Evidence that a secondary binding and protecting site for Factor VIII on von Willebrand Factor is highly unlikely

Sylviane LAYET,* Jean-Pierre GIRMA,*† Bernadette OBERT,* Edith PEYNAUD-DEBAYLE,*
Nicolas BIHOREAU† and Dominique MEYER*

*INSERM U.143, Hôpital de Bicêtre, 94275 Le Kremlin-Bicêtre Cédex, Paris, and †TM-Innovation, Les Ulis, France

A binding domain for Factor VIII (F.VIII) has been previously identified on the *N*-terminal portion of human von Willebrand Factor (vWF) subunit [amino acids (AA) 1–272]. In order to characterize other possible structures of vWF involved in its capacity to bind and to protect F.VIII against human activated protein C (APC), we used a series of purified vWF fragments overlapping the whole sequence of the subunit. Among those were fragments SpIII (dimer; AA 1–1365), SpII (dimer; AA 1366–2050) and SpI (monomer; AA 911–1365) generated by *Staphylococcus aureus* V8 proteinase, a P34 species (monomer; AA 1–272) obtained with plasmin, a monomeric 39/34 kDa disperse fragment (AA 480–718) and a tetrameric III-T2 fragment (AA 273–511/674–728) produced from SpIII by trypsin. Three other fragments without precise extremities were located using selected monoclonal antibodies to vWF. Two *C*-terminal fragments of 270 and 260 kDa, overlapping SpI and SpII, were respectively generated from vWF with trypsin and protease 1 from *Crotalus atrox* venom. An *N*-terminal 120 kDa fragment, overlapping P34 and 39/34 kDa fragments, was produced by protease 1. Our results show that vWF bound to F.VIII and protected it from degradation by APC in a dose-dependent way. Among the *C*-terminal and central vWF fragments (SpII, tryptic 270 kDa, 260 kDa, SpI, 39/34 kDa and III-T2), none had the capacity to bind or to protect F.VIII, even at high concentrations. The three *N*-terminal fragments (SpIII, 120 kDa and P34) bound to F.VIII in a dose-dependent and saturable fashion. SpIII and the 120 kDa fragment had the capacity to protect F.VIII in a dose-dependent way. In contrast, the P34 species did not significantly protect F.VIII, even when using high concentrations of the fragment. In conclusion, the *N*-terminal end of vWF subunit (AA 1–272) plays a crucial role in binding to F.VIII, but requires additional structures of the 120 kDa fragment to protect it against APC. In addition, the presence of a secondary binding and/or protecting domain on other portions of the vWF subunit (potentially destroyed during the proteolysis of vWF) is highly unlikely.

INTRODUCTION

Human von Willebrand Factor (vWF) is a glycoprotein that appears as a series of multimers with molecular masses from 500 to more than 15000 kDa [1]. Each multimer is composed of an association of unique subunits (275 kDa) of 2050 amino acids (AA) linked together by disulphide bridges, the protomer being the homodimer. vWF plays two major roles. It promotes platelet adhesion to the subendothelium at high shear rates [2]. vWF also serves as carrier for Factor VIII (F.VIII), stabilizing its pro-coagulant activity *in vivo* [3] and *in vitro* [4]. A vWF-binding domain has been identified on the light chain of F.VIII [5,9] and is localized between residues 1670 and 1689 [7]. No primary vWF-binding site has been identified on the heavy chain of F.VIII [6,10]. The complexation of the light chain of F.VIII with vWF was demonstrated to stabilize F.VIII against inactivation by activated protein C (APC) [11,12] or activation and inactivation by Factor Xa [13].

The precise mechanism by which vWF prevents proteolytic F.VIII activation and inactivation is not well understood. Similarly, little is known about the structure(s) of vWF involved in F.VIII stabilization. A major F.VIII-binding domain has been identified on the *N*-terminal end of the vWF subunit between residues 1 and 272 [14,15]. By using expressed cDNA fragments and monoclonal antibodies (MAbs) to vWF, the binding site was localized within the 106 *N*-terminal AAs of the subunit [16] between residues Thr-78 and Thr-96 [17]. Other studies have

suggested that each subunit present in multimers of vWF is able to bind F.VIII [6,10,18]. However several pieces of evidence suggest that a discrepancy exists between the binding ability of vWF and its capacity to protect F.VIII. In normal plasma or in vWF-deficiency states, a ratio of 1 unit of F.VIII per unit of vWF is generally observed [19] corresponding to the occupancy of only a few per cent of the available vWF subunits. F.VIII in plasma with higher F.VIII/vWF ratio demonstrated a decreased stability as compared with normal plasma [20]. Recent studies by Leyte *et al.* [8] established that the binding sites on vWF for F.VIII exhibit multiple classes of affinity, and the high-affinity binding was restricted to only a few per cent of the vWF subunits. A study by Koedam *et al.* [11] demonstrated that, although additional F.VIII does bind to vWF, it is no longer protected against proteolysis by APC. Other observations from the same group [21] and from Fay *et al.* [22] suggested that the binding domain located on the *N*-terminal end of vWF (AA 1–272) is not by itself sufficient to protect F.VIII against APC. In addition, findings by Koedam *et al.* [21] indicated that a second domain (or group of domains) which binds and protects F.VIII is present on the *C*-terminal part of vWF.

Human F.VIII is a glycoprotein synthesized as a single-chain precursor of 2332 AAs [23] circulating in plasma as a non-covalent complex with vWF. F.VIII appears as a series of proteolytic heterodimeric molecules which are linked through a metal-ion bridge and composed of a *N*-terminal heavy chain varying between 90 and 210 kDa and of a common *C*-terminal

Abbreviations used: F.VIII, Factor VIII; F.VIIIa, activated Factor VIII; VIII:C, coagulant activity of Factor VIII; vWF, von Willebrand Factor; vWF:Ag, von Willebrand Factor-related antigen; APC, activated protein C; MAb, monoclonal antibody; i.r.m.a., immunoradiometric assay; AA, amino acid; TBS, Tris-buffered saline (25 mM-Tris/HCl/0.15 M-NaCl, pH 7.4); iPr₂P-F, di-isopropyl fluorophosphate; Tos-Phe-CH₂Cl, tosylphenylalaninylchloromethane ('TPCK').

† To whom correspondence should be addressed.

light chain of 80 kDa (AA 1649–2332). F.VIII plays a major role in the intrinsic pathway of blood coagulation. After activation of F.VIII, F.VIIIa acts as a cofactor and associates with Factor IXa, phospholipids and Ca^{2+} to form the tenase complex which activates Factor X to Factor Xa. Production of F.VIIIa by thrombin has been shown to proceed through specific cleavages at residues 372 and 740 of the heavy chain and concomitantly at residue 1689 of the light chain [24,25]. This leads to an active trimeric complex composed of two complementary species from the heavy chain, a ~ 50 kDa fragment (AA 1–372) and a ~ 45 kDa fragment (AA 373–740), and a shortened light chain of ~ 72 kDa (AA 1690–2332). Similarly, in the presence of phospholipids, Factor Xa also activates F.VIII to F.VIIIa through cleavages identical with those produced by thrombin. Inactivation of F.VIIIa appears to be related to further proteolysis with either Factor Xa at residues 336 and 1721 [24] or thrombin at residue 336 [26] or to spontaneous dissociation and/or conformational change of F.VIIIa [24,25,27].

Procoagulant activity of F.VIII or F.VIIIa is also regulated by APC (11,24,28,29). A binding site for APC has been identified on the light chain of F.VIII between residues 2009 and 2018 [30]. In the presence of phospholipids, the heavy chain of F.VIII is the primary substrate of the enzyme [24,28,29], which also cleaves F.VIII or F.VIIIa at residue 336 [24,26]. Finally, using synthetic peptides, a domain of F.VIII interacting with phospholipids has been localized on the light chain between residues 2303 and 2332 [31].

In the present study we used a series of proteolytic fragments of vWF overlapping the whole sequence of the subunit to study the structures of vWF involved in its capacity to bind or to protect F.VIII against inactivation by APC. We showed that the sequence lying between AAs 1 and 272 of vWF possesses the capacity to bind F.VIII, but that additional sequences are required to protect it against inactivation by APC. We also showed that no secondary domain interacting with F.VIII exists on the C-terminal end of vWF.

MATERIALS AND METHODS

Materials

Sephadex, Sepharose and Sephacryl gels, DEAE-Sephacel, heparin-Sepharose and Mono Q columns were purchased from Pharmacia (Uppsala, Sweden). Whatman DE 52 DEAE-cellulose was from Whatman International (Maidstone, Kent, U.K.). Ultrogel ACA 54 was from IBF (Villeneuve-la-Garenne, France). Plasmin (EC 3.4.21.7) and dispase (EC 3.4.24.4) were obtained from Boehringer (Meylan, France). Tosylphenylalanyl-chloromethane (Tos-Phe- CH_2Cl ; 'TPCK')-treated trypsin (EC 3.4.21.4), *Crotalus atrox* venom, BSA, di-isopropyl fluorophosphate ($\text{iPr}_2\text{P-F}$) and phenylmethanesulphonyl fluoride were from Sigma (La Verpillière, France). *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) was from Miles Laboratories (Naperville, IL, U.S.A.). APC (EC 3.4.21.-) and cephalin were purchased from Diagnostica STAGO (Asnières, France). UH 100/10 membranes and nitrocellulose membranes were obtained from Schleicher und Schüell (Dassel, Germany). PM 10 Diaflo membranes were from Amicon (Danvers, MA, U.S.A.). Na^{125}I was from Amersham International (Amersham, Bucks., U.K.) and Iodo-Gen was from Pierce Chemical Co (Rockford, IL, U.S.A.).

Immunopurification of F.VIII

F.VIII was purified by immunoadsorption from F.VIII concentrates produced by the Centre National de Transfusion Sanguine (CNTS) (Les Ulis, France) using a MAb to F.VIII (MAb 463-A8) as previously described [32]. F.VIII concentrates

were dissolved at 37 °C in 0.02 M-imidazole/HCl/0.15 M-NaCl, pH 6.8 (imidazole-buffered saline), containing 0.1 M-lysine, 0.35 M- CaCl_2 and 2 mM- $\text{iPr}_2\text{P-F}$ and applied to MAb 463-A8 coupled (0.5 mg of IgG/ml of beads) to Sepharose 4B. After 3 h at 22 °C the gel was packed on to a column and washed with the same buffer to remove vWF. Bound F.VIII was eluted with 50 % ethylene glycol/0.02 M-imidazole/HCl/1 M-NaCl/0.4 M- CaCl_2 , pH 7, containing 2 mM-phenylmethanesulphonyl fluoride and 10 mM-histidine. Purified F.VIII was stored in the eluting buffer for 1 month at -20 °C without loss of activity.

Purification of vWF

Human vWF was purified by the method of Thorell & Blombäck [33] from commercial F.VIII concentrates provided by the CNTS as previously reported [34]. The concentrates were dissolved in imidazole-buffered saline containing 1 mM- $\text{iPr}_2\text{P-F}$, and the solution was dialysed against 25 mM-Tris/HCl/0.15 M-NaCl, pH 7.4 (TBS), containing 2 M-glycine. The precipitate was centrifuged at 10000 g for 20 min at 22 °C. The supernatant was concentrated by precipitation with 12 % (w/v) poly(ethylene glycol) 4000. After centrifugation at 10000 g for 15 min at 4 °C, the pellet was dissolved in imidazole-buffered saline containing 0.35 M- CaCl_2 to dissociate vWF from F.VIII. vWF was then isolated by gel filtration on Sepharose CL-4B equilibrated in the same buffer. Purified vWF was dialysed against imidazole-buffered saline, concentrated by precipitation with 40 % satd. $(\text{NH}_4)_2\text{SO}_4$ and stored at -80 °C.

Proteolysis of vWF by *S. aureus* V8 proteinase and purification of SpIII, SpII and SpI fragments

Purified vWF was digested with V8 proteinase and fragments were purified as previously reported [35]. The proteolytic fragments SpIII (dimer; AA 1–1365), SpII (dimer; AA 1366–2050) and SpI (monomer; AA 911–1365) were separated by ion-exchange chromatography on DEAE-Sephacel and further purified by gel filtration on Sephadex G-150 and immunoadsorption on to selected MAbs, directed against vWF, coupled to Sepharose 2B. Purified fragments were stored at -80 °C until use.

Proteolysis of SpIII by plasmin: purification of P34 fragment

Plasmin was added to purified SpIII in 50 mM- $\text{Na}_2\text{HPO}_4/\text{H}_3\text{PO}_4$ /80 mM-NaCl, pH 7.4, at an enzyme/substrate ratio of 1:20 (w/w). Digestion was allowed to proceed for 20 h at 22 °C. The reaction was terminated by adding $\text{iPr}_2\text{P-F}$ (2 mM final concn.). P34 fragment (monomer; AAs 1–272) was purified by the method of Hamilton *et al.* [36] with slight modifications; the digest was freeze-dried and dissolved in TBS containing 6 M-guanidinium chloride and submitted to gel filtration on Sephacryl S-200 HR equilibrated in the same buffer. The protein peak containing P34 fragment was extensively dialysed against TBS and concentrated by anion-exchange chromatography on a Mono Q column. Purified P34 fragment was stored at -80 °C until use.

Proteolysis of SpIII by trypsin: purification of III-T2 fragment

Purified SpIII (1 mg/ml) in TBS was digested with Tos-Phe- CH_2Cl -treated trypsin at an enzyme/substrate ratio of 1:50 for 18 h at 22 °C. The reaction was stopped by addition of 2 mM- $\text{iPr}_2\text{P-F}$. The digest was subjected to chromatography on heparin-Sepharose equilibrated in TBS. The pass-through containing the tetrameric III-T2 fragment comprising the two sequences AA 273–511 and AA 674–728 [37] was submitted to gel filtration on Sephacryl S-200 HR using TBS containing 6 M-guanidinium chloride as eluant. After extensive dialysis against TBS and freeze-drying, purified III-T2 fragment was separated by gel filtration through Sepharose CL-4B and elution with TBS.

Proteolysis of vWF by dispase: purification of a 39/34 kDa fragment

Purified vWF was digested in TBS containing 0.2 mM-CaCl₂ by dispase by the method of Andrews *et al.* [38] at an enzyme/substrate ratio of 1:5 (w/w) for 18 h at 22 °C. The reaction was stopped by adding 0.01 M-EDTA. A 39/34 kDa fragment (monomer; AA 480–718) was isolated by adsorption on to heparin-Sepharose equilibrated in TBS. After washing, the 39/34 kDa fragment was eluted by increasing the NaCl concentration from 0.15 to 0.5 M. Further purification was performed by gel filtration on Sephadex G-150 equilibrated in TBS. The 39/34 kDa fragment was concentrated by dialysis using UH 100/10 membranes and stored at –80 °C until use.

Proteolysis of vWF by trypsin: purification of a 270 kDa fragment

Purified vWF was hydrolysed with Tos-Phe-CH₂Cl-treated trypsin at an enzyme/substrate ratio of 1:40 (w/w) for 10 min at 37 °C in 0.02 M-Tris/HCl/1 mM-CaCl₂, pH 7.8. The reaction was terminated by adding 1 mM-iPr₂P-F and 1 mM-phenylmethanesulphonyl fluoride. Among the proteolytic fragments, a C-terminal 270 kDa fragment was purified; following dialysis against TBS, the digest was incubated in batch with MAb 203 coupled to Sepharose 2B for 16 h at 22 °C. The gel was then packed on to a column and extensively washed with the same buffer. Bound 270 kDa fragment was eluted with 0.1 M-glycine/HCl/1 M-NaCl, pH 2.4. The pH was immediately neutralized with 1.5 M-Tris/HCl buffer, pH 8.8. The purified fragment was dialysed against TBS and stored at –80 °C before use.

Purification of protease 1 from *C. atrox* venom

Protease 1 was purified from *C. atrox* venom by the method described by Pandya [39]. The freeze-dried venom (100 mg) was dissolved in 5 ml of 0.01 M-K₂HPO₄/H₃PO₄ buffer, pH 8, and applied on DEAE-cellulose. The pass-through protein peak containing protease 1 was concentrated by ultrafiltration on PM 10 Diaflo membranes. Protease 1 was further purified by gel filtration on Ultrogel ACA 54. Protease 1 was stored in TBS at –20 °C. On SDS/PAGE, protease 1 appears as a single polypeptide chain of 26 kDa.

Proteolysis of vWF by protease 1 from *C. atrox* venom: purification of 120 and 260 kDa fragments

Purified vWF in TBS was hydrolysed with protease 1 as described by Bakhshi & Kirby [40] for 3 h at 37 °C using an enzyme/substrate ratio of 1:50 (w/w). Proteolysis was stopped by addition of 2 mM-EDTA and 2 mM-iPr₂P-F. Digestion of vWF yielded two major fragments with apparent molecular masses of 120 and 260 kDa.

The two fragments were separated by gel filtration on Sephacryl S-300 equilibrated in TBS. The 120 kDa fragment was then separated from the bulk of contaminants and concentrated by ion-exchange chromatography on Mono Q using a stepwise increase of NaCl concentration (0.15 and 1 M-NaCl). The 120 kDa fragment was eluted at 1 M-NaCl. It was dialysed against TBS containing 6 M-guanidinium chloride and submitted to gel filtration on Sephacryl S-300 equilibrated in the same buffer. The 120 kDa peak was then extensively dialysed against TBS and concentrated on a Mono Q column.

The 260 kDa fragment was further isolated from contaminants and residual vWF by ion-exchange chromatography on a Mono Q column by using a 0–1 M-NaCl gradient in 25 mM-Tris/HCl buffer, pH 7.4. The 260 kDa fragment and vWF were eluted at 0.15 and 0.35 M-NaCl respectively.

Purified 120 kDa and 260 kDa fragments were stored in TBS at –80 °C before use.

MAbs and polyclonal antibodies

A series of MAbs directed against vWF was produced and characterized as previously described [41,42]. Among those, MAbs 8, 203, 328, 418, 543 and 549 were used in the present study. MAbs 418 and 543 are directed to fragment P34 (AA 1–272). MAb 418 inhibits the binding of vWF to F.VIII [14]. MAb 328 recognizes the 39/34 kDa dispase fragments (AA 480–718) and inhibits the binding of vWF to GPIb [14]. MAbs 203 and 549 recognize SpI (AA 911–1365) [43]. MAb 8 binds to the C-terminal fragment SPII (AA 1366–2050) [43]. MAb 463-A8, directed against the F.VIII light chain, was produced as previously reported [44].

IgG fractions were isolated from ascites fluids by the method of Fine & Steinbuch [45].

A polyclonal antibody to vWF was raised in rabbits and rendered monospecific as previously described [46]. The IgG fraction was prepared [45], and the specific antibody was immunoadsorbed on to vWF coupled to Sepharose as described in [46].

After labelling, monoclonal and polyclonal antibodies to vWF were used for the detection of fragments by immunoblotting and for the estimation of vWF:Ag by immunoradiometric assay (i.r.m.a.) [46]. MAbs 8, 418 and 203 and MAb 463-A8 were coupled to Sepharose and used for immunopurification of vWF fragments and F.VIII respectively.

Radiolabelling

vWF, proteolytic fragments and IgG were radiolabelled with Na¹²⁵I as described in [47]. The specific radioactivity of vWF and proteolytic fragments varied from 0.2 to 2 µCi/µg of protein. Radiolabelled IgG was used for immunoblotting and for radioimmunoassay at a specific radioactivity of about 10 µCi/µg.

Binding of F.VIII to immobilized vWF or proteolytic fragments

A solid-phase binding assay was performed in U-shaped 96-well poly(vinyl chloride) microtitre plates (Dynatech, Marne-La-Coquette, France). The wells were coated for 16 h at 4 °C with 100 µl (10 µg/ml) of purified vWF or proteolytic fragment in 0.05 M-Na₂CO₃, pH 9.8. After washing with 25 mM sodium barbital/acetate buffer, pH 7.3 (Michaelis buffer), containing 0.2% BSA (Michaelis buffer/BSA), residual sites were saturated by incubating BSA (1%) for 1 h at 37 °C. F.VIII potentially present was then removed by incubating 0.4 M-CaCl₂ in Michaelis buffer for 30 min. After washing, 100 µl of selected concentrations [0.03–3 units of Factor VIII coagulant activity (VIII:C)/ml] of purified F.VIII in Michaelis buffer/BSA containing 40 mM (final concn.)-CaCl₂ were incubated for 1 h at 37 °C. After extensive washing, 25 µl of TBS containing vWF (10 µg/ml) were added, and bound F.VIII activity was quantified *in situ* by using the chromogenic assay described below. Non-specific binding was estimated by using BSA-coated wells.

Protection of F.VIII against inactivation by APC

The assay was performed in a final volume of 1 ml of Michaelis buffer/BSA containing 2 mM (final concn.)-CaCl₂. Aliquots (400 µl) of purified F.VIII at a concentration of 1.25 units of VIII:C/ml (final concn. 0.5 unit/ml) were incubated for 15 min at 37 °C with 480 µl of serial dilutions of vWF or proteolytic fragments at a final concentration ranging from 0.01 to 150 µg/ml. After the addition of 100 µl of a 1:150 dilution of cephalin and incubation for 15 min at 37 °C, the reaction was started by adding 20 µl of purified APC (final concn. 0.8 µg/unit of VIII:C). After 1 h, aliquots (25 µl) were removed and diluted

Fig. 1. SDS/PAGE analysis of purified F.VIII and products of digestion of vWF

(a) Purified F.VIII. Electrophoresis was performed on SDS/PAGE under non-reducing conditions using a 6–12% linear gradient of polyacrylamide. The proteins were revealed by silver staining as described in the Materials and methods section. (b) Purified vWF was treated by protease 1 from *C. atrox* venom at an enzyme/substrate ratio of 1:50 for 3 h at 37 °C, and 260 and 120 kDa fragments were purified as described in the Materials and methods section. Electrophoresis was performed by SDS/PAGE, using a 3.5–20% linear gradient of polyacrylamide. Analysis of unreduced digest (lanes 1–5): lane 1, Coomassie Blue staining; lane 2, electroblotting followed by staining by ^{125}I -MAB 8; lane 3, MAB 549; lane 4, MAB 418; lane 5, MAB 328; lanes 6 and 7, analysis and Coomassie Blue staining of purified 260 and 120 kDa fragments under non-reducing conditions; lanes 8 and 9, the same as lanes 6 and 7, but under reducing conditions. (c) Purified vWF was digested with trypsin (enzyme/substrate ratio of 1:40) for 10 min at 37 °C and the 270 kDa fragment was purified as described in the Materials and methods section. Electrophoresis was performed by SDS/PAGE on a 3.5–20% linear gradient of polyacrylamide. Analysis of unreduced digest (lanes 1–5): lane 1, Coomassie Blue staining; lane 2, electroblotting followed by staining by ^{125}I -MAB 8; lane 3, MAB 549; lane 4, MAB 418; lane 5, MAB 328; lane 6, analysis and Coomassie Blue staining of purified 270 kDa fragment under non-reducing conditions; lane 7, the same as lane 6, but under reducing conditions. The positions of the molecular mass (*M*) markers are indicated at the left.

20-fold at 4 °C with TBS containing 10 µg of vWF/ml. Residual F.VIII activity was measured by a chromogenic assay and compared with the activity of F.VIII incubated with vWF or proteolytic fragments under similar experimental conditions but in the absence of APC. In control experiments the activity of APC was compared at the end of incubation to that used as starting material by allowing 100 µl aliquots of the reaction mixture in wells of microtitre plates to react with 25 µl of 1 mM chromogenic substrate S2366 (pyroglutamylprolylarginine *p*-nitroanilide hydrochloride) (in 50 mM-Tris/HCl/80 mM-NaCl, pH 8, containing 4 mM- CaCl_2 for 1 h at 37 °C. The amount of APC was estimated by the absorbance at 405 nm.

Assay of F.VIII

F.VIII activity was measured using a chromogenic assay (Stachrom VIII:C, Diagnostica STAGO) as follows. Test samples (25 µl) were applied to U-shaped 96-well plates. Solutions of Factor X (25 µl) and Factor IXa (25 µl) containing synthetic phospholipids were added and incubated for 6 min at 37 °C. The chromogenic substrate CBS4803 (acetyl-D-leucyl-glycylarginine *p*-nitroanilide hydroacetate) (25 µl) was then added, and after 6 min the reaction was stopped by adding 50% (v/v) acetic acid (25 µl). The absorbance was read at 405 nm by using a Titertek Multiscan (Flow Laboratories, Helsinki, Finland). Serial dilutions of normal plasma pool in diluting buffer (Diagnostica STAGO) were used as standard. Purified F.VIII was tested in comparison by using as starting material a concentration of about 1 unit/ml in diluting buffer containing 10 µg of purified vWF/ml.

SDS/PAGE and Western blotting

SDS/polyacrylamide gels (with 3.5–20 or 6–12% linear polyacrylamide gradients) were performed as described by Laemmli

[48] in a vertical slab-gel system. Two dimensional (non-reducing/reducing) PAGE was performed by using a Laemmli slab gel (7.5% polyacrylamide) and unreduced samples in the first dimension. After migration the selected lane was cut, immersed in 2% (v/v) glycerol/100 mM-dithiothreitol/1% SDS/0.125 M-Tris/HCl buffer, pH 6.8, for 1 h at 37 °C, and placed at the top of a vertical slab gel composed of a stacking gel (7.5% polyacrylamide) and a 7.5–20% polyacrylamide gradient running gel.

SDS/agarose/PAGE was performed using a continuous buffer system as previously described [49].

After electrophoresis the proteins were stained with Coomassie Blue or electrotransferred on to nitrocellulose membranes as described by Towbin *et al.* [50] and revealed by using a labelled antibody. Radiolabelled material was revealed by autoradiography. In other experiments the purity of labelled proteins was estimated by slicing each lane of the dried gel into 5 mm pieces and counting them for radioactivity. Purified F.VIII was revealed using a silver-staining protocol as described by Morrissey [51]. Markers included high- and low-molecular-mass standards (Bio-Rad Laboratories, Richmond, CA, U.S.A., and Pharmacia), purified IgG (160 kDa), fibrinogen (340 kDa) and fibronectin (440 kDa).

RESULTS

Characterization of purified F.VIII and vWF

When F.VIII concentrates were applied on to MAB 463-A8 coupled to Sepharose, about 60% of the F.VIII was bound. Elution from the MAB using a 50% solution of ethylene glycol yielded a recovery of ~60%. The F.VIII preparations contained ~200 units of VIII:C/ml (~6000 units of VIII:C/mg) and

Fig. 2. Schematic representation of proteolytic fragments of vWF

The Figures shows the subunit composition of the fragments, their localization within the vWF subunit and their reactivity towards selected MAbs to vWF. SpIII, SpII and SpI were obtained after digestion of vWF by V8 proteinase. P34 fragment was produced by digestion of SpIII with plasmin. The 39/34 kDa fragment was isolated after digestion of vWF with dispace. III-T2 and the 270 kDa fragments were generated from SpIII and vWF respectively by trypsin digestion. The 120 and 260 kDa species were produced by digestion of vWF by protease 1 from *C. atrox*.

0.2 unit of von Willebrand Factor-related antigen (vWF:Ag)/ml. Immunopurified F.VIII appears as a series of bands when analysed by SDS/PAGE under non-reducing conditions (Fig. 1a). The bands corresponding to the heavy chain were observed at 210, 180, 160, 130 and 115 kDa respectively. A major band at 80 kDa corresponded to the light chain. No other band could be detected; in particular, a total lack of material with molecular masses 70, 50 and 45 kDa demonstrated the absence of activated F.VIII in the preparations.

The preparations of purified vWF contained ~ 200 units of vWF:Ag/mg. The content in fibrinogen and fibronectin was less than 0.1 % (w/w); VIII:C represented less than 0.01 unit/mg. Purified vWF appears as a set of 11–12 bands with molecular masses from 500 to over 10000 kDa when analysed unreduced by SDS/agarose/PAGE (results not shown).

Isolation and characterization of 120 and 260 kDa fragments

The kinetics of the digestion of vWF by protease 1 using an enzyme/substrate ratio of 1:50 showed the formation of two

major fragments with molecular masses of 120 and 260 kDa. Both species increased to a maximum within 2 h. They were then stable to prolonged digestion (24 h) and thus appeared to be final products. As indicated by blue staining of SDS/PAGE gels and Western blots using MAbs (results not shown) the 260 kDa fragment appeared as a primary species. By contrast, the 120 kDa fragment appeared to be derived from a transient 140 kDa fragment which did not interact with MAbs specific for the 260 kDa fragment (results not shown).

An analysis of the 3 h digest is presented in Fig. 1. The 260 kDa fragment was recognized by MAbs 8 and 549, but it did not interact with MAbs 418 and 328 (Fig. 1b). Thus the 260 kDa fragment was localized in the C-terminal part of vWF subunit and overlapped SpI and SpII (Fig. 2). The 120 kDa fragment was recognized by MAbs 418 and 328, but not by MAbs 8 and 549 (Fig. 1b). Thus it belonged to the N-terminal end of vWF subunit (Fig. 2).

After purification the 260 kDa fragment appeared as a single band by SDS/PAGE under non-reducing conditions (Fig. 1b).

Fig. 3. Binding analysis of purified F.VIII to purified vWF or vWF fragments

vWF or fragments were immobilized on to wells of microtitre plates ($\sim 0.05 \mu\text{g}/\text{well}$) as described in the Materials and methods section. After washing in the presence of 0.4 M-CaCl_2 , $100 \mu\text{l}$ of purified F.VIII in Michaelis buffer/BSA containing 40 mM-CaCl_2 were incubated for 1 h at 37°C . Bound F.VIII was estimated by using a chromogenic assay. Each point represents the mean $\pm$ S.D. for n experiments. Non-specific binding was estimated by using BSA-coated wells (Δ). The immobilized ligand was: (a): $\bullet$, vWF ($n = 12$); $\circ$, SpIII ($n = 7$); $\blacktriangle$, SpII ($n = 3$); (b): $\blacklozenge$, 120 kDa fragment ($n = 5$); $\blacktriangle$, 260 kDa fragment ($n = 4$); $\square$, 270 kDa species ($n = 4$); (c): $\diamond$, P34 ($n = 8$); $\blacktriangle$, 39/34 kDa fragment ($n = 3$); $\blacksquare$, III-T2 ($n = 3$); $\square$, SpI ($n = 4$).

By immunoblotting and i.r.m.a., the purified 260 kDa fragment reacted with MAbs 8 and 549, whereas epitopes 418 and 328 were no longer detectable in the preparation (results not shown). After reduction the purified 260 kDa fragment appeared composed of five species with molecular masses of 106, 95, 86, 78 and 48 kDa (Fig. 1b). Purified 120 kDa fragment appeared as a single band, with trace amounts of a 86 kDa species (Fig. 1b). Both fragments recognized MAb 418, and the purified 120 kDa species reacted with MAb 328. Epitopes 8 and 549 were not detectable by immunoblotting and i.r.m.a. in the preparations (results not shown). Under reducing conditions the mixture also exhibited five components of molecular masses 42, 38, 35, 30 and 27 kDa (Fig.

1b). As demonstrated by two-dimensional (non-reducing/reducing) PAGE, all the five species belonged to both the 120 kDa and the 86 kDa fragments (results not shown). MAb 543 recognizing reduced P34 reacted against the 30 and 27 kDa subunits (results not shown).

Isolation and characterization of a tryptic 270 kDa fragment

Proteolysis of vWF by trypsin generated four main fragments of molecular masses 270, 190, 130 and 34 kDa (Fig. 1c). Immunoblots showed that MAb 418 reacted with the 34 kDa fragment. The recognition of the 190 and 130 kDa fragment by MAb 328 indicated that both overlapped and were derived from the central part of the vWF subunit. The 270 kDa fragment reacted with MAbs 549 and 8, but not with MAbs 418 and 328. Thus the 270 kDa fragment belonged to the C-terminal end of vWF subunit and overlapped the cleavage site of V8 proteinase at residues 1365–1366 (Fig. 2).

The immunopurified 270 kDa tryptic fragment appeared as a single band by SDS/PAGE under non-reducing conditions (Fig. 1c). In addition, by immunoblotting with MAbs 549 and 8, only the 270 kDa species was detected. Epitopes 418 and 328 were no longer present in the preparations (results not shown).

After reduction, the purified 270 kDa fragment appeared to be composed of multiple species ranging from 118 to 33 kDa, with major bands at 118, 88, 68, 57, 48/44 and 33 kDa (Fig. 1c).

Characterization of P34, III-T2 and 39/34 kDa fragments

When analysed by SDS/PAGE and Coomassie Blue staining, P34 fragment appeared as a major band with a molecular mass of 34 kDa and several faint bands, barely seen, at ~ 68 , ~ 50 , ~ 25 and ~ 20 kDa (results not shown). Quantification performed using the labelled preparation and counting of the gel for radioactivity indicated that the P34 species represented 89% of the loaded material. Taken together, species with molecular masses of 68 and 50 kDa accounted for 5% of the radioactivity, whereas 6% of it was located on the lowest-molecular-mass fragments. On immunoblotting, purified P34 and 68 kDa species reacted with MAb 418; by i.r.m.a., using MAb 328 and 418 as first and second antibody respectively, the presence of epitope 328 could be detected, corresponding to an equivalent weight of SpIII of about 0.2%.

When analysed by SDS/PAGE, preparations of purified III-T2 exhibited a major band with a molecular mass of 87 kDa (using Coomassie Blue staining), with trace amount bands of 67 and 50 kDa. When immunoblotting with MAb 328, only the major 87 kDa fragment was revealed. Epitopes 418, 549 and 8 could not be detected in the purified material.

The purified 39/34 kDa disperse fragment appeared as a single band when SDS/PAGE gels were Coomassie Blue-stained, and it represented more than 99.5% of the material, as judged by using labelled preparations. Immunoblotting using MAb 328 revealed only the 39/34 kDa fragment, and epitopes 418, 549 and 8 were not detectable in the preparations (results not shown).

F.VIII binding to immobilized vWF or proteolytic fragments

Preliminary experiments using ^{125}I -vWF or purified fragments established that coating of proteins on to microtitre wells was dose-dependent, with a plateau ($\sim 0.05 \mu\text{g}/\text{well}$ of immobilized protein) reached when the concentration was $\sim 5 \mu\text{g}/\text{ml}$ ($0.5 \mu\text{g}/\text{well}$). In the following experiments, coating was performed at a protein concentration of $10 \mu\text{g}/\text{ml}$ ($1 \mu\text{g}/\text{well}$), and the purified proteolytic fragments were compared with vWF for their ability to interact with purified F.VIII in the binding assay.

Binding data obtained using increasing concentrations of F.VIII and immobilized vWF or fragments as ligand are presented in Fig. 3. When vWF was used, binding of F.VIII was

Fig. 4. Protection of F.VIII against APC by vWF or proteolytic fragments

F.VIII (final concn. of 0.5 unit/ml) premixed with vWF or a proteolytic fragment (final concn. ranging from 0.01 to 150 $\mu\text{g/ml}$) was incubated with APC (0.4 $\mu\text{g/ml}$) for 1 h at 37 °C in the presence of phospholipids. The reaction was stopped by 20-fold dilution and cooling to 4 °C. Residual F.VIII was estimated by a chromogenic assay. Results are expressed as a percentage (mean $\pm$ s.d. for n experiments) of the F.VIII activity of samples incubated in the absence of APC. F.VIII was incubated in the presence of: (a): ●, vWF ($n = 5$); ○, SpIII ($n = 3$); □, SpII ($n = 2$); (b): ◆, 120 kDa ($n = 3$); △, 260 kDa fragment ($n = 3$); (c): ◇, P34 ($n = 7$); ▲, III-T2 ($n = 2$); △, 39/34 kDa fragment ($n = 3$); □, Spl ($n = 3$).

concentration-dependent and saturable. The total amount of bound F.VIII increased from 1 to 4.7 munits of VIII:C/well as the concentration of F.VIII increased from 0.03 to 3 units/ml (3–300 munits/well). Non-specific binding increased to 0.1 munit/well at the highest concentration of F.VIII tested. Thus, assuming a molecular mass of 275 kDa for vWF (0.05 $\mu\text{g/well}$) and 260 kDa for F.VIII, with an activity of ~ 6000 units/mg, our data indicated that only $\sim 1\%$ of the vWF subunits participated in the interaction. Among the nine purified fragments tested, only those containing epitope 418, i.e. SpIII, 120 kDa and P34 fragments, had the ability to bind F.VIII in a dose-dependent and saturable fashion. The dose-response curves (Fig. 3) reached a maximum of 3.1, 0.5 and 0.6 munit/well for SpIII, 120 kDa and P34 fragments respectively when the F.VIII concentration was 3 units/ml (300 munits/well). These data suggested a similar binding capacity for SpIII and vWF. About 1% of the SpIII

subunit (molecular mass 170 kDa; 0.05 $\mu\text{g/well}$) bound F.VIII. In contrast, 120 kDa and P34 fragments exhibited a decreased binding capacity for F.VIII as compared with vWF. Assuming molecular masses of 60 and 34 kDa respectively, only 0.06 and 0.04% of the subunits interacted with F.VIII.

In contrast, binding of F.VIII to other fragments, either overlapping SpIII, i.e. III-T2, 39/34 kDa and Spl fragments, or located on the C-terminal part of vWF subunit, i.e. SpII, 260 and 270 kDa species, was not significantly distinct from the non-specific binding upon repeated testing (Fig. 3).

Protection of F.VIII against inactivation by APC by vWF or proteolytic fragments

When F.VIII (0.5 unit/ml) was incubated for 1 h in the presence of APC, VIII:C decreased to 10% of the activity estimated in the absence of the proteinase. Fig. 4(a) shows that increasing the concentration of vWF prevented the inactivation of F.VIII in a dose-dependent way. A maximal effect corresponding to a recovery of 100% of VIII:C was observed with a vWF concentration of 10 $\mu\text{g/ml}$ (3.6×10^{-8} mol/l). The half-maximal effect was reached at ~ 1 $\mu\text{g/ml}$ (3.6×10^{-9} mol/l). Among the nine purified fragments tested, only SpIII and 120 kDa fragments had the ability to protect F.VIII in a dose-dependent fashion (Figs. 4a and 4b). A maximal protection of more than 95% occurred when the concentration reached 10 and 50 $\mu\text{g/ml}$ (5.8×10^{-8} and 8.3×10^{-7} mol/l) of SpIII and 120 kDa fragments respectively. The half-maximal effect was observed at 1 $\mu\text{g/ml}$ (5.8×10^{-9} mol/l) for SpIII and 5 $\mu\text{g/ml}$ (8.3×10^{-8} mol/l) for the 120 kDa fragment. By contrast, the smaller fragment P34 used at a concentration as high as 150 $\mu\text{g/ml}$ and other fragments (i.e. 39/34 kDa, III-T2, Spl, 260 and 270 kDa fragments) were unable to inhibit the inactivation of F.VIII by APC (Figs. 4b and 4c). Control experiments performed using the chromogenic substrate S2366 to estimate the amidolytic activity of APC demonstrated that neither vWF nor its fragments had the capacity to inhibit APC in the various steps of the present study (results not shown).

DISCUSSION

In plasma, vWF binds to F.VIII and stabilizes its procoagulant activity [3,20]. This binding capacity has been related to the presence of specific sites on both molecules. By using synthetic peptides and selected MAbs to F.VIII, a binding domain for vWF has been localized on the light chain of F.VIII between AA 1670 and 1689 [5–9]. Similarly, using proteolytic fragmentation of vWF and selected MAbs, a binding site for F.VIII has been identified on the N-terminal part of vWF between residues 1 and 272 [14,15] and the specific role of the sequence Thr-78–Thr-96 in the interaction has been suggested [17]. Other studies, by Koedam *et al.* [21], confirmed the presence of a binding domain of vWF for F.VIII within the N-terminal part of the molecule. They suggested, in addition, that a second site interacting with F.VIII existed close to, or overlapping, the site of cleavage of the vWF subunit by V8 proteinase (residues 1365–1366).

Little is known about the mechanism by which binding of vWF to F.VIII induces the stabilization of F.VIII activity by protecting it against proteolytic degradation. However, several observations performed *in vivo* [4] or *in vitro* [11] indicated that the presence of bound vWF was not sufficient to prevent F.VIII degradation, thus suggesting that a specific conformation of the vWF subunit which bound to F.VIII was required. In addition, when comparing the binding and protecting properties against APC of a series of vWF fragments containing one of the two potential domains interacting with F.VIII, Koedam *et al.* [21] and Fay *et al.* [22] concluded that structures adjacent to those

involved in the binding were necessary to confer a protecting capacity to fragments.

In the present study we attempted to discriminate better the vWF structures involved in the binding to F.VIII from those required for its protection against proteolytic degradation. For this purpose a series of fragments of vWF were produced, purified and tested both in a solid-phase system for their capacity to bind to purified F.VIII and in a liquid-phase assay for their ability to protect F.VIII against APC. Two criteria were followed for the choice of fragments: (1) they overlapped the whole sequence of vWF subunit; and (2) each of them overlapped sites of proteolytic cleavage responsible for the generation of other fragments and thus for destruction of potential interactive F.VIII domains.

The presence of a binding domain for F.VIII on the *N*-terminal end of vWF was confirmed using SpIII, a homodimer of the sequence 1–1365 [14], P34 fragment, a monomer extending from AA 1 to 272 [14,15] and a tryptic 120 kDa fragment of intermediate length characterized by using MAbs. The three fragments, which all overlapped the sequence AA 1–272 and contained the 418 epitope known to be involved in the binding of vWF to F.VIII, specifically bound F.VIII in a dose-dependent and saturable way. However, analysis on a molar basis of the respective binding curves obtained with similar amounts of coated proteins strongly suggested that the three species recognized F.VIII with different capacities. On the one hand we observed that only $\sim 1\%$ of the subunits of vWF and SpIII fragment participated in this interaction. Our values for vWF are in agreement with those previously observed by Leyte *et al.* [8] and suggest that not all the vWF subunits are equivalent with regard to F.VIII binding. In addition, the similarity of the binding parameters that we observed for SpIII and vWF indicated that the difference between subunits of vWF remained in the smaller SpIII molecule. We thus conclude that such a difference cannot be simply explained by a steric hindrance modulating the accessibility of F.VIII to subunits according to their location either inside or outside a globular vWF protein. In contrast, this can be related to the existence of at least two distinct native conformations of the *N*-terminal end of the subunits. Only one, relative to a small fraction of the subunits ($\sim 1\%$), would confer a high affinity for F.VIII.

The 120 kDa and P34 fragments also significantly bound to F.VIII and exhibited equivalent binding parameters. Their binding capacity was diminished, however, as compared with vWF or SpIII. This is assumed to reflect significant conformational changes induced during the purification procedures, because both species were derived from vWF or SpIII by a striking shortening of the amino acid sequence after extreme proteolytic degradation and were purified under denaturing conditions. In addition, the similarity of the binding properties of the two fragments also supported that the 120 kDa species did not contain an additional binding site for F.VIII within its *C*-terminal part, in comparison with the P34 fragment.

Our experiments on the binding to F.VIII performed with other fragments of vWF also confirmed this latter conclusion. Taken together, the three species, P34, III-T2 and 39/34 kDa fragments which extend from AA 1 to 728, virtually encompass the entire length of the 120 kDa species, but exhibit a single F.VIII-binding domain on the P34 fragment. In addition, analysis of molecular masses of the reduced forms of the 120 kDa fragment and their reactivity against MAbs to vWF, as well as the comparison with the position of P34, III-T2 and 39/34 kDa species in the vWF subunit, also suggested that the three fragments overlapped the sites of cleavages occurring within the structure of the 120 kDa fragment. We thus assume that the destruction by proteolysis of a potential secondary interactive

domain with F.VIII, located in the *C*-terminal portion of the 120 kDa fragment, is highly speculative.

Similarly, we observed a total failure to bind to F.VIII of four fragments derived from the *C*-terminal portion of vWF subunit, i.e. the tryptic 270 kDa fragment, the 260 kDa fragment generated by protease 1, SpI and SpII. In addition, the complementarity of SpI and SpII in the sequence of vWF, the reactivity of the 270 and 260 kDa species towards MAbs to vWF and the molecular masses of their reduced forms indicated that the 270 and the 260 kDa fragments overlapped the site of cleavage located between SpI and SpII (AA 1365–1366); conversely, the complementary fragments SpI and SpII overlapped the site of cleavage occurring within the 270 or 260 kDa fragments. We thus again assume that no potential secondary F.VIII binding site of vWF has been proteolysed during the generation of the *C*-terminal fragments and therefore that no F.VIII-binding site of vWF exists outside the sequence of the P34 fragment.

Our study on the protection of F.VIII against APC by vWF and a series of proteolytic fragments clearly indicated that binding to F.VIII is the prerequisite condition for its stabilization. We observed that, among the six fragments devoid of a binding site for F.VIII, none had the capacity to protect it, even when used at high concentrations. Similarly we observed that vWF or fragments having the dual property of binding and protecting lost the two capacities in parallel when blocking their binding site with MAb 418 (not shown). In addition we found that, on a molar basis, vWF and SpIII had a similar capacity to protect F.VIII. A total protection of 0.5 unit/ml of F.VIII was reached with ~ 4 and 6×10^{-8} mol/l respectively, whereas the same effect required about 10 times more 120 kDa fragment ($\sim 8 \times 10^{-7}$ mol/l). Within the accuracy of the method, these values are in close agreement with the binding parameters that we observed. In addition, comparison of these values, together with the characterization of the purified material by gel electrophoresis and reactivity with MAbs to vWF, clearly indicates that the protecting effect induced by the 120 kDa species cannot result from the presence of contaminating material in the preparation. Our results also confirm and extend previous observations from others [21,22] that the presence of the binding site is not sufficient in itself to carry a protecting capacity. We showed that the P34 fragment contained a binding domain for F.VIII with an equivalent affinity to that of the 120 kDa fragment. In contrast, we observed that P34 fragment was totally unable to protect F.VIII against proteolytic degradation by APC, even at high concentrations. Assuming that similar binding characteristics implied a similar capacity to protect F.VIII, we expected that a total inhibition of the effect of APC would be obtained when the concentration of P34 fragment was $\sim 10^{-6}$ mol/l ($\sim 30 \mu\text{g/ml}$). However, even at a five times higher concentration, P34 fragment did not exhibit any significant protecting effect. These results clearly show that additional structures present in the 120 kDa fragment are required to protect F.VIII. Our study on the reactivity of this fragment for MAbs to vWF suggested that it did not overlap SpI (AA 911–1365). We thus assume that a sequence located between AA 272 and 911 should play a major role in the mechanism leading to the protection of F.VIII. In agreement with findings from others [21,22], the protecting capacity that we observed is clearly dependent upon the size of the substrate, strongly suggesting that it may act by steric hindrance at the level of either the binding site of APC or phospholipids on the light chain of F.VIII or at the level of the cleavage site by APC on the heavy chain.

In conclusion, the present study demonstrates that no F.VIII-binding domain exists on the vWF subunit outside the sequence AA 1–272. The capacity to bind to F.VIII appears highly dependent upon a specific conformation found in $\sim 1\%$ of the

native vWF subunits. The capacity to bind to F.VIII is a prerequisite condition for the protein to acquire the ability to protect F.VIII against APC. An additional sequence located between P34 and Spl fragment appears necessary, however, to confer the protecting capacity. We do not yet know its exact role in the mechanism of the binding/protecting of F.VIII and its smallest active form in the vWF subunit. Such information should follow from more precise study on the structure of the 120 kDa fragment and its proteolytic degradation within the context of the interaction with F.VIII.

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