von Willebrand Factor

A REDUCED AND ALKYLATED 52/48-kDa FRAGMENT BEGINNING AT AMINO ACID RESIDUE 449 CONTAINS THE DOMAIN INTERACTING WITH PLATELET GLYCOPROTEIN Ib*

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From the Departments of Basic and Clinical Research and Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037 and the Department of Biochemistry, University of Washington, Seattle, Washington 98195

We have purified a reduced and alkylated tryptic fragment of von Willebrand factor (vWF) which migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a 52/48-kDa doublet, but behaved as a single 46-kDa species after partial deglycosylation. After extensive treatment with denaturants, the 52/48-kDa polypeptide retained its ability to inhibit ristocetin-induced platelet aggregation in the presence of native vWF, as well as aggregation induced by de-N-acetylated vWF alone. Therefore, the 52/48-kDa polypeptide interacts with the platelet glycoprotein Ib receptor even in the absence of ristocetin. Both the 52/48- and the 46-kDa species inhibited ristocetin-induced binding of the intact molecule to platelets, but did not affect thrombin-induced binding. Determination of the NH2-terminal sequence of both members of the doublet gave identical results: VTLNPSDPEHCQ. This provided additional evidence that differences between the doublet constituents were only of carbohydrate composition and established the position of this peptide within the vWF polypeptide chain of approximately 2050 amino acid residues as beginning with the residue tentatively designated 449. These studies suggest that native conformation is not necessary for binding of vWF to platelets at the glycoprotein Ib receptor and that a linear amino acid sequence following residue 449 defines a domain responsible for this interaction.

von Willebrand Factor (vWF) plays a critical role in the early stages of hemostasis by promoting the adherence of platelets to denuded subendothelium. Ristocetin-induced binding of vWF to the platelet glycoprotein (GP) Ib receptor is an in vitro reflection of a central event in this process (1). Asialo vWF binds to the GPIb receptor in the absence of ristocetin or any other agonist and induces platelet aggregation in a manner considered to mimic more closely the in vivo interaction of vWF with platelets (2). ADP and thrombin also induce binding of vWF to platelets, but this interaction involves a receptor localized on GPIIb/IIIa (3).

Recently, a monoclonal antibody which blocked ristocetin-induced vWF binding to platelets has been shown to immunoprecipitate a tryptic fragment of vWF (4). This fragment was composed of a major 56/52-kDa doublet and a minor 49-kDa polypeptide, suggesting that the ristocetin-induced binding domain was restricted to a relatively small portion of the molecule.

We have now isolated a tryptic fragment of vWF that migrates in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, as a polypeptide of apparent molecular mass 52/48 kDa. After 70% deglycosylation by endo-P-N-acetylglucosaminidase F (endo F), a single band with apparent 46-kDa mobility was seen. Following reduction, alkylation, and extensive treatment with denaturants, this polypeptide completely inhibited asialo vWF-initiated platelet aggregation as well as ristocetin-induced aggregation in the presence of intact vWF. Moreover, it inhibited ristocetin-induced binding of intact vWF to platelets, but did not interfere with thrombin- or ADP-induced binding. Amino-terminal sequencing of the fragment indicated that it began with residue 449 of the intact, approximately 2050-residue vWF subunit. These studies provide direct evidence that a domain necessary for the binding of vWF to the platelet GPIb receptor is contained within this fragment. Since native conformation is not required for this function, it is likely that a linear amino acid sequence following residue 449 defines this domain.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies against Reduced and S-Carboxymethylated vWF Subunit.—Monoclonal antibodies against vWF were produced in mice and identified in a solid phase enzyme-linked assay as previously described (5), with the exception that the vWF used as immunogen was reduced as described below. Of 36 clones which reacted with intact, as well as reduced and S-carboxymethylated vWF, three (RG21, RG42, and RG46) were identified which blocked platelet aggregation and binding of 125I-vWF to platelets induced by ristocetin.

2The number of amino acids comprising the intact vWF subunit and the value of 275 kDa for the vWF subunit molecular mass was estimated by K. Titani, L. H. Ericsson, K. Takio, S. Kumar, M. W. Chopek, and K. Fujikawa following elucidation of a tentative primary amino acid sequence and assuming approximately 15% carbohydrate content of the vWF molecule. Residue numbers described herein should be regarded as tentative.
Purification of vWF for Trypsin Cleavage Studies—vWF was obtained by immunoadsorbant chromatography from commercial factor VIII concentrate (Armour Pharmaceutical, Kankakee, IL), using an anti-vWF monoclonal antibody coupled to Sepharose 4B (Pharmlab), as described (5). The vWF bound to the antibody was eluted with 3 M NaSCN dissolved in 0.01 M imidazole, 0.15 M NaCl buffer, pH 6.8, containing 0.1 M L-lysine HCl and 0.02% NaN3. The eluted vWF was dialyzed extensively against 0.05 M Tris, 0.15 M NaCl, pH 7.3 (PBS) and then concentrated by ultrafiltration with an Amicon PM-30 membrane (Amicon Corp. Danvers, MA). After centrifugation at 10,000 x g for 30 min at room temperature, the preparation was subjected to HPLC size exclusion chromatography using two Bio-Rad columns (60 x 2.15 cm) mounted in series, one TSK G4000 SW and the other G3000 SW. The columns were equilibrated with 0.2 M Na acetate, pH 5.5, containing 5% dimethyl sulfoxide, and run at a flow rate of 4 ml/min. The void volume fractions were pooled and stored at -70 °C. This material lacked the largest vWF multimers on SDS-agaroose electrophoresis (6). After reduction, polypeptides of 225, 176, and 140 kDa were identified by SDS-5% PAGE (7). Each polypeptide reacted with monoclonal antibodies to vWF after Western blotting onto nitrocellulose membranes (8).

Trypsin cleavage was performed in 0.2 M Na acetate buffer, pH 7.0, containing 0.02% NaN3, at 37 °C for 2 h, using 2,500 units of a trypsin (bovine pancreatic Type I, 15,000 units/mg, Sigma)/mg of vWF. Thirty mg of trypsin-cleaved vWF was then subjected to HPLC size exclusion chromatography, using two Bio-Rad columns (60 x 2.15 cm) mounted in series, one TSK G3000 SW and one G2000 SW, equilibrated in 0.2 M Na acetate, 5% dimethyl sulfoxide, pH 5.5.

Reduced and Alkylated—Lyophilized protein was dissolved in 0.1 M Na2EDTA, pH 8.6, containing 7 M guanidine-HCl and 0.01 M NaEDTA. An equal amount (w/w) of dithiothreitol to protein was added. After 1-h incubation at 37 °C, a 2.7-fold (w/w) excess iodoacetamide to dithiothreitol was added to the mixture, followed by incubation for an additional 30 min at room temperature in the dark. The alkylated samples were extensively dialyzed against TBS at 4 °C. Parial deglycosylation with endo F (9) was accomplished by incubating 50 units of enzyme/mg of protein in 0.1 M sodium phosphate buffer, 5 mM Na2EDTA, 0.02% NaN3, pH 6.1, for 18 h (10).

Purification of vWF for Binding Studies and Preparation of Asialo vWF—For this purpose, vWF was isolated from cryoprecipitate (gift of the American Red Cross, Bethesda, MD) using a method previously described (11). Analysis by SDS-agaroose electrophoresis (6) showed that this material contained the largest vWF multimers. On reduced SDS-PAGE, the 225-, 176-, and 140-kDa peptides were all present, but the largest represented a higher proportion than in the vWF before reduction and alklylation. Several protein peaks were seen, but only one was associated with significant ristocetin cofactor activity (measured as described in Ref. 11). Included fractions retaining this activity were pooled as Fraction B (Fig. 1). Upon analysis in unreduced SDS-PAGE, Fraction B consisted predominantly of two polypeptides with migration rates corresponding to 120 and 76 kDa, respectively (Fig. 1, inset). Fraction B was concentrated by ultrafiltration, lyophilized, and then reduced and S-carboxymethylated in 7 M guanidine-HCl. In a typical experiment, 14.7 mg of reduced and alkylated Fraction B was obtained from 97.5 mg of immunopurified vWF. No ristocetin cofactor activity remained after reduction and alkylation. However, 150 µg/ml reduced and alkylated Fraction B inhibited 50% of the ristocetin-induced binding of intact 125I-vWF to platelets (Fig. 2). A similar inhibition required only 9 µg/ml intact vWF, but 1500 µg/ml reduced and alkylated undigested vWF. In contrast to the unreduced sample, reduced and alkylated Fraction B contained a number of peptide bands on SDS-10–15% gradient polyacrylamide gel electrophoresis, the largest having an apparent molecular mass of 52 kDa (compare Fig. 3 and Fig. 1, inset). In order to determine which reduced and alkylated polypeptide was likely to be responsible for inhibition of binding of intact 125I-vWF, Western blotting studies were performed with three monoclonal antibodies (RG21, RG42, and RG46) which inhibited ristocetin-induced platelet aggregation and binding of vWF to platelets. These all reacted with the doublet of 52/48 kDa, and with some additional minor bands, but not with any of the other major components present in Fraction B (Fig. 3). Since their pattern of reactivity with minor bands differed, it is likely that these three monoclonal antibodies were to different epitopes. Three monoclonal antibodies to vWF which lacked the ability to inhibit ristocetin-induced platelet aggregation failed to react with the 52/48-kDa doublet, although they did react with other vWF peptides (not shown).

Isolation of the 52/48-kDa doublet was achieved by subjecting reduced and alkylated Fraction B to HPLC chroma-
von Willebrand Factor Platelet Glycoprotein Ib Binding Domain

FIG. 2. Effect of the vWF 52/48-kDa peptide doublet, and its partially deglycosylated 46-kDa form, on ristocetin-induced binding of $^{125}$I-vWF to platelets: comparison with intact vWF and Fraction B. Upper panel, native vWF, reduced and alkylated (R-A) Fraction B, and reduced and alkylated intact vWF were the competing ligands. Middle panel, the 52/48-kDa doublet, as shown in Fig. 5 (lane IV), was used as the competing ligand. The data shown are the aggregate from three experiments, each with a different preparation of the 52/48-kDa doublet. Bottom panel, the 52/48-kDa peptide and its 46-kDa partially deglycosylated form were compared as competing ligands with the other peptides shown in Fig. 5, as well as with reduced and alkylated undigested vWF. In all panels, specific binding is shown.

tofocusing, salt gradient elution, and size exclusion chromatography, all in the presence of 6 M urea (Figs. 4 and 5). The other peptides present in Fraction B were also largely separated one from another (Fig. 5). In a typical experiment, 2.6 mg of the 52/48-kDa doublet was recovered from 14.7 mg of Fraction B, with an overall yield from intact vWF of 2.7%. The concentration of 52/48-kDa doublet necessary to achieve 50% inhibition of ristocetin-induced $^{125}$I-vWF binding to platelets was approximately 0.52 $\mu$M. None of the other peptides showed significant inhibition at any concentration tested (Fig. 2). The IC$_{50}$ for reduced and alkylated intact vWF was 6.5 $\mu$M. The concentration of the 52/48-kDa doublet which completely inhibited ristocetin-induced binding of $^{125}$I-vWF (1.47 $\mu$M) did not discernibly inhibit thrombin-induced binding.

The 52/48-kDa doublet, at a concentration of 75 $\mu$g/ml (1.5 $\mu$M), completely inhibited aggregation of platelets induced by asialo vWF in platelet-rich plasma (Fig. 6). At a concentration of 500 $\mu$g/ml (10 $\mu$M), the 52/48-kDa doublet completely inhibited aggregation of washed platelets induced by ristocetin in the presence of purified native vWF (Fig. 6).

The 52/48-kDa doublet contained 99 $\mu$g of total hexose (measured as described in Ref. 16) per mg of protein. Treatment with endo F resulted in 70% removal of the total hexose and in the partially deglycosylated fragment then migrating as a single 46-kDa species in SDS-PAGE (Fig. 7). This form of the fragment was equally effective in blocking ristocetin-induced binding of intact vWF as the fully glycosylated counterpart (Fig. 2).

FIG. 3. SDS-10–15% gradient PAGE of peptides contained in Fraction B after reduction and alkylation. The upper panel shows a Coomassie blue-stained gel. The lower gel is an autoradiograph of an identical gel run in parallel but subjected to Western blotting and reacted with the monoclonal antibody R6-46. This anti-vWF antibody inhibits ristocetin-induced platelet aggregation and blocks ristocetin-induced binding of vWF to platelets. Lane 1 is the entire Fraction B. Lanes 2–8 are the individual peptides of Fraction B obtained by electroelution (17) from polyacrylamide gels. The molecular mass standards are indicated on the right.

FIG. 4. HPLC chromatofocusing of reduced and alkylated Fraction B. Seven mg of Fraction B were applied to a Mono-P HR 5/20 column (Pharmacia) in 0.025 M BisTris (Sigma), 6 M urea, adjusted to pH 7.1 with imidodiacetic acid. The limit buffer was 5% (v/v) polybuffer 74 (Pharmacia) in 6 M urea, adjusted to pH 3.8 with imidodiacetic acid. The gradient was run at a flow rate of 1 ml/min. After the pH of the gradient reached 3.8, a 0–1 M NaCl gradient in the previous limit buffer was run at 1 ml/min. Fractions were pooled, as indicated, and each was chromatographed on two HPLC TSK G3000 SW columns (30 $\times$ 0.75 cm) mounted in series and equilibrated in 0.025 M BisTris, 6 M urea, 0.15 M NaCl, pH 5.8. Pools I, II, and IV each gave one major protein peak, whereas pool III gave two. All protein peaks were well separated from the polybuffer peak.
The calculated molecular mass of each peptide is shown on the dialyzed in T3S prior to electrophoresis. The amount of protein applied to each lane was 2, and the IV shown in Fig. 4. (Platelet-rich plasma polyacrylamide gels after electroelution, both polypeptides with platelet-poor plasma, and the 62/48-kDa doublet, or buffer gels by the method of Hunkapiller control concentrations of all constituents are indicated in the figure. Aggregation induced appeared identical to those comprising the purified 52/48-kDa doublet prior to electroelution. The first 12 amino acids aggregation induced by ristocetin (10^7/ml washed platelets for 5 min in a Lumi aggregometer) at 0.1 mmol/l was then added and incubated at 37 °C for 5 min. Aggregation was initiated with 25 µl of asialo vWF. The final concentrations of all constituents are indicated in the figure.

Fig. 6. Effect of the vWF 52/48-kDa doublet on platelet aggregation induced by ristocetin (left) and asialo vWF (right). For ristocetin-induced aggregation, purified native vWF and the 52/48-kDa doublet, or buffer as a control, were incubated with 3 x 10^7/ml washed platelets for 5 min in a Lumi aggregometer (ChronoLog Corp., Havertown, PA) cuvette in a volume of 500 µl. Ristocetin (10 µl) was then added and the degree of aggregation determined. Aggregation induced by asialo vWF was performed with citrated platelet-rich plasma (2). Platelet count was adjusted to 4 x 10^9/ml with platelet-poor plasma, and the 52/48-kDa doublet, or buffer as a control (75 µl), was then added and incubated at 37 °C for 5 min. Aggregation was initiated with 25 µl of asialo vWF. The final concentrations of all constituents are indicated in the figure.

The calculated molecular mass of each peptide is shown on the right.

of each were as follows: VTLNPSDPEHCQ. This sequence was compared to a tentative amino acid sequence of the molecule as determined by one of us (Dr. K. Titani) and was found to correspond in its entirety to the sequence beginning with valine 449 and ending with glutamine 460. This sequence was not found elsewhere in the molecule.

DISCUSSION

The studies reported here provide direct evidence that a restricted domain of the 275-kDa subunit of vWF mediates interaction with the platelet GPIb receptor. This domain lies within the NH2-terminal region of the single, approximately 2050-amino acid residue vWF polypeptide chain and begins with residue 449. It mediates the interaction of vWF with GPIb both in the presence or the absence of ristocetin, as shown by the fact that the 52/48-kDa doublet isolated by us blocked platelet aggregation induced by native vWF in the presence of ristocetin, as well as aggregation induced by asialo vWF alone. Asialo vWF binds to the GPIb receptor and initiates platelet aggregation in the absence of ristocetin or any other platelet agonist (2, 11).

Previous work by others has demonstrated that vWF need not be intact to interact with the GPIb receptor. Pasquini and Hershgold (18), as well as Andersen and McKee (19), showed that ristocetin cofactor activity was retained in spite of extensive proteolytic digestion of vWF with plasmin. Martin and co-workers (20) demonstrated that some of this activity was retained in a tryptic fragment comprised of 73-, 46-, and 43-kDa chains. More recently, Girma et al. (21) demonstrated that a 315-kDa fragment bound to ristocetin-treated platelets but not to thrombin-treated platelets. After reduction, a 170-kDa peptide was identified as the principal constituent of this fragment, although the binding properties of the reduced form were not reported. In studies performed by Sixma et al. (4) a tryptic fragment containing 56-, 52-, and 49-kDa chains was immunoprecipitated by a monoclonal antibody to vWF. This antibody inhibited binding of intact vWF to platelets in the presence of ristocetin. However, the ability of the tryptic fragment or any of its constituent chains to inhibit binding was not described. Nor did any of the previous studies provide evidence that the GPIb binding domain of vWF could interact with platelets in the absence of added platelet agonists.

The 52/48-kDa peptide described here also reacted with
monoclonal antibodies which blocked ristocetin-induced platelet aggregation and prevented ristocetin-induced binding of intact vWF to the GPIb receptor. However, presence of the epitopes for these antibodies on the 52/48-kDa fragment does not necessarily prove that a domain important to ristocetin-induced binding is also present on this polypeptide. Antibodies can inhibit the function of a molecule by changing its conformation and do not need to interact at the functional domain being altered to do this (22). Direct demonstration that the binding domain resided on the 52/48-kDa doublet, and its 46-kDa partially deglycosylated form was accomplished here by showing that the fragment completely inhibited ristocetin-induced binding of intact vWF to platelets.

Since 70% deglycosylation of the 52/48-kDa doublet resulted in its migrating as a single 46-kDa species on SDS-PAGE, it is likely that the two components differed only in carbohydrate composition. Additional evidence that this is so was the identical NH₂-terminal sequence of both components. Failure of deglycosylation to alter the ability of this fragment to block ristocetin-induced binding of intact vWF is consistent with previous studies showing little or no effect of deglycosylation on ristocetin-induced binding of native ¹²⁵I-vWF to platelets (10).

The reduced and alkylated intact vWF had a 10-fold greater IC₅₀ for inhibition of ristocetin-induced ¹²⁵I-vWF binding than the 52/48-kDa doublet. The reason for this difference is not clear at present. Further experimentation and a better knowledge of the molecular basis of the vWF-GPIb interaction will be required to clarify this experimental observation. Our findings, however, suggest that other properties of the vWF subunit, not directly related to the portion containing the GPIb binding domain, may affect its interaction with platelets.

The ability of the 52/48-kDa peptide to prevent ristocetin-dependent or ristocetin-independent binding of vWF to GPIb was retained after reduction and alkylation in the presence of 7 M guanidine-HCl and subsequent chromatography in 6 M urea. The survival of inhibitory activity in spite of extensive treatment with these denaturants suggests that native conformation is not necessary for interaction of this vWF fragment with platelets. It is likely, therefore, that a linear amino acid sequence following residue 449 mediates the interaction of vWF with its GPIb platelet receptor.

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REFERENCES