Mapping the Glycoprotein Ib-binding Site in the von Willebrand Factor A1 Domain*

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Received for publication, March 17, 2000
Published, JBC Papers in Press, April 11, 2000, DOI 10.1074/jbc.M002292200

The von Willebrand factor (vWF) mediates platelet adhesion to exposed subendothelium at sites of vascular injury. It does this by forming a bridge between subendothelial collagen and the platelet glycoprotein Ib-IX-V complex (GPIb). The GPIb-binding site within vWF has been localized to the vWF-A1 domain. Based on the crystal structure of the vWF-A1 domain (Emsley, J., Cruz, M., Handin, R., and Liddington, R. (1998) J. Biol. Chem. 273, 10396–10401), we introduced point mutations into 16 candidate residues that might form all or part of the GPIb interaction site. We also introduced two mutations previously reported to impair vWF function yielding a total of 18 mutations. The recombinant vWF-A1 mutant proteins were then expressed in Escherichia coli, and the activity of the purified proteins was assessed by their ability to support flow-dependent platelet adhesion and their ability to inhibit ristocetin-induced platelet agglutination. Six mutations located on the front and upper anterior face of the folded vWF-A1 domain, R524S, G561S, H563T, T594S/E596A, Q604R, and S607R, showed reduced activity in all the assays, and we suggest that these residues form part of the GPIb interaction site. One mutation, G561S, with impaired activity occurs in the naturally occurring variant form of von Willebrand’s disease-type 2M underscoring the physiologic relevance of the mutations described here.

von Willebrand factor (vWF) is a plasma glycoprotein that plays an important role in primary hemostasis (1, 2). vWF mediates the adhesion of platelets to sites of injury by forming a bridge between components of the subendothelium and platelet receptor sites on glycoproteins (GP) Ib/IX/V and Ib/IIIa. The interaction with vWF stabilizes adherent platelets and permits them to remain attached under the shear stresses encountered in the arterial circulation (3–6). Although plasma vWF does not bind to circulating platelets, the interaction can be induced by the addition of the antibiotic ristocetin or the snake venom protein botrocetin, by subjecting a platelet-vWF suspension to shear stress, or by immobilization of vWF onto vascular subendothelium or other surfaces. It has been assumed, but not proven, that modulation of GPIb/IX binding activity involves conformational changes in vWF, with exposure of functional sites that are normally hidden when the protein is in solution.

The vWF-binding site for platelet GPIb is localized in the first of three repeated A domains (vWF-A1). We previously reported the crystal structure of a recombinant vWF-A1 domain (7). The A1 domain (amino acids 479–717) contains 2 cysteine residues that form an intramolecular disulfide bond (Cys509-Cys695). The amino acid sequence residing between the two cysteines, Ser510-Leu694, adopts a globular structure in which the hydrophobic β sheet forms a central core that is surrounded by amphipathic α helices (7). The vWF-A1 domain of vWF has been studied extensively, and it is well established that the GPIb-binding site resides within this region (8–12). Several attempts have been made to identify those amino acid residues that are critical for vWF-A1 function. However, despite these studies, which have employed deletion or alanine scanning mutagenesis (13, 14), only a double mutation at Glu596 and Lys509 has been reported to impair the binding of vWF to platelet GPIb/IXV (14). Other mutations impair either the binding or the activity of the modulator botrocetin.

Three naturally occurring missense mutations have been identified in the A1 domain, which impair hemostasis. Patients carrying these three mutations (G561S, F606I, and I662F) have a variant form of von Willebrand’s disease called type 2M disease (15, 16) which is characterized by low vWF antigen levels and disproportionately low ristocetin cofactor activity but normal vWF multimer structure. Paradoxically, despite a clear hemostatic defect, botrocetin-induced binding of patient vWF to platelets remains normal. The unusual phenotype induced by these mutations has been reproduced by the study of recombinant vWF carrying the type 2M mutations (15, 16). This disparity between the clinical and laboratory findings suggests that additional testing of the type 2M vWD mutants, perhaps under flow conditions, may be necessary to understand how these mutations perturb hemostasis.

We have previously reported the expression and characterization of a recombinant vWF-A1 protein that inhibits the interaction of full-length vWF with GPIb (17). We observed that recombinant vWF-A1 binds to GPIb without the need for modulators and is an effective inhibitor of ristocetin-induced platelet agglutination. As previously noted, we have also determined the crystal structure of the vWF-A1 domain (7).

In the current study, we used the crystal structure of the vWF-A1 domain to identify candidate residues that might interact with the GPIbα polypeptide in the platelet GPIb-IX-V complex. Residues that were located on the surface of the
domain and might serve as contact sites between vWF and the GPIb polypeptide subunit were selected for mutagenesis. We then expressed recombinant mutant vWF-A1 and analyzed both the inhibition of ristocetin-dependent platelet agglutination and the support of flow-dependent platelet adhesion by these mutants. We have identified a cluster of amino acids on the front and upper anterior faces of the vWF-A1 domain which, we believe, constitute the GPIb interaction site and mediate platelet attachment to vWF.

EXPERIMENTAL PROCEDURES

Construction of vWF-A1 Mutants—Mutations were introduced into vWF-A1 cDNA with a PCR-based mutagenesis strategy. Two rounds of PCR amplification were performed. First, each oligonucleotide primer with the desired nucleotide substitution was combined with an end primer and amplified. Second, the resultant DNA fragment was then combined with the opposite end primer to produce the vWF-A1 cDNA fragment. The outside primers introduced BamHI and HindIII restriction sites for cloning. The PCR product was digested with BamHI and HindIII and inserted into pQEG as described previously (17).

The amino acid residues mutated were as follows: RS245S, S526R, E557Q, D605S, D605R, G618S (type 2M vWD), H563T, Q583R, K585E, Q589E, E589A, Q604R, S605R, S605T, E605R, and K606E. Most of the mutant amino acids was sequenced to confirm both the presence of the desired mutation and the lack of any other mutations.

Expression and Purification of the Recombinant Proteins—E. coli M15 [pREP4] (Qiagen, Chatsworth, CA) containing each of the pQEG-vWF-A1 variants was cultured overnight at 37 °C in 30 ml of 50 g/liter tryptone, 15 g/liter yeast extract, 25 μg/ml kanamycin. The overnight culture was diluted 1:30 and grown to an A600 of 0.8. The culture was adjusted to 1.5 mM isopropyl β-D-thiogalactopyranoside and incubated for 4 h at 37 °C. The cells were then harvested and resuspended in 25 ml of lysis buffer (50 mM Tris-Cl, 0.1 mM NaCl, 1 mg/ml EDTA, pH 8.0) containing a final concentration of 250 μg/ml lysozyme and allowed to stand for 15 min at 4 °C. The bacterial cells were lysed in the presence of 1.25 mg/ml deoxycholic acid and 7 μg/ml DNase I. The lysate was centrifuged at 12,000 × g for 15 min and the washed pellet was washed in lysis buffer containing 3 mM urea, 2.5% Triton X-100, and 10 mM EDTA followed by recentrifugation.

For the purification of the vWF-A1 proteins, the washed pellet was solubilized by the addition of 6.5 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.5. The solubilized proteins were diluted 40-fold in 50 ml of 35 mM Tris-HCl, 500 mM NaCl, 0.2% Tween 20, pH 7.8. They were passed over a Ni2+ -chelated Sepharose (Amersham Pharmacia Biotech) column equilibrated with 25 ml of Tris-HCl, 200 mM NaCl, pH 7.8 buffer. vWF-A1 proteins eluted from the column with 350 mM imidazole. The isolated proteins were adsorbed to and eluted from a heparin-Sepharose column (Amersham Pharmacia Biotech). The highly purified proteins were dialyzed against 25 ml of Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.8.

Platelet Agglutination Assay—Ristocetin-induced platelet agglutination was carried out on siliconized glass cuvettes at 37 °C with constant stirring at 1,200 rpm in a 4-channel aggregometer (Bio/Data Corp., Horsham, PA). A suspension of platelet-rich plasma (PRP) containing 2 μM each of vWF-A1 mutant was prepared. After 5 min incubation at 37 °C, agglutination was initiated by the addition of ristocetin (Sigma) (18). Blood was collected from healthy adult donors into syringes or competition studies, whole blood was incubated with 100 μg/ml monoclonal antibody 6D1 ascites or 2 or 4 μg/ml wild type vWF-A1 protein, respectively, for 15 min at room temperature before the attachment assay.

Platelet Agglutination Assays in a Parallel Plate Flow Chamber—We also used a parallel plate flow chamber that was assembled as described by the manufacturer (Glycotech, Rockville, MD). Purified recombinant vWF-A1 protein (diluted to 150 μg/ml with 25 mM Tris, 150 mM NaCl, pH 7.4) was added to a glass coverslip and incubated for 1 h at 37 °C. Coated coverslips were subsequently rinsed and incubated with TBS.

The adsorbed proteins were measured by enzyme-linked immunosorbent assay. Briefly, after rinsing the unadsorbed A1 protein, residual binding sites were blocked with 3% bovine serum albumin, 0.05% Tween 20 in TBS (TBS-T) for 30 min at 37 °C. Coverslips were then incubated with the recombinant A1, protein, with 25 μg/ml wild type vWF-A1 protein, respectively, for 15 min at room temperature before the attachment assay.

Platelet Adhesion Assays in Capillary Tubes—We also used a parallel plate flow chamber that was assembled as described by the manufacturer (Glycotech, Rockville, MD). Purified recombinant vWF-A1 protein (diluted to 150 μg/ml with 25 mM Tris, 150 mM NaCl, pH 7.4) was added to a glass coverslip and incubated for 1 h at 37 °C. Coated coverslips were subsequently rinsed and incubated with TBS.

The adsorbed proteins were measured by enzyme-linked immunosorbent assay. Briefly, after rinsing the unadsorbed A1 protein, residual binding sites were blocked with 3% bovine serum albumin, 0.05% Tween 20 in TBS (TBS-T) for 30 min at 37 °C. Coverslips were then incubated with the recombinant A1, protein, with 25 μg/ml wild type vWF-A1 protein, respectively, for 15 min at room temperature before the attachment assay.

The vWF-A1-coated coverslips formed the lower surface of the chamber, and a silicone rubber gasket determined the flow path height of 254 μm. The flow chamber was assembled and filled with TBS. A syringe pump (Harvard Apparatus Inc., Holliston, MA) was used to aspirate blood through the flow chamber. The flow rate of 0.48 and 0.6 ml/min produced a wall shear rate of 300 and 1500 s−1, respectively (18). Blood was collected from healthy adult donors into syringes containing 3.8% of sodium citrate as anticoagulant. Blood was then perfused for 2 min, and the coated coverslip was washed with TBS. Attached platelets were observed with phase contrast objectives and recorded with a VCR (Sony). The number of adherent platelets was determined by overlaying on a 36-square grid on at least 6–8 frames and counting and averaging the number of platelets in 12 randomly selected squares. The data points represent the average of 2–3 individual experiments. For some experiments, the whole frame was counted. For antibody inhibition studies or competition studies, whole blood was incubated with 100 μM monoclonal antibody 6D1 or 2 or 4 μM wild type vWF-A1 protein for 5 min at 37 °C before the attachment assay.

Protein Quantitation—Protein concentrations were determined by the BCA method (Pierce). Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis gels assessed the purity of the fragments (19).

RESULTS

Platelet Adhesion to Capillary Tubes Coated with Multimeric vWF or Recombinant vWF-A1 Domain—Platelets adhered to immobilized multimeric vWF and to the recombinant A1 domain at all flow rates tested. In fact, highly purified recombinant A1, when immobilized, seemed to support platelet adhesion as well as multimeric vWF at 600 s−1 (Fig. 1). Since it was impossible to determine the molecular weight of vWF multimers, which are heterogeneous, it was difficult to make a precise comparison. However, at a concentration of 20 μg/ml, multimeric vWF supported the adhesion of 180 ± 23 platelets/mm2 surface area at a flow rate of 600 s−1. A concentration of 100 μg/ml of vWF-A1 supported 260 ± 15 platelets/mm2 at the same flow rate. After 5 min of perfusion over surfaces coated with either protein, only single adherent platelets were observed in the flow chamber.

Effect of Mutations Based on the Crystal Structure of the vWF-A1 Domain—By solving the crystal structure of vWF-A1, we were able to inspect the structural model and select amino
acid residues that might serve as contact sites for GPIb. The selections were made based on their position within the folded domain and the orientation and chemical nature of their side chains. Based on experience with other proteins, we hypothesized that the residues that mediate binding were most likely to be solvent-accessible and on the surface of the A1 domain. We also avoided residues that we could show were involved in internal hydrogen or hydrophobic interactions that, if modified, would perturb the global structure of the domain. In addition, we also created one of the mutations causing type 2M vWD, R524S. Sequence analysis revealed that the protein containing the mutation E596A, which was previously reported to be essential for the GPIb-vWF interaction (14), resulted with an additional mutation, T594S. Despite the mis-sense mutation, we proceeded to report its effect in this study.

Mutant vWF-A1 proteins were expressed and purified, as described for wild type vWF-A1. The average yield of the mutant proteins was between 2 and 4 mg/liter of bacterial culture. All the proteins migrated identically to wild type vWF-A1 protein, when analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions (data not shown). As previously reported for the wild type vWF-A1 protein, differential migration under reduced and non-reduced conditions provided evidence for the formation of a disulfide bond between Cys509 and Cys695 (17). During the process of purification, all of the mutants were adsorbed to and eluted from heparin-Sepharose columns, suggesting that none of the mutations impaired heparin binding. This was expected, as the heparin-binding site is located in a different part of the A1 domain (20). In addition, we tested the reactivity of the mutants with a monoclonal antibody, LJ-RG-46 (21), which recognizes an epitope on vWF-A1 that is not in the same area as the point mutations. They bound normal compared with wild type (data not shown).

The ability of the recombinant vWF-A1 proteins to compete with multimeric vWF for binding to the GPIb complex was then analyzed. As previously reported 2 μM wild type vWF-A1 protein completely inhibited ristocetin-induced platelet agglutination (RIPA) (17). By comparison, the introduction of mutations G561S, H563T, T594S/E596A, Q604R, S607R, and Q628R greatly impaired the ability of the mutant proteins to compete with multimeric vWF in the RIPA assay (Fig. 2). Mutations R524S, S526R, E557Q, D560S, Q583R, K585E, R629E, and H656E inhibited RIPA between 40 and 60%, whereas the mutations D560R, Q590R, L659, and K660E inhibited RIPA between 25 and 35%.

To better evaluate the effect of these mutations in a more physiologically relevant setting, we next studied the ability of mutant proteins to support flow-dependent platelet adhesion at a low shear rate of 50 s⁻¹. In some cases there was a close correlation between the two assays. Those mutations that retained less than 25% of their inhibitory activity in the RIPA assays, D560R, G561S, H563T, T594S/E596A, Q604R, and S607R, completely lost their ability to support capillary tube adhesion (Fig. 3A). E557Q and D560S, which inhibited RIPA by 40%, supported 60% of wild type adhesion. There were also some minor discrepancies, which could be explained by the fact that the flow assay does not require a modulator like ristocetin. R524S, which inhibited RIPA by 50%, only supported 10% of normal platelet adhesion. Q590R and R629E were fully active in the adhesion assay but retained only 50% of their RIPA inhibitory activity. The remaining mutants retained only 25% of wild type adhesive activity. It is important to point out that mutation of the same six residues Gly561, His563, Thr584/Glu586, Gln590, and Ser607 had the most marked effect on both the RIPA and the low shear adhesion assays.

Fig. 3A also shows that the interaction of platelets with immobilized vWF-A1 can be blocked by the addition of the monoclonal anti-GPIb antibody 6D1 or by the addition of soluble wild type vWF-A1 protein. As seen in Fig. 3B, the results obtained at calculated shear rates of 600 s⁻¹ were comparable to those obtained at a shear rate of 50 s⁻¹.

There are several limitations to the capillary tube flow sys-

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tem. A large volume of perfusate is needed to obtain high shear rates, and the system uses extensively washed platelets, without added red blood cells. Accordingly, we repeated the studies in a parallel plate perfusion chamber under both intermediate (300 s$^{-1}$) and high shear (1500 s$^{-1}$) conditions using whole blood as the source of platelets. Fig. 4 shows a representative photomicrograph of attached platelets after 2 min of perfusion with whole blood at high shear stress. Mutation G561S completely abolished the ability of vWF-A1 to support platelet adhesion and E557Q partially inhibited adhesion. Mutation K660E supported near normal platelet adhesion.

Fig. 5A shows the quantitative analysis of attached platelets at intermediate shear (300 s$^{-1}$) and Fig. 5B at high shear (1500 s$^{-1}$) rates. The five mutant proteins with no adhesive activity at low (50 s$^{-1}$) shear stress gave similar results at intermediate and high shear. There were several new and discrepant observations at higher shear. Mutations S526R, K585E, and R629E that inhibited RIPA by 40–50% and had 25–100% adhesive activity at low shear retained 25–95% activity at the higher shear rates. However, mutations E557Q, D560S, Q583R, and H656E that also retained 45–60% inhibitory activity in the RIPA and had a 70 to 30% activity at low shear fell to 10 to 0%
at the two higher shear rates. Interestingly, R524S that inhibited RIPA by 50% had no activity at the two measured shear rates. Unexpectedly, K660E, which inhibited RIPA by 30% and had a 25% activity at low shear, had more activity at intermediate (55%) and high (90%) shear. Q590R, Q628R, and L659R, which retained 25–100% activity at low shear, fell to 0% activity at the two higher rates of shear stress. The type of amino acid substitution, as well as the position of the substitution, can influence the results. Conversion of Asp560 to Ser rather than Arg reduced activity at low shear to 50% of wild type activity and completely abolished activity at higher shear. In contrast, D560R, which had no adhesive activity at low shear, resulted with >40% activity at higher shear.

The inhibition of platelet adhesion at high shear stress with soluble wild type vWF-A1 protein or monoclonal antibody 6D1 was similar to low shear conditions (Fig. 5B). Protein-coated coverslips analyzed by enzyme-linked immunosorbent assay with the anti-polyhistidine antibody demonstrated that the adsorption of protein on coverslip was similar for all the recombinant proteins (data not shown).

**DISCUSSION**

It is well established that vWF mediates the flow-dependent adhesion of platelets to vascular subendothelium via its A1 domain (6). In previous studies, we reported the expression of a recombinant vWF-A1 protein that binds directly to GPIb and competes with multimeric vWF in the RIPA assay (17). In this study we have demonstrated that recombinant vWF-A1 protein also supports flow-dependent platelet adhesion to capillary tubes and microslides in an *ex vivo* perfusion chamber as effectively as full-length vWF multimers. Since each vWF-A1 molecule only contains a single GPIb/IX/V interaction site, this suggests that a single contact between a platelet and vWF may be sufficient to arrest flow and permit stable flow-dependent adhesion. This was somewhat surprising, as it is well known that high molecular weight vWF multimers are more effective in RIPA assays and are necessary for optimal hemostasis *in vivo* (22).

Although each vWF multimer contains multiple vWF-A1 domains and, therefore, potential platelet-binding sites, the relatively compact globular shape of vWF may limit the number of A1 domains that can participate in hemostasis. We and others (23, 24) have previously reported that vWF has a loosely coiled oblate ellipsoid with average dimensions of 250 × 50 Å. There is now evidence, obtained by atomic force microscopy, that some unfolding of vWF occurs when it is immobilized and subjected to shear stress (25). However, the calculated size of the unfolded form of vWF is still substantially less than what one would predict for fully extended vWF polymers, suggesting that even in immobilized vWF multimers only a fraction of the total number of vWF-A1 interaction sites may be available for platelet adhesion.

Another explanation is that the platelets in the perfusion chamber are interacting with multiple independent vWF-A1 domains that are immobilized and arrayed on the capillary tube. This could occur if some fraction of the immobilized vWF-A1 molecules were spaced optimally and mimicked the
geometry of available vWF-A1 domains in the immobilized polymeric vWF. The data derived from the flow chamber studies also provide evidence that the folding and conformation of the isolated vWF-A1 domain is similar to that found in full-length vWF as both support flow-dependent platelet adhesion equally well.

We have used the two previously described flow systems as they provided an efficient and physiologically relevant method to study the function of small quantities of recombinant mutant proteins. The analysis is also simplified as it utilizes recombinant vWF-A1 protein rather than the more heterogeneous vWF multimers. Using flow-dependent adhesion as one of the functional end points, since the recombinant vWF-A1 domain expressed is fully active, also eliminates the need for a modulator like ristocetin. The close correlation between the reduced capacity of mutant forms of vWF-A1 to support adhesion and inhibit RIPA helps to validate the RIPA assays and supports our contention that the residues we have identified play a role in platelet adhesion.

There is some clinical data suggesting a region of vWF-A1 that could mediate binding to platelet GPIb. There are three mutations, G561S, F606I, and I662F, that have been described in patients that have nearly normal levels of vWF protein but significant impairment of hemostasis and clinical bleeding, a variant called type 2M von Willebrand’s disease. These A1 domain mutations impair vWF binding to GPIb but do not affect vWF binding to collagen and heparin (15, 16). Interestingly, although these mutations decrease ristocetin-induced platelet agglutination, the 2M vWF mutant proteins can still be activated by botrocetin (15, 16). Our study is the first to analyze the effect of the type 2M mutation, G561S, on the isolated vWF-A1 domain. The result obtained with this mutant confirmed the clinical observation of a hemostatic defect. The importance of this residue can be explained by inspection of the crystal structure (Fig. 6) which demonstrates that Gly561 is surface-exposed and lies at the center of the putative GPIb-binding surface (see below). The two other amino acid residues implicated in type 2M vWD, Phe606 and Ile662, are buried in the hydrophobic core of the domain, so that their effect on GPIb binding must be indirect, perhaps via destabilization of the global fold.

Kroner and Frey (13) and Matsushita and Sadler (14) using deletion and alanine-scanning mutagenesis of the vWF-A1 domain attempted to define the GPIb contact site. Since studies with full-length vWF require modulators like ristocetin and botrocetin to induce vWF binding to platelet GPIb, they encountered mutations that impaired modulator binding that were not located in the GPIb-binding site. However, the mutation of two residues, Glu596 and Lys599, clearly reduced the vWF interaction with GPIb (14). In this study, mutations of Thr594 and Glu596 also confirmed the observations reported by these other groups who used recombinant full-length vWF in their studies. Interestingly, Glu596 lies very close to Gly561 in the three-dimensional structure.
The residues chosen for mutagenesis in our study were completely exposed on the surface of the domain and did not form hydrogen bonds, salt bridges, van der Waals interactions or have other contact with the rest of the domain. Mutations were carefully designed to avoid creating additional bonding interactions. As we were trying to disrupt a large interface with a point mutant, the mutations were designed to be as radical as possible (either uncharged-to-charged or charge reversal), with the introduced side chain larger rather than smaller than the wild type residue. In practice, most of the residues were changed to either glutamate or arginine.

Six of the 18 mutant proteins showed impaired activity at all shear rates tested, whereas 4 others retained activity under all conditions. These results define a putative GPIb-binding surface encompassing the front and upper faces of the vWF-A1 domain (Fig. 6). This surface is adjacent to but distinct from the surfaces implicated in heparin and botrocetin binding and from the site of the vWD type IIB mutations, which cluster on the lower surface of the domain. The location is also distinct from the epitope of the function-blocking antibody, NMC-4, which binds to the right-hand face of the domain (26).

Six additional mutants showed activity at low shear but greatly reduced activity (<10%) at high shear, and one (K660E) showed increased activity with increased shear. This suggests, first, that the functional assay employed is critical. RIPA, a popular assay of vWF function, proved to be the least sensitive assay. Flow-dependent adhesion, particularly at high shear (1500 s\(^{-1}\)), was the most stringent assay and picked up the highest percentage of mutants with impaired vWF function. A possible molecular explanation for these shear-dependent results comes from our studies of the homologous integrin I domain. Recent studies have shown that the I domain of integrin \(\alpha\beta_1\) switches conformation to a high affinity state when it engages ligand and that this involves a reorganization of the upper surface of the domain (27). Furthermore, mutations to residues that change position in the two conformations have different effects on binding activity under static versus flow conditions, suggesting that adhesion under static conditions detects binding to the low affinity conformation (28). If the vWF A1 domain undergoes analogous conformational changes, it could explain the shear-dependent results and the contrasting effects of certain mutations. The crystal structure of a complex between A1 and a suitable fragment of GPIbα will be required to test this hypothesis and to interpret completely the mutational data.

Nevertheless, it is reassuring that a mutation introduced in the critical area that we have defined markedly reduces the ability of the A1 domain to support flow-dependent adhesion and also causes a bleeding diathesis in patients with type 2M vWD. This not only helps validate that we have identified the appropriate region of the vWF-A1 domain but that our ex vivo analytic model using isolated recombinant domain proteins is valid. Finally, these studies demonstrate that it is possible to make accurate predictions about protein function from the analysis of three-dimensional protein structure and to confirm these postulates by site-specific mutagenesis and functional analysis of the recombinant polypeptides derived from a much larger homopolymer.

Acknowledgments—We thank Dr. Simon C. Robson (Beth Israel Deaconess Medical Center, Boston) and Dr. Zaverio M. Ruggeri (Scripps Research Institute, La Jolla, CA) for providing monoclonal antibody LJ-RG-46 and Dr. Barry Coller (Mt. Sinai School of Medicine, New York) for providing the monoclonal antibody 6D1. We also acknowledge Drs. Bruce Ewenstein and Robert Wise for their insights and helpful discussions during the course of this work. We thank Anne McLeod for providing the conformational specific antibody and Dogarí Estavillo and Rafal Barezak for technical assistance.

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