Functional analysis of a type IIB von Willebrand disease missense mutation: Increased binding of large von Willebrand factor multimers to platelets

glycoprotein Ib/ristocetin/endothelial cells/recombinant protein

KATHLEEN A. COONEY*, SUSAN E. LYONS†, and DAVID GINSBURG‡§

Departments of *Internal Medicine and †Human Genetics, ‡Program of Cellular and Molecular Biology, and §Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI 48109-0650

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ABSTRACT Type IIB von Willebrand disease is an autosomal dominant bleeding disorder characterized by the selective loss of high molecular weight von Willebrand factor (vWF) multimers in plasma, presumably due to their abnormally increased reactivity with platelets. We and others have recently identified a panel of missense mutations clustered in the platelet glycoprotein Ib binding domain of vWF from patients with type IIB von Willebrand disease. We now report functional analysis of one of the most frequent type IIB missense mutations, Arg-543 → Trp (vWF R543W). vWF from a human umbilical vein endothelial cell culture heterozygous for the vWF R543W mutation showed markedly increased binding of large vWF multimers to platelets in the presence of a low dose of ristocetin compared to vWF from a normal control culture. Recombinant vWF containing the vWF R543W mutation expressed in COS-7 cells also demonstrated increased binding of large vWF multimers. Mixed multimers obtained by cotransfection of mutant and wild-type cDNAs showed partial dominance of the vWF R543W mutation. Thus these data demonstrate that the vWF R543W mutation alone is sufficient to confer increased binding of large vWF multimers to platelets in a dominant fashion and that no other factors relating to vWF posttranslational processing or secretion in endothelial cells are required for this effect.

von Willebrand factor (vWF) is a multimeric glycoprotein that has several critical roles in hemostasis. In addition to carrying factor VIII in the circulation, vWF binds platelets to the subendothelium at the site of vascular injury to initiate formation of a hemostatic plug. vWF is synthesized in endothelial cells and megakaryocytes as a pre-pro-polypeptide of 2813 amino acids and, subsequently, undergoes extensive posttranslational modifications, including propeptide cleavage, glycosylation, and multimerization. Relatively small vWF multimers are secreted constitutively into the circulation from endothelial cells, whereas the entire spectrum of multimers is stored and released from Weibel–Palade bodies. In plasma, vWF circulates as multimers ranging in size from 400 to 20,000 kDa at a concentration of 5–10 μg/ml (1, 2).

Within the 2050-amino acid mature vWF protein, discrete functional domains have been localized through the study of vWF proteolytic fragments and monoclonal antibodies. Fujimura et al. (3) have demonstrated that the portion of vWF responsible for binding to platelet glycoprotein Ib (GpIb) is contained within the tryptic fragment extending from Val-449 to Lys-728 in the mature protein. This same tryptic fragment also contains binding sites for heparin and collagen (4–6).

don Willebrand disease (vWD) is an autosomally inherited bleeding disorder resulting from defects in vWF. Qualitative abnormalities in vWF have been associated with the type II variants of vWD (7). In contrast to the other forms of vWD, patients with type IIB typically present with a variable degree of thrombocytopenia in addition to a moderately severe bleeding disorder (8, 9). Laboratory evaluation of type IIB patients commonly reveals disproportionately low ristocetin cofactor activities compared to vWF antigen levels in addition to enhanced ristocetin-induced platelet aggregation. Ristocetin is a positively charged antibiotic that induces binding of vWF to platelet GpIb. The highest molecular weight vWF multimers are notably absent from the plasma of patients with type IIB vWD, although the full spectrum of multimers is present in platelets (10). Type IIB vWD must be distinguished from platelet-type vWD (or pseudo-vWD), an intrinsic platelet defect that presents with a nearly identical clinical phenotype. A potential mutation in the GpIb α chain gene has recently been reported in a family with this disorder (11). In contrast, the defect in type IIB vWD resides within the vWF molecule itself. Plasma high molecular weight vWF multimers of type IIB vWD patients are hypothesized to bind spontaneously to platelets, with subsequent clearance from the circulation (12) accounting for the characteristic multimer pattern and thrombocytopenia. In support of this hypothesis, De Marco and coworkers (13) have demonstrated that vWF purified from a patient with type IIB vWD can induce spontaneous platelet aggregation not seen with normal vWF. This spontaneous platelet aggregation was blocked by a monoclonal antibody against platelet GpIb. Furthermore, vWF secreted from type IIB endothelial cells was shown to contain a full range of vWF multimers and to bind spontaneously to platelets (15).

We recently reported the identification of four missense mutations occurring within the GpIb binding domain of patients with type IIB vWD (16). The demonstration of one of these four mutations in each of 14 patients with type IIB vWD from 11 unrelated families, the absence of each of these single nucleotide changes from a large panel of normal DNA samples, and the observation that one of these mutations (vWF R543W) has occurred on at least two distinct genetic backgrounds provided compelling genetic evidence that these mutations are responsible for the type IIB vWD phenotype. Three of the four mutations were also identified in an independent panel of patients by Randi et al. (17). Recently, two additional type IIB vWD mutations have also been described including a methionine insertion at amino acid 540

Abbreviations: vWD, von Willebrand disease; vWF, von Willebrand factor; GpIb, glycoprotein Ib; HUVEC, human umbilical vein endothelial cell.

*To whom reprint requests should be addressed at: Howard Hughes Medical Institute, 1150 W. Medical Center Dr., 4520 Medical Science Research Building I, Ann Arbor, MI 48109-0650.

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We now report functional analysis of vWF containing the vWF R543W substitution, one of the most common type IIB mutations accounting for approximately one-third of type IIB vWD families. Analysis of vWF containing the R543W substitution obtained from a human umbilical vein endothelial cell (HUVEC) culture and recombinant material expressed in COS-7 cells demonstrates that the single nucleotide substitution resulting in R543W is sufficient to cause the increased binding of large vWF multimers to platelets characteristic of type IIB vWD.

METHODS

Expression of Recombinant vWF. The polymerase chain reaction (PCR) was used to amplify exons 28 sequences from patient B1 (16) using primers A and C as described (16, 20). The Pst I-Bal I fragment (nucleotides 4029–4368 with the adenine of the initiator ATG codon numbered as +1) was inserted into a series of ligation steps into full-length vWF in the eukaryotic expression vector pMT2 (20–22). The entire Pst I-Bal I PCR-derived fragment was sequenced to ensure insertion of the mutation and to exclude PCR errors. Transfection of 20 μg of plasmid DNA into COS-7 cells was performed as described (21, 22). Forty hours after transfection, cells were washed twice in phosphate-buffered saline and cultured in 3 ml of serum-free Dulbecco’s modified Eagle’s medium (JRH Biosciences, Kansas City, MO) containing 0.5% bovine serum albumin, 1% ITS growth supplement (Collaborative Research) in a 100-mm plate. Medium was collected after 24 h and EDTA and phenylmethylsulfonyl fluoride were added to final concentrations of 5 mM and 2 mM, respectively.

HUVEC Culture. A HUVEC culture was established from an umbilical cord (23) obtained at birth from patient B5. This HUVEC culture was shown to be heterozygous for the vWF R543W mutation (16). For preparation of conditioned medium, cells were grown for 24 h in M199 medium (GIBCO) supplemented with heparin (0.1 mg/ml), 0.1% bovine serum albumin, and endothelial cell growth supplement (Collaborative Research; 0.1 mg/ml). Medium was collected and EDTA/phenylmethylsulfonyl fluoride was added as above.

Platelet Binding Assay. The amount of vWF in the cell medium was quantified by a sandwich ELISA as described (21). Values were normalized to a standard curve generated from purified vWF (kindly provided by P. Bockenstedt, University of Michigan Medical School). For platelet binding, 15 ng of vWF in conditioned medium was incubated with and without formalin-fixed platelets (BioData, Hatboro, PA, 1 × 10^7 platelets per 100 μl of incubation mixture) in the presence or absence of ristocetin in Tris-buffered saline (TBS; 140 mM NaCl/10 mM Tris-HCl, pH 7.5). After a 30-min incubation without stirring, the mixture was layered over a 20% (wt/vol) sucrose cushion and centrifuged for 4 min at 12,000 × g. Quantification of the vWF present before and the vWF left unbound after platelet incubation was performed by ELISA as above.

Multimer Analysis. Conditioned medium was incubated with and without platelets and ristocetin, and the platelets were pelleted through a sucrose gradient as described above. vWF remaining in the supernatant was analyzed by vertical nondenaturing agarose gel electrophoresis with minor modifications of the procedure of Raines et al. (24). After electrophoresis and capillary transfer of material to poly(vinylidene difluoride) membranes (Millipore), membranes were blocked in TN/Blotto [25 mM Tris-HCl, pH 7.5/0.5 M NaCl/5% (wt/vol) Carnation instant milk] for 1 h, incubated with a 1:500 dilution of peroxidase-conjugated rabbit anti-human vWF antibody (Dakopatts, Glostrup, Denmark) in TN/Blotto, and then washed three times in TNT (25 mM Tris-HCl, pH 7.5/0.5 M NaCl/0.1% Triton X-100). Blots were developed using a commercially available kit for chemiluminescence (Amersham) (25) and exposed to Kodak XAR film for 5 sec to 20 min.

RESULTS

To study the effect of the R543W mutation on vWF function, mutant vWF from HUVECs that had been demonstrated to be heterozygous for the R543W mutation (16) was obtained and compared to vWF from normal HUVECs. As shown in Fig. 1A, vWF from the IIB HUVECs demonstrates markedly increased binding to platelets, notably in the presence of low concentrations of ristocetin (0.1–0.3 mg/ml). In addition, a small amount of spontaneous binding (10%) could be detected. The binding of both mutant and wild-type vWF could be partially blocked by preincubation with the anti-platelet GpIb antibody 6D1 (data not shown).

The multimeric composition of mutant HUVEC vWF bound to platelets was subsequently analyzed by nondenaturing agarose gel electrophoresis. No significant binding of wild-type vWF to platelets was detected in the absence of ristocetin or in the presence of ristocetin at 0.25 mg/ml (Fig. 2A). However, in the presence of ristocetin at 1 mg/ml, most of the large wild-type vWF multimers were cleared from the supernatant. The low level of spontaneous binding of IIB HUVEC vWF to platelets seen in the more-sensitive ELISA assay was not detected by multimer analysis (Fig. 2B).

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Effect of vWF R543W on vWF-platelet binding. Platelet binding assays were performed using vWF from wild-type (WT) and IIB HUVEC lines (A) and vWF from COS-7 cells transfected with wild-type (vWF WT), mutant (vWF R543W), or both (vWF R543W:WT) vWF cDNAs (B). The amount of unbound vWF remaining in the supernatant after incubation with platelets (Pits) in the presence or absence of ristocetin is expressed as a percentage of a control incubation in the absence of platelets and ristocetin. In A data (average and standard deviation) were derived from transfections of at least two independently derived DNA constructs and in B data were from two parallel cell cultures. Points without error bars represent standard deviations of <2%. Approximately 20 pg of vWF was detected from the formalin-fixed platelets alone (data not shown).
However, in the presence of low concentrations (0.25 mg/ml) of ristocetin, there was a significant decrease in the amount of large mutant HUVEC vWF multimers remaining in the supernatant after platelet incubation compared to the normal control. With ristocetin at 1 mg/ml, nearly all of the mutant vWF was cleared from the supernatant.

To determine whether the R543W substitution alone was sufficient to account for the observed increased platelet binding of mutant HUVEC vWF, recombinant vWF containing the R543W substitution (vWF R543W) was expressed by transient transfection of COS-7 cells and compared to wild-type vWF. vWF R543W exhibited slight spontaneous platelet binding and markedly increased platelet binding in the presence of low concentrations of ristocetin. Additionally, vWF obtained by a cotransfection of a 1:1 mixture of vWF R543W and wild-type vWF DNA constructs showed enhanced platelet binding in the presence of a low dose of ristocetin that was intermediate to that of wild-type and pure mutant vWF. No vWF was detected in medium from COS-7 cells transfected with the control pMT2 vector alone and ristocetin in the absence of platelets did not result in clearance of vWF from the supernatant (data not shown).

Multimer analysis of nonreduced recombinant vWF containing the R543W mutation also showed no spontaneous platelet binding (Fig. 3). However, there was a selective loss of the high molecular weight multimers after incubation with platelets in the presence of low concentrations of ristocetin (0.25 mg/ml). No such change was evident with the recombinant wild-type vWF control under the same conditions.

**DISCUSSION**

Type IIB is distinguished from other forms of vWF by enhanced ristocetin-induced platelet aggregation and often by thrombocytopenia. Granlinsk et al. (27) have described four patients exhibiting a type IIB vWD phenotype with the additional feature of spontaneous platelet aggregation. Analysis of purified vWF from one of the four patients revealed an ~50% reduction in the sialic acid and galactose concentration (27). Asialo-vWF had been shown (28, 29) to cause spontaneous platelet aggregation and it was hypothesized that carbohydrate moieties may be important in the pathogenesis of type IIB vWD. De Marco et al. (30) subsequently purified vWF from three additional patients/families with type IIB vWD and found that all three had normal sialic acid content. They further reported that the spontaneous platelet aggregation induced by type IIB vWF could be blocked by an antibody against platelet GpIIb or by a 52/48-kDa tryptic fragment from normal vWF containing the GpIIb binding domain.

Recognition of the importance of the vWF GpIIb binding domain in type IIB vWD directed the search for potential mutations to this region. The putative GpIIb binding domain is contained within the tryptic vWF fragment extending from Val-449 to Lys-728 (3). Mohri et al. (31) demonstrated that two synthetic peptides from Lys-474 to Pro-488 and from Leu-694 to Pro-708 partially inhibited the binding of native vWF to platelets, and they hypothesized that the intrachain disulfide bond between Cys-509 and Cys-695 (32) would bring these two remote amino acid segments into close proximity forming part of the GpIIb binding site. The six reported type IIB vWD mutations are all tightly clustered within a 40-amino acid segment in the disulfide loop from Cys-509 to Cys-695 (16–19). The absence of any N- or O-linked glycosylation sites within this region (33) further suggests that glycosylation does not play a major role in the pathogenesis of type IIB vWD. In addition to GpIIb binding, the segment of vWF forming the disulfide loop and containing the type IIB vWD mutations has also been demonstrated to be important for collagen binding (5, 6, 34).

Ware et al. (18) have reported functional analysis of the type IIB mutation vWF W550C. A recombinant vWF protein fragment spanning residues 441–733 and containing this mutation blocked the binding of an anti-GpIIb antibody to platelets in the absence of ristocetin, whereas the wild-type fragment showed no effect. Although intact multimeric vWF was not examined, this observation suggests that the W550C mutation accounts for spontaneous platelet binding in the type IIB vWD patient.

The studies reported here demonstrate that the single amino acid substitution vWF R543W is sufficient to confer increased binding of large vWF multimers to platelet GpIIb resulting in the type IIB vWD phenotype. The enhanced platelet binding of recombinant vWF is remarkably similar to vWF prepared from the HUVEC line containing the same vWF R543W mutation. Thus these data demonstrate that the vWF R543W mutation alone is sufficient to produce the specific type IIB "gain-of-function" phenotype and that no
other factors relating to vWF posttranslational processing or secretion in endothelial cells are required for this effect.

DeMarco et al. (14) showed that type IIB vWF purified from patient plasma bound to platelets with greater affinity than normal vWF despite absent high molecular weight multimers in the former. Our data demonstrate that this increased affinity is most marked for high vWF multimers. This could be due to a simple additive effect of the multiple platelet binding sites present in these large multimers or possible cooperativity between individual binding sites. These observations are consistent with previous studies of fractionated normal plasma vWF multimers (35, 36).

Cotransfection studies using a 1:1 molar mixture of mutant and wild-type DNA demonstrate that vWF R543W is partially dominant over wild-type vWF in vitro. This is consistent with the apparent autosomal dominant inheritance of most cases of type IIB vWD and also suggests that the homozygous state might result in a more severe clinical phenotype. Such homozygosity for type IIB vWD has not yet been described and could potentially be lethal. There has been one report of a family with two daughters with type IIB vWD from two phenotypically normal parents (37). It is possible that the genetic abnormality in this family is autosomal recessive, with each parent having a subclinical type IIB vWD defect that is only evident in the homozygous state. Alternatively, this could represent germline mosaicism as demonstrated in another type IIB vWD family (38).

Human diseases due to quantitative and/or qualitative loss of a functional protein can arise from multiple distinct molecular defects including point mutations and gene deletions. In the best studied example, the hemoglobinopathies, more than 200 mutations have been reported in the 146-amino acid human β-globin chain (39). However, the unique sickle cell anemia phenotype is always due to the same nucleotide substitution in codon 6, although this mutation appears to have arisen at least four times. By analogy, the common quantitative type I and III vWD phenotypes might be expected to occur through a large number of distinct molecular defects, whereas only a limited number of missense mutations may have the potential to disrupt the unique "gain-of-function" of type IIB vWD. Consistent with this hypothesis, a panel of four missense mutations accounts for >90% of type IIB vWD patients (16–19) and several of these mutations have occurred by independent mutational events (16, 17). The apparent small number of potential type IIB vWD mutations should facilitate the development of highly sensitive and specific DNA-based diagnosis and classification for this disorder.

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