Proteolytic Interactions of Factor IXa With Human Factor VIII and Factor VIIIa

By Barry J. Lamphear and Philip J. Fay

Factor IXa was shown to inactivate both factor VIII and factor VIIIa in a phospholipid-dependent reaction that could be blocked by an antifactor IX antibody. Factor IXa-catalyzed inactivation correlated with proteolytic cleavages within the A1 subunit of factor VIIIa and within the heavy chain (contiguous A1-A2-B domains) of factor VIII. Furthermore, a relatively slow conversion of factor VIII light chain to a 68-Kd fragment was observed after prolonged incubation. Sites of cleavage were identified within the A1 domain at Arg336-Met337 and within the factor VIII light chain at Arg1719-Asn1720. Factor IXa failed to cleave isolated factor VIII heavy chains, yet cleaved isolated factor VIII light chain. In addition, the purified A1/A3-C1-C2 dimer derived from factor VIIIa was a substrate for factor IXa; however, cleavage of the A1 subunit occurred at less than 30% of the rate of cleavage of A1 in trimeric factor VIIIa. These data suggest that factor VIII light chain contributes to the binding site for factor IXa and also support a role for a heavy chain determinant located within the A2 subunit in the association of factor VIIIa with factor IXa. Furthermore, the capacity of factor IXa to proteolytically inactivate its cofactor, factor VIIIa, suggests a mode of regulation within the intrinsic tenase complex.

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MATERIALS AND METHODS

Reagents. Human factor VIII concentrates (Koate-HP) were generously provided by Dr M Mozen (Cutter Division of Miles Laboratories, Berkley, CA). Factor VIII-deficient plasma was purchased from George King Biomedical, Inc (Overland Park, KS). Homogeneous human factor IXa and human α-thrombin (2,900 U/mg) were obtained from Enzyme Research Laboratories, Inc (South Bend, IN). The Murine monoclonal antibody R8B12, specific for the COOH-terminal portion of the A2 domain of factor VIII, has been described. Hirudin (~2,000 U/mg) was purchased from Sigma Chemical Co (St Louis, MO).

Proteins. Factor VIII was prepared from Koate-HP, as described previously. Purification of factor VIII subunits and factor VIIIa dimer (A1/A3-C1-C2) from plasma-derived factor VIII was as described previously.

Assays. Factor VIIIa activity was measured by one-stage clotting assay with factor VIII-deficient plasma. The inactivation of factor VIII and factor VIIIa, as well as cleavage of factor activity occurs spontaneously without further proteolysis owing to pH-dependent dissociation of the A2 subunit from a stable but inactive A1/A3-C1-C2 dimer. Factor Xa has been reported to activate factor VIII, yielding the same cleavage fragments as those generated by thrombin. Subsequent inactivation correlates with further cleavage of factor VIIIa. Factor IXa has also been implicated in activation of factor VIII; however, the extent of activation is reduced compared with either thrombin or factor Xa. Factor VIII(a) is proteolytically inactivated by the potent anticoagulant activated protein C. The sites of protease cleavage within factor VIII(a) occur at residues 562, which bisect the A2 domain, and within the A1 domain at residue 336.

Previously, we observed that reconstituted factor VIIIa was transiently stabilized by factor IXa and subsequent loss of cofactor activity appeared to correlate with proteolysis. In this report, we further characterize the proteolytic interaction between factor IXa- and factor VIII-derived substrates with respect to cofactor activity and covalent alteration. Additionally, the use of several factor VIII-derived substrates adds insight into interactions between factor VIII(a) and factor IXa.

VIII-derived substrates, by factor IXa was performed in buffer A (0.02 mol/L HEPES, pH 7.2, 0.15 mol/L NaCl, 0.005 mol/L CaCl₂, and 0.01% Tween 20). Factor IXa (650 nM) was preincubated with hirudin (50 U/mL) for 30 minutes prior to combining with factor VIII-derived substrates to inhibit any potential thrombin contamination. All reactions were run at 22°C and contained phospholipid in the form of inosithin at concentrations noted in the figure legends. Samples were removed at indicated times and assayed for factor VIII activity, or they were analyzed by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by either silver staining or Western blotting. Under the reaction conditions used, factor IXa had no effect on the plasma-based assay for factor VIIa activity. The effect of the antifactor IX antibody on inactivation of factor VIII was performed by preincubating factor IXa (650 nmol/L) either in the presence or absence of antibody (119 µg/mL) for 60 minutes at 22°C before combining with factor VIII. Thrombin activation of factor VIIa was performed in buffer A containing 225 mmol/L NaCl. Factor VIIa (66 µg/mL) was incubated with thrombin (1 µg/mL) at 22°C. Assuming an average molecular weight of 220,000 for factor VIIA and 36,600 for thrombin, this represents approximately 1:1 molar ratio of thrombin to factor VII in the reaction. Under these conditions, factor VIIa activity was maximal by 2 minutes. At 10 minutes, the factor VIIa was added to reactions for subsequent inactivation by factor IXa. These reactions contained hirudin (1 to 5 U/mL) to inhibit thrombin activity. Protein concentration was determined by the Coomassie dye binding method of Bradford.

Electrophoresis. SDS-PAGE was performed using the Laemmli buffer system22 and a Bio-Rad (Richmond, CA) minigel electrophoresis system. Electrophoresis was for 1 hour at 150 V, and protein bands were visualized after either staining with silver nitrate or Western blotting. For the latter analysis, unstained gels were transferred to polyvinylidene difluoride (PVDF) membrane using the procedure of Matsudaira.24 Bands on the membranes that reacted to the R8B12 antibody were visualized as described previously.16 Densitometric scans were performed using an LKB Ultrascan Laser densitometer with peak integrator (Piscataway, NJ).

NH₂-terminal sequence analysis of electrophoretically resolved factor VIII polypeptides. Factor VIII (260 µg/mL, 0.14 mL) was reacted with factor IXa (5.6 µg/mL) in buffer A containing 400 µg/mL phospholipid at 22°C until less than 5% of the initial factor VIII activity remained. The reaction was subjected to SDS-PAGE using a 10% separating gel. After electrophoresis, the resolved fragments were transferred to PVDF membrane that was then briefly stained with Coomassie blue.26 Bands corresponding to the ~68-, ~48-, and ~45-Kd fragments were excised and subjected to 10 cycles of automated amino-terminal sequence analysis using an Applied Biosystems’ pulsed-liquid phase sequenator (model 477A; Foster City, CA).

RESULTS

Inactivation of factor VIII by factor IXa. Heterodimeric factor VIII was treated with varying levels of factor IXa in a reaction containing calcium in the presence or absence of phospholipid (Fig 1). In the absence of phospholipid, no change in factor VIII activity was observed (Fig 2). In the presence of phospholipid, a loss of cofactor activity was observed, with the rate of inactivation dependent on factor IXa concentration. At lower factor IXa concentrations, a lag was observed before inactivation (data not shown). Factor IXa used for these and subsequent reactions was preincubated with hirudin to inhibit any possible trace levels of thrombin in the factor IXa preparation. To further confirm that the inactivation was due to factor IXa and not a contaminating protease, factor IXa was preincubated with an antifactor IX polyclonal antibody before reaction with factor VIII. Inactivation was blocked after preincubation of factor IXa with the antibody (Fig 2). Therefore, the inactivation of factor VIII appeared specific for factor IXa.

A reaction time course of inactivation of factor VIII by factor IXa in the presence of phospholipid was analyzed by
SDS-PAGE followed by silver staining, with the results shown in Fig 3. Inactivation was rapid, with cleavages observed within both factor VIII heavy and light chains. Initial cleavages resulted in conversion of heavy chains to smaller-sized fragments. Most notable were the conversion of the 210-Kd species to a 170-Kd fragment, and the concomitant appearance of two lower molecular weight fragments of 45 and 48 Kd. Assuming equivalent staining characteristics for the low molecular weight fragments, the 45-Kd fragment was the relatively more abundant cleavage product. Disappearance of heavy chains and appearance of the 45- and 48-Kd fragments correlate with cofactor inactivation. By 1 hour, an additional band at 43 Kd appears and increases as the 48-Kd band decreases, suggesting this fragment may be produced from additional cleavage within the 48-Kd peptide. A doublet fragment of 68/66 Kd was also generated, concomitant with the disappearance of the factor VIII light chain doublet. This fragment was slightly smaller than the light chain–derived A3-C1-C2 fragment obtained from thrombin cleavage of factor VIII. The 43- and 68-Kd bands appeared subsequent to factor VIII inactivation.

Reactivity of the factor IXa–generated factor VIII fragments to a monoclonal antibody, R8B12, was tested. This antibody, which recognizes a COOH-terminal epitope located within the A2 domain of factor VIII heavy chain, reacted with factor VIII heavy chains and the 170- and 48-Kd fragments, suggesting these fragments contain this epitope (Fig 4). In addition, faint bands of 83 and 67 Kd also reacted with this antibody. These bands did not represent cross-reactivity to factor VIII light chain and light chain cleavage product, respectively, because no reactivity to light chain in the control lane was detected. Therefore, these bands likely represent COOH-terminal heavy chain–derived cleavage products from the 110- and 120-Kd heavy chain species that were likely obscured in the silver stained gel by the presence of light chain and light chain cleavage product. The 43-Kd fragment produced after prolonged incubation of factor VIII with factor IXa also reacted to the R8B12 antibody (data not shown), further suggesting that this fragment results from additional cleavage within the 48-Kd fragment.

Amino-terminal sequence analysis. A concentrated sample of factor VIII (1.18 μmol/L) was reacted with factor IXa (0.13 μmol/L) under similar reaction conditions as described above, except phospholipid concentration was elevated to 400 μg/mL. Factor VIII activity was monitored by clotting assay, and the reaction was terminated when less than 5% of the original activity remained. Fragments from the digest were resolved after SDS-PAGE, transferred to PVDF membranes, and stained with Coomassie blue, as described in Materials and Methods. Identification of the 48-Kd fragment was further confirmed through Western blotting of a gel sample run in parallel by reaction to the R8B12 monoclonal antibody. Excised bands were subjected to 10 cycles of automated amino-terminal sequence analysis, and the results are shown in Table 1.

The results indicate that the 45-Kd fragment was
PROTEOLYSIS OF FACTOR VIII(A) BY FACTOR IXA

Table 1. Amino-Terminal Sequences of Factor VIII Fragments After Proteolysis by Factor IXa

<table>
<thead>
<tr>
<th>Cycle No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Q</td>
<td>S</td>
<td>G</td>
<td>V</td>
<td>P</td>
<td>Q</td>
<td>-</td>
</tr>
<tr>
<td>Factor VIII residues 1719-1729* (R)</td>
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<td>R</td>
<td>A</td>
<td>Q</td>
<td>S</td>
<td>G</td>
<td>-</td>
<td>V</td>
<td>P</td>
<td>Q</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>K</td>
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<td>D</td>
</tr>
<tr>
<td>Factor VIII residues 336-346* (R)</td>
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<td>E</td>
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<td>A</td>
<td>D</td>
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<td>-</td>
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<td>Y</td>
<td>Y</td>
<td>L</td>
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<td>Y</td>
<td>L</td>
<td>G</td>
<td>A</td>
<td>V</td>
</tr>
</tbody>
</table>

Abbreviation: - , not identified.

*Data from Vehsr et al.

derived from the NH2-terminus of the heavy chains. The apparent size of this fragment was consistent with cleavage at Arg336-Met337. Cleavage at this site by factor IXa was confirmed by NH2-terminal sequence analysis of the ~48-Kd fragment that initiated at Met337 and most likely originated from the carboxy-terminus of the ~90-Kd heavy chain species. The relative low abundance of this product as compared to fragment A1 suggests that it did not originate from all heavy chains species, and therefore further suggests that factor IXa does not efficiently cleave the A2-B domainal junction region of factor VIII at Arg740-Ser741, a site rapidly cleaved by thrombin, and proposed for activated protein C, and factor Xa. A second protease site, located at Arg1719-Asn1720, converts factor VIII light chain to the ~68-Kd fragment.

Interaction of factor IXa with factor VIII subunits. To determine if proteolysis of factor VIII requires intact heterodimeric factor VIII, isolated factor VIII light chain or heavy chain subunits were evaluated as substrates for factor IXa (Fig 5). Factor IXa catalyzed slow cleavage of light chain, yielding the ~68-66-Kd fragment observed in reactions with intact factor VIII and confirmed the light chain origin for this fragment. No cleavage of isolated heavy chains was detected (data not shown), suggesting a light chain requirement for proteolysis of this component. These data suggest that a portion of the binding site for factor IXa is located on factor VIII light chain, because factor IXa can bind and cleave light chain in the absence of heavy chains.

To assess the contribution of the factor VIII light chain in the binding of the protease, factor IXa inactivation of factor VIII was performed either in the absence or presence of a seven-fold molar excess of factor VIII light chain over factor VIII heterodimer. If light chain competes with factor VIII for binding to factor IXa, then a reduced rate of factor VIII inactivation should occur. However, under these conditions, no change in the rate of factor VIII inactivation was observed (Fig 6). This suggests that the binding of factor IXa with factor VIII light chain is weak relative to association with intact heterodimer and that factor IXa can bind and cleave light chain in the absence of heavy chains.

Factor IXa cleavage of factor VIIIa and A1/A3-C1-C2. Because it is the activated form of factor VIII, factor VIIIa, that serves as a cofactor for factor IXa, analysis of the proteolytic activity toward factor VIIIa substrates was performed. Thrombin-activated factor VIIIa was combined with factor IXa in a reaction containing calcium and phospholipid (Fig 7). A rapid decay of cofactor activity was detected that correlated with proteolysis of the A1 subunit and the appearance of the ~45-Kd fragment that migrated just above the A2 subunit, presumably the result of cleavage at Arg336-Met337. The rate of loss of A1 subunit, as judged by laser densitometric scans of the gel, paralleled the rate of decay of factor VIIIa activity (data not shown). This result...
confirmed our earlier observation of factor IXa inactivation of reconstituted factor VIIIa. Although no additional change in the mobility of factor VIIIa subunits was apparent, the A2 subunit appeared to migrate as a less distinct band after prolonged reaction with factor IXa. The apparent lack of cleavage of the factor VIIIa A3-C1-C2 subunit at the Arg1719-Asn1720 site likely reflects the short time course and reduced enzyme to substrate ratio used in this experiment compared with the reaction conditions showing cleavage of the factor VIII light chain (Fig 3).

Isolated A1/A3-C1-C2 dimer was also used as a substrate for the protease with cleavage of the A1 subunit to the 45-Kd fragment (Fig 7). Because the same site appeared to be cleaved in factor VIIIa and the A1/A3-C1-C2 dimer, the relative rates of cleavage of the A1 subunit in the two factor IXa substrates were compared. Figure 8 illustrates the relative rates of disappearance of the A1 subunit in these reactions as determined by densitometry scans of the stained gels. The initial rate of cleavage of A1 in the heterotrimer was greater than threefold faster than that observed for the A1/A3-C1-C2 dimer. This result suggests that, although the A2 subunit is not essential for factor IXa association with factor VIIIa and subsequent cleavage of the A1 subunit, it may enhance the interaction of protease with substrate.

**DISCUSSION**

Recently, we reported that factor IXa slowly inactivated factor VIIIa that had been reconstituted from isolated A1/A3-C1-C2 dimer and A2 subunit. In this study we have further investigated the interactions of factor IXa with factor VIII(a) by examining the proteolytic activity of factor IXa toward several factor VIII-derived substrates. Both factor VIII and factor VIIIa were inactivated by factor IXa. Inactivation of factor VIII was dependent on phospholipid and specific for factor IXa, because it was blocked by antifactor IX antibody.

Our results suggest no activation of factor VIII by factor IXa. This is in contrast to the work of Rick, who showed that factor IXa catalyzed a modest (2- to 6-fold) activation of factor VIII. The effect required relatively high ratios of factor IXa to factor VIII and was independent of the presence of phospholipid. Neuschwandt and Jesty reported factor IXa activated factor VIII at a slow rate, as measured by the potentiation of acetylated factor X activation. Our experiments do not rule out the possibility that factor VIII fragments produced by factor IXa possess transient cofactor activity. However, studies described in this report indicate that the major proteolytic effect of factor IXa on factor VIII(a) correlates with inactivation.

Factor IXa also inactivated factor VIII (and factor VIIIa) via cleavage at residue 336, resulting in the appearance of a ~45-Kd fragment, originating from factor VIII heavy chain NH2-terminus, and a series of fragments ranging from ~48 to ~170 Kd, derived from the COOH-termini of the heterogeneous heavy chains. These conclusions are supported by the following evidence. (1) Cleavage of factor...
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interaction, then factor IXa interaction with low concentrations of factor VIIIa, as observed in the earlier porcine studies (~1 nmol/L components) and in the partially reconstituted human heterotrimers, could in fact be stabilizing relative to the spontaneous dissociation of A2 subunit at these concentrations. On the other hand, reaction of factor IXa with significantly higher concentrations of factor VIIIa in the latter studies, where much of the material exists as heterotrimer, could be observed as solely inactivating as a result of slow cleavage of the A1 subunit. The role for factor IXa inactivation of factor VIIIa(a) is unknown but may serve to regulate factor VIIIa activity during factor X generation by the intrinsic tenase complex. Self-damping of the activation of factor X by factors IXa and VIIIa has been previously observed, and proteolytic inactivation of factor VIIIa by factor IXa may contribute to this effect. Further study is also necessary to evaluate the importance of this mode of regulation relative to other anticoagulant pathways such as the protein C system.

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