Support of Ristocetin-Induced Platelet Aggregation by Procoagulant-Inactive and Plasmin-Cleaved Forms of Human Factor VIII/von Willebrand Factor

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Human factor VIII/von Willebrand factor (fVIII/vWF) was purified to homogeneity as defined by electrophoretic and immunologic criteria and tested for fVIII procoagulant and ristocetin cofactor activities. As little as 0.4 µg/ml of purified fVIII/vWF fully aggregated washed human platelets in the presence of ristocetin. Purified fVIII/vWF, whether thrombin-activated or -inactivated, and fVIII/vWF with its procoagulant activity abolished by a human inhibitor, supported ristocetin-induced platelet aggregation as effectively as native fVIII/vWF. Similarly, purified hemophiliac fVIII/vWF protein and fVIII/vWF-like protein isolated from normal serum supported the aggregation of platelets in the presence of ristocetin. FVIII/vWF extensively degraded by human plasmin and exposed to denaturing solvents also supported platelet aggregation in the presence of ristocetin. Plasmin hydrolyzed fVIII/vWF to yield noncovalently bonded fragments that could be separated into two pools by gel filtration in guanidine hydrochloride. After dialysis into dilute neutral buffers, the pool of high molecular weight fragments supported significant ristocetin-induced platelet aggregation and cross-reacted with an antibody to native fVIII/vWF. Hence, unlike fVIII procoagulant activity, the ristocetin cofactor activity and antigenicity of fVIII/vWF are retained, despite changes in the structure and conformation of the molecule. These results suggest caution about the interpretation of procoagulant activity:fVIII/vWF antigen ratios or procoagulant activity:ristocetin cofactor activity ratios in whole plasma, fVIII/vWF concentrates or purified preparations of fVIII/vWF, since small amounts of minimally degraded, procoagulant-inactive fVIII/vWF could markedly alter such values.

MATERIALS AND METHODS

Reagents

Reagent grade chemicals were used without further purification. Unless specifically noted, the buffer used in these studies was 0.15 M NaCl-0.05 M Tris hydrochloride-0.1 M 6-aminocaproic acid-0.02% sodium azide, pH 7.35.

Factor VIII Assay

fVIII procoagulant activity was measured by a kaolin-activated partial thromboplastin time method using hemophilic plasma with 0.1% fVIII activity. The fVIII procoagulant activity in units per

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millilitre was computed from a standard curve obtained by assaying NIH reference plasma (0.76 U of FVIII procoagulant activity per ml).

**Protein Concentrations**

These were estimated by the absorbance at 280 nm corrected for light scattering by subtracting the absorbance at 320 nm and expressed in absorbance units. Alternatively, protein concentrations were determined from amino acid compositions or determined by the method of Lowry et al.\textsuperscript{17} using crystalline bovine serum albumin (Sigma, St. Louis, Mo.) as a standard. Using protein concentrations calculated from amino acid compositions or determined by the method of Lowry et al.,\textsuperscript{17} an extinction coefficient $E_{280}^{	ext{mm}}$ of 12.3 was obtained.

**Electrophoresis**

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed essentially as described elsewhere.\textsuperscript{16,18} Gels were stained for protein or carbohydrate, destained, and stored as previously described.\textsuperscript{16} Molecular weight assignments were made by comparing the electrophoretic mobility of the bands observed to the mobility of proteins of known molecular weight.\textsuperscript{16}

**fVIII/vWF Purification**

The starting material was either fresh-frozen or outdated human plasma, cryoprecipitate, glycine-precipitated FVIII/vWF (Method IV, Hyland Laboratories, San Cotino Calif.) or intermediate-purity FVIII/vWF concentrates (kindly supplied by Dr. M. Wickerhauser, American National Red Cross, Bethesda Md.) In each case, partially purified FVIII/vWF was prepared by the method of Newman et al.\textsuperscript{16} using the modifications previously described.\textsuperscript{16} FVIII/vWF-like protein was also prepared from human plasma by recalcifying 400 ml of fresh-frozen plasma with 1.0 M CaCl\textsubscript{2} to a final concentration of 0.025 M and allowing it to clot in 2 plastic centrifuge bottles for 2 hr at 37°C. The fibrin clot was freed from the sides of the bottles and then centrifuged at 6000 $g$ for 20 min at 22°C. The serum was decanted and frozen at $-90°C$ for 12 hr; it was then crushed and thawed at $4°C$ in the presence of 6% ethanol. A gelatinous precipitate was recovered after centrifugation at 6000 $g$ for 10 min at 4°C. The precipitate was resuspended in 50 ml of 0.02 $M$ Tris-0.2 $M$ EACA, pH 7.0, and further purified as previously described,\textsuperscript{16} except that the bentonite adsorption step and the first polyethylene glycol precipitation were omitted. In each case, the product of the second polyethylene glycol (Carbowax 4000, Union Carbide Corp., So. Charleston W.Va.) precipitation was washed with cold 8% ethanol and redissolved in the Tris-NaCl buffer to a final protein concentration of $-15$ mg/ml.

The FVIII/vWF preparations were then further purified by gel filtration at room temperature, on 4% agarose (Biogel A-15m, Bio-Rad Laboratories, Richmond Calif.) as previously described.\textsuperscript{13,15} The void volume fractions were pooled and the protein quantitatively precipitated at $4°C$ with one-third volume of 40% polyethylene glycol. The precipitate was washed 4 times with ice-cold 8% ethanol, redissolved in Tris-NaCl buffer to an absorbance of at least 3.0 at 280 nm, and stored frozen at $-20°C$ for subsequent studies. All of these FVIII/vWF preparations were homogeneous by SDS-gel electrophoretic criteria and gave the same pattern, shown in Fig. 1, for normal FVIII/vWF. In the absence of reducing reagents, only the top of the gel stained for protein, and no other bands were seen; after reduction by $\beta$-mercaptoethanol, a single band of 195,000 daltons was observed. In addition, the FVIII/vWF preparations gave a single line on immunodiffusion or immunoelectrophoresis against an unadsorbed rabbit antiserum.

**Enzymatic Treatment of Purified FVIII/vWF**

Thrombin-activated or thrombin-inactivated FVIII/vWF was prepared by incubating FVIII/vWF with purified human thrombin (supplied by Dr. D. L. Aronson, Bureau of Biologics, FDA) in final concentrations of 53 $\mu$g FVIII/vWF protein/ml and 0.05 NIH Units thrombin/ml at 37°C. At maximum activation (usually 1–3 min incubation) or at maximum inactivation (usually 4–6 hr incubation, sufficient hirudin was added to neutralize exactly the thrombin in the reaction mixture. The exact amount of hirudin added was determined from its stoichiometry with thrombin, using the thrombin clotting time of purified fibrinogen solutions. These solutions were then tested immediately for FVIII coagulant and vWF platelet-aggregating activities.

Plasmin digests of FVIII/vWF were typically prepared as follows:

\[
\text{NR R} \quad \frac{1}{[\text{FVIII/vWF}]} \quad \text{Absorbance units}^{-1}
\]

![Fig. 1. Ristocetin cofactor activity of purified normal FVIII/vWF expressed as the reciprocal of the initial velocity (v) of ristocetin-induced platelet aggregation, defined as the change in percent transmittance (%T) per minute versus the reciprocal of the final FVIII/vWF concentration (absorbance at 280 nm). Each point represents the mean of seven determinations that were performed at different times and with different preparations of FVIII/vWF protein. The range for each separate point barely exceeded the deviation from the line shown for the mean values. Linearity was regularly observed for final FVIII/vWF concentrations in a range of 8 \times 10^{-4}–10^{-2} absorbance U; however, concentrations as low as 5 \times 10^{-4} absorbance U could be estimated. SDS-5% polyacrylamide gel analyses of nonreduced (NR) and reduced (R) FVIII/vWF protein used in these experiments are also shown: the subunit molecular weight (MW) is estimated to be 195,000 daltons.}
to each milliliter purified FVIII/vWF with an absorbance at 280 nm of \( \approx 3.0 \) 20 \( \mu l \) of highly purified human plasminogen (2 mg/ml 22 CTA U/ml) and 5 Ploug U of urokinase (Leo Pharmaceuticals, Copenhagen, Denmark) were added. For experiments that measured the immunologic or platelet-aggregating properties of plasmin-digested FVIII/vWF with time, samples were removed at selected intervals. \( \beta \)-Nitrophenyl-\( \beta \)-guanidino-benzoate was then added to a 200-fold molar excess with respect to the plasmin concentration; the samples were quick-frozen in a dry-ice-ethanol bath and stored at \( -20^\circ C \) until all could be analyzed at one time. For SDS-gel electrophoretic studies, proteolysis was stopped by adding an equal volume of 2% SDS-10 M urea-0.02 M sodium phosphate, pH 7.1, and immediately boiling for 2-3 min. To prepare “terminal” plasmin digests of FVIII/vWF, the mixture was incubated at 37°C for 24 hr and arbitrarily defined as terminal based on the following: (1) FVIII procoagulant activity was totally abolished; (2) the 195,000-dalton subunit band was completely cleaved to lower molecular weight species; and (3) no major changes in the SDS-gel pattern occurred with further incubation. To separate the high and low molecular weight fragments in the terminal digest, the digestion mixture was made 3 M in guanidine hydrochloride (extreme purity, Heico Co., Delaware Water Gap, Pa.) by adding the dry salt and was incubated for 24 hr against 100 times its volume of 6 M guanidine hydrochloride in Tris-NaCl. Fractions beneath the two protein peaks were pooled separately and dialyzed at 4°C against several changes of appropriate buffer before use. The completeness of the separation was established by SDS-gel electrophoresis and immunologic studies.

Immunologic Studies

Plasma from a hemophiliac patient with a known specific FVIII procoagulant inhibitor titer of 1:512 was mixed with alumina gel (Calbiochem, San Diego, Calif.) in a volume ratio of 10:1, stirred for 5 min at room temperature, and centrifuged at 6000 g. Then, purified FVIII/vWF (\( \approx 44 \mu g/ml \)) was incubated at 37°C with 0.1 vol of the adsorbed plasma and the disappearance of FVIII procoagulant activity monitored over time. The FVIII activity decreased rapidly to 10% of the initial activity after 17 min. After 3 hr, \( \approx 97% \) of the FVIII procoagulant activity was inhibited, and the mixture was then tested for ristocetin cofactor at several selected dilutions, the most concentrated of which was 4.4 \( \mu g/ml \).

An IgG fraction of rabbit antiserum to human FVIII/vWF, which inhibited both the FVIII and ristocetin cofactor activities of FVIII/vWF, was used to perform immunodiffusion and immunoelectrophoresis studies by standard techniques.21

Platelet Aggregation Studies

Fresh human platelets were collected, washed by the method previously described by us,3 from hemophiliacs suspended in 0.05 M Tris-0.15 M NaCl to a final concentration of 200,000/cumm. Platelet aggregation was monitored using a Chrono-log aggregometer (Chrono-log Corp., Havertown, Pa.) as described earlier.14 The maximum slope of the curve recording percent transmittance versus time was determined and defined as the initial velocity of platelet aggregation. The initial velocities increased in direct proportion to the FVIII/vWF level until a final FVIII/vWF concentration of 10 \( \mu g/ml \) was achieved. Importantly, the initial velocities were found to be much more sensitive to small changes in FVIII/vWF concentration than the total change in light transmittance.4 Final FVIII/vWF protein concentrations as low as 0.4 \( \mu g/ml \) could be easily quantitated by this method.

RESULTS

Ristocetin Cofactor Activity of Highly Purified Normal FVIII/vWF

In Fig. 1, the ristocetin-induced platelet-aggregating activity of native FVIII/vWF is depicted by expressing the reciprocal of the initial velocity of aggregation as a function of the reciprocal of the FVIII/vWF concentration (absorbance at 280 nm). The velocity of ristocetin-induced platelet aggregation increased with FVIII/vWF concentration up to a final FVIII/vWF concentration in the assay cuvette of \( \approx 0.01 \) absorbance unit, after which there was no measurable increase in the rate of platelet aggregation. The line shown in Fig. 1 depicts the means of experiments performed on 7 different days; the deviation of the points from the line is about the same as that observed on any given day. In the presence of ristocetin, FVIII/vWF also promoted the clumping of platelets that had been washed and stored at room temperature for 48 hr or platelets that had been frozen and thawed 3 times. Washed membranes prepared from sonicated platelets and stored at \( -90^\circ C \) for 3 yr also formed visible clumps in this system, as did formalin-fixed platelets. However, none of these aged platelet preparations aggregated as well as freshly washed platelets in the presence of FVIII/vWF and ristocetin.

Ristocetin Cofactor Activity of Procoagulant-Inactive FVIII/vWF and Plasmin-Degraded FVIII/vWF

To determine whether the ability to support ristocetin-induced platelet aggregation requires FVIII/vWF with FVIII procoagulant activity, several forms of FVIII/vWF without FVIII procoagulant activity were tested for ristocetin cofactor activity. Preparations of FVIII/vWF-like protein from hemophilic plasma or from normal human serum had less than 1% of the specific FVIII procoagulant activity of native FVIII/vWF, yet, as shown in Fig. 2, hemophilic FVIII/vWF had \( \approx 100% \) and serum FVIII/vWF had at least 70% of normal ristocetin cofactor activity. The procoagulant activity of purified normal FVIII/vWF was completely inhibited by the alumina gel-adsorbed human FVIII procoagulant inhibitor plasma, but full ristocetin cofactor activity was retained. In these experiments, any contribution of the inhibitor plasma to the ristocetin cofactor activity was subtracted. Next, thrombin-activated FVIII/vWF with \( \approx 210% \) of the control FVIII procoagulant activity and thrombin-inactivated FVIII/vWF with only \( \approx 10% \) of the control FVIII procoagulant activity were tested for ristocetin cofactor activity and found to give essentially the same double-reciprocal plot as native FVIII/vWF. As
previously reported by ourselves and others, digestion of purified native FVIII/vWF by plasmin for 24 hr completely inactivated the procoagulant activity.\textsuperscript{13,22} Unlike the other modified forms of FVIII/vWF protein, plasmin-inactivated FVIII/vWF did not retain full ristocetin cofactor activity, as indicated by the somewhat steeper line in the double-reciprocal plot in Fig. 2. Comparison of $1/s$ for plasmin-inactivated FVIII/vWF to $1/s$ for normal FVIII/vWF indicated that the plasmin-inactivated FVIII/vWF only had $\sim 70\%$ of the ristocetin cofactor activity of normal FVIII/vWF or the other modified forms of FVIII/vWF described above.

Degradation of FVIII/vWF by Plasmin as a Function of Time

The observation that essentially terminal plasmin digests of FVIII/vWF retain $\sim 70\%$ of control levels of ristocetin cofactor activity prompted a closer examination of the effects of plasmin on the structure–function relationships of FVIII/vWF. The upper panel of Fig. 3 (A) shows the loss of FVIII procoagulant activity with

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Comparison of the ristocetin cofactor activities of normal FVIII/vWF (○), hemophilic FVIII/vWF (■), "serum FVIII/vWF" (▵), FVIII/vWF inactivated by a human inhibitor (△), thrombin-activated FVIII/vWF (▲), thrombin-inactivated FVIII/vWF (□), and plasmin-inactivated FVIII/vWF (○). Except for plasmin-cleaved FVIII/vWF, the reciprocal of the initial velocity of platelet aggregation as a function of the reciprocal of the protein concentration gave values that were essentially indistinguishable from those observed for normal native FVIII/vWF. The steeper slope of the line for plasmin-cleaved FVIII/vWF corresponded to $\sim 70\%$ of the vWF activity of native FVIII/vWF.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Time-dependent structure–function relationships as FVIII/vWF is digested by plasmin at 37°C. (A) The gels on the left are, respectively, those of nonreduced (NR) and reduced (R) FVIII/vWF. Before reduction, the bulk of the protein was excluded from the gel; after reduction and using a protein load that emphasizes minor components, a principal band of $\sim 195,000$ daltons was observed as well as several minor high molecular weight bands. After 3 min of digestion by plasmin, the 195,000-dalton band was still observed, and what were previously minor bands became intensified, suggesting that these were early plasmin cleavage fragments. After 60, 240, and 1440 min of incubation, the reduced gel patterns were all about the same, containing no bands $\sim 103,000$ daltons. The SDS-gel of nonreduced FVIII/vWF after 1440 min of digestion by plasmin shows a diffuse pattern of protein stain that enters the top of the gel and a second discrete band of $\sim 38,000$ daltons. Under the digestion conditions, here, only $\sim 25\%$ of the initial FVIII procoagulant activity remained at 5 min, although the 195,000-dalton subunit was clearly the dominant band at this time; FVIII procoagulant activity was completely abolished by 15 min. (B) Immunodiffusion of plasmin-digested FVIII/vWF with time. The native FVIII/vWF control gave a single line when tested against the rabbit antiserum in the center well. At 15 min of incubation, the plasmin-cleaved FVIII/vWF gave two immunoprecipitin lines; the inner line began disappearing by about 3 hr of digestion. With continued digestion, the outermost immunoprecipitin line became more diffuse and migrated closer to the center well.}
\end{figure}
time when highly purified native fVIII/vWf was incubated at 37°C with a final plasmin concentration of 0.68 CTA U/ml. By 5 min, the fVIII procoagulant activity of the incubation mixture was decreased to about 30% of its starting level, and by 15 min, the procoagulant activity was totally destroyed. In contrast, samples removed from the digestion mixture at 1 and 24 hr retained ~95% and ~70%, respectively, of the starting level of ristocetin cofactor activity. Samples of the incubation mixture were also analyzed by SDS-5% polyacrylamide gel electrophoresis before and after reduction with β-mercaptoethanol. In Fig. 3A the nonreduced SDS-gel pattern of terminally plasmin-degraded fVIII/vWf shows that most of the protein remained in the top portion of the gel and a poorly staining band of ~38,000 daltons could also be seen. Both the protein at the top of the gel and the ~38,000-dalton band gave a positive reaction for carbohydrate when stained with the periodic acid-Schiff reagent. Considerable variation was observed in the staining intensity of the ~38,000-dalton band, suggesting slow degradation by plasmin with the removal of groups important to Coomassie staining. Thus, the gel shown in Fig. 3, in which the ~38,000-dalton band is fairly faint in intensity, might be somewhat more extensively digested than the one in Fig. 4A, possibly due to small differences in incubation conditions.

Immunologic studies lent further support to the interpretation of the gel patterns of nonreduced plasmin-degraded fVIII/vWf. As shown in Fig. 3B, the time course of the digestion of fVIII/vWf by plasmin was also monitored by immunodiffusion against rabbit antiserum to native fVIII/vWf. Before exposure to plasmin, the fVIII/vWf protein gave a single immunoprecipitin line, but after 15 min of incubation with plasmin, two immunoprecipitin lines were observed that persisted at all subsequent time points. By 1440 min, however, the more rapidly migrating line had become much fainter than at the earlier time points. While the more slowly migrating line was never resolved into more than one component, as might be expected from the SDS-gel heterogeneity of the high molecular weight fragments, the diffuseness of the 1440-min time point suggested some degree of heterogeneity. In every case, there was a reaction of identity between samples in adjacent wells; furthermore, both the 15-min digest and the 1440-min digest exhibited reactions of identity with native fVIII/vWf.

In Fig. 3, SDS-gel analyses of reduced samples of plasmin-digested fVIII/vWf protein showed predominantly the 195,000-dalton band for the first few
minutes of digestion; after 3 min, minor bands of <200,000–100,000 were observed. These intermediates were very transient and progressively disappeared as bands attributable to lower molecular weight fragments of the 195,000-dalton subunit became more intense, such that by 240 min, the dominant bands had no molecular weights of approximately 103,000, 88,000, 33,000, and 17,500 daltons. When compared to those of Atichertakarn et al.22 and Guisasola et al.,23 the predominance of lower molecular weight bands in our 240-min digestion mixture suggests that cleavage by plasmin was more complete in our study than in theirs. As indicated above, FVIII procoagulant activity was completely abolished within the very first few minutes of digestion, despite the fact that the 195,000-dalton band remained dominant. In contrast, ~70% or more of the ristocetin cofactor activity remained at 24 hr, despite complete degradation of the 195,000-dalton subunits to species of 103,000 daltons or less.

**Isolation and Preliminary Studies of the Fragments Produced When FVIII/vWf was Digested by Plasmin**

As described in the preceding section, major high molecular weight fragments of ~200,000–400,000 daltons and a single low molecular weight fragment of ~38,000 daltons were observed on SDS-gel analyses of the nonreduced terminal digestion mixture. When we gel-filtered the digestion mixture in neutral buffers with and without high concentrations of NaCl, we separated at least two protein peaks, as has been reported by other workers.22,24 Under these conditions it was possible to purify the large fragment free of the 38,000-dalton fragment; however, to purify the 38,000-dalton fragment free of the high molecular weight fragments, gel filtration in 6 M guanidine hydrochloride was necessary. The top panel of Fig. 4 (A) shows the chromatogram that resulted when a terminal plasmin digest of FVIII/vWf was filtered on 4% agarose in 6 M guanidine hydrochloride–0.05 M Tris–0.15 M NaCl, pH 7.35. A large peak of protein was eluted in the void volume and a much smaller protein peak was eluted later. The SDS-electrophoretic results shown in Fig. 4A indicated that the protein in the void volume contained the higher molecular weight bands, while the small peak of poorly absorbing protein corresponded to the single-chain ~38,000-dalton fragment. Based on SDS-gel analyses, the two peaks of protein were completely resolved and the ~38,000-dalton band appeared to be pure. The patterns observed when these peaks were analyzed on highly crosslinked 12.5% SDS-polyacrylamide gels indicated that the high molecular weight fragments were responsible for the complex subunit structure observed when the reduced whole digestion mixture was analyzed on SDS-5% gels. As shown on the SDS-12.5% gels in Fig. 4A, the mobility of the ~38,000-dalton fragment after reduction was slightly less than on the nonreduced gel, indicating that it does not contain a smaller disulfide-linked fragment, although it might contain a small disulfide loop.

The bottom panel of Fig. 4(B) shows the immunoprecipitin reactions of the isolated fragments of plasmin-digested FVIII/vWf with rabbit antiserum to normal human FVIII/vWf. Again, by double diffusion, the digest mixture gave two immunoprecipitin lines with the rabbit antiserum to native FVIII/vWf. The isolated large and small species each formed single immunoprecipitin lines that gave reactions of identity with the more intense and less intense lines, respectively, of the 120-min and terminal digests. By immunoelectrophoresis, the larger fragments had a mobility identical to the major components of the digest mixture, while that of the smaller fragment corresponded to the minor component. The large and small species had opposite electrophoretic mobilities at pH 8.8, with the larger species moving in the direction of intact FVIII/vWf. Thus, the immunologic studies support the homogeneity of the isolated small fragment and confirm the interpretation of the parallel immunologic and gel electrophoretic studies of the whole digest presented in the preceding section.

**DISCUSSION**

The ability to stimulate human platelet aggregation in the presence of ristocetin appears to be an unique characteristic of the FVIII/vWf protein. The rate of aggregation increases with FVIII/vWf final concentrations up to a value of 10 µg/ml. Hence, ristocetin cofactor activity is a sensitive measure of low concentrations of FVIII/vWf, and by the method described here, as little as 0.4 µg/ml of FVIII/vWf protein gives measurable aggregation. As suggested by earlier studies on whole plasma,1,5,6 increases or decreases in FVIII procoagulant activity had little effect on the ristocetin cofactor activity. Except for plasmin-digested FVIII/vWf, which had ~70% of the ristocetin activity, each of the other procoagulant-inactive FVIII/vWf species had about the same level of ristocetin cofactor activity observed for native FVIII/vWf protein.

Several investigators, including us, have shown that gel filtration of highly purified FVIII/vWf protein on 4% agarose in 0.25 M CaCl2 separates the procoagulant-active moiety from procoagulant-inactive moieties, the latter representing ~99% of the protein and containing virtually all of the ristocetin cofactor activity.1,11,12,14,15 Hence, the electrophoretic and immuno-
logic properties of purified plasma fVIII/vWf represent primarily those of vWF protein, and our data permit some conclusions to be drawn about the relationship of ristocetin cofactor activity to molecular structure. Except for plasmin-inactivated fVIII/vWf, the other altered forms of fVIII/vWf examined here have a subunit structure indistinguishable by SDS-gel electrophoresis from that of native fVIII/vWf. However, the presence of substantial ristocetin cofactor activity in plasmin-degraded fVIII/vWf indicates that an intact 195,000-dalton subunit is not necessary for vWF activity. The time-dependence of the degradation of the 195,000-dalton subunit by plasmin showed that after 45 min of digestion, most of the subunits were cleaved to species of 103,000 daltons or less; yet at this time, the degraded fVIII/vWf retained ~95% of its ristocetin cofactor activity. After 24 hr of digestion, with absolutely no trace of subunits larger than 103,000 daltons, fVIII/vWf still had ~70% of its starting ristocetin cofactor activity. These data convincingly demonstrate that fVIII/vWf protein with intact 195,000-dalton subunits is not necessary for ristocetin cofactor activity and that fVIII procoagulant and ristocetin cofactor activities can clearly be "separated" by minimal proteolysis of the fVIII/vWf protein.

Separation and preliminary characterization of the noncovalently bonded fragments produced by plasmin digestion of fVIII/vWf gave further information about the structural requirements for ristocetin cofactor activity. Of interest was our finding that even the separated pool of large fragments possessed a small amount of ristocetin cofactor activity (~10%). That this occurred following exposure to 6 M guanidine hydrochloride, a strong denaturant, suggests that secondary and tertiary structural requirements for ristocetin cofactor activity are either minimal or can be partially restored following denaturation. It is possible that as the guanidine hydrochloride is removed by dialysis, self-association of the fVIII/vWf fragments gives rise to ristocetin cofactor activity.

All of the modified forms of fVIII/vWf examined cross-reacted with rabbit antibodies to normal human fVIII/vWf. Significantly, both the large and the small plasmin fragments cross-reacted with rabbit antibody to the normal human protein, the larger fragment giving a reaction of identity with normal fVIII/vWf. Hence, these results strongly imply that the antigenicity of the fVIII/vWf molecule, like ristocetin cofactor activity, is well preserved despite exposure of the protein to agents that destroy fVIII procoagulant activity and extensively degrade its structure. When considered with the observation that both antigenicity and ristocetin cofactor activity are commonly reduced in von Willebrand disease, our results are compatible with the suggestion that the antigenic sites and the vWF-active site(s) are the same or closely related. Coupled with the recent report suggesting that fVIII/vWf from patients with classical von Willebrand disease may have an abnormal carbohydrate structure, the observations presented in this article are perfectly consistent with recent findings from our laboratory that indicate that ristocetin cofactor activity is related to the carbohydrate structure of the fVIII/vWf protein.

It must be emphasized that the results presented here demonstrate that ristocetin cofactor activity or antigenicity can be "separated" from the fVIII procoagulant activity by selectively inactivating the latter. Studies purporting to purify vWF free of fVIII must clearly demonstrate that fVIII-procoagulant activity is not merely inactivated during preparation. For example, we and others have reported that EDTA, which has been used in the separation process, rapidly destroys fVIII procoagulant activity—a point apparently overlooked in recently published comments about the isolation of vWF free from fVIII procoagulant activity.

Finally, recent studies have demonstrated that antigen levels as well as fVIII and ristocetin cofactor activity levels can be affected by enzymatic digestion. For example, Atichartakarn et al. have shown that digestion of fVIII/vWf by plasmin causes an apparent increase in the amount of fVIII/vWf antigen by quantitative immunoelectrophoresis. Using whole plasma, Lian et al. correlated the increased antigen level with an increase in mobility of plasmin-cleaved fVIII/vWf by crossed immunoelectrophoresis. Since diagnostic tests, such as those for carrier detection, frequently utilize the ratio of fVIII procoagulant activity to ristocetin cofactor activity or to antigen level, our results and theirs underscore the need for minimizing the degradation of fVIII procoagulant activity in face of a stable or an apparently increased level of ristocetin cofactor activity or fVIII/vWf antigen concentration.

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Support of ristocetin-induced platelet aggregation by procoagulant-inactive and plasmin-cleaved forms of human factor VIII/von Willebrand factor

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