Identification of Aspartic Acid 514 through Glutamic Acid 542 as a Glycoprotein Ib–IX Complex Receptor Recognition Sequence in von Willebrand Factor. Mechanism of Modulation of von Willebrand Factor by Ristocetin and Botrocetin†

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ABSTRACT: As the first step in hemostasis, the binding of von Willebrand factor (vWF) to the platelet membrane glycoprotein (GP) Ib–IX complex is essential for platelet adhesion at high-shear blood flow. This interaction in vivo requires the prior binding of vWF to the subendothelial matrix, a process which exposes a normally cryptic binding site on vWF for the GP Ib–IX complex. This process can be mimicked in vitro by modulators such as ristocetin or the snake venom protein botrocetin or by desialation of vWF. We have previously localized the GP Ib binding site on vWF to a monomeric dispase fragment which extends from Leu-480/Val-481 to Gly-718 in the primary sequence of mature vWF [Andrews, R. K., Gorman, J. J., Booth, W. J., Corino, G. L., Castaldi, P. A., & Berndt, M. C. (1989) *Biochemistry 28*, 8326–8336]. This fragment also contains a distinct binding site for botrocetin. Analysis of synthetic peptides corresponding to hydrophilic stretches of sequence within this fragment indicated that the sequence Asp-514–Glu-542 represents a major adhesive sequence involved in receptor recognition. This peptide inhibited both the ristocetin- and botrocetin-mediated binding of vWF to either platelets or purified GP Ib–IX complex (IC_{50} ~50–200 μM) as well as the asialo-vWF- and bovine vWF-dependent agglutination of platelets. Both the N- and C-terminal halves of the peptide were inhibitory but less so than the intact peptide. This peptide also inhibited botrocetin binding to vWF, suggesting that botrocetin modulates vWF–GP Ib interaction by binding in close proximity to the vWF adhesion sequence. Two proline-rich peptides, Cys-474–Pro-488 and Leu-694–Pro-708, only inhibited the ristocetin-mediated binding of vWF to receptor, suggesting that they encompass a ristocetin modulation site. In support of this, irrelevant peptides containing proline-rich repeats were also effective inhibitors of ristocetin-dependent vWF receptor interaction and together with the proline-rich vWF peptides also inhibited the ristocetin-dependent flocculation of fibrinogen. Interaction of the proline-rich peptides with ristocetin was confirmed by ultraviolet difference spectroscopy. The combined results are consistent with an electrostatic model for vWF activation which involves interaction with a modulator exposing an adjacent GP Ib–IX complex binding domain encompassing the sequence Asp-514–Glu-542 of vWF.

Adhesion of platelets to exposed subendothelial matrix initiates the hemostatic process. At high blood shear rate (>800 s^{-1}), platelet adhesion involves the recognition of subendothelial bound von Willebrand factor (vWF)[1] (Sakariassen et al., 1979; Baruch et al., 1991) by a specific platelet receptor, the glycoprotein (GP) Ib–IX complex [for a review, see Booth et al. (1990)]. In contrast, plasma vWF does not bind to the GP Ib–IX complex. It is believed that vWF bound to subendothelial matrix undergoes a conformational change that exposes a previously cryptic binding site that is now recognized by the GP Ib–IX complex (Sakariassen et al., 1984), although conformational changes in the GP Ib–IX complex may also potentially be involved in mediating vWF-dependent platelet adhesion. This activation of vWF can be mimicked in vitro by specific modulators, such as ristocetin and botrocetin (Berndt et al., 1988; Andrews et al., 1989a). Alternatively, alteration of the structure of vWF by removal of sialic acid (De Marco & Shapiro, 1981) or by proteolysis (Fujimura et al., 1986; Andrews et al., 1989b) results in a form of vWF or vWF fragment which can spontaneously bind to the GP Ib–IX complex. Ristocetin is a glycopeptide antibiotic isolated from the culture medium of *Nocardia lurida* which binds to the D-Ala-D-Ala dipeptide sequence on Gram-positive bacteria (Nieto & Perkins, 1971; Kalman & Williams, 1980). Botrocetin is a 25-kDa disulfide-linked dimeric protein first purified by our laboratory from the venom of the South American pit viper, *Bothrops jararaca* (Andrews et al., 1989a). Recently, Fujimura et al. (1991a) purified two structurally and functionally distinct forms of botrocetin from *B. jararaca* venom, a related, more active dimeric form and a single-chain, 28-kDa monomeric form. All three forms of botrocetin have been shown to bind vWF (Andrews et al., 1989a; Read et al., 1989; Fujimura et al., 1991a).

Mature vWF consists of 2050 amino acid residues and circulates in plasma as a series of disulfide-linked multimers between 1×10^6 and 20×10^6 in molecular weight (Titani et al., 1986). The binding site for GP Ib–IX complex has been localized to a dimeric 52/48-kDa tryptic fragment of vWF which extends from Val-449 to Lys-728 (Fujimura et al., 1986). This sequence also contains the binding site for botrocetin (Andrews et al., 1989a). We subsequently further

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[1] Abbreviations: BSA, bovine serum albumin; Da, dalton; GP, glycoprotein; IC_{50}, concentration of inhibitor giving 50% inhibition; vWF, von Willebrand factor.

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Table I: Structure of vWF Peptides

sequence[a]	structure
514–542	DLVFLLDGSSRLSEAEFEVLKAFVVDMME
564–581	AYIGLKDRKRPSELRRIA
607–628	SKIDRPEASRIALLLMASQEPQ
628–646	QRMSRNFVRYVQGLKKKKV
655–674	PHANLKQIRLIEKQAPENKA
677–694	LSSVDELEQQRDEIVSYL
474–488[b]	CQEPGGLVVPPTDAP
694–708[b]	LCDLAPEAPPPTLPP
514–529	DLVFLLDGSSRLSEAE
520–539	DGSSRLSEAEFEVLKAFVVD
527–542	EAEFEVLKAFVVDMME
700–705	EAPPPT

[a] Position in primary structure of mature vWF (Titani et al., 1986). [b] Peptide sequences reported by Mohri et al. (1988).

localized the GP Ib–IX complex binding site on vWF to a monomeric 39/34-kDa dispase fragment which extends from either Leu-480 or Val-481 to Gly-718. This fragment contained a single intrachain disulfide bond between cysteinyl residues at positions 509 and 695 (Andrews et al., 1989b). In 1988, Mohri et al. identified two peptide sequences, Cys-474–Pro-488 and Leu-694–Pro-708, which blocked the ristocetin-dependent binding of vWF and the binding of asialo-vWF to washed human platelets. These two sequences would be brought into proximity by the disulfide bond between Cys-509 and Cys-695. In this regard, Mohri et al. (1988) reported that the effect of the two peptides on vWF–GP Ib–IX complex interaction was synergistic. In contrast, Girma et al. (1990) could not confirm this synergistic effect and, in addition, found that these two peptides had no effect on the botrocetin-dependent binding of vWF to platelets. In order to resolve these discrepancies, we have started to address two fundamental questions: First, how do ristocetin and botrocetin modulate vWF so that it becomes adhesive for platelets? Second, what is(are) the adhesive sequence(s) involved in binding to its platelet receptor, the GP Ib–IX complex? Our approach has been to examine the effect of synthetic peptides corresponding to hydrophilic stretches of sequence within the 39/34-kDa vWF fragment. The combined data suggest that Cys-474–Pro-488 and Leu-694–Pro-708 may not be directly involved in receptor recognition but may be important in the recognition of ristocetin and have identified the sequence Asp-514–Glu-542 as a major adhesive sequence mediating vWF–GP Ib–IX complex interaction.

MATERIALS AND METHODS

Materials. The vWF peptides listed in Table I were custom synthesized by Auspep Pty. Ltd., Melbourne, Australia. The amino and carboxy termini were blocked by acetyl and amide groups, respectively. The peptides were purified to homogeneity by reverse-phase HPLC and the composition of the peptides was confirmed by amino acid analysis. Two additional peptides, pEGLPPGPPIPP and KPPTPPPEPET, were purchased from Sigma, St. Louis, MO. The former is known as bradykinin potentiator C and the latter corresponds to the C-terminal sequence of SV40 tumor antigen. N^{α},N^{ϵ}-Diacetyl-Lys-D-Ala-D-Ala, bovine serum albumin (BSA) (fraction V), and *B. jararaca* venom were also purchased from Sigma. Ristocetin and *Vibrio cholerae* neuraminidase were obtained from Calbiochem, La Jolla, CA. Fibrinogen (grade L) was obtained from KabiVitrum, Stockholm, Sweden. Lyophilized human factor VIII concentrate was the gift of the Commonwealth Serum Laboratories, Melbourne, Australia.

Proteins. vWF was purified from factor VIII concentrate as previously described (Booth et al., 1984; Andrews et al., 1989a), concentrated to 1 mg/mL, and stored in 0.01 M Tris, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide, pH 7.4 (TSA buffer) at −70 °C. vWF was iodinated by the Iodobead method according to the manufacturer's instructions [Pierce, Rockford, IL; 1 mg of protein (mCi of sodium [^{125}I]iodide)$^{-1}$ iodobead^{-1}] and routinely had a specific radioactivity of 500–1000 cpm/ng. Labeled protein was separated from free label by chromatography on Sephadex G-25 (Pharmacia) equilibrated with TSA buffer. A monomeric 39/34-kDa proteolytic fragment of human vWF (Leu-480/Val-481–Gly-718) was obtained by dispase treatment of purified vWF and purified by heparin-affinity chromatography as previously described (Andrews et al., 1989b). Human vWF and the 39/34-kDa dispase fragment were desialated using *V. cholerae* neuraminidase as reported (Andrews et al., 1989b). The 39/34-kDa dispase fragment was reduced and alkylated as described previously in detail (Andrews et al., 1989b). Bovine vWF (Andrews et al., 1989b), botrocetin (Andrews et al., 1989a), and the human platelet membrane GP Ib–IX complex (Berndt et al., 1985, 1988) were all purified as previously described in detail elsewhere.

Binding Analyses. Analysis of the binding of ^{125}I-labeled vWF to botrocetin coupled to Matrex pel 102 beads (Amicon, Danvers, MA; ~5 μg of botrocetin/mg of beads) was performed essentially as previously described (Andrews et al., 1989a). The assay volume of 100 μL consisted of botrocetin-coupled beads (0.7 mg), BSA (1 mg/mL), and ^{125}I-labeled vWF (1 μg/mL) in TSA buffer. Experiments addressing the effect of native or reduced and alkylated 39/34-kDa dispase fragment on the vWF–botrocetin reaction were performed using a final fragment concentration of 10^{-7}–10^{-5} M in TSA buffer. In experiments to examine the effect of small peptides on binding, the assays incorporated 0.7 volume of 0.05 M Hepes and 0.15 M sodium chloride, pH 7.4 (HS buffer) or peptide (10^{-5}–10^{-3} M) in HS buffer. The botrocetin-coupled beads were preincubated with buffer or vWF fragment or peptide for 15 min prior to the addition of ^{125}I-labeled vWF. The assays were terminated after 15 min by centrifugation (8750*g*, 2 min, 22 °C), followed by aspiration of the supernatant. Radiolabel in the pellet was measured using a γ-counter. Nonspecific binding was estimated by incorporating a 50-fold excess of unlabeled vWF in a parallel assay.

The reconstitution assay for the ristocetin- and botrocetin-dependent binding of ^{125}I-labeled vWF to GP Ib–IX complex coated beads has been previously described (Berndt et al., 1988; Andrews et al., 1989a). The assay volume of 100 μL consisted of GP Ib–IX complex coated beads (5 mg/mL), BSA (1 mg/mL), ^{125}I-labeled vWF (1 μg/mL), and either TSA buffer or ristocetin (1 mg/mL) or botrocetin (50 μg/mL) in HS buffer or peptide [(5×10^{-6}) – 10^{-3} M] in HS buffer. The latter constituted 0.5 volume of the assay. After preincubation of the peptide for 15 min with the GP Ib–IX complex coated beads, the assay was commenced by the addition of either the ristocetin or the botrocetin. After 30 min at 22 °C, the assay was terminated by centrifugation and aspiration of the supernatant as described above.

The assay for the ristocetin- and botrocetin-dependent binding of ^{125}I-labeled vWF to human platelets has also been previously described (Berndt et al., 1988; Andrews et al., 1989a). The assay was similar to the reconstitution assay but incorporated washed platelets (2×10^7/mL) in place of beads and was performed in a final volume of 200 μL for 1 h. In both the platelet and bead assays, nonspecific binding was determined by omitting ristocetin and botrocetin or by incorporating

a 50-fold excess of unlabeled vWF in parallel assays.

Aggregation Studies. Aggregation was performed in citrated platelet-rich plasma in a final volume of 500 μL, as previously described (Andrews et al., 1989b). The platelet count was adjusted to give 10^8/mL in the assay. Platelet aggregation was induced by asialo-vWF or purified bovine vWF (final concentrations 100 and 10 μg/mL, respectively) or by bovine plasma (0.1 volume). Peptides were tested at a final concentration of 0.5 mM by the addition of 0.2 volume of a 2.5 mM stock in HS buffer.

Ristocetin-Dependent Flocculation of Fibrinogen. The effect of peptides on the ristocetin-dependent flocculation of fibrinogen was evaluated according to the method of Scott et al. (1991). Briefly, ^{125}I-labeled fibrinogen (2.0 mg/mL) was incubated with ristocetin (0.7 mg/mL) in the absence or presence of peptide (0.01–1 mM, final concentration) at 22 °C for 10 min. After microfugation at 10000*g* for 2 min, the supernatant was counted to assess the degree of fibrinogen flocculation.

Ultraviolet Difference Spectroscopy. Complex formation between peptides and ristocetin was evaluated by ultraviolet difference spectroscopy using a Hitachi 220A spectrophotometer according to the method of Nieto and Perkins (1971). A baseline was recorded between 400 and 240 nm with 1×10^{-4} M ristocetin in 0.05 M Hepes buffer, pH 7.4, in the front and reference cuvettes of the spectrophotometer. Equivalent volumes of buffer and peptide (0.025–0.4 mM, final concentration) in buffer were then added to the reference and front cuvettes, respectively, and the difference spectrum was recorded. The observed spectral changes were stable over a time period of 60 min. None of the peptides employed in these experiments absorbed at these wavelengths at concentrations of peptide up to 0.4 mM.

RESULTS

Identification of Asp-514 to Glu-542 as a Glycoprotein Ib–IX Complex Receptor Recognition Sequence in von Willebrand Factor. Previous studies from our laboratory have localized the GP Ib–IX complex binding domain of vWF to a dispase proteolytic fragment which extends from Leu-480 or Val-481 to Gly-718 in the primary structure of vWF. In order to further define the adhesion sequence of vWF involved in receptor recognition, we have examined the effect on vWF–GP Ib–IX complex interaction of peptides corresponding to either hydrophilic stretches of sequence within this fragment or peptides corresponding to highly conserved sequences within proteins that contain A-domains (Colombatti & Bonaldo, 1991) (Table I). For comparison, two peptides, Cys-474–Pro-488 and Leu-694–Pro-708, previously identified to inhibit ristocetin-dependent binding of vWF to platelets (Mohri et al., 1988) were also included in this analysis. One peptide, Asp-514–Glu-542, was found to be inhibitory in all the assay systems employed to assess vWF–GP Ib–IX complex interaction.

In initial experiments, the first eight peptides listed in Table I were tested for their effect on the binding of ^{125}I-labeled vWF either to washed platelets or to purified GP Ib–IX complex, immobilized onto Immunobeads, in the presence of either ristocetin or botrocetin (Berndt et al., 1988, Andrews et al., 1989a). Three of the vWF peptides inhibited ristocetin-dependent binding of vWF to platelets or GP Ib–IX beads with an IC_{50} less than 1 mM (Figure 1). The remaining five vWF peptides either showed no inhibitory effect at 1 mM or weakly inhibited but with an IC_{50} greater than 1 mM (the highest peptide concentration tested; data not shown). The

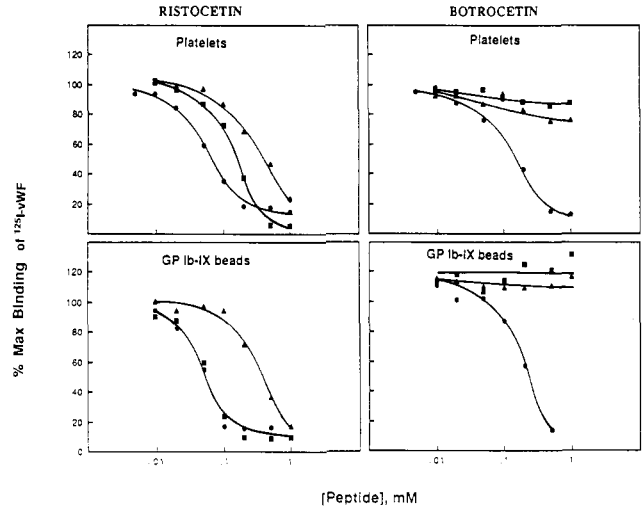

FIGURE 1: Inhibition of ristocetin- and botrocetin-dependent binding of ^{125}I-labeled von Willebrand factor to platelets and GP Ib–IX complex coated beads by vWF peptides: (●) Asp-514–Glu-542, (▲) Cys-474–Pro-488, and (■) Leu-694–Pro-708. The data are representative of three separate experiments using different platelet donors.

inhibitory peptides included the previously described vWF peptides Cys-474–Pro-488 and Leu-694–Pro-708 (Mohri et al., 1988) as well as another vWF sequence, Asp-514–Glu-542. Ristocetin-induced binding of vWF to platelets was most sensitive to this latter peptide (IC_{50} = 70 μM) compared with vWF 694–708 (IC_{50} = 150 μM) and vWF 474–488 (IC_{50} = 400 μM). The corresponding IC_{50} values for the GP Ib–IX bead assay were 50, 50, and 400 μM, respectively. These IC_{50} values for the vWF peptide 514–542 are approximately an order of magnitude higher than the IC_{50} value (5 μM) for the inhibition of ristocetin-dependent binding of vWF by the intact 39/34-kDa vWF dispase fragment (Andrews et al., 1989b). In contrast to the ristocetin-dependent binding data, botrocetin-induced binding of vWF to platelets and to GP Ib–IX beads was inhibited by only one of the eight vWF peptides, namely, the Asp-514–Glu-542 peptide (Figure 1; data for three peptides only are shown). Neither the Cys-474–Pro-488 or the Leu-694–Pro-708 peptide inhibited botrocetin-dependent vWF binding at peptide concentrations up to 1 mM (Figure 1). These data confirm the report of Girma et al. (1990), who also found that these two peptides were ineffective in inhibiting botrocetin-dependent binding of vWF to platelets. The IC_{50} values for Asp-514–Glu-542 inhibition of botrocetin-dependent vWF binding, 150 μM for the platelet assay and 200 μM for the GP Ib–IX bead assay, were similar but higher than those for the inhibition of ristocetin-dependent vWF binding, indicating that botrocetin-dependent vWF binding to receptor was less sensitive to inhibition by this peptide.

Since the data of Figure 1 do not discriminate between whether various peptides inhibit the interaction between vWF and its platelet receptor, the GP Ib–IX complex, or vWF and modulator, we therefore also examined the effect of the vWF peptides on asialo-vWF or bovine vWF induced platelet agglutination in citrated platelet-rich plasma as a model for modulator-independent binding of vWF to platelets. In this assay, plasma vWF does not bind to platelets in the absence of modulator (ristocetin or botrocetin) and therefore does not compete with either asialo-vWF or bovine vWF. Of the three vWF peptides which inhibited ristocetin-dependent binding of vWF to platelets (Figure 1), the peptide Asp-514–Glu-542 fully inhibited asialo-vWF induced platelet aggregation at concentrations ≥200 μM (Figure 2), whereas the peptides

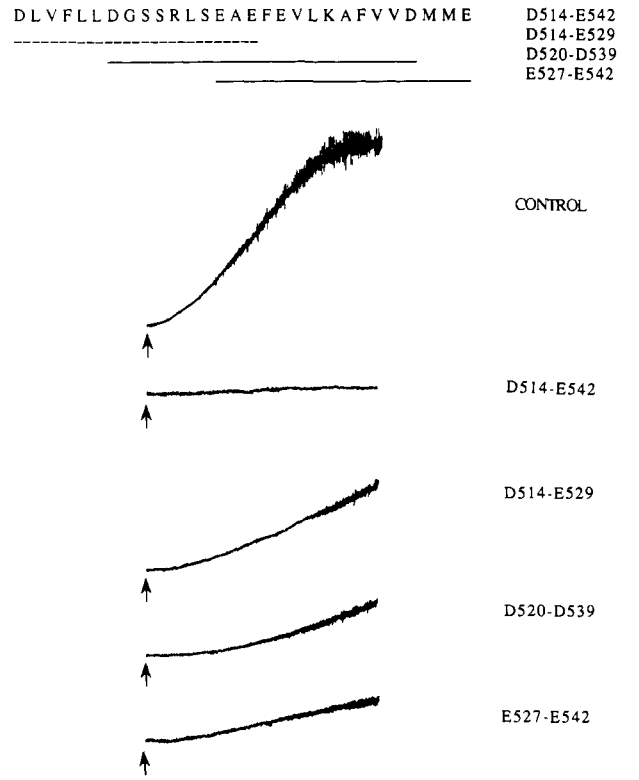

FIGURE 2: Effect of vWF peptides (0.5 mM) on asialo-vWF-induced platelet agglutination. The traces represent relative transmittance on the vertical axis, as a measure of the extent of aggregation, and time horizontally, where the full width is 5 min. The arrows show the time of addition of asialo-vWF (final concentration, 100 μg/mL). The peptides included in each assay are shown to the right of each trace, and the peptide sequences are illustrated at the top of the figure. The results are representative of three separate experiments using different platelet donors.

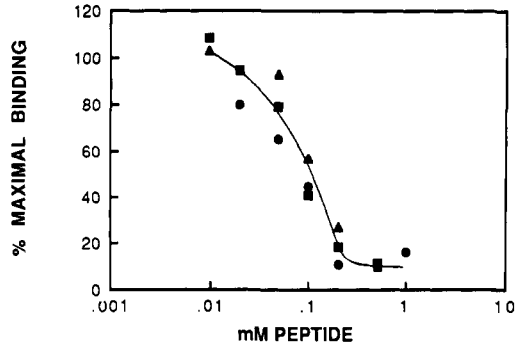

FIGURE 3: Inhibition of ristocetin-dependent binding of ^{125}I-labeled von Willebrand factor to GP Ib–IX complex coated beads by Leu-694–Pro-708 (●), by an equimolar mixture of Leu-694–Pro-708 and Cys-474–Pro-488 (▲), or by an equimolar mixture of Leu-694–Pro-708 and Asp-514–Glu-542 (■). The *x*-axis corresponds to the total concentration of inhibitory peptide.

Table II: Peptide Structures Inhibiting Ristocetin-Dependent Binding of vWF to Receptor

vWF sequence 474–488	CQEPGGLVVPPTDAP
vWF sequence 694–708	LCDLAPEAPPPTLPP
bradykinin potentiator C[a]	pEGLPPGPPIPP
C-terminal sequence of SV40 tumor antigen	KPPTPPPEPET

[a] pE = pyroglutamate.

Cys-474–Pro-488 and Leu-694–Pro-708 had little or no effect at a final concentration of 500 μM. The remaining five vWF peptides also failed to inhibit asialo-vWF-induced platelet aggregation at this concentration (data not shown). The maximal rate of agglutination of platelets by bovine vWF (10 μg/mL) was inhibited 75% by 500 μM Asp-514–Glu-542 peptide and 35% by 500 μM Cys-474–Pro-488, the remaining peptides having no effect at this concentration (data not shown).

Truncated forms of the Asp-514–Glu-542 peptide (Table I) were at least an order of magnitude less effective as inhibitors of vWF-GP Ib–IX complex interaction. The peptides Asp-514–Glu-529, Asp-520–Asp-539, and Glu-527–Glu-542 at 1 mM had no inhibitory effect on either the ristocetin- or botrocetin-dependent binding of vWF to platelets or GP Ib–IX beads. This result is consistent with the data of Mohri et al. (1988) that overlapping 15-mers through this region of vWF did not inhibit ristocetin-dependent binding of vWF to platelets. These three peptides, however, at 0.5 mM partially inhibited asialo-vWF-induced platelet aggregation (Figure 2). Since both the Asp-514–Glu-529 and Glu-527–Glu-542 peptides had similar inhibitory activity, it would appear that both the N- and C-terminal halves of the Asp-514–Glu-542 peptide are required for full biological activity.

It has been previously reported that the vWF peptides Cys-474–Pro-488 and Leu-694–Pro-708 acted synergistically in inhibiting ristocetin-dependent binding of vWF to platelets (Mohri et al., 1988), a result not confirmed by Girma et al. (1990). In the present study, we also examined the effect of this combination of vWF peptides and the combination of Asp-514–Glu-542 and Leu-694–Pro-708, added in equimolar amounts, on the ristocetin-dependent binding of vWF to purified GP Ib–IX complex. The data of Figure 3 compared to that of Figure 1 suggest that the inhibitory effect of these peptides in combination is additive rather than synergistic. Similar results were obtained for the corresponding platelet binding assay (data not shown).

Ristocetin Modulation of vWF Binding. The inhibition of ristocetin-dependent binding of vWF by the vWF peptides 474–488 and 694–708 and their failure to substantially affect other assays of vWF–GP Ib–IX complex interaction suggests that these peptides may be interfering with the modulator function of ristocetin. These vWF peptides share significant sequence similarities (Table II), are relatively hydrophobic, and are rich in aliphatic amino acids such as glycine, alanine, leucine, and valine, as well as proline. In addition, seven of the 10 prolines are clustered as Pro-Pro or Pro-Pro-Pro, suggesting a high degree of inflexibility and "turn" in the peptide structure at these points. To examine whether the inhibition of ristocetin-dependent vWF–receptor interaction by these vWF peptides could be due to ristocetin recognition of these proline repeats, we evaluated the effect of two random peptides containing this structural feature (Table II). As for the vWF peptides 474–488 and 694–708, both random peptides were effective inhibitors of the ristocetin-dependent binding of vWF to the GP Ib–IX complex but had no effect on the corresponding botrocetin-dependent binding of vWF (Figure 4) and little effect on asialo-vWF platelet agglutination (data not shown), suggesting that all four peptides (Table II) were interfering with the modulator function of ristocetin rather than directly inhibiting vWF–GP Ib–IX complex interaction.

Two additional lines of evidence support this conclusion. Scott et al. (1991) recently reported that the flocculation of fibrinogen by ristocetin was due to binding of dimeric ristocetin to fibrinogen. Peptides containing the consensus sequence XPGX′ inhibited ristocetin-dependent fibrinogen flocculation by presumably binding to ristocetin and competing for the ristocetin binding sites on fibrinogen (Scott et al., 1991). The proline-rich vWF peptides, 474–488 and 694–708, also inhibited the ristocetin-dependent flocculation of fibrinogen

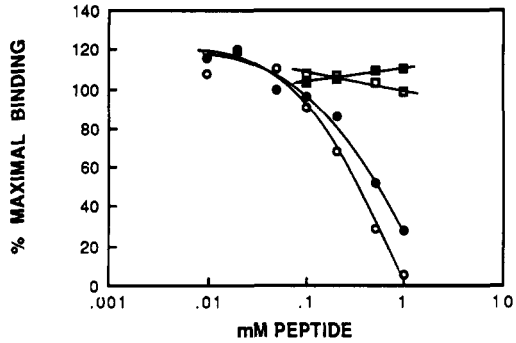

FIGURE 4: Effect of random peptides pEGLPPGPPIPP (●, ■) and KPPTPPPEPET (○, □) on the ristocetin-dependent (circles) and botrocetin-dependent (squares) binding of ^{125}I-labeled von Willebrand factor to GP Ib–IX complex coated beads.

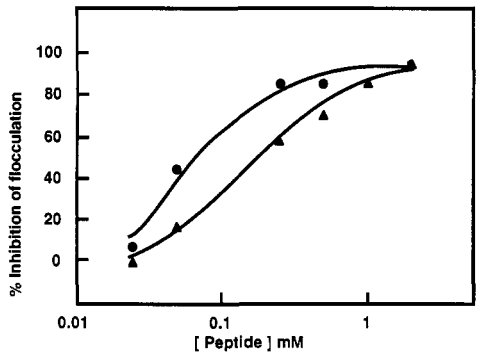

FIGURE 5: Inhibition of ristocetin-dependent flocculation of ^{125}I-labeled fibrinogen at 22 °C by vWF peptides (●) Leu-694–Pro-708 and (▲) Cys-474–Pro-488. Ristocetin and fibrinogen were at final concentrations of 0.7 and 2.0 mg/mL, respectively.

(Figure 5) with similar potency as observed for their effect on the ristocetin-dependent binding of vWF to receptor (Figure 1). This finding is consistent with both these vWF peptides binding ristocetin. The two random peptides containing proline-rich repeats (Table II) also inhibited the ristocetin-dependent flocculation of fibrinogen. In contrast, other vWF peptides (Table I) had little or no effect in this assay at concentrations up to 1 mM (data not shown). Finally, direct evidence for complex formation between the proline-rich peptides and ristocetin was obtained using ultraviolet difference spectroscopy (Nieto & Perkins, 1971). The vWF peptide, 694–708, gave concentration-dependent changes in the difference spectrum of ristocetin at peptide concentrations from 0.025 to 0.4 mM (Figure 6). Similar spectral changes were observed with the other proline-rich peptides but not with vWF peptides lacking the proline-rich repeats (data not shown). The observed spectral changes are consistent with formation of peptide–ristocetin complexes in which the spectral characteristics of the aromatic groups in ristocetin (Kalman & Williams, 1980) are perturbed by bound peptide (Nieto & Perkins, 1971).

Ristocetin as an antibiotic functions by binding to the D-Ala-D-Ala dipeptide sequence on Gram-positive bacteria (Nieto & Perkins, 1971). However, N^{α},N^{ϵ}-diacetyl-Lys-D-Ala-D-Ala had no inhibitory effect on the ristocetin-dependent binding of vWF to receptor at concentrations up to 1 mM, indicating that ristocetin recognition of proline-containing sequences (Scott et al., 1991) probably involves a different binding site on the glycopeptide antibiotic. The smaller vWF peptide, EAPPPT (Table I), was also ineffective as an inhibitor of ristocetin-dependent vWF binding to receptor at peptide concentrations up to 1 mM and did not form a complex with ristocetin as evaluated by ultraviolet difference spectroscopy,

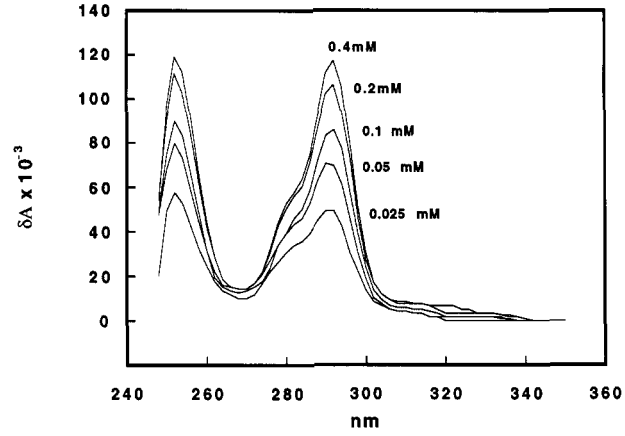

FIGURE 6: Ultraviolet difference spectroscopy of the ristocetin–peptide Leu-694–Pro-708 complex. Reference and sample cuvettes both contained 0.1 mM ristocetin in 0.05 M Hepes buffer, pH 7.4. For each spectrum, the sample cuvette also contained the indicated concentration of peptide.

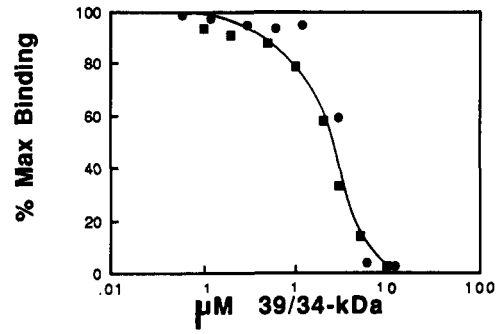

FIGURE 7: Inhibition of binding of ^{125}I-labeled von Willebrand factor to immobilized botrocetin by the 39/34-kDa vWF fragment (■) and by reduced and alkylated 39/34-kDa vWF fragment (●).

suggesting that either a minimum chain length is necessary for effective binding of ristocetin to proline-rich peptides or that more than one proline recognition structure is required per peptide for effective interaction.

Botrocetin Modulation of vWF Binding. It has previously been shown that the 39/34-kDa vWF dispase fragment inhibits botrocetin-dependent vWF binding to purified GP Ib–IX complex and that prior reduction and alkylation of this fragment does not result in loss or diminution of inhibitory activity (Andrews et al., 1989b). This suggests that neither the botrocetin or GP Ib binding domains on the 39/34-kDa fragment depend upon tertiary structural constraints imposed by the disulfide bond linking Cys-509 and Cys-695. The data in Figure 7 confirm that reduction and alkylation of the 39/34-kDa vWF fragment does not significantly alter its ability to compete with ^{125}I-labeled vWF for binding to botrocetin immobilized on beads. For this experiment, complete reduction and alkylation of the 39/34-kDa vWF fragment was confirmed by its decreased mobility on SDS–polyacrylamide gels under nonreducing conditions (Andrews et al., 1989b). The vWF–botrocetin binding assay was also used to identify potential botrocetin-binding peptide sequences within the 39/34-kDa vWF fragment. Of the first eight vWF peptides listed in Table I, only the peptides Asp-514–Glu-542 (IC_{50} = 180 μM) and Cys-474–Pro-488 (IC_{50} = 900 μM) inhibited binding of vWF to immobilized botrocetin. By contrast, the vWF peptide Leu-694–Pro-708 did not inhibit binding at peptide concentrations up to 1 mM (Figure 8). Truncated derivatives of the Asp-514–Glu-542 peptide (Table I) were also ineffective in inhibiting the interaction between botrocetin and vWF (data not shown).

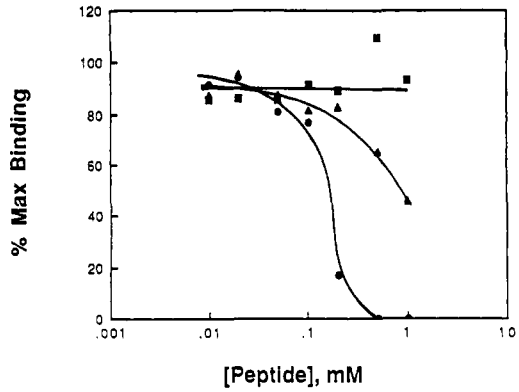

FIGURE 8: Inhibition of binding of ^{125}I-labeled von Willebrand factor to immobilized botrocetin by vWF peptides: (●) Asp-514–Glu-542, (▲) Cys-474–Pro-488, and (■) Leu-694–Pro-708.

DISCUSSION

Platelet adhesion to exposed vascular subendothelium represents the first event in hemostasis. At moderate to high shear rates, this adhesion is dependent upon von Willebrand factor (vWF) and a specific vWF receptor on platelets, the glycoprotein (GP) Ib–IX complex (Booth et al., 1990). Previous studies using in vitro modulators of vWF adhesive function, such as ristocetin or botrocetin, or vWF activated by desialation have localized the GP Ib–IX complex receptor binding domain of vWF to the first A-domain repeat sequence between Cys-509 and Cys-695 and adjacent N- and C-terminal sequences (Fujimura et al., 1986; Andrews et al., 1989b). In particular, Mohri et al. (1988) identified two peptide sequences, Cys-474–Pro-488 and Leu-694–Pro-708, which inhibited synergistically the ristocetin-dependent binding of vWF and the binding of asialo-vWF to platelets. They suggested that these two peptides brought into proximity by an intrasubunit disulfide bond between Cys-509 and Cys-695 collectively encompassed a GP Ib–IX complex receptor recognition site. Recent evidence, however, suggests that this model is too simplistic and does not fully explain all the available data. First, in contrast to their inhibitory effects on ristocetin-dependent vWF–receptor interaction, these two vWF peptides have no inhibitory effect on botrocetin-dependent binding of vWF to either platelets or purified GP Ib–IX complex (Girma et al., 1990; Fujimura et al., 1991b; Sugimoto et al., 1991; this study). Second, a heterodimeric vWF fragment, III-T2, spanning residues 273–511 and 674–728, inhibits ristocetin- but not botrocetin-dependent vWF–receptor interaction (Fujimura et al., 1991b; Sugimoto et al., 1991). Finally, several anti-vWF monoclonal antibodies have been described which also inhibit ristocetin- but not botrocetin-dependent binding of vWF to the GP Ib–IX complex whereas other anti-vWF monoclonal antibodies block vWF–receptor interaction induced by either modulator (Berndt et al., 1988; Andrews et al., 1989a; Girma et al., 1990; Fujimura et al., 1991b). This suggests either that different regions of vWF are involved in receptor recognition induced by different modulators, that additional sequences within the Cys-509–Cys-695 sequence are involved in receptor recognition, or that the mechanisms of modulation by ristocetin and botrocetin are distinct. In the present study, we have attempted to resolve these ambiguities by analyzing the effects of vWF peptides corresponding to hydrophilic stretches of sequence within the first A-domain of vWF.

A key finding of this study has been the identification of a previously unrecognized sequence, Asp-514–Glu-542, as a potential GP Ib–IX complex receptor recognition sequence in vWF. Asp-514–Glu-542 peptide was chosen for analysis because this sequence shows a high degree of homology in proteins that contain A-domains (Colombatti & Bonaldo, 1991). This peptide inhibited both the ristocetin- and botrocetin-dependent binding of vWF to platelets and purified GP Ib–IX complex as well as the vWF-dependent aggregation of platelets induced by asialo-vWF and bovine vWF, supporting an adhesive role for this sequence in mediating vWF–GP Ib–IX complex interaction. Additional support for this conclusion derives from a comparison of the IC_{50} value (50 μM) for the inhibition of ristocetin-dependent binding of vWF to purified GP Ib–IX complex by the Asp-514–Glu-542 peptide with the IC_{50} value (5 μM) for the corresponding inhibition by the 39/34-kDa vWF fragment, Leu-480/Val-418–Gly-718. This difference of only an order of magnitude between the vWF peptide and the considerably larger vWF proteolytic fragment suggests that the Asp-514–Glu-542 peptide encompasses a major portion of the receptor recognition sequence. The present data, however, do not formally exclude the possibility that the Asp-514–Glu-542 peptide is inhibitory because it interacts with vWF rather than with receptor. In apparent contradiction to our findings, Fujimura et al. (1991b) reported that the vWF peptide fragment 449–549, at 100 μM completely inhibited ristocetin-dependent binding of vWF to platelets without affecting botrocetin-dependent binding. An analysis of the data of Figure 1, however, suggests that at this concentration, the Asp-514–Glu-542 peptide is relatively ineffective in inhibiting the botrocetin-dependent binding of vWF to either platelets (IC_{50} = 150 μM) or purified GP Ib–IX complex (IC_{50} = 200 μM).

In contrast to the vWF peptide Asp-514–Glu-542, which was found to be inhibitory in all the assay systems employed to assess vWF–GP Ib–IX complex interaction, the vWF peptides Cys-474–Pro-488 and Leu-694–Pro-708 (Mohri et al., 1988) were found to only effectively inhibit the ristocetin-dependent binding of vWF to receptor. Since ristocetin has been demonstrated to bind to vWF (Scott et al., 1991), it seemed possible that the selective inhibitory effect of these two vWF peptides might be due to interference with the modulator function of ristocetin rather than at the level of vWF–receptor interaction. This conclusion was directly supported by three lines of evidence. First, random peptides that contained proline repeats were also effective inhibitors of ristocetin-dependent binding of vWF to receptor but did not inhibit botrocetin-dependent binding of vWF to receptor or asialo-vWF-induced platelet aggregation. Second, all four peptides containing proline-rich repeats (Table II) inhibited the ristocetin-dependent flocculation of fibrinogen. Finally, ultraviolet difference spectroscopy confirmed that complex formation occurred between ristocetin and the proline-rich peptides.

Scott et al. (1991) recently demonstrated that the functional form of ristocetin that mediates vWF binding is the ristocetin dimer and that the ristocetin recognition site in proteins is a β-turn of the form XPGX′. This consensus sequence occurs in only two of the four peptides, however, although all four peptides contain proline repeats (Table II) and inhibited the ristocetin-dependent binding of vWF to platelets and purified GP Ib–IX complex and the ristocetin-dependent flocculation of fibrinogen, suggesting that the sequences XPPX′ or XPPPX′ are also probable ristocetin recognition sites. Each of the four inhibitory peptides therefore contains at least two potential ristocetin binding sites which would be expected to stabilize the interaction with ristocetin dimer. In this context, it is of interest that the peptide EAPPPT containing a single potential

ristocetin recognition site was ineffective as an inhibitor over a similar concentration range as the other four peptides. Additional support for the conclusion that ristocetin binding to proline-rich repeats in the vWF peptides 474–488 and 694–708 explains their inhibitory effect on ristocetin-dependent vWF–GP Ib–IX complex interaction derives from a recent report by Ware et al. (1991), who constructed mutant recombinant domains of vWF. In a subsequent study, they altered the triplet of proline residues (702–704) to a triplet of either arginine or aspartic acid residues and expressed the mutant vWF domains in CHO cells (Azuma et al., 1991). Neither charge mutant bound to GP Ib in the presence of ristocetin, although botrocetin-dependent binding was normal and the reduced and alkylated vWF fragment (441–730) also bound normally to GP Ib. These results suggest that the proline triplet at 702–704 is critical for the normal modulator function of ristocetin but not for vWF–GP Ib–IX complex interaction.

Recent studies have localized the botrocetin modulator site on vWF to within the first A-domain. In this study, we confirmed that this site resides within the sequence Leu-480/Val-481–Gly-718 (Andrews et al., 1989b) since the 39/34-kDa vWF fragment inhibited the interaction between vWF and immobilized botrocetin. The integrity of the Cys-509–Cys-695 disulfide bond was not important for botrocetin recognition since reduction and alkylation of the 39/34-kDa fragment did not affect its ability to inhibit vWF–botrocetin interaction. The inability of III-T2 vWF fragment, 273–511 and 674–728, to inhibit this interaction suggests that the botrocetin modulator site probably resides between residues 512 and 673 in the primary structure of vWF (Sugimoto et al., 1991). In this regard, Sugimoto et al. (1991) have identified three noncontiguous peptides, 539–553, 569–583, and 629–643, which inhibited the binding of botrocetin to immobilized vWF by 60–75% at a concentration of 0.5 mM, although, somewhat surprisingly, none of the three peptides inhibited botrocetin-dependent binding of vWF to platelets. Two of these peptides overlap with peptides from the present study, 564–581 and 628–646, which we found did not inhibit botrocetin-vWF interaction. The reason for this discrepancy is not clear, although it may be noted that Sugimoto et al. (1991) found that peptides that included the tetralysine sequence at 642–645 were ineffective as inhibitors of botrocetin–vWF interaction. In the present study, we have identified an additional peptide sequence, Asp-514–Glu-542, which also inhibited the binding of vWF to immobilized botrocetin (IC_{50} = 180 μM). Since this peptide also inhibits the botrocetin-dependent binding of vWF to platelets and the purified GP Ib–IX complex, the present data do not discriminate between whether the peptide is affecting botrocetin–vWF interaction, vWF–GP Ib–IX complex interaction, or both. The latter, however, is the most likely possibility since we found that this peptide was a general inhibitor of vWF–GP Ib–IX complex interaction.

Although the mechanism(s) involved in vWF activation is(are) poorly understood, the present data and recent studies on the molecular mechanism of Type IIb von Willebrand's disease are consistent with an electrostatic model for this event. The Cys-509–Cys-695 disulfide bond partitions this region of vWF into two distinct domains. Sequences N- and C-terminal to this disulfide bond are exclusively negatively charged, whereas the sequence within the disulfide loop is predominantly positively charged. It is known that ristocetin must be positively charged to exert its vWF cofactor activity (Coller, 1987). It is therefore conceivable that the binding of ristocetin to the negatively charged vWF sequences 474–488 and 694–708 could alter electrostatic interactions between this segment of the vWF molecule and positively charged residues between position 510 and 694 exposing the Asp-514–Glu-542 sequence and possibly other vWF sequences in an active conformation for binding to the GP Ib–IX complex. Removal of negatively charged sialic acid residues from N- and O-linked glycosylation sites at residues Asn-468, Thr-485, Thr-492, Thr-493, Ser-500, Thr-705, and Thr-714 provides a similar electrostatic model for the activity of asialo-vWF. vWF from patients with Type IIb von Willebrand's disease can bind spontaneously to the GP Ib–IX complex in the absence of modulators and is more sensitive to ristocetin (Sadler, 1991). In individual patients, this activation of the vWF appears to be due to a single point mutation at one of at least five loci, R543 → W, 545 → C, W550 → C, V553 → M, and R578 → Q (Cooney et al., 1991; Randi et al., 1991; Ware et al., 1991). Three of these point mutations involve loss of a positively charged arginine residue and all reside within a highly basic segment of vWF adjacent to the Asp-514–Glu-542 sequence, again consistent with an electrostatic model for vWF activation. Finally, peptide studies (Sugimoto et al., 1991; this study) indicate that botrocetin recognizes several sequences that reside within this region of the vWF molecule, Asp-514–Gln-583, suggesting that botrocetin modulation of vWF function may also involve an electrostatic mechanism. Current studies in our laboratory are aimed at further defining the adhesive and modulator sequences in vWF and the mechanism and control of vWF activation. In this regard, in the following paper (Knott et al., 1992), we report the solution structure of the Asp-514–Glu-542 peptide as determined by nuclear magnetic resonance spectroscopy.

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Registry No. VWF, 109319-16-6; sequence 514–542, 143902-05-0; sequence 564–581, 143902-06-1; sequence 607–628, 143902-07-2; sequence 628–646, 143902-08-3; sequence 655–674, 143902-09-4; sequence 677–694, 143902-10-7; sequence 474–488, 117723-30-5; sequence 694–708, 117132-58-8; sequence 614–529, 143902-11-8; sequence 520–539, 143902-12-9; sequence 527–542, 143902-13-0; sequence 700–705, 143902-14-1; CQEPGGLVVPPTDAP, 117723-30-5; p^{E}GLPPGPPIPP, 30953-20-9; KPPTPPPEPET, 75813-50-2; ristocetin, 1404-55-3; botrocetin, 85537-36-6.