von Willebrand Factor (vWF) is a multimeric protein that mediates platelet adhesion to exposed subendothelium at sites of vascular injury (1). The A1 domain of vWF (vWF-A1) forms the principal binding site for platelet glycoprotein Ib (GpIb), an interaction that is tightly regulated. We report here the crystal structure of the vWF-A1 domain at 2.3-Å resolution. As expected, the overall fold is similar to that of the vWF-A3 and integrin I domains. However, the structure also contains N- and C-terminal arms that wrap across the lower surface of the domain. Unlike the integrin I domains, vWF-A1 does not contain a metal ion-dependent adhesion site motif. Analysis of the available mutagenesis data suggests that the activator botrocetin binds to the right-hand face of the domain containing helices α5 and α6. Possible binding sites for GpIb are the front and upper surfaces of the domain. Natural mutations that lead to constitutive GpIb binding (von Willebrand type Ib disease) cluster in a different site, at the interface between the lower surface and the terminal arms, suggesting that they disrupt a regulatory region rather than forming part of the primary GpIb binding site. A possible pathway for propagating structural changes from the regulatory region to the ligand-binding surface is discussed.

Crystal Structure of the von Willebrand Factor A1 Domain and Implications for the Binding of Platelet Glycoprotein Ib*

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von Willebrand Factor (vWF)† is a multimeric protein that mediates platelet adhesion to exposed subendothelium at sites of vascular injury (1). The adhesive properties of vWF are tightly regulated so that plasma vWF does not normally interact with circulating platelets. vWF, however, will bind to platelets after it is “activated” by poorly understood conformational changes that occur after it binds to the vessel wall. A reduction in the plasma concentration of vWF or mutations that impair binding, activation, or assembly of vWF multimers cause von Willebrand disease (vWD), a common bleeding disorder characterized by decreased platelet adhesion and mucocutaneous bleeding (2).

vWF-mediated adhesion of platelets to the vessel wall, under the high flow/shear conditions present in circulating blood, is mediated by sequences within the first (A1 domain) and third (A3 domain) A type repeats of vWF. The A1 domain (residues 479–717) binds to platelet glycoprotein Ib-IX-V complex (GpIb), subendothelial heparans, cell surface sulfatides (reviewed in Ref. 3), and the non-fibrillar collagen type VI (4). The vWF-A3 domain contains the principal site for binding the fibrillar collagens types I and III (5, 6).

Although initially noted in the primary sequence of vWF, the A domain has been subsequently discovered in a large number of cell matrix-associated or adhesive proteins and receptors (7). For example, varying numbers of A domains are found in several of the atypical, short chain collagens. A single A domain is inserted into the sequence of several integrin receptors, where it is generally referred to as the I domain. A/I domains are frequently involved in either cell adhesion or cell ligand interactions. In 1995, we reported the crystal structure of the first family member, the I domain of the leukocyte integrin αMβ2 (8), and the crystal structures of several A domains have now been solved (9–12). This work has provided new insights into how A domains mediate cellular adhesion and facilitates detailed structure-function studies. All A domains have a very similar structure comprising a variant of the dinucleotide-binding fold. The integrin I domains contain a metal ion-dependent adhesion site (MIDAS) on the upper face of the domain that is an important element of ligand binding (8, 13–15). In contrast, the vWF-A3 domain does not bind metal and does not require metal for binding to collagen (10).

To advance studies of vWF binding to platelet GpIb and to gain more understanding of the molecular switches that activate vWF, we have solved the crystal structure of the vWF-A1 domain and in this paper correlate its structure with existing biochemical and mutational data.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—Recombinant vWF-A1 containing residues 475–709 of mature vWF and 12 residues at the N terminus from the expression vector (MRGSHHHHHHGS) was expressed in Escherichia coli, refolded, and purified as follows. Our previously published technique (16) was used to transform cells, induce protein, and harvest inclusion bodies. Next, the washed pellet was solubilized by the addition of 6.5 m guanidine hydrochloride in 50 m M Tris-HCl, pH 7.5. The solubilized protein was diluted 40-fold in 50 m M Tris-HCl, 500 m M NaCl, 0.2% Tween 20, pH 7.8. It was passed over an Ni2+-chelated Sepharose (Pharmacia) column equilibrated with 25 m M Tris-HCl, 200 m M NaCl (pH 7.8) buffer. vWF-A1 protein eluted from the column with 350 m M imidazole. The isolated protein was absorbed to and eluted from a Heparin-Sepharose column (Amerham Pharmacia Biotech). The highly purified protein was dialyzed against 25 m M Tris-HCl, 150 m M NaCl, 0.05% Tween 20, pH 7.8. This protein failed to produce crystals suitable for x-ray analysis.

The protein (0.4 mg/ml) was next treated with immobilized α-chymotrypsin (Sigma) in 0.1% tris, pH 8.0, 0.15 M NaCl, 0.1% Triton X-100 for 24 h at 4 °C with constant agitation, loaded onto a Heparin-Sepharose column (Pharmacia) equilibrated with 0.1 M tris, pH 8.0, 0.15 M NaCl, and then eluted with 0.1 M tris, pH 8.0, 0.6 M NaCl. Finally, the protein was diluted 3-fold with water and concentrated to 4 mg/ml. The molec-
ular mass estimated by SDS-polyacrylamide gel electrophoresis reduced from 27 to 24 kDa after chymotrypsin digestion. In the crystal structure (see below), the C terminus is ordered to within 4 residues of the C terminus of the expressed domain, and at the N terminus the first residue visible in the electron density map is Asp986. Chymotrypsin cleaves specifically after aromatic residues, and Tyr495 at the N terminus is the only aromatic residue that is not visible in the final electron density map. Cleavage after Tyr495 and the loss of 33 residues from the N terminus gives a predicted size of 24.5 kDa, consistent with the size estimated by SDS-polyacrylamide gel electrophoresis. Other aromatic residues are presumably protected from cleavage by the folded conformation of the domain. Cleavage did not detectably perturb vWF-A1 binding to GpIB or its ability to inhibit ristocetin-induced platelet aggregation by full-length vWF (data not shown).

Cysteine residues are presumably protected from cleavage by the folded conformation of the domain. Cleavage did not detectably perturb vWF-A1 binding to GpIB or its ability to inhibit ristocetin-induced platelet aggregation by full-length vWF (data not shown).

Data Collection, Structure Determination, and Refinement—A single crystal was transferred into a cryoprotectant buffer consisting of 0.1 M Tris, pH 8.5, 8% polyethylene glycol 8000. Larger crystals grew to dimensions 0.1 mm × 0.1 mm × 1 mm in the presence of 5 mM CdCl2 and belong to the space group P61 with cell dimensions a = b = 86.4 Å, c = 68.1 Å, α = β = 90°, γ = 120°. The asymmetric unit contains one vWF-A1 domain and 57% solvent.

Molecular replacement was performed using the crystal structure of the integrin α2-1 domain (11), stripped of loops and side chains, as the search model. The highest peak in the cross-rotation function gave the correct orientation of the monomer. The translation function gave a strong peak in space group P61, than in P62, establishing the correct enantiomorph. Rigid body refinement using XPLOR (19) led to an R factor of 52% and a correlation coefficient of 0.45. Using the mercury derivative data and model-derived phases, a difference Fourier revealed the position of two mercury atoms. These sites were refined using HEAVY (20), and phases were calculated using MLPHARE (phasing power = 0.41), leading to an experimental map that was solvent flattened using DM. The heavy atom-derived and molecular replacement-derived maps were then averaged using the program RAVE (21). This averaged map was of higher quality than either of the component maps and was readily interpretable. An initial round of model building using program O (22) allowed insertion of 173 residues including some side chains. This model was subjected to positional refinement using XPLOR, resulting in an R factor (Rwork) of 42.3% for the working set of 26.7%, and 99.8% completeness. Figure 3 and 4 includes part of the salt-bridge network (see below). (b) The “upper” face lies at the C-terminal end of the β-sheet and is composed of loops connecting the sheet to the flanking helices.

Table I

| Resolution | No. of observations | Rwork* | Rfree | Dmap |
|------------|---------------------|--------|-------|------|
| 10–2.3 Å  | 11849 (9144)        | 8.3%   | 13.2/2.4 | 7.9/2.5 |
| 10–2.8 Å  | 7174 (9953)         | 13.4%  | 9.0/2.5 |

* Rwork = ΣI – 〈I〉/ΣI, where 〈I〉 = average intensity from multiple observations of symmetry related reflections. Rmsd = root mean square deviation from ideal values.

Table II

Location of von Willebrand disease type IIb mutations

| Mutants | Location | Environment |
|---------|----------|-------------|
| Natural |          |             |
| Pro963  | N-terminal arm | Packs against Tyr508, stabilizes N-terminal arm |
| His965  | N-terminal arm | Salt bridge to Glu542 in α1; part of salt-bridge network |
| Arg985  | α1-β turn | Hydrogen bond to Cys509; packs against Ser512; stabilizes N-terminal arm |
| Arg987  | α1-β turn | Packs against Arg545 |
| Tyr500  | Strand ββ | Packs against the disulfide, Arg446 and Leu577 from β3 |
| Val511  | Strand βB | Packs against Arg446 and Leu577 from β3 |
| Val521  | Helix α3 | Packs against N-terminal arm |
| Val532  | Helix α3 | Water-mediated salt bridge to Glu501 in N-terminal arm |
| Pro747  | End of helix α7 | Packs into hydrophobic core |
| Arg787  | End of helix α7 | Packs into hydrophobic core |
| Ala698  |          |             |
| Scanning |          |             |
| Glu507-Glu501 | N-terminal arm | Packs against lower surface |
| His509-Asp506 | N-terminal arm | Packs against lower surface |
| Arg585-Glu509 | Helix α7 | Salt bridges to Asp528 and Arg543 in α1; part of salt-bridge network |
| Arg511 | N-terminal arm | Salt bridges to Asp608 and Glu700 |
| Cys509 | Disulfide bridge | At base of domain; mutation destroys disulfide bridge |
Salt Bridges and Buried Charges—

The vWF-A1 domain contains a large number of charged amino acids. Some of these form an elaborate salt-bridge network that wraps around the periphery. In integrin I domains, this upper face contains two positively charged residues, Arg524 and His656, packed together at the center of an otherwise polar but charged surface (~20 Å in diameter, with charged groups at the periphery. In integrin I domains, this upper face contains the MIDAS motif, consisting of three closely apposed loops that together coordinate a magnesium ion. However, the homologous loops of vWF-A1 are not suitable for metal coordination.

(c) The "lower" face includes the N- and C-terminal arms and the loops connecting the α-helices to the N-terminal end of the β-sheet. (d) The "right-hand" face includes helices α4, α5, and α6 flanking one side of the β-sheet, and is highly acidic. (e) The "left-hand" face (not shown) includes helices α1, α3, and α7 flanking the opposite side of the β-sheet and is highly basic. (f) The "back" face (not shown) includes helices α6, α7, and strand βF.

Salt Bridges and Buried Charges—

The vWF-A1 domain contains a large number of charged amino acids. Some of these form an elaborate salt-bridge network that wraps around the lower rim of the domain (Fig. 4a). These stabilizing salt bridges may explain the sensitivity of this region to alanine mutagenesis (23). There are four buried acidic residues: Asp514 at the bottom of strand βA and an arginine from the α4-βD turn is seen in all of the Aβ domain structures that have been studied, suggesting that this is an important element of folding; Asp292 is buried below the upper face of the domain without a stabilizing salt-bridge partner. This residue is homologous in sequence to the aspartic acid of the DxSxS integrin MIDAS motif; it has been suggested that the side chain of Arg224 might form a salt bridge with the buried aspartic acid, substituting for the metal ion, but our crystal structure shows that instead Arg224 points outward into solution, packing against the side chain of His656 from the βE-α6 loop.

N- and C-terminal Arms and von Willebrand Disease Type IIB Mutations—

Upstream from the N-terminal strand, βA, the chain makes a 90° turn, with Cys506 three residues from the turning point. Further upstream, the side chains of Phe507, Tyr508, and Ile499 pack against hydrophobic elements on the surface of the domain, and His505 and Glu501 make stabilizing salt bridges with helices α1 and α3. At the C terminus, the α7 helix extends a turn beyond the disulfide bridge, followed by an extended structure that packs against the hydrophobic side of the domain as far as Ala701; in addition, Glu700 makes a salt bridge with Arg711 near the N terminus. Beyond this, a sequence of three prolines in an extended conformation protrudes downward from the body of the domain.

Ordered electron density exists for all residues that have been identified as natural mutation sites leading to the type IIB phenotype ("gain of function," constitutive binding to GpIb). All of these sites lie on the lower surface of the domain at the interface between the body of the domain and the N- and C-terminal arms (see Table II and Fig. 3). Our crystal structure shows that in the wild-type protein, these residues are all involved in salt bridges or hydrophobic packing. The most likely effect of these mutations is that they disrupt the interface between the N- and C-terminal arms and the body of the.
domain. Scanning mutagenesis has led to the identification of further mutants with a similar phenotype (23). These lie in the same region and include a triple-alanine mutation in the middle of the C-terminal helix (RDE687–689), which breaks salt-bridge contacts with helix α1 and the salt-bridge network. Mutations of Cys509 to Gly or Arg, which break the disulfide bridge, also lead to constitutive GpIb binding (24).

Comparison with the vWF-A3 Domain—The central β-sheets of the A1 and A3 domains overlap very closely, with an RMS deviation on main chain atoms of 0.55 Å for 40 residues (similar comparisons with the integrin I domains also give values in the range 0.5–0.6 Å). Extending the overlap to the entire domain gives an RMS deviation of 1.4 Å for 165 residues in equivalent structural environments, as defined in MULTIFIT (25). The resulting alignment has 21% amino acid identity and is shown in Figs. 1 and 2. The A3 crystal structure lacks the N- and C-terminal extensions found in A1, but the disulfide bridge is similarly located. The α helices are generally similar in length and orientation, with the major differences restricted to the loops connecting strands and helices on the upper and lower surfaces of the domain. vWF-A1 and vWF-A3 both lack the α2 helix found in the integrin αMβ2 and αLβ2 I domains. In A1, helix α7 is preceded by a turn of 3₁₀ helix, whereas in A3, the 3₁₀ helix is replaced by an α helix that is longer by 3 residues. The surface charge distribution is less asymmetric in A3 than in A1, lacking the basic patch on the right-hand face of A1.

The shape of the upper surface of the domain is affected by three changes in the surface loops. (a) At the top of the βA strand, arginine Arg524 in A1 points out into solution, whereas the equivalent A3 residue, Ser2938, points inward. The side chain of the next residue, Leu525, points into the interior of the protein; homologous residues in the integrin I domains are similarly oriented. This contrasts with A3, in which Phe3939 points out into solution. These two differences give the βA-α1 turn a quite different conformation in A1 than in A3. (b) There is a 1-residue insertion in the α3-α4 loop of A1, which wraps over the top of the βB-βC hairpin, allowing space for the side chain of His500 that replaces a glycine in A3. (c) A1 has a 4-residue insertion in the βD-α5 loop, which includes a turn of 3₁₀ helix. The lower surface is affected by the following changes: (i) in the βC-α3 loop, Leu568 packs more closely into the hydrophobic core than the larger tryptophan in A3, causing a shift of the entire loop; (ii) the end of helix α4 has a different conformation, and the α4-βD loop is two residues shorter in A1; (iii) The α6-βF turn adopts a different conformation.

**DISCUSSION**

Using our crystal structure of the vWF-A1 domain, coupled with the analysis of naturally occurring and experimentally introduced mutations, we can begin to map the binding sites for GpIb and activators like botrocetin and ristocetin. In addition, with an understanding of the type IIb vWD “gain-of-function”
mutations in relation to the ligand-binding surfaces, we can begin to formulate models of the activation process.

**Botrocetin and Ristocetin Binding Sites**—Two groups have performed scanning mutagenesis on the A1 domain (23, 26) which can be used to help localize the binding sites for these activators. Matsushita and Sadler (23) measured the direct binding of botrocetin to vWF. Ignoring those mutations that involve buried charges and probably cause misfolding of the A1 domain, the remaining mutation sites leading to reduced (50% normal) binding are located in helices $a_5$ and $a_6$ and adjoining structures: Arg 636 in helix $a_5$, Arg 629 and Arg 632 in the 310 helix immediately preceding $a_5$, and RLIEK 663–667 in the neighboring $a_6$ helix. These data strongly suggest that botrocetin binds to the right-hand face of the domain (Figs. 3 and 4). Mutation of the lysine cluster KKKK642–645 also reduced botrocetin binding (23), but Kroner and Frey (26) report normal botrocetin-induced function for this mutant.

Matsushita and Sadler (23) reported four mutants with a selective loss-of-function: loss of ristocetin-induced GpIb binding but normal botrocetin-induced binding. These map near to or near the upper/front surface of the domain, in contrast to the natural mutant (Gly$^{561} \rightarrow$ Ser) has the same phenotype (27). Gly$^{561}$ is part of the tight $\beta B$-$\beta C$ turn at the upper front/edge of the domain. Its main chain torsion angles are not unusual, and modeling suggests that the mutant side chain can point out into solution without severe distortion of the main chain. The ability of botrocetin to overcome the binding deficiency in these mutants may arise from the stabilizing effect of its tight binding to an adjacent surface of the A1 domain. Kroner and Frey (26) reported three further mutations with the same phenotype, in the region of the type IIb mutations and the lysine cluster KKKK$^{642-645}$, adjacent to the N- and C-terminal arms. The arms contain proline-rich segments that have been implicated in ristocetin binding (28). It is therefore possible that selective loss-of-function in these mutants arises from defective ristocetin binding.

**The GpIb Binding Site**—Data from various sources, when linked together, provide some clues to the location and extent of the GpIb interaction site within the A1 domain. First, previously reported studies from our laboratory on the functional properties of a vWF-A1/A3 chimera that contains the N-terminal half of vWF-A1 (as far as Leu$^{598}$ in the middle of helix $a_4$), but still binds GpIb normally (6), strongly suggest a role for the front half of the domain. Second, the majority of mutations that lead to a selective loss-of-function (described above) cluster at or near the upper/front surface of the domain, in contrast to the

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**Fig. 4. Four faces of the vWF-A1 domain, defined from the orientation in Fig. 3.** Atoms are shown as spheres, drawn with BALLS, RASTER3D, and RENDER (33, 34). Arginines and lysines are in blue, aspartic acids and glutamic acids are in red, and histidines are in green; all other residues are in gray. The proposed binding site for botrocetin includes the residues marked “B,” and a possible binding site for heparin is circled. a, front face (same view as Fig. 3). Sites of mutations with impaired GpIb binding (Gly$^{561}$, Glu$^{596}$, and Lys$^{599}$) are labeled. b, upper face at the C-terminal end of the $\beta$-sheet. c, lower face including the N-terminal arm. d, right-hand face, including helices $a_5$ and $a_6$. 
gain-of-function mutants, which cluster on the lower surface (see below). Third, Matsushita and Sadler reported that the double alanine mutation at Glu306 and Lys509 on the front surface (helix a4) showed reduced GpIb binding (without affecting botrocetin binding) and suggested that it might form part of the GpIb binding site. Two further natural mutations (Type IIIm) with impaired GpIb binding lie in helix a4 and the following loop, but both residues (Phe606 and Arg611) are buried in our crystal structure and the mutations probably lead to destabilization of the folded interface. Overall, the data point to a role for the upper/front surfaces of the domain in GpIb binding, but further work is clearly required to confirm this location.

Type IIb Mutations and Regulation of GpIb Binding—The vWD type IIb mutations, which lead to constitutive binding of vWF to GpIb, are all located at or near the interface between the lower surface of the domain and the N- and C-terminal arms (Fig. 3), and the mutations are expected to break salt bridges and hydrophobic contacts that stabilize this interface. Because these are so numerous, are “gain-of-function,” and map to both sides of the interface, it is very unlikely that this region forms a binding surface for GpIb; rather, it is more likely to be involved in the regulation of binding affinity. The separation between this region and the most likely GpIb binding surface begs the question of how structural changes are communicated between the two sites.

A possible clue comes from studies on the homologous integrin I domain. Whereas ligand binding sites have been shown to lie on the upper surface of the domain, Zhang and Plow (29) have made αM/αL I domains chimeras, in which the swapping of sequences in the lower surface of the domain (at sites homologous to the type IIb mutations in vWF-A1) leads to constitutive high affinity ligand binding. A plausible pathway for communicating structural changes from one face of the I domain to the opposite face is provided by the two crystal structures of the integrin αM I domain observed by Lee et al. (30). In these structures, a change in the shape and charge distribution of the upper ligand-binding face that would influence its affinity for ligand is propagated via a large (10 Å) downward shift of the C-terminal helix, α7, to the lower face of the domain. The more extended conformation of the domain was identified with the high affinity state.

Although we have no evidence for such a conformational change in the vWF-A1 domain, we note that several type IIb mutations map either to the C-terminal helix or to a residue that salt-bridges to the helix (Table II). We expect that these mutations disrupt salt bridges and hydrophobic interactions that lock the helix against the body of the domain. Although the N and C termini remain linked by a disulfide bridge in type IIb vWD, a substantial downward shift of the C-terminal helix is theoretically possible, requiring a concerted movement of the N-terminal arm. Thus, by destabilizing the folded conformation of the terminal arms, the type IIb mutations could shift the equilibrium toward the extended conformation. A conformational switch of this kind, in which the high affinity state is identified with a more extended conformation of the A1 domain, could be triggered when high flow/shear unfolds immobilized vWF (31).

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