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Subunit Structure and Function of Porcine Factor Xa-Activated Factor VIII†

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ABSTRACT: Factor Xa and thrombin (factor IIa) activate factor VIII (fVIII) by different proteolytic pathways. Thrombin cleaves fVIII at Arg372 between the A1 and A2 domains, at Arg740 between the A2 and B domains, and at Arg1689 between the B and A3 domains to form an A1/A2/A3-C1-C2 heterotrimer. We now report a stable porcine $fVIIIa_{Xa}$ preparation obtained by Mono S HPLC at pH 6. NH_2-terminal sequence analysis of purified subunits of $fVIIIa_{Xa}$ revealed that factor Xa cleaves fVIII at Arg219 within the A1 domain and at Arg490 within the A2 domain, as well as at Arg372, Arg740, and Arg1689. Analytical ultracentrifugation of the $fVIIIa_{Xa}$ preparation yielded results consistent with a single, 148 kDa species, similar to previous results with $fVIIIa_{IIa}$ [Lollar, P., & Parker, C. G. (1989) *Biochemistry 28*, 666−674]. Thus, the major species in the $fVIIIa_{Xa}$ preparation contains five subunits, including fragments of the A1 and A2 domains that remain noncovalently bound. Fluorescence anisotropy measurements indicated there was no difference in the affinity of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ for a fluorescent dye-labeled, active-site-blocked derivative of porcine factor IXa. Additionally, the $fVIIIa_{Xa}$ preparation bound dye-labeled factor IXa with 1:1 stoichiometry, indicating that all $fVIIIa_{Xa}$ molecules in the preparation can bind factor IXa. However, $fVIIIa_{Xa}$ had 4-fold less procoagulant activity than $fVIIIa_{IIa}$. Kinetic analysis of fVIIIa cofactor activity using purified factor IXa and factor X suggested this difference is due to greater activity of $fVIIIa_{IIa}$ relative to $fVIIIa_{Xa}$ within the intrinsic fXase complex, rather than a difference in their stabilities.

Factor VIII (fVIII)[1] is the plasma glycoprotein that is decreased or absent in hemophilia A. Internal sequence homology in fVIII defines a sequence of domains designated NH_2-A1-A2-B-A3-C1-C2-COOH (Vehar et al., 1984; Toole et al., 1984). FVIII circulates in plasma bound noncovalently to von Willebrand factor. After limited proteolysis by thrombin (factor IIa) or factor Xa, activated fVIII (fVIIIa) binds factor IXa on phospholipid membranes to form the blood coagulation intrinsic pathway factor X activation complex (intrinsic fXase).

Although fVIII is synthesized as a 2332 amino acid, single-chain protein, it is subject to intracellular proteolysis prior to secretion (Kaufman et al., 1988). This produces a series of calcium-linked heterodimers formed by cleavage at Arg740, between the A2 and B domains, at several sites within the B domain, and at Arg1648, between the B and A3 domains (Fass et al., 1982; Vehar et al., 1984; Toole et al., 1984; Eaton et al., 1986, 1987; Fay et al., 1986). The heterodimer formed by cleavage at Arg740 and Arg1648 is designated A1-A2/*ap*-A3-C1-C2 where *ap* refers to an acidic peptide bounded by Glu1649 and Arg1689. Heterodimers formed by variable cleavage within the B domain and at Arg1648 are designated A1-A2-B^{var}/*ap*-A3-C1-C2. The A1-A2 and A1-A2-B^{var} fragments are known as fVIII heavy chains. The *ap*-A3-C1-C2 fragment is called the fVIII light chain.

Heterodimeric fVIII has no detectable cofactor activity in a purified system consisting of factor IXa, factor X, and phospholipid membranes (Lollar et al., 1985). Thrombin activates fVIII by catalyzing proteolytic cleavages between the A1 and A2 domains at Arg372, between the A2 and B domains at Arg740, and between the B and A3 domains at Arg1689 (Eaton et al., 1986). Cleavages at Arg372 and Arg740 form the A2 subunit, which is essential for fVIIIa function (Lollar & Parker, 1989, 1990; Fay et al., 1991a). Cleavage at Arg1689 is responsible for dissociation from von Willebrand factor but is not necessary for the development of cofactor activity (Hill-Eubanks et al., 1989; Aly & Hoyer, 1992; Regan & Fay, 1995). Thrombin-activated fVIII ($fVIIIa_{IIa}$) is a 160 kDa A1/A2/A3-C1-C2 heterotrimer (Eaton et al., 1987; Lollar & Parker, 1989; Fay et al., 1991a). At the plasma concentration of fVIII (≈1 nM) at pH 7.4, $fVIIIa_{IIa}$ spontaneously loses activity (Hultin & Jesty, 1981; Lollar et al., 1984, 1985; Lollar & Fass, 1984) due to reversible dissociation of the A2 subunit (Lollar & Parker, 1990; Fay et al., 1991a; Lollar et al., 1992).

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[1] Abbreviations: fVIII, factor VIII; fVIIIa, factor VIIIa (referring to either factor Xa-activated or thrombin-activated forms); factor IIa, thrombin; $fVIIIa_{IIa}$, thrombin-activated factor VIII; $fVIIIa_{Xa}$, factor Xa-activated factor VIII; Fl-M-FPR-factor IXa, fluorescein-5-maleimidyl-D-phenylalanyl-prolyl-arginyl-factor IXa; PCPS, phosphatidylcholine/phosphatidylserine; intrinsic fXase, phospholipid membrane-bound complex of fVIIIa and factor IXa; buffer A, 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 5 mM $CaCl_2$, and 0.01% poly(oxyethylene)-sorbitan monoleate (Tween 80), pH 6.0; buffer B, 0.15 M NaCl, 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mM $CaCl_2$, pH 7.4. Porcine sequence numbers are defined by sites that are homologous to human fVIII (Healey et al., 1996; Wood et al., 1984).

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In contrast to thrombin, the activation of fVIII by factor Xa is membrane-dependent. Factor Xa also cleaves fVIII at Arg372, Arg1689, and Arg740 (Eaton et al., 1986). However, additional cleavages are made in the A1 and A2 subunits that have not been identified (Lollar et al., 1985; Eaton et al., 1986). The peak level of fVIIIa activity produced by factor Xa is significantly lower than that produced by thrombin (Lollar et al., 1985; Neuenschwander & Jesty, 1992), although the functional difference between $fVIIIa_{IIa}$ and $fVIIIa_{Xa}$ has not been fully characterized.

The analysis of human and porcine $fVIIIa_{IIa}$ structure and function has been facilitated by methods to isolate it in stable form (Lollar & Parker, 1989; Curtis et al., 1994). We now report a method for producing a stable preparation of porcine $fVIIIa_{Xa}$, identification of factor Xa-catalyzed cleavages within the A1 and A2 domains, definition of the subunit composition of $fVIIIa_{Xa}$, and characterization of some of its functional differences from $fVIIIa_{IIa}$.

EXPERIMENTAL PROCEDURES

Materials. Activated partial thromboplastin reagent was purchased from General Diagnostics (Morris Plains, NJ). fVIII-deficient plasma was purchased from George King Biomedical, Inc. (Overland Park, KA). Pooled normal porcine plasma was prepared as described previously (Lollar & Parker, 1991). Mono S (HR 5/5) chromatography resin was purchased from Pharmacia (Piscataway, NJ). Recombinant tick anticoagulant protein was a gift from Dr. Sriram Krishnaswamy, Emory University. Unilamellar phosphatidylcholine/phosphatidylserine (PCPS) (75/25 w/w) vesicles were prepared as described previously (Barenholz et al., 1977).

Isolation of Proteins. All steps were done at room temperature unless otherwise indicated. Published procedures were used to isolate porcine factors IXa, X, Xa, and thrombin (Lollar et al., 1984; Lollar & Parker, 1989; Duffy & Lollar, 1992), fluorescein-5-maleimidyl-D-phenylalanyl-prolyl-arginyl-factor IXa (Fl-M-FPR-factor IXa) (Duffy et al., 1992), and fVIII and $fVIIIa_{IIa}$ (Lollar et al., 1993).

To isolate porcine $fVIIIa_{Xa}$, a 10 mL activation mixture was prepared containing 1 μM porcine fVIII, 100 μM PCPS, and 100 nM factor Xa in 0.43 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.006% Tween 80, and 0.04% poly(ethylene glycol) (PEG) 8000, pH 7.4. After 12 min, fVIII activation was stopped by the addition of the factor Xa-specific inhibitor, tick anticoagulant protein to 440 nM. The activation time was determined by preliminary small-scale experiments that established the time to reach optimum procoagulant activity. The activation mixture was diluted with 4.8 volumes of 40 mM MES, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 6.0, and immediately loaded at 2 mL/min onto a Mono S HR 5/5 column. The column was attached to system buffers 5 mM MES, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 6.0 (buffer A), and buffer A plus 1 M NaCl and equilibrated in Buffer A plus 0.1 M NaCl. After the column was washed with buffer A plus 0.1 M NaCl until the absorbance at 280 nm was less than 0.003, the column was further washed with 30 mL of buffer A plus 0.5 M NaCl and then 10 mL of buffer A plus 0.7 M NaCl . $FVIIIa_{Xa}$ was eluted with a 10 mL linear gradient from 0.7 to 1 M NaCl in buffer A, followed by 5 mL of buffer A plus 1.8 M NaCl at a flow rate of 1 mL/min. Fractions were analyzed for absorbance at 280 nm and for procoagulant activity as described below. Additionally, fractions were analyzed for factor Xa by using the chromogenic substrate Spectozyme Xa and for tick anticoagulant protein by inhibition of purified porcine factor Xa. There was no detectable factor Xa or tick anticoagulant protein in any of four preparations of $fVIIIa_{Xa}$. Fractions were analyzed for PCPS by measuring the optical density at 320 nm. One preparation contained detectable amounts of the trailing edge of a PCPS peak and was repurified by additional Mono S HPLC of the pooled $fVIIIa_{Xa}$ activity peak. The molar yield of $fVIIIa_{Xa}$ from four preparations averaged 70%. The $fVIIIa_{Xa}$ preparation was stable for at least 2 months when stored at 4 °C in elution buffer. Additionally, $fVIIIa_{Xa}$ preparations could be frozen at −80 °C and thawed without loss of activity.

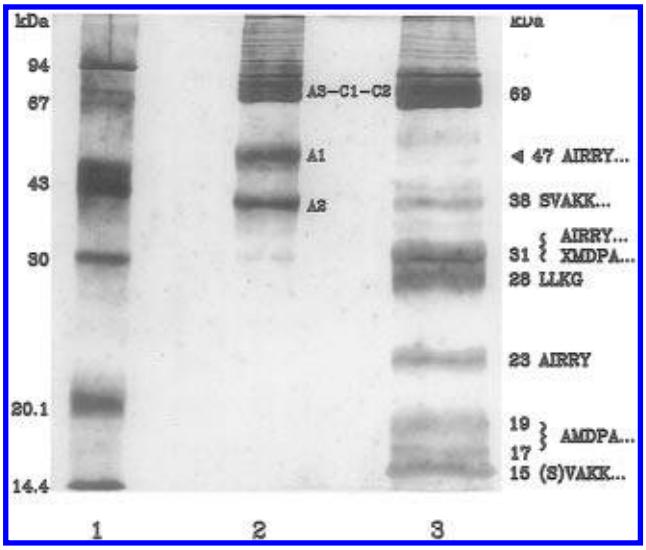

FIGURE 1: SDS−PAGE of porcine $fVIIIa_{Xa}$. Lane 3, Mono S peak material (1 μg) was subjected to SDS−PAGE followed by silver staining as described in Experimental Procedures. NH_2-terminal sequences of the fragments are shown to the right. Lane 1, molecular weight markers. Lane 2, purified porcine $fVIIIa_{IIa}$ (1 μg).

Electrophoresis. SDS−PAGE was done using a buffer system as described (Laemmli, 1970) followed by silver staining of the proteins (Morrissey, 1981). The apparent molecular masses of factor Xa-derived proteolytic fragments of fVIII were estimated by comparison to molecular weight standards shown in Figure 1.

$FVIIIa_{Xa}$ Peptide Isolation. Reduced and carboxamidomethylated factor Xa-derived proteolytic fragments of fVIII were isolated by preparative SDS−polyacrylamide gel electrophoresis at 4 °C using a MiniPrep Cell (Bio-Rad, Hercules, CA). The Laemmli buffer system, a 6-cm, 11% resolving gel, and a 1-cm 4% stacking gel were employed. $FVIIIa_{Xa}$, 0.2−0.5 mL, 0.1−0.2 mg (0.8−1.3 nmol), in 62 mM Tris, 1% SDS, 5% glycerol, and 0.001% bromphenol blue, pH 6.8, was loaded and stacked at 250 V for 1 h and then resolved at 400 V for 10−12 h. Fractions (0.2−0.4 mL) were eluted at 70−100 μL/min and then were analyzed by SDS−PAGE and silver staining. Fractions containing purified fragments were pooled and loaded onto Pro-Spin poly(vinylidene difluoride) membrane cartridges (Applied Biosystems, Foster City, CA) prewet with 25 μL of methanol. Membranes were washed four times with 50 μL of water and then dried by centrifugal evacuation prior to NH_2-terminal sequencing.

*NH_2-Terminal Sequencing.*Protein fragments electroblotted onto the Pro-Spin membranes were sequenced using an Applied Biosystems Model 477A pulsed-liquid protein sequencer (Hewick et al., 1981), and phenylthiohydantoin amino acids were separated and identified on-line using an Applied Biosystems Model 120A PTH analyzer as described (Pohl, 1994).

Sedimentation Analysis. Experiments were done using a Beckman Optima XL-A analytical ultracentrifuge equipped with a UV spectrophotometer for detection of protein at 280 nm. Samples contained 0.19 mg/mL of the $fVIIIa_{Xa}$ preparation in 0.60 M NaCl, 5 mM MES, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 6.0. An An60Ti rotor containing a double sector cell was used. The solvent sector contained matched Mono S column buffer.

Sedimentation velocity runs were done at 60 000 rpm and 20 °C. Scans of A_{280} vs radial position were taken at 4 min intervals. The sedimentation coefficient, *s*, was calculated by second moment analysis and was corrected to the value predicted for sedimentation in pure water at 20 °C ($s_{20,w}$) as described (Cantor & Schimmel, 1980).

Equilibrium sedimentation was done by using the high-speed meniscus depletion technique (Yphantis, 1964) at 15 000 rpm and 20 °C. The effective reduced molecular weight, σ, was calculated by nonlinear least-squares analysis of the dependence of A_{280} on squared radial position, r^2:

$$A_{280}(r) = A_{280}(r_0)\exp\left[\frac{\sigma(r^2 - r_0^2)}{2}\right]$$

where r_o is a reference radius in the cell. The molecular weight, M, was then calculated using the relationship

$$\sigma = \frac{M(1 - \bar{v}\rho)\omega^2}{RT}$$

where $\bar{v}$ is the partial specific volume, ρ is the solution density, ω is the angular velocity, *R* is the gas constant, and *T* is the absolute temperature. The effective reduced molecular weights of porcine $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ are indistinguishable (*vide infra*), which indicates that their polypeptide compositions are identical. Therefore, a partial specific volume of 0.73 mL/mg, and an extinction coefficient at 280 nm of 1.6 $(mg/mL)^{-1}cm^{-1,}$ which were previously determined for $fVIIIa_{IIa}$ (Lollar & Parker, 1989), were used for $fVIIIa_{Xa}$.

Coagulation Assays. FVIII and fVIIIa ($fVIIIa_{Xa}$ and $fVIIIa_{IIa}$) were measured by one-stage and two-stage coagulation assays, respectively, using activated partial thromboplastin time assay and human fVIII-deficient plasma as a substrate (Lollar et al., 1993). In both assays, 1 unit is defined as the amount of activity in 1 mL of normal pooled citrated porcine plasma.

Kinetics of Factor X Activation by Intrinsic fXase. The steady-state kinetics of factor X activation by intrinsic fXase were measured in 0.15 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, and 0.01% Tween 80 or 0.1% PEG 8000, pH 7.4 (buffer B), at 20−22 °C. The concentrations of factor X, factor IXa, fVIIIa, and PCPS used are given in the figure legends. At 15, 30, 45, and 60 s after initiation of the reaction, the product, factor Xa, was measured by mixing 20 μL aliquots of the activation solution with 80 μL of 0.4 mM Spectrozyme Xa in 0.15 M NaCl, 20 mM HEPES, 50 mM EDTA, and 0.1% PEG 8000, pH 7.4. The rate of Spectrozyme Xa hydrolysis catalyzed by factor Xa was measured in a kinetic microtiter plate reader (V_{max}, Molecular Devices). Initial rates of *p*-nitroanilide release were determined at 405 nm under conditions in which less than 10% Spectrozyme Xa hydrolysis had occurred. Factor Xa concentrations were determined by interpolation on a standard curve that was prepared by using a preparation of purified porcine factor Xa. The molar concentration of the factor Xa standard was determined by active-site titration (Lollar et al.,, 1985; Chase & Shaw, 1970). Under the conditions used, the velocity of factor X formation was linear and was calculated by linear regression.

Fluorescence Measurements. Steady-state fluorescence anisotropy measurements of Fl-M-FPR-factor IXa were made at 25 °C using an SLM-8000 spectrofluorometer in T-format as described previously (Duffy et al.,, 1992). All measurements were made in buffer B. PCPS, factor X, $fVIIIa_{Xa}$, and/or $fVIIIa_{IIa}$ were present at concentrations as described in the figure legends.

Data Analysis. The microscopic dissociation constant, K_d, and the stoichiometry, *n*, of the fVIIIa−Fl-M-FPR-factor IXa interaction were determined by nonlinear least-squares regression of the fluorescence anisotropy data as previously described (Duffy et al.,, 1992) using the quadratic equation

$$B = \frac{(nE_0 + K_d + C_0) - [(nE_0 + K_d + C_0)^2 - 4nE_0C_0]^{1/2}}{2}$$

where *B* is the concentration of bound complex and E_0 and C_0 are the nominal concentrations of factor IXa (enzyme) and fVIIIa (cofactor), respectively.

The K_d for the binding of fVIIIa to factor IXa also was estimated by measuring the initial velocity of factor X activation by varying factor IXa at fixed concentrations of fVIIIa and by assuming the concentration of complex is proportional to the initial velocity. The K_d was determined using the above quadratic formula and by fixing the stoichiometry at 1.

The Michaelis−Menten parameters K_m and V_{max} were measured by measuring the initial velocity of factor X activation as a function of factor X concentration at fixed concentrations of factor IXa, fVIIIa, and PCPS and fitting the data by unweighted nonlinear least-squares analysis to the equation

$$v_i = \frac{V_{max}S}{K_m + S}$$

where *S* is the substrate (factor X) concentration and $V_{max} = k_{cat}E_0$ and where E_0 is the total concentration of enzyme. For intrinsic fXase, only the factor IXa−fVIIIa complex contributes significantly to the velocity, so that $E_0 = B$, where *B* is the concentration of bound factor IXa−fVIIIa complex defined above.

Nonlinear least-squares regression analysis was done using the Marquardt algorithm (Bevington, 1969). Data are expressed as the mean ± 1 SD.

RESULTS

Isolation of $fVIIIa_{Xa}$. In preliminary experiments, we found that activation of 1 μM porcine fVIII by 100 nM factor Xa in the presence of 100 μM PCPS at pH 7.4 resulted in a

peak of procoagulant activity at 10−12 min. $FVIIIa_{Xa}$ activity then declined by 50% in approximately 1 h. For preparative purposes, fVIII was activated to peak levels, followed by inactivation of factor Xa with tick anticoagulant protein and then Mono S HPLC. A single peak of activity emerged from the Mono S column in 0.8 M NaCl that coincided with the A_{280} peak (not shown).

Identification of Factor Xa-Catalyzed Cleavages in Porcine fVIII. The $fVIIIa_{Xa}$ preparation was analyzed by SDS−PAGE and compared to $fVIIIa_{IIa}$ (Figure 1). The 47 kDa A1 and 38 kDa A2 subunits were nearly completely degraded to bands with apparent molecular masses of 15, 17, 19, 23, 28, and 31 kDa, consistent with earlier reports (Lollar et al.,, 1985). Incubation of fVIII with factor Xa for 21 h under these conditions resulted in further degradation of the 31 kDa fragment, but no further degradation of the other fragments. The formation of the 69 kDa A3-C1-C2 band has previously been shown to be due to cleavage at Arg1689 (Eaton et al., 1986).

The 15, 17−19, 23, 28, and 31 kDa fragments were isolated by preparative SDS−PAGE and subjected to NH_2-terminal sequence analysis. The sequences were identified relative to the sequence of porcine fVIII (Table 1). The sequence of the 23 kDa fragment corresponds to the NH_2-terminus of the A1 domain. The 17−19 kDa bands were not resolved and produced a single sequence consistent with cleavage at Arg219. The polypeptide molecular masses predicted by cleavage of the A1 domain at Arg219 and Arg372 are 24 700 and 17 292 Da, respectively, in good agreement with the 17−19 kDa and 23 kDa masses estimated by SDS−PAGE.

The porcine fVIII A1 domain contains potential glycosylation sites at Asn214 and Asn240 (Healey et al., 1996). It is likely that at least one of these sites is glycosylated because the electrophoretic mobility of the A1 subunit during SDS−PAGE is considerably lower than that of the A2 subunit (Figure 1), even though the predicted molecular mass of the A1 subunit actually is slightly lower than the A2 subunit (41 992 vs 42 088 Da). The A1 subunit of human fVIII also migrates anomalously slowly, which has been shown by endoglycosidase digestion to be due to glycosylation of the A1 domain (Kaufman et al., 1988). Glycosylation may explain the apparent heterogeneity of the 17−19 kDa preparation when only a single sequence is obtained.

The 15 and 28 kDa fragments are derived from the A2 domain. The former corresponds to the NH_2-terminus and the latter corresponds to the polypeptide generated by cleavage at Arg490. The polypeptide molecular masses predicted by the porcine sequence corresponding to the 15 and 28 kDa fragments are 13 371 and 28 717 Da, respectively, which is in good agreement with the masses estimated by SDS−PAGE.

The 31 kDa fragment contained a major sequence corresponding to the Ala1−Arg219 A1-derived fragment and a minor sequence corresponding to the Ala220−Arg372 A1-derived fragment. The association of these fragments in the presence of SDS may be a dynamic process that produces an electrophoretic mobility between that of the A1 subunit and the 23 kDa band. Alternatively, glycosylation at Asn214 could produce an Ala1−Arg219 A1-derived polypeptide with reduced electrophoretic mobility. Another possibility is that factor Xa cleaves fVIII at an additional site in the A1 domain COOH-terminal to Arg219 and that the fragment was not identified by our analysis.

Table 1: NH_2-Terminal Sequence Analysis of Porcine $FVIIIa_{Xa}$[a]

cycle	amino acid 1 (pmol)	amino acid 2 (pmol)
	15 kDa Fragment:	
1	Ser (15)	Ala (7)
2	Val (37)	
3	Ala (36)	
4	Lys (31)	
5	Lys (31)	
	sequence: (Ser)-Val-Ala-Lys-Lys	
	placement: Ser373−Lys377	
	17−19 kDa Fragments	
1	Ala (19)	
2	Met (17)	Ile (7)
3	Asp (19)	
4	Pro (19)	Leu (6)
5	Ala (19)	
6	Pro (16)	His (1)
7	Ala (14)	
8	Arg (14)	
9	Ala (14)	
10	Gln (11)	
	sequence: Ala-Met-Asp-Pro-Ala-Pro-Ala-Arg-Ala-Gln	
	placement: Ala220−Gln229	
	23 kDa Fragment:	
1	Ala (43)	
2	Ile (43)	Asp (3)
3	Arg (45)	Pro (5)
4	Arg (47)	Glu (4)
5	Tyr (34)	Ala (6)
	sequence: Ala-Ile-Arg-Arg-Tyr	
	placement: Ala1−Tyr5	
	28 kDa Fragment	
1	Leu (7)	Ala (4)
2	Leu (9)	Met (3)
3	Lys (5)	Asp (3)
4	Gly (8)	
	sequence: Leu-Leu-Lys-Gly	
	placement: Leu491−Gly494	
	31 kDa Fragment	
1	Ala (27)	
2	Ile (19)	Met (5)
3	Arg (21)	Asp (7)
4	Arg (23)	Pro (8)
5	Tyr (15)	Ala (9)
6	Tyr (16)	Pro (7)
7	Leu (17)	Ala (9)
8	Gly (17)	Arg (8)
9	Ala (20)	
10	Val (13)	Gln(6)
	sequence 1: Ala-Ile-Arg-Arg-Tyr-Tyr-Leu-Gly-Ala-Val	
	placement 1: Ala1−Val10	
	sequence 2: X-Met-Asp-Pro-Ala-Pro-Ala-Arg-X-Gln	
	placement 2: Ala220−Gln228	

[a] Sequences were determined as described in Experimental Procedures and were placed using published porcine fVIII sequences (Toole et al., 1986; Lubin et al., 1994; Healey et al., 1996).

Thus, in addition to cleavages at Arg372, Arg740, and Arg1689 that are also catalyzed by thrombin, factor Xa cleaves porcine fVIII at Arg219 and Arg490. The sequences around the factor Xa cleavage sites in the A1 and A2 domains are shown in Table 2. Also shown are the alignments of human and porcine fVIII in these regions.

Sedimentation Analysis of $fVIIIa_{Xa}$. The subunit composition of porcine $fVIIIa_{IIa}$ has been established by analytical ultracentrifugation as an A1/A2/*ap*-A3-C1-C2 heterotrimer (Lollar & Parker, 1989). Velocity ultracentrifugation of porcine $fVIIIa_{IIa}$ is consistent with a single species ($s_{20,w}$ =

Table 2: Sequences around Factor Xa Cleavage Sites in the A1 and A2 Domains of Porcine fVIII[a]

```
A1          210       220       230
Porcine  SWHSARNDSWTRAMDPAPARAQPAMHTV
         ::::     :      : : ::: : ::::
Human    SWHSETKNSLMQDRDAASARAWPKMHTV

A2               490       500
Porcine  DVSALHPGRLLKGWKHLKDMPILPGETF
         :: :     :: :: ::::: :::::: :
Human    DVRPLYSRRLPKGVKHLKDFPILPGEIF
```

[a] The P_1 arginine is indicated in boldface italic type.

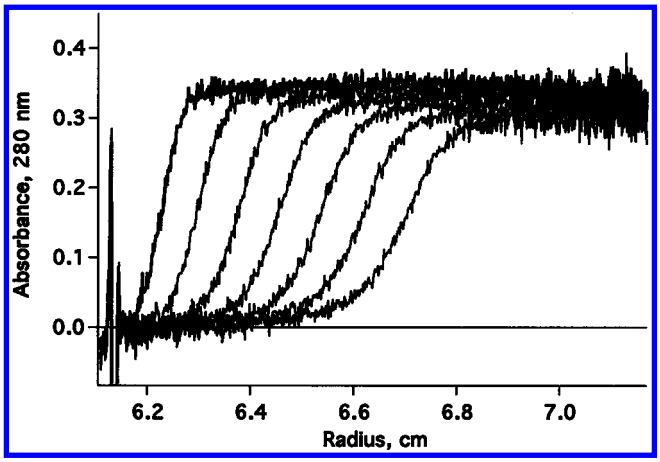

FIGURE 2: Velocity sedimentation of porcine $fVIIIa_{Xa}$. The $fVIIIa_{Xa}$ preparation (0.19 mg/mL) in 0.60 M NaCl, 5 mM MES, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 6.0, was sedimented at 60 000 rpm in a Beckman XLA analytical ultracentrifuge as described in Experimental Procedures. Shown is the superposition of scans that were obtained at 4 min intervals.

7.2 S). Equilibrium ultracentrifugation yields an apparent molecular mass that ranges from 145 to 161 kDa, depending on the loading concentration, which is consistent with the molecular weight of the heterotrimer predicted by polypeptide composition.

Sedimentation velocity analysis of the $fVIIIa_{Xa}$ preparation revealed a single boundary migrating with a sedimentation coefficient of 6.4 S (Figure 2), which when corrected for solvent density yielded an $s_{20,w}$ of 7.2 S. Sedimentation equilibrium analysis was consistent with a single 148 kDa species (Figure 3). Thus, the hydrodynamic properties of the $fVIIIa_{Xa}$ preparation are indistinguishable from those of $fVIIIa_{IIa}$ and are consistent with a model in which cleavage of fVIII by factor Xa within the A1 and A2 domains does not lead to dissociation of any of the fragments (Figure 4).

Functional Comparison of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$. The procoagulant activity of porcine $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ were measured using porcine plasma as a standard as described in Experimental Procedures. The specific activity of the $fVIIIa_{Xa}$ preparation was 11 600 units/A_{280} (18 600 units/mg). Porcine $fVIIIa_{IIa}$ was isolated using the same chromatography system and assay reagents for comparison and had a specific activity of 45 300 units/A_{280} (72 500 units/mg), consistent with previously published results (Lollar & Parker, 1989). Thus, the specific procoagulant activity of the $fVIIIa_{Xa}$ preparation was 4-fold less than the $fVIIIa_{IIa}$ preparation.

To determine if $fVIIIa_{Xa}$ is less active in a system using purified components, its ability to act as a cofactor for factor

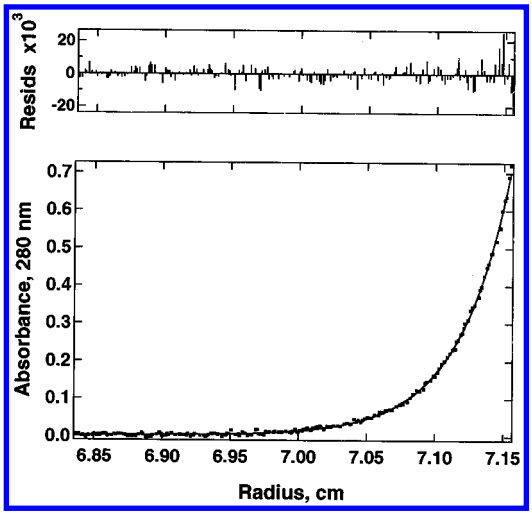

FIGURE 3: Equilibrium sedimentation of porcine $fVIIIa_{Xa}$. The $fVIIIa_{Xa}$ preparation, 0.19 mg/mL in 0.60 M NaCl, 5 mM MES, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 6.0, underwent analytical ultracentrifugation at 15 000 rpm as described in Experimental Procedures. In the lower panel, the curve represents a nonlinear least-squares fit to the data, assuming a single sedimenting species. In the upper panel are plotted the residuals from the least-squares fit.

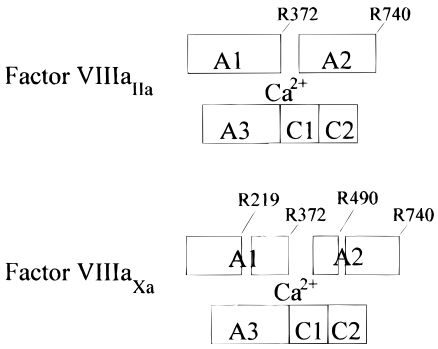

FIGURE 4: Subunit composition of porcine $fVIIIa_{Xa}$ species with comparison to $fVIIIa_{IIa}$.

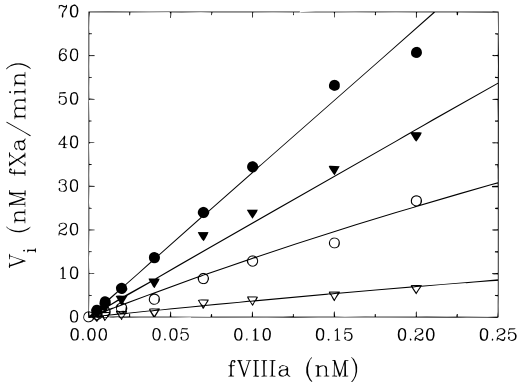

FIGURE 5: $FVIIIa_{Xa}$-dependent and $fVIIIa_{IIa}$-dependent activation of factor X by factor IXa. Reaction mixtures (0.25 mL) contained 0.25 nM factor IXa (open symbols) or 5 nM factor IXa (closed symbols) and $fVIIIa_{Xa}$ (triangles) or $fVIIIa_{IIa}$ (circles) as indicated, along with 1 μM factor X and 5 μM PCPS in buffer B plus 0.01% Tween 80. The initial velocity of factor Xa formation was measured as described in Experimental Procedures.

IXa during membrane-dependent factor X activation was evaluated. Figure 5 shows the dependence of the initial velocity of factor X activation on $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ concentration at two fixed concentrations of factor IXa. The data are linear with respect to fVIIIa, indicating that factor

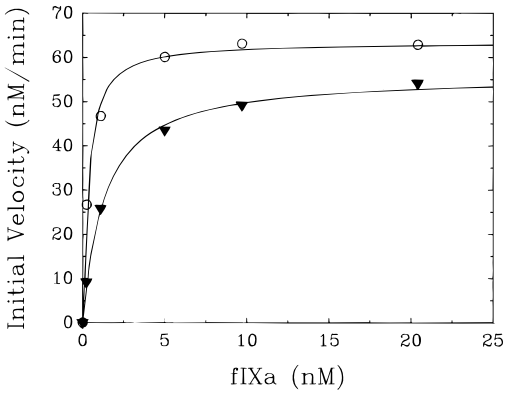

FIGURE 6: Factor IXa dependence of factor X activation at fixed concentrations of $fVIIIa_{Xa}$ or $fVIIIa_{IIa}$. Reaction mixtures (0.25 mL) contained 0.2 nM $fVIIIa_{Xa}$ (triangles) or 0.2 nM $fVIIIa_{IIa}$ (circles) and factor IXa as indicated, along with 5 μM PCPS and 1 μM factor X in buffer B plus 0.01% Tween 80. The initial velocity of factor Xa formation was measured as described in Experimental Procedures. The curves represent nonlinear least-squares fits according to the binding parameters shown in Table 3.

Table 3: Binding Parameters for the Interaction of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ with Factor IXa and Fl-M-FPR-Factor IXa[a]

Kinetic Measurements		
species	K_d (nM)	$v_{i,sat}$[b]
$fVIIIa_{Xa}$/factor IXa	1.2 ± 0.1	56.0 ± 1.0
$fVIIIa_{IIa}$/factor IXa	0.26 ± 0.04	63.5 ± 1.3

Fluorescence Measurements			
Species	K_d (nM)	n	Δr_{sat}[c]
$fVIIIa_{Xa}$	1.0 ± 0.3	1.14 ± 0.049	0.074 ± 0.001
$fVIIIa_{Xa}$ + factor X	2.7 ± 0.7	1.00 ± 0.074	0.086 ± 0.002
$fVIIIa_{IIa}$	1.9 ± 0.5	0.82 ± 0.04	0.078 ± 0.003
$fVIIIa_{IIa}$ + factor X	1.5 ± 0.3	1.48 ± 0.024	0.116 ± 0.002

[a] Binding parameters were determined as described in Experimental Procedures. [b] Initial velocity at the saturating level of fVIIIa−factor IXa complex. [c] Anisotropy increase at the saturating level of fVIIIa-Fl-M-FPR−factor IXa complex.

IXa is not significantly saturated with fVIIIa under these conditions. At the lower concentration of factor IXa, the slope of the $fVIIIa_{IIa}$ line is 3.8-fold greater than the slope of the $fVIIIa_{Xa}$ line. At the higher concentration of factor IXa, the ratio of the slopes decreases to 1.7.

This phenomenon was investigated further by fixing the concentration of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ and varying the concentration of factor IXa. The initial velocity of factor X activation reaches a plateau, consistent with saturable binding of fVIIIa by factor IXa (Figure 6). Assuming the initial velocity is proportional to the concentration of bound fVIIIa-factor IXa and assuming a stoichiometry of 1 for the binding of fVIIIa to factor IXa from fluorescence measurements (*vide infra*), it is possible to estimate the apparent dissociation constants for the binding of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ to factor IXa. This analysis indicates that $fVIIIa_{Xa}$ binds factor IXa more weakly than does $fVIIIa_{IIa}$ (Table 3). However, at saturation, the $fVIIIa_{Xa}$−factor IXa and $fVIIIa_{IIa}$−factor IXa complexes activate factor X nearly equally well.

The activation of factor X by the intrinsic fXase obeys Michaelis−Menten kinetics (van Dieijen et al., 1981; Lollar et al., 1985; Neuenschwander & Jesty, 1988; Duffy & Lollar, 1992). Phospholipid increases the catalytic efficiency of factor IXa by lowering the K_m for the reaction, whereas the major effect of fVIIIa is to increase the V_{max} (van Dieijen et al., 1981). Figure 7 compares the factor X dependence of

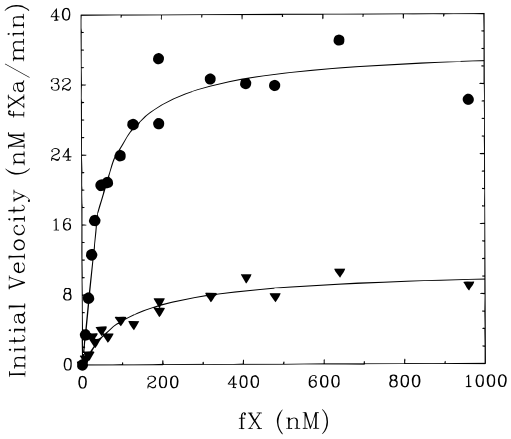

FIGURE 7: Michaelis−Menten parameters for the activation of factor X by factor IXa/$fVIIIa_{Xa}$ and factor IXa/$fVIIIa_{IIa}$. Reaction mixtures (0.25 mL) contained 0.25 nM factor IXa, 0.2 nM $fVIIIa_{Xa}$ (triangles) or 0.2 nM $fVIIIa_{IIa}$ (circles), 5 μM PCPS, and factor X as indicated in buffer B plus 0.01% Tween 80. The initial velocity of factor Xa formation was measured as described in Experimental Procedures. The curves represent nonlinear least-squares fits to the Michaelis−Menten equation, yielding $K_m = 112 \pm 22$ nM and 43.1 ± 6.6 nM and $V_{max} = 10.8 \pm 0.7$ nM/min and 36.1 ± 1.3 nM/min for $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$, respectively.

the initial velocity of factor X activation by factor IXa in the presence of PCPS and either $fVIIIa_{IIa}$ or $fVIIIa_{Xa}$. The concentrations of factor IXa and fVIIIa, 0.25 nM and 0.2 nM, respectively,were selected to produce relatively large differences between $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ (Figure 5). Under these conditions, the defect associated with $fVIIIa_{Xa}$ is due to both an 2.6 fold-increase in K_m and a 3.6-fold decrease in V_{max}.

Stability of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$. The A2 subunit of $fVIIIa_{IIa}$ is in reversible equilibrium with the A1/A3-C1-C2 dimer (Lollar & Parker, 1990, 1991; Fay et al., 1991a; Lollar et al., 1992). A2 subunit dissociation leads to loss of fVIIIa activity and accounts for the labile activity of $fVIIIa_{IIa}$ in dilute solution. The dissociation rate constant decreases when the pH is lowered from 7.4 to 6.0. To determine whether the difference in procoagulant activity might also be a function of lability, we diluted $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ to 1 nM and measured their kinetics of decay at pH 6.0 and pH 7.4 (Figure 8). The experiments were done by diluting decay mixtures into a solution containing a molar excess of factor IXa and then measuring the initial velocity of factor X activation. Under these conditions, the initial velocity of factor X activation is directly proportional to the concentration of remaining active fVIIIa. Figure 8 shows that $fVIIIa_{Xa}$ was actually more stable than $fVIIIa_{IIa}$ at both pH 6.0 ($t_{1/2}$ 20 min vs $t_{1/2}$ 10 min) and pH 7.4 ($t_{1/2}$ 7 min vs $t_{1/2}$ 5 min). Thus, the relatively low activity of $fVIIIa_{Xa}$ does not appear to be due to faster dissociation of its A2 subunit.

Binding of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ to Factor IXa. The membrane-dependent binding of $fVIIIa_{IIa}$ to a fluorescent, active-site-blocked derivative of porcine factor IXa, Fl-M-FPR-factor IXa, can be measured from the increase in fluorescence anisotropy associated with the binding event (Duffy et al., 1992; Mutucumarana et al., 1992). Addition of factor X to the $fVIIIa_{IIa}$/Fl-M-FPR-factor IXa complex produces an additional increase in anisotropy, despite the fact that Fl-M-FPR-factor IXa is blocked at the active site (Lollar et al., 1994). The increase in anisotropy may result from the binding of factor X to Fl-M-FPR-factor IXa and

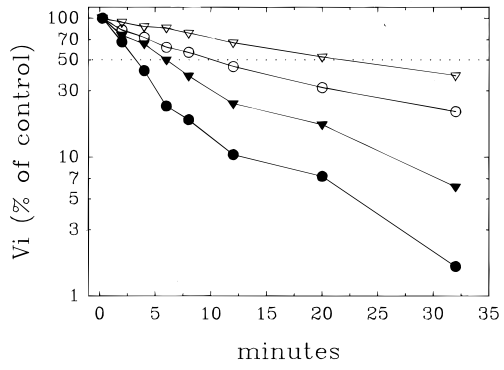

FIGURE 8: Decay of porcine $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ at pH 6.0 and 7.4. $FVIIIa_{Xa}$ (triangles) or $fVIIIa_{IIa}$ (circles) was diluted to 1 nM in 0.15 M NaCl, 5 mM MES, 5 mM $CaCl_2$, and 0.1% PEG 8000, pH 6.0 (open symbols), or 0.15 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.1% PEG 8000, pH 7.4 (closed symbols). At various times, fVIIIa samples were further diluted to 0.25 nM into activation mixtures (0.25 mL) containing 2 nM factor IXa, 20 μM PCPS, and 0.3 μM factor X in buffer B plus 0.01% Tween 80.The initial velocity of factor Xa formation was measured as described in Experimental Procedures and was normalized to the initial starting value..

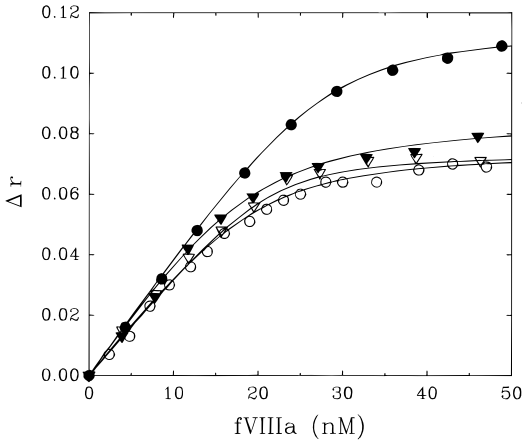

FIGURE 9: $FVIIIa_{Xa}$-dependent and $fVIIIa_{IIa}$-dependent increase in fluorescence anisotropy (Δr) of Fl-M-FPR-factor IXa in the presence and absence of factor X. Solutions (0.5 mL) contained 19 nM porcine Fl-M-FPR-factor IXa and 100 μM PCPS, varying concentrations of porcine $FVIIIa_{Xa}$ alone (open triangles), $FVIIIa_{Xa}$ plus 300 nM factor X (closed triangles), $fVIIIa_{IIa}$ alone (open circles), or $fVIIIa_{IIa}$ plus 300 nM factor X (closed circles). The curves are drawn by nonlinear least-squares fits to the binding isotherm described in Experimental Procedures according to binding parameters shown in Table 3.

sandwiching of the fluorescein dye between the two proteins, resulting in further hindrance of rotational motion of the dye probe.

In the absence of factor X, $fVIIIa_{Xa}$ produces fluorescence changes that are very similar to those produced by $fVIIIa_{IIa}$ (Figure 9). The stoichiometries for both $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ in the presence and absence of factor X are near unity, indicating that all molecules in both preparations can bind factor IXa (Table 3). The dissociation constants for $fVIIIa_{Xa}$ in the presence and absence of factor X were slightly lower than for $fVIIIa_{IIa}$. Thus, the lower activity of $fVIIIa_{Xa}$-associated intrinsic fXase cannot be attributed to weaker binding of $fVIIIa_{Xa}$ to factor IXa. However, in contrast to $fVIIIa_{IIa}$, factor X produces only a slight additional increase in anisotropy of Fl-M-FPR-factor IXa in the presence of saturating concentrations of $fVIIIa_{Xa}$ (Table 3), indicative of a qualitative difference in the assembly of $fVIIIa_{Xa}$-associated and $fVIIIa_{IIa}$-associated intrinsic fXase complexes.

DISCUSSION

The kinetics of porcine fVIII activation by factor Xa and by thrombin differ in both the proteolytic cleavage pattern and the amount of activity generated. Thrombin produces three subunits, A1 (Ala1−Arg372), A2 (Ser373−Arg740), and A3-C1-C2 (Ser1649−Tyr2332), which resist further proteolytic degradation (Lollar & Parker, 1989). In contrast, the activation of porcine fVIII by factor Xa is associated with very little formation of the A1 subunit, although the intact A2 subunit does accumulate as an intermediate (Lollar et al., 1985).

In this study we developed a method to produce a stable preparation of porcine $fVIIIa_{Xa}$ and compared some of its structural and functional features to $fVIIIa_{IIa}$. The procoagulant activity of the $fVIIIa_{Xa}$ preparation is 4-fold lower than that of porcine $fVIIIa_{IIa}$. The preparation contains very little uncleaved A1 subunit but does contain detectable uncleaved A2 subunit (Figure 1). NH-terminal sequence analysis reveals that factor Xa cleaves porcine fVIII at Arg219 and Arg490, in addition to cleavages at Arg372, Arg740, and Arg1689 that are also catalyzed by thrombin (Table 1).

Despite the proteolytic degradation of the A1 and A2 subunits, the hydrodynamic properties of the $fVIIIa_{Xa}$ preparation are similar to those of $fVIIIa_{IIa}$. Therefore, the novel proteolytic fragments produced by factor Xa remain bound. The five-chain species formed by cleavages at Arg219, Arg372, Arg490, Arg740, and Arg1689 is shown as the middle structure in Figure 4. Additionally, a minor four-chain species lacking cleavage at Arg490, shown as the bottom structure in Figure 4, is also likely to be present.

Arg490 in porcine fVIII is in a region that is homologous to the Arg484−Ile508 segment in human fVIII recognized by clinically significant alloimmune and autoimmune anti-fVIII antibodies (Healey et al., 1995). These antibodies bind $fVIIIa_{IIa}$ and inhibit intrinsic fXase noncompetitively (Lollar et al., 1994). Since these observations define a functionally important region, cleavage at Arg490 in porcine fVIII may be partly responsible for the decreased activity of $fVIIIa_{Xa}$ compared to $fVIIIa_{IIa}$. The activity in the $fVIIIa_{Xa}$ preparation is an average of molecules with and without cleavage at Arg490. Since we have not isolated a species consisting of cleavages at Arg219, Arg372, Arg740, and Arg1689 but not Arg490 (Figure 4), we do not know its relative specific activity. Additionally, the presence of a small amount of this species may contribute significantly to the specific activity of the preparation.

In contrast to porcine fVIII, the activation of human fVIII by factor Xa is associated with the formation of substantial amounts of intact A1 subunit (Eaton et al., 1986). Thus, if cleavage occurs at a site homologous to porcine Arg219, it is slow compared with cleavage at Arg372. Factor Xa also cleaves human fVIII at Arg1721 in the A3 domain (Eaton et al., 1986). This reaction is slow compared with other cleavages of human fVIII by factor Xa and its functional significance is unknown. In contrast, under conditions in which factor Xa completely cleaves porcine fVIII at Arg1689 within 12 min, we observed no cleavage at Arg1721 over 21 h.

Additionally, the proteolysis of human fVIII by factor Xa is associated with cleavage near the COOH-terminal end of the A1 domain at a site that has been tentatively identified as Arg336 (Eaton et al., 1986). Activated protein C, which proteolytically inactivates human and porcine fVIII and $fVIIIa_{IIa}$, catalyzes cleavages at Arg336 and Arg562 in human fVIII (Fay et al., 1991b). Cleavage at Arg336 promotes dissociation of the A2 subunit (Fay et al., 1991b, 1993), which is essential for the activity of human and porcine $fVIIIa_{IIa}$ (Lollar & Parker, 1990, 1991). Cleavage at Arg562 disrupts the factor IXa binding site (Fay et al., 1994; O'Brien et al., 1995).

These results indicate there are differences between factor Xa-catalyzed cleavages in human and porcine fVIII that may be functionally significant. The human and porcine A1 and A2 domains are 79% and 84% identical, respectively. However, there is considerably more divergence of sequence around the porcine fVIII factor Xa cleavage sites (Table 2). Eleven of the 14 residues in the porcine sequence Ala212−Pro225 surrounding the factor Xa cleavage site at Arg219 differ from human fVIII. Additionally, seven of the 12 residues in the porcine sequence Ser484−Trp495 surrounding the factor Xa cleavage site at Arg490 differ from human fVIII. This is consistent with the observation that factor Xa catalyzes different proteolytic cleavage patterns in porcine and human fVIII (Lollar et al., 1985; Eaton et al., 1986).

The comparison of human and porcine fVIII shows that the latter has approximately 6-fold more specific procoagulant activity (Lollar et al., 1992). Part of this difference may be because the porcine A2 subunit dissociates more slowly from the parent $fVIIIa_{IIa}$ molecule than in the homologous human situation (Lollar et al., 1992). However, differences between human and porcine $fVIIIa_{Xa}$ species also could be partly responsible for the differences in procoagulant activity. The comparison of human and porcine fVIII is important because both are used clinically to treat factor VIII deficiency (Hay & Lozier, 1995). The assay used to define a unit of porcine fVIII for dosage purposes is based on an arbitrary standard due to the difference between human and porcine fVIII. Isolation and characterization of human $fVIIIa_{Xa}$ are required for further study, as well as are methods for determining the relative procoagulant contributions of $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$.

Besides its decreased procoagulant activity compared to $fVIIIa_{IIa}$, $fVIIIa_{Xa}$ also has less activity in a purified system when assayed as a cofactor for factor IXa during factor X activation. The difference in cofactor activity decreases as the formation of fVIIIa−factor IXa complexes approaches saturation (Figures 6 and 7), which suggests weaker binding of $fVIIIa_{Xa}$ to factor IXa compared to binding of $fVIIIa_{IIa}$ (Table 3). Consistent with this, the V_{max} for factor X activation, which is proportional to the concentration of fVIIIa−factor IXa complexes, is lower in the presence of $fVIIIa_{Xa}$ compared to $fVIIIa_{IIa}$ (Figure 7).

However, fluorescence anisotropy measurements are not consistent with the simple interpretation that the membrane-dependent binary interaction between $fVIIIa_{Xa}$ and factor IXa is weaker than the corresponding $fVIIIa_{IIa}$−factor IXa interaction (Figure 9). It is possible that the functional difference between $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ depends on the substrate, factor X. The increase in the K_m for factor X activation for $fVIIIa_{Xa}$ compared with $fVIIIa_{IIa}$, which may indicate poorer binding of factor X, is consistent with this. Additionally, the qualitative difference between factor X binding to $fVIIIa_{Xa}$-associated intrinsic fXase and $fVIIIa_{IIa}$-associated intrinsic fXase manifested by a decrease in the limiting fluorescence anisotropy (Table 3), is consistent with the possibility that the defect in $fVIIIa_{Xa}$ depends on substrate.

The decreased cofactor activity of $fVIIIa_{Xa}$ does not necessarily account for its decreased procoagulant activity. However, apart from its measurable functions related to the intrinsic fXase complex, the only other known determinant of fVIIIa activity *in vitro* is its lability in dilute solution (Hultin & Jesty, 1981; Lollar et al., 1984, 1992; Lollar & Fass, 1984; Lollar & Parker, 1990). Because porcine $fVIIIa_{Xa}$ is less labile than $fVIIIa_{IIa}$ (Figure 8), this mechanism cannot account for the difference in procoagulant activity. The difference in lability between $fVIIIa_{Xa}$ and $fVIIIa_{IIa}$ was not detected in previous kinetic studies in the porcine system (Lollar et al., 1985). However, analysis of the kinetics of human fVIII activation and inactivation suggests that $fVIIIa_{Xa}$ has less cofactor activity and decays more slowly than $fVIIIa_{IIa}$ (Neuenschwander & Jesty, 1992), similar to the results of the present study. These results were interpreted using a model in which fVIIIa is in conformational equilibrium between active and inactive states and only the active state is labile. According to this model, the equilibrium constant for formation of the active conformer is more favorable for $fVIIIa_{IIa}$ than $fVIIIa_{Xa}$, resulting in higher cofactor activity but attendant faster decay. Our results are consistent with this model.

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