sequence are O-sulfated in the native receptor isolated from circulating human platelets.

Previous studies have identified that the vWF-binding site lies within an ∼40-kDa N-terminal globular domain comprising the first 293 amino acids of GP Ibα (Handa et al., 1986; Berndt et al., 1988; Vicente et al., 1988; Andrews et al., 1989b). This domain is comprised of seven leucine-rich repeats and their N- and C-terminal flanking sequences and a highly negatively-charged stretch of amino acids between Asp-269 and Asp-287, DEGDTDLYDYYPEED-TEGD (López et al., 1987; Titani et al., 1987). Our analysis of the potential role of this negative charge cluster in GP Ib−IX function was considerably enhanced by our identification of a novel metalloproteinase, termed mocarhagin, from the Mozambiquan spitting cobra, *Naja mocambique mocambique*, that specifically cleaved GP Ibα between Glu-282 and Asp-283 as the sole detectable proteolytic event on platelets and abolished vWF binding. We have identified a second major substrate for mocarhagin, the P-selectin receptor, PSGL-1, on neutrophils (De Luca et al., 1995). Mocarhagin cleaves a 10 amino acid peptide from the mature N-terminus of PSGL-1, abolishing P-selectin binding. In both GP Ibα and PSGL-1, the cleavage site occurs within a negative charge cluster, at the N-terminal side of an aspartate residue, and to the C-terminal side of three potential sulfated tyrosine residues, determinants which may explain the exquisite substrate specificity of the enzyme. This apparent specificity for a negative charge cluster is further suggested by the inhibition of mocarhagin by heparin. N-Terminal sequence analysis showed that mocarhagin was homologous to members of the metalloproteinase−disintegrin protein family described primarily in viper venoms (Gould et al., 1990; Paine et al., 1992; Gomis-Rüth et al., 1993).

Analysis of N-terminal peptide proteolytic fragments of GP Ibα generated by trypsin, mocarhagin, and cathepsin G suggests that the amino acid sequence Tyr-276−Glu-282 is important for botrocetin-dependent recognition of vWF. The mocarhagin N-terminal peptide fragment (His-1−Glu-282) inhibited botrocetin-dependent binding of vWF to platelets to a comparable degree to the larger tryptic fragment (His-1−Arg-293) and glycocalicin (IC_{50} ∼0.3 μM), whereas the cathepsin G fragment (His-1−Leu-275) was an order of magnitude less effective (IC_{50} ∼3 μM). Since the cathepsin G fragment, His-1−Leu-275, could still almost completely inhibit botrocetin-dependent binding of vWF, either distinct or overlapping sequences between His-1 and Leu-275 must also be involved in addition to Tyr-276−Glu-282 in vWF recognition. These results were supported by studies with anti-GP Ibα monoclonal antibodies. Two anti-GP Ibα

monoclonal antibodies, SZ2 and ES85, preferentially inhibited botrocetin- dependent, relative to ristocetin-dependent, binding, and had epitopes in the sequence Tyr-276−Arg-293. In contrast, the monoclonal antibody AK2, that mapped into the His-1−Leu-275 fragment, blocked both botrocetin-dependent and ristocetin-dependent binding of vWF to platelets as do a range of previously reported anti-GP Ibα antibodies (Berndt et al., 1988; Andrews et al., 1989b). Ristocetin-dependent binding of vWF apparently primarily requires the His-1−Leu-275 sequence, a result in accord with the recent findings of Marchese et al. (1995).

Isolation of the Tyr-276−Glu-282 peptide from the native protein using mocarhagin and cathepsin G allowed the first direct analysis of tyrosine sulfation in the native receptor by UV absorbance spectroscopy and mass spectrometry. This detailed analysis showed that Tyr-278 and Tyr-279 were >85% sulfated and that Tyr-276 was ∼50% sulfated. Our results, however, do not exclude the possibility that Tyr-276 was partially desulfated during preparation or analysis. Further, the native receptor used for analysis was derived from a pool of multiple donors, raising the possibility that the observed heterogeneity may reflect variation between donors. Previous studies have provided evidence for sulfation of GP Ib using recombinant GP Ibα or its fragments expressed in mammalian cell lines. Dong et al. (1994) showed that Tyr-276, Tyr-278, and Tyr-279 of GP Ibα were sulfated in the GP Ib−IX complex expressed in CHO cells and that a sulfate-deplete receptor was less efficient than wild type in binding vWF in the presence of ristocetin. Marchese et al. (1995) mutated these tyrosine residues to phenylalanine in a recombinant His-1 to Ala-302 fragment of GP Ibα expressed in CHO cells and reported that the sulfate-depleted fragment showed diminished ristocetin-dependent and markedly reduced botrocetin-dependent vWF binding. Synthetic peptides corresponding to the sequences Ser-251−Tyr-279 and Gly-271−Glu-285 (but lacking sulfated tyrosines) inhibited ristocetin-dependent vWF binding to platelets, but were less effective against botrocetin-dependent or asialo-vWF binding (Vicente et al., 1990). Other synthetic peptides, Thr-81−Leu-95 and Leu-136−Leu-150, preferentially inhibited ristocetin-dependent vWF binding (Vicente et al., 1990). Katagiri et al. (1990) identified residues Asp-235−Lys-262 as a putative vWF-binding domain, but only ristocetin-dependent binding was examined. However, all of the peptides studied were inhibitory only at concentrations (IC_{50}s of ∼0.5 mM) much greater than those required for inhibition by glycocalicin or the ∼45-kDa tryptic fragment, suggesting that optimal vWF binding requires a nonlinear, conformational-dependent recognition site and/or posttranslational receptor modification such as tyrosine sulfation. Murata et al. (1991) selectively mutated single negatively-charged amino acid residues to neutral residues within a His-1-Ala-302 recombinant GP Ibα fragment. Loss of anionic residues between Asp-252 and Asp-277 abolished botrocetin-dependent vWF binding, and greatly decreased ristocetin-dependent binding. In contrast, mutant GP Ibα fragments lacking anionic residues between Glu-281 and Asp-287 showed a specific loss of botrocetin-dependent vWF binding, with normal ristocetin-dependent binding. The extent to which these mutations were affecting native receptor conformation, however, was not clear. We have previously identified a GP Ibα-binding sequence, Asp-514−Glu-542, within the A1 domain of vWF (Berndt et al., 1992). Sobel et al. (1992) identified a proximal sequence in the A1 domain of vWF, Tyr-565−Ala-587, as a binding site for heparin. A simple model consistent with the above findings would be that the heparin-binding sequence of vWF interacts with the heparin-like anionic/sulfated tyrosine region of GP Ibα while the Asp-514−Glu-542 vWF sequence interacts with a second GP Ib-binding site.

GP Ibα also contains a high-affinity binding site for α-thrombin within the N-terminal peptide domain (Ganguly & Gould, 1979; Moroi et al., 1982; Harmon & Jamieson, 1986; Okumuru et al., 1978; Yamamoto et al., 1986). Several lines of evidence suggest that the negative charge cluster forms the thrombin-binding site. First, the sequence of this region is homologous to other thrombin-binding proteins such as fibrinogen, the thrombin receptor, thrombomodulin, factors V and VIII, and hirudin (Fenton et al., 1993), and tyrosine sulfation of both factor V (Pittman et al., 1994) and hirudin (Niehrs et al., 1990) is required for maximal interaction with thrombin. Second, synthetic peptides based on the amino acid sequence of GP Ibα Phe-216−Asp-274 inhibited thrombin-induced platelet aggregation, and a monoclonal antibody (TM60) that inhibited both ristocetin- and thrombin-induced platelet aggregation recognized peptide Asp-249−Asp-274 (Katagiri et al., 1990). Synthetic peptides based on sequences within Ala-244−Asp-287 inhibited thrombin binding to platelets or low-dose thrombin-induced aggregation, and Tyr to Ala substitutions within these anionic sequences decreased thrombin binding to glycocalicin (De Marco et al., 1994; Gralnick et al., 1994). Similarly, individually substituting phenylalanine for Tyr-276, Tyr-278, and Tyr-279 in recombinant His-1−Ala-302 fragments of GP Ibα inhibited the ability of the fragments to bind thrombin (Marchese et al., 1995). Finally, both the mocarhagin fragment (His-1−Glu-282) and the trypsin fragment (His-1−Arg-293) bound thrombin directly in a ligand blot assay, whereas the cathepsin G fragment (His-1−Leu-275) failed to bind thrombin, and two monoclonal antibodies with epitopes including the Tyr-276−Glu-282 sequence or affected by cleavage at Leu-275/Tyr-276 (ES85 and SZ2) blocked binding of thrombin to GP Ibα on fixed platelets (this study).

In summary, characterization of mocarhagin as a novel metalloproteinase that selectively cleaves the platelet vWF receptor and determining its cleavage site have definitively localized the vWF-binding site to residues His-1−Glu-282 of GP Ibα. In conjunction with cathepsin G, mocarhagin has enabled identification of the GP Ibα sequence Tyr-276−Glu-282 as an important recognition motif for binding of vWF and thrombin to the GP Ib−IX complex on platelets, and demonstrated unequivocally that three tyrosine residues within this sequence can be sulfated in the native molecule. The data support a potential bimodal model for the vWF-GP Ib−IX complex interaction involving the anionic/sulfated tyrosine sequence, and provide the basis for understanding the mechanism of platelet adhesion at the molecular level.

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