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Inactivation of Factor VIII by Factor IXa

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ABSTRACT: Factor VIII (FVIII) is the nonproteolytic cofactor for FIXa in the tenase complex of blood coagulation. FVIII is proteolytically activated by thrombin and FXa in vitro to form a heterotrimer with full procoagulant activity. Activated protein C inactivates thrombin-activated FVIII through cleavage adjacent to position Arg 336 in the cofactor. We have investigated the interaction of FIXa and FVIII and subjected FVIII polypeptides to N-terminal amino acid sequence analysis. Contrary to previous reports, we were unable to demonstrate the activation of FVIII by FIXa. Incubation of these two proteins at equimolar or close to equimolar concentrations resulted in the inactivation of FVIII, coincident with cleavage of the FVIII heavy chain adjacent to Arg 336 and the light chain adjacent to Arg 1719. These cleavages were detected in the presence or absence of thrombin, indicating that FIXa does not stabilize thrombin-activated FVIIIa. APC cleaved FVIII at the same position in the heavy chain, and simultaneous incubation of FVIII, APC, and FIXa did not result in stabilization of the cofactor. We conclude that FIXa does not play a role in the stabilization or activation of FVIII.

Factor VIII (FVIII) is the nonproteolytic cofactor for FIXa, a serine protease which activates factor X (FX) in the central reaction of the coagulation cascade (Mann & Krishnaswarmy, 1990). FVIII must itself undergo limited proteolysis in order to participate in the intrinsic tenase complex, and this activation is effected in vitro by thrombin and by FXa (Eaton et al., 1986). Thrombin cleaves arginyl-serine bonds in FVIII at positions 372-373, 740-741, and 1689-1690 to generate a three-chain metal ion-bridged polypeptide with full cofactor activity. Site-directed mutagenesis of recombinant FVIII and characterization of naturally occurring variant molecules have demonstrated that the cleavages at positions 372-373 and 1689-1690 are essential for the expression of FVIII activity (Pittman & Kaufman, 1988; Eaton et al., 1986; O'Brien & Tuddenham, 1989; O'Brien et al., 1990; Arai et al., 1990). FXa also activates FVIII following cleavage adjacent to positions 372, 740, 1689, and 1721, and, in addition, the enzyme will further cleave FVIII at position Arg 336-Ser 337,

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inactivating the cofactor. This cleavage is also made by activated protein C (APC), a physiological inactivator of FVIIIa.

It has been reported that FIXa will also activate, and subsequently inactivate, partially purified FVIII (Rick, 1982). In other studies it has been shown that thrombin-activated porcine FVIIIa is stabilized by the presence of FIXa and phospholipid (Lollar et al., 1984) and that very high concentrations of FIX or FIXa protect FVIII from inactivation by APC (Bertina et al., 1984, Walker et al., 1987; Rick et al., 1990).

In this study, we have investigated the interaction of highly purified human plasma-derived FIXa (pdFIXa) with highly purified recombinant human FVIII (rFVIII) in order to identify cleavages mediated by the enzyme using amino acid sequence analysis of FIXa-generated FVIII polypeptides. In contrast to previously published data, we found that pdFIXa inactivated rFVIII in the presence of phospholipid and calcium ions. We were unable to demonstrate activation of the cofactor by FIXa, nor was the thrombin-activated cofactor stabilized by the presence of FIXa. We therefore conclude that FIXa does not play a role in the activation or stabilization of FVIII.

Materials and Methods

Electrophoresis reagents were from Bio-Rad Laboratories, Richmond, CA. Sulpho-NHS-biotin and streptavidin-HRP conjugate were from Pierce Europe, Luton, U.K. The chemiluminescent substrate for western blots (ECL reagent) was from Amersham International, Amersham, U.K. All reagents for protein sequence analysis were from ABI, Warrington, U.K. All other reagents were from Sigma, Poole Dorset, U.K., and were reagent grade or better. Protein estimations were performed by the method of Bradford (1976) using bovine serum albumin as standard. One stage FIX and FVIII coagulation assays were performed as described elsewhere (Rotblat et al., 1983) using citrated normal pooled plasma from 10 healthy volunteers as standard. FIX deficient plasma substrate was immunodepleted as described elsewhere (Goodall et al., 1982), and FVIII deficient plasma was purchased from Diagen Thame, Oxon, U.K. Monoclonal antibodies (MoAb) C6 and C10 were the gifts of Dr. A. Goodall, Royal Free Hospital, London. MoAb C7F7 was the gift of Dr. D. Eaton, Genentech, Inc., San Francisco, CA. MoAb 3A6 anti-FIX monoclonal antibody was the gift of Dr. Y. Ohkubo, Nara Medical College, Nara, Japan.

Purification of Proteins. Recombinant human FVIII, the gift of Dr. J. Lawrence and Dr. Shu Len Lui, Baxter Healthcare, Inc., Duarte, CA, was purified from culture medium by a combination of monoclonal antibody affinity chromatography and anion exchange chromatography. The protein was stored at −20 °C in 0.025 M Tris-HCl, 0.15 M NaCl, and 50% glycerol (v/v), pH 7.5. Stored aliquots of FVIII used in the experiments described here had a specific activity of 4068 units FVIII:C/mg by one stage FVIII:C assay and protein estimation using a standard preparation of bovine serum albumin (Pierce Europe, Luton, U.K.). No loss of activity was detected over a period of several months under these conditions. Western blot analysis demonstrated that since no breakdown products were detected after prolonged storage of FVIII at −20 °C, the material was not denatured on storage.

Human plasma-derived FIX was purified from pooled citrated normal plasma by the method of Smith (1980) using a immunoaffinity column kindly provided by Dr. Smith. The purified protein was resolved as a single band on SDS-PAGE silver-stained gels, with N-terminal amino acid sequence analysis confirming a single sequence. Purified FIX had a specific activity of 201 units/mg prior to activation. FIX antigen was measured using a commercial chromogenic assay with a detection limit of 5 ng/mL (ELISA IX:AG Boehringer Mannheim GmbH, Mannheim, Germany). No FX was detectable by standard one-stage coagulation assay. Protein C was not detected in a commercial chromogenic assay for protein C (Immunochrom PC, Immuno Ltd., Kent, U.K.). FIX was activated by incubation with FXIa coupled to controlled pore glass (XIa-CPG) (Ohkubo et al., 1990). Following incubation of FIX in 0.05 M Tris-HCl, 0.15 M NaCl, and 5 mM $CaCl_2$, pH 7.4, with Xla-CPG for 16 h at room temperature, the FIXa coagulant activity increased 25-fold and all the protein was fully proteolyzed to the two-chain enzyme, as assessed by SDS-PAGE.

Human thrombin, specific activity 3000 units/mg, was from Sigma, Poole Dorset, U.K. Protein C (181.25 units/mg, Calbiochem, Nottingham, U.K.) was homogeneous by SDS-PAGE. This was activated with the activator from *Agkistrodon contortrix* venom (Protac, Calbiochem).

von Willebrand factor (vWF) was prepared from high purity FVIII concentrate (the gift of Cutter Laboratories, San Francisco, CA) as follows: one bottle of concentrate was reconstituted in 5 mL of 0.05 M Tris-HCl and 1 M NaCl, pH 7.4. This was then applied to a 16 × 60 cm Superose 6 Prep-Grade column (Pharmacia, Milton Keynes, U.K.) equilibrated in the same buffer. The column was developed isocratically at a flow rate of 0.75 mL/min with 2-mL fractions being collected. Void volume fractions were pooled, concentrated to a volume of 3 mL, and made 0.24 M in $CaCl_2$. This material was then rechromatographed on the same column equilibrated with 0.05 M Tris-HCl and 0.15 M NaCl (TBS) containing 0.24 M $CaCl_2$. Void volume fractions were collected and buffer exchanged into TBS, pH 7.4, on a PD 10 column (Pharmacia). This vWF preparation contained less than 0.01 unit of FVIII:C/mL by one-stage coagulation assay. vWF was assayed by ELISA (ELISA vWF, Boehringer Mannheim Coagulation Diagnostics, Mannheim, Germany). Peak fractions contained 46 units of vWF:Ag/mL and a protein concentration of 180 μg/mL. SDS-PAGE analysis resolved a predominant band at 250 kDa on reduction. This material was used immediately for the assays described to avoid precipitation on freeze/thawing.

N-Terminal Protein Sequence Analysis of FVIII Cleavage Products Following Digestion with FIXa. To allow digestion to go to completion for N-terminal sequence analysis, 15 μg of recombinant human FVIII in 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4 (TBS), was made 10 mM in $CaCl_2$ in a total volume of 0.5 mL. 10% (v/v) phospholipid (rabbit brain cephalin-RBC, Sigma) was then added with 2.5 μg of FIXa. Following incubation for 2 h at 37 °C, the reaction was stopped by the addition of five volumes of ice-cold acetone, and the mixture was centrifuged at 13000*g* for 2 min. The supernatant was discarded and the precipitate resuspended in 20 μL of SDS-PAGE reducing sample buffer and heated to 100 °C for 1 min. Aliquots were stored frozed prior to electrophoresis. For sequencing, the digest was resolved on SDS-PAGE 7.5%-12.5% gradient discontinuous gels (Rotblat et al., 1985) with 0.1 mM sodium thioglycolate added to the cathodic buffer. Molecular mass markers were run simultaneously. Following electrophoresis at 35 mA/300 V, the gel was electroblotted onto PVDF membranes (Applied Biosystems, Inc.) using a transblot apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturers' instructions. The membrane was stained with Coomassie blue, destained for 30 min in 50% methanol, and soaked in distilled water prior

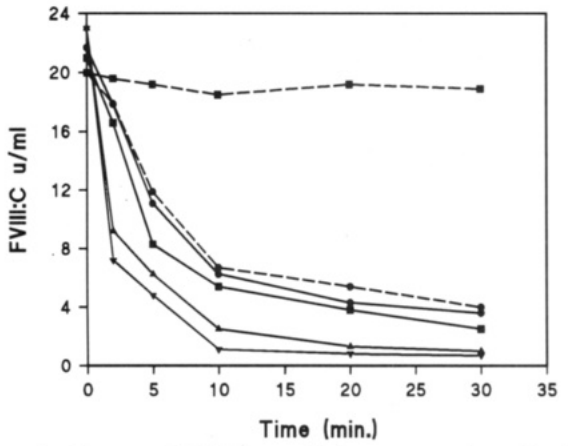


FIGURE 1: One-stage FVIII:C coagulation assay performed on incubation mixtures containing FVIII, FIXa, phospholipid, and calcium ions. FVIII (325 ng) was diluted in 50 μL of TBS, pH 7.4, containing 10 mM $CaCl_2$; 10 μL of mixed brain phospholipids (rabbit brain cephalin) was then added. Various concentrations of FIXa were then added as follows: (●) 75 ng (approximately equimolar concentration FIXa and FVIII); (■) 150 ng (2-fold molar excess of FIXa over FVIII); (▲) 300 ng (4-fold molar excess); (▼) 600 ng (8-fold molar excess). (■--■) Purified vWF (8 μg) was also added to FVIII which was incubated at 37 °C for 30 min prior to the addition of 150 ng of FIXa. Samples were incubated for 30 min at 37 °C with the one-stage FVIII:C assay being performed at times 0, 2, 5, 10, 20, and 30 min after the addition of FIXa. The experiment using a 4-fold molar excess of FIXa was also repeated at an incubation temperature of +4 °C (●--●).

to air drying. Individual bands were excised and placed in a tangential-flow cartridge for sequencing in an ABI 473A protein sequencer. The pulsed liquid delivery of reagents was optimized for the altered geometry of the cartridge. Data from the on-line liquid chromatograph were analyzed with ABI 610A software to identify and quantify PTH-amino acids in each cycle of Edman degradation.

Results

Interaction of IXa with FVIII As Assessed by One-Stage FVIII:C Coagulation Assay. Following the incubation of FVIII with FIXa, a dose-dependent decrease in the FVIII activity was detected using a one-stage FVIII coagulation assay (Figure 1). At approximately equimolar concentration of FIXa and FVIII (325 ng of FVIII to 77 ng of FIXa), FVIII activity declined from 21.7 units/mL prior to the addition of FIXa, to 3.6 units/mL 30 min after the addition of the enzyme. At an 8-fold molar excess of FIXa over FVIII the activity declined to 0.83 unit/mL over the same period.

In the same assay, the same preparation of FVIII was also incubated with vWF (1:1 unit ratio of FVIII:C to vWF:Ag) for 30 min at 37 °C prior to the addition of a 2-fold molar excess of FIXa over FVIII. No inactivation of FVIII:C activity was recorded (Figure 1). Thus, the addition of FIXa to FVIII/vWF complex at a concentration that had been shown to inactivate purified FVIII did not result in the inactivation of the cofactor. Incubation of FVIII with FIXa at 4 °C decreased the rate of inactivation, but activation of the cofactor was not detected, indicating that the lack of detectable activation was not the result of rapid proteolysis occurring before the first assay time point (Figure 1).

Aliquots were also removed from a reaction mixture containing homogeneous FIX and FXIa-CPG at various time points to generate a range of partially activated FIXa preparations. The degree of activation of FIX was assessed by SDS–PAGE analysis (Figure 2). Activation of FIX to FIXa was concomitant with the appearance of the two-chain disulfide-linked enzyme which resolved as a single band under nonreducing conditions. Approximately 50% of the zymogen was converted to FIXa at the 1-h time point (Figure 2B, lane 3). Zymogen FIX (Figure 2B, lane 1) was incubated with FVIII at a 2-fold molar excess in the presence of calcium ions and phospholipid. No inactivation was detected by time course FVIII:C assay (Figure 2A). Significant inactivation of FVIII was detected when FVIII was incubated with FIXa that had been incubated with FXIa–CPG for 30 min, and progressively faster inactivation rates were detected when a 2-fold molar excess of 50% activated FIXa and 100% activated FIXa was incubated with FVIII under the same conditions (Figure 2A). These data indicate that the inactivation of FVIII by FIXa

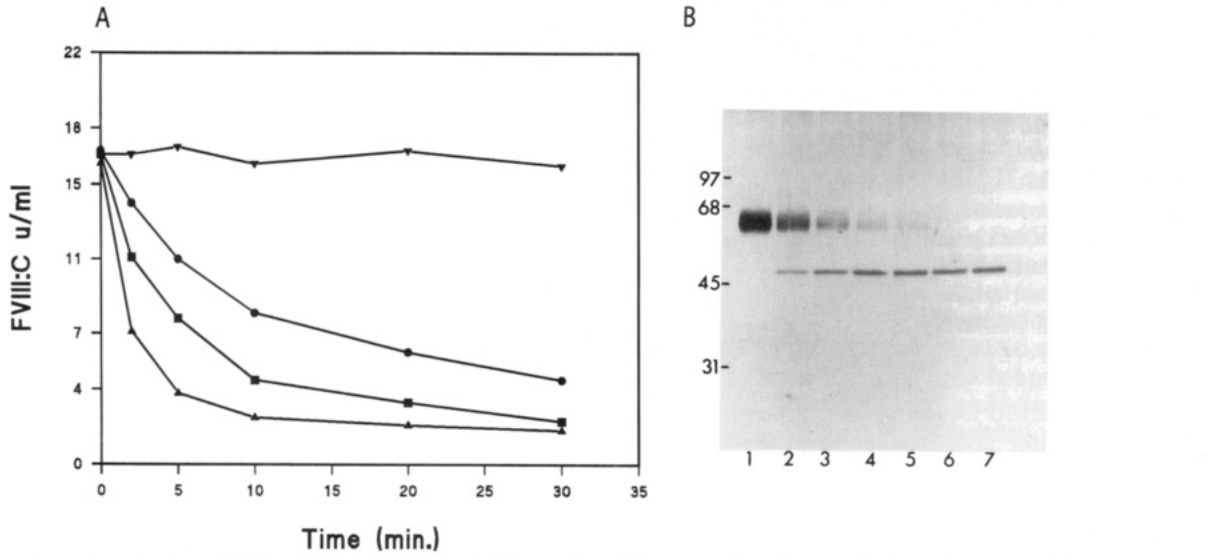
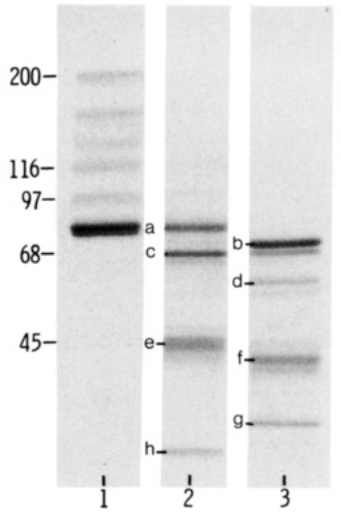


FIGURE 2: Effect of partial activation of FIX and zymogen FIX on FVIII activity. FIX was activated by incubation with FXIa-CPG as described under Materials and Methods. Samples were removed and subjected to nonreducing SDS–PAGE analysis and silver stained on a 10% homogeneous gel as shown in panel B. (lane 1) FIX prior to the addition of FXIa–CPG; (lane 2) 30-min postincubation with FXIa–CPG; (lane 3) 1 h; (lane 4) 1.5 h; (lane 5) 2 h; (lane 6) 2.5 h; (lane 7) 3 h. Molecular weight markers are shown on the left-hand side of the gel ($\times 10^3$). (Panel A) FVIII (200 ng) was incubated with a 2-fold molar excess of (a) zymogen FIX (▼); (b) FIXa incubated with FXIa for 30 min (approximately 25% activated) (●); (c) FIXa incubated with FXIa for 1 h (50% activated) (■); and (d) FIXa incubated with FXIa for 3 h (100% activated) (▲). Samples were incubated for 30 min at 37 °C in TBS, pH 7.4, containing 10% (v/v) phospholipid and 10 mM $CaCl_2$. Aliquots were removed and assayed in a one-stage FVIII coagulation assay.



BAND	Mr(K)	N-TERMINAL AMINO ACID SEQUENCE
a	80	$^{FVIII-1653}$XXLQ(S)DQEEIDYD^{1665}
b	70	$^{FVIII-1690}$(S)FQKKTRHYFIAA^{1702}
c	68	$^{FVIII-1721}$NRAQSGXVPQFKKVV^{1735}
d	50	$^{FVIII-1}$(A)(T)RRYYLGAVELS^{13}
e	45	$^{FVIII-1}$AXRRYYLGAVELSWDY^{16}
f	40	$^{FVIII-373}$(S)VAKKHPKTXVHYIAA^{388}
h	30	$^{FIXa-181}$XVGGEDAXPGQFP^{193}

FIGURE 3: Sequence of FIXa-derived FVIII polypeptides. FVIII was incubated with FIXa described under Materials and Methods. An aliquot of the sequenced digest was subjected to electrophoresis on a 7.5–12.5% gradient gel and stained with Coomassie blue. The sequence determinations for the major proteolytic products are shown in the table below the gel. Band letters on the gel refer to the letters in the table. X depicts a residue where no PTH-amino acid could be identified. Parentheses denote tentative assignments. (Lane 1) Purified FVIII control. (Lane 2) FVIII + FIXa. (Lane 3) FVIII + thrombin. Note that band g is thrombin (as determined by the migration distance on control gels) and h is the heavy chain of FIXa determined by sequence analysis and the migration distance on control gels. Molecular weight markers ($\times 10^3$) are shown along the left-hand side of the gel. The location of amino acids in the native molecule is given in the superscript at the beginning of each sequence. The average initial yield was 12.56 pmol. The average repetitive yield was 95.4%.

was dependent on prior activation of zymogen FIX to FIXa and that the inactivation rates were dependent on the concentration of added enzyme.

N-Terminal Sequence Analysis of FVIII Degradation Products Following Incubation with FIXa. Following incubation of FVIII with FIXa, samples were subjected to SDS–PAGE, electroblotted, and the individual polypeptide bands excised and subjected to N-terminal amino acid sequence analysis. Approximately 50% of the FVIII light chain was unproteolyzed (Figure 3 lane 2, band a). A 68-kDa fragment generated by proteolysis at position 1719 (Figure 3, lane 2, band c) was also resolved and sequenced. This cleavage, the result of proteolysis of a scissile bond between residues Arg 1719 and Asn 1720, has not been previously reported. The heavy chain series of bands derived from the N-terminal portion of the molecule were proteolyzed to generate a major cleavage product at 45 kDa with the mature N-terminus of the cofactor (Figure 3, lane 2, band e) and weakly staining bands at 30–40 kDa which represent diffuse degradation peptides. On several occasions we were able to identify a new N-terminal sequence beginning at residue 373 in this series of polypeptides, showing that FIXa is also capable of cleaving FVIII at position 372, but this band was not well resolved by SDS–PAGE. This cleavage has been shown to be essential for the activation of FVIII by thrombin (Pittman & Kaufman 1988). FIXa is also able to proteolyze the cofactor at this position, but to a much reduced extent, such that the product of this cleavage, the 40-kDa fragment, could only be resolved using immunochemical detection methods (vide infra).

In keeping with earlier reports identifying APC-mediated FVIII cleavage products (Eaton et al., 1986), the cleavage at position 336 is inferred rather than identified since this polypeptide has the same N-terminus as the mature protein. The polypeptide resolved at approximately 30 kDa (band h) was sequenced and shown to be the N-terminus of the heavy chain of FIXa.

FVIII was also subjected to digestion with thrombin for comparison with FIXa cleavage products (Figure 3, lane 3). On thrombin digestion, bands at 70 kDa, 50 kDa, and a doublet at 40 kDa were resolved and sequenced. As reported previously, the new N-termini were at position 1690 (Figure 3, lane 3, band b), position 1 (band d), and position 373 (band f) (Eaton et al., 1986). The cleavage of the second A domain into a doublet at approximately 40 and 36 kDa only occurs after prolonged exposure of FVIII to thrombin (O'Brien et al., 1990). Both species had the same N-terminal residue. The proteolytic degradation products of FVIII/FIXa cleavage are therefore consistent with the gradual inactivation of the cofactor following cleavage adjacent to Arg 336 in the heavy chain of the molecule and slow cleavage of the light chain adjacent to Arg 1719.

Inhibition of FIXa-Catalyzed FVIII Proteolysis and Inactivation by a FIX-Specific Monoclonal Antibody. In order to confirm that the proteolysis of FVIII by FIXa was not caused by contaminating proteases such as FXa or thrombin, a monoclonal antibody to FIX (3A6), which completely inhibited the activity of FIXa in one stage coagulation assays, was preincubated with the FIXa preparation prior to the addition of radiolabeled human FVIII. Proteolysis was then assessed by SDS–PAGE and autoradiography. FIXa without 3A6 MoAb proteolyzed FVIII under these conditions as shown in Figure 4 (lane b). The addition of 3A6 MoAb to the FIXa prior to addition of [^{125}I]FVIII abolished the proteolytic effect (lane c). A monoclonal antibody to the B domain of FVIII, C10, did not inhibit the cleavage (Figure 4, lane d). These results confirm that FIXa alone is responsible for the proteolysis reported in this study.

Simultaneous Incubation of Purified FVIII with Thrombin and FIXa. In order to investigate the effect of simultaneous addition of thrombin and FIXa, recombinant FVIII was incubated with phospholipid, calcium, FIXa (molar ratio 1:1.6 FVIII/FIXa), and a catalytic concentration of thrombin. Aliquots were removed at various time points for FVIII:C assay and SDS–PAGE analysis. In addition, FVIII was incubated with either FIXa or thrombin alone at the same concentration of reactants. Time course analysis of these reactions is depicted in Figure 5. The heavy chain of FIXa is marked with an arrow. Incubation of FVIII with FIXa resulted in a progressive loss of FVIII:C activity, which was concomitant with the appearance of the 45-kDa band on the gel (Figure 5A). Cleavage adjacent to Arg 336 could therefore be observed within 1 min of the addition of the enzyme. The secondary cleavage of the FVIII light chain adjacent to Arg 1719 was only apparent following prolonged incubation times. Most of the FVIII:C activity was abolished at the 60-min time

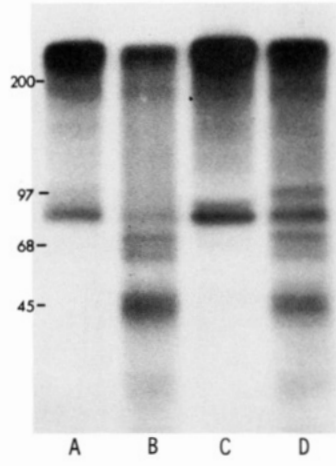


FIGURE 4: Incubation of FVIII with monoclonal antibody-inhibited FIXa. One microgram of protein A purified 3A6 monoclonal antibody to FIX or C10 monoclonal antibody to FVIII was incubated with 1 μg of FIXa in 50 μL of TBS and 10 mM $CaCl_2$ containing 10% (v/v) phospholipid for 30 min at 37 °C. [^{125}I]FVIII (10 μL) was then added to each aliquot, and a further incubation for 1 h was allowed to proceed. Samples were then acetone precipitated and subjected to SDS–PAGE and autoradiography. (Lane a) [^{125}I]FVIII. (Lane b) [^{125}I]FVIII + FIXa. (Lane c) [^{125}I]FVIII + FIXa + MoAb 3A6. (Lane d) [^{125}I]FVIII + FIXa + MoAb C10.

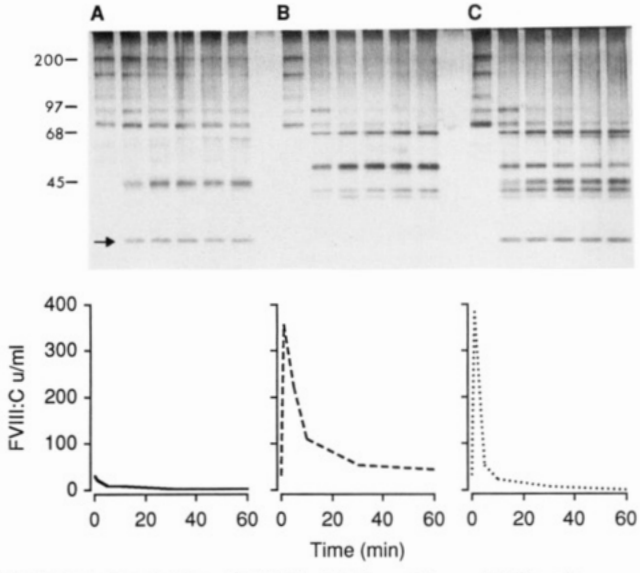


FIGURE 5: Incubation of FVIII with thrombin and FIXa. Recombinant FVIII (4.5 μg) was incubated with thrombin and FIXa in 120 μL of TBS and 10 mM $CaCl_2$, pH 7.4, containing 10% (v/v) phospholipid. Aliquots were removed at 1, 5, 10, 30, and 60 min following the addition of the enzymes and diluted in TBSA for one-stage coagulation assay or precipitated with ice-cold acetone and resuspended in 10 μL of SDS–PAGE reducing sample buffer. SDS–PAGE was carried out on a 10–15% gradient slab gel, which was stained with silver. (Panel A) Incubation of FVIII with FIXa (1.25 μg) with the activity time course shown below the relevant gel section. (Panel B) FVIII incubated with 1.125 units of human thrombin. (Panel C) FVIII incubated with the same concentrations of FIXa and thrombin simultaneously. The migration positions of the molecular mass markers are shown at the side of the gel. The band marked with an arrow is the heavy chain of FIXa.

point (Figure 5A). There was no generation of the 50-kDa fragment, which is an essential component of the active cofactor complex, nor was the 40-kDa A2 domain product of Arg 372–Ser 373 cleavage readily apparent. Even at the 1-min time point, none of these species was detectable on silver-stain analysis, confirming that FIXa does not activate FVIII.

Incubation of FVIII with thrombin under the same conditions resulted in an increase, and subsequent decay, of FVIII

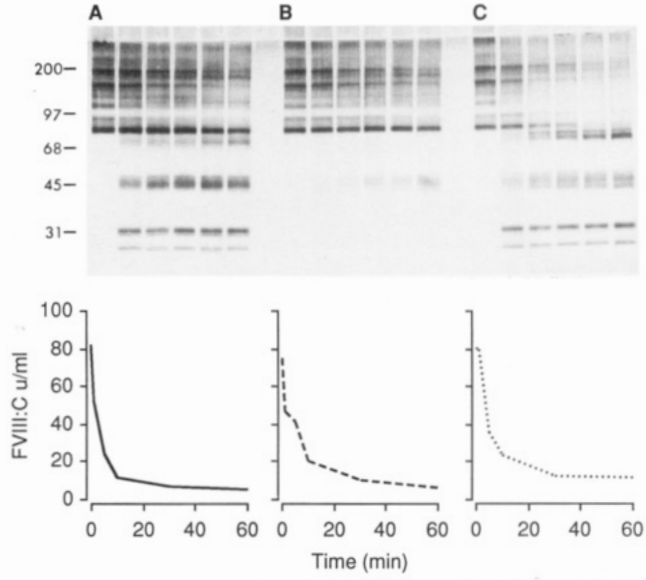


FIGURE 6: Incubation of FVIII with APC and FIXa. Recombinant FVIII (4.5 μg) was incubated with APC and FIXa in 120 μL of TBS and 10 mM $CaCl_2$, pH 7.4, containing 10% (v/v) phospholipid. Aliquots were removed at 1, 5, 10, 30, and 60 min following the addition of the enzymes and diluted in TBS containing 0.1% BSA for one-stage coagulation assay or precipitated with ice-cold acetone and resuspended in 10 μL of SDS-PAGE reducing sample buffer. SDS–PAGE was carried out on a 7.5–12.5% gradient slab gel, which was stained with silver. (Panel A) Incubation of FVIII with FIXa (1.25 μg) with FVIII:C activity shown below the gel section. (Panel B) FVIII incubated with 0.192 μg of APC. (Panel C) FVIII incubated with 1.25 μg of FIXa and 0.192 μg of APC. The bands below the 31-kDa marker in panels A and C are the heavy and light chains of FIXa.

activity (Figure 5B). This was coincident with the proteolysis of the heavy chains of FVIII to the 50- and 40-kDa doublet polypeptides and the simultaneous appearance of the 70-kDa fragment derived from the 80-kDa light chain. Significant residual FVIII:C activity was detectable at the 60-min time point. Simultaneous incubation of thrombin, FIXa, and FVIII resulted in an initial activation and then subsequent rapid inactivation of the cofactor, with the generation of the 70-, 68-, 50-, 45-, and 40-kDa fragments (Figure 5C). It is therefore evident that the incubation of FVIII with FIXa does not affect the extent of activation by thrombin, but inactivation proceeds rapidly through cleavage adjacent to position 336 in the FVIII heavy chain and possibly through further proteolysis of the 70- to the 68-kDa chain.

Simultaneous Incubation of FVIII, FIXa, and APC. Inactivation of FVIII by APC has been shown to occur concomitantly with the cleavage of the FVIII heavy chain adjacent to position 336, resulting in the generation of a 45-kDa polypeptide (Eaton et al., 1986). In order to compare the proteolysis effected by both enzymes, FVIII was incubated with FIXa alone, APC alone, and simultaneously with both FIXa and APC. Time course analysis by SDS–PAGE and coagulation assay is shown in Figure 6. Incubation of FVIII with a 1:1.6 molar ratio of FVIII to FIXa resulted in the characteristic loss of activity following proteolysis of the heavy chain of FVIII and the appearance of the 45-kDa fragment. Progressive loss of the light chain and the appearance of the 68-kDa fragment was also observed (Figure 6A). Incubation of FVIII with a catalytic concentration of APC resulted in a similar decay of FVIII activity and the appearance of the 45-kDa species, with no proteolysis of the 80-kDa light chain (Figure 6B). Simultaneous incubation of FVIII, FIXa, and APC resulted in an amalgam of these proteolytic cleavages

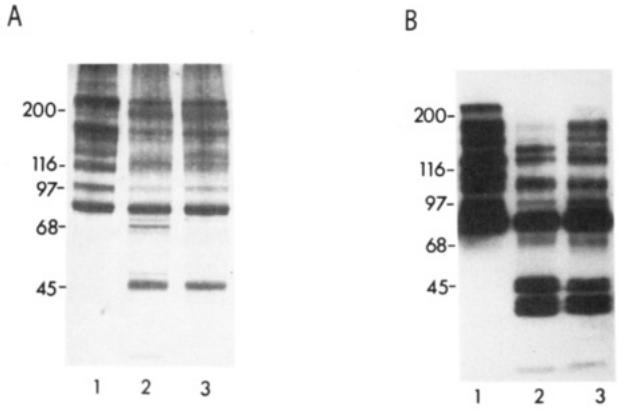


FIGURE 7: Comparison of the cleavages in the FVIII heavy chain mediated by FIXa and APC. (A) SDS–PAGE silver-stain analysis of FVIII incubated with FIXa and APC. FVIII (36 ng) was incubated with an equimolar concentration of FIXa for 30 min at 37 °C. A second aliquot was incubated with 5 ng of APC. Both reaction mixtures contained 10% mixed brain phospholipids and 10 mM $CaCl_2$. Following incubation, samples were acetone precipitated and subjected to electrophoresis on a 10% homogeneous PAGE gel, which was stained with silver. (Lane 1) FVIII. (Lane 2) FVIII + FIXa. (Lane 3) FVIII + APC. (B) Western blot of FVIII incubated with APC and FIXa. Recombinant FVIII (36 ng) was incubated with 5 ng of APC and an equimolar concentration of FIXa in TBS, pH 7.4, 10% (v/v) phospholipid and 10 mM $CaCl_2$. Samples were incubated for 40 min at 37 °C and acetone precipitated and subjected to SDS–PAGE and western blotting. The blot was probed with biotinylated MoAb C6 and C7F7 simultaneously. Biotinylated bound antibody was detected using steptavidin coupled to horseradish peroxidase and an enhanced chemiluminescent substrate according to the manufacturers' instructions (Amersham International, U.K.). (Lane 1) FVIII. (Lane 2) FVIII + FIXa. (Lane 3) FVIII + APC.

and the rapid loss of FVIII activity (Figure 6C). There was little evidence of a synergistic effect on the inactivation of FVIII by FIXa and APC since the inactivation rates were similar in all the experiments at these concentrations of reactants. Thus, at concentrations of FIXa which resulted in the inactivation of FVIII, no protection against inactivation by APC was detected.

Comparison of the Cleavage of the FVIII Heavy Chain by APC and FIXa. Both FIXa and APC proteolyzed the FVIII heavy chain in a similar manner, such that a single heavy chain band was resolved at approximately 45 kDa on silver-stain analysis (Figure 7A). Since this represents the amino-terminal portion of the molecule, the pattern resolved by silver or Coomassie staining did not account for the A2 domain of FVIII following digestion by FIXa and APC. Therefore FVIII was incubated with APC and FIXa, and the A2 domain was detected by western blot analysis. This methodology allowed detection of trace quantities of immunoreactive polypeptides that were not detected by protein staining, through the use of an amplified biotin/streptavidin detection system (Figure 7B). The blot was probed with an anti-A2 domain monoclonal antibody C6, which reacted with the heavy chain series of polypeptides and C7F7, an anti-light chain MoAb, with the characteristic polypeptides resolved prior to incubation of the cofactor with either APC or FIXa (Figure 7B). The C6 antibody does not react with the A1 domain of FVIII which is not stained. Following FIXa digestion, two doublet polypeptides were resolved at approximately 45 and 38 kDa. The identical pattern was resolved on incubation of FVIII with APC (Figure 7B, lane 3). These bands most likely represent the A2 domain with the small acidic region from positions 337–740 and the A2 domain alone from positions 373–740. These results support the hypothesis that FIXa is capable of effecting proteolysis of FVIII at position 372–373, but the

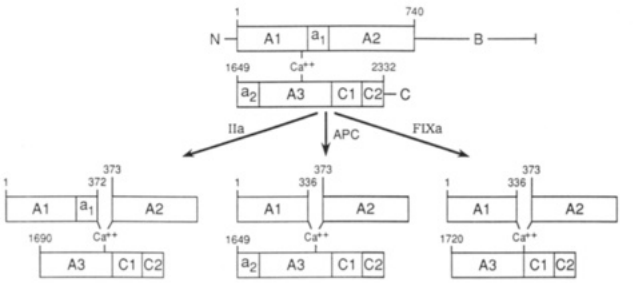


FIGURE 8: Proteolysis of human FVIII mediated by various enzymes. The B domain of FVIII is not required for the in vitro or in vivo procoagulant function of FVIII and is released from the molecule by all three enzymes following proteolysis at position 740. Cleavages mediated by thrombin (IIa) and activated protein C (APC) were first described by Eaton et al. (1986). APC is shown to cleave FVIII at position 372–373 in addition to the 336–337 site. a1 and a2 refer to the small acidic domains in the heavy and light chains of FVIII. N and C refer to the N-terminus and C-terminus of the molecule, respectively.

extent of this cleavage is very low, with resolution of these fragments only possible on immunochemical staining. Interestingly, APC proteolyzes the FVIII heavy chain in the same way as FIXa and, thus, probably proteolyzes the 372–373 site also. These cleavages are depicted in Figure 8. Thrombin generates a heterotrimeric activated cofactor following cleavage of FVIII at positions 372–373 and 1689–1690. It is inferred that APC and FIXa cleave FVIII adjacent to position 336, generating a 45-kDa polypeptide and, to a much lesser extent, adjacent to position 372. While the latter is one of the activation cleavages, it is clearly insufficient to activate FVIII in the absence of the generation of the 50-kDa fragment. Cleavages mediated by FIXa and APC appear to be identical, with the exception of the FVIII light chain proteolysis effected by FIXa alone adjacent to position 1719 (Figure 8).

Discussion

In this study we have investigated the interaction between highly purified recombinant FVIII and plasma derived FIXa. In an early study using partially purified coagulation factors, it was reported that FIXa activated FVIII in complex with, or isolated from, von Willebrand Factor (Rick, 1982). A 6-fold activation of FVIII and the FVIII/vWF complex was detected using high concentrations of FIXa, in a manner which was qualitatively similar to the activation of FVIII by thrombin. In addition, FIXa-activated FVIII was reported to be resistant to subsequent thrombin activation. Using highly purified rFVIII and pdFIXa, we were unable to detect the activation of FVIII by the enzyme. A dose-dependent loss of FVIII activity on incubation of the cofactor with FIXa was consistently recorded, which was concomitant with the proteolysis of the heavy chain adjacent to position 336. This is the same inactivation cleavage of the FVIII heavy chain that is mediated by APC (Eaton et al., 1986). Proteolysis of the FVIII heavy chain by FIXa appears to generate identical polypeptides to those resolved following the APC-catalyzed inactivation of FVIII.

It is probable that both enzymes are also capable of proteolyzing the scissile bond between residues 372 and 373. This is one of the activation cleavage sites in FVIII (Eaton et al., 1986). Despite this, neither FIXa nor APC activates the cofactor. FIXa does not generate the 50-kDa fragment from the N-terminus of the heavy chain, which is an essential component of the heterotrimeric activated form of FVIII (Lollar & Parker, 1989). In addition, FIXa does not generate the 70-kDa light chain fragment that is also required for the

expression of FVIII procoagulant activity in vitro and in vivo (Pittman & Kaufman 1988; O'Brien & Tuddenham, 1989). FIXa uniquely cleaves the FVIII light chain at position 1719 to generate a 68-kDa fragment. FXa cleaves FVIII between positions 1721 and 1722 to generate a similar size polypeptide (Pittman & Kaufman, 1988; Eaton et al., 1986). The time course analysis of the inactivation of FVIII with FIXa demonstrated that proteolysis of the light chain occurred after the loss of FVIII coagulant activity, and so the significance of this cleavage, if any, is unclear.

The inactivation of FVIII by FIXa occurred at approximately equimolar concentrations of reactants or at higher FIXa concentrations. Several studies have been reported in which FVIII has been incubated with up to 70-fold molar excess of FIXa (Rick, 1982; Lollar et al., 1984; Walker et al., 1987). We reasoned that while FIXa circulates in molar excess over FVIII in plasma (Kane & Davie, 1988), only a small proportion of the zymogen is activated to FIXa during clot formation (Houghie & Baugh, 1983). FVIII and FIXa probably form a 1:1 stoichiometric complex similar to that formed by FVa and FXa in prothrombinase (Tucker et al., 1983). This hypothesis is supported by a recent report describing the kinetic analysis of the activation of FX by a 1:1 stoichiometric complex of FVIII and FIXa assembled on the surface of peripheral blood monocytes (McGee & Lee, 1991). The present experiments were largely conducted at close to equimolar concentrations of reactants to more closely reflect physiological conditions.

The observation that FIXa catalyzes the inactivation of FVIII is not consistent with several earlier reports that suggest that FIXa stabilizes thrombin-activated FVIII and protects the cofactor from inactivation by APC. Lollar et al. (1984) used porcine ^{125}I-labeled FVIII and a high molar excess of FIXa and were able to demonstrate the stabilization of the thrombin activated cofactor, with no apparent change in the proteolytic profile on SDS–PAGE seen with thrombin alone. We detected the generation of the 45-kDa polypeptide on simultaneous incubation of FVIII with FIXa and thrombin, which infers that the 336–337 cleavage occurs in the presence of both enzymes and militates against the hypothesis that FIXa stabilizes the thrombin-activated human cofactor. Interestingly Lollar and co-workers were unable to detect any activation of porcine FVIII by FIXa in a plasma-free chromogenic assay.

It has been reported that FIXa prevents APC inactivation of partially purified FVIII (Bertina et al., 1984; Rick et al., 1990). The purity of the reagents used in those experiments did not allow the analysis of cleavage products by SDS–PAGE. Using highly purified reactants, FVIII is not protected from inactivation by APC in the presence of equimolar concentrations of FIXa. SDS–PAGE analysis of FVIII incubated with FIXa and a catalytic concentration of APC indicated that the 336–337 scissile bond was cleaved with the loss of FVIII:C procoagulant activity in the presence of both enzymes. The discrepancy between our results and previous studies may be due to species differences, differences in reaction conditions, or, perhaps most likely, the purity of the reactants. FVIII in complex with vWF is not proteolyzed by FIXa, and so inactivation of the cofactor by the enzyme may occur only after FXa or thrombin release FVIII from vWF upon cleavage at position 1689. A corollary of this is that partially purified FVIII contaminated with vWF would be resistant to inactivation by FIXa. vWF also protects FVIII from APC-catalyzed inactivation (Fay et al., 1991). The APC binding site on FVIII has been localized to residues 2009–2018 in the light chain of the molecule. Since FIXa and APC proteolyze FVIII in a similar way and vWF protects the cofactor from proteolysis by both enzymes, it is possible that the binding site for FIXa, like that for APC, is located in the light chain.

Since thrombin and FXa are generated after FIXa in the intrinsic coagulation cascade, it has been argued that FIXa may be the first protease to activate FVIII in vivo, allowing subsequent back-activation of the cofactor by FXa or thrombin. Our results are not consistent with this hypothesis. The experiments reported by Pieters et al. (1989) suggest that thrombin is the sole activator of FVIII in tissue factor-activated plasma. Therefore extrinsic pathway-generated thrombin may be the initial FVIII-activating enzyme in vivo. Subsequent to thrombin activation, FVIII participates in the tenase complex but becomes susceptible to inactivation by activated protein C and, as we have now shown, by FIXa. This latter reaction may explain why the majority of individuals with partial protein C deficiency do not have a thrombotic tendency (Miletich et al., 1987).

Registry No. FVIII, 9001-27-8; FIXa, 37316-87-3; APC, 42617-41-4; thrombin, 9002-04-4.

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Multiple Mechanisms Regulate the Proliferation-Specific Histone Gene Transcription Factor HiNF-D in Normal Human Diploid Fibroblasts†

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ABSTRACT: The proliferation-specific transcription factor complex HiNF-D interacts with sequence specificity in a proximal promoter element of the human H4 histone gene FO108, designated Site II. The occupancy of Site II by HiNF-D has been implicated in proper transcription initiation and as a component of the cell cycle regulation of this gene. In the present study we have investigated the role of the HiNF-D/Site II interaction in controlling the level of H4 histone gene transcription during modifications of normal cellular growth. HiNF-D binding activity is present at high levels in rapidly proliferating cultures of human diploid fibroblasts and is reduced to less than 2% upon the cessation of proliferation induced by serum deprivation of sparsely populated fibroblast cultures. Density-dependent quiescence also abolishes HiNF-D binding activity. Downregulation of transcription from the H4 gene occurs concomitant with the loss of the HiNF-D/Site II interaction, further suggesting a functional relationship between Site II occupancy and the capacity for transcription. Serum stimulation of quiescent preconfluent cells results in an increase in HiNF-D binding activity as the cells are resuming DNA synthesis and H4 histone gene transcription. Density-inhibited quiescent cells respond to serum stimulation with only a minimal increase in the HiNF-D binding activity, 30% of maximal levels. However, H4 histone gene transcription is stimulated to a level equal to that detected in extracts of the sparsely populated serum-stimulated cultures. These results suggest that there is a threshold level of HiNF-D binding activity necessary for the activation of H4 histone gene transcription. Additionally, these findings suggest that there may be a mechanism repressing HiNF-D binding activity in the density-inhibited cultures which is not operative in the sparsely populated, serum-deprived cultures. Density-inhibited cultures may have reached a state analogous to the initial steps of differentiation and have invoked a series of mechanisms to decrease expression of proliferation-specific factors. Serum stimulation is able to overcome the one mechanism downregulating HiNF-D in both sparsely populated and density-inhibited quiescent cultures but is unable to reverse the repression of proliferation-specific factors that occurs in density-inhibited cultures. These results are consistent with the presence of at least two levels of control over the HiNF-D/Site II interaction which are responsive to and reflect the proliferative state of the cell and the extent to which the cell exhibits properties associated with differentiation.

The control of growth-related genes is central to maintaining stringent cell cycle control and regulating the transition between proliferation and a commitment to differentiate. Understanding molecular mechanisms operative in regulating expression of cell-cycle- and cell-growth-related genes should provide insights into the loss of growth control in transformed and tumor cells. The human H4 histone gene FO108 (Sierra et al., 1982) is a cell-cycle-dependent, growth-related gene for which expression is temporally and functionally coupled to DNA synthesis (Plumb et al., 1983). Regulation of this coupling is mediated at both the transcriptional and post-transcriptional levels (Baumbach et al., 1987). This H4 histone gene exhibits a basal level of transcription throughout the cell cycle, with a 2–3-fold enhanced level of transcription during the early stages of S phase (Baumbach et al., 1987; Ramsey-Ewing et al., 1992).

Two in vivo protein–DNA interactions, Sites I and II, have been delineated within the first 200 nucleotides upstream of the H4-FO108 transcription initiation site (Pauli et al., 1987, 1988). At least three independent protein factors have been detected that bind to these domains in vitro (van Wijnen et al., 1989, 1991; Wright et al., 1992): HiNF-C, an Sp1-like protein, and HiNF-E, an ATF-related transcription factor, bind adjacent to each other in the Site I domain. The interactions at Site I do not change during the cell cycle nor during the complete shutdown of H4 histone gene transcription that occurs during differentiation (Pauli et al., 1987; Stein et

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