Analysis of the Structure and Function of the von Willebrand Factor A1 Domain Using Targeted Deletions and Alanine-Scanning Mutagenesis†

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Received April 30, 1996; Revised Manuscript Received July 8, 1996⊗

ABSTRACT: von Willebrand factor (vWF) mediates the primary adhesion of platelets to sites of vascular damage through interaction with glycoprotein Ib (GPIb) of the platelet GPIb/IX complex. To investigate the vWF/GPIb interaction we introduced both in-frame deletions and substitutions into the vWF A1 domain. The introduction of nine sequential 20-amino acid deletions within the Cys509-Cys695 loop of the A1 domain caused the defective secretion of vWF from mammalian cells, and resulted in multimeric vWF without platelet-binding activity. In other experiments we substituted alanine for charged amino acids (residues 524, 534, 549, 552, 569−573, and 642−645) in proposed functional domains within the Cys509-Cys695 loop. All six substitution mutants showed normal secretion from transfected mammalian cells and bound to fixed platelets in the presence of botrocetin. In contrast, only mutants vWF-R524A and vWF-K549A showed significant binding to platelets in the presence of ristocetin. Mutant vWF-K549A showed increased platelet-binding at suboptimal concentrations of both botrocetin and ristocetin. These results suggest that the substituted amino acids do not play a critical role in the activation of vWF by botrocetin or in the direct interaction of vWF with the GPIb/IX complex. However, the charged amino acids at positions 534, 552, 569−573, and 642−645 do play an important role in the ristocetin-induced binding of vWF to platelets. The interaction of vWF with heparin was significantly reduced by substitution of Lys residues 642−645, indicating that these residues may form part of a heparin-binding domain in the carboxy-terminal half of the Cys509-Cys695 loop.

von Willebrand factor (vWF)[1] is a multimeric plasma and platelet protein that performs essential functions in hemostasis as the carrier of coagulation factor VIII, and as the ligand for primary platelet adhesion at sites of vascular damage (Montgomery & Coller, 1994; Sadler, 1995). Although vWF does not normally interact with platelets in the circulation, the binding of vWF to components of the subendothelial matrix in the presence of high shear forces results in platelet adhesion, which is followed by platelet activation and subsequently thrombus formation. The acquisition of platelet-binding activity by vWF is thought to require conformational changes within vWF, but the precise mechanism involved is not known. *In vitro*, the binding of vWF to platelets in suspension can be induced by the antibiotic ristocetin (Howard & Firkin, 1971) or by the snake venom protein botrocetin (Read et al., 1978).

The primary adhesion of platelets to vWF occurs through glycoprotein Ib (GPIb) of the platelet GPIb/IX complex. The GPIb-binding site in vWF was localized to a homodimeric tryptic fragment of vWF spanning residues 449 to 728[2] (Fujimura et al., 1986, 1987), and to a smaller monomeric fragment generated by dispase digestion that spans residues 480/481 to 718 (Andrews et al., 1989b). Analysis of this region using peptides and inhibitory antibodies suggested that GPIb bound to vWF through two short sequences (residues 474−488 and 694−708) adjacent to the 185-amino acid loop defined by Cys509 and Cys695 (Mohri et al., 1988). However, deletion of residues 441−507 and 697−733 did not reduce the ability of the intact Cys509-Cys695 segment to interact with GPIb (Sugimoto et al., 1993). This suggests that residues on either side of the loop may play a role in regulating the interaction of vWF with GPIb and that the binding site for GPIb resides within the loop. This is consistent with the identification of residues Asp514-Glu542 as a potential binding site for GPIb (Berndt et al., 1992), and with the identification of three discontinuous segments within the Cys509-Cys695 loop that may interact with botrocetin (Sugimoto et al., 1991). Other studies identified potential binding sites for heparin (Sobel et al., 1992), collagen (Roth et al., 1986), and sulfatides (Christophe et al., 1991) in this region.

The vWF A1 domain is the site of numerous von Willebrand disease mutations (vWD) that affect the interaction of vWF with the GPIb/IX complex. Mutations responsible for type 2B vWD, which is defined by the heightened reactivity of vWF with GPIb, are located in this region and cluster between residues 540 and 578 (Ginsburg & Sadler, 1993), although a few type 2B mutations are located in the regions on either side of the loop (Hilbert et al., 1994, 1995a;

† This research was supported by American Heart Association (Wisconsin) Grant-in-Aid 92-GB-12 (P.A.K.) and National Institutes of Health Grant HL50520 (P.A.K.).

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⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: vWF, von Willebrand factor; vWD, von Willebrand disease; GPIb, glycoprotein Ib, wt, wild type; ELISA, enzyme-linked immunosorbent assay, HBSS, Hanks' buffered saline solution; mAb, monoclonal antibody; BSA, bovine serum albumin; mw, molecular weight.

[2] vWF amino acids are numbered using the first amino acid of mature vWF as the amino acid number one. Nucleotides are numbered assigning +1 to the major transcription start site, which is 250 nucleotides before the first nucleotide of the translation initiation codon as recommended by the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (Sadler, 1994).

S0006-2960(96)01031-8 CCC: $12.00

Rabinowitz et al., 1993; Holmberg et al., 1993). The A1 domain also contains mutations that cause reduced reactivity with GPIb. These mutations are clustered in the carboxy-terminal half of the loop and include one in-frame deletion (Mancuso et al., 1996) and several missense mutations (Rabinowitz et al., 1992; Hilbert et al., 1995b).

Electrostatic interactions appear to play an important role in regulating the affinity of vWF for GPIb. For example, most of the type 2B vWD mutations, which result in the increased affinity of vWF for GPIb, are clustered in a positively-charged region of the amino-terminal portion of the Cys509-Cys695 loop (Ginsburg & Sadler, 1993). Three of the type 2B mutations involve the substitution of the positively-charged amino acid Arg. In addition, asialo vWF binds spontaneously to GPIb (De Marco & Shapiro, 1981), and various polyionic compounds, including heparin (Sobel et al., 1991) and synthetic peptides containing basic amino acids (Mohri et al., 1993), inhibit the vWF/GPIb interaction.

To further investigate the interaction of the vWF with the GPIb/IX complex on platelets we used two different approaches involving site-directed mutagenesis of the vWF A1 domain. In the first set of experiments we introduced sequential non-overlapping 20-amino acid deletions within the Cys509-Cys695 loop to investigate whether discrete functional domains could be identified and to determine whether the intact Cys509-Cys695 loop is required for the interaction of full-length vWF with GPIb. The results from this approach suggest that the deleted amino acids do not contain discrete functional domains and that the continuity of the Cys509-Cys695 loop is critical for the normal synthesis of vWF and the function of A1 domain. In the second approach, we used alanine-substitution mutagenesis to investigate the role of selected charged amino acids in proposed functional domains within the Cys509-Cys695 loop. Our results show that while none of the substituted amino acids is required for the botrocetin-induced binding of vWF to platelets, several of the charged residues are required for the ristocetin-induced binding of vWF to GPIb. These studies also identify a potential heparin-binding site in the carboxy-terminal half of Cys509-Cys695 loop.

EXPERIMENTAL PROCEDURES

Plasmids and Mutant Construction. Mutations were introduced into vWF cDNA using mutagenic primers and single-stranded DNA (Kunkel, 1987). Mutagenesis was performed on plasmid pCITEα18vWF1, which was constructed by inserting an *Nae*I/*Nco*I vWF cDNA fragment (nucleotides 3858 to 4731) into *Msc*I/*Nco*I-digested pCITE (Novagen, Madison, WI), creating pCITEvWF. For single-stranded DNA production, a fragment of pCITEvWF containing the vWF insert was inserted into pUC18 (Pharmacia Biotech, Uppsala, Sweden), creating pCITEα18vWF1. The sequence of the mutant cDNA fragments was determined using Sequenase (Life Technologies, Gaithersburg, MD). Mutant cDNA fragments were inserted into the full-length vWF expression vector pvW198.1 (Kroner et al., 1991) using a two-step cloning procedure (Kroner et al., 1992). Plasmid DNA was purified using either the Plasmid Maxi Kit (Qiagen, Chatsworth, CA) or Biggerprep (5Primer→3Prime, Boulder, CO).

Transfection of Cells. COS-7 cells (ATCC CRL1651) grown in RPMI plus 10% fetal calf serum were transfected using Lipofectamine (Felgner et al., 1987). In brief, 1.5×10^6 COS-7 cells were plated in 100 mm dishes and grown overnight. After washing the cells twice with HBSS, 8 mL of optiMEM media (Life Technologies, Gaithersberg, MD) containing 5 μg of plasmid DNA and 60 μg of Lipofectamine (Life Technologies, Gaithersberg, MD) was added, and the cells were incubated at 37 °C for 6 h. After removal of the DNA/Lipofectamine mixture, 7 mL of culture medium was added and the cells were incubated for an additional 60 h at 37 °C. Conditioned media was harvested, cleared by centrifugation (10 000*g* for 10 min at 4 °C), and stored in aliquots at −80 °C. Intracellular vWF was isolated from transfected cells by adding 500 μL of modified HEPES-Tyrode buffer (20 mM HEPES, 140 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 1 mM NaH_2PO_4, pH 8.0) containing 0.2% Triton X-100, 5 mM EDTA, 5 mM NEM, 0.1 mg/mL leupeptin, and 1 mM PMSF and incubating on ice for 15 min. The cell lysate was removed, and the plates were washed with an additional 375 μL of lysis buffer. The cell lysate from the two washes was combined, cleared by centrifugation (14 000*g* at 5 °C for 10 min) and then stored at −80 °C.

vWF ELISA. The concentration of vWF in conditioned media and cell lysates was determined by antigen-capture ELISA using vWF monoclonal antibody (mAb) AvW1 (Montgomery et al., 1986) as the capture antibody and a rabbit anti-vWF polyclonal antibody as the primary detecting antibody. The anti-vWF polyclonal antibody was detected using a goat anti-rabbit IgG polyclonal antibody coupled to horseradish peroxidase (Pierce, Rockford, IL) and the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). Absorbance data were obtained using a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA) and analyzed using SOFTmax software (Molecular Devices Corp., Menlo Park, CA). Absorbance values were converted to ng of vWF/mL using a standard curve generated from dilutions of pooled normal plasma. The reactivity of vWF with mAb AvW3 (Kawai & Montgomery, 1987) was performed using AvW3 as the capture antibody.

Immunoprecipitation, SDS−PAGE, and SDS−Agarose Electrophoresis. vWF was immunoprecipitated from conditioned media or cell lysate using vWF mAb AvW1 coupled to Sepharose-4B (Pharmacia Biotech, Piscataway, NJ). Monomeric vWF was analyzed by SDS−PAGE in 4%−12% polyacrylamide gels. vWF was transferred to nitrocellulose and visualized using the ECL detection system (Amersham Corp., Arlington Heights, IL). The primary detecting antibody was rabbit anti-vWF IgG, and the secondary detecting antibody was goat anti-rabbit IgG coupled to horseradish peroxidase (Pierce, Rockford, IL). The multimeric structure of immunopurified vWF was determined according to Raines et al. (1990) except that electrophoresis was performed at 150 V for 30 min, followed by electrophoresis at 70 V for 12−14 h at 15 °C. vWF was transferred to PVDF transfer membrane (Immobilon P, Millipore Corp., Bedford, MA) and visualized as described above.

Platelet-Binding Assay. The binding of vWF to fixed platelets was measured using a modification of the assay developed by Scott and Montgomery (1991). vWF mAb

AvW1 was labeled with ^{125}I using Iodobeads (Pierce, Rockford, IL). For the assay, samples with vWF in excess of 100 ng/mL were diluted in Tris-Saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 1% BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) to reach a final vWF concentration of 100 ng/mL. BSA was present in the reaction to reduce background and to prevent the precipitation of vWF in the presence of ristocetin (Scott et al., 1991). Samples with a vWF concentration of less than 100 ng/mL were concentrated by ultrafiltration using Centricon-30 or Centriprep-30 concentrators (Amicon, Beverly, MA) and then diluted to 100 ng/mL. The recovery of vWF after ultrafiltration was greater than 90%, and the multimeric profile was not altered. For each reaction 10 μL of ^{125}I-labeled AvW1 (approximately 1.0×10^4 cpm) was added to microfuge tubes containing 160 μL of conditioned media or pooled normal plasma and incubated at room temperature (rt) for 30 min. After incubation, 20 μL of fixed platelets (1.0×10^6 platelets/mL in Tris-Saline; BIO/DATA Corp., Horsham, PA) were added to each tube, followed by 10 μL of ristocetin sulfate (H. Lundbeck A/S, Copenhagen, Denmark), botrocetin, or Tris-Saline. Botrocetin was purified according to Andrews et al. (1989a). After gentle mixing by vortexing, the tubes were incubated for 30 min at rt with rocking. Platelets were pelleted by centrifugation (10 min at 14 000*g*), and the upper one-half of the supernatant was transferred to a clean tube. The amount of radioactivity in the pellet (a) and supernatant (b) fractions was determined using a γ counter. The percent of radioactivity bound to platelets was calculated using the formula $[(a - b)/a + b]100$. The maximum binding of total radioactivity to platelets was typically 60%−70% and was the same as that seen in the presence of excess vWF. No precipitation of vWF occurred in reactions without platelets, and the background using either Tris-Saline or conditioned media was generally 5% of the total counts added.

Collagen-Binding Assay. The interaction of vWF with collagen was determined in an ELISA assay using type III collagen to capture vWF. Type III collagen was a gift from Dr. Thomas Kunicki (The Scripps Research Institute, La Jolla, CA) and was purified as described by Miller and Rhodes (1982). Type III collagen was dialyzed in buffer containing 50 mM sodium borate, 0.8 M NaCl (pH 8.2), and stored at 5 °C. For the ELISA, collagen was diluted to 5 μg/mL in carbonate buffer (50 mM sodium carbonate, pH 9.6) and 50 μL was added to each well of a microtiter plate. After incubation overnight at 5 °C, the plates were washed, 50 mL of vWF (100 ng/mL) was added to each well, and the plate was incubated for 1 h at rt. vWF bound to collagen was detected as described above.

Heparin-Binding Assay. The binding of vWF to heparin was determined using heparin-Sepharose CL-4B (Pharmacia Biotech Inc., Piscataway, NJ). All incubations were at rt. vWF was labeled by adding 27 μL of ^{125}I-labeled AvW1 (1000 cpm/μL) to 400 μL of conditioned media containing vWF at 100 ng/mL. After incubation for 30 min, 160 μL of each AvW1/vWF mixture was aliquoted into two microfuge tubes. One set of tubes received 20 μL of soluble heparin (100 mg/mL in ddH_2O, sodium salt, grade I-A from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, MO), and the other set received 20 μL of ddH_2O. After incubation for 15 min, 50 μL of heparin-Sepharose (50% by volume) was added and the tubes were incubated for an additional 30 min with rocking. Heparin-Sepharose was pelleted by centrifugation at 14 000*g* for 10 min, and the amount of vWF bound was determined as described above.

Table 1: Deletion Mutations in the vWF A1 Domain

mutant	amino acids deleted[a]	amino acids deleted[b]
vWF-Δ1	Arg511−Phe530	Arg1274−Phe1293
vWF-Δ2	Glu531−Trp550	Glu1294−Trp1313
vWF-Δ3	Val551−Asp570	Val1314−Asp1333
vWF-Δ4	Arg571−Gln590	Arg1334−Gln1353
vWF-Δ5	Val591−Asp610	Val1354−Asp1373
vWF-Δ6	Arg611−Met630	Arg1374−Met1393
vWF-Δ7	Ser631−Pro650	Ser1394−Pro1413
vWF-Δ8	Val651−Pro670	Val1414−Pro1433
vWF-Δ9	Glu671−Ile690	Glu1434−Ile1453

[a] vWF amino acids are numbered using the first amino acid of mature vWF as amino acid number one. [b] Corresponding amino acid numbers in mature vWF using the initiating Met as the first amino acid as recommended by the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (Sadler, 1994).

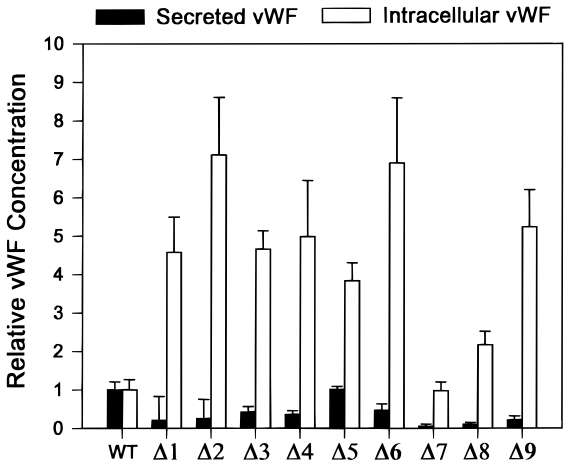

FIGURE 1: Secretion of vWF deletion mutants. The concentration of vWF in conditioned media and cell lysates was determined by antigen-capture ELISA. The concentration of the mutant vWF is plotted relative to the concentration of secreted or intracellular wt vWF, which were normalized to 1. The average concentration of wt vWF in conditioned media (7.0 mL volume) and cell lysates (0.8 mL volume) was 25.7 ± 5.4 and 8.8 ± 2.3 ng/mL, respectively. WT indicates wt vWF. The deletion mutants are defined in Table 1. The data shown represent the average of six independent transfections of COS-7 cells. The error bars represent the standard error.

RESULTS

Construction and Expression of vWF Deletion Mutants. To determine whether the continuity of the vWF A1 domain is important for the interaction of full-length vWF with the GPIb/IX complex on platelets, and to identify discrete functional domains, we constructed nine vWF mutants containing consecutive non-overlapping deletions of 20 amino acids between residues Cys509 and Cys695 (Table 1). The mutated cDNA fragments were inserted into the full-length vWF expression vector pvW198.1, and both mutant and wild type (wt) vWF were transiently expressed in mammalian cells. Analysis of conditioned media by antigen-capture ELISA showed that, with the exception of vWF-Δ5, which showed normal secretion, each of the deletion mutations caused the reduced secretion of vWF compared to wt vWF (Figure 1). The extent of the secretory defect varied with each mutant and ranged from 5% of wt

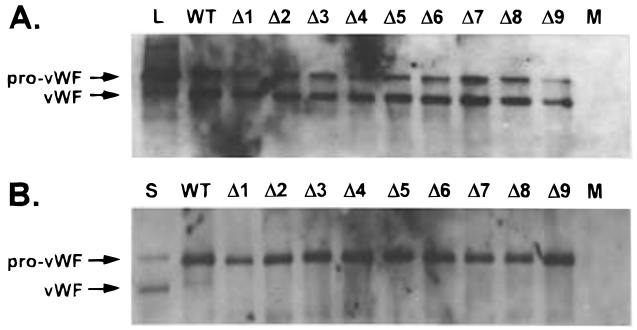

FIGURE 2: Monomeric structure of vWF deletion mutants. Expressed wt or mutant vWF was immunopurified from conditioned media (A) or cell lysates (B) and analyzed by SDS−PAGE and Western blotting as described in the Experimental Procedures. Pro-vWF indicates the uncleaved precursor of vWF. S and L identify wt vWF isolated from the conditioned media or cell lysates of transfected COS-7 cells, respectively. WT indicates wt vWF. M indicates immunoprecipitations performed with conditioned medium or cell lysate from mock-transfected cells. The deletion mutants are defined in Table 1. In panel A, lane L contains approximately 20 ng of vWF, and the remaining lanes each contain approximately 35 ng of vWF. In panel B, lane S contains approximately 30 ng of vWF, and the remaining lanes each contain 16 ng of vWF.

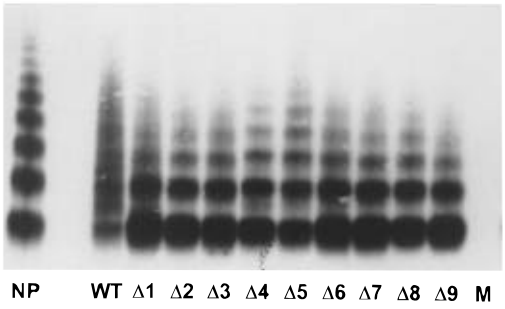

FIGURE 3: Multimeric structure of vWF deletion mutants. vWF was immunopurified from the conditioned media of transfected COS-7 cells or pooled normal plasma (NP) and analyzed by SDS−agarose electrophoresis and Western blotting as described in the Experimental Procedures. WT indicates wt vWF, and M indicates immunoprecipitations performed with conditioned media from mock-transfected cells. The deletion mutants are defined in Table 1. Each lane contains approximately 14 ng of vWF.

Table 2: Alanine-Subsititution Mutations in the vWF A1 Domain

mutant	amino acids substituted[a]	amino acids substituted[b]
vWF-R524A	Arg524	Arg1287
vWF-K534A	Lys534	Lys1297
vWF-K549A	Lys549	Lys1312
vWF-R552A	Arg552	Arg1315
vWF-KDRKR(569−573)5A	Lys569−Arg573	Lys1332−Arg1336
vWF-KKKK(642−645)4A	Lys642−Lys645	Lys1405−Lys1408

[a] vWF amino acids are numbered using the first amino acid of mature vWF as amino acid number one. [b] Corresponding amino acid numbers in mature vWF using the initiating Met as the first amino acid as recommended by the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (Sadler, 1994).

vWF for vWF-Δ7 to 46% of wt vWF for vWF-Δ6. Analysis of cellular lysates showed that, except for vWF-Δ7, each of the deletion mutants showed an increase in the intracellular level of vWF compared to wt vWF. While the intracellular level of vWF-Δ7 was similar to the intracellular level of wt vWF, the increase over wt vWF for the other mutants ranged from 2-fold higher for vWF-Δ8 to 7-fold higher for vWF-Δ2 and vWF-Δ6. Compared to an adjusted ratio of 1.0 for wt vWF, the ratio of intracellular to secreted vWF for the mutant proteins ranged from 3.8 for vWF-Δ5 to 28.4 for vWF-Δ2.

We further investigated the altered secretion profile of the mutant proteins by analyzing immunopurified secreted and intracellular vWF by SDS−PAGE (Figure 2). The secreted form of each of the mutant proteins was similar to wt vWF, and consisted of both full-length mature vWF and pro-vWF (Figure 2A). A similar analysis of intracellular protein showed that, like wt vWF, the mutant intracellular vWF was almost exclusively pro-vWF (Figure 2B). Multimeric analysis showed that the immunopurified intracellular form of both wt and mutant vWF consisted primarily of pro-vWF dimers (data not shown). These results suggest that each of the deletions in the Cys509-Cys695 loop of the A1 domain prevents the efficient transit of vWF to the trans-Golgi, where the pro-vWF dimers are processed to mature vWF, and dimers associate to form high molecular weight (mw) multimers (Vischer & Wagner, 1994). Analysis of secreted protein demonstrated that, with the exception of vWF-Δ5, which showed a normal multimer profile, each of the deletion mutants showed reduced levels of high mw multimers (Figure 3).

The effects of the deletion mutations on the function of the vWF A1 domain were determined for the secreted form of each mutant protein using platelet-binding assays. In multiple assays each of the mutant proteins failed to bind to fixed platelets either spontaneously or in the presence of concentrations of ristocetin or botrocetin capable of inducing maximal binding of wt vWF to platelets (data not shown). In addition, each of the mutant proteins failed to react with vWF mAb AvW3, a conformation-dependent antibody that blocks the binding of vWF to platelets (data not shown). These results suggest that each of the 20-amino acid deletions in the Cys509-Cys695 loop induced conformational changes in the A1 domain that prevented the interaction with the GPIb/IX complex, either by directly affecting the GPIb binding site or by preventing activation by ristocetin or botrocetin.

Construction and Expression of vWF Alanine-Substitution Mutants. To further investigate the role of charged amino acids in the structure and function of the vWF A1 domain, we used alanine-scanning mutagenesis (Cunningham & Wells, 1989) to construct six mutants in which alanine was substituted for primarily positively-charged amino acids in proposed functional domains within the Cys509-Cys695 loop (Table 2 and Figure 4). Four mutants contain single amino acid substitutions. vWF-R524A and vWF-K534A contain substitutions in a proposed GPIb/IX-binding site (Berndt et al., 1992), and vWF-K549A and vWF-R552A contain substitutions in the region where most type 2B vWD mutations are clustered (Ginsburg & Sadler, 1993). In addition, two mutants, vWF-KDRKR(569−573)5A and vWF-KKKK(642−645)4A, contain multiple substitutions in two different proposed botrocetin-binding sites, respectively (Sugimoto et al., 1991). vWF mutant KDRKR569A also resides in a proposed heparin-binding domain (Sobel et al., 1992).

In contrast to the deletion mutants, each of the alanine-substitution mutants showed normal secretion from COS-7 cells (data not shown). Analysis of the immunopurified secreted protein by SDS−PAGE showed that each of the

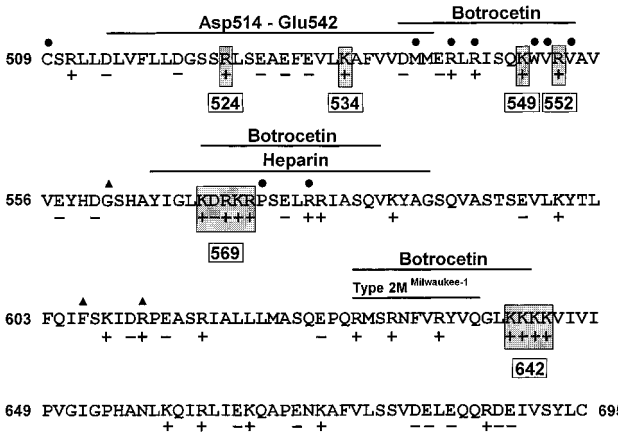

FIGURE 4: Alanine-substitution mutations in the vWF A1 domain. This figure shows the amino acid sequence of mature vWF from Cys509 to Cys 695. The amino acids changed to alanine are shown in gray. The positions of the substituted amino acids in mature vWF are shown below each substitution. Where multiple alanines were inserted, the number below identifies the position of the first amino acid in the group. Acidic and basic amino acids are identified by − and + signs, respectively. Lines above the amino acid sequence identify proposed binding sites for the GPIb/IX complex (Berndt et al., 1992), botrocetin (Sugimoto et al., 1991), and heparin (Sobel et al., 1992). Circles identify the locations of type 2B vWD mutations, which result in enhanced binding to platelets (Ginsburg & Sadler, 1993), and triangles identify type 2M vWD mutations, which result in reduced binding to platelets (Rabinowitz et al., 1992; Mancuso et al., 1991; Hilbert et al., 1995b). Type 2M:Milwaukee-1 identifies a deletion of amino acids Arg629-Gln639 in a vWD patient that results in the loss of ristocetin- and botrocetin-induced platelet-binding activity (Mancuso et al., 1996). The nomenclature for the vWF alanine-substitution mutants is defined in Table 2.

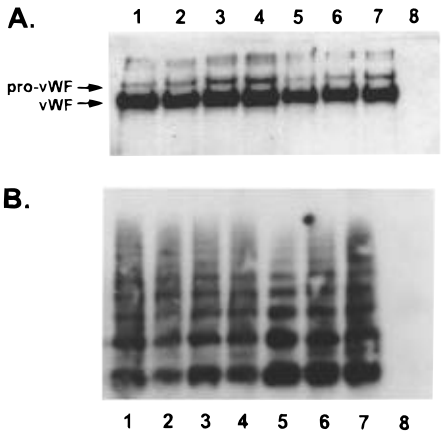

FIGURE 5: Monomer and multimer analysis of the vWF alanine-substitution mutants. vWF was immunopurified from the conditioned media of transfected COS-7 cells and analyzed by SDS−PAGE (A) or SDS−agarose electrophoresis (B). vWF was visualized after transfer to nitrocellulose (A) or PVDF membrane (B) as described in the Experimental Procedures. In A Pro-vWF identifies the uncleaved vWF precursor. WT refers to wt vWF. lane 1, wt vWF; lane 2, vWF-R524A; lane 3, vWF-K534A; lane 4, vWF-K549A; lane 5, vWF-R552A; lane 6, vWF-KDRKR(569−573)-5A; lane 7, vWF-KKKK(642−645)4A; lane 8, mock. "Mock" refers to immunoprecipitations performed with the conditioned media of mock-transfected cells. In panel A each lane contains approximately 120 ng of vWF. In panel B each lane contains approximately 21 ng of vWF.

mutant proteins was secreted primarily as full-length mature vWF (Figure 5A). With the exception of vWF-R552A, the multimeric structure for each of the mutant proteins was similar to wt vWF (Figure 5B). vWF-R552A consistently showed a moderate reduction in the accumulation of high mw multimers accompanied by an increase in the accumulation of low mw multimers.

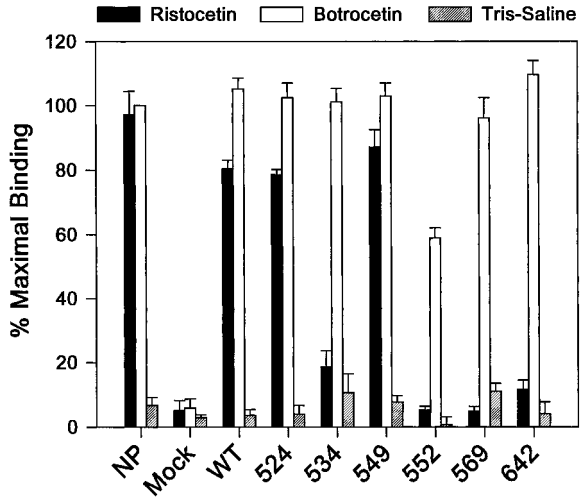

FIGURE 6: Binding of the vWF alanine-substitution mutants to platelets. Conditioned media from transfected COS-7 cells or pooled normal plasma containing vWF at 100 ng/mL were incubated with ^{125}I-labeled vWF mAb AvW1 and then added to a solution containing fixed platelets and either ristocetin (1.2 mg/mL) or botrocetin (5.0 μg/mL). The values plotted were normalized to the amount of normal plasma (NP) vWF bound to platelets in the presence of botrocetin. The spontaneous binding of vWF to platelets was determined in reactions to which Tris-Saline was added. The data shown represent the average of three experiments using the conditioned media from three independent transfections. The error bars represent the standard deviation. The vWF mutants are identified as described in Figure 4 and Table 2.

Binding of vWF Alanine-Substitution Mutants to Platelets. Each of the alanine-substitution mutants bound to platelets in the presence of botrocetin (5 μg/mL). With the exception of vWF-R552A, which showed 60% of maximal binding, the binding was similar to wt vWF (Figure 6). In contrast, only mutants vWF-R524A and vWF-K549A showed appreciable binding to platelets in the presence of ristocetin (1.2 mg/mL). The ristocetin-induced binding of mutants vWF-R524A and vWF-K549A to platelets was similar to wt vWF. Mutant vWF-K534A showed 19% of maximal binding, and the remaining mutants showed 12% or less maximal binding. None of the alanine-substitution mutants showed evidence of spontaneous binding to platelets.

Figure 7 shows dose−response curves for the binding of the alanine-substitution mutants to platelets in the presence of botrocetin (Figure 7A,B) or ristocetin (Figure 7C). Compared to wt vWF, only two mutants, vWF-R552A and vWF-K549A, show altered botrocetin dose−response curves. At low concentrations of botrocetin vWF-K549A showed an increased binding to platelets that is similar to that seen by mutations responsible for type 2B vWD. Conversely, vWF-R552A showed reduced binding to platelets at all botrocetin concentrations. Ristocetin dose−response platelet-binding assays were performed with vWF-R524A and vWF-K549A, the only mutant proteins showing significant binding to platelets at an optimal concentration of ristocetin. Both of these mutant proteins showed altered binding profiles. vWF-R524A showed moderately-reduced binding to platelets in the presence of 0.6 mg/mL ristocetin. The multimeric profile of vWF-R524A is similar to wt vWF (Figure 5B), so the reduced binding to platelets in the presence of a suboptimal concentration of ristocetin does not appear to be due to the loss of high mw multimers. vWF-K549A showed

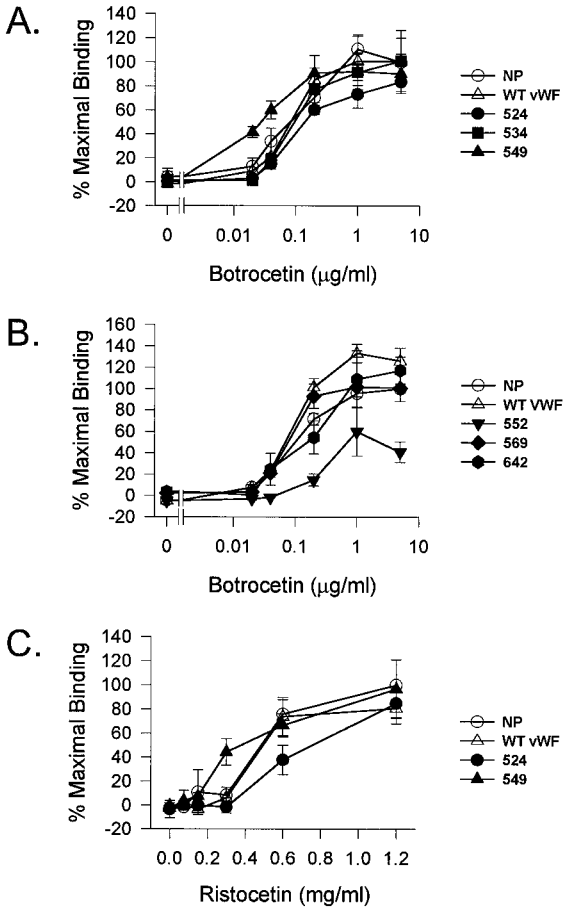

FIGURE 7: Ristocetin and botrocetin dose−response curves. Platelet-binding assays were performed with conditioned media from transfected COS-7 cells or pooled normal plasma as described in Figure 6 with the exception that the final botrocetin concentrations (panels A and B) ranged from 0.02 to 5.0 μg/mL and the final ristocetin concentrations (panel C) ranged from 0.075 to 1.2 mg/mL. NP identifies assays performed with pooled normal plasma. The results from each plot were normalized to the amount of NP vWF bound to platelets in the presence of botrocetin at 5.0 μg/mL (panels A and B) or ristocetin at 1.2 mg/mL (panel C). The data shown represents the average of three experiments using the conditioned media from three independent transfections. The error represents the standard deviation. The vWF mutants are identified as described in Figure 4 and Table 2.

significantly increased binding to platelets in the presence of 0.3 mg/mL ristocetin. While normal plasma vWF and wt vWF showed background binding to platelets at this concentration of ristocetin, vWF-K549A showed binding that was nearly 50% of the maximum.

Binding of vWF Alanine-Substitution Mutants to Heparin. We performed binding assays with heparin-Sepharose to determine whether the alanine-substitution mutations affect the interaction of vWF with heparin (Figure 8). Although mutants vWF-R524A, vWF-K534A, vWF-K549A, and vWF-KDRKR(569−573)5A showed normal binding to heparin, mutants vWF-R552A and vWF-KKKK(642−645)4A showed binding that was 55% and 30% of wt vWF, respectively. The addition of soluble heparin to the binding assays demonstrated the specificity of the vWF/heparin interaction by reducing the binding of all vWF samples to heparin-Sepharose to background levels.

Binding of vWF Alanine-Substitution Mutants to Collagen. The effect of the alanine-substitutions on the vWF/collagen interaction was tested by ELISA using type III collagen as

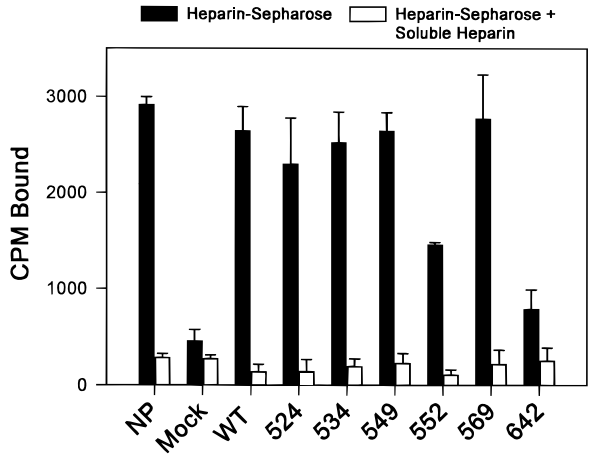

FIGURE 8: Binding of the vWF alanine-subsititution mutants to heparin. Conditioned media from transfected COS-7 cells or pooled normal plasma containing vWF at 100 ng/mL were incubated with ^{125}I-labeled vWF mAb AvW1, and then added to a solution containing heparin-Sepharose. After incubation, the heparin-Sepharose was pelleted by centrifugation and the amount of radioactivity bound was determined as described in the Experimental Procedures. The specificity of binding was shown by including soluble heparin in the binding reaction. NP is pooled normal plasma. The data shown represent the average of three experiments using the conditioned media from three independent transfections. The error represents the standard deviation. The vWF mutants are identified as described in Figure 4.

the capturing protein. In this assay the capture of each of the mutant proteins by type III collagen was similar to the capture of wt vWF (data not shown).

Reactivity of vWF Alanine-Substitution Mutants with mAb AvW3. vWF mAb AvW3 is an inhibitory antibody that blocks the binding of vWF to the platelet GPIb/IX complex (Kawai & Montgomery, 1987). The epitope for AvW3 has not been mapped, but it is likely that it resides within the A1 domain of vWF. To localize the epitope for AvW3 we performed antigen-capture ELISA to test the reactivity of AvW3 with each of the alanine-substitution mutants. With the exception of vWF-R552A, each of the mutant proteins showed nearly equal reactivity with mAb AvW3 and AvW1 (data not shown). vWF-R552A showed normal reactivity with AvW1 but substantially reduced reactivity with AvW3.

DISCUSSION

vWF Biosynthesis. As the A1 domain is not required for the efficient secretion of vWF from transfected cells (Sixma et al., 1991), we were surprised to find that each of the vWF deletion mutants we analyzed showed defective secretion. In addition, these proteins are not recognized by the conformation-specific vWF mAb AvW3 and do not bind to platelets in the presence of ristocetin or botrocetin. Thus, it seems likely that the presence of a 20-amino acid deletion anywhere in the Cys509-Cys695 loop leads to a misfolded protein that is retained within the cell and poorly secreted. The nearly exclusive presence of dimeric pro-vWF within transfected cells suggests that the mutant proteins are retained in the endoplasmic reticulum or Golgi complex, and only inefficiently enter the trans-Golgi, which is the likely site of vWF endoproteolytic cleavage and multimerization (Vischer & Wagner, 1994). The difference in the ratio of intracellular to secreted protein for the different deletion mutants suggests that other defects resulting in reduced synthesis and/or increased degradation may be involved as well.

Our results imply that natural mutations in the A1 domain could contribute to low plasma levels of vWF by interfering with vWF biosynthesis. If the secretory defect is due to the abnormal conformation of the A1 domain, then the secreted mutant protein could also fail to interact with the GPIb/IX complex on platelets. We and others recently identified a type 2M vWD patient that is heterozygous for a deletion mutation causing an in-frame deletion of 11 amino acids (Arg629-Gln639) in the A1 domain (Mancuso et al., 1996). This patient shows reduced levels of plasma vWF with disproportionately-reduced ristocetin cofactor activity. The expressed mutant vWF is partially retained within transfected cells, and secreted vWF does not interact with fixed platelets in the presence of ristocetin or botrocetin. Although vWF biosynthesis is generally unaffected by the introduction of point mutations [this study and Matsushita and Sadler (1995)], naturally occurring mutations affecting the secretion of recombinant vWF have been described. Hilbert et al. (1995b) recently reported that type 2 vWD mutations Arg611Cys and Arg611His cause a significant decrease in the secretion of vWF from transfected cells. Reduced secretion was also observed in co-transfection experiments including wt vWF, indicating a possible dominant-negative effect of these mutations.

Interaction of vWF with GPIb. All of the vWF deletion mutants failed to interact with platelets either spontaneously or in the presence ristocetin or botrocetin. The general defect in secretion shown by these proteins, and the lack of recognition by vWF mAb AvW3 suggest that the loss of platelet-binding activity is most likely due to misfolding of the A1 domain. As native vWF also fails to interact spontaneously with the GPIb complex on platelets, we cannot exclude the possibility that the GPIb-binding site in some of the mutant proteins is intact, and that the failure to react with platelets *in vitro* is due solely to the loss of reactivity with ristocetin or botrocetin. However, our results are consistent with studies that found that *Escherichia coli*-expressed monomeric A1 domain fragments which contain 15−21-amino acid deletions within the Cys509-Cys695 loop also failed to interact with platelets (Sugimoto et al., 1993). Taken together, these results strongly suggest that the continuity of the Cys509-Cys695 loop is critical for the interaction of full-length multimeric vWF with the GPIb/IX complex.

Each of the alanine-substitution mutants showed normal binding to platelets in the presence of 5 μg of botrocetin/mL, with the exception of mutant vWF-R552A, which showed binding that was about 60% of wt vWF. These results demonstrate that the substituted amino acids are not critical either for the interaction of vWF with botrocetin or for the binding of vWF to GPIb. In botrocetin dose−response assays only mutants vWF-K549A and vWF-R552A showed platelet-binding profiles different from wt vWF. While the reduced binding of vWF-R552A to platelets was also evident at suboptimal concentrations of botrocetin, vWF-K549A displayed enhanced binding at low botrocetin concentrations similar to that observed for type 2B vWD mutations, which further supports the role of this region of the A1 domain in regulating the interaction of vWF with GPIb. The moderate decrease in binding observed with vWF-R552A could reflect the reduced interaction of vWF with botrocetin, the inability of vWF-bound botrocetin to "activate" vWF, or the alteration of the vWF GPIb-binding site. Although Arg552 may be important for one or more of these interactions, defects in each of these mechanisms could result from a general conformational change induced by the R552A substitution. Although the botrocetin-induced binding of vWF to GPIb is not thought to be dependent on multimer size (Brinkhous et al., 1983), Christophe et al. (1994) demonstrated that type 2A plasma vWF, which lacks high mw vWF multimers, showed reduced binding to platelets in the presence of botrocetin. Thus, it is also possible that the reduced botrocetin-induced platelet-binding activity shown by vWF-R552A is a result of the moderate defect in multimerization.

Mutants vWF-KDRKR(569−573)5A and vWF-KKKK-(642−645)4A reside in or near two of the proposed positively-charged botrocetin-binding domains spanning residues 569−583 and 629−643, respectively (Sugimoto et al., 1991). Botrocetin has a net negative charge at physiological pH (Fujimura et al., 1991), and if the binding of botrocetin to vWF is dependent primarily on electrostatic interactions, then the substitution of clusters of charged amino acids in these binding sites would be expected to reduce botrocetin-induced binding of vWF to platelets. However, vWF-KDRKR(569−573)5A and vWF-KKKK(642−645)4A show normal botrocetin-induced binding to platelets. The phenotype of vWF-KKKK(642−645)4A is consistent with the results of Sugimoto et al. (1991), who found that although Lys residues 642 and 643 form part of the botrocetin-binding domain spanning residues 629−643, a peptide spanning residues 639−653, which contains Lys642−645, does not inhibit the botrocetin-induced binding of vWF platelets. Residues 569−573 (KDRKR) constitute five of the eight charged residues in the botrocetin-binding domain spanning residues 569−583, yet substitution of these residues with alanine has no effect on the botrocetin-induced binding of vWF to platelets. Although electrostatic interactions may be an important feature of the binding of vWF by botrocetin, the charged residues we analyzed are not essential for this interaction.

Only mutants vWF-R524A and vWF-K549A showed significant binding to platelets in the presence of ristocetin. While both mutants showed normal binding to platelets at 1.2 mg of ristocetin/mL, vWF-K549A showed enhanced binding to platelets at 0.6 mg of ristocetin/mL, which is similar to the phenotype resulting from type 2B vWD mutations. Conversely, vWF-R524A showed moderately decreased binding at 0.6 mg of ristocetin/mL. The selective loss of ristocetin-induced binding to platelets by mutants vWF-K534A, vWF-R552A, vWF-KDRKR(569−573)5A, and vWF-KKKK(642−645)4A is similar to the phenotype produced by a type 2M vWD mutation at residue 561 (Rabinowitz et al., 1992). The failure of the mutant proteins to interact with platelets in the presence of ristocetin most likely is not due to the disruption of the GPIb binding site on vWF, as each of these mutants binds to platelets in the presence of botrocetin. However, ristocetin and botrocetin appear to promote the binding of vWF to platelets by distinct mechanisms (Girma et al., 1990; Sugimoto et al., 1991), and ristocetin and botrocetin may induce the formation of different GPIb-binding sites on vWF. If ristocetin and botrocetin induce the same binding site, then the defect in the mutant proteins is either due to the reduced interaction with ristocetin or to the inability of ristocetin to induce a conformational change that exposes the GPIb-binding site.

While this study was in progress Matsushita and Sadler (1995) published the results of a comprehensive study of the vWF A1 domain using a similarly strategy of mutagenesis. Comparison of the relevant constructs can provide additional information concerning the roles of specific amino acids in regulating the interaction of vWF with GPIb. In this study mutant R524A binds to platelets in the presence of both ristocetin and botrocetin. The comparable mutant of Matsushita and Sadler (1995), designated (520−524)2A, binds to platelets in the presence of botrocetin but not ristocetin. These results suggest that Asp520 may be an important residue for the ristocetin-induced binding of vWF to platelets. In addition, when the K549A and R552A mutations are combined in the single mutant (549−552)2A (Matsushita & Sadler, 1995), the combined mutant lacks both ristocetin- and botrocetin-induced binding to GPIb. Since mutants vWF-K549A and vWF-R552A are both capable of binding to platelets, these results suggest that the combined mutations result in a conformational change that prevents interaction with GPIb. The two mutants vWF-KDRKR-(569−573)5A and vWF-KKKK(642−645)4A were expressed as similar constructs in both studies, and, although the phenotypes of the vWF-KDRKR(569−573)5A mutants are similar, the phenotypes of the vWF-KKKK(642−645)-4A mutants are different. In this study vWF-KKKK(642−645)4A shows normal botrocetin-induced binding to platelets but no ristocetin-induced binding. In the Matsushita and Sadler study (1995), mutant (642−645)4A also lacks ristocetin-induced platelet-binding activity, but binding to platelets in the presence of half-maximal concentrations of botrocetin is only 20% of wt vWF. This difference could reflect minor differences in the expression systems or assay conditions.

Identification of a Heparin-Binding Site. Recent studies suggest that the heparin-binding domain in vWF plays a role in regulating the vWF/GPIb interaction, and may overlap with the GPIb-binding site (Andrews et al., 1995). Although heparin-binding sites in vWF have been identified both in the amino-terminus of the mature protein (Fretto et al., 1986) and in the A1 domain (Fujimura et al., 1987; Mohri et al., 1989), studies with recombinant vWF suggest that the binding site in the A1 domain is the only functional binding site in full-length vWF (Sixma et al., 1991). Sobel et al. (1992) further localized the heparin-binding site in the A1 domain to residues Tyr565-Ala587. A second sequence in this region, Asn633-Val648, contains a significant cluster of basic residues, but a peptide containing these residues does not bind to heparin or suppress the inhibition of the vWF/platelet interaction by heparin (Sobel et al., 1992). Our study suggests, however, that the Asn633-Val648 segment does contribute to the vWF/heparin interaction in full-length multimeric vWF. When the core basic residues in Asn633-Val648 are replaced by alanine [mutant vWF-KKKK(642−645)4A], heparin-binding is reduced by 70% compared to wt vWF. In contrast, mutant vWF-KDRKR(569−573)5A, in which the core basic residues of the Tyr565-Ala587 consensus sequence are replaced with alanine, shows normal heparin-binding activity. These results do not exclude the participation of the Tyr565-Ala587 consensus sequence in the vWF/heparin interaction, but they show that the core basic residues (Lys569-Arg573) are not essential for this function.

Margalit et al. (1993) suggest that heparin-binding domains may have both charge and conformational restraints. Thus, segments of vWF that contain appropriately-spaced basic residues as defined by consensus heparin-binding sequences may not be in the appropriate conformation in the native protein for interaction with heparin. Comparison of structurally-defined heparin-binding sequences revealed common spatial motifs in which two basic residues are separated by six or twelve residues depending on whether the protein is folded into an α-helix or a β-sheet, respectively (Margalit et al., 1993). A β-sheet motif is present in the proposed Tyr565-Ala587 heparin-binding domain, and both an α-helix and β-sheet motif are present in the proposed Asn633-Val648 heparin-binding domain. Interestingly, alignment of the vWF A1 domain sequence with the sequence for the related A domain of the integrin CR3, for which the crystal structure has been determined (Lee et al., 1995), shows that an α-helix and β-sheet are likely to be present within the Asn633-Val648 consensus sequence. Conversely, the predicted β-sheet in the Tyr565-Ala587 consensus sequence is more likely to contain α-helices, which may reduce the ability of this segment to interact with heparin.

Mutant vWF-R552A also showed reduced binding to heparin. Although R552A is part of a β-sheet motif, the lower heparin-binding activity shown by vWF-R552A may be due to the reduced accumulation of high mw multimers, which is known to affect the vWF/heparin interaction (de Romeuf & Mazurier, 1993). Although the presence of lysine at position 549 is consistent with several heparin-binding domains (Margalit et al., 1993), the substitution of alanine at this position had no effect on heparin-binding by vWF. Studies with additional mutants may clarify the relevance of these motifs and identify other residues that are important for the interaction of vWF with heparin.

Localization of the AvW3 Epitope. vWF mAb AvW3 is an inhibitory antibody that blocks the binding of vWF to the GPIb/IX complex (Kawai & Montgomery, 1987). The inability of AvW3 to recognize vWF deletion mutants vWF-Δ1 through vWF-Δ9 by antigen-capture ELISA suggests that AvW3 recognizes a complex epitope that is dependent on the native conformation of the vWF A1 domain. These results are consistent with the inability of AvW3 to recognize SDS-denatured multimeric vWF or reduced vWF (Kawai & Montgomery, 1987). Mutant R552A was the only alanine-substitution mutant to show markedly reduced activity with AvW3. Although Arg552 could be a critical residue in the AvW3 epitope, it is more likely that the R552A substitution causes a general conformational change in the A1 domain that reduces reactivity with AvW3.

ACKNOWLEDGMENT

We thank Dr. Robert Montgomery for many helpful discussions during the course of these experiments. We also thank Cathy LaRue for assisting in the construction and characterization of the vWF deletion mutants, Bernard Gasch and Matthew Montgomery for constructing the alanine-substitution mutants, and Drs. Paul Foster, Paul Scott, and Jonathan Rosenberg for critical review of the manuscript.

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BI9610313