Purification and Characterization of Factor VIII 1,689-Cys: A Nonfunctional Cofactor Occurring in a Patient With Severe Hemophilia A

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We have purified the factor VIII from a CRM* Hemophilia A plasma (90 U/dL VIII:Ag but 0 U/dL VIII:C) and analyzed the protein before and after thrombin activation by Western blotting with monoclonal antibodies (MoAbs). Normal or patient citrated plasma was ultracentrifuged, cryo-ethanol-precipitated and chromatographed on Sepharose 6B. The void volume fractions were reduced and subjected to ion exchange chromatography yielding material of specific activity ~1,000 U/mg protein (VIII:C or VIII:Ag). Factor VIII purified in this way from normal plasma is fully activatable by thrombin with proteolytic fragmentation as previously described by F. Rotblat et al (Biochemistry 24: 4294, 1985). Factor VIII 1,689-Cys has the normal distribution of factor VIII light and heavy chains prior to thrombin activation. After exposure to thrombin the heavy chain polyptides were fully proteolyzed but the light chain was totally resistant to cleavage. This is consistent with the demonstration in the patient’s leucocyte DNA of a C to T transition in codon 1,689 converting Arg to Cys at the light chain thrombin cleavage site as previously described by J. Gitschier et al (Blood 72:1022, 1988). Unbleached light chain of Factor VIII 1,689-Cys is not released from von Willebrand factor (vWF) by thrombin, but this is not the sole cause of the functional defect since the protein purified free of vWF has no coagulant activity. We conclude that the functional defect in factor VIII 1,689-Cys is a consequence of failure to release the acidic peptide from the light chain upon thrombin activation.

MATERIALS AND METHODS

Prestained mol wt standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bethesda Research Laboratories, MD. Electrophoresis reagents were from BioRad, Richmond, CA. Coatest MoAb to FVIII was a gift from Dr Dan Eaton, Genentech Inc, South San Francisco, CA. C8, C5, and RF1 antibodies were provided by Dr Alison Goodall, Royal Free Hospital, London. Sepharose 6B and DEAE Sephadex A50 were provided by Pharmacia, Piscataway, NJ. All other chemicals of reagent grade or better were provided by Sigma, Poole, Dorset, UK. Protein estimations were performed by the method of Bradford1 using bovine serum albumin (BSA) as standard. FVIII assays were performed using a chromogenic substrate assay (Kabi Coatest FVIII) according to the method of Carlebjörk et al,2 and by one stage FVIII assay.3 Immunoradiometric assay for FVIII:Ag was performed as described previously,4 SDS-PAGE and Western blots were performed as described previously.5 Proteins were radiolabeled with 0.5 mCi (18.5 MBq) 125I/10 μg using iododeb (Pierce Europe, Luton, UK) according to the manufacturer’s instructions. Centricon 30 microconcentraters were from Amicon (Danvers, MA).

Micropurification of FVIII. In a typical experiment, blood was collected into 3.8% trisodium citrate dihydrate (Sigma), 1:9 vol/vol, and centrifuged to collect platelet-poor plasma. Benzamidine hydrochloride (Sigma) was added to a final concentration of 5 mmol/L and the plasma snap-frozen in an ethanol/dry-ice bath and stored at -70°C. Plasma (100 to 200 mL) was then thawed at 37°C and centrifuged at 18,000 x g in a Sorvall RC5B centrifuge in an SS34 From the Haemostasis Research Group, Clinical Research Centre, Middlesex, England.

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The protein circulates in plasma as a heterodimer comprised of a variable length heavy chain (A1/A2/B) and a light chain (A3/C1C2). Activation cleavages by thrombin and FXa that generate an active species with the domain structure A1/A2 + A3/C1C2 are indicated. The epitopes for MoAb C8, C5, and C7F7 are indicated by arrows. Since C7F7 binds to an epitope within the acidic region of the light chain between positions 1,648 and 1,689, reactivity is lost after thrombin activation of FVIII. In contrast C5 binds to the light chain before and after thrombin proteolysis. C8 binds to an epitope on the A2 domain and thus binds the heavy chain prior to thrombin activation, and to the mol wt 40-kD chain following proteolysis.

The resuspended cryoprecipitate was applied to a 100 x 1.5-cm column of Sepharose 6B equilibrated with IBS pH 6.9. The column was developed isocratically at a flow rate of 0.15 mL/min. Three-milliliter fractions were collected and assayed for FVIII activity in a one-stage FVIII assay or FVIII antigen by IRMA. Fractions containing peak activity or antigen were pooled and made 35 mmol/L 2S mercaptoethanol prior to application to a 2 x 0.5-cm column of DEAE Sephadex A-50, preequilibrated with IBS pH 6.9. The sample was applied at a flow rate of 0.2 mL/min and a single flow through fraction collected. The column was washed down to baseline absorbance at 280 nm and step-eluted with IBS without benzamidine hydrochloride containing 1 mol/L NaCl. Two-milliliter fractions were collected and assayed for FVIII activity or antigen. Peak activity or antigen fractions were then pooled and stored at -70°C.

For analysis of thrombin activation of FVIII, the DEAE pool was divided into aliquots that were then made 2.5 mmol/L CaCl₂. Thrombin at a 1:2 wt/wt ratio was added to one aliquot that was then placed with a control aliquot without thrombin at 37°C for 16 hours. Fractions of each aliquot (300 μL) were then precipitated with 1.2 mL ice cold acetone and collected by centrifugation in an MSE microfuge (MSE, Loughborough, UK) at 12,000 x g for two minutes. Acetone was discarded and samples resuspended in SDS-PAGE reducing sample buffer and boiled for five minutes. Twenty microliters of each sample was then applied to each lane of a 7.5% homogeneous SDS-PAGE gel and subjected to Western blotting as described previously.

Binding of FVIII light chain to vWF. To 1-mL aliquots of citrated platelet poor plasma or serum, 25 μL of radiolabeled anti-light chain MoAb (C5) was added, and the mixture incubated for one hour at 37°C. The samples were then applied to a 0.5 x 45-cm column of Sepharose 6B preequilibrated with 0.05 mol/L tris HCl, 0.15 mol/L NaCl pH 7.5 (TBS) at a flow rate of 20 mL/h. One-milliliter fractions were collected and counted in an NE 1600 multihed gamma counter (Nuclear Enterprises, Edinburgh) for two minutes. Normal plasma used in these experiments was prepared from a pool of six normal donors. Serum was prepared by adding 3 U of human thrombin to 1 mL plasma and incubating the sample for 30 minutes at 37°C. Following removal of the clot a further 3 U of thrombin was added and incubation repeated for another 30 minutes.

RESULTS

Purification of normal and CRM⁺ FVIII. In order to characterize normal and CRM⁺ FVIII molecules utilizing Western blot analysis it was necessary to develop a micro-purification scheme prior to electrophoresis. Initial experiments using matrix-bound MoAbs to vWF were not successful as a result of the low degree of purification achieved. Overloading contaminant proteins on gels led to nonspecific signal generation and band distortion and rendered blots uninterpretable. Further purification of the eluates from these columns was possible using matrix-bound antibodies to FVIII, but yields were low and denaturing buffers used to...
elute FVIII from the columns had to be removed prior to activation of FVIII by thrombin, leading to further losses. The successful protocol was devised using 100 to 200 mL of plasma as starting material and involved the use of ultracentrifugation to remove lipid, cryoprecipitation, and gel filtration chromatography (Table 1). Void volume fractions from the Sepharose 6B column contained the high mol wt vWF/FVIII complexes that can be disrupted by reduction without loss of FVIII activity. Anion exchange chromatography was used as a final step in the purification, FVIII being step-eluted with 1 mol/L NaCl. The eluate from this column contained purified FVIII with typical specific activities of 500 to 1,000 U/mg. Recovery from starting plasma was regularly about 10%, yielding sufficient FVIII from 200 mL of plasma for ten separate Western blot analyses.

Western blot analysis of normal and CRM+ FVIII. Two MoAbs were used as probes for FVIII in Western blots. MoAb C8 binds to the heavy chain of the molecule (Fig 1) and has been mapped to an epitope on the A2 domain. Prior to thrombin activation of normal FVIII purified as described earlier, this antibody reacts with the mol wt 210 to 90 kD series of polypeptides (Fig 2A, lane 1). Following thrombin activation only the mol wt 40-kD A2 domain can be visualized (Fig 2A, lane 2), indicating that under the experimental conditions used proteolysis of FVIII had gone to completion.

This material was also analyzed using MoAb C7F7, an antibody that has been mapped to the mol wt 80-kD light chain of FVIII. Epitope is close to the N terminal region of the light chain (Fig 1), either in the acidic region between residues 1,648 to 1,689 or spanning the thrombin cleavage site at position 1,689. This is inferred from the fact that the antibody binds to the mol wt 80-kD chain and not the mol wt 73-kD thrombin-derived, activated moiety. Normal FVIII purified as described earlier reacted with this antibody prior to thrombin activation (Fig 2B, lane 1) and a single mol wt 80-kD band was detected. Following thrombin activation no binding was detected (Fig 2B, lane 2). These data indicated that FVIII purified from a small volume of plasma and subjected to Western blot analysis could be characterized using two MoAbs, such that the thrombin-mediated cleavages at positions 372, 740, and 1,689 could be monitored.

CRM+ FVIII was then purified from 120 mL of plasma from a hemophilia A patient as described above. Prior to thrombin activation MoAb C8 reacted with the normal array of polypeptides at mol wt 210 to 90 kD (Fig 2C, lane 1). Following thrombin proteolysis MoAb C8 bound to the mol wt 40-kD thrombin-derived A2 domain (Fig 2C, lane 2). This demonstrated that the cleavages at positions 372 and 740 occurred as normal in this molecule, and that the proteolysis of the heavy chain had gone to completion. C7F7 reacted with the mol wt 80-kD chain prior to thrombin treatment of this material (Fig 2D, lane 1) but in contrast to normal FVIII, reactivity was still detected following exposure to thrombin (Fig 2D, lane 2), demonstrating that cleavage at position 1,689 does not occur in this molecule.

In order to evaluate the carrier status of the patient’s mother, FVIII was purified from 85 mL of her plasma and subjected to Western blot analysis. Prior to thrombin activation, the FVIII from this individual cross-reacted with C7F7 antibody (Fig 2E, lane 1); following thrombin treatment, no mol wt 80-kD band could be detected, indicating that no detectable CRM+ FVIII circulates in her plasma (Fig 2E, lane 2).

Binding of FVIII light chain to vWF. High molecular weight complexes of vWF and FVIII are excluded from Sepharose 6B gel matrix emerging in the void volume fractions of filtration columns packed with this resin. This property was used to evaluate the binding of the light chain to vWF prior to, and after thrombin treatment. Incubation of normal pooled plasma and radiolabeled C5 antithrombin antibody resulted in the formation of radioiodinated complexes that were detected as a peak of radioactivity in the void volume fractions of the column. This peak was abolished when serum was used in place of plasma, demonstrating that the FVIII light chain no longer binds to the vWF complex after thrombin activation (Fig 3), since C5 binds to thrombin-cleaved light chain. The CRM+ plasma and serum were then separately incubated with radiolabeled C5 antibody and chromatographed as described earlier. Both plasma and serum contained high molecular mass complexes as determined by the elution of radioactive peaks in the void volume fractions (Fig 3), demonstrating that the light chain of FVIII 1,689-Cys remains bound to vWF after thrombin proteolysis.

CRM+ FVIII eluted from vWF immobilized on a matrix-bound MoAb to vWF (RF1) had no detectable procoagulant activity (VIII:Ag 100 U x 103/mL VIII procoagulant activity <10 U x 103/mL). Furthermore, irreversible dissociation of the CRM+ FVIII/vWF complex using 35-mmol/L 2 mercaptoethanol did not restore procoagulant activity (VIII:Ag 900 U x 103/mL VIII procoagulant activity <10 U x 103/mL). Under these conditions normal FVIII procoagulant activity was unaffected.

DISCUSSION

We have developed a method for purifying CRM+ FVIII obtained from approximately 100 to 200 mL of patients’ plasma, and of characterizing it by Western blot analysis.
Fig 2. Western blot analysis of normal and CRM* human FVIII. FVIII was partially purified as described under Materials and Methods. Control samples were incubated for 16 hours with buffer while other samples were incubated with thrombin at a 1:2 wt/wt ratio. Following acetone precipitation and resuspension in SDS-PAGE reducing sample buffer, samples were boiled for five minutes and applied to a 7.5% homogeneous gel. Following electrophoresis, the gels were electroblotted onto nitrocellulose membrane for one hour at 0.8 A. Membranes were blocked with NET buffer containing 0.25% gelatin and 0.1% BSA for one hour. Ten micrograms of protein A purified C8 or C7F7 MoAb was then added to 50 mL of NET buffer and the membranes incubated in these solutions for 16 hours at room temperature. Following two washes of 100 mL of NET buffer, the membranes were incubated with 50 mL of Goat anti-mouse Ig radiolabeled with $^{125}$I in 50 mL of NET buffer for two hours. The membranes were then washed with NET buffer, dried, and exposed to Kodak Xomat RP film (Kodak, Rochester, NY) for 16 hours at $-70^\circ$C with DuPont Cronex plus intensifying screens. (A) Western blot of normal human FVIII with MoAb C8 as probe. Lane 1, FVIII; lane 2, thrombin-treated FVIII. Molecular mass markers shown $\times 10^3$. (B) Normal human FVIII with MoAb C7F7 as probe. Lane 1, FVIII; lane 2, thrombin-treated FVIII. (C) CRM* FVIII with MoAb C8 as probe. Lane 1, CRM* FVIII; lane 2, thrombin-treated CRM* FVIII. (D) CRM* FVIII with MoAb C7F7 as probe. Lane 1, CRM* FVIII; lane 2, thrombin-treated CRM* FVIII. (E) FVIII from the mother of the propositus with MoAb C7F7 as probe. Lane 1 FVIII; lane 2 thrombin-treated FVIII.
Factor VIII 1,689-Cys has a missense mutation at position 1,689 leading to the substitution of an arginine by a cysteine residue. This was first detected by differential oligonucleotide melting and direct sequencing following the amplification of the patient’s leucocyte DNA by polymerase chain reaction. Studies using human FVIII derived from normal plasma have indicated that proteolysis of FVIII light chain at this position is concomitant with the expression of cofactor activity. Recently, site-directed mutagenesis has demonstrated that the genetic analysis. We conclude that the propositus in this kindred has a de novo mutation arising in his mother’s germ line. It follows that the mutation 1,689 Arg-Cys is the sole abnormality in his FVIII and the cause of its functional defect. The reason for the abnormal activity to antigen ratio in the plasma of the patient’s mother remains unknown.

Chromatographic profiles of normal plasma and serum samples preincubated with radiolabeled anti-light chain antibodies were dissimilar. Although a small proportion of the radioactive label could be detected in the void volume fractions of the serum profile, this was markedly less than that seen with plasma. We take this to indicate that mol wt 80-kD light chain dissociates from vWF on thrombin activation in agreement with several other reports. FVIII 1,689-Cys light chain evidently remains bound to vWF after thrombin treatment, as the elution profiles of the radiolabel were essentially identical in serum and plasma. A similar chromatograph of CRM+ serum was assayed for FVIII:Ag using an IRMA based on a human polyclonal anti-FVIII antibody. A peak of FVIII:Ag was detected in the void volume fractions of this column that was absent in the case of normal human serum chromatographed under identical conditions (data not shown). It has been suggested that the function of the thrombin-mediated 1,689 cleavage is simply to release FVIII from vWF. But while this is clearly a consequence of proteolysis at this position, it would appear not to be sufficient in itself to activate FVIII, since purified CRM+ FVIII has no activity even in the absence of vWF. Therefore, proteolysis at this position has a role in activating the molecule in addition to releasing it from the carrier protein.

There are few descriptions in the literature of functional properties of other CRM+ FVIII molecules. FVIII Leiden has been shown to have a reduced affinity for phospholipid-
bound FIXa in the intrinsic FX-activating complex. Mertens et al used kinetic analysis to characterize the molecular defect in the protein. Furlong et al\(^{2}\) used matrix-bound MoAbs to partially purify FVIII/vWF complex for Western blot analysis. Two MoAbs directed against an epitope on the FVIII light chain were used to detect a portion of FVIII purified from plasma or concentrate. While Western blots of concentrate-derived material proved successful, FVIII purified directly from plasma contained cross-reacting contaminant bands as a result of the low purity of the FVIII eluted from these columns. The approach was successful in identifying the FVIII light chain in several CRM* hemophilia A plasmas. However, the functional properties of these molecules could not be addressed due to the use of denaturing steps in the purification as described.

The characterization of naturally occurring CRM* FVIII proteins should lead to further insights into the function of this complex protein and is complementary to genetic analysis, and to the characterization of recombinant FVIII modified by site-directed mutagenesis and synthesized in vitro. Through use of the methodology described here, it will be possible to characterize other mutations that lead to synthesis of function-impaired or nonfunctional FVIII molecules. It may also be possible to detect partial primary sequence deletions, transpositions, or insertions in CRM* molecules through Western blot analysis, mutations that might not be readily identified by genetic analysis without sequencing the entire FVIII coding region. To attempt to gain insight into the molecular defects in other CRM* FVIII molecules, we are currently using a combination of differential oligonucleotide binding to polymerase chain reaction-amplified hemophilic DNA and protein characterization.

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Purification and characterization of factor VIII 1,689-Cys: a nonfunctional cofactor occurring in a patient with severe hemophilia A

DP O'Brien and EG Tuddenham