Proteolysis of Blood Coagulation Factor VIII by the Factor VIIa−Tissue Factor Complex: Generation of an Inactive Factor VIII Cofactor†

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ABSTRACT: Activation of factor VIII by thrombin occurs via limited proteolysis at R^{372}, R^{740}, and R^{1689}. The resultant active factor VIIIa molecule consists of three noncovalently associated subunits: A1-a1, A2-a2, and A3-C1-C2 (50, 45, and 73 kDa respectively). Further proteolysis of factor VIIIa at R^{336} and R^{562} by activated protein C subsequently inactivates this cofactor. We now find that the factor VIIa−tissue factor complex (VIIa−TF/PL), the trigger of blood coagulation with restricted substrate specificity, can also catalyze limited proteolysis of factor VIII. Proteolysis of factor VIII was observed at 10 sites, producing 2 major fragments (47 and 45 kDa) recognized by an anti-factor VIII A2 domain antibody. Time courses indicated the slow conversion of the large fragment to 45 kDa, followed by further degradation into at least two smaller fragments. N-Terminal sequencing along with time courses of proteolysis indicated that VIIa−TF/PL cleaved factor VIII first at R^{740}, followed by concomitant cleavage at R^{336} and R^{372}. Although cleavage of the light chain at R^{1689} was observed, the majority remained uncleaved after 17 h. Consistent with this, only a transient 2-fold increase in factor VIII clotting activity was observed. Thus, heavy chain cleavage of factor VIII by VIIa−TF/PL produces an inactive factor VIII cofactor no longer capable of activation by thrombin. In addition, VIIa−TF/PL was found to inactivate thrombin-activated factor VIII. We hypothesize that these proteolyses may constitute an alternative pathway to regulate coagulation under certain conditions. In addition, the ability of VIIa−TF/PL to cleave factor VIII at 10 sites greatly expands the known protein substrate sequences recognized by this enzyme−cofactor complex.

Factor VIII is a large plasma glycoprotein ($M_r \approx 280\,000$) that is required as a cofactor for the activation of factor X by factor IXa. Factor VIII is secreted into the plasma in a two-chain form (heavy chain $M_r \approx 200\,000$; light chain $M_r \approx 80\,000$) held together largely though ionic interactions involving divalent metal (i.e., Ca^{2+}). The domainal structure of factor VIII can be represented as A1-a1-A2-a2-B-a3-A3-C1-C2, where the heavy chain is comprised of the A1-a1-A2-a2-B domains, and the light chain is comprised of the a3-A3-C1-C2 domains (*1*).

Full expression of factor VIII cofactor activity requires its activation via limited proteolysis. In vivo, this activation is thought to be mediated largely by thrombin, though factor Xa can efficiently catalyze this reaction in a phospholipid-dependent manner in vitro (*2, 3*). Factor VIII activation by thrombin involves hydrolysis of three peptide bonds: R^{372}−S^{373} and R^{740}−S^{741} in the heavy chain (at the a1/A2 and a2/B junctions, respectively), and R^{1689}−S^{1690} in the light chain (at the a3/A3 junction). It has been shown that hydrolysis at both R^{372} and R^{1689} is sufficient for generation of factor VIIIa activity (*4*), while hydrolysis at R^{740} may serve only to release the bulky B domain from the activated molecule. Thus, the final thrombin-activated factor VIII molecule [factor $VIIIa_{(T)}$][1] is a noncovalently associated heterotrimer consisting of the A1-a1 subunit ($M_r \approx 50\,000$), the A2-a2 subunit ($M_r \approx 45\,000$), and the A3-C1-C2 subunit ($M_r \approx 73\,000$). Activation of factor VIII by factor Xa can also occur (*5*) but involves cleavage at R^{1721} in the light chain rather than at R^{1689}. The functional consequence of this alternate light chain cleavage is unclear, but likely involves the generation of factor VIIIa activity (*1*).

Once activated, factor VIIIa can undergo further proteolysis by either factor Xa (*5*), factor IXa (*6, 7*), or activated protein C (*5, 8*), resulting in its inactivation. All three of these proteases cleave at R^{336} to separate the A1 and a1 domains. Additionally, activated protein C cleaves at R^{562} to bisect the A2 domain, which also results in loss of activity (*9*).

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[1] Abbreviations: TF, tissue factor; PC, L-α-phosphatidylcholine (egg white); PS, L-α-phosphatidyl-L-serine (bovine brain); PE, L-α-phosphatidylethanolamine (bovine liver); TF/PL, TF relipidated into phospholipid vesicles composed of 10 mol % PS, 60 mol % PC, and 30 mol % PE; VIIa−TF/PL, enzyme−cofactor complex of factor VIIa and relipidated TF; IXa−VIIIa, enzyme−cofactor complex of factor IXa and factor VIIIa; BSA, bovine serum albumin; factor VIII, unactivated factor VIII; factor $VIIIa_{(T)}$, thrombin-activated factor VIII; factor $VIII_{(VIIa-TF)}$, factor VIII treated with VIIa−TF/PL; vWf, von Willebrand's factor.

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It is commonly accepted that the complex of factor VIIa and tissue factor (VIIa−TF/PL) is the primary initiator of blood coagulation under normal circumstances and in many pathological conditions (for a review, see reference *10*). The formation of this activating complex on suitable phospholipid surfaces triggers blood coagulation largely by activating factor X through limited proteolysis (hydrolysis of the R^{194}−Ile^{195} bond). While this reaction is generally considered the major driving force for initiating coagulation, the VIIa−TF/PL complex is also known to proteolytically activate factor IX (*11, 12*) (hydrolysis of the R^{145}−Ala^{146} and R^{180}−Val^{181} bonds) and factor VII (*13*) (hydrolysis of the R^{152}−Ile^{153} bond). Both of these reactions serve to greatly amplify the coagulant response; the former by generating a secondary activator of factor X (IXa−VIIIa), and the latter by increasing levels of VIIa−TF/PL via a positive feedback loop. Aside from these three known natural substrates of VIIa−TF/PL (four cleavage sites in total), no natural cleavage sites have been reported for this highly specific enzyme−cofactor pair until recently. Safa et al. (*14*) were able to demonstrate that VIIa−TF/PL can also catalyze proteolysis of factor V at four sites to produce a factor V molecule with altered properties. We now demonstrate the ability of VIIa−TF/PL also to catalyze the proteolysis of factor VIII. We find that VIIa−TF/PL recognizes and cleaves factor VIII at 10 sites throughout the factor VIII molecule, resulting in an inactive factor VIII cofactor that is incapable of activation by thrombin.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (BSA; fraction V and fatty acid free), recombinant hirudin, and octyl-β-D-glucopyranoside were from Calbiochem. Purified phospholipids were from Avanti Polar Lipids, Inc. (Alabaster, AL). All phospholipids were stored at high concentrations (10 mg/mL) in chloroform under Argon at −20 °C. The chromogenic substrate S-2222 (*N*-benzoyl-I-E-G-R-*p*-nitroaniline) was from Chromogenix AB (DiaPharma Group Inc., Franklin, OH). Human factor VIII-deficient plasma was from George King Bio-Medical (Overland Park, KS). The anti-factor VIII A2 domain antibody (530p) was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY), and the sheep anti-human factor VIII polyclonal antibody was from Cedarlane Labs Ltd. (Hornby, Ontario, Canada). All other reagents were of the highest quality available from various suppliers.

Protein Preparations. Recombinant human factor VIIa and TF (TF_{1-244}, membrane-anchored TF lacking most of the carboxyl-terminal cytoplasmic tail) were prepared as previously described (*15, 16*). The TF was relipidated into phospholipid membranes composed of 10% PS, 60% PC, and 30% PE by detergent dialysis using octyl-β-D-glucopyranoside (*17*) as previously described for both full-length TF (*15*) and TF_{1-244} (*16*), but in the absence of any carrier protein. Blank phospholipid vesicles were prepared in a similar fashion by omitting the TF.

Human factor X was isolated from plasma (*18*) and used to prepare acetylated factor X as previously described (*19*). This modified factor X has the unique properties of still being capable of activation by IXa−VIIIa and retaining latent amidolytic activity. More importantly, the generated acetylated factor Xa no longer supports clotting and lacks the ability to feedback activate factor VIII (*19*). Recombinant human full-length factor VIII was a generous gift of Dr. Roger Lundblad of Baxter Healthcare Corp. (Duarte, CA). Human vWf was purchased from Haematologic Technologies, Inc. (Essex Junction, VT), bovine α-thrombin was a gift of Dr. Charles T. Esmon, and the factor VIIa inhibitor XK1 (a factor X light chain−TFPI Kunitz domain 1 chimera) was a gift of Dr. George Broze, Jr. (Washington University, St. Louis, MO). The inhibitory anti-TF monoclonal antibody 5G9 (*20*) was purified from ascites fluid by protein A affinity chromatography.

Factor VIII Clotting Assays. These assays were modified from a standard one-stage clotting assay to involve two stages essentially as described by Hultin and Jesty (*21*). The first stage involved a 37 °C timed incubation of 210 units/mL factor VIII in HBSA buffer (20 mM Hepes−NaOH, pH 7.5, 100 mM NaCl, 0.1% BSA) with the indicated concentrations of thrombin or VIIa−TF/PL. Incubations using VIIa−TF/PL contained 5 mM $CaCl_2$, a limiting amount of factor VIIa (typically 10 nM), plus a saturating amount of TF/PL (20 nM TF relipidated into 150 μM phospholipid vesicles composed of 10 mol % PS, 60 mol % PC, and 30 mol % PE). Timed aliquots were removed from this incubation and quickly diluted 500-fold into HBSA buffer containing either 1 unit/mL hirudin (for thrombin reactions) or 400 nM 5G9 (an anti-TF inhibitory antibody). One microliter of this diluted sample was then immediately added to a timed, preincubated clotting assay (stage II).

The clotting assay was done as follows. Factor VIII-deficient plasma (50 μL) was preincubated with 49 μL of HBSA and 50 μL of Sigma APTT reagent for 8 min before the timed addition of the 1 μL aliquot from stage I. Clotting was subsequently initiated by the immediate addition of 50 μL of 25 mM $CaCl_2$. Clotting times were measured using an ST4 Coagulometer (Diagnostica Stago) and converted into milliunits per milliliter of factor VIIIa activity by comparison to a standard curve prepared using pooled normal human plasma (assuming a plasma concentration of 1 unit/mL factor VIII).

Amidolytic Assays of Factor VIIIa Activity. Experiments examining the ability of factor $VIII_{(VIIa-TF)}$ to be activated by thrombin were done in four stages. The first stage involved the timed incubation of 50 nM factor VIII with 10 nM factor VIIa, 20 nM TF/PL (150 μM phospholipid vesicles composed of 10 mol % PS, 60 mol % PC, and 30 mol % PE), and 5 mM $CaCl_2$ at 37 °C in HBSA buffer. Timed aliquots were removed directly into HBSA buffer containing 1 nM thrombin (factor VIII activation step, stage II) and allowed to incubate for 30 s before removing an aliquot of this mixture directly into a preincubating stage III reaction tube. Stage III reaction tubes each contained 100 μM blank phospholipid vesicles (10% PS, 30% PE, and 60% PC), 5 mM $CaCl_2$, 1 unit/mL hirudin, 400 nM 5G9 anti-TF antibody, and 50 pM factor IXa. The stage III reaction was then immediately started by the subsequent addition of acetylated factor X to 100 nM final concentration. Generation of acetylated factor Xa was followed by discontinuous assay over 2 min, with the acetylated factor Xa activity in each sample being measured in stage IV using S-2222 and monitoring the absorbance at 405 nm using a Vmax microplate reader (Molecular Devices, Menlo Park, CA)

essentially as previously described (*3*). The initial rate of acetylated factor Xa generation (millioptical density units per minute per minute) was then determined for each timed aliquot from stage I and used as a monitor of factor VIII activation. All data have been normalized to the initial activity of factor VIII.

SDS−PAGE and Western Blotting. Reactions for these experiments were done for the indicated times at 37 °C in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, with 5 mM $CaCl_2$ using 25 nM factor VIII, ± 10 nM factor VIIa and 20 nM TF/PL (150 μM phospholipid vesicles). Timed aliquots were removed and stopped by the addition of an equal volume of reducing SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.05% bromophenol blue final) and then boiled for 5 min. Samples were separated by SDS−PAGE (either 10% polyacrylamide or 4−20% gradient polyacrylamide gels) and transferred to nitrocellulose or PVDF membranes (Micron Separations Inc., Westborough, MA) using a wet-blot apparatus (CBS Scientific Co., Del Mar, CA) at 18 V for 2 h. For Western blotting, membranes were blocked with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and then incubated with either a sheep anti-human factor VIII polyclonal antibody or an anti-A2 domain monoclonal antibody (530p) for 2 h at 37 °C with gentle agitation. Blots were washed 3 times with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and then developed using the appropriate VECTASTAIN ABC amplification system (Vector Labs, Burlingame, CA).

Protein Sequencing. N-Terminal sequencing of the various proteolytic fragments of factor VIII was done from Coomassie-stained, air-dried PVDF membrane sections using an Applied Biosystems (Procise Model 492) protein sequencer (in the laboratory of Dr. Kenneth Jackson, Molecular Biology Resource Facility, W. K. Warren Medical Research Institute, Oklahoma City). Regions chosen for sequencing were identified by Coomassie staining of the PVDF membrane and alignment with a corresponding silver-stained gel and Western-blotted membranes (refer to Figure 4). Sequencing was done for either 5 or 8 cycles, and the data shown (refer to Table 1) are merged from three separate proteolysis/sequencing reactions. In most cases, multiple amino acid sequences were identified for each sequencing reaction. All of the sequences identified were found to correspond to sequences within factor VIII, and were readily decipherable from one another by comparison to the known factor VIII sequence (*22*) using the FINDPATTERNS program in Wisconsin Package Version 9.1 [Genetics Computer Group (GCG), Madison, WI].

RESULTS

Proteolysis of Factor VIII by VIIa−TF/PL. To investigate whether factor VIIa could catalyze proteolysis of factor VIII, factor VIII was incubated with either factor VIIa alone, TF/PL alone, or VIIa−TF/PL for 3 h at 37 °C. The samples were then analyzed by SDS−PAGE and Western blotted using a polyclonal antibody to factor VIII (Figure 1A). The appearance of at least two major factor VIII fragments (roughly 47 and 45 kDa) only in the presence of both factor VIIa and TF/PL indicated that proteolysis of factor VIII occurred and that a complex of factor VIIa with

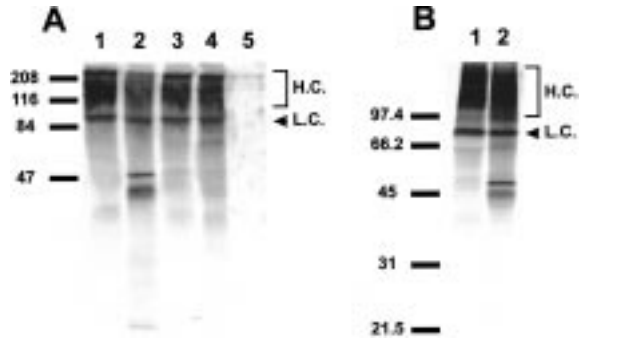

FIGURE 1: Western blot of proteolysis of factor VIII by VIIa−TF/PL. Panel A: The factor VIII starting material is shown in lane 1. The arrows on the right indicate the factor VIII light chain (L.C.) and heterogeneous factor VIII heavy chain (H.C.). Following a 3 h incubation of factor VIII with VIIa−TF/PL, at least two major fragments are observed migrating at roughly 45 and 47 kDa (lane 2). In contrast, a 3 h incubation of factor VIII with factor VIIa alone (lane 3) or TF/PL alone (lane 4) does not result in proteolysis. Lane 5 is a negative blotting control (VIIa−TF/PL with no factor VIII). Panel B: Factor VIII proteolysis by VIIa−TF/PL in the presence (lane 1) and absence (lane 2) of the VIIa−TF/PL inhibitor XK1. Samples were separated on a 10% polyacrylamide−SDS gel (56 ng of factor VIII per lane) and Western blotted using a sheep anti-human factor VIII polyclonal antibody. Positions of molecular weight markers are indicated on the left.

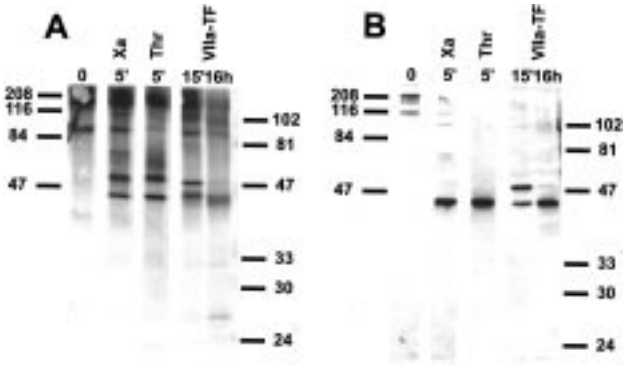

FIGURE 2: Comparison of factor VIII proteolysis by factor Xa, thrombin, and VIIa−TF/PL. Western blot analysis of factor VIII proteolysis using a polyclonal antibody to factor VIII (panel A) and an anti-A2 domain monoclonal antibody (panel B). The factor VIII starting material is shown in lane 1. Incubation of factor VIII with either 5 nM factor Xa for 5 min (lane *2*) or 0.1 nM thrombin for 5 min (lane 3) produces the expected activated heavy chain fragments migrating at roughly 50 kDa (A1-a1 subunit) and 45 kDa (A2-a2 subunit). In contrast, the two major fragments generated from proteolysis of factor VIII by 10 nM VIIa−TF/PL for 15 min and 16 h (lanes 4 and 5) differ in mobility from the factor Xa- or thrombin-derived fragments. Samples were separated on a 10% polyacrylamide−SDS gel (112 ng of factor VIII per lane). Positions of high- and low-range molecular weight markers are indicated.

TF/PL was required. Furthermore, preincubation of VIIa−TF/PL with an inhibitor specific for the VIIa−TF/PL complex (*23*) effectively inhibited proteolysis of factor VIII (Figure 1B), thus demonstrating the absolute requirement for factor VIIa and ruling out the possibility of a contaminating protease.

The two fragments observed by proteolysis of factor VIII with VIIa−TF/PL were similar to the expected heavy chain fragments of the normal thrombin-activated factor VIII molecule. However, direct comparison of factor VIII cleavage by VIIa−TF/PL, factor Xa, and thrombin (Figure 2A) indicated that the factor VIII fragments derived from proteolysis with VIIa−TF/PL differed in size from the

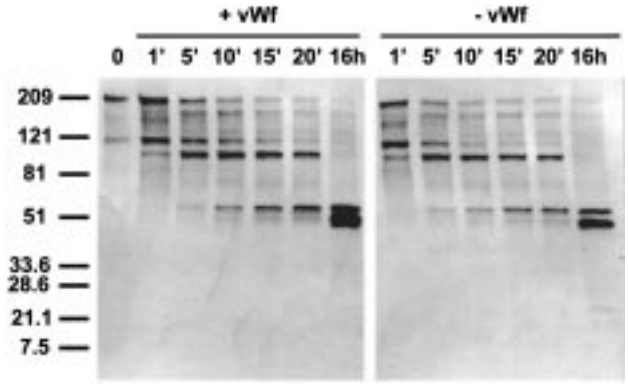

FIGURE 3: Effect of von Willebrand's factor on factor VIII proteolysis by VIIa−TF/PL. Factor VIII (25 nM) was incubated with 10 μg/mL vWf (left panel) or HBSA buffer (right panel) for 30 min at 37 °C in the presence of 5 mM Ca^{2+} before addition of VIIa−TF/PL (10 nM complex). Samples were removed at the indicated times, subjected to 4−20% gradient SDS−PAGE (56 ng of factor VIII per lane), and then Western blotted using an anti-A2 domain monoclonal antibody. Positions of molecular range markers are indicated.

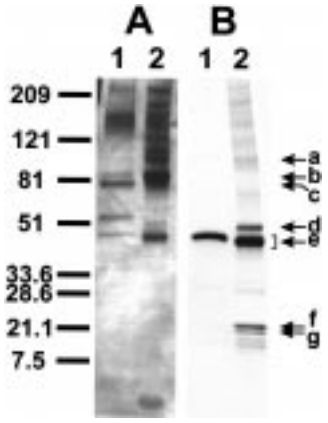

FIGURE 4: Separation and identification of VIIa−TF/PL-derived proteolytic fragments of factor VIII. Thrombin-activated factor VIII (lanes 1, shown for comparison) or a 16 h digest of 238 nM factor VIII with 10 nM VIIa−TF/PL (lanes 2) was separated on a 4−20% gradient polyacrylamide−SDS gel and visualized by silver staining (panel A) or monoclonal (anti-A2 domain) Western blot analysis (panel B). Lettered arrows point to bands that were cut out and sequenced from a corresponding PVDF membrane containing only the VIIa−TF/PL-treated material (refer to Table 1 for sequence data). Lanes contained 500 ng of factor VIII for staining and blotting, and 500 ng−5 μg of factor VIII for sequencing.

expected heavy chain fragments of factor $VIIIa_{(T)}$. The heavy chain fragments of factor $VIIIa_{(T)}$ migrated at roughly 50 kDa (A1-a1 subunit) and 45 kDa (A2-a2 subunit), as did the fragments derived from factor Xa activation of factor VIII. Consistent with this, an anti-A2 domain antibody only recognized the lower of these two bands (Figure 2B). In contrast, the factor VIII fragments obtained by proteolysis with VIIa−TF/PL were both recognized by the anti-A2 antibody. In addition, prolonged incubation resulted in the conversion of these two bands to a doublet migrating at roughly 45 and 43 kDa, of which only the 45 kDa fragment was recognized by the anti-A2 domain antibody.

Since factor VIII in plasma is noncovalently associated with vWf, we examined the ability of VIIa−TF/PL to catalyze the proteolysis of factor VIII that had been reconstituted with plasma levels of vWf (10 μg/mL). The factor VIII was allowed to preincubate with vWf for 30 min at 37 °C in the presence of 5 mM Ca^{2+} prior to addition of VIIa−TF/PL. Since the levels of factor VIII and vWf used in this experiment are more than 10-fold higher than their reported K_d for binding (*24, 25*), one would expect greater than 90% of the factor VIII to be complexed with vWf under these conditions. The results are shown in Figure 3 and demonstrate that under the conditions used, proteolysis of the factor VIII/vWf complex by VIIa−TF/PL occurs rapidly and is nearly indistinguishable from a parallel experiment done in the absence of vWf.

N-Terminal Sequencing and Fragment Identification. To identify the sites of cleavage by VIIa−TF/PL within factor VIII, the various fragment bands obtained from factor VIII proteolysis in the absence of vWf were isolated from PVDF membranes and subjected to N-terminal sequencing. Appropriate bands were identified for sequencing by comparing a silver-stained gel of the reaction mixture to Western blots using the polyclonal and the anti-A2 domain antibodies (Figure 4). The sequence data obtained for each membrane section are summarized in Table 1. In all but two cases, multiple N-terminal sequences were obtained. Upon analysis, all of these sequences were readily decipherable from one another, and all corresponded to sequences within factor VIII. Sequences were obtained corresponding to cleavages at the known activation sites in the heavy chain at R^{740} and R^{372}, and in the light chain at R^{1689}. In addition, sequences were obtained corresponding to cleavage at K^{36}, R^{336}, and R^{562} in the heavy chain, at R^{1652} in the light chain, and at K^{1098}, R^{1313}, and R^{1548} in the B domain. Sequences corresponding to the N-terminus of the heavy chain and the light chain of factor VIII were also identified.

Correlation of Proteolytic Fragments and Factor VIII Activity. To further characterize the proteolysis of factor VIII by VIIa−TF/PL, a time course of proteolysis was carried out to (i) assess the order of cleavage at the various sites in the heavy chain, (ii) identify potential transient fragments, and (iii) correlate proteolysis with the potential generation of factor VIIIa activity.

By combining the known sites of cleavage (Table 1) with the migration and time of appearance of the various heavy chain fragments on gels (Figure 5A), it was determined that cleavage at R^{740} likely occurs first to generate an initial transient intermediate heavy chain fragment (90 kDa; A1-a1-A2-a2). This product is further hydrolyzed at R^{336} and R^{372} to generate the a1-A2-a2 (α) and A2-a2 fragments, which migrate at 47 and 45 kDa, respectively. It should be noted that the A1 subunit (45 kDa), which is expected to comigrate with the A2-a2 subunit (cf. Figure 4), is not visualized in blots using the anti-A2 domain antibody (Figure 5A). Prolonged incubation of factor VIII with VIIa−TF/PL resulted in full conversion of the α fragment to the A2-a2 domain fragment, followed by further proteolysis at R^{562} (minimally) to produce the low molecular weight β and γ fragments (ca. 23−21 kDa). These fragments are expected to be similar in size and were neither efficiently separated on the gel nor recognized well by the anti-A2 domain antibody. Thus, it is unclear which of the identified small fragments is visualized on the blots.

The potential activity of $VIII_{(VIIa-TF)}$ was examined in a parallel experiment and compared to factor $VIIIa_{(T)}$ in a modified two-stage factor VIII clotting assay (Figure 5B). As expected, incubation of factor VIII with thrombin resulted

Table 1: Amino-Terminal Sequence Data of Factor $VIII_{(VIIa-TF)}$ Proteolytic Fragments[a]

PVDF section	estimated molecular mass (kDa)	cycle no.[b] 1	2	3	4	5	6	7	8	domain	cleavage site[c]	expected fragment[d]
a	90	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	A2/B junction	Arg^{740}	741−1648
b	82	Thr	Thr	Leu	Gln	Ser	−	−	−	a3 domain	Arg^{1652}	1653−2332
		Glu	Ile	Thr	Arg	Thr	Thr	Leu	Gln	B/a3 junction	*N*-$term_{LC}$	1649−2332
		Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	A2/B junction	Arg^{740}	741−1548
c	80	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	a3/A3 junction	Arg^{1689}	1690−2332
d	48	Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	A1/a1 junction	Arg^{336}	337−740 [α]
		Met	Leu	Phe	Leu	Pro	Glu	Ser	Ala	B domain	Lys^{1098}	1099−1548
e	43	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	a1/A2 junction	Arg^{372}	373−740
		Ser	Phe	Pro	Phe	Asn	Thr	Ser	Val	A1 domain	Lys^{36}	37−372
		Ala	Thr	Arg	Arg	Tyr	Tyr	Leu	Gly	A1 domain	*N*-$term_{HC}$	1−336
f	23	?	Val	Ala	Lys	Lys	−	−	−	a1/A2 junction	Arg^{372}	373−562 [β]
		Ala	Leu	Lys	Gln	Phe	Arg	Leu	Pro	B domain	Arg^{1313}	1314−1548
		Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	A2 domain	Arg^{562}	563−740 [γ]
		Met	Leu	Phe	Leu	Pro	Glu	Ser?	Ala?	B domain	Lys^{1098}	1099−1313
		Val	Ala	Thr	Glu	Ser	Ser	Ala	Lys	B domain	Arg^{1548}	1549-(?)
g	21	Gly	Asn	Gln	Ile	Met	−	−	−	A2 domain	Arg^{562}	563−740 [γ]
		Met	Leu	Phe	Leu	Pro	Glu?	Ser?	Ala	B domain	Lys^{1098}	1099−1313
		Ala	Leu	Lys	Gln	Phe	Arg?	Leu	Pro	B domain	Arg^{1313}	1314−1548
		Ala	Thr	Arg	Arg	Tyr	Tyr	Leu	Gly	A1 domain	*N*-$term_{HC}$	1−(?)

[a] The data shown are combined from three separate blots and sequence determinations (done for either 5 or 8 cycles) from PVDF membranes. Most membrane sections contained multiple amino acid sequences that were deciphered into those shown, and which match the indicated regions and sites within factor VIII (see also Figures 4 and 7). [b] Question marks indicate either no amino acid determined for that cycle or low levels of the indicated amino acid. [c] Indicated residues are immediately amino-terminal to the hydrolyzed bond. *N*-$term_{HC}$ and *N*-$term_{LC}$ refer to the mature amino termini of the heavy chain and light chain, respectively, in the unactivated factor VIII starting material. [d] Expected fragments are deduced from the corresponding sites of proteolysis and the migration of each fragment on gels. The indicated carboxy-terminal amino acid positions are based on other sites of proteolysis by the factor VIIa−tissue factor complex and the full-length factor VIII molecule (2332 amino acids). Question marks indicate no corresponding cleavage site was found that would result in the expected size fragment.

in an initial rapid increase in factor VIII clotting activity (roughly 10-fold). In contrast, incubation of factor VIII with VIIa−TF/PL showed only a modest increase in activity (2-fold) within the first 60 s followed by a steady decline in activity thereafter. This deficiency in activity was paralleled in factor X activation assays in a purified system (not shown) and is consistent with the primary order of cleavage being R^{336} followed by R^{372} to generate an inactive factor VIII heavy chain.

The lack of activity of $factor\ VIII_{(VIIa-TF)}$ in both a clotting assay and a factor X activation assay prompted us to examine the potential ability of thrombin to activate $factor\ VIII_{(VIIa-TF)}$ based on the premise that the more efficient cleavage of the factor VIII light chain by thrombin may produce a more active cofactor. The results (Figure 6A) clearly show, however, that VIIa−TF/PL proteolysis of factor VIII prevented its subsequent activation by thrombin in a time-dependent manner. Greater than 50% of the factor VIII was rendered resistant to thrombin activation within 10 min, with an approximate initial rate (loss of activity) of roughly 5.6 nM factor VIII min^{-1} (nM VIIa−TF/PL)$^{-1}$ under these conditions.

In addition to the ability of VIIa−TF/PL to prevent thrombin activation of factor VIII, VIIa−TF/PL was also found to be capable of proteolytic inactivation of factor $VIIIa_{(T)}$. When factor $VIIIa_{(T)}$ was incubated with 10 nM VIIa−TF/PL, proteolysis of the A1-a1 domain of factor $VIIIa_{(T)}$ was observed within the first minute to yield a smaller band that comigrated with the A2-a2 domain (Figure 6B). Based on the cleavage sites determined above, this smaller band is consistent with the A1 domain.

DISCUSSION

Proteolysis of Factor VIII. The data presented above demonstrate cleavage of factor VIII by VIIa−TF/PL. Proteolysis was found to occur at numerous sites throughout the factor VIII molecule, with the following 10 sites being positively identified by N-terminal sequencing: K^{36}, R^{336}, R^{372}, R^{562}, R^{740}, K^{1098}, R^{1313}, R^{1548}, R^{1652}, and R^{1689} (Figure 7). Based on the time course of proteolysis, the appearance of heavy chain fragments, the sites of cleavage, and the decay of factor VIII activity, we conclude that VIIa−TF/PL inactivates factor VIII primarily via cleavage at R^{336} within the factor VIII heavy chain. This cleavage, along with that at R^{740}, generates a transient factor VIII α fragment (a1-A2-a2) that is slowly converted to the 45 kDa A2-a2 fragment via subsequent proteolysis at R^{372}. Prolonged incubation with VIIa−TF/PL produces smaller degradation products that have no apparent further effect on factor VIII activity. Proteolysis at these other identified sites within factor VIII are mostly limited to regions in the B domain (K^{1098}, R^{1313}, and R^{1548}), resulting in additional low molecular weight fragments that comigrate with the β and γ fragments. Proteolysis at K^{36} also seems to occur only with prolonged incubation times (cf. Figure 4A) to produce an N-terminally truncated A1 subunit.

It is notable that at no time did we observe a factor VIII fragment consistent with the A1-a1 subunit of $factor\ VIIIa_{(T)}$ (cf. Figure 2A). Thus, we find no direct physical evidence for cleavage at R^{372} before R^{336} under these conditions. It is therefore likely that R^{740} and R^{336} are the preferred VIIa−TF/PL cleavage sites with proteolysis at R^{372} either closely following or occurring concomitant with cleavage at R^{362}.

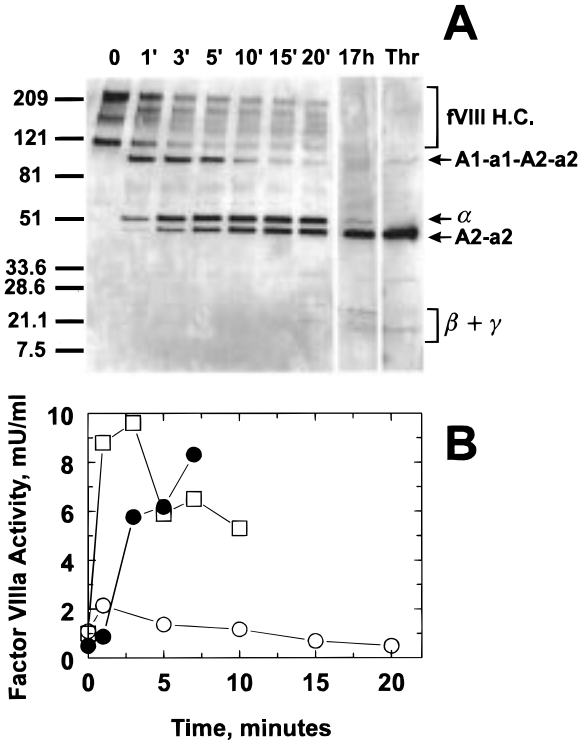

FIGURE 5: Time course of factor VIII proteolysis by VIIa−TF/PL. Panel A: Factor VIII proteolysis by VIIa−TF/PL was followed for the indicated times (0−20 min and 17 h) by Western blot analysis using an anti-A2 domain antibody. Samples were separated on a 4−20% gradient polyacrylamide−SDS gel. Each lane contained 56 ng of factor VIII. For comparison, factor $VIIIa_{(T)}$ is shown in lane 9 (Thr). Panel B: A parallel experiment examining the clotting activity of factor VIII after incubation with 1 nM thrombin (□), 0.2 nM thrombin (●), or 10 nM VIIa−TF/PL (○) for the indicated times. Incubation of factor VIII with VIIa−TF/PL produced only a modest increase in activity (2-fold) within the first 60 s followed by a steady decline in activity thereafter. This lack of activity was paralleled in factor X activation assays in a purified system (not shown).

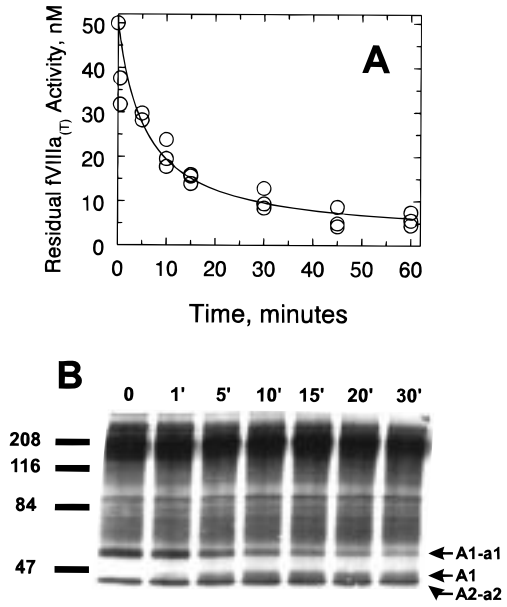

FIGURE 6: Panel A: Resistance of factor $VIII_{(VIIa-TF)}$ to activation by thrombin. Incubation of 50 nM factor VIII with VIIa−TF/PL (10 nM) resulted in a time-dependent inactivation of factor VIII with respect to activation by 1 nM thrombin. Greater than 50% of the factor VIII was inactivated within 10 min, with an approximate initial rate (loss of activity) of roughly 5.6 nM factor VIII min^{-1} (nM VIIa−TF/PL)$^{-1}$. All data have been normalized to the initial activity of the factor VIII. Panel B: Proteolytic inactivation of factor $VIIIa_{(T)}$ by VIIa−TF/PL. Factor VIII was treated with 1 nM thrombin for 5 min and then quenched with 1 unit/mL hirudin. VIIa−TF/PL was then added (10 nM final), and timed samples were removed and subjected to SDS−PAGE and Western blotting using a polyclonal antibody as described for Figure 1. Proteolysis of the A1-a1 domain of factor $VIIIa_{(T)}$ was observed within the first minute, and yielded a smaller band consistent with the A1 domain only. No proteolysis of the A2-a2 band was observed over this time course, and no proteolysis was observed when VIIa−TF/PL was omitted from the reaction (not shown). Molecular weight markers are indicated on the left.

However, without invoking a required conformational change in the factor VIII molecule upon cleavage at R^{336}, there seems no obvious reason a priori why proteolysis at R^{336} should necessarily precede proteolysis at R^{372}. Thus, it remains a distinct possibility that a small subpopulation of factor VIII molecules may exist transiently in an active state with cleavages only at R^{372} and R^{740}. This would be consistent with the small (2-fold) transient increase in factor VIII clotting activity observed in Figure 5B.

The cleavages at R^{336} and R^{562} in the factor VIII heavy chain are the same as those previously reported to be catalyzed by activated protein C (*9*), and the rates of factor VIII inactivation by VIIa−TF/PL and activated protein C are comparable. However, unlike activated protein C (*9*), VIIa−TF/PL was found to prefer proteolysis at R^{336} to proteolysis at R^{562}. In addition, also unlike activated protein C, VIIa−TF/PL was found to cleave factor VIII at the known activation sites in the heavy chain, and at two sites in the light chain (R^{1652} and R^{1689}). The potential effect of the light chain cleavages on the activity of the factor VIII mixture is unclear since inactivation of factor VIII via the heavy chain cleavages occurred on a much shorter time scale. However, based on previous studies with both factor Xa (*5*) and factor IXa (*6, 7*)—both of these enzymes cleave the factor VIII light chain at sites different from the thrombin activation site of R^{1689} (R^{1719} for factor Xa and R^{1721} for factor IXa)—the light chain cleavages by VIIa−TF/PL would not be expected to have much effect on factor VIIIa activity in the absence of heavy chain cleavage (*1*).

Cleavage Sites of VIIa−TF/PL. Given the well-documented high selectivity and specificity of VIIa−TF/PL, it is quite surprising and unexpected that this enzyme−cofactor complex can recognize and proteolyze factor VIII as well as was observed. Until recently, only 4 natural human substrate cleavage sites were known for VIIa−TF/PL. When combined with the 4 new sites identified in factor V (*14*), the 10 sites in factor VIII more than quadruple the previously known VIIa−TF/PL natural human recognition sites (Table 2). Of the 10 sites in factor VIII, only 3 (R^{336}, R^{740}, and R^{372}) are attacked rapidly by VIIa−TF/PL (the associated products appear within the first minute) while cleavage at the other sites is observed only in prolonged incubations (≥20 min). The data strongly suggest that cleavage at R^{740} and R^{336} are the primary sites of attack by VIIa−TF/PL, with cleavage at R^{372} occurring either immediately after or concomitant with cleavage at R^{336}.

Four unique sites of cleavage in factor VIII were identified for VIIa−TF/PL: K^{36}, K^{1098}, R^{1548}, and R^{1652}. With regard to the K^{36} cleavage site in factor VIII, this cleavage generates an N-terminally truncated A1 domain. Whether or not this

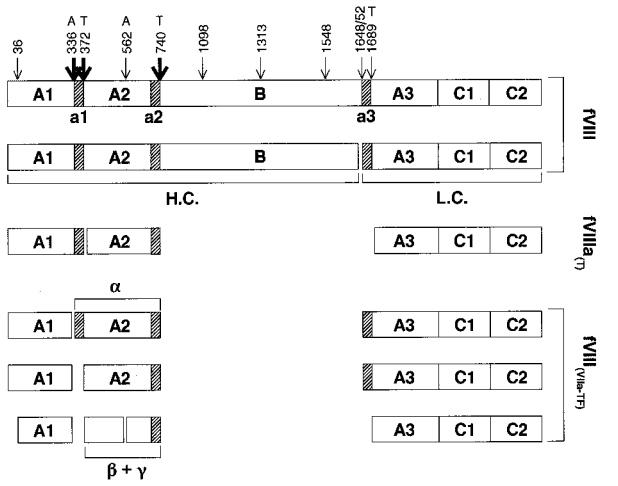

FIGURE 7: Schematic diagram of the VIIa−TF/PL cleavage sites within factor VIII. Proteolysis of factor VIII by VIIa−TF/PL was found to occur at all three thrombin activation sites (indicated by "T"; R^{372}, R^{740}, and R^{1689}) and both activated protein C sites (indicated by "A"; R^{336} and R^{562}). Additional sites of cleavage by VIIa−TF/PL were found at K^{36}, K^{1098}, R^{1313}, R^{1548}, and R^{1652}. The three primary sites of cleavage by VIIa−TF/PL are indicated by boldface arrows (R^{336}, R^{372}, and R^{740}). Associated heavy and light chain fragments of factor VIII that result from cleavage by thrombin or VIIa-T/PL are indicated.

Table 2: Cleavage Sites of the Known Natural Human Substrates of VIIa−TF/PL

	cleavage site sequence										
protein substrate[a]	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	ref
factor VII	K	P	Q	G	R	I	V	G	G	K	(*30*)
factor X	N	N	L	T	R	I	V	G	G	Q	(*31*)
factor IX (R^{145})	S	K	L	T	R	A	E	T	V	F	(*32*)
factor IX (R^{180})	N	D	F	T	R	V	V	G	G	E	(*32*)
factor V (R^{679})	V	M	A	T	R	K	M	H	D	R	(*14*)
factor V (R^{709})	A	L	G	I	R	S	F	R	N	S	(*14*)
factor V (R^{1018})	P	L	S	P	R	T	F	H	P	L	(*14*)
factor V (R^{1192})	E	L	I	Q	R	N	L	S	P	A	(*14*)
factor VIII (K^{36})	P	R	V	P	K	S	F	P	F	N	*b*
A factor VIII (R^{336})*	E	P	Q	L	R	M	K	N	N	E	*b*
T factor VIII (R^{372})*	F	I	Q	I	R	S	V	A	K	K	*b*
A factor VIII (R^{562})	S	V	D	Q	R	G	N	Q	I	M	*b*
T factor VIII (R^{740})*	A	I	E	P	R	S	F	S	Q	N	*b*
factor VIII (K^{1098})	M	S	F	F	K	M	L	F	L	P	*b*
factor VIII (R^{1313})	Q	R	S	K	R	A	L	K	Q	F	*b*
factor VIII (R^{1548})	V	P	F	L	R	V	A	T	E	S	*b*
factor VIII (R^{1652})	R	E	I	T	R	T	T	L	Q	S	*b*
T factor VIII (R^{1689})	N	Q	S	P	R	S	F	Q	K	K	*b*

[a] Asterisks designate the preferred VIIa−TF/PL cleavage sites in factor VIII. Sites in factor VIII cleaved by thrombin and activated protein C are indicated with either a "T" or an "A", respectively. [b] Cleavage site determined in the present study (amino acid sequence as per ref *22*).

cleavage alone would inactivate factor VIII is unknown. The cleavages at the K^{1098} and R^{1548} sites are within the B domain, and likely have no effect on factor VIII activity. The fourth unique site (R^{1652}) is in the a3 domain of the light chain, and is only three residues away from the mature light chain *N*-terminus. The potential role of this cleavage and its potential effect on factor VIII activity are also unknown.

Perhaps the most striking of these VIIa−TF/PL cleavage sites are the two sites containing K residues in the P_1 position. It is not unprecedented for trypsin-like serine proteases to be able to hydrolyze peptides with K in this position. The complex of factor VIIa with soluble tissue factor has been previously shown to recognize a small tripeptidyl substrate with K in the P_1 position, albeit with roughly 8-fold lower activity than the R-containing analogue (*26*). Additionally, two Kunitz-type inhibitors that react with VIIa−TF/PL (basic pancreatic trypsin inhibitor and the first Kunitz domain of tissue factor pathway inhibitor) each contain K in the P_1 position of the reactive site loop. Nonetheless, based on the known extraordinary selectivity of VIIa−TF/PL toward protein substrates, it seems unusual that VIIa−TF/PL would exhibit proteolytic activity at these sites.

The ability of VIIa−TF/PL to cleave factor VIII at all the required activation sites is curious, and seeds the speculation that under certain circumstances (i.e., where cleavage at the inactivation sites is protected) it may be possible for VIIa−TF/PL to generate an active factor VIIIa species. It has been demonstrated that factor IXa can, in certain circumstances, protect factor VIII/VIIIa from inactivation by activated protein C (*27*, *28*). However, when we examined potential factor VIII activity in a purified system using acetylated factor X as the substrate, proteolysis by VIIa−TF/PL in the presence of factor IXa (10 nM) showed no apparent increase in activity compared to when factor IXa was absent (data not shown). Nonetheless, it remains theoretically feasible that under certain conditions, factor VIIIa activity could be generated by VIIa−TF/PL. This is currently under investigation.

The significance of factor VIII inactivation by VIIa−TF/PL is currently unclear, but may be speculated to contribute to an additional control mechanism for factor X activation under certain conditions. When compared to the rapid activation of factor VIII by thrombin, inactivation of factor VIII by VIIa−TF/PL is relatively slow. Thus, this may preclude VIIa−TF/PL inactivation of factor VIII from being of import during normal hemostasis. However, it is interesting to note that high factor VIII levels have been associated with ischemic heart disease and myocardial infarction (*29*). It is feasible, therefore, that VIIa−TF/PL may play an important part in controlling and/or maintaining circulating factor VIII levels, or controlling factor VIII activity in nonhemostatic settings and under conditions where activated protein C is not available.

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