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Defective dimerization of von Willebrand factor subunits due to a Cys → Arg mutation in type IID von Willebrand disease

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ABSTRACT **The same heterozygous T → C transition at nt 8567 of the von Willebrand factor (vWF) transcript was found in two unrelated patients with type IID von Willebrand disease, with no other apparent abnormality. In one family, both alleles were normal in the parents and one sister; thus, the mutation originated *de novo* in the proposita. The second patient also had asymptomatic parents who, however, were not available for study. The structural consequences of the identified mutation, resulting in the Cys^{2010} → Arg substitution, were evaluated by expression of the vWF carboxyl-terminal domain containing residues 1366–2050. Insect cells infected with recombinant baculovirus expressing normal vWF sequence secreted a disulfide linked dimeric molecule with an apparent molecular mass of 150 kDa before reduction, yielding a single band of 80 kDa after disulfide bond reduction. In contrast, cells expressing the mutant fragment secreted a monomeric molecule of apparent molecular mass of 80 kDa, which remained unchanged after reduction. We conclude that Cys^{2010} is essential for normal dimerization of vWF subunits through disulfide bonding of carboxyl-terminal domains and that a heterozygous mutation in the corresponding codon is responsible for defective multimer formation in type IID von Willebrand disease.**

von Willebrand disease (1) is a genetically transmitted disorder of hemostasis characterized by quantitative or qualitative defects of von Willebrand factor (vWF), an adhesive protein essential for normal platelet function and the carrier of procoagulant factor VIII in blood (2). According to the revised classification endorsed by the Subcommittee on vWF of the International Society on Thrombosis and Hemostasis (3), which evolved from the classification previously proposed by Ruggeri and Zimmerman (1), quantitative defects are recognized as either partial (type 1) or severe (type 3), and qualitative defects (type 2) are divided into four groups with distinctive phenotypes (2A, 2B, 2M, and 2N). Type 2 von Willebrand disease includes several subtypes characterized by alterations of the vWF multimer pattern (1). In some instances (4), the recognition of missense mutations responsible for specific defects has provided fundamental insights into the complex structure–function relationships of vWF, most notably with regard to the regulation of binding to platelet glycoprotein Ib (5, 6), the interaction with factor VIII (7–9), and the stability or large multimers (10, 11).

Types IIC (12) and IID (13) of von Willebrand disease, presently considered subtypes of type 2A, are unique in that they exhibit a gross derangement of vWF structure and abnormal subunit processing after release into the circulation (14, 15), suggesting their origin in mutations that alter the biosynthesis of the molecule. In particular, the phenotype of type IID von Willebrand disease includes autosomal dominant hereditary transmission of a moderate to severe hemorrhagic diathesis, prolonged bleeding time, normal factor VIII procoagulant (FVIII:C), and vWF antigen (vWF:Ag) levels, but markedly reduced ristocetin cofactor activity (vWF:RC) due to the lack of large vWF multimers in plasma. Candidate mutations in type IIC von Willebrand disease have been identified in the propeptide region of pro-vWF (16, 17), an amino-terminal domain cleaved from the mature vWF subunit during the course of posttranslational processing and thought to be necessary for normal multimer assembly (18–20). We have now found in two unrelated patients that a heterozygous Cys^{2010} → Arg mutation in the mature vWF subunit causes the type IID von Willebrand disease phenotype. Recombinant expression of mutant vWF fragments also demonstrated that the mutation is responsible for defective disulfide bonding of C-terminal domains, thus impairing dimer formation. These results define a key structural feature for the synthesis of normal vWF multimers of large size.

MATERIALS AND METHODS

Patients. The proposita in family 1 had her first severe bleeding episodes at age 13. She also reported frequent epistaxis in early childhood. The hospital admission prompting these studies was due to heavy menorrhagia. The proposita in family 2 was originally described by Nilsson and Cronberg (21) as having an atypical form of von Willebrand disease. Easy bruising, epistaxis, severe bleeding after tooth extraction, and heavy menorrhagia were her clinical symptoms. All subjects were aware of the experimental nature of these studies and gave their informed consent according to the Declaration of Helsinki; they had been medication-free for the week preceding blood drawing.

Diagnostic Tests. The bleeding time was measured with a modified Ivy technique (22) using a simplate device (23). Plasma containing 0.011 M trisodium citrate was used for the determination of FVIII:C (24), vWF:Ag (25), vWF:RC (26), and collagen binding activity (vWF:CBA) (27). The distribution of vWF multimers was evaluated by SDS/agarose gel electrophoresis (28) with luminographic visualization (29).

Mutation Screening. In this paper, nucleotides of the vWF cDNA sequence are numbered from the major transcription cap site (+1) located 250 nt upstream of the first nucleotide in the ATG initiation codon. Amino acid residues are numbered from the amino-terminal Ser (residue 1) to the carboxyl-terminal Lys (residue 2050) in the mature vWF subunit; correspondence with residues in the pre-pro-vWF sequence is obtained by adding 763 to the residue number in the mature subunit. High molecular weight genomic DNA was prepared from leukocytes (30) and used for the amplification of vWF gene sequences by PCR (31). Exons 1–22 and 35–52 were

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Abbreviations: vWF, von Willebrand factor; FVIII:C, normal factor VIII procoagulant; vWF:Ag, vWF antigen; vWF:RC, vWF ristocetin cofactor; vWF:CBA, vWF collagen binding activity.
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amplified by using primers designed with a computer program (32); exons 23–34, with sequence homologous to the pseudogene on chromosome 22, were amplified by using the previously described primers (33). In particular, the primers used for the amplification of exons 49–52, encoding domains possibly involved in the dimerization of vWF (34, 35), had the following sequences—exon 49: 5′-ACA CTG TCT CTG ACT TTA CC-3′ (sense primer), 5′-GAG ACA GTA AAG AGG AAA GC-3′ (antisense primer); exon 50: 5′-GAG TTC GGA GCT AAA AAT TGG C-3′ (sense primer), 5′-TTG CTA ATG GGT TTC AAG GAG C-3′ (antisense primer); exon 51: 5′-ACC TAT TTC CAG CCC AGT GAG G-3′ (sense primer), 5′-CAC CCA GCC CTT ATT GAA GC-3′ (antisense primer); exon 52: 5′-AGA TCA GAC CTG CCT TGC TTG G-3′ (sense primer), 5′-ACA AGA GCA GAA CAT GCA GAG G-3′ (antisense primer). (Primer sequences for the amplification of other exons can be provided on request.) The amplified sequences, the majority of them comprising single exons, were flanked by sufficient intronic DNA to allow the detection of splice site mutations. Annealing temperatures were 54°C for exon 49, 63°C for exon 50, 63°C for exon 51, and 65°C for exon 52. PCR was performed in the Trio-Thermoblock (Biometra, Göttingen, Germany) using reagents and *Taq* polymerase from GIBCO/BRL. Double-stranded or single-stranded PCR products were analyzed by nondenaturing PAGE combined with silver staining (36). Samples with aberrant electrophoretic patterns were further analyzed by direct sequencing of single-stranded DNA obtained by asymmetric PCR. The results were confirmed first by sequencing the complementary strand and then after direct cloning of the PCR product into the vector PCR I (Invitrogen), according to the manufacturer's instructions. Sequencing was performed using the Sequenase 2.0 kit (United States Biochemical).

Construction of Baculovirus Expression Vectors. Synthesis of the carboxyl-terminal portion of the mature vWF subunit in *Spodoptera frugiperda* (Sf9) cells (Invitrogen) was directed by the recombinant baculovirus designated as JB10. The corresponding secreted protein has the amino acid sequence of the proteolytic fragment II derived from digestion of purified multimeric vWF with *Staphylococcus aureus* V8 protease (37). The plasmid pJB10 was used to produce the recombinant virus and contained the following elements in a 5′ to 3′ direction: (*i*) a consensus ribosomal binding site; (*ii*) the initiating vWF Met codon, ATG, and the remainder of the vWF signal peptide sequence; (*iii*) coding sequence for the first 3 amino acid residues of the vWF propeptide; and (*iv*) coding sequence for mature vWF residues Glu^{1366} to Lys^{2050} and the translation termination codon, TGA (38). The plasmid pJB10 was constructed as follows. The first three elements were amplified by PCR with *Xho* I (5′) and *Not* I (3′) flanking restriction sites and cloned into the plasmid pBS/KS$^-$ (Stratagene); the new plasmid was designated pKMSP. A fragment coding for residues Glu^{1366} to Lys^{2050} of the mature vWF subunit ($vWF^{1366-2050}$), with *Not* I restriction sites at each end, was amplified by PCR from a full-length vWF cDNA template and then cloned into pKMSP as a *Not* I restriction fragment; the resulting plasmid was designated pJB4. Site-directed mutagenesis (39) was used to join the codon for residue 3 of the vWF propeptide with the codon for Glu^{1366} of the mature subunit. The resulting plasmid, designated pJB6, was examined by DNA sequence analysis to confirm that the coding sequence for residues 1–25 of pre-pro-vWF (22 residues of the signal peptide and 3 residues of the propeptide) was fused to the coding sequence for residues 1366–2050 of the mature subunit.

The vWF fragment in pJB6 was removed by *Xho* I digestion of the 5′ cloning site followed by filling in of the cohesive end and digestion of the 3′ cloning site with *Not* I and cloned into the baculovirus expression vector pVL1393 (Invitrogen) which was previously digested with *Sma* I and *Not* I. The resulting plasmid was designated pJB10 (wild type).

The vector for expression of the C2010R mutation in recombinant vWF fragment II was prepared by introducing the identified candidate mutation into the plasmid pJB6 through site-directed mutagenesis using a uracil-containing template (39); the resulting mutant plasmid, confirmed by DNA sequencing, was designated pJB6E52M. The insert was removed from pJB6E52M as described above and cloned into pVL1393 previously digested with *Sma* I and *Not* I. The resulting plasmid was designated pJB12.

Generation of Recombinant Viruses and Transfection of Sf9 Cells. Large-scale preparations of the plasmids pJB10 and pJB12 were purified by CsCl-ethidium bromide gradient centrifugation. Recombinant virus for the direction of protein synthesis in Sf9 cells was produced by homologous recombination, as described (40). Three micrograms of plasmid DNA from each vector was preincubated with 1 μg of linearized *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) in the presence of cationic liposomes (Invitrogen) and the mixture was then cotransfected into Sf9 cells. After 5 days, the culture supernatants were harvested. Plaque assays were performed on dilutions of the culture supernatants, and recombinant viral plaques were identified and isolated. Isolation and purity of recombinant viruses was confirmed through PCR amplification of the inserts using primers corresponding to the flanking polylinker sites. Additional plaque purification of the recombinant viruses was done as needed.

Expression of Recombinant vWF Protein Fragments in Sf9 Cells. The recombinant viruses were amplified in serum-free cell culture media (Excell 400; JRH Biosciences, Lenexa, KS) with 0.1% penicillin–streptomycin–fungizone mixture (BioWhittaker) until a multiplicity of infection of 10 or greater was achieved. Supernatants from 7-day cultures following recombinant virus infection were collected and recombinant proteins were precipitated from the serum-free medium by addition of 60% (wt/vol) polyethylene glycol 4000 (Fluka) in 400 mM citrate buffer (pH 5.5), to yield a final concentration of 15% polyethylene glycol. After centrifugation of the supernatant/polyethylene glycol mixture (15,000 × *g*, 30 min at 22–25°C), the supernatant was decanted and the pellet dissolved in phosphate-buffered saline buffer (pH 7.4). The total protein concentration of the resuspended pellet was determined by bicinchoninic acid assay (Pierce). Prior to electrophoresis, 10 μg of protein from each dissolved pellet was boiled for 1 min in sample buffer (nonreduced) or for 10 min in sample buffer plus 100 mM dithiothreitol (reduced). A precast Tris·glycine/6% polyacrylamide gel (NOVEX, San Diego) was used for SDS/PAGE analysis at constant voltage of 125 V for 1.5 hr, followed by electrotransfer of the proteins onto a nitrocellulose membrane (41). The membrane was blocked in a solution of Blotto (42), consisting of 5% dry milk in phosphate-buffered saline (pH 7.4) with 250 μM phenylmethylsulfonyl fluoride and 0.02% Thimerosal (Sigma), for 30 min before a 2-hr incubation with murine ascites diluted 1:1000 in fresh Blotto. The ascites contained either the murine monoclonal antibody LJ-2.2.9, for analysis of nonreduced samples, or the M31 pool, for analysis of reduced samples. The former was generated against intact vWF (43) and recognizes an epitope in the carboxyl-terminal domain (Val^{1927}–Lys^{2050}) of the molecule; the latter is a mixture of monoclonal antibodies directed against cyanogen bromide fragment M31 of mature vWF (44), corresponding to residues 1481–1693 (45). After incubation with the primary antibody, the membrane was washed three times with fresh Blotto before a 1-hr incubation with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Zymed) diluted 1:2000 in Blotto. Protein bands were visualized by luminescence after application to the membrane of ECL reagent for Western blots (Amersham).

RESULTS

Diagnosis of Type IID von Willebrand Disease. The results of relevant tests in the two propositae are shown in Table 1. The bleeding time in the proposita of family 1 was >20 min (normal, <8 min); all other hemostatic parameters tested were normal, with the exception of a low vWF:Ag/vWF:RC ratio and a markedly reduced vWF:CBA. The diagnosis of type IID von Willebrand disease was unequivocal on the basis of the typical multimeric structure reported for this vWF variant (Fig. 1). Of note, both her parents had normal hemostatic parameters (Table 1) and normal vWF multimers (Fig. 1), suggesting that the modality of genetic transmission did not follow the expected autosomal dominant mode, or that a *de novo* mutation had occurred. In the proposita of family 2, the bleeding time was >30 min and both vWF:Ag and vWF:RC were decreased (Table 1). As in family 1, analysis of vWF multimers (not shown) supported the diagnosis of type IID von Willebrand disease. Both her parents had normal hemostatic parameters and no history of bleeding; however, they were not available for vWF multimer analysis and genetic studies, nor were other members of this family.

Mutation Screening and Identification of a Candidate Mutation Causing Type IID von Willebrand Disease. The search for the molecular defect in the proposita of family 1 comprised screening of the entire coding regions of the vWF gene. Only one candidate mutation was found, a T → C transition at nucleotide 8567 in exon 52, resulting in the Cys^{2010} → Arg substitution. The patient was heterozygous for this mutation while her mother, her father, and her sister only displayed wild-type sequence (Fig. 2). Biological maternity and paternity were confirmed by haplotype analysis of intragenic polymorphisms (results not shown). Additionally, father and patient shared a silent mutation (a T → C transition) only 8 bp on the 3′ side of the candidate mutation. After cloning the exon 52 PCR products from father and patient, the silent mutation and the candidate mutation were both localized on the same chromosome, indicating the *de novo* origin of the observed T8567C transition in the paternal germ line. The mutation created a new restriction site (*MspA1* I). No additional deviant sequences were detected in the remaining 51 exons of vWF, except for those associated with known polymorphisms that were transmitted according to the expected inheritance pattern. The same heterozygous mutation of residue 2010 was also detected in the proposita of the Swedish family, but not on 100 chromosomes of anonymous control persons from northern Germany.

Expression of Wild-Type and Mutant Recombinant vWF Fragment II. Synthesis and secretion of normal vWF fragment II in Sf9 insect cells was directed by the recombinant virus JB10, which was produced by homologous recombination of AcMNPV with the expression vector pJB10 (Fig. 3); synthesis and secretion of fragment II with the Cys^{2010} → Arg mutation was directed by the recombinant virus JB12, identical to JB10 except for the T → C type IID transition. Recombination of

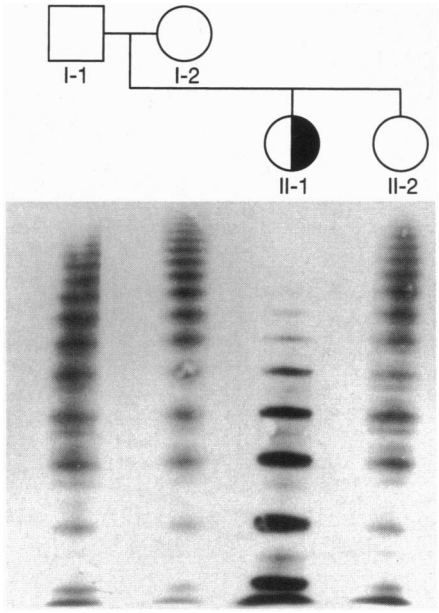

FIG. 1. Analysis of vWF multimers by SDS/agarose gel electrophoresis in the members of family 1. Visualization of vWF multimers following electrophoresis in high-resolution gels and capillary transfer onto nitrocellulose membrane was obtained with a chemiluminescent immunostaining. Note in the proposita of family 1 (patient II-1) the absence of high molecular weight multimers, the lack of normal satellite bands, and the presence of aberrant bands, all typical of subtype IID von Willebrand disease. Note also that the father (I-1), mother (I-2), and sister (II-2) of the proposita exhibit normal distribution of vWF multimers.

AcMNPV with the two expression plasmids carrying vWF sequence was efficient, yielding numerous recombinant clones that were used to transfect Sf9 cells. Recombinant protein was secreted into the serum-free cell culture medium in sufficient amounts to be extracted by polyethylene glycol precipitation. Analysis of the product of JB10 infected cells by SDS/PAGE followed by Western blotting and immunoluminescent detection with the anti-vWF monoclonal antibody LJ-2.2.9 (which reacts only with nonreduced vWF) revealed a single band of ≈150 kDa under nonreducing conditions (Fig. 4). After reduction of disulfide bonds, the product shifted to an approximate molecular mass of 80 kDa and could be detected with the monoclonal antibody pool M31 (Fig. 4). Thus, recombinant vWF fragment II is secreted by Sf9 cells as a homodimer, in agreement with the known structure of fragment II derived from proteolysis of native vWF. Moreover, in studies not reported here, we also found that expression of the fragment II cDNA in mammalian Chinese hamster ovary cells yields the same dimeric molecule obtained from insect cells, albeit at a 10- to 100-fold lower concentration, providing additional evidence that the latter can synthesize and assemble human

Table 1. Relevant laboratory parameters in the two families studied

Patient	Bleeding time, min	vWF:Ag, units/ml	vWF:RC, unit/ml	vWF:CBA, units/ml	F VIII:C, units/ml
Family 1					
I-1	5	0.78	0.76	0.64	1.30
I-2	6	1.07	0.82	1.40	0.72
II-1	>20	1.19	0.73	0.07	0.91
II-2	4	0.78	0.76	0.88	1.00
Family 2					
II-1	>30	1.25	0.32	n.d.	0.74

The upper limit for normal bleeding time is 8 min. The units used to express factor VIII and vWF-related measurements are arbitrary and referred to average normal plasma giving the result of 1 unit/ml in each assay. n.d., Not determined.

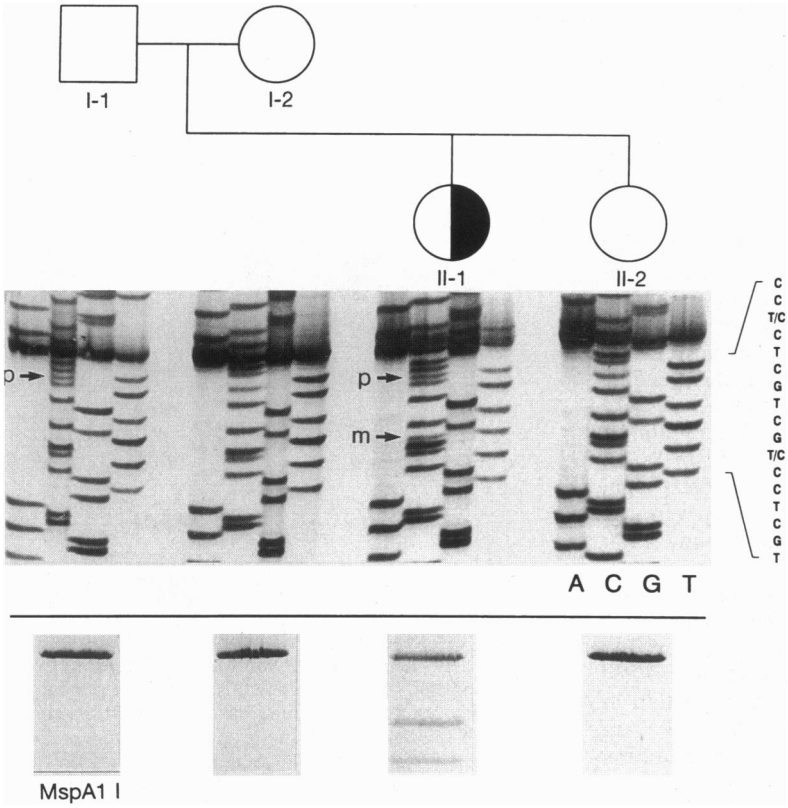

FIG 2. DNA sequence and restriction enzyme digest analysis in the members of family 1. A heterozygous T8567C transition in exon 52 of the vWF gene is apparent in the proposita (indicated by m in the lane of the sequencing gel corresponding to patient II-1) but in no other member of the family. The new *MspA1* I restriction site created by the nucleotide substitution confirms the occurrence of the heterozygous mutation only in the proposita (*Lower*). Father (I-1) and proposita also display a silent T → C transition (identified by p) 8 bp apart from the candidate mutation.

vWF correctly. In contrast to the findings obtained with the JB10 virus, which contain normal vWF sequence, cells infected with the JB12 virus secreted a protein with the same apparent molecular mass of 80 kDa both before and after reduction of disulfide bonds (Fig. 4). These results are compatible with the concept that fragment II containing the Cys^{2010} → Arg amino acid substitution is secreted as a monomer, indicating that the identified type IID mutation prevents the dimerization of vWF subunits occurring through disulfide bonding of carboxyl-terminal domains.

DISCUSSION

Our findings provide evidence that the Cys^{2010} → Arg substitution in the vWF subunit is the cause of type IID von Willebrand disease characterized by absence of the large, functionally relevant vWF multimers. The results presented here are in agreement with the dominant mode of inheritance of this phenotype (13, 46). In one of our patients, the causative heterozygous mutation was documented to have occurred *de novo* in the paternal allele. Moreover, the same mutation was found in a second unrelated patient whose parents, who were not available for definitive genetic analysis, were also asymptomatic, again suggesting the possibility of a *de novo* origin. Because type IID patients are rare, probably accounting for <1% of all cases of von Willebrand disease, the independent occurrence of the same nucleotide transition in two unrelated individuals suggests the possibility that most if not all such patients share the same molecular defect. The IID phenotype, therefore, may be strictly dependent on the abolition of an interchain disulfide bond involving Cys^{2010}, at variance with the fact that the more common von Willebrand disease IIA and IIB subtypes are caused by several distinct mutations, albeit clustered in specific domains of the vWF molecule (4).

The model proposed for the intracellular process of vWF multimer assembly envisions dimer formation as an early event occurring after removal of the signal peptide from the initial translation product and requiring interchain disulfide bond formation at the carboxyl termini of pro-VWF subunits (47). Dimerization was initially thought to be an absolute prerequisite for the subsequent formation of intersubunit disulfide bridges linking amino-terminal domains of dimers and leading to progressive growth of the polymer chain. Structural evidence obtained with direct visualization of purified vWF multimers (48) is in agreement with these concepts, but studies with recombinant truncated mutants have suggested the possibility of alternative pathways of intersubunit assembly. In fact, a vWF molecule truncated after domain D3 (with residue Gly^{478} of the mature subunit representing the carboxyl terminus) still displayed intermolecular disulfide bonding, proving that the pairing of free sulfhydryl groups between amino-terminal domains of vWF may proceed independently of disulfide bond formation between carboxyl-terminal domains (49). In agreement with this concept, it has also been demonstrated that mammalian cell expression of a recombinant vWF fragment comprising the carboxyl-terminal portion of domain D3 and the entire domain A1 (residues 441–730 of the mature subunit) leads to secretion of properly folded dimeric molecules displaying the platelet glycoprotein Ibα-binding function associated with native vWF (5, 38). Thus, experimental evidence indicates that intersubunit assembly may occur though amino terminal domains even in the absence of carboxyl-terminal disulfide bridges, a key concept to understand how mutant type IID subunits may become part of vWF multimers.

The results presented here demonstrate that Cys^{2010} is essential for the formation of intersubunit bridges in the fragment II region of native vWF (35, 37). As in the case of the D3 domain, where the substitution of any one of three closely spaced Cys residues within the sequence Cys^{459}-Gln-Ile-Cys-His-Cys^{464} results in abolition of interchain bonding (50), substitution of the single Cys^{2010} within the carboxyl terminal sequence Cys^{2008}-Ser-Cys-Cys^{2011} abrogates dimerization. Therefore, it is possible that Cys^{2010} is directly involved in an intermolecular disulfide bridge, pairing with the corresponding residue in a second subunit. Indeed, the sequence between

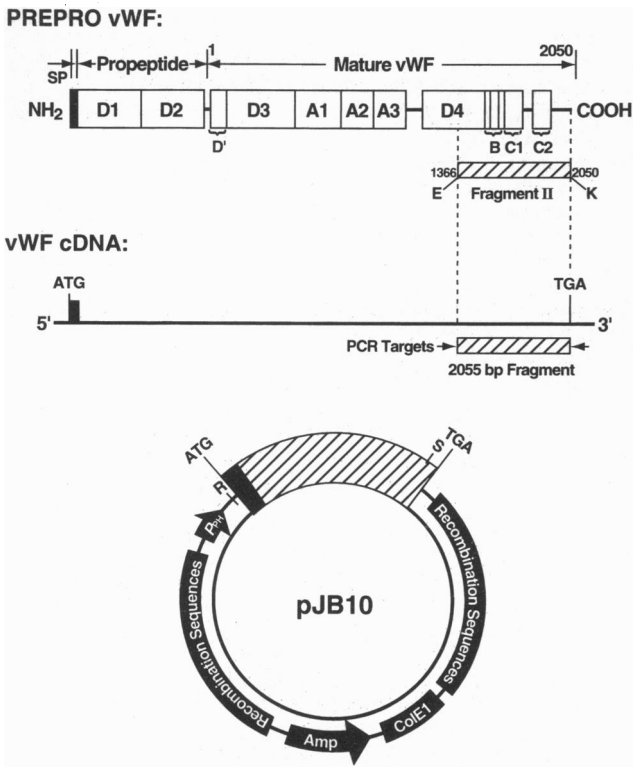

FIG. 3. Schematic representation of the structure of vWF and of the plasmid pJB10 used to express recombinant fragment II. (*Upper*) The vWF precursor (pre-pro-vWF) is organized in repeating domains as determined by linear amino acid sequence. vWF is synthesized as a 2813-residue precursor subunit composed of a 22-residue signal peptide (SP), a 741-residue pro-peptide, and a 2050-residue mature vWF subunit. Recombinant fragment II (hatched bar) contains part of the D4 domain as well as domains B1, B2, B3, C1, and C2. (*Lower*) The site of insertion of the vWF fragment II coding sequence in the expression plasmid pJB10 is shown. The expression plasmid pJB12 is identical except for the presence of the candidate mutation in the coding sequence.

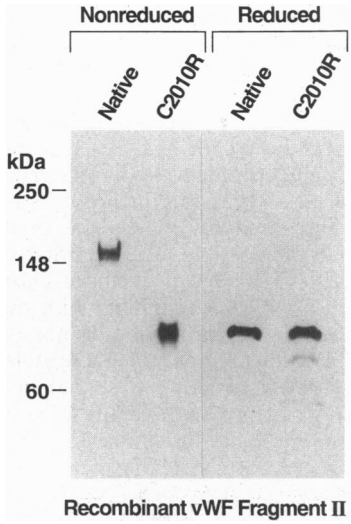

FIG. 4. Immunoblotting analysis of normal and mutant recombinant vWF fragment II. These two species secreted by infected Sf9 cells were analyzed by immunoblotting with the monoclonal antibodies LJ-2.2.9 (reacting only with nonreduced protein) and M31 pool (reacting well even after reduction of disulfide bonds). The immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG and a fixed amount of substrate, followed by autoradiography. Normal recombinant vWF fragment II is a 150-kDa protein before reduction, converted to an 80-kDa protein after disulfide bond reduction. In contrast, the corresponding mutant recombinant fragment containing the candidate mutation is a single chain structure with an apparent molecular mass of 80 kDa both before and after reduction of disulfide bonds.

residues 1927 and 2050 must contain intersubunit bonds because the carboxyl-terminal domain obtained by tryptic cleavage of multimeric vWF at Arg^{1926}-Val^{1927} is dimeric (51). Because this region contains 16 Cys residues and no free sulfhydryl groups exist in normal vWF (35, 52), the number of normal intersubunit bonds must be even to satisfy the pairing of all cysteines. The absolute requirement for Cys^{2010} in the process of dimerization may indicate that more than one intersubunit bridge is needed for the stability of dimers and/or that this specific Cys residue occupies a critical conformational position necessary for correct folding of the carboxyl-terminal domain and, in turn, influencing the subsequent process of dimerization. Of note, the correct intersubunit assembly of isolated fragment II, in addition to the previously demonstrated similar properties of amino-terminal vWF fragments (5, 38, 49), confirms that domain expression in heterologous systems is a valid approach to dissect the structure of complex parent molecules. In view of the occurrence of independent dimerization of amino- and carboxyl-terminal domains, expression of isolated fragment II rather than intact vWF subunit proved essential to demonstrate unequivocally the location of Cys residues involved in intermolecular bonding. Indeed, expression of full-length vWF with the type IID Cys → Arg mutation, either in mammalian or insect cells, would predictably still result in dimer formation due to independent disulfide bonding between amino-terminal domains (49), thus obscuring the structural significance of the Cys^{2010} residue. We also considered it unnecessary to attempt coexpression of normal and mutant vWF subunits, since the consequences of such a situation are already clearly illustrated by the modality of genetic transmission of the structural vWF abnormality in type IID von Willebrand disease patients, who are heterozygous for the Cys → Arg substitution with obvious dominant negative effects.

The finding that a single Cys → Arg heterozygous mutation can profoundly alter vWF multimer formation clarifies the molecular mechanisms of this process. It is generally assumed that the protomeric unit of vWF polymers is a dimer of mature subunits linked at the C termini, and that protomers assemble into multimers by bonding at the N termini (48); therefore, the number of subunits in a vWF polymer should always be even and the amino-terminal domain of the capping subunit at either end should not be involved in any intersubunit bridge. It follows from this model that abnormal subunits unable to dimerize at C termini should not be incorporated into vWF polymers and, thus, should not interfere with the process of multimer assembly. Moreover, even if N-terminal-linked dimers of abnormal subunits may exist, according to previous findings (5, 38, 49), their incorporation into multimers should also be prevented by the inability to form disulfide bridges through the C-terminal domain. In contrast, the dominant negative nature of the IID phenotype indicates that mutant subunits with the Cys^{2010} → Arg substitution become part of nascent polymers, impairing their assembly and structure, even in the presence of normal vWF precursor in a proportion that should not be less than 1:1. Because the Cys^{2010} → Arg mutation obliterates intermolecular bonds within the entire 685-residue length of the carboxyl-terminal fragment II region, disulfide bridges linking abnormal subunits to multimers must be located exclusively in amino-terminal domains. The derangement of vWF biosynthesis in type IID von Willebrand disease may thus be explained by assuming that mutated subunits synthesized in normal quantity can form intermolecular bonds at reactive N termini that would usually be less favored than dimerization at C termini but can, nevertheless,

lead to incorporation into forming polymers. When this happens, the presence of a dysfunctional carboxyl-terminal domain at one of the growing ends of the polymeric chain blocks further addition of protomers on that side, yielding an odd subunit number; if the same process occurs on both sides of the forming multimer, the length of the polymer becomes fixed and the subunit number remains even. This arrest can occur at any moment during the random incorporation of protomers into polymers and obviously makes the synthesis of large multimers virtually impossible. The fact that the multimeric pattern of type IID vWF typically includes aberrant bands not seen in normal (13, 46) is compatible with the existence of multimers containing an odd subunit number. Thus, the $Cys^{2010} \rightarrow Arg$ mutation provides a suitable molecular basis for the alteration of vWF structure observed in type IID von Willebrand disease, identifying a key residue involved in the process of normal multimer biosynthesis.

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