Impaired Intracellular Transport Produced by a Subset of Type IIA von Willebrand Disease Mutations*

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Type IIA von Willebrand disease (vWD) results from abnormalities in von Willebrand factor (vWF) characterized by absence of plasma high molecular weight (HMW) vWF multimers. In this report, 5 distinct point mutations were identified in 6 Type IIA vWD families. A total of 7 mutations, all clustered within a 124-amino acid segment of the vWF A2 domain, now account for 9 of a panel of 11 Type IIA families. In COS-7 cells, 3 single amino acid substitutions, Val844→Asp, Ser743→Leu, and Gly742→Arg, impaired the transport of vWF multimers between the endoplasmic reticulum and the Golgi complex, with more profound effects on the secretion of HMW multimers than lower molecular weight forms. In contrast, 2 substitutions, Arg834→Trp and Gly742→Glu, resulted in secretion of HMW multimers similar to wild-type vWF. The vWF structure observed within patient platelets correlated closely with the synthesis pattern seen for the corresponding mutants in COS-7 cells. These findings demonstrate that structural alterations within the A2 domain of vWF can produce the characteristic phenotype of Type IIA vWD via two distinct molecular mechanisms.

von Willebrand factor (vWF) is a multimeric plasma glycoprotein that plays a central role in hemostasis (1). vWF acts both as a carrier for coagulation factor VIII in the plasma and as a mediator of initial platelet adhesion to the subendothelium after vascular injury. The human vWF gene spans 178 kilobases on chromosome 12 with a partial pseudogene located on human chromosome 22 (2, 3). A number of distinct functional domains have been identified within vWF, including regions binding to Factor VIII, to the platelet receptors GpIb and GpIIb-IIIa, and to components of the extracellular matrix, such as collagen and heparin (4). Von Willebrand disease (vWD) is the most common inherited bleeding disorder in humans. Over 20 distinct vWD phenotypes have been described, all manifesting as either quantitative (Type I) or qualitative (Type II) abnormalities in plasma vWF (5).

vWF is synthesized as a 2813-amino acid prepropolypeptide in endothelial cells and megakaryocytes, where it subsequently undergoes a complex series of processing steps prior to storage or secretion. Cleavage of the signal sequence and propeptide results in a mature 2050-amino acid vWF monomer. Biosynthesis and processing of vWF has been characterized in cultured endothelial cells and includes dimerization, glycosylation, sulfation, propeptide cleavage, and multimerization to form molecular species consisting of up to 100 subunits. vWF molecules dimerize within the endoplasmic reticulum (ER) via disulfide bond formation at the carboxyterminal end. In the Golgi and post-Golgi compartments, the prosesence is cleaved and disulfide linkages are formed at the amino terminus to generate high molecular weight (HMW) multimers (6,7). The propeptide has been shown to be required for multimer assembly, although cleavage is not necessary for this function (8, 9). Wise et al. (8) have postulated that some Type II vWD variants could be due to mutations in the propeptide resulting in failure to form HMW multimers. These large polymers are thought to be most critical to the function of vWF as a mediator of platelet adhesion (1). Type IIA vWD is autosomal-dominant in inheritance and is characterized by the absence of HMW vWF multimers in plasma. There is evidence that extracellular proteolysis of vWF may be responsible for the loss of HMW multimers in a subset of Type IIA vWD patients (10–12), whereas the mechanism in others is unknown.

Several single base missense mutations potentially responsible for Type IIA vWD have been identified (13–15). In this study, we extend our analysis to an additional 8 Type IIA vWD families and report the identification of apparent missense mutations in 6. To investigate the molecular mechanism responsible for the absence of HMW multimers in Type IIA vWD plasma, we have characterized the synthesis and assembly of vWF containing each of 5 Type IIA vWD mutations, Val844→Asp(V844D), Arg834→Trp(R834W), Ser743→Leu(S743L), Gly742→Glu(G742E), and Gly742→Arg(G742R).

**EXPERIMENTAL PROCEDURES**

**Patient Material**—Nine patients with Type IIA vWD from 8 unrelated families were studied (A1–A10). A10 has been reported previously (10). Patients were classified by referring physicians as having Type IIA vWD based on a moderately severe clinical bleeding disorder, prolonged bleeding time, decreased Factor VIII procoagulant activity, decreased vWF antigen, decreased ristocetin co-factor activity, and a vWF multimer pattern consistent with Type IIA vWD.

High molecular weight DNA was prepared from patient peripheral blood samples as previously described (2).
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**PCR and DNA Sequence Analysis—**DNA PCR was performed as previously described, using allele-specific oligonucleotide primers to amplify approximately 1.3 kilobases of genomic DNA containing Exon 28 from the authentic chromosome 12 vWF gene without introducing novel restriction sites. Primer A was CTTTGACATCTTACACCCAGGCAACTGGTCT (5′-3′) and primer B was CAGCGATCAGCAAGCCGGTCT (5′-3′). The amplified products were purified single-stranded using Sequenase (U. S. Biochemicals). Single-stranded DNA template was isolated by using allele-specific oligonucleotide primers to previously described (13, 16) or directly sequenced. For the latter, asymmetric PCR was performed as previously described (18, 19) using each PCR primer alone for a second round of PCR with 1 μl of the first round product as template. Single-stranded DNA template was isolated by purification over a Qiagen column according to the manufacturer's protocol (Qiagen). DNA sequence analysis was performed on the amplified product using Sequenase (U. S. Biochemicals).

**Expression Vector Construction—**Identified Type IIA vWD mutations were inserted into full length vWF cDNA in the expression vector pMT2 (20). The expression vector containing wild-type full length vWF was E. coli Escherichia coli MT2 derived from pMT2 using restriction enzymes EcoRI and HindIII. Restriction enzyme digestion for three previously identified DNAs were first amplified by PCR and screened by digestion of PCR products confirmed the same mutation in these patients. DNA sequencing analysis was performed using the Sequenase (U. S. Biochemicals) kit.

**Cell Culture and Transfection—**COS-7 cells were maintained in Dulbecco's modified Eagle's medium (JRH Biosciences), supplemented with 10% fetal bovine serum (Gibco Laboratories). Transfections were performed as previously described (21). Cell medium and cell lysates were harvested between 48 and 60 h post-transfection. Cell medium was collected and used for determining the amount of protein secreted into the medium. Cell lysates were harvested by sonicating the cell pellets in 1.5-3 ml of Nonidet P-40 lysis buffer. Phenylmethylsulfonyl fluoride at a final concentration of 2 mM was added to the lysis buffer.

**ELISA—**VWF was quantitated by a sandwich ELISA using ELISA using 1:1000 anti-vWF (immunoglobulin fraction, Dakopatts) as the coating antibody and 1:4000 peroxidase-conjugated anti-vWF (immunoglobulin fraction, Dakopatts) as the detecting antibody, according to manufacturer's instructions. β-Galactosidase (β-Gal) was quantitated by an ELISA utilizing 1:500 anti-β-Gal antibody (Cappel) as the coating antibody and a 1:4000 dilution of anti-β-Gal conjugated to biotin, followed by 5 minutes of streptavidin-peroxidase conjugate (Boehringer Mannheim) as the detecting system. To conjugate biotin to anti-β-Gal, anti-β-Gal was incubated with d-biotinyl-l-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) at a weight/weight ratio of 1:1. IgG+biotin at room temperature for 4 h followed by dialysis against 50 mM Tris-HCl, pH 7.5. ELISAs were developed with o-phenylenediamine as the colorimetric substrate and quaternized at A540 on a Dynatech MR650 ELISA reader.

**Results—**Identification of Type IIA vWD Missense Mutations—Patient DNAs were first amplified by PCR and screened by restriction enzyme digestion for three previously identified mutations (13, 14). Three patients (A8, A9, A10) showed loss of the BstEII site associated with the A2 mutation (designated patient 2 in Ref. 13), a C→T substitution at nucleotide 4789 which results in a single amino acid substitution, Arg4425 → Trp (R3484W) (Fig. 1). Direct sequence analysis of A8, A9, and A10 PCR products confirmed the same mutation in these patients.

**Plasma and platelet samples were prepared and analyzed on non-reducing SDS-agarose gels at the Mayo Clinic, Rochester, MN. Platelet samples were first prepared from blood by modification of the method of Ref. 24. Platelet pellets were resuspended in 0.5 ml of 2.5 mM EDTA, pH 7.2, and subjected to 5 cycles of freeze, thaw, and vortex mixing, followed by the addition of 0.2 ml of 10% SDS and 0.2 ml of 20% Triton X-100 and heating to 60°C for 15 min. Plasma and platelet multiuser analysis was performed as previously described (25).
patients. A9 is the son of A8. Using a frequent neutral polymorphism, C4641T (+) and A transition at nucleotide 4514 (A3) result in substitutions of Glu and Arg, respectively, for Gly742. In the adjacent codon, a C transition at nucleotide 4517 (A3) substitutes Leu for Ser. A T→C transition at nucleotide 4619 was identified in A7, altering amino acid 777 from Leu to Pro. In an additional two patients (A5 and A11), no abnormalities in Exon 28 sequence were found. All 5 mutations were observed to be heterozygous with the normal sequence, and 149 normal alleles screened by PCR for each patient were negative. Including the three previously reported mutations (13, 14), we have now identified 7 distinct mutations. We have also determined the locations of these mutations within the vWF molecule are shown schematically in Fig. 1. All 7 mutations are clustered within a 124-amino acid segment within the A2 repeat of vWF.

**Point Mutations Have Heterogeneous Effects on Secretion of vWF**—In order to examine the biosynthesis and secretion of vWF containing the Type IIA mutations, COS cells were transfected with a vWF expression plasmid (pMT2vWF) containing each of five mutations indicated by boxes in Fig. 1. A β-galactosidase expression vector (pJ3P-Gal) was co-transfected. Cellular protein levels at only 3.5% of WT-vWF, while the S743L and G742R mutations resulted in extracellular vWF levels at 14% and 22% of wild-type, respectively. Steady state levels of intracellular vWF were elevated compared to WT-vWF for the mutant V844D, while the other mutants had levels similar to WT-vWF.

To further examine the cellular mechanism for these variable levels of secreted vWF, transfected cells were pulse-labeled, and the vWF in cell media and lysates were immunoprecipitated and analyzed by electrophoresis and autoradiography. At each time point, quantities of vWF were measured by ELISA and normalized for transfection efficiency. Average values with standard deviations from two experiments are represented here as percentages of WT-vWF.

**FIG. 1.** Schematic diagram of Type IIA vWD mutations. Primers A, B, and C (indicated by arrows) were used to amplify Exon 28 sequence without amplification of the pseudogene sequence. Exon 28, which encodes A1 and A2 vWF repeats, is depicted as a gray bar, and the surrounding intron sequence as black boxes.

**FIG. 2.** ELISA analysis of steady state vWF from COS-7 transfected cells. COS-7 cells were placed in serum-free medium 48 h post-transfection. Cell medium and lysate were harvested after an additional 24 h. The quantities of vWF in cell lysates and media were measured by ELISA and normalized for transfection efficiency. Average values with standard deviations from two experiments are represented here as percentages of WT-vWF.

**FIG. 3.** Immunoprecipitation of pulse-chase labeled samples. COS-7 cells were transfected with 15 μg of pWTvWF or 15 μg of the mutant constructs and 15 μg of pJ3β-Gal. Pulse-chase experiments were performed as described under “Materials and Methods.” Radiolabeled cell lysates and cell media samples were immunoprecipitated with polyclonal antisera against vWF and β-Gal, followed by analysis on SDS-polyacrylamide gels. A, COS-7 cells transfected with pWTvWF. B, COS-7 cells transfected with pR834W. C, COS-7 cells transfected with pV844D. β-Gal present in cell lysates was used as an internal control for transfection efficiency and immunoprecipitation. The small quantity of β-Gal detected in the cell medium at 48 and 66 h likely represents protein released from dead cells.
transfected with control vector pMT2 alone in COS-7 cells. Were treated with blotting and detection by chemiluminescence. Through 6% reducing SDS-polyacrylamide gels, followed by electrophoresis. Pulse-labeled vWF accumulated and then decreased (Figs. 3C and 4A), indicating that the mutant V844D, S743L, and G742R proteins are degraded in an intracellular compartment. vWF Secretory Mutants Are Blocked in Transport from the Endoplasmic Reticulum to the Golgi Complex—COS cells transfected with pWTvWF secreted both pro- and mature vWF (Fig. 3A) consistent with previous studies (27). Cell lysates contained a single form of vWF which appeared slightly smaller than the pro-vWF seen in cell medium and was Endo H-sensitive, indicating that it had not undergone the complete carbohydate processing that occurs in the Golgi complex (Fig. 5). vWF forms that had passed through the ER to the Golgi compartments were not detectable in cell lysates, indicating that exit of pro-vWF from the ER is the rate-limiting step in the synthetic process. Thus, after exit from the ER, vWF travels rapidly through the Golgi compartments to be secreted.

All of the mutants contained a single vWF species in cell lysates that appeared identical in size with WTvWF on reducing gels. For all mutants, intracellular vWF was observed to be Endo H-sensitive, indicating that these proteins were retained in a compartment prior to the medial Golgi. These results suggest that intracellular transport of the secretory mutants, V844D, S743L, and G742R is blocked between the ER and the Golgi complex. vWF present in the cell medium from all transfections was Endo H-resistant, indicating that it represented secreted vWF rather than the release of intracellular vWF.

Many proteins that are retained within the ER have been found to be associated with resident ER binding proteins (28). The binding protein BiP (GRP78) associates with partially assembled complexes and misfolded or incorrectly glycosylated proteins in the ER (29-31). vWF has been shown to be transiently associated with BiP (29). Therefore, mutants blocked in ER to Golgi transport were examined for binding to BiP as a mechanism for altered secretion. Cell lysates were collected after either a 15-min labeled pulse or a pulse followed by a 16-h cold chase, and immunoprecipitated with antibodies to vWF or BiP. A small amount of both wild-type and mutant vWF proteins were co-immunoprecipitated with BiP at these time points, but there was no significant difference in the quantities of BiP-associated vWF between WTvWF and any of the mutant forms (Fig. 6). Thus, an increased association with BiP does not appear to occur in conjunction with retarded transport of these mutant forms of vWF.

Severity of the Secretory Impairment Correlates with Decreased Secretion of High Molecular Weight Multimers—Synthesis of multimers was examined by analysis on nonreducing 1.5% SDS-agrose gels. In cell lysates from pulse-chase experiments, WTvWF was assembled into multimers over a 24-h chase period (data not shown). VWF in cell lysates was completely Endo H-sensitive, these data indicate that multimer assembly begins in the ER in COS cells. Analysis of intracellular steady state multimers for each mutant revealed a pattern identical with WTvWF (Fig. 7A), consisting of up to four lower molecular weight bands. Failure to observe fully assembled multimers in these constitutively secreting cells is probably due to the rapid transport of vWF to the extracellular compartment after exit from the ER.

Examination of vWF in the cell medium demonstrated that cells transfected with the vWF mutants pV844D, pS743L, and pG742R secreted decreased quantities of vWF (Fig. 7B),
while cells transfected with pR834W or pG742E secreted vWF comparable to WTvWF, consistent with the normal transport of this mutant seen in COS cells. The normal multimer patterns observed in COS cells and in platelets for R834W and G742E may be due to the absence from the transfection system of a factor(s) responsible for multimer loss and protection from this factor(s) within the platelet in vivo. Taken together, these observations suggest that the absence of plasma vWF HMW multimers associated with R834W and G742E occurs in plasma after secretion, while in the remaining mutants the defect results from aberrant intracellular transport.

**DISCUSSION**

The 7 Type IIA vWD amino acid substitutions identified to date (Fig. 1) are clustered within a small segment of the vWF molecule, but share no other obvious biochemical similarity. Three of the mutations are acidic, one basic, and three neutral. The mutations in patients A4 and A6 are in the same G742 codon, one substituting an acidic and the other a basic residue. No consistent alterations in computer-predicted secondary structure are evident. Of the seven substitutions, two represent C→T transitions at CpG dinucleotides, a proposed hot spot for mutation within the human genome (32). Significantly, one of these C→T transitions (R834W) was previously reported (10) to contain HMW multimers, also consistent with the normal transport of this mutant seen in COS cells. The normal multimer patterns observed in COS cells and in platelets for R834W and G742E may be due to the absence from the transfection system of a factor(s) responsible for multimer loss and protection from this factor(s) within the platelet in vivo. Taken together, these observations suggest that the absence of plasma vWF HMW multimers associated with R834W and G742E occurs in plasma after secretion, while in the remaining mutants the defect results from aberrant intracellular transport.
of available laboratory tests. Although it is apparent from these studies that a number of different mutations can give rise to Type IIA vWD, the tight clustering of mutations, as well as the occurrence of at least one of these mutations in multiple patients may eventually permit precise DNA-based diagnosis and classification of this disorder.

Although Type IIA vWD has generally been defined as a single subtype of vWD characterized by loss of plasma vWF HMW multimers, analysis of mutant vWF biosynthesis reveals significant heterogeneity. Interestingly, in 1983, Weiss and co-workers (24) proposed three subgroups of Type IIA vWD based on platelet vWF multimer pattern. Our data now provide a molecular basis for these early observations. We thus propose a reclassification of Type IIA vWD into two groups, each characterized by a distinct molecular mechanism. In Group I, single amino acid substitutions result in defects in intracellular transport and decreased vWF secretion. Three of the five Type IIA vWD mutations reported here were transport-defective, resulting in reduced secretion, most pronounced in the HMW versus low molecular weight vWF multimer species. In Group II, synthesis and transport are indistinguishable from WTvWF, and the loss of plasma HMW vWF multimers may result from extracellular proteolysis after secretion. No obvious structural motifs define each group or appear to differentiate the two groups. Group I mutations can result from either acidic (V844D), basic (G742R), or neutral (S743L) substitutions. Of note, the Group II mutation G742E occurs in the same codon as the Group I defect G742R and immediately adjacent to a second Group I mutation, S743L.

The Group II mutations, R834W and G742E, were both transported and secreted normally from COS cells. In addition, platelet lysates from patients with these mutations contained intact HMW multimers. Interestingly, endothelial cells from a Type IIA vWD patient produced and secreted the full range of multimers, but larger multimers were unstable in culture medium (33). Additionally, in some Type IIA vWD patients, collection of Type IIA vWD blood directly into protease inhibitors has been reported to preserve large multimers (10, 11, 34). The first patient reported to have this type of defect (10) (A10 in this study) was found to have the previously identified R834W mutation (13). This same mutation has been observed in at least three unrelated families on two different chromosomal backgrounds, indicating that it has arisen at least twice independently. Taken together, these data strongly suggest that this single amino acid change is an authentic Type IIA vWD mutation and that extracellular proteolysis is responsible for the loss of vWF multimers in these patients. The proteolytic cleavage site generating the 176-kDa carboxyl-terminal vWF fragment associated with some Type IIA vWD has been localized (35) to the Tyr424-Met425 peptide bond in the vicinity of the clustered Type IIA vWD mutations (Fig. 1); however, the responsible protease has not yet been identified.

The Group I mutations V844D, S743L, and G742R demonstrated defects in transport resulting in quantitative and qualitative defects in vWF. While other examples of genetic disease resulting from impaired intracellular transport have been described (36–38), they are typically characterized by purely quantitative defects and recessive inheritance. In a heterozygote, expression of the normal allele is generally unaffected by failure of the mutant protein to be transported. For vWD, the multimeric nature of vWF provides a mechanism for a mutation of this type to function in a “dominant-negative” fashion. The dominant inheritance and qualitative loss of HMW multimers characteristic of Type IIA vWD could result from selective retention of HMW forms containing greater numbers of mutant subunits. While an increased interaction of the Group I mutants with BiP was not evident, binding to other resident ER proteins cannot be excluded (28).

Both subgroups of Type IIA vWD may result from a spectrum of alterations to the same sensitive surface structure. While vWF has a very high cysteine content (8.3% of the total amino acids), there are no cysteines between residues 696 and residue 905 (17). Additionally, epitope mapping studies with a panel of monoclonal antibodies suggest that this region may contain an immunodominant epitope(s) (39). As noted above, this domain appears highly accessible to plasma protease(s) (35). Taken together, these data suggest that this region is highly exposed on the surface of vWF. Group I mutations may disrupt this structure, increasing the interactions of this domain with ER proteins, and resulting in markedly delayed transport through the secretory pathway. Other substitutions (Group II) may escape detection by resident ER proteins and thus may allow vWF secretion but result in increased sensitivity to extracellular protease(s) within the same exposed region.

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