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### Research Article

von Willebrand factor (vWF) is a large, multimeric glycoprotein that helps platelets adhere to vascular subendothelium. Although vWF binding to platelet receptors and connective tissue constituents is of fundamental importance in adhesion, there is little information regarding the nature of these vWF binding sites. In this paper, we have compared the structural requirements for vWF binding with platelet glycoprotein Ib (Gplb), heparin, and collagen and have shown that fragments derived from large vWF multimers retain biologic activity. We have shown that a 440,000-D subunit produced by disulfide reduction and alkylation of vWF polymer binds to platelet Gplb. When analyzed by polyacrylamide gel electrophoresis and Sepharose CL6B chromatography, the 440,000-D vWF oligomer is a dimer of the 220,000 subunit of fully reduced native vWF. This vWF dimer competes with <sup>125</sup>I-vWF for binding to Gplb with an IC<sub>50</sub> of 100 micrograms/ml (227 nM). The Gplb binding domain on vWF was further localized by digestion of native vWF polymers with Staphylococcal V8 protease. A 285,000-D fragment of vWF multimer was separated from heterogeneous 210,000-225,000-D fragments by its ability to bind to heparin. The 285,000-D fragment that bound to heparin Sepharose was composed of two disulfide-linked 175,000- and 115,000-D polypeptides. The heterogeneous fragments contained disulfide-linked 96,000, 66,000, and 53,000-D polypeptides when analyzed on polyacrylamide gel electrophoresis. The 285,000-D fragment competed with <sup>125</sup>I-vWF for binding [...]

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# Structural Basis of von Willebrand Factor Binding to Platelet Glycoprotein Ib and Collagen

## Effects of Disulfide Reduction and Limited Proteolysis of Polymeric von Willebrand Factor

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### Abstract

von Willebrand factor (vWF) is a large, multimeric glycoprotein that helps platelets adhere to vascular subendothelium. Although vWF binding to platelet receptors and connective tissue constituents is of fundamental importance in adhesion, there is little information regarding the nature of these vWF binding sites. In this paper, we have compared the structural requirements for vWF binding with platelet glycoprotein Ib (GpIb), heparin, and collagen and have shown that fragments derived from large vWF multimers retain biologic activity. We have shown that a 440,000-D subunit produced by disulfide reduction and alkylation of vWF polymer binds to platelet GpIb. When analyzed by polyacrylamide gel electrophoresis and Sepharose CL6B chromatography, the 440,000-D vWF oligomer is a dimer of the 220,000 subunit of fully reduced native vWF. This vWF dimer competes with  $^{125}\text{I}$ -vWF for binding to GpIb with an  $\text{IC}_{50}$  of 100  $\mu\text{g}/\text{ml}$  (227 nM).

The GpIb binding domain on vWF was further localized by digestion of native vWF polymers with Staphylococcal V8 protease. A 285,000-D fragment of vWF multimer was separated from heterogeneous 210,000–225,000-D fragments by its ability to bind to heparin. The 285,000-D fragment that bound to heparin Sepharose was composed of two disulfide-linked 175,000- and 115,000-D polypeptides. The heterogeneous fragments contained disulfide-linked 96,000, 66,000, and 53,000-D polypeptides when analyzed on polyacrylamide gel electrophoresis. The 285,000-D fragment competed with  $^{125}\text{I}$ -vWF for binding to GpIb with an  $\text{IC}_{50}$  of 22  $\mu\text{g}/\text{ml}$  (77 nM), while the other fragments did not compete for binding. Neither the vWF dimer nor the proteolytic fragments competed with native  $^{125}\text{I}$ -vWF polymer for binding to collagen.

### Introduction

The von Willebrand factor (vWF)<sup>1</sup> is a heterogeneous multimeric plasma protein that facilitates adhesion of platelets to injured

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1. *Abbreviations used in this paper:* GpIb, glycoprotein Ib;  $\text{IC}_{50}$ , concentration that inhibits binding by 50%; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, 0.05 M Tris, 0.15 M NaCl, 0.02% azide, pH 7.4; vWF, von Willebrand factor; vWF<sub>d</sub>, von Willebrand factor dimer.

vessel wall (1–3). Although the mechanism by which vWF promotes adhesion has not been fully delineated, there is evidence that vWF links platelets to the vessel wall by binding to platelet glycoprotein Ib (GpIb), as well as to exposed subendothelial collagen (4–9). In previous studies, we, and others, have demonstrated vWF binding to GpIb and competition for binding by a soluble proteolytic fragment of GpIb, glycolocalin (10, 11). In addition, there are several reports of vWF binding to fibrillar collagen (7–9, 12–14), and we have recently reported the binding and Factor XIII-dependent cross-linking of vWF to monomeric collagen (Bockenstedt, P., and R. I. Handin, manuscript submitted for publication).

There is evidence that vWF has distinct domains that bind either to platelet surface glycoproteins or components of connective tissue (15–19). Using a series of monoclonal antibodies directed against specific epitopes on vWF, two groups (15, 16) have demonstrated selective inhibition of vWF binding to collagen or to platelet receptors on GpIb and GpIIb-IIIa. In addition, Girma et al. (18) have used limited proteolysis of vWF polymer to produce fragments with selective binding to either GpIb or the IIb/IIIa complex. The current studies were undertaken to further localize the binding sites on vWF for collagen and GpIb and to develop methods to isolate fragments of vWF polymer containing these functional domains.

### Methods

*Materials.* Iodobeads were obtained from Pierce Chemical Co., Rockford, IL.  $^{125}\text{I}$ -Na was obtained from New England Nuclear, Boston, MA. Sephacryl S 1000 and Sepharose CL6b were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Affigel Gelatin Sepharose, high molecular weight standards, and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories, Richmond, CA. Heparin Sepharose (0.92 mg heparin per ml hydrated gel) and dextran sulfate Sepharose (0.62 mg dextran sulfate,  $M_r$  500,000 per ml hydrated gel) were obtained from Bethesda Research Laboratories, Gaithersburg, MD. Ristocetin was obtained from Bio/Data Corp., Hatboro, PA. Bovine serum albumin, polyethylene glycol  $M_r$  8,000 and  $M_r$  20,000, beta-mercaptoethanol, dextran sulfate  $M_r$  500,000, aprotinin, and phenylmethylsulfonyl fluoride (PMSF), were obtained from Sigma Chemical Co., St. Louis, MO. Staphylococcal V8 protease was obtained from Miles Laboratories, Inc., Naperville, IL. Human fibronectin was a generous gift from Dr. Jan McDonagh, Beth Israel Hospital, Boston, MA. All other chemicals were reagent grade or better.

*Formalin-fixed platelets.* These were prepared as previously described (20) and resuspended in 50 mM Tris, 0.15 M NaCl, 0.02% sodium azide, pH 7.4 at a final concentration of  $6 \times 10^5$  platelets per microliter. The ability of native vWF polymers to agglutinate formalin-fixed platelets was determined in the presence of 1 mg/ml ristocetin (21, 22) and agglutination was monitored on a Payton dual-channel aggregometer (Payton Scientific, Inc., Buffalo, NY). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method (23).

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**vWF purification.** vWF was purified by a modification of the procedure of Kao et al. (4). 5 liters of plasma obtained within 2–3 d of collection from the American Red Cross Blood Services-Northeast Region (Boston, MA), frozen at  $-20^{\circ}\text{C}$  for 24 h, and thawed at  $4^{\circ}\text{C}$  for 18 h was used as the starting material. The resulting cryoprecipitate was collected by centrifugation at 6,500 *g* for 20 min, resuspended in 20 mM Na citrate, pH 6.1, and reprecipitated twice with final concentrations of 5 and 12% polyethylene glycol ( $M_r$  8,000) (PEG) as previously described. The final precipitate was collected by centrifugation at 6,500 *g* for 30 min, the pellet surface was washed twice with ice-cold 0.02 M Tris, 0.15 M NaCl, 8% ethanol, pH 7.4 to remove residual PEG, and was resuspended in 10–15 ml of 0.05 M Tris, 0.15 M NaCl, 0.02% azide, pH 7.4 (TBS), and applied to a  $2.5 \times 90$  cm siliconized glass column containing Sephacryl S 1000. 4-ml fractions, collected at a 70 ml/h flow rate, were analyzed for ristocetin-dependent platelet agglutinating activity (21), protein concentration by the method of Lowry (24), and for purity by 5% SDS-PAGE after disulfide bond reduction, as described above (23). Those fractions demonstrating a single 220,000-D band on 5% SDS-PAGE were pooled and concentrated by precipitation with 35% (wt/vol) ammonium sulfate followed by centrifugation at 34,800 *g* for 30 min at  $4^{\circ}\text{C}$ . The precipitate was then resuspended in a small volume of TBS and dialyzed extensively against TBS. Purified vWF was then stored at  $-20^{\circ}\text{C}$  in 250- $\mu\text{l}$  aliquots for up to 3 wk.

**Iodination of vWF.** vWF was iodinated using the iodobead technique. One iodobead was preincubated with 0.5–1.0 mCi  $^{125}\text{I}$ -Na and 100  $\mu\text{l}$  of TBS for 10 min at room temperature, followed by addition of 500  $\mu\text{g}$  of purified vWF. After 10 min, the iodination was stopped by transfer of the vWF solution to a  $0.7 \times 10$  cm Sephadex G-25 column preequilibrated with a 1 mg/ml bovine serum albumin solution in TBS and eluted with TBS. Typical specific radioactivity of vWF was 0.20–0.25  $\mu\text{Ci}/\mu\text{g}$  protein.

**Preparation of reduced-alkylated vWF.** 3 mg of purified native vWF was incubated with 10 mM beta-mercaptoethanol at room temperature for 3 h and then carboxymethylated with 40 mM iodoacetamide for 20 min. The reduced, alkylated vWF was then gel-filtered on a 1 cm  $\times$  92 cm Sepharose CL6b column in 50 mM Tris, 0.5 M NaCl, 0.02% sodium azide, pH 7.4. 1-ml fractions were collected and the absorbance at 280 nm was measured. The vWF oligomer produced by reduction and alkylation typically eluted at fractions 55–65. The desired fractions were pooled and concentrated by dialysis against 20% polyethylene glycol  $M_r$  20,000 in TBS. Samples of the vWF oligomer were analyzed under reducing and nonreducing conditions on 4.3% SDS-PAGE.

**Affinity chromatography.** Studies of vWF binding to dextran sulfate ( $M_r$  500,000) and heparin substituted Sepharose columns were performed at room temperature on  $0.9 \times 6$  cm siliconized glass columns containing 6 ml of gel. The columns were equilibrated with 0.05 M Tris, 0.15 M NaCl, pH 7.4 containing 0.1% bovine serum albumin (wash buffer). 10  $\mu\text{g}$  of  $^{125}\text{I}$ -vWF in 0.5 ml of the same buffer were applied to each column and 2 ml of buffer eluted to distribute the sample throughout the column volume. 1 h later, the column was washed with 50 ml of the same buffer at a flow rate of 10 ml/h. The effluent, collected in 2.5 ml fractions, was monitored for radioactivity. Once background levels of radioactivity were achieved, a 50-ml linear gradient of 0.15–1.5 M NaCl in 0.05 M Tris, pH 7.4 containing 0.1% albumin was applied at a flow rate of 20 ml/h. The effluent, collected in 2.5 ml fractions, was again monitored for radioactivity.

In some experiments, the heparin sepharose column eluates were analyzed on 5% SDS-PAGE. In those experiments, all wash and eluting buffers contained 0.01% albumin. 120  $\mu\text{l}$  of ice-cold 100% trichloroacetic acid (TCA) were added to 500  $\mu\text{l}$  of each elution fraction. The samples were then centrifuged at 10,000 *g* for 4 min. The supernatant was removed, and the radioactivity in the precipitate was measured in a Beckman gamma 8,000 spectrometer (Beckman Instruments, Inc., Fullerton, CA). The TCA precipitate was washed three times with ice-cold 100% acetone and then dissolved in Laemmli sample buffer for analysis on 5% SDS-PAGE.

**Staphylococcal V8 digestion.** Purified native vWF polymers were incubated with Staphylococcal V8 protease at a ratio of 1  $\mu\text{g}$  protease to 350  $\mu\text{g}$  protein at  $37^{\circ}\text{C}$ . In some experiments, 30- $\mu\text{l}$  aliquots were removed

from the digest at varying time intervals over 4 h, mixed with 10 U/ml aprotinin and 25  $\mu\text{l}$  sample buffer, and heated to  $100^{\circ}\text{C}$  for 5 min for analysis on 8.75% SDS-PAGE under reducing conditions. After 4 h, 10 U/ml aprotinin and 5 mM PMSF were added, and the protein digest was applied to a 5 ml heparin Sepharose column and allowed to equilibrate for 30–60 min at room temperature. The unbound protein fragments were then eluted with 25 ml of 50 mM Tris, 0.15 M NaCl 0.02% azide, pH 7.4 at a flow rate of 10 ml/h. Protein fragments bound to heparin Sepharose were eluted with 30 ml of 50 mM Tris, 1.0 M NaCl, pH 7.4 at a flow rate of 10 ml/h. The 1.0-M and 0.15-M NaCl eluates were then individually dialyzed against distilled water, frozen in a dry ice acetone bath, lyophilized, and stored at  $-20^{\circ}\text{C}$  until further use. Lyophilized samples were resuspended in TBS containing 10 U/ml aprotinin and 5 mM PMSF.

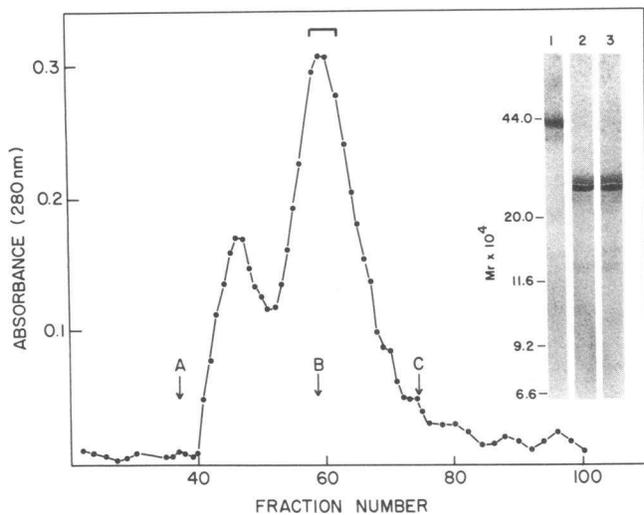
**Platelet binding assay.** Varying concentrations of native vWF polymer, reduced, alkylated vWF oligomer, or proteolytic fragments were added to assays containing 1  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -vWF polymer or reduced, alkylated oligomer, 50  $\mu\text{l}$  of formalin-fixed platelets (600,000 platelets/ $\mu\text{l}$ ), and sufficient TBS to bring the final volume to 200  $\mu\text{l}$ . Incubations were initiated by the addition of 0.5 mg/ml ristocetin with stirring. After 60 min, 100  $\mu\text{l}$  of each assay was layered on 200  $\mu\text{l}$  30% sucrose cushions in 400  $\mu\text{l}$  conical polypropylene tubes and platelet-bound  $^{125}\text{I}$ -vWF was separated from free by centrifugation at 10,000 *g* for 4 min at  $4^{\circ}\text{C}$ . The supernatant was aspirated, the tips containing platelet pellets sliced off, and total radioactivity determined in a Beckman gamma spectrometer (Beckman Instruments, Inc.).

**Collagen binding assay.** 100  $\mu\text{l}$  of acid-soluble calfskin collagen in 20 mM Na citrate, pH 6.1 (1.8 mg/ml) were incubated in individual microtiter wells for 90 min at room temperature. Residual unbound collagen was removed by aspiration and the wells were washed three times with TBS. The wells were then filled completely with a 2 mg/ml albumin solution in TBS and incubated for 1 h at room temperature (15). The wells were then decanted and were ready for immediate use in collagen binding assays. 1  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -vWF, varying concentrations (0–700  $\mu\text{g}/\text{ml}$ ) of vWF polymer, dimer, or vWF digest fragment to be tested, and sufficient TBS to bring the final assay volume to 100  $\mu\text{l}$  were added to the collagen-coated microtiter wells. After 1 h at room temperature, the wells were aspirated dry, washed three times with TBS, and bound radioactivity was measured in a gamma spectrometer.

## Results

Previous studies of vWF binding to the platelet have utilized heterogeneous fractions of polymeric vWF as radioligands (25–28). Binding of these vWF fractions to GpIb requires the addition of the cationic antibiotic, ristocetin (4). In addition, binding is saturable and reversible with virtually all the polymeric  $^{125}\text{I}$ -vWF bound to the platelet in the presence of ristocetin readily displaced by nonradiolabeled vWF polymer (4, 25, 26). We have recently shown that vWF polymers also bind to collagen monolayers in a saturable, reversible, and specific fashion (Bockenstedt, P., and R. I. Handin, manuscript submitted for publication). In the current studies, an oligomer of vWF was produced from vWF polymer by limited disulfide reduction and alkylation. As shown in Fig. 1 (inset, lane 1), this oligomer has an apparent  $M_r$  of 440,000 on 4.3% SDS-PAGE. This mobility is consistent with that of a dimer of the 220,000-D subunit that makes up native vWF polymer. Upon denaturation and further reduction, a single 220,000  $M_r$  band is produced, which is identical to the 220,000-D subunit obtained by fully reducing native vWF polymer (inset, lanes 2 and 3).

To determine whether this vWF dimer (vWF<sub>d</sub>) retains the tertiary and quaternary structure necessary for binding to GpIb or to collagen, vWF<sub>d</sub> was used as a competing ligand in both the platelet and collagen binding assays. As shown in Fig. 2 A,

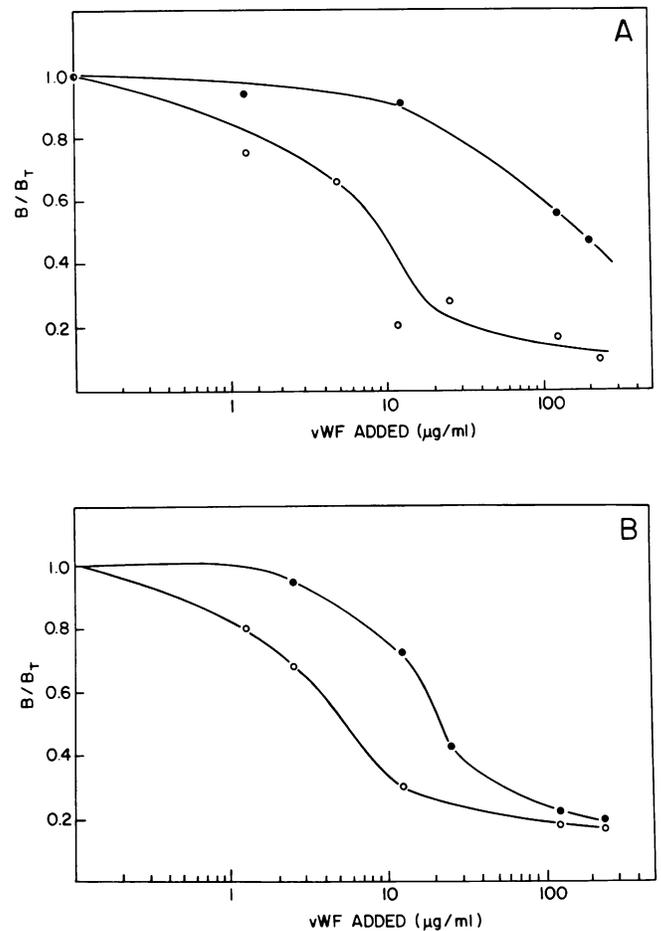


**Figure 1.** Chromatography of partially reduced, alkylated vWF on Sepharose CL6b. 3 mg vWF polymer were incubated with 10 mM beta-mercaptoethanol for 3 h at room temperature followed by 40 mM iodoacetamide for 20 min and chromatographed on a 120 ml Sepharose CL6b column in 50 mM Tris, 0.5 M NaCl, 0.02% sodium azide, pH 7.4. 1-ml fractions were collected at a flow rate of 10 ml/h. The arrows indicate elution positions of molecular weight markers: A, Blue dextran; B, ferritin; C, catalase. *Inset*, 4.3% SDS-PAGE of: 1, peak 2 unreduced; 2, peak 2 fully reduced; 3, vWF polymer fully reduced. The bar over peak 2 delineates the fractions that were pooled, concentrated, and used in subsequent studies.

vWF<sub>d</sub> competes with <sup>125</sup>I-vWF polymer for ristocetin-dependent binding to formalin-fixed platelets with an IC<sub>50</sub> of 100 μg/ml (250 nM). In a subsequent experiment, shown in Fig. 2 B, vWF and vWF<sub>d</sub> were competed against <sup>125</sup>I-vWF<sub>d</sub>. In this experiment, the IC<sub>50</sub> for polymer and dimer was 4±0.5 μg/ml (~4.2 nM) and 19±2 μg/ml (47 nM), respectively, assuming an average M<sub>r</sub> of 1.2 × 10<sup>6</sup> for polymeric vWF.

In contrast to the results obtained with the platelet, vWF<sub>d</sub> does not compete with <sup>125</sup>I-vWF polymer for binding to collagen monolayers (Fig. 3). This suggests that the domain on vWF responsible for binding to collagen is unfavorably altered during the reduction and alkylation procedure used to produce vWF<sub>d</sub> or that collagen binding requires a higher order oligomer.

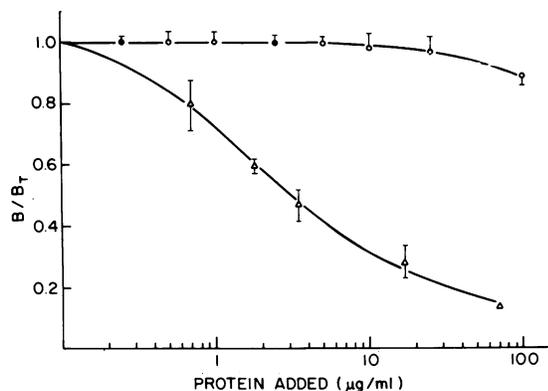
Previous studies from our laboratory have demonstrated that vWF<sub>d</sub> can reassociate into larger oligomers when incubated at the concentrations used in our assay (29). Thus, the effective free concentration of vWF dimer available for binding to the platelet may be reduced by the formation of noncovalently linked oligomers. This creates a complex set of equilibria with both dimer-dimer and dimer-GpIb interactions. To eliminate the possibility that vWF<sub>d</sub> reassociate during the binding studies, and to further localize the regions on vWF that bind to GpIb and collagen, proteolytic fragments of vWF were produced by incubation of vWF polymer with Staphylococcal V8 protease. As shown in Fig. 4, during incubation with Staphylococcal V8 protease, there was progressive loss of the 220,000-D subunit of vWF. Two major fragments of 175,000 and 115,000 D and a doublet at 48,000/43,000 were produced within 15–30 min of digestion. Minor bands of 66,000, 96,000, and 150,000 were also apparent within 15 min of digestion but remained constant in amount over the 4-h incubation. After 4 h of incubation, all of the 220,000-D polypeptide had been digested into smaller



**Figure 2.** Effect of increasing concentrations of vWF polymer and vWF<sub>d</sub> on ristocetin-dependent binding of <sup>125</sup>I-vWF polymer (A) and <sup>125</sup>I-vWF<sub>d</sub> (B). 1 μg/ml <sup>125</sup>I-vWF polymer (A) or <sup>125</sup>I-vWF<sub>d</sub> (B) was incubated with varying concentrations of nonradioactive vWF polymer or vWF<sub>d</sub>, formalin-fixed platelets, and 1 mg/ml ristocetin for 1 h at room temperature. Platelet-bound radioactivity was separated by centrifugation through 30% sucrose. ○, vWF polymer; ●, vWF<sub>d</sub>. Results indicate the mean±SEM of five separate experiments performed in duplicate.

fragments. After thiol reduction, the major bands had mobilities of 175,000, 105,000, and less than 53,000 D when analyzed by 8.75% SDS-PAGE.

We encountered difficulty separating the unreduced fragments produced by Staphylococcal V8 protease digestion by standard gel filtration techniques. Thus, we took advantage of the known affinity of vWF for glycosaminoglycans and designed a chromatographic technique to separate the fragments by their ability to bind to glycosaminoglycan-substituted Sepharose columns. Fig. 5 shows the proteolytic fragments eluted from heparin Sepharose after digestion with Staphylococcal V8 protease. Lanes 1 and 4 are unreduced and reduced samples of the vWF fragments that did not bind to the heparin Sepharose column (fragment 1). The heterogeneous 210,000–225,000-D bands in lane 1 are composed of several disulfide-linked 45,500-, 60,000-, and 110,000-D fragments that can be separated after thiol reduction and electrophoresis on 8.75% polyacrylamide gels (lane 4). Lanes 2 and 3 contain unreduced and reduced samples of the vWF fragment that bound to the heparin Sepharose column (fragment



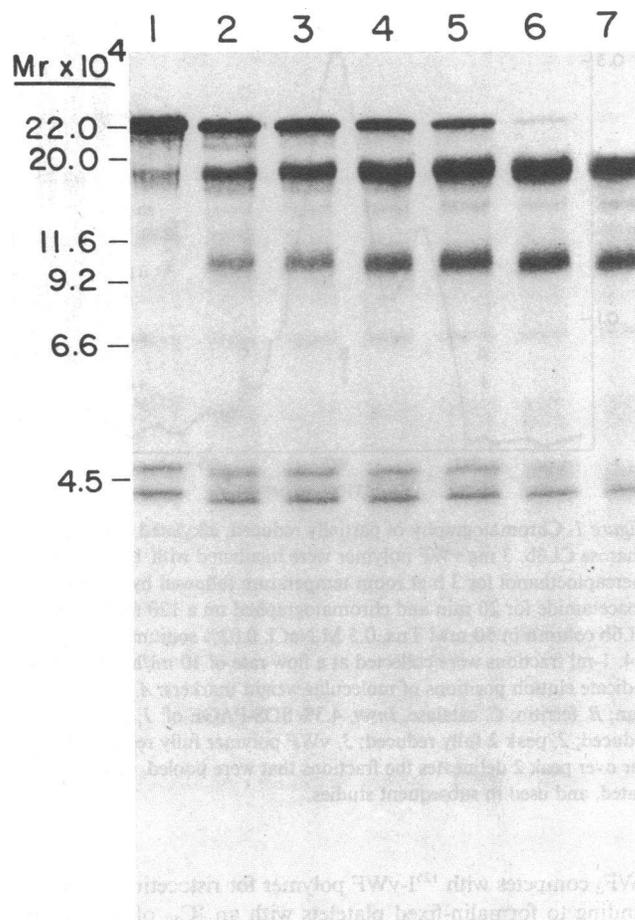
**Figure 3.** Effect of increasing concentrations of vWF polymer and vWF<sub>d</sub> on the binding of <sup>125</sup>I-vWF polymer to collagen-coated microtiter wells. 1 µg/ml <sup>125</sup>I-vWF polymer and varying concentrations of nonradioactive vWF polymer or vWF<sub>d</sub> were incubated in collagen-coated microtiter wells for 1 h at room temperature. Unbound radioactivity was removed by aspiration, and the wells were then washed three times with TBS. Residual bound radioactivity was measured in a gamma spectrometer. Δ, vWF polymer; ○, vWF<sub>d</sub>. Results indicate the mean ± SEM of three separate experiments performed in duplicate.

2). Fragment 2, which is quite homogeneous, has a molecular weight of 285,000 and is composed of two disulfide-linked 175,000- and 115,000-D fragments. Faint 96,000- and 70,000-D bands are thought to be derived from 115,000-D fragments that have been further proteolyzed. These fragments are probably identical to the two major 175,000- and 105,000-D bands noted in the unfractionated Staphylococcal V8 digest (Fig. 4).

To determine whether any of these proteolytic products retained functional activity, we tested fragments 1 and 2 for their ability to compete with native <sup>125</sup>I-vWF for binding to platelet GpIb and collagen monolayers. As shown in Fig. 6 A, fragment 1, which was not retained by heparin Sepharose, did not compete with <sup>125</sup>I-vWF polymer for binding to GpIb. Fragment 2, however, which had an estimated *M<sub>r</sub>* of 285,000 and was retained on heparin Sepharose, competed with native <sup>125</sup>I-vWF for binding to GpIb with an IC<sub>50</sub> of 22 ± 3 µg/ml (77 nM). Neither of these fragments competed with <sup>125</sup>I-vWF polymer for binding to collagen monolayers (Fig. 6 B). These studies suggest that the domains on vWF that bind to GpIb and heparin are located on a 285,000-D proteolytic fragment derived from polymeric vWF, while binding to collagen, which is lost by thiol reduction or proteolysis, requires the more complex quaternary structure present in vWF polymer.

## Discussion

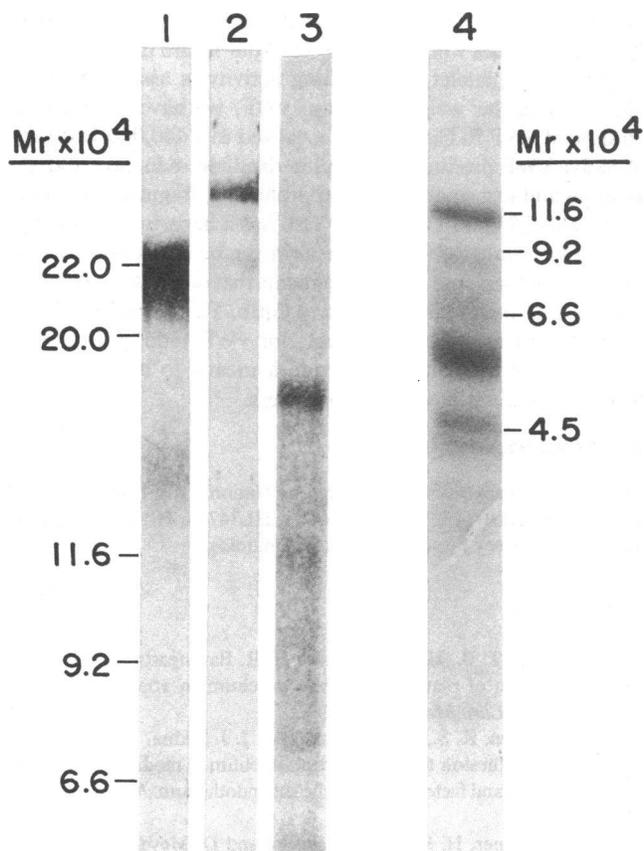
Although vWF is multifunctional and binds to platelet GpIb (5, 6, 25, 26), GpIIb-IIIa (30–32), components of the subendothelium such as collagen (7–9, 16) and Factor VIII:C (28, 33), the domain on native vWF polymer that binds to each of these receptors or proteins has not been localized. One approach that has been used to localize these domains is to dissociate the polymers into smaller oligomers by thiol reduction. Although previous studies have demonstrated the binding of reduced forms of vWF to platelets, no attempt was made to obtain a preparation of vWF oligomers of homogeneous molecular weight or to separate oligomers from unreduced polymeric vWF (34–37). As



**Figure 4.** Analysis of digestion of vWF by Staphylococcal V8 protease by 8.75% SDS-PAGE. 350 µg vWF were incubated with 1 µg of Staphylococcal V8 protease at 37°C. At various time intervals, 30-µl aliquots were removed, mixed with 10 U/ml aprotinin, 5 mM PMSF, and 25 µl of Laemmli sample buffer and boiled for 5 min for analysis on SDS-PAGE. Lane 1, 15 min; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 2 h; lane 6, 3 h; lane 7, 4 h.

our studies indicate (Fig. 1), reduction and alkylation is seldom complete, and a small amount of high molecular weight polymer may remain unless a purification scheme is designed to specifically remove these polymers. We have shown here that a vWF dimer, produced by reduction and alkylation, binds to platelet GpIb. In addition, when this dimeric form was used as a radioligand, both unlabeled dimer and polymer competed for binding. These data suggest that the binding domain for GpIb is retained in a 440,000-mol-wt subunit of polymeric vWF. The disparity in IC<sub>50</sub> between native polymer and dimer may reflect cooperativity in binding between multiple disulfide-linked subunits in the native polymer leading to multiple platelet interactions for each vWF polymer or, alternatively, may reflect non-covalent self-association between the reduced, alkylated subunit dimers to form aberrant vWF polymers with only a limited number of platelet binding sites.

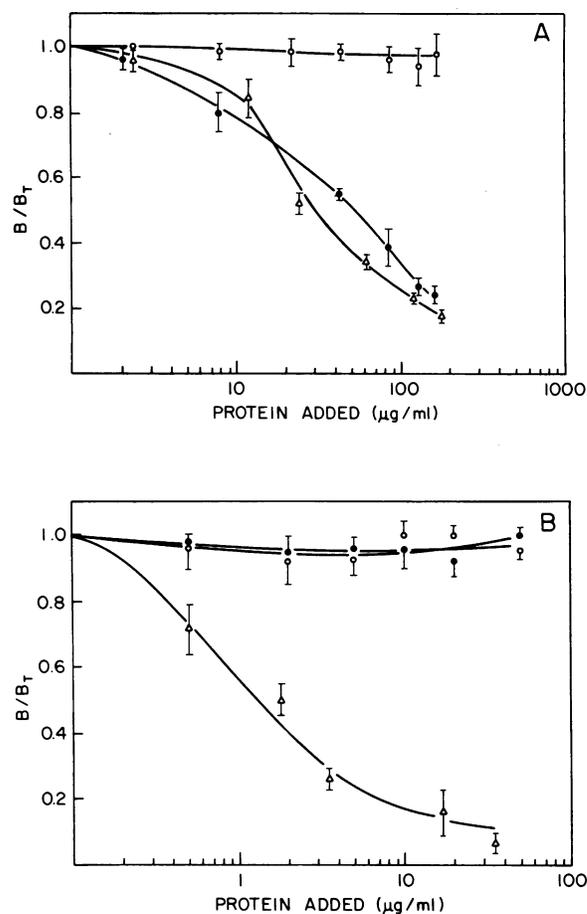
Sixma et al. (38) have shown that a reduced, alkylated form of vWF can bind to the subendothelium and enhance platelet adhesion at low but not high shear rates. In contrast, we were unable to show that our vWF<sub>d</sub> species binds to collagen coated microtiter wells. This may be due to steric interference with the



**Figure 5.** 4.3% SDS-PAGE analysis of vWF proteolytic fragments isolated by chromatography on heparin Sepharose. 3 mg of vWF polymer were digested with 10  $\mu$ g Staphylococcal V8 protease for 4 h at 37°C as previously described and applied to a 5 ml heparin Sepharose column. A portion of the vWF appeared in the wash through volume (fragment 1). Bound vWF fragments were then eluted with 1 M NaCl (fragment 2). Fragments 1 and 2 were dialyzed against distilled H<sub>2</sub>O, lyophilized, and resuspended in 1 ml TBS containing 10 U/ml aprotinin and 5 mM PMSF for analysis on 4.3% SDS-PAGE. Lane 1, fragment 1 unreduced; lane 2, fragment 2 unreduced; lane 3, fragment 2 reduced; lane 4, fragment 1 reduced and analyzed by 8.75% SDS-PAGE.

collagen binding site by carboxymethyl groups present in the reduced, alkylated form of vWF or, more likely, may suggest that the component to which the dimeric species bound in the studies of Sixma and colleagues was different from collagen. They used everted vessel segments in their studies and did not identify the subendothelial component to which the vWF bound. The vWF subunit could have bound to endogenous subendothelial vWF within the matrix, as they have recently reported (40), or to a tissue glycosaminoglycan or other matrix component.

Because of the problems encountered in using vWF<sub>d</sub> as a probe for vWF binding to macromolecules, we approached the problem of isolating functional vWF domains through proteolytic digestion of native polymer. Martin et al. (19) have shown that a 30,000-mol-wt tryptic fragment of vWF retains ristocetin-dependent platelet binding activity. Because of the large size and heterogeneous nature of vWF, reproducible digestion of vWF and separation of proteolytic fragments by standard gel filtration techniques can be difficult. Based on the observation of Suzuki



**Figure 6.** (A) Effects of increasing concentrations of vWF polymer, and vWF fragments 1 and 2 on ristocetin-dependent binding of <sup>125</sup>I-vWF polymer to platelets.  $\Delta$ , vWF polymer;  $\circ$ , fragment 1;  $\bullet$ , fragment 2. Results indicate the mean  $\pm$  SEM of four separate experiments performed in duplicate. (B) Effects of increasing concentrations of vWF polymer and vWF digest fragments 1 and 2 on the binding of <sup>125</sup>I-vWF polymer to collagen monolayers.  $\Delta$ , vWF polymer;  $\circ$ , fragment 1;  $\bullet$ , fragment 2. Results indicate the mean  $\pm$  SEM of three separate experiments performed in duplicate.

et al. (40) that vWF binds to heparin and dextran sulfate-substituted Sepharose, we designed an affinity chromatographic technique for the isolation of vWF fragments based on their affinities for sulfated glycosaminoglycans. As described above, we have identified a 285,000-D proteolytic fragment of vWF that both binds to heparin Sepharose and retains GpIb binding activity. This species, fragment 2, may be similar to the fragment isolated by Girma et al. (18) using a monoclonal anti-vWF antibody. The 210,000–225,000-mol-wt fragments that were not retained by heparin Sepharose did not bind to platelet GpIb or to collagen. It is possible that these fragments contain the vWF domain that binds to platelet GpIIb-IIIa. This was not evaluated.

Comparison of relative binding affinities of vWF modified by proteolytic digestion or by reduction and alkylation to native polymers is difficult because of the heterogeneity in molecular weight of native vWF. The smallest oligomer of native vWF capable of binding to platelets or the subendothelium has yet to be defined, and there is some suggestion that larger molecular weight forms may have several available binding sites. Thus, to

make comparisons between the  $IC_{50}$  of  $vWF_d$ , fragment 2, and native  $vWF$  used in this study, it is necessary to make assumptions for the molecular weight and number of available platelet binding sites per  $vWF$  polymer. It is important to emphasize that plasma  $vWF$  multimers vary in estimated relative molecular weight from 440,000 to over  $20 \times 10^6$  D and that mixtures of these heterogeneous multimers have been used in previously published radioligand binding assays. However, assuming a mean molecular weight of  $1.2 \times 10^6$  for native  $vWF$ , and one GpIb binding site per molecule,  $vWF$  polymer would have an  $IC_{50}$  of 4.2 nM when  $^{125}I$ - $vWF$  polymer is the radioligand in ristocetin-dependent platelet-binding assays. In comparison,  $vWF_d$  and proteolytic fragment 2 would have an  $IC_{50}$  of 250 nM and 77 nM, respectively. If, however, we assume that a functional GpIb binding site exists on each 220,000-mol-wt subunit, then the  $IC_{50}$  for polymer would become 23 nM, a figure that more closely approximates the  $IC_{50}$  for fragment 2. In those experiments in which native  $vWF$  and  $vWF_d$  competed with  $^{125}I$ - $vWF_d$  for binding to GpIb, the  $IC_{50}$  for native polymer and  $vWF_d$  was 23 nM and 43 nM, respectively.

These discrepancies in  $IC_{50}$  may be explained in several ways. First, they may reflect a selective binding advantage for polymeric  $vWF$  which may have multiple high-affinity binding sites on each  $1.2 \times 10^6$  D polymer. Alternatively, the reduction and alkylation or proteolytic digestion procedures may reduce the binding affinities of  $vWF_d$  and fragment 2, respectively, resulting in a greater  $IC_{50}$  in competition assays with  $^{125}I$ - $vWF$  polymer. Finally, with  $vWF_d$ , a complex equilibrium between  $vWF_d$ - $vWF_d$  and  $vWF_d$ - $vWF$  polymer may reduce the effective concentrations of  $vWF_d$  available for binding to GpIb. It is clear that the apparent affinities of oligomers of native  $vWF$  produced by reduction and alkylation and protease digestion are influenced by the choice of radioligands and the assumptions made for the molecular weight of the heterogeneous native  $vWF$  polymer.

Although we have used heparin as a convenient tool for isolation of proteolytic fragments of  $vWF$ , the observed affinity of  $vWF$  polymer for heparin may merit additional investigation. The ability of  $vWF$  to bind to heparin has been previously noted, but incompletely defined (40, 41). Two other adhesive proteins present in extracellular matrix, fibronectin and laminin, also share similar affinities for heparin and dextran sulfate sepharose (41-45). The binding of heparin to fibronectin enhances the binding of fibronectin to collagen (43, 44). Laminin also has binding sites for tissue glycosaminoglycans as well as type IV collagen (45). In similar fashion, this specific, high affinity interaction of  $vWF$  with glycosaminoglycans may explain one of the mechanisms by which  $vWF$  becomes bound to the subendothelium. It is conceivable that the binding of  $vWF$  to heparin-like molecules within the subendothelium may actually facilitate interaction of  $vWF$  domains with platelet surface receptors or, conversely, the binding of  $vWF$  to the platelet receptor may facilitate the binding to the subendothelium. In addition, tissue glycosaminoglycans have been implicated in the polymerization process of matrix fibronectin (46) and collagen (47). Thus, heparin- $vWF$  interactions may be important in polymerization of  $vWF$  from dimer into oligomers at the endothelial cell surface. Since the species and amount of extracellular matrix glycosaminoglycans can be influenced by cell culture conditions, the interaction of  $vWF$  with certain glycosaminoglycans may explain the discrepancy in  $vWF$  polymer size in the medium of cultured endothelial cells described by several laboratories (48-50). Lastly, the affinity of  $vWF$  for sulfated glycosaminoglycans like heparin

may have no role in  $vWF$ -mediated platelet adhesion *in vivo*. These possibilities will be the subject of our future investigations.

Although platelet agglutinating activity is associated with higher molecular weight forms of  $vWF$ , we have shown that binding of  $vWF$  to GpIb can be localized to a 440,000-D dimer of native  $vWF$  produced by limited disulfide reduction and alkylation and to a smaller 285,000 proteolytic fragment of native  $vWF$ . This 285,000-D fragment also has a heparin binding domain. The affinity of  $vWF$  for heparin can be used to purify this fragment and separate it from fragments that do not have heparin binding activity and do not bind to GpIb. These studies help to define the structural requirements for  $vWF$  binding to GpIb, collagen, and heparin and provide a means to localize these binding domains within  $vWF$  polymers.

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