Independent Assembly and Secretion of a Dimeric Adhesive Domain of von Willebrand Factor Containing the Glycoprotein Ib-binding Site*

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von Willebrand factor (vWF) is a multimeric glycoprotein that supports platelet adhesion on thrombogenic surfaces as part of the normal hemostatic response to vascular injury. We have employed a domain-specific expression strategy to analyze the biosynthetic processing steps and minimum structural requirements for assembly of the platelet receptor glycoprotein Ib-binding domain of vWF. A chimeric cDNA that codes for the vWF signal peptide and a segment of vWF internal primary sequence, residues 441-730, directs the secretion of a functional vWF fragment from mammalian cells. The recombinant molecule intrinsically assembles through intermolecular disulfide bond formation into a dimeric adhesive domain without contributions from other regions of vWF, including propeptide, previously indicated as essential for vWF multimer assembly. Prevention of N-linked glycosylation on the recombinant domain does not impair dimer formation or the ability to support platelet aggregation. These results identify a minimum structural element for vWF subunit assembly and provide new insights into the processing steps to produce vWF multimers and adhesive domains.

Platelet adhesion on damaged vascular surfaces is dependent upon von Willebrand factor (vWF), 1 a multimeric glycoprotein of plasma and the subendothelial matrix that serves as a bridging molecule between platelets and the subendothelium (1). vWF interacts with molecules of the vessel wall, in particular collagen and heparin-like glycosaminoglycans (2-6), and following vascular injury binds to the platelet glycoprotein (GP) Ib-IX receptor complex (7, 8). The interaction between platelet GP Ib-IX and vWF is a primary event of hemostasis that under blood flow conditions of high-shear stress culminates in thrombus formation (9).

The vWF primary translation product consists of a 22-residue signal peptide, a 741-residue propeptide, and a 2050-residue mature subunit (10, 11). After extensive post-translational processing, intermolecular disulfide bond formation among some of the 169 Cys residues of the vWF mature subunit produces multimeric vWF (12). Depending upon the extent of multimer formation, plasma vWF is composed of a population of molecules that have been estimated to range up to 50 individual subunits (1). The extent of vWF multimer formation is directly related to hemostatic efficacy (13, 14). Although vWF exists as a heterogeneous population of molecules, considerable progress has been made in localizing specific regions containing the crucial binding sites for normal hemostasis. When isolated under nonreducing conditions, a homodimeric 116-kDa tryptic fragment of plasma vWF, comprising subunit residues 449-728, inhibits binding of radiolabeled plasma vWF to platelet GP Ib-IX, heparin, and collagen (4, 15), and like plasma vWF, can support platelet aggregation through interaction with GP Ib-IX (16). Thus, the 116-kDa tryptic fragment purified as an isolated domain retains crucial functions that are characteristic of native vWF.

To investigate the structural features that govern the adhesive properties of the 116-kDa region of vWF, we constructed a recombinant plasmid to synthesize independently the primary sequence of the 116-kDa tryptic fragment. The expressed molecule undergoes post-translational processing, including intermolecular disulfide bond formation, to produce a homodimeric domain. Moreover, the expressed domain recapitulates native vWF function as demonstrated by its ability to support agonist-induced GP Ib-mediated platelet aggregation and heparin binding. The covalent assembly of a dimeric domain identifies a vWF sequence with the minimum structural elements for assembly into an independent domain and demonstrates an alternative methodology to characterize the processing steps and functional properties of this complex adhesive protein.

EXPERIMENTAL PROCEDURES

Plasmid Construction—A recombinant plasmid, pAD4/WT, was constructed to direct the synthesis in mammalian cells of vWF mature subunit residues Arg449-Asn728, corresponding closely to the monomeric subunit of the vWF 116-kDa tryptic fragment (residues Val449-Lys728). pAD4/WT contains a chimeric vWF cDNA fragment with the following five structural elements in a 5' to 3' direction: 1) a Kozak consensus translation initiation sequence, CCACC (17); 2) the initiating vWF methionine codon, ATG, and coding sequence for the remainder of the 22-residue vWF signal peptide; 3) coding sequence for 3 amino acid residues from the amino terminus of the vWF propeptide; 4) coding sequence for vWF amino acid residues Arg449-Asn728; and 5) a translation termination codon, TGA. The coding sequence for vWF signal peptide was included in the cDNA fragment to direct cellular secretion of the synthesized molecules, and the coding sequence for the first 3 residues of vWF propeptide was included to leave the signal peptidase recognition sequence intact.

To generate pAD4/WT, a vWF full-length cDNA clone (provided by Dr. Dennis Lynch, Dana Farber Cancer Institute, Boston MA) was used as template in a polymerase chain reaction (PCR) (18) to generate a 376-base pair fragment containing a Sall restriction site.
Domain-specific Expression of von Willebrand Factor

The kozak sequence, and coding sequence for precursor vWF residues 1–122. Oligonucleotide vWF<sub>160</sub>R<sup>4</sup> TGGCAGCCCAACATGATTCCT GCCAG<sup>A</sup>) contains a SaII restriction site (italic) 5' to the kozak sequence (underlined) followed by the vWF Met<sup>V</sup> (ATG) codon.

The second oligonucleotide of the PCR, vWF<sub>125</sub> (CTTACGTATTTGATCACACCCCT<sup>G</sup>) corresponds to nucleotides 2/352–2/356 (ss-)
coding scheme of BamHI restriction site (underlined). The PCR product was cleaved with SaII and XhoI, cloned into M13mp18 and designated pAD1.

The coding sequence corresponding to vWF mature subunit residues Arg<sup>256</sup> – Asn<sup>263</sup> was also generated by PCR using oligonucleotides that add BamHI restriction sites to the ends of the amplified fragment. The two oligonucleotides for this PCR (ACGGATCCGCCGTT-
TTGCCCTCAGA<sup>G</sup>) and (GGATCCCATGGTTCTCTTG-
GG<sup>G</sup>) correspond to nucleotide numbers 23/196–23/214 and 24/999–
24/1018, respectively (the additional 5' nucleotides in italics were included to add BamHI restriction sites on the 5' end of each molecule). The amplified fragment was cloned into the BamHI site of pAD1 (BamHI site of pAD1 is within the polylinker of M13mp18).

The vector contains the coding sequence corresponding to vWF mature subunit residues Asn<sup>7</sup> – Arg<sup>120</sup>. The SaII/XhoI fragment of the pAD3 was cloned into the SaII and XhoI sites of pBS/KS<sup>-</sup> (Stratagene, La Jolla, CA). The acquired sequence of the pBS/KS<sup>-</sup> polylinker sequence allowed the removal of the vWF insert after digestion with XhoI 5' (5' of the vWF initiating Met codon) and NotI (3' to the artificial vWF stop codon). The XhoI/NotI fragment was cloned into the XhoI/NotI site of the expression plasmid pCDNA<sup>E</sup> (Invitrogen), which was previously digested with XhoI/XbaI. The resultant plasmid was designated pAD4/WT.

A similar expression plasmid was generated to establish transformants that constitutively secrete the vWF antigen. The SaII/XhoI fragment of pAD3 was cloned into the SaII and XhoI sites of pBS/KS<sup>-</sup> (Stratagene, La Jolla, CA). The acquired sequence of the pBS/KS<sup>-</sup> polylinker sequence allowed the removal of the vWF insert after digestion with XhoI 5' (5' of the vWF initiating Met codon) and NotI (3' to the artificial vWF stop codon). The XhoI/NotI fragment was cloned into the XhoI/NotI site of the expression plasmid pCDNA<sup>E</sup>, which contains a neomycin resistance gene inserted in the parent plasmid pCDM8 (20).

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The in vitro expression of the m13 recombinant, designated plasmid pAD3, was determined to verify the organization of the coding elements and the absence of any spontaneous mutagenic errors. The SaII/XhoI insert of the pAD3 was cloned into the eukaryotic expression plasmid, pCDNA<sup>E</sup> (Invitrogen), which was previously digested with XhoI/XbaI. The resultant plasmid was designated pAD2.

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of the anti-GP Ibα monoclonal antibody LJ-Ib1 (28) or the anti-GP Ibα/IIa antibody LJ-CPS (29) was added at a final concentration of 100 μg/ml and incubated with platelets for 1 min at 37 °C before the addition of ristocetin.

Tunicamycin Treatment of Stable Transformants—Stable transformants secreting vWF antigen were incubated overnight in 10% DMEM containing tunicamycin (0.8 μg/ml) (Sigma). Cells were washed twice with PBS and incubated in DMEM containing tunicamycin (6.4 μg/ml) for another 24 h. Culture medium was harvested and concentrated up to 300-fold by centrifugation (Centricon 30, Amicon) prior to assay. Control stable transformants were treated identically except that the culture medium lacked tunicamycin. For platelet aggregation assays from tunicamycin-treated and untreated cells (Fig. 5B), the quantity of NMC-4 antigen was determined from densitometric scans of a Western blot autoradiograph (Fig. 5A). As determined from the densitometric scans, equivalent amounts of NMC-4 antigen were used in the platelet aggregation assay.

RESULTS

Expression of the 116-kDa Domain of vWF—To direct the independent expression and secretion of the 116-kDa domain of vWF, we constructed the expression plasmid pAD4/WT (Fig. 1). The chimeric vWF cDNA fragment within pAD4/WT contains coding sequence for vWF signal peptide and the first 3 residues of vWF propeptide fused to the coding sequence for vWF subunit residues Arg441-Asn730. The signal peptide and 3 residues of the propeptide were included to facilitate secretion and minimize structural constraints that might block signal peptidase cleavage and secretion as a result of the chimeric attachment of residues 441–730. pAD4/WT also contains a termination codon immediately following the Asn730 codon.

COS-1 cells were transfected with pAD4/WT, radiolabeled with [35S]methionine, and the culture medium was immunoprecipitated with the anti-vWF monoclonal antibody, NMC-4. The epitope recognized by NMC-4 is dependent upon a disulfide-bond-dependent conformation present within the vWF 116-kDa tryptic fragment and native vWF (16, 22). SDS-PAGE analysis of the immunoprecipitation products under nonreducing conditions revealed a predominant species of approximately 116 kDa secreted by cells transfected with pAD4/WT, whereas control transfections using pCDNA1 were negative for vWF antigen (Fig. 2A). Electrophoresis under reducing conditions revealed a 52-kDa but not the 116-kDa species, as expected from the dissociation of dimeric 116-kDa molecules into their monomeric components (Fig. 2A). The radiolabeled 116-kDa antigen could be detected as early as 15 min after the removal of [35S]methionine from transfected cells (Fig. 2B). Thus, the vWF signal peptide directs the secretion of the internal vWF domain, designated r116, and the molecule is immunoprecipitated with an anti-vWF monoclonal antibody that recognizes native vWF conformation. The presence of at least one intermolecular disulfide bond is demonstrated by a predicted increase in electrophoretic mobility after reduction of disulfide bonds.

Functional Properties of r116—To characterize r116 more completely, we inserted the vWF chimeric insert of pAD4/WT into a similar vector, pCDNAneo, which allows the selection of stable transformants resistant to the aminoglycoside antibiotic, G418. The resultant r116 expression plasmid, pAD5/WT, was transfected into CHO-K1 cells and stable transformants were selected that constitutively secrete r116. r116 was immunoprecipitated from the culture medium of stable transformants using an NMC-4 immunofinity column. As illustrated in Fig. 3, immunopurified r116 can successfully compete with [35S]vWF for binding to formalin-fixed platelets in the presence of ristocetin with an IC50 value of approximately 1 μM. Likewise, r116 cannot inhibit [35S]vWF binding to heparin with an IC50 value of approximately 7.5 μM (Fig. 3).

![Figure 1](https://via.placeholder.com/150)

**Fig. 1. Structural components of pAD4/WT.** A schematic representation of the precursor vWF molecule (prepro-vWF) is represented in a linear array that illustrates the repeating primary structure of vWF revealed by the linear amino acid sequence (11, 39). vWF is synthesized as a 2813-residue molecule with a signal peptide (SP), a 741-residue propeptide, and 2050-residue mature vWF subunit. The C terminus of the D3 linear domain and the entire A1 domain are represented in a homodimeric 116-kDa tryptic fragment of plasma vWF containing binding sites for the platelet receptor GP Ib-IX, collagen, and heparin (6). The monomeric subunit of the 116-kDa fragment is a heterogeneous 52/48-kDa fragment extending from Val196 to Lys737 of the vWF mature subunit and is illustrated below the corresponding region of the mature subunit (16). Portions of the vWF cDNA were fused to generate the recombinant plasmid, pAD4/WT, for synthesizing the primary sequence of the 116-kDa fragment. Briefly, the coding sequence for the vWF initiating methionine (ATG), signal peptide, and three N-terminal residues of propeptide were joined to coding sequence closely corresponding to the 52/48-kDa monomeric subunit fragment of the 116-kDa tryptic fragment. A translation termination codon (TGA) was added 3' to the Asn730 codon. The expression plasmid contains a cytomegalovirus promoter (CMV promoter) and a polyadenylation signal (poly(A) signal) to drive the expression of the vWF fragment.

![Figure 2](https://via.placeholder.com/150)

**Fig. 2. SDS-PAGE analysis of 35S-labeled vWF proteins immunoprecipitated from the culture media of transfected COS-1 cells.** Panel A, COS-1 cells were transfected with pCDNA1 (M) or pAD4/WT (W) plasmid. Cells were metabolically labeled with [35S]methionine, and the culture medium was immunoprecipitated with the anti-vWF monoclonal antibody NMC-4. Immunoprecipitated samples were analyzed by 10% SDS-PAGE and autoradiography under nonreducing (NR) or reducing (R) conditions. The migration of protein standards is indicated. Panel B, after a 4-h incubation with [35S]methionine, fresh medium was applied to cells and immunoprecipitated with NMC-4 after the indicated time of incubation (15 min, 2 h, 6 h, 24 h). Immunoprecipitated products were analyzed as described above under nonreducing and reducing conditions.
purified r116 was evaluated for its ability to inhibit I-vWF (2 pg/ml) values are approximately 1 to fibrillar collagen at 16 platelets in the presence of ristocetin (1 mg/ml). Ristocetin was added at the position of the arrow, and washed platelets (2 cated concentrations of immunopurified r116 were incubated with illustrated as a percent of the binding in the absence of r116 plotted... Data points are the mean of duplicate determinations. r116 was added to fibrillar collagen at 16 µM at the highest concentration tested. IC50 values are approximately 1 µM and 7.5 µM for platelet GP Ib and heparin, respectively.

Unexpectedly, at all concentrations tested (up to 16 µM) immunopurified r116 does not compete with 125I-vWF for binding to type I collagen. In the same collagen inhibition assays, an inhibition of 80% was observed with addition of the anti-vWF monoclonal antibody MR5 (100 µg/ml), a known inhibitor of vWF-collagen binding (3). A similar 80% inhibition of 125I-vWF binding could be demonstrated by MR5 in the presence of 12 µM r116 further illustrating the apparent lack of interaction between r116 and fibrillar collagen or between r116 and MR5 (data not shown). Thus, of the three reported functional properties of the tryptic 116-kDa fragment, binding to platelet GP Ib, heparin, and collagen, r116 recapitulates two of these functions but has no apparent affinity for type I collagen.

Immunopurified r116 was further evaluated for its ability to support platelet aggregation in the presence of ristocetin (Fig. 4). The aggregation response increases with increasing concentrations of r116 and is dependent upon the addition of ristocetin, a property shared with native vWF. Preincubation of platelets with the monoclonal antibody LJ-Ibl1, which binds the α-subunit of the GP Ib-IX complex and blocks vWF binding to GP Ib-IX (28), abolished the aggregation response, whereas incubation with the anti-GP Ib/IIa antibody LJ-CP8 (29) did not, consistent with a vWF-GP Ibα-specific interaction (Fig. 4B).

Prevention of N-Linked Glycosylation on r116 Does Not Impair Dimer Formation or Platelet Aggregation—The presence of N-linked carbohydrate side chains within r116 was investigated by treating pAD5/WT stable transformants with tunicamycin. Western blot analysis demonstrates that the expressed r116 from untreated cells can be resolved into species with slightly different electrophoretic mobilities, whereas r116 synthesized in the presence of tunicamycin is a single major species (Fig. 5A). The results demonstrate that the presence of N-linked carbohydrate side chains is not a prerequisite for the formation of intermolecular disulfide bonds to produce r116. The heterogeneity of species observed in the absence of tunicamycin demonstrates that N-linked glycosylation on r116 is a heterogeneous process, presumably due to incomplete processing of N-linked side chains or complete lack of N-linked side chains on some of the monomeric species. In untreated cells, whereas after tunicamycin treatment a single major species is observed under reducing conditions, r116 synthesized in the presence of tunicamycin can support platelet aggregation. No platelet aggregation was observed in the absence of ristocetin for either sample or with concentrated culture media from nontransfected CHO-K1 cells.
subunits of r116. Glycosylation within the analogous 116-kDa tryptic fragment of plasma vWF is also heterogeneous and explains the doublet produced (52/48 kDa) after reduction of the tryptic fragment (5).

The functional contribution of N-linked glycosylation on r116 was evaluated in platelet aggregation assays. The relative quantities of r116 from tunicamycin-treated and untreated cells were determined from densitometric scans of the Western blot illustrated in Fig. 5A, and equal amounts of NMC-4 antigen were assayed for their ability to support GP Ib-mediated platelet aggregation. The results demonstrate that r116 from tunicamycin-treated cells retains the ability to support GP Ib-mediated platelet aggregation (Fig. 5B), and like r116 produced in the absence of tunicamycin, requires ristocetin to support platelet aggregation. Control culture media from untransformed CHO-K1 cells did not induce platelet aggregation.

**DISCUSSION**

The role of vWF in hemostasis is to serve as an anchoring substrate for platelet-subendothelium and platelet-platelet interactions. In spite of the structural complexity of multimeric plasma vWF, a single 116-kDa tryptic fragment has been characterized containing vWF-binding sites for the platelet GP Ib-IX receptor complex, collagen, and heparin (3, 4, 6, 15). In the present study, we have obtained evidence that the 116-kDa fragment of vWF can be assembled and secreted as an independent molecule with the ability to: 1) fold into a disulfide-dependent conformation analogous to that of the corresponding domain of native vWF; 2) intrinsically assemble into a dimeric molecule without contributions from adjacent regions of vWF or N-linked carbohydrate side chains; and 3) retain function as shown by its ability to support agonist-induced GP Ib-mediated platelet aggregation and interact with heparin.

The observed self-assembly of the monomeric subunits of r116 provides information on the regulation of intracellular processing to produce multimeric vWF. Previous structural analyses of tryptic vWF fragments had identified the intermolecular disulfide bonds responsible for vWF multimers to be within two regions of vWF, an N-terminal region (residues 283–695) and a C-terminal region (residues 1908–2050) containing 30 and 18 Cys residues, respectively (12). Wagner et al. (30) proposed that the initiating step of multimer formation is dimerization of precursor vWF molecules via C-terminal disulfide bonds. Studies with recombinant molecules suggested that the formation of multimers, after dimer formation, is dependent on the 741-residue vWF propeptide, presumably by promoting the formation of intermolecular disulfide bonds within the N-terminal region (31, 32). More recently, a truncated recombinant molecule containing the vWF propeptide and residues 1–470 of the mature subunit has been shown to form dimeric molecules through N-terminal intermolecular disulfide bonds (33). This result demonstrates that dimer formation as a result of C-terminal intermolecular disulfide bonds is not a prerequisite for N-terminal intermolecular disulfide bonds. The expression of r116 demonstrates that vWF residues N-terminal to Gly404, including vWF propeptide, are not required for the synthesis of a functional dimeric domain.

As defined by Titani et al. (34), plasma vWF contains 1 N-linked and 8 O-linked carbohydrate side chains associated with each monomeric subunit of the 116-kDa tryptic fragment. The large disulfide-loop (Cys500-Cys605) within each monomeric subunit is flanked by two clusters of O-linked carbohydrate side chains, four within Thr534-Ser550 and four within Thr707-Thr724. Some heterogeneity of vWF glycosylation has been reported (35) and explains the doublet produced after reduction of the 116-kDa tryptic fragment (5). A structural role for N-linked glycosylation within plasma vWF has been suggested based on tunicamycin treatment of cultured human endothelial cells and the observed lack of native vWF dimer formation and subsequent multimer formation (36). Our results from the tunicamycin treatment of transformed CHO-K1 cells demonstrate that N-linked glycosylation of r116 is also heterogeneous but the structural and/or functional consequences of N-linked glycosylation within r116 are not apparent. r116 lacking N-linked side chains is still secreted as a functional dimeric domain.

The use of r116 in GP Ib inhibition assays is similar to previous assays performed with vWF tryptic fragments. Using a 116-kDa tryptic fragment, Mohri et al. (16) reported an IC50 value of 0.05 μM as compared to 5 μM reported by Fujimura et al. (22) using a similar fragment. A 100-fold discrepancy in IC50 values between two independently prepared tryptic fragments could reflect the extent of proteolysis within the purified fragments. By comparison, r116 had an IC50 value of approximately 1 μM and represents an intermediate value as compared to the tryptic fragments. The requirement of agonist to support r116 binding to GP Ib is analogous to the situation with plasma vWF and demonstrates that r116 mimics a characteristic functional property of plasma vWF. We have recently demonstrated that a r116 domain containing an amino acid substitution associated with type IIB von Willebrand’s disease can bind GP Ib in the absence of agonist (37) analogous to reduced and S-carboxymethylated molecules lacking the disulfide bond-dependent conformation (15, 37).

Based on the previous characterization of vWF tryptic fragments (3, 4, 6, 38), an unexpected result is the inability of r116 to inhibit binding of 125I-vWF to type I collagen. Several possible explanations for this result can be proposed and will be pursued in our continuing characterization of r116. An obvious explanation would be structural differences, such as glycosylation or tertiary structure, between r116 and the tryptic 116-kDa fragment. It is possible that during the immunopurification, as compared to the procedures associated with the isolation of a tryptic fragment, r116 has been structurally altered resulting in a dysfunctional collagen-binding site. It should also be remembered that vWF contains another collagen-binding domain within residues 911–1114 (4) (corresponding closely to the vWF A3 domain), and this sequence contains the epitope recognized by an anti-vWF monoclonal that inhibits vWF-collagen binding (3). If the collagen-binding site within the A3 domain is a high affinity binding site, it is possible that trace contaminants of this sequence or even trace contaminants of multimeric vWF like molecules or constituents of connective tissue that may noncovalently bind collagen. Such a phenomenon would help explain why some reports have suggested that native conformation is not required for this domain to interact with collagen (3, 24, 38).

Site-directed mutagenesis of the r116 domain will provide an opportunity to precisely define the structural elements that are responsible for normal vWF function. In the expression systems that we have characterized, approximately 50-
fold more r116 is synthesized by pAD5/WT, than constructs expressing prepro-vWF. The increased expression levels insure that sufficient quantities of variant recombinant molecules can be produced for detailed structural and functional studies.

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