Comparative Analysis of Type 2b von Willebrand Disease Mutations: Implications for the Mechanism of von Willebrand Factor Binding to Platelets

By Kathleen A. Cooney and David Ginsburg

von Willebrand factor (vWF) is a multimeric glycoprotein that forms an adhesive link following vascular injury between the vessel wall and its primary ligand on the platelet surface, glycoprotein Ib (GpIb). Type 2b von Willebrand disease (vWD) is a qualitative form of vWD resulting from enhanced binding of vWF to platelets. Molecular characterization of the vWF gene in patients with type 2b vWD has resulted in identification of a panel of mutations associated with this disorder, all clustered within the GpIb binding domain in exon 28 of the vWF gene. We have expressed six of the most common type 2b vWD mutations in recombinant vWF and show that each mutation produces a similar increase in vWF binding to platelets in the absence or presence of ristocetin. Furthermore, expression of more than one type 2b vWD mutation in the same molecule (cis) or in different molecules within the same multimer (trans) failed to produce an increase in vWF platelet binding compared with any of the individually expressed mutations. Taken together, these data support the hypothesis that the vWF GpIb binding domain can adopt either a discrete "on" or "off" conformation, with most type 2b vWD mutations resulting in vWF locked in the on conformation. This model may have relevance to other adhesive proteins containing type A domains.

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THE MULTIMERIC coagulation protein, von Willebrand factor (vWF), is synthesized in megakaryocytes and endothelial cells as a 2,813-amino acid prepropeptide that undergoes a complex series of posttranslational modifications before storage or secretion. The vWF protein dimerizes in the endoplasmic reticulum via formation of C-terminal disulfide bonds. Multimerization occurs by N-terminal disulfide bonds as the protein transits the Golgi and post-Golgi compartments, and the multimeric protein is directed toward specialized storage granules: the a-granule in the platelet and the Weibel-Palade body in the endothelial cell. vWF is secreted into the plasma and circulates as a spectrum of multimers, some as large as 20,000 kD (for review, see Ruggeri et al.1).

The primary receptor for the interaction of vWF with the platelet is glycoprotein Ib (GpIb). The GpIb binding domain is contained within a vWF tryptic fragment extending from Val449 to Lys728.2 A disulfide bond between Cys509 and Cys695 forms a loop structure3,4 that has been shown to be critical to the platelet-binding function of vWF.5,6 vWF does not spontaneously bind to platelet GpIb in the circulation. It is hypothesized that vWF undergoes a conformational change in vivo that promotes binding to platelet GpIb as a result of an initiating event, such as the binding of vWF to collagen in the subendothelium. Alternatively, conformational changes in platelet GpIb, such as those resulting from GpIb mutations associated with pseudo-von Willebrand disease, may also lead to enhanced vWF binding.

The vWF-platelet interaction has been studied in vitro by the use of various pharmacologic agents to promote binding. Ristocetin,8 a positively charged antibiotic, and botrocin,9 a protein isolated from venom obtained from the South American pit viper, Bothrops jararaca, both promote binding of vWF to GpIb in a dose-dependent fashion. Asialo-vWF10 and various vWF proteolytic fragments11,12 also bind spontaneously to platelet GpIb and have been used as tools to further analyze this interaction.

von Willebrand disease (vWD) is a common bleeding disorder due to either quantitative (types 1 and 3) or qualitative (type 2) defects in vWF.13 Type 2b vWD is characterized by a relative decrease in plasma high-molecular-weight vWF multimers, thrombocytopenia, and enhanced ristocetin-induced platelet aggregation in vitro. We and others14,15 have identified a number of missense mutations associated with this disorder, all localized within a segment of vWF exon 28 containing the GpIb binding domain. Functional analysis has shown that each of these mutations, when expressed in recombinant vWF, results in an increase in both spontaneous and ristocetin-induced platelet binding.16-23 Even though four of the common mutations result in loss of a basic residue (vWFH505D, vWFR543W, vWFR545C, and vWFR578Q), no unifying molecular mechanism has been proposed to account for the increased platelet binding caused by these mutations. In this report, we analyze six individual type 2b vWD mutations using the same assay conditions. Although these mutations result in varying degrees of spontaneous platelet binding, nearly identical enhanced platelet binding is observed in the presence of ristocetin. Moreover, introduction of more than one 2b vWD mutation into the same vWF molecule fails to further enhance the platelet binding function of vWF. Based on these observations, we propose a model of vWF function in which there is an abrupt switch between distinct "on" and "off" conformations of the GpIb binding domain.

MATERIALS AND METHODS

Expression vector construction. Individual and multiple type 2b vWD mutations were inserted into full-length vWF cDNA in the eukaryotic expression vector, pMT2, using the commercial pSEL-ECT system (Promega Corp, Madison, WI). To simplify plasmid...
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>vWF</th>
<th>Codon</th>
<th>Oligonucleotides</th>
<th>Sequence/ (reverse complement)</th>
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The first two columns list plasmid and recombinant vWF names, respectively, for each type 2b vWD mutant construct. Amino acids are numbered relative to the mature vWF protein. Also included in the column labeled “codon” is the numbering system recommended by the International Society on Thrombosis and Haemostasis, in which the first methionine in the signal peptide is codon 1. The fourth and fifth columns list the name and sequence of oligonucleotides used to create the constructs. The sequence of each oligonucleotide is written 5'-3' on the (+) strand, and the substituted nucleotide is underlined.

construction, a cloning cassette (pvWFsal) was created by inserting unique SalI sites at positions 4151 and 4705 (using the numbering system recommended by the Subcommittee on vWF of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis)25. These novel SalI sites were created by subcloning a BamH1-KpnI vWF fragment (nucleotides 2967 to 3002) into pGEM and using the polymerase chain reaction (PCR) to alter the third nucleotide in codons 1301 and 1485. The substitutions (underlined) were contained within the PCR primers: primer A, GAAATGTCAAGGCTCTGGTGTGCACATG (+) strand nucleotides 4130-4159; primer B, GGCGCCGCGGCTGCCACGC-CGGCCGCGGCTGCCACGC (-) strand nucleotides 4720-4694. Neither substitution changed the predicted amino acid sequence. The BamH1-KpnI fragment was subcloned in several steps in full-length vWF in pMT2. To derive the mutant plasmids, vWF was amplified from genomic DNA obtained from a normal individual using primers A and B, containing the SalI sites. The PCR product was digested with SalI and cloned into the vector pALTER. Site-directed mutagenesis was performed according to manufacturer’s instructions, and the resulting fragments were inserted into pvWFsal. The plasmids and corresponding mutagenesis oligonucleotides are depicted in Table 1. All fragments derived by PCR or mutagenesis were sequenced to confirm the desired substitutions and exclude polymerase errors. The corresponding mutagenesis oligonucleotides are depicted in Table 1. The secreted forms of vWF were secreted in the absence of platelets to determine the amount of non-specific precipitation of vWF by ristocetin. A dose-dependent precipitation of vWF by ristocetin was observed with each rvWF, varying from 13% at 0.25 mg/mL to a maximum of 25% at 1 mg/mL. This value was subtracted from the amount of total precipitation in each experiment such that the data shown represent specific binding of vWF to platelets.

**Multimer analysis.** Multimer analysis of recombinant vWF was performed by vertical agarose electrophoresis, capillary transfer to poly(vinylidene difluoride) membranes (Millipore Corp, Bedford, MA), and visualization with a peroxidase-conjugated rabbit anti-human vWF antibody (Dakopatts, Carpenteria, CA) and chemiluminescence (Amersham Corp, Arlington Heights, IL) as previously described.17

**RESULTS**

**Comparative analysis of individual type 2b vWD mutations.** To determine the relative severity of individual type 2b vWD missense mutations, six previously identified mutations were inserted into full-length vWF in the eukaryotic expression vector, pMT2, and expressed in COS-7 cells. Analysis of platelet binding function for recombinant vWFs containing each of the previously reported type 2b vWD mutations, vWF543W, vWF545C, vWF578Q, and vWF578Q, is shown in Fig 1A. These substitutions represent the four most common type 2b mutations, which account for greater than 80% of reported cases.15 Although small differences in spontaneous platelet binding are observed, the binding of each of these recombinant mutant proteins in the presence of doses of ristocetin up to 1 mg/mL is nearly identical and distinctly greater than that of vWFWT. In our assay system, the maximal amount of vWF binding that can be induced with or without the agonist ristocetin is approximately 60% and 70% of the total. This is consistent with the observations of other investigators using similar assays.16,20,27 Values above 100% are probably due to an increment of vWF background associated with the addition of formalin-fixed platelets (data not shown).

Two different mutations have been reported at the same nucleotide position: vWVF551L in a family with typical type 2b vWD18 and vWVF551F in a patient with clinical type 2a vWD.23 Expression of recombinant vWF containing each of these individual mutations was performed, and mutant vWFs

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were compared in a platelet binding assay (Fig 1B). Substitution of leucine or phenylalanine for valine at position 551 results in nearly equivalent spontaneous binding of vWF to platelets. Binding to platelets in the presence of low-dose ristocetin is also similar. These binding curves are also nearly identical to those shown for the four most common type 2b vWD mutations (Figs 1A).

**Expression of two type 2b vWD mutations in cis and trans.** The next set of experiments was designed to test the hypothesis that vWF containing two type 2b vWD mutations would demonstrate an additive or synergistic gain of function. Mutant vWF molecules containing two type 2b vWD mutations (cis) were created and compared with vWF multimers obtained by cotransfection of two single-mutant plasmids in which each monomer in a given multimer should contain either one or the other type 2b vWD mutation (trans). In addition, homogeneous single-mutant multimers were combined to make a mixture in which each subunit of a given multimer was obtained following cotransfection with equal amounts of "cis" and "trans" vWF multimers. This construct closely resembles mixed multimers. Unfortunately, the exact composition of mixed multimers cannot be readily determined. The three classes of double-mutant recombinant vWFs were subjected to the platelet binding assay (Fig 2B) described earlier. At each dose of ristocetin, vWF containing two type 2b vWD mutations (cis) demonstrated less platelet binding compared with either mutation expressed individually, in trans, or in mixed multimers.

Recombinant vWF containing three mutations in cis is poorly secreted from COS-7 cells. A third construct containing three type 2b vWD mutations (vWFR543W/R545C/R578Q) was assembled to determine the effects of multiple type 2b vWD mutations expressed in cis. This construct failed to produce significant quantities of secreted recombinant vWF (<110 ng/100-mm plate, approximately 3 times less than other constructs) when transfected into COS-7 cells. Similar results were obtained with transfection of this plasmid into COS-1 cells or A293T cells (data not shown). Furthermore, multimer analysis of secreted vWF from COS-7 cells following transfection with pWFR543W/R545C/R578Q (cis) revealed a loss of high-molecular-weight multimers compared with vWFT or vWF obtained following cotransfection with equal amounts of pWFR543W, pWFR545C, and pWFR578Q (trans) (Figs 3A, lane 6). vWF containing type 2b vWD mutations vWFR543W, vWFR545C, and vWFR578Q in cis fails to bind platelet GPIb. vWFR543W/R545C/R578Q (cis) was subsequently analyzed in a platelet binding assay and compared with the same three mutations expressed in a cotransfection (trans) and obtained from mixing pure multimer populations (mixed). vWFR543W/R545C/R578Q failed to bind to platelets either spontaneously or in the presence of ristocetin (up to 1 mg/mL; Fig 4). By comparison, both trans and mixed multimers bound equally well to platelets in the absence or presence of ristocetin.

**DISCUSSION**

Eleven distinct mutations in the vWF gene (10 missense mutations and one methionine insertion) have been associated with type 2b vWD, and a number of these mutations have been verified by functional analysis using recombinant vWF. These reports suggest that type 2b vWD mutations result in varying degrees of spontaneous vWF binding to platelets but similar enhanced platelet binding in the presence of ristocetin. However, direct comparison of the effects of these mutations is limited, due to the different assay systems used by various laboratories to measure vWF binding to platelets. In this report, we demonstrate that six individual
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**A**

Two mutations studied here represent different amino acid substitutions at position 551. The vWFV551L mutation was originally reported by Donnér et al.\(^1\) in a patient classified as type 2b vWD. As predicted, recombinant vWF containing this mutation demonstrates spontaneous binding to platelets and enhanced binding to platelets in the presence of low-dose ristocetin compared with vWFWT (Fig 1B). The vWFV551F mutation was previously reported by Ribba et al.\(^2\) in a patient with phenotypic characteristics consistent with type 2a vWD. However, the patient was noted to be thrombocytopenic during episodes of bleeding. Recombinant vWFV551F was shown by these investigators to have spontaneous platelet binding but no increased binding in the presence of ristocetin. Our data analyzing vWFV551F confirm spontaneous binding to platelets as shown by Ribba et al., and we have also demonstrated enhanced platelet binding in the presence of low-dose ristocetin. Taken together, substitution of either leucine or phenylalanine for valine at position 551 results in recombinant proteins with nearly identical platelet binding properties, yet the patients in whom these mutations were initially reported have distinct clinical phenotypes. Recently, Piao et al.\(^3\) reported three unrelated type 2b vWD patients with the same R578G mutation but different degrees of thrombocytopenia and clinical bleeding. Therefore, it is likely that other genetic and nongenetic factors significantly modulate phenotypic expression of these mutations in vivo.

Many studies have been performed using proteolytic frag-
The repetitive A domains, first recognized in vWF, have subsequently been identified in a number of other proteins involved in hemostasis, cell adhesion, immunity, and development of the extracellular matrix. In immunity, and development of the extracellular matrix, the loop structure is critical for the adhesive functions of vWF with GpIb. They further showed that two terminal regions of mature vWF (aa 1 to 272) and Arg578. These latter data suggest that the specific type 2b amino acid substitutions at these two positions may be responsible for the associated gain of function, rather than the loss of charge. Alternatively, the additional alanine substitutions present in cis in these scanning mutant proteins may have suppressed the effect of alanine substitutions at Arg543 and Arg578.

Fig 4. Analysis of three type 2b vWD mutations in cis, trans, and mixed multimers. Platelet binding assays were performed in triplicate, and each point represents the mean ± SD (if ±5%) of these 3 experiments. vWFRS43W/R646C/R578G (cis) was obtained from transfection of 2 independently derived vWF expression vectors containing all 3 mutations and is shown as two separate points (— x — and — ■ —).

These previous observations and our current data support a model for vWF function in which the vWF GpIb binding domain contains within the A1 repeat can adopt one of two distinct conformations: "on" and "off". In this model, native vWF in the circulation is predominantly in the off conformation and is thus not associated with the platelet surface. It is hypothesized that following vessel injury, the appropriate signal, perhaps binding to collagen or another subendothelial ligand under shear conditions, may switch the A1 domain into the on conformation, leading to GpIb binding and formation of a platelet plug. Type 2b vWD mutations may all result in a shift of the vWF GpIb binding domain into this on conformation, accounting for the similar increase in binding observed with all six mutations and the lack of an additive or synergistic effect between mutations. Alterations in surface charge in asialo-vWF and interaction of vWF with ristocetin and possibly also botrocetin may result in a similar switch of the vWF A1 domain into the on conformation. Tornai et al recently described a murine monoclonal antibody, IC1E7, that increases ristocetin-induced platelet aggregation. This antibody maps to the aminoterminal region of mature vWF (aa 1 to 272). Thus, there appear to be a variety of distinct stimuli that can modulate the conformational change and act as the "physiologic switch" that allows vWF to interact with platelet GpIb. Matsushita and Sadler recently proposed a similar model in which specific segments of the vWF A1 domain cooperate to inhibit GpIb binding. They suggest that specific mutations and modulators exert their effects on vWF binding to GpIb by relieving this inhibition.

This two-conformation model may also have relevance to several integrins and other proteins that contain type A modules with adhesive functions. It has been shown that interactions between Mac-1 and complement component iC3b, as well as VLA-1 and type VI collagen, are dependent on divalent cations. The crystal structure of the A domain from the α-subunit of integrin CR3 (CD11b/CD18) has recently been solved, and appears to contain an exposed Mg²⁺ coordination site. Although the interaction of vWF and platelet GpIb is not known to be dependent on divalent cations, the effect of the positively charged antibiotic ristocetin may be operating through a homologous mechanism.
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REFERENCES


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