Variable content of von Willebrand factor mutant monomer drives the phenotypic variability in a family with von Willebrand disease

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KEY POINTS

1. Von Willebrand disease is characterized by variable expressivity, even within families with the same *VWF* mutation

2. The content of mutant monomers in the final multimeric structure may explain the observed variability

ABSTRACT

Von Willebrand disease (VWD) is an inherited bleeding disorder characterized by incomplete penetrance and variable expressivity. We evaluated a 24-member pedigree with VWD type 2 caused by a T>G mutation at position 3911 that predicts a methionine to arginine (M1304R) change in the platelet-binding A1 domain of von Willebrand factor (VWF). This mutation manifests as an autosomal-dominant trait, with clinical and biochemical phenotypic variability among affected individuals, including differences in bleeding tendency and VWF quantity, activity, and multimer pattern. Sequencing of all *VWF* coding regions in three affected individuals did not identify additional mutations. When expressed in heterologous cells, the M1304R was secreted in lower quantities, failed to drive formation of storage granules, and was defective in multimerization and platelet binding. When co-transfected in equal quantities with the wild-type cDNA, the mutant cDNA depressed VWF secretion, although multimerization was only mildly affected. A llama nanobody (AU/VWFa-11) that detects the mutant A1 domain demonstrated highly variable binding to VWF from different affected members, indicating that the VWF contained different percentages of mutant monomers in different individuals. Thus, the observed variability in VWD phenotypes could in part be determined by the extent of mutant monomer incorporation in the final multimer structure of plasma VWF.

INTRODUCTION

Von Willebrand disease (VWD) is the most common congenital bleeding disorder of humans and is caused by quantitative or qualitative defects in von Willebrand factor (VWF), a large plasma glycoprotein required for effective platelet adhesion at sites of blood vessel injury. The *VWF* gene spans approximately 178 kb and maps to chromosome 12p13.3.[1-3] Exons 1-17 encode the signal peptide and propeptide and exons 18-52 encode the mature VWF polypeptide and 3′ untranslated region. A pseudogene with homology to exons 23-34 of *VWF* maps to chromosome 22 and contains several nonsense and splice-site mutations, suggesting that it is unable to generate functional transcripts.[4,5]

VWF is synthesized by endothelial cells and megakaryocytes and either secreted constitutively or stored in endothelial Weibel-Palade bodies and platelet α-granules, from which it is released during activation.[6-8] During synthesis, VWF monomers first homodimerize and undergo several posttranslational modifications in the endoplasmic reticulum, then form large multimers through polymerization in the *trans* Golgi, with mature multimers ranging in mass from 500 to 20,000 kDa.[9,10]

The current clinical classification of VWD characterizes mild and severe quantitative defects of VWF as VWD types 1 and 3, respectively, whereas qualitative defects of VWF are classified as VWD type 2.[11] VWD type 2 can be the result of abnormal multimerization, impaired secretion of high-molecular-weight multimers, increased ADAMTS13 proteolysis after secretion, enhanced or decreased interaction of VWF with the platelet receptor glycoprotein (GP) Ib-IX-V, or defective binding to collagen, or coagulation factor VIII.[12]

The complexity of VWD is underscored by the variability observed in both the clinical phenotype (mucocutaneous bleeding) and the biochemical phenotype (represented by VWF levels and

activity). Although considered a monogenic disease, VWD is characterized by incomplete penetrance and variable expressivity, even in families with a single causative *VWF* mutation.[13,14]

We have identified a 24-member family with 11 individuals affected with VWD caused by a mutation in exon 28 of the *VWF* gene. Among the affected members there was considerable phenotypic variability: in bleeding manifestations, VWF levels, and VWF activity but most remarkably in multimer distribution. We thoroughly investigated the potential genetic and molecular mechanisms involved in this variability and demonstrated variable content of the mutant subunit in plasma VWF among affected individuals as a potential explanation.

MATERIALS AND METHODS

Participants. Twenty-four members of a single European-American multigenerational pedigree (11 VWD patients and 13 control relatives) were recruited. The study received institutional review board approval from the University of Iowa and the University of Colorado Anschutz Medical Campus, and informed consent was obtained for all participants, in accordance with the Declaration of Helsinki. Further details regarding diagnosis and treatment of this family can be found in Supplemental Data.

VWF analysis. Assays to examine VWF antigen, multimers, collagen, nanobody AU/VWFa-11 and platelet binding, VWF propeptide antigen, factor VIII coagulant activity, ADAMTS13 activity, recombinant VWF expression and VWF-storage granules in mammalian cells are described in the Supplemental Data.

Bleeding scores. To determine the bleeding phenotypes of pedigree members, we employed two different bleedings scores. One is a modified bleeding questionnaire that was originally designed and validated by the Epidemiology Branch of the Centers for Disease Control and Prevention (CDC) to screen women with VWD (Supplemental Data).[15] The other one is the current ISTH/SSC bleeding score that was administered to seven of the eleven affected members.[16]

Isolation of genomic DNA and *VWF* sequencing. Genomic DNA was isolated from whole blood using a commercially available kit (Qiagen). Because VWF ristocetin cofactor (VWF:RCo) and VWF antigen (VWF:Ag) in several pedigree members suggested the diagnosis of VWD type 2, the sequencing strategy for *VWF* focused on exon 28, which harbors the majority of type 2 mutations. PCR and sequencing primers were designed to discriminate and selectively amplify *VWF* versus its corresponding pseudogene.[5] PCR was performed on genomic DNA by standard methods, and Sanger sequencing was performed using BigDye V3.1 on an ABI3730xl

sequencer (Life Technologies). The sequencing data were analyzed using Sequencher V4.9 software (Gene Codes Corporation). To rule out the presence of additional mutations in the VWF gene, we sequenced all exons and introns for *VWF* in three affected members and one control relative at the Partners Healthcare Center for Genetics and Genomics (Harvard Medical School, Boston, MA).

ABO Genotyping. ABO genotyping was performed by amplifying and sequencing exon 6 and part of exon 7 of the *ABO* gene using primer pairs 1 and 3 as described by Mizuno et al.[17]

Molecular Modeling. The crystal structure of the wild-type VWF A1 (PDB: 1AUQ) was used as the starting point for the molecular dynamics (MD) simulations. The mutant A1 structure was obtained by analogy by replacing the side chain of methionine with that of arginine at position 1304. Coordinates for the mutated side chain were constructed and minimized with 100 steps of steepest descent using the program CHARMM.[18] The MD simulations were performed with the program NAMD[19] using the CHARMM all-hydrogen force field (PARAM22)[18] and the TIP3P model of water. The details are described in Supplemental Data.

Allele-specific mRNA assay. RNA was isolated from platelets of the studied family members and cDNA was synthesized from 200 ng of RNA for each individual. Standard locus-specific PCR using *VWF* exon 28 primers (5-GAGCCCCACCACTCTGTATG-3 and 5-TGCCCGCATACTTCACCT-3) was performed (Details in Supplemental Data). Ratios of allele-specific mRNA were calculated as previously described.[20]

RESULTS

Pedigree of a family with von Willebrand disease

The family we studied contains 24 members from 3 generations, with 11 individuals carrying the diagnosis of VWD based on low VWF levels (Figure 1A). The disease was transmitted in autosomal-dominant fashion. The affected individuals exhibited low VWF antigen levels (Figure 1B), low ratios of ristocetin cofactor to VWF antigen (≤ 0.7), and decreased VWF binding to recombinant GPIbα and collagens type III and VI (Figures 1F–1I). All but two affected individuals had ratios of VWF propeptide to VWF antigen lower than 3, indicating normal VWF clearance (Figure 1E). All affected individuals had decreased factor VIII activity (Figure 1C) but normal ADAMTS13 activity (Figure 1D). Demographic parameters and VWF measurements are compiled in Supplemental Table S1.

Phenotypic variability among affected pedigree members

The bleeding scores, obtained using two clinical bleeding assessment tools, differed significantly among affected family members (Supplemental Table S1). VWF multimer patterns also varied significantly among affected family members with some individuals having severely reduced quantities of high-molecular-weight multimers, whereas others exhibited multimer patterns almost indistinguishable from those of the unaffected members (Figure 2B). The multimer patterns remained similar over time for most of the affected members (Supplemental Figure S4) and no increases in satellite bands were detected on a high-resolution gel indicating that the loss of high-molecular-weight multimers is not due to excessive ADAMTS13 cleavage (Supplemental Figure S5). The distribution of multimers did not correlate with the bleeding scores (Figure 2). We then measured the binding of nanobody AU/VWFa-11 to plasma VWF from the affected individuals. The nanobody detects an epitope within the A1 domain exposed when the A1 domain is decrypted or in a platelet-binding conformation.[21,22] In addition, the

nanobody binds at elevated levels to type 2B[19] and type 2M VWF.[23] We found that AU/VWFa-11 binds at elevated levels to VWF from 9 patients. Nanobody binding was heterogeneous among the different family members and did not correlate with the bleeding scores (Figure 2A).

VWD in this family is caused by a mutation (M1304R) in VWF

Sequencing of exon 28 revealed a T>G substitution at position 3911 of the *VWF* gene that predicts a change of methionine to arginine at position 1304 (M1304R) in the A1 domain of VWF. The mutation segregates with the VWD phenotype (defined by VWF:RCo < 20 U/dL, Supplemental Table S1) in all affected members and is not present in the unaffected individuals. Complete sequencing of *VWF* in three affected individuals in the pedigree (individuals I.1, III.4 and III.6) revealed 5 sequence variations in addition to the M1304R mutation (Supplemental Table S2). All are previously reported single nucleotide polymorphisms (SNP). Interestingly, individuals I.1 and III.4 were heterozygous for the D1472H variant in exon 28 that has been associated with lower values for VWF:RCo. This finding is of no clinical significance as it is due to an artifact related to the assay. All exonic non-synonymous SNPs found in these individuals were then genotyped in all members of the pedigree. All but two affected individuals (III.6 and III.7) were heterozygotes for the D1472H variant. None of the remaining variants segregated with the disease phenotype. ABO blood group status, a known modifier of VWF levels, did not significantly influence levels in individuals affected by the mutation (Supplemental Table S1). Although the putative causative mutation, M1304R, has not been previously reported, mutations in the same region are known to cause type 2B VWD.[24]

Molecular modeling of the structural consequences of the M1304R mutation

Within the 3-dimensional crystal structure of the A1 domain, Met1304 side chain is surrounded by multiple hydrophobic residues (Figure 3A). Substitution of methionine with arginine introduces a larger and positively charged side chain into a tightly packed hydrophobic

environment that may destabilize the folding of the A1 domain. We simulated the effect of the M1304R mutation on the A1 structure using molecular dynamics (MD). We first replaced the methionine 1304 side chain with that of arginine to create a mutant A1 structure, and then performed MD simulations at room temperature for 50-ns on the structures of both the wild-type and mutant A1. Analysis of the simulation trajectories showed that the backbones (Cα) of the residues around position 1304 are more flexible in the mutant A1 than in the wild type (Figure 3B, shaded area). When examining the time course of the structural changes, we found that the α-helix that contains residue 1304 in the mutant A1 domain deviates more from the starting configuration than does the wild-type A1 domain (represented by increased CαRMSD in Figure 3C). These results indicate that the arginine substitution at position 1304 kinetically destabilizes the A1 structure. Consistent with this, using the method of free energy perturbation, we estimated that the folding free energy of the mutant is less favorable than the wild type by 36 kcal/mol (Supplemental Figure S1), suggesting that mutant A1 is also thermodynamically less stable than the wild type. This conformational change may affect both the interactions of the VWF A1 domain with platelet GPIbα and nanobody AU/VWFa-11 binding.

Interestingly, the substitution of Met1304 with valine, a hydrophobic residue with a smaller side chain than methionine, results in gain of platelet-binding function, and defines a VWD type 2B phenotype (http://www.vwf.group.shef.ac.uk). To compare the differences caused by Val or Arg substitution at position 1304, we applied the same free energy perturbation protocol to examine the changes in stability of the A1 domain caused by M1304V mutation. We found that M1304V also reduces the stability of the A1 domain, albeit to a much smaller degree, i.e., 5 kcal/mol (see Supplemental Data under free energy perturbation calculations). This is consistent with previous equilibrium refolding experiments showing that type 2B mutations are associated with a slight destabilization of the A1 domain.[25] Thus, it is plausible that M1304V destabilizes the A1 domain in the vicinity of the mutated site enough to allow it to bind platelets, while M1304R drastically

destabilizes the A1 domain, causing it to unfold or misfold, and resulting in loss of function and complete abrogation of platelet binding.

Biosynthetic defect associated with the VWF M1304R mutation

To assess the impact of the M1304R mutation on VWF synthesis, we transfected VWF cDNAs encoding either wild type or M1304R mutant VWF into human embryonic kidney cells (HEK293) and examined VWF secretion, granule storage, multimeric structure, and platelet-binding functions. The cells transfected with mutant VWF had a markedly reduced concentration of VWF in the cell supernatant compared to those transfected with the wild-type cDNA (17% of wild type; $P < 0.001$), but both cells had similar quantities of VWF in the cell lysates (Figure 4A). The secreted mutant VWF consisted primarily of low-molecular weight multimers (Figure 5B, lane 1), whereas the wild-type contained the full range of multimer sizes (Figure 5B, lane 5). The mutant VWF did not bind platelets in the presence of ristocetin or botrocetin (Figures 5D and 5E, when %DNA of mutant is 100%).

Cells expressing the mutant displayed a diffuse fine granular VWF staining pattern with occasional very small round granules (Figure 4B); in contrast, cells expressing wild-type VWF showed well-defined cigar-shaped structures characteristic of Weibel-Palade bodies (Figure 4C). These data show that the M1304R mutation causes defects in intracellular VWF processing, such as secretion, multimerization, and granule storage, as well as defective platelet binding.

Increased incorporation of wild-type monomers improves VWF synthesis and functions

To determine the impact of different levels of mutant incorporation on VWF synthesis and function, we co-transfected HEK293T cells with the mutant and wild-type VWF cDNAs in different ratios. As the percentage of wild-type cDNA increased, the amount of VWF secreted and the multimer size of the secreted VWF also increased (Figures 5A and 5B). Because the

wild-type VWF has a myc-His tag not present in the mutant, we were able to differentially detect the two forms of VWF on multimer gels (Figure 5C). When expressed alone, the M1304 mutant was secreted only as small multimers (Figure 5C, M1304R: lane 1). However, in the presence of just 25% wild-type cDNA, the average multimer size of the secreted VWF markedly increased (Figure 5C, M1304R: lane 2). Both wild-type and mutant VWF were readily detected in the multimers in the presence of 25% and 50% of wild-type cDNA (Figure 5C, lanes 2 and 3). Consistent with the improvement of VWF secretion and multimerization, both ristocetin- and botrocetin-induced VWF binding to platelets increased as the percentage of the wild-type cDNA increased (Figures 5D–5E). These findings indicate that the degree of wild-type monomer incorporation into VWF multimers not only impacts intracellular VWF processing but also its ability to bind platelets.

The phenotypic variability observed in the affected family members is not due to a hypomorphic wild-type *VWF* allele

The results above show that VWF secretion, multimerization, and functions were affected by the extent of incorporation of mutant and wild-type monomers into the final VWF multimers. Therefore, it is possible that phenotypic heterogeneities among the affected individuals are caused by different expression levels of mutant or normal *VWF* alleles. It is now recognized from large studies of families with VWD that in addition to characterized VWD mutations, several polymorphisms modify VWF levels and may modify VWF activity.[13,26-28] We therefore measured the levels of mutant and normal VWF mRNA in affected and unaffected members of the pedigree to investigate the potential contribution of a hypomorphic normal allele to the observed phenotypic variability. The levels of the normal mRNA were similar among the affected individuals carrying the M1304R variant, ranging from 73% to 78% of total VWF mRNA, ruling out a contribution of a hypomorphic allele (Supplemental Figure S2).

DISCUSSION

We have characterized the molecular pathogenesis and clinical and biochemical phenotypes of VWD in a large family in which the disease is caused by a mutation that converts Met1304 to Arg within the VWF A1 domain. The family was remarkable for displaying a wide phenotypic variability; we identified one cause of this variability as being due to the fact that the plasma VWF multimers of affected individuals displayed a wide range of mutant monomer content (Figure 2A).

In the current classification of VWD, different individuals in this family would be given dissimilar diagnoses, based on differences in their clinical laboratory data (Figure 2). For example, III.3 would be classified as type 1 (VWF:RCo/VWF:Ag ≥ 0.7 and normal multimer distribution), II.1, II.3, II.5, II.7, III4, and III.6 would be classified as type 2A (VWF:RCo/VWF:Ag < 0.7 and loss of high molecular weight multimers), and I.1, I.3, and III.7 would be classified as type 2M (VWF:RCo/VWF:Ag < 0.7 and normal multimer distribution). We reviewed the medical records of 5 affected individuals and found that over the last 20 years they have been described either as having type 1, types 2A or 2M based on their VWF levels and multimer distribution, suggesting that the variability we observed in our study persists over time.

Of interest, a potential diagnosis of type 2B VWD is also suggested by the location of the mutation. Type 2B VWD has been described as due to either substitution of valine or insertion of an additional methionine at position 1304, in addition to several other mutations in surrounding amino acids. In this family, type 2B VWD was ruled out by the presence of normal platelet counts in all affected individuals and lack of an enhanced response to low-dose ristocetin (data not shown). Further, recombinant M1304R VWF did not bind platelets in the presence of ristocetin or botrocetin (Figures 5D–5E), consistent with a type 2M phenotype.

Potential explanations for the phenotypic variability that are purely genetic are either based on the presence of a hypomorphic normal allele (an allele that produces reduced quantities of its protein product) in addition to the M1304R mutant or the presence of genetic variants that when associated with the mutation would either reduce or increase VWF levels. A hypomorphic *VWF* allele was recently described as a determinant of plasma VWF levels.[29] We ruled out the possibility that a hypomorphic normal allele contributed to the observed variability by demonstrating that all affected members had similar ratios of normal to mutant mRNA (Supplemental Figure S2). This effect appeared to be uniform among all affected individuals. We also looked for other genetic variants that might contribute to the phenotypic variability by sequencing the entire coding regions and exon/intron boundaries of *VWF* in three affected individuals. We then sequenced the identified variants in every member of the pedigree. None of the sequence variants were found in all the affected members, although the D1472H variant occurred in all but two. This variant was reported by Flood et al.[30] to be associated with lower VWF:RCo levels, a consequence of defective binding of ristocetin to VWF in the VWF:RCo assay but not associated with increased bleeding. In this family, however, this variant does not affect VWF:RCo in the affected or unaffected individuals. Based on these results, it is highly unlikely that the observed variability results from a combination of the rare M1304R mutation and common genetic variants in *VWF*.

Another possible explanation for the observed variability in laboratory and clinical phenotypes is that the composition of plasma VWF varies between individuals. The plasma VWF from the affected members displayed a wide range of binding of AU/VWFa-11 (Figure 2A), which preferentially recognizes the mutant monomers and whose binding is not affected by VWF multimer size (Supplemental Figure S3). This indicates that plasma VWF from different affected members varies in its quantity of mutant monomers. This difference could account for differences observed in the functional assays. This is supported by our *in vitro* studies showing

that when expressed alone, the mutant VWF was poorly secreted (Figure 4A) and inadequately multimerized (Figure 5B, lane 1), and did not bind to platelets (%DNA of mutant = 100% in Figures 5D–5E). Each of these parameters improved as the percentage of wild-type monomer incorporated into the multimers increased (Figure 5).

Variability in mutant content may have its origins in the early stages of VWF synthesis, one scenario being depicted in Supplemental Figure S6. Heterozygous cells produce both normal and mutant monomers, the latter in lower quantities. The monomers can form three types of dimers, normal–normal, normal–mutant, and mutant–mutant, and these dimers are transported to the Golgi to form multimers. During this process, the protein quality control (QC) system in the ER monitors the quality of newly synthesized VWF monomers or dimers and removes misfolded ones.[31] Some of the misfolded proteins are transported to the Golgi, where they are retained or degraded. Our data support a mechanism in which the mutant-containing dimers and multimers have a higher probability of being recognized as misfolded and removed by the QC system. This mechanism would account for the marked reduction in the secretion of the mutant VWF when it is expressed alone (Figure 4A).

In the family we studied, the variability of mutant content in plasma VWF among affected individuals could be related to genetically determined variations in the QC system that result in more or less efficient removal of mutant monomers and dimers during VWF synthesis. In addition, the extent to which the mutant-containing multimers are cleared by the QC system could also vary among individuals.

Therefore, there are several potential mechanisms that can lead to the variable multimer pattern and nanobody binding observed in the family described here. An individual could have an efficient protein QC system that removes most of the mutant-containing VWF monomers and dimers, yielding plasma VWF made mostly of normal monomers with a final multimeric

distribution of low molecular weight forms. These multimers will exhibit low nanobody binding because they contain few mutant monomers. In the current clinical laboratory classification this individual would be classified as having VWD type 2A. In this family, this particular situation is best represented by patients II.3 and II.5, both of whom were diagnosed with VWD type 2A based on clinical laboratory data (Figure 2). Conversely, some individuals may have a less efficient QC system, allowing for mutant monomers and dimers to be incorporated into the multimers. This will result in plasma VWF that contains different amount of mutant monomers and dimers leading to variable multimer sizes and nanobody binding. When the multimer pattern is normal, the individuals would be classified as having VWD type 2M or VWD type 1. In both scenarios, mutant monomers in plasma VWF would be detected by increased nanobody binding. Examples of this mechanism would be represented by all the affected members of this family except for patients II.3 and II.5 in Figure 2.

In summary, our findings suggest that phenotypic variability in one family afflicted with VWD results from variable incorporation of mutant monomers into VWF multimers. The mutant content of an individual's plasma VWF will determine the bleeding and multimer phenotype of that individual's VWD and how the disease is classified. In this family, different individuals carrying the same mutations could be classified as type 1, type 2A, or type 2M. Cellular modifiers such as molecular chaperones in the ER and Golgi that play a role on VWF biosynthesis and trafficking may explain the variable mutant incorporation observed in this family.

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AUTHORSHIP

Contributions: J.C. designed and performed experiments, analyzed data and co-wrote the manuscript; J.D.H designed and performed experiments, analyzed data, and co-wrote the manuscript; S.H. and R.M. analyzed data and edited the manuscript; P.J., V.H.F. and R.W. performed experiments; G.I. designed and performed experiments, analyzed data, and edit the manuscript; D.W.C. designed experiments, interpreted data, and edited the manuscript; J.A.L. and J.D.P. directed the project, designed experiments and interpreted data, and co-wrote the manuscript.

DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1: Pedigree and biochemical analysis. A. *A three-generation pedigree with VWD.* Shaded symbols represent affected individuals diagnosed with VWD based on the levels of VWF antigen and ristocetin cofactor activities. Compared to the non-affected family members, the affected individuals on average had a decreased level of VWF in plasma (**B**)**,** a lower ratio of ristocetin cofactor activity to VWF antigen (**F**), decreased binding to recombinant GPIbα (**G**), collagen III (**H**), and collagen VI (**I**), and decreased FVIII coagulation activity (**C**), but a slightly higher ratio of VWF propeptide to VWF antigen (**E**), and similar ADAMTS13 activity (**D**). The bottom and top of the boxes represent the first and third quartiles, respectively, of the values, the line inside the box indicates the median, and the end of the whiskers show the range of values that lie within 1.5 fold of the interquartile range of the median.

Figure 2: Phenotypic heterogeneity among the affected family members. A. *Nanobody binding.* Binding of the AU/VWFa-11 nanobody to plasma VWF was higher in most of the affected family members than in pooled normal plasma (PNP), and binding varied considerably among the affected individuals. **B** *VWF multimers.* Multimer patterns of plasma VWF varied significantly among the affected individuals; while some samples lack both high and intermediate sized molecular weight forms, others showed normal multimer patterns. Individual III.4 was an infant and a bleeding survey was not completed for this subject. For each of the VWD patients 1 μl of plasma was loaded; for the PNP control, 0.5 μl of plasma was loaded.

Figure 3. The potential destabilizing effects on the VWF A1 domain caused by the M1304R mutation. A. *Location of Met1304 in the crystal structure of the wild-type A1.* The side chain of Met1304 is surrounded by hydrophobic residues in the wild-type VWF A1 structure. **B.** *Flexibility of the mutant (M1304R) and wild-type A1 structures in molecular dynamics (MD) simulations.* Root-mean-square fluctuations (RMSF) of Cα atoms averaged over the last 40 ns

of the simulations (total 50 ns) are shown. The backbone α carbons (Cα) of the mutant A1 (magenta-solid line) in the simulations fluctuate more around the mean than Cα of wild-type A1 (black dashed line). The shaded area indicates the residues at or around the mutation site. **C.** *Time course of backbone deviation of the α-helix that contains residue 1304.* Root-mean-square deviations (RMSD) were monitored over time for the backbone of the α-helix comprising residues 1290 to 1305; mutant (magenta), wild-type (black). The mutant structure deviated more from the initial structure than did the wild-type, represented by increased CαRMSD.

Figure 4: Defects in secretion and pseudo Weibel-Palade body formation of recombinant M1304R VWF. A. *Expression of recombinant mutant or wild-type VWF*. HEK293T cells were transfected with cDNAs for either wild-type or mutant VWF and the concentrations of recombinant VWF were assessed in both conditioned media (supernatant) and cell lysate by ELISA. The VWF concentration in the media (supernatant) was markedly lower for the mutant than for the wild-type, although the intracellular concentrations were similar (lysate). The concentration of wild-type VWF was normalized to 100% and that of the mutant VWF was presented relative to this value. Each bar represents the mean ± standard deviation of at least three separate experiments. VWF inside the transfected cells was examined by immuno-staining and confocal microscopy (**B** and **C**). **B.** Cells transfected with M1304R VWF showed diffuse intracellular VWF staining indicating that the mutant was defective in forming storage granules. **C.** Cells transfected with wild-type VWF showed punctate staining of VWF localized to granules resembling Weibel-Palade bodies.

Figure 5. Increased expression of wild-type VWF corrects the biosynthetic and functional defects caused by the M1304R mutation. cDNAs for wild-type and mutant VWF were co-transfected at different ratios into HEK293T cells and biosynthetic and functional parameters were examined. **A.** *VWF concentration in the condition medium of transfected cells.*

As the ratio of wild-type to mutant cDNA increased, VWF concentration in the condition medium also increased. **B.** *Recombinant VWF multimer patterns.* The mutant VWF multimers lack the medium and high molecular weight multimers (lane 1). As the percentage of wild-type cDNA increased, the multimer size of the recombinant VWF also increased (lanes 2-4). **C.** *Detection of mutant and wild-type VWF in the multimers.* The recombinant M1304R VWF (no tag) is detected by antibody AVW-5. The wild-type VWF with myc-His tags was co-expressed with the mutant. The presence of the myc-His tags disrupts the AVW5 epitope and the antibody can no longer recognize the wild-type VWF, which is detected by anti-myc antibody. Mutant VWF (red) was detected in all multimer bands when coexpressed with the wild-type (green) (lanes 2 and 3 in **C**). When the mutant cDNA comprised only 25% of the transfected cDNA, the mutant protein was detected only in low molecular weight multimers (lane 4 in **C**). **D.** *Ristocetin-induced platelet binding.* **E.** *Botrocetin-induced platelet binding.* In both **D** and **E**, the M1304R mutation resulted in defective platelet binding, which tended to normalize with increasing percentage of the wild-type cDNA in co-transfection.