Requirements of von Willebrand Factor to Protect Factor VIII From Inactivation by Activated Protein C

By Stefan J. Koppelman, Maggy van Hoeij, Tom Vink, Hanneke Lankhof, Marion E. Schiphorst, Conchi Damas, André J. Vlot, Robert Wise, Bonno N. Bouma, and Jan J. Sixma

The interaction of factor VIII with von Willebrand factor (vWF) was investigated on a quantitative and qualitative level. Binding characteristics were determined using a solid-phase binding assay and protection of factor VIII by vWF from inactivation by activated protein C (aPC) was studied using three different assays. Deletion mutants of vWF, a 31-kD N-terminal monomeric tryptic fragment of vWF that contained the factor VIII binding site (T31) and multimers of vWF of different size were compared with vWF purified from plasma. We found that deletion of the A1, A2, or A3 domain of vWF had neither an effect on the binding characteristics nor on the protective effect of vWF on factor VIII. Furthermore, no differences in binding of factor VIII were found between multimers of vWF with different size. Also, the protective effect on factor VIII of vWF was not related to the size of the multimers of vWF. A 20-fold lower binding affinity was observed for the interaction of T31 with factor VIII, and T31 did not protect factor VIII from inactivation by aPC in a fluid-phase assay. Comparable results were found for a mutant of vWF that is monomeric at the N-terminus (vWF-dPRO). The lack of multimerization at the N-terminus may explain the decreased affinity of T31 and vWF-dPRO for factor VIII. Because of this decreased affinity, only a small fraction of factor VIII was bound to T31 and to vWF-dPRO. We hypothesized that this fraction was protected from inactivation by aPC but that this protection was not observed due to the presence of an excess of unbound factor VIII in the fluid phase. Therefore, vWF, T31, and vWF-dPRO were immobilized to separate bound factor VIII from unbound factor VIII in the fluid phase. Subsequently, the protective effect of these forms of vWF on bound factor VIII was studied. In this approach, all forms of vWF were able to protect factor VIII against inactivation by aPC completely. We conclude, in contrast with earlier work, that there is no discrepancy between binding of factor VIII to vWF and protection of factor VIII by vWF from inactivation by aPC. The protective effect of T31 was not recognized in previous studies due to its low affinity for factor VIII. The absence of multimerization observed for T31 and vWF-dPRO may explain the low affinity for factor VIII. No other domains than the binding site located at the D' domain were found to be involved in the protection of factor VIII from inactivation by aPC.

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Submitted June 13, 1995; accepted October 18, 1995.

Supported in part by the Netherlands Heart Foundation (Grant No. 90.092).

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0006-4971/96/8706-0040$3.00/0

Blood, Vol 87, No 6 (March 15), 1996: pp 2292-2300

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A

Fig 1. (A) vWF is represented as a bar consisting of four repeating domains. The signal peptide (SP) and the pro-peptide (D1-D2) are cleaved off before secretion, resulting in the mature vWF molecule: NH2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-COOH. For the dimerization, disulfide bonds are formed between the C-termini of the subunits, multimerization takes place at the D3 domains. For the clarity of this representation, only one disulfide bridge is depicted at the sites of dimerization and multimerization. The factor VIII binding site is located on the D' domain, whereas the D', D3, A1, A2, and a part of the A3 domain are necessary for protection of factor VIII against inactivation by aPC. The black parts show the deleted domains.

To obtain optimal protection, a ratio factor VIII to vWF of 1 to 1 in units (1 to 50 on a molar base) was required. This may explain the in vivo ratio of factor VIII and vWF.

It is generally accepted that the factor VIII binding site is located on the first 272 amino acids of vWF, but, strikingly, a fragment representing this binding site was not sufficient to protect factor VIII against aPC-mediated degradation. The difference in protection against inactivation by aPC between these two fragments may be explained by the presence of the D', D3, A1, A2, and A3 domain in the 120-
kD fragment. Another possibility is that the size\textsuperscript{31,34} and/or dimeric state\textsuperscript{34} of the 120-kD fragment are important in the protection of factor VIII against inactivation by aPC.

This study was performed to elucidate the mechanism by which vWF protects factor VIII against inactivation by aPC. Using a tryptic fragment and several mutants of vWF (Fig 1B) we found, in contrast with earlier work, that binding of factor VIII to the small tryptic fragment representing the first 272 amino acids of vWF is sufficient to protect factor VIII against aPC-catalyzed degradation.

**MATERIALS AND METHODS**

**Materials.** Purified recombinant factor VIII was a generous gift from Dr D. Pittman (Genetics Institute, Cambridge, MA). Factor VIII concentrations are commonly expressed in units per mL. One unit represents the amount of factor VIII activity in 1 mL of pooled normal human plasma. Assuming a molecular weight of 250 kD and a plasma concentration of 0.2 \mu g/mL, 1 U/mL corresponds to 1 nmol/L. Factor VIII concentrates used for the purification of vWF were from the Red Cross Blood Bank (Friesland, The Netherlands) and were, for this purpose, not heat-treated. Protein-G Sepharose was obtained from Pharmacia (Uppsala, Sweden). The factor VIII Coatest and chromogenic substrate S2366 were obtained from Chromogenix (Stockholm, Sweden). Ninety-six-well plates were from Costar (Cambridge, MA). Bovine serum albumin (BSA) and bovine brain cephalin were purchased from Sigma (St Louis, MO). Bio-Gel A15 was from BioRad (Richardson, CA). All other chemicals were of the highest grade available.

**Antibodies.** MoAb CLB-CAGA directed against the light chain of factor VIII and MoAb CLB-41 directed against the C-terminus of vWF were kind gifts of Dr J.A. van Mourik (Central Laboratory of The Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands). An enzyme-linked immunosorbent assay (ELISA) to measure vWF was constructed using polyclonal antibodies (PoAbs) and peroxidase-conjugated PoAbs directed against vWF (DAKO, Glostrup, Denmark) with normal pooled plasma used as a standard. T31 was measured using the same ELISA, with purified T31 as a standard.

**Construction of mutants of vWF.** vWF that was dimeric at the C-terminus (vWF-dPRO) was described earlier.\textsuperscript{15} The polymerase chain reaction (PCR) gene fusion technique\textsuperscript{16} was used to construct the other deletion mutants of vWF. Amino acids 478 to 716, 730 to 910, or 910 to 1113 were deleted to obtain vWF lacking the A1 domain (vWF-dA1), the A2 domain (vWF-dA2), or the A3 domain (vWF-dA3), respectively. Deletion constructs were cloned in the furin cDNA containing baby hamster kidney cells were used for transfection. Stable cell lines were selected with methotrexate. Recombinant vWF was expressed in medium containing 1% Ultroser G and tested for vWF antigen and factor VIII antigen. Multimer analyses were performed as described.\textsuperscript{17} The factor VIII-free, vWF-containing fractions were dialyzed against 50 mmol/L N\textsubscript{2}-hydroxyethyl-\textsubscript{6}ylypipеразин-\textsubscript{2}антиксронный сульфат (HEPS), pH 7.4, containing 150 mmol/L NaCl. Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed one band at 240 kD and a faint triplet at 40-48-60 kD (probably representing fibrinogen, approximately 1% of the total amount of protein). T31 was prepared from purified vWF as described\textsuperscript{15} and shown on a nonreduced SDS-PAGE a doublet at 31 kD. Protein C was purified and activated as described previously.\textsuperscript{40} aPC showed on SDS-PAGE a doublet at approximately 60 kD. Protein concentrations were measured with the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions with BSA as a standard.

**Factor VIII-vWF binding assay.** Microtiter wells were coated overnight with 1 \mu g/mL MoAb CLB-41 directed against vWF in 50 mmol/L Na\textsubscript{2}CO\textsubscript{3}, pH 9.5 at 4°C. The wells were washed once with 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl (TBS) containing 0.1% Tween-20. After blocking with TBS containing 0.1% Tween-20 and 3% BSA for 1 hour at 37°C, the wells were incubated with different forms of vWF (0.25 nmol/L [0.005 U/mL]) in TBS containing 0.1% Tween-20 and 3% BSA for 2 hours at 37°C. The concentrations of vWF before and after incubation were measured with a vWF ELISA. By subtracting both concentrations and taking into account the volume of the wells, the amount of vWF bound to the wells was calculated. The wells were washed three times with TBS containing 0.2% Tween-20 and subsequently incubated with factor VIII (0 to 20 nmol/L) in TBS containing 0.1% Tween-20, 3% BSA, and 3 mmol/L CaCl\textsubscript{2} for 1 hour at 37°C. After washing three times with TBS containing 0.1% Tween-20, bound factor VIII was quantified using the factor VIII Coatest (Chromogenix) with purified factor VIII as a reference. When normal plasma was used as a source of vWF, a 1/200 dilution of plasma in TBS containing 0.1% Tween-20 and 3% BSA was used instead of purified vWF. After washing, endogenous factor VIII was removed by incubation with TBS containing 0.1% Tween-20, 3% BSA, and 250 mmol/L CaCl\textsubscript{2} for 30 minutes at 37°C. In another series of experiments, purified vWF or T31 was coated directly to the wells in 50 mmol/L Na\textsubscript{2}CO\textsubscript{3}, pH 9.6, overnight at 4°C. Nonspecific binding of factor VIII to the wells was determined in the absence of vWF and was less than 10%. Dissociation constants and stoichiometries were calculated by Scatchard analysis of the data using Enzfitter software.\textsuperscript{19}

**Inactivation of factor VIII by aPC in solution.** Factor VIII (1.1 nmol/L [1.1 U/mL]) was incubated with vWF (0 to 165 nmol/L [0 to 3.3 U/mL]) for 30 minutes at 37°C in 100 \mu L of 50 mmol/L HEPS, pH 7.4, containing 0.3% BSA, 150 mmol/L NaCl, and 3 mmol/L CaCl\textsubscript{2}. After the addition of 10 \mu L of a 1/10 dilution of cephalin and incubation for 30 minutes, the reaction was started by the addition of aPC (4 nmol/L final concentration). Samples of 5 \mu L were collected and immediately diluted 100-fold in ice-cold TBS containing 0.3% BSA and 5 nmol/L [0.1 U/mL] vWF to prevent further inactivation of factor VIII by aPC. Once diluted, factor VIII was stable for at least 1 hour. Factor VIII was measured using the factor VIII Coatest. Control experiments using the chromogenic substrate S2366 showed that neither form of vWF inhibited aPC directly.

**Inactivation of factor VIII bound to vWF immobilized on microtiter plates.** Wells were coated with vWF (0.5 nmol/L [0.1 \mu g/mL]), vWF-dPRO (0.5 nmol/L [0.1 \mu g/mL]), MoAb CLB-CAGA (33 nmol/L [5 \mu g/mL]), or T31 (400 nmol/L [10 \mu g/mL]) overnight in 50 mmol/L Na\textsubscript{2}CO\textsubscript{3}, pH 9.6, at 4°C. Wells were washed three times with TBS containing 0.1% Tween-20. After blocking with TBS containing 0.1% Tween-20 and 3% BSA for 1 hour at 37°C, factor VIII (25 nmol/L [25 U/mL]) was incubated for 1 hour at 37°C in TBS containing 0.1% Tween-20, 3% BSA, and 3 mmol/L CaCl\textsubscript{2}. The wells were washed three times with TBS containing 0.1% Tween-20, and bound factor VIII was incubated with 0 to 4 nmol/L aPC for 5 minutes. Microtiter plates were emptied and the remaining factor VIII was measured using the factor VIII Coatest. Non-specific binding of factor VIII to the wells was determined in the absence of vWF and was less than 10%.
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RESULTS

The binding of factor VIII to the different forms of vWF. The stoichiometry and binding affinity of the interaction of factor VIII with vWF was studied using a solid-phase binding assay. Various amounts of purified factor VIII were incubated for 1 hour on immobilized vWF and bound factor VIII was measured using a chromogenic assay. This solid-phase binding assay was previously used to study the interaction between factor VIII and vWF.14,21 Figure 2 shows the saturation curve of the binding of factor VIII to vWF purified from plasma. Scatchard analysis of the data showed a single class of high-affinity binding sites with a dissociation constant of 0.3 ± 0.03 mmol/L. The stoichiometry at saturation was 1 mol of factor VIII per 50 mol of vWF (1.0 U factor VIII per 1.0 U vWF). The characteristics of the binding between factor VIII and the different forms of vWF were tested using this binding assay. Table 1 shows the dissociation constants and stoichiometries at saturation. vWF captured directly from plasma yielded the same binding characteristics as purified vWF, indicating that the purification procedure did not affect the binding of factor VIII. Also, multimers of vWF of different size were tested on their ability to bind factor VIII. Three fractions obtained from a gel filtration column were used. These fractions show on a Western blot of a nonreducing agarose gel, stained for vWF, heavy, intermediate, and light multimers, respectively (data not shown). The binding affinities and stoichiometries for the different multimers were the same as for unfractionated vWF. Fragments comparable with T31 were earlier shown to contain the factor VIII binding site.33,42 This monomeric form of vWF (10 fmol/well [0.2 mU/well]) for 1 hour at 37°C. Bound factor VIII was measured with a chromogenic assay using normal plasma as a standard curve.

Table 1. Affinity and Stoichiometry of the Interaction of Factor VIII With Different Forms of vWF

<table>
<thead>
<tr>
<th>Form of vWF</th>
<th>kd ± SD (nmol/L)</th>
<th>Stoichiometry Factor VIII-vWF (mol/mol ± SD)</th>
<th>U/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture from normal plasma</td>
<td>0.352 ± 0.011</td>
<td>0.022 ± 0.003</td>
<td>1.1</td>
</tr>
<tr>
<td>Purified from plasma (pool)</td>
<td>0.30 ± 0.03</td>
<td>0.020 ± 0.004</td>
<td>1.0</td>
</tr>
<tr>
<td>Purified from plasma (pool)*</td>
<td>0.33 ± 0.04</td>
<td>0.205 ± 0.001</td>
<td>1.0</td>
</tr>
<tr>
<td>Purified different multimers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavy</td>
<td>0.38 ± 0.13</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.41 ± 0.14</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>0.37 ± 0.07</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>31-kD N-terminal fragment*</td>
<td>5.2 ± 0.6</td>
<td>0.0004 ± 0.0006</td>
<td></td>
</tr>
</tbody>
</table>

| Recombinant forms            |                  |                                             |     |
| Full length                  | 0.27 ± 0.04      | 0.024 ± 0.003                              | 1.2 |
| Delta A1                     | 0.29 ± 0.03      | 0.018 ± 0.001                              | 0.9 |
| Delta A2                     | 0.25 ± 0.03      | 0.024 ± 0.002                              | 1.2 |
| Delta A3                     | 0.25 ± 0.06      | 0.022 ± 0.003                              | 1.1 |
| Delta D4                     | 0.51 ± 0.05      | 0.026 ± 0.002                              | 1.3 |
| Delta PRO                    | 2.6 ± 0.5        | 0.018 ± 0.004                              | 0.8 |

Factor VIII (0 to 10 nmol/L) was incubated on different forms of immobilized vWF (10 fmol/well [0.2 mU/well]) for 1 hour at 37°C. Bound factor VIII was measured with a chromogenic assay using normal plasma as a standard curve.

Abbreviation: ND, not determined.

*Coated directly on the well.
factor VIII activity using the factor VIII Coatest as described in the three separate experiments.

The presence of aPC was added and the reaction was started after 30 minutes by the addition of aPC (4 nmol/L final concentration). In the control situation, no aPC was added (A). Samples were collected and assayed for factor VIII activity using the factor VIII Coatest as described in the Materials and Methods. Data points represent the mean ± SD of three separate experiments.

Although no binding affinities of fragments like T31 for factor VIII were published, the stoichiometry that we have found is comparable with the stoichiometry described by Layet et al. To investigate whether other N-terminal domains of vWF contributed to the factor VIII binding, mutants of vWF lacking the A1, A2, or A3 domain were tested on factor VIII. These recombinant vWF molecules had the same binding properties as vWF purified from plasma (Table 1). vWF-dPRO had a 10-fold lower affinity for factor VIII compared with the mature molecule, whereas the stoichiometry of the binding was not different (Table 1). The multimeric composition of vWF-dA2 and vWF-dA3 was similar to that of vWF in normal plasma, whereas vWF-dA1 consisted of primarily intermediate and small multimers (data not shown). The vWF-dPRO preparation consisted primarily of dimeric vWF.

Protection of factor VIII from inactivation by aPC by different forms of vWF in a fluid-phase assay. Factor VIII (1 nmol/L, 1 U/mL) was incubated with 4 nmol/L aPC in the presence of an optimal concentration of cephalin. Figure 3 shows a time course of the inactivation of factor VIII by aPC. Factor VIII activity decreased with a half-life of approximately 8 minutes, whereas vWF (50 nmol/L [1 U/mL]) completely protected factor VIII from inactivation by aPC (Fig 3). Because factor VIII in the absence of vWF is a labile molecule, we followed the factor VIII activity in the absence of both vWF and aPC. During the time course of the experiment, no significant decrease of factor VIII activity was observed. To investigate the protective effect of vWF in more detail, several concentrations of vWF were used.

Figure 4 shows the protection of factor VIII by vWF against aPC-mediated proteolytical degradation. A concentration of 1 nmol/L (1 U/mL) factor VIII is optimally protected by 50 nmol/L (1 U/mL) of vWF, whereas lower concentrations of vWF only partially protected factor VIII from inactivation by aPC. As a measure for the protection against aPC, the concentration of vWF necessary for half-maximal protection was determined. For vWF purified from plasma, this concentration was 6 ± 1 nmol/L (0.12 ± 0.02 U/mL; Fig 4). T31 did not protect factor VIII against aPC (Fig 4). Based on the affinity of T31 for factor VIII, one might expect that T31 should provide some protection, but, due to the low stoichiometry of this interaction, no protection of T31 against aPC was found. The different mutants of vWF were also tested for their ability to protect factor VIII against aPC. Table 2 shows the concentrations of the different forms of vWF at which half-maximal protection against aPC was reached. No protection by T31 and vWF-dPRO could be shown, whereas the deletion of the A1, A2, or A3 domain did not affect the protective effect. There was no difference in protection found between the high, intermediate, and low multimers (Table 2).
Protection of factor VIII against proteolytic degradation is closely related to the binding to vWF in vivo and in vitro. As described above, T31 and vWF-dPRO have decreased affinities for factor VIII as compared with the mature molecule (Table 1). Also, they did not protect factor VIII against aPC (Table 2). Because of the decreased affinity of vWF-dPRO and T31 for factor VIII and the low stoichiometry of the interaction between factor VIII and T31, only a small fraction of factor VIII may be bound to these forms of vWF. We hypothesized that this fraction was protected against aPC, but that this protective effect was not observed, due to the presence of an excess of unbound factor VIII. Therefore, we developed an assay that allowed us to investigate the inactivation of factor VIII bound to T31 or to vWF-dPRO in the absence of free factor VIII. The different forms of vWF were coated on microtiter plates and incubated with factor VIII. The coat concentrations were chosen such that the amount of bound factor VIII was the same for all forms of vWF and that the amount of factor VIII was the same as in the solid-phase binding assay. All forms of vWF protected factor VIII from inactivation by aPC completely (Fig 5), indicating that T31 and vWF-dPRO protected factor VIII as well as mature vWF. The possibility that aPC was not able to inactivate factor VIII in this assay was excluded because factor VIII bound to the microtiter plate via an MoAb directed against its light chain was degraded by 70%. Because binding of factor VIII to vWF immobilized on a microtiter plate was shown to be different from binding of factor VIII to vWF in solution, we also used a fluid-phase assay to separate factor VIII bound to vWF from free factor VIII. Factor VIII was incubated with the T31, vWF-dPRO, or mature vWF. Bound factor VIII was then isolated by incubation with Sepharose-linked polyclonal antibodies against vWF and subsequent centrifugation through a sucrose layer. The pellet was immediately resuspended, diluted to 50 pmol/L, and then treated with aPC and assayed for remaining factor VIII activity. Dilution to 50 pmol/L does not induce dissociation of the complex within the experimentation time (data not shown). Figure 5 shows that all forms of vWF protected factor VIII equally well. Factor VIII bound to Sepharose via an MoAb directed against the factor VIII light chain (CLB-CAGA) was not protected against aPC in this assay. The results of the experiments with isolated bound factor VIII indicate that binding of factor VIII to T31 or to vWF-dPRO is sufficient for protection against aPC.

**DISCUSSION**

Survival of factor VIII in vivo strongly depends on complex formation with vWF. Free factor VIII is rapidly cleared from the circulation, whereas factor VIII in complex with vWF decays with the half-life of vWF. Earlier work showed that vWF protects factor VIII against inactivation by aPC. The inactivation of factor VIII by aPC is a phospholipid-dependent process and, because the binding sites for both phospholipid and vWF on factor VIII are located in close spatial proximity on the light chain, masking of the phospholipid binding site on factor VIII by vWF may explain the protective effect of vWF. The binding site for factor VIII is located on the first 272 amino acids of vWF, but N-terminal fragments (amino acids 1-272, T313 and SPII-T431 or 1-298, P34) containing this binding site are not sufficient to protect factor VIII against inactivation by aPC. The difference between the 120-kD and T31 or P34 is not only the presence of the D3, A1, A2, and a part of the A3 domain, but also that the 120-kD fragment is dimeric at the N-terminus. Therefore, the discrepancy in binding and protection of factor VIII may be explained in two ways. First, the D3, A1, A2, or A3 domain may play an important role in the protection of factor VIII. Second, dimerization may be required for optimal protection. To elucidate the mechanism of protection of factor VIII from aPC-mediated inactivation, we tested several forms of vWF on their ability to bind factor VIII and to protect it against inactivation by aPC.

Using a solid-phase binding assay, we found that factor VIII bound to vWF with a kd of 0.30 nmol/L, corresponding to previously described values. Although solid-phase binding assay may not represent true equilibrium binding, we found in a previous study the same binding affinities using a fluid-phase binding assay. More recent work showed that the kd found using the fluid-phase binding assay represented indeed the ratio of $k_{diss}$ and $k_{prot}$ indicating that our solid-phase binding assay is suitable to determine binding affinities. Comparing multimers of vWF of different size, we found the same affinities for factor VIII for the different multimers. Deletion of the A1, A2, or A3 domain did not alter the binding characteristics, but deletion of the pro-peptide of vWF resulted in a 10-fold lower affinity. The pro-peptide of vWF is required for the multimerization of the vWF subunits at the N-terminus and deletion of this pro-peptide resulted in the expression of vWF, which was dimerized at the C-terminus (vWF-dPRO) while the N-termi-
Fig 5. Comparison of three factor VIII protection assays. (1) Factor VIII degradation in solution. (2) Degradation of factor VIII immobilized on microtiter plates via different forms of vWF or via an antibody directed against factor VIII (MoAb CLB CAgA). (3) Degradation of factor VIII immobilized on Sepharose beads via different forms of vWF or via an antibody directed against factor VIII (MoAb CLB CAgA). The first solid bar on the left represents the control incubation for the degradation of factor VIII in solution (factor VIII in the absence of either aPC or vWF). The second bar on the left shows the amount of factor VIII left after incubation with aPC in solution. These two controls were not performed for the degradation of factor VIII on microtiter plates or Sepharose beads, because a ligand (vWF or an anti-factor VIII antibody) is necessary to immobilize factor VIII. (4) Factor VIII degradation in solution. Factor VIII was incubated with different forms of vWF or MoAb CLB CAgA in solution for 30 minutes at 37°C. Cephalin was added and, after 30 minutes, the reaction was started by the addition of aPC as described in the Materials and Methods. (5) Factor VIII degradation on microtiter plates. Factor VIII bound to the different forms of vWF or MoAb CLB CAgA immobilized on microtiter plates was treated with aPC in the presence of cephalin as described in the Materials and Methods. (6) Factor VIII degradation on Sepharose beads. Factor VIII was incubated in the presence or absence of vWF, T31, or vWF-dPRO for 1 hour at 37°C. Subsequently, a suspension of protein G Sepharose-linked PoAbs against vWF or MoAb CLB CAgA was added. After 30 minutes of mixing at room temperature, bound factor VIII was collected by centrifugation and was treated with aPC in the presence of cephalin as described in the Materials and Methods. For all three assays, the remaining factor VIII activities were measured with the factor VIII Coatest and expressed as a percentage of the control situation without aPC. Bars represent the mean ± SD of three separate experiments. Because of insufficient amounts of vWF-dPRO, fluid-phase experiments with vWF-dPRO were performed two times and data points represent the mean. Separate values did not differ more than 10% of the mean.

The ability of vWF-dPRO to bind factor VIII is disrupted. One of us reported that the factor VIII binding is unaltered, whereas Leyte et al. reported defective factor VIII binding by vWF-dPRO. This difference is most likely explained by the different cell types used for the expression of both forms of vWF-dPRO or by the presence of an extra Ala residue in vWF-dPRO described by Leyte et al. We used vWF-dPRO described by Wise et al. and found that it was able to bind factor VIII, although with a 10 times lower affinity than normal vWF, either purified from plasma or full-length recombinant vWF. The N-terminal fragment T31 that contained the factor VIII binding site had also a lower affinity for factor VIII as compared with normal vWF. Binding of factor VIII to fragments similar to T31 was described earlier, but the affinity of the interaction was not reported. The stoichiometry that we have found the interaction between factor VIII and T31 is comparable with the stoichiometry described by Layet et al. They found that this stoichiometry was decreased 20- to 50-fold as compared with mature vWF. This decrease in stoichiometry was explained by significant conformational changes due to proteolytical degradation.

Because a 120-kD N-terminal fragment both binds and protects factor VIII quite as well as normal vWF, the low affinity that we have found for the interaction of T31 with factor VIII may be explained in two ways. First, other domains of vWF may be required for optimal binding of factor VIII. However, this is not likely because deletion of the A1, A2, or A3 domain did not influence the binding of factor VIII. Deletion of the D3 domain was not possible because this domain contains the cysteine residues involved in the disulphide bond formation required for the multimerization. Because vWF-dPRO, which contains all domains of the mature vWF, also expressed a low affinity for factor VIII, a possible role for the D3 domain in the binding of factor VIII is unlikely. The other explanation for the decreased binding affinity may be that the multimerization at the N-terminus of the vWF subunits is important for the factor VIII binding. This is in agreement with the binding characteristics of vWF-dPRO, which is also not multimerized at the N-terminus. Based on these results, we suggest that dimerization at the N-terminus of the vWF subunits increases the binding affinity for factor VIII. However, this dimerization is not a requirement to bind factor VIII.

We found that vWF was able to protect factor VIII against aPC in the fluid phase. As a standard for protection of factor...
VIII by vWF, we measured the concentration of vWF necessary for half-maximal protection. Because factor VIII binds to both phospholipid surfaces and vWF, the presence of phospholipid decreases the binding affinity of vWF for factor VIII. Therefore, values found for half-maximal protection cannot be compared with the binding affinity as presented in Table 1. Deletion of either the A1, A2, or the A3 domain did not affect the protective effect of vWF. No differences in protection were found between multimers of vWF of different size. However, vWF that was dimeric at the C-terminus and that expressed a monomeric N-terminus (vWF-dPRO), did not protect factor VIII against inactivation by aPC. The same results were obtained for T31, which is also monomeric at the N-terminus. Based on the kd for the interaction between T31 and factor VIII of 5.2 nmol/L described in this study, one might expect that 100 nmol/L T31 should protect 1 nmol/L factor VIII to some extent. However, this was not observed (Fig 4). Because of the low stoichiometry of the binding of factor VIII to T31, protection of factor VIII by T31 is expected at a molar excess of T31 of at least 2,500. T31 and vWF-dPRO have low affinity for factor VIII as compared with normal vWF and the interaction of factor VIII with T31 expressed a low stoichiometry. These parameters predict that, in a mixture of factor VIII and T31 or vWF-dPRO, only a small fraction of factor VIII is bound to T31 or vWF-dPRO. The presence of an excess of unbound factor VIII, which is not protected against inactivation by aPC, may mask a possible protective effect on the bound fraction of factor VIII. These considerations devalue the assay that we and others have used to investigate a possible protective effect of vWF fragments on factor VIII. Therefore, two assays were developed in which factor VIII bound to the different forms of vWF was separated from free factor VIII. In the first assay, factor VIII was bound to the different forms of vWF coated on microtiter plates. Factor VIII bound to all forms of vWF used in this study appeared to be completely protected against aPC. The binding of factor VIII to vWF immobilized on a surface is different from the binding in solution. Although the same affinity is found in both systems, the stoichiometry of the binding in the fluid-phase is 20-fold higher. The underlying mechanism for the discrepancy of factor VIII binding in the solid- and fluid-phase assay is not entirely understood. To exclude possible effects of the immobilization of the different forms of vWF, a fluid-phase assay was developed in which bound factor VIII was separated from unbound factor VIII. In this assay, factor VIII bound to the different forms of vWF was isolated by incubation with antibodies against vWF bound to Sepharose beads and subsequent centrifugation through a sucrose layer. This assay expresses fluid-phase binding characteristics because vWF is immobilized to a less rigid carrier. Using this fluid-phase assay, we found that factor VIII bound to any form of vWF was completely protected against aPC.

We conclude, in contrast with earlier work, that factor VIII bound to the 31-kD monomeric N-terminal fragment of vWF is protected against degradation by aPC. Because of the low affinity of this fragment for factor VIII, this protective effect cannot be observed in fluid-phase assays. The low binding affinity of this fragment for factor VIII may be explained by the monomeric state of its N-terminus. We suggest that the multimerization of vWF at the N-terminus is necessary for the expression of a high-affinity binding site for factor VIII, whereas the degree of multimerization does not affect the binding of factor VIII.

ACKNOWLEDGMENT

We thank Drs R.J. Kaufman and D.D. Pittman for the recombinant factor VIII.

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