Enhanced Thrombin Sensitivity of a Factor VIII-Heparin Cofactor II Hybrid*

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Generation of thrombin at a site of vascular injury is a key event in the arrest of bleeding. In addition to the conversion of fibrinogen into the insoluble fibrin, thrombin can initiate a number of positive and negative feedback mechanisms that either sustain or down-regulate clot formation. We have modulated the thrombin sensitivity of human blood coagulation factor VIII, an essential cofactor in the intrinsic pathway of blood coagulation. We have substituted an acidic region of factor VIII corresponding to amino acid sequence Asp713–Ala736 of the thrombin inhibitor heparin cofactor II. Functional analysis of the resulting factor VIII-heparin cofactor II hybrid, termed des-(868–1562)-factor VIII-HCII, revealed an increase in procoagulant activity as measured in a one-stage clotting assay. Incubation of purified des-(868–1562)-factor VIII-HCII with different amounts of thrombin showed that this protein was more readily activated by thrombin when compared with des-(868–1562)-factor VIII, a control protein lacking amino acid sequence Ile65–Leu80 of heparin cofactor II. This was manifested by an increase in the second order rate constant of activation by thrombin for des-(868–1562)-factor VIII-HCII (12.0 ± 0.48 × 10⁶ M⁻¹ s⁻¹) compared with des-(868–1562)-factor VIII (1.77 ± 0.21 × 10⁶ M⁻¹ s⁻¹). Our data suggest that amino acid sequence Ile65–Leu80 of heparin cofactor II endows factor VIII with increased sensitivity towards thrombin which results in accelerated clot formation.

Selective interaction of thrombin with a number of blood coagulation factors is essential to ascertain control over both pro- and anticoagulant pathways (1–7). Determination of the three-dimensional structure of thrombin has greatly contributed to our current knowledge on the action of this serine protease (8, 9). Studies on the three-dimensional structure of a complex of thrombin and hirudin, an inhibitor derived from the leech Hirudo medicinalis, revealed that a positively charged area, the so-called anion binding exosite I of thrombin, interacts strongly with a stretch of negatively charged amino acids present at the carboxyl terminus of hirudin (10, 11). Similar areas of negatively charged amino acids are present in the thrombin receptor, thrombomodulin and heparin cofactor II and have been shown to interact with anion binding exosite I of thrombin (12–15). Studies using synthetic peptides corresponding to the negatively charged amino acids of the proteins mentioned above have shown that the affinity of these peptides for thrombin varies considerably (15, 16). These observations suggest that the ability of thrombin to interact with different components of the hemostatic system is, at least in part, determined by the affinity of anion binding exosite I of thrombin for stretches of negatively charged amino acids present on its substrates. Modulation of the pro- and anticoagulant activities of thrombin may be accomplished by simply exchanging negatively charged areas between the different substrates of this serine protease.

Factor VIII is an essential cofactor for factor IXa in the conversion of factor X to factor IXa in the intrinsic pathway of blood coagulation (3). Molecular cloning of the factor VIII cDNA revealed that factor VIII consists of a series of homologous domains which can be represented as follows: A1-A2-B-A3-C1-C2 (17, 18). Proteolytic processing of factor VIII at amino acid positions Arg1775, Arg2060, and Arg2089 (19, 20). Consequently, factor VIII circulates in plasma as a metal ion-linked heterodimer consisting of a heavy chain (A1-A2-B) and light chain (A3-C1-C2). Activation of factor VIII by thrombin proceeds through limited proteolysis at amino acid positions Arg1775, Arg2060, and Arg2089 (19, 20). Consequently, activated factor VIII consists of a heterotrimer of the separate A1 and A2-domains together with a thrombin cleaved light chain (21, 22). We have shown previously that an acidic region corresponding to amino acid sequence Asp1136–Arg1139 of factor VIII is involved in the activation of factor VIII by thrombin (23, 24). Here, we have replaced amino acid sequence Asp1136–Ala1139 of factor VIII by amino acid sequence Ile1136–Leu1140 of heparin cofactor II, a potent inhibitor of thrombin (25). Functional importance of amino acid sequence Ile1136–Leu1140 of heparin cofactor II in the inhibition of thrombin has been suggested by analysis of heparin cofactor II derivatives that lack this particular amino acid sequence (26). The factor VIII-heparin cofactor II hybrid constructed, designated des-(868–1562)-factor VIII-HCII, was expressed in mouse fibroblast C127 cells, and analysis of the properties of the resulting protein suggests that amino acid sequence Ile1136–Ser1138 of heparin cofactor II endows factor VIII with increased sensitivity towards thrombin.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, DNA modifying enzymes, culture media, trypsin, and antibiotics were obtained from Life Technologies, Inc. (Breda, The Netherlands). Pfu polymerase and the vector pGEM-T were purchased from Stratagene (Cambridge, United Kingdom). Oligonucleotide primers were synthesized using an automated DNA synthesizer (Applied Biosystems type 381A, Foster City, CA). The “Sequenase” kit was from the U.S. Biochemical Corp. The synthetic substrate Pefachrome Xa was obtained from Pentapharm AG (Basel, Switzerland). Hirudin (from leeches), L-ω-phosphatidyl-L-serine, L-ω-phosphatidyl choline, type I-EH were obtained from Sigma. Monodonal antibody MAb530, directed against the heavy chain of factor VIII, was obtained from Seralabs (Sussex, United Kingdom). Glu-Gly-Arg-chloromethyl ketone and Phe-Pro-Arg-chloromethyl ketone were obtained from Calbiochem.

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A. Construction and characterization of the factor VIII-heparin cofactor II hybrid. A schematic representation of des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII. In both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII part of the B-domain of factor VIII is deleted. Thrombin cleavage sites at amino acid position Arg777, Arg780, and Arg789 are indicated by arrows. Areas containing a large number of negatively charged amino acids are marked by hatched boxes. Amino acid sequence Val798–Asn799 of factor VIII (I) and the corresponding part of des-(868–1562)-factor VIII-HCII (II) is given. Amino acid sequence lle53–Leu59 derived of heparin cofactor II is surrounded by a box. Sulfated tyrosines are indicated by asterisks. B. activity of des-(868–1562)-factor VIII-HCII in a one stage clotting assay. The clotting time of factor VIII-deficient plasma containing 2.2, 4.4, and 8.8 pmol of des-(868–1562)-factor VIII-HCII (I) and des-(868–1562)-factor VIII (II) was measured using a one-stage clotting assay. Conditioned medium of cells transfected with either des-(868–1562)-factor VIII-HCII or des-(868–1562)-factor VIII cDNA was used as a source of recombinant factor VIII. The concentration of factor VIII employed in the clotting assay was determined by measuring the amount of factor VIII light chain employing monoclonal antibodies CLB-Cag 12 and CLB-Cag 117 as outlined under “Experimental Procedures.” Mean values (± S.D.) of five experiments are given.

Construction of a Factor VIII-Heparin Cofactor II Hybrid—Construction of the factor VIII-heparin cofactor II hybrid was performed using the previously described plasmid pCLB-dB695 as a template (23). The plasmid pCLB-dB695-HCII was constructed by overlap extension mutagenesis using oligonucleotide primer H3 (5′-TCTAGTCTGAGGACTGAC-3′); position 2173–2190 of factor VIII; nucleotide 208–209 of heparin cofactor II; sense) and oligonucleotide primer H3 (5′-ATACAACATAGAACCTCCG-3′; nucleotide 2173–2190 of factor VIII; nucleotide 208–209 of heparin cofactor II; antisense) were used to amplify a 510-bp fragment that contains nucleotide 1683–2190 of factor VIII and nucleotide 208–209 of heparin cofactor II. Both the 510-bp fragment and the 129-bp fragment were purified, and 1 ng of both fragments was used as a template in a polymerase chain reaction employing primer F8-1 and H2. The resulting fragment was digested with BamHI and HindII and yielded a 410-bp fragment in which nucleotide 2191–2265 of factor VIII has been replaced by nucleotide 208–297 of heparin cofactor II. The BamHI-HindII fragment containing part of the heparin cofactor II cDNA was used to replace the corresponding fragment of pCLB-dB695. The resulting plasmid was designated pCLB-dB695-HCII, and the sequence of the BamHI-HindII fragment containing part of the heparin cofactor II cDNA was verified by oligonucleotide sequencing.

Tissue Culture and Transfection—Mouse fibroblasts were transfected with pCLB-dB695-HCII and pCLB-dB695 as described previously (27). Production of factor VIII was monitored by measuring both factor VIII activity and factor VIII antigen. Factor VIII cofactor activity was addressed by the ability of factor VIII to function as a cofactor for the factor IXa-dependent formation of factor Xa, employing a chromogenic substrate for factor Xa (Coatest Factor VIII, Chromogenix, Möndal, Sweden). Factor VIII antigen was determined using monoclonal antibody CLB-Cag 12, directed against the factor VIII heavy chain; MAS 530 (Seralab, Sussex, United Kingdom) directed against the heavy chain of factor VIII; lanes 3 and 6. CLB-Cag 117 as described previously (23). Normal plasma from a pool of 20 donors was used as a standard. Large scale culturing of transfected cells was performed in cell factories according to the instructions of the manufacturer (Nunc A/S, Roskilde, Denmark).

Biochemical Characterization of Factor VIII—Des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII (previously designated Factor VIII-del(868–1562) (23) were purified from conditioned medium essentially as described previously (28). The specific activity of the purified factor VIII ranged from 2000 to 4500 units/mg which is similar to that of factor VIII preparations derived from plasma. Activation of des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII by thrombin was monitored as described previously (24, 27). To prevent feedback activation of factor VIII by activated factor X, acetylated factor X was used in our studies (29). Phospholipid vesicles (consisting of 50% phosphatidylcholine and 50% phosphatidylserine; final concentration 100 μM) were incubated for 10 min at 37 °C in a Ca²⁺-containing buffer (50 mM Tris-HCl, pH = 7.5, 100 mM NaCl, and 25 mM CaCl₂). Subsequently, 0.1 mM of factor IXa, 0.2 μM acetylated factor X, and 0.2 mM sequence Ile53–Leu59 of heparin cofactor II, and amino acid sequence lle53–Leu59 of factor VIII employing liver cDNA as a template (the first nucleotide of both cDNAs corresponds to the first nucleotide of the translation initiation codon). Oligonucleotide primer F8-1 (5′-TCTAGTCTGAGGACTGAC-3′; nucleotide 1683–1703 of factor VIII; sense) and oligonucleotide primer H3 (5′-ATACAACATAGAACCTCCG-3′; nucleotide 2173–2190 of factor VIII; nucleotide 208–209 of heparin cofactor II; antisense) were used to amplify a 129-bp fragment that contained nucleotide 1683–2190 of factor VIII and nucleotide 208–209 of heparin cofactor II. Both the 510-bp fragment and the 129-bp fragment were purified, and 1 ng of both fragments was used as a template in a polymerase chain reaction employing primer F8-1 and H2. The resulting fragment was digested with BamHI and HindII and yielded a 410-bp fragment in which nucleotide 2191–2265 of factor VIII has been replaced by nucleotide 208–297 of heparin cofactor II. The BamHI-HindII fragment containing part of the heparin cofactor II cDNA was used to replace the corresponding fragment of pCLB-dB695. The resulting plasmid was designated pCLB-dB695-HCII, and the sequence of the BamHI-HindII fragment containing part of the heparin cofactor II cDNA was verified by oligonucleotide sequencing.

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Factor VIII-Heparin Cofactor II Hybrid

RESULTS AND DISCUSSION

We have shown previously that efficient activation of blood coagulation factor VIII by thrombin is dependent on the presence of amino acid sequence Asp713–Arg740 of the factor VIII heavy chain (23). Here, we have replaced amino acid sequence Asp713–Ala736 of des-(868–1562)-factor VIII by amino acid sequence Ile65–Leu680 of heparin cofactor II (Fig. 1A). The modified factor VIII cDNA was expressed in C127 cells, and the properties of the resulting hybrid protein were compared with des-(868–1562)-factor VIII, a B-domain deleted factor VIII which has been previously characterized (23) (Fig. 1A). Characterization of the conditioned medium of transfected cells expressing des-(868–1562)-factor VIII-HCII revealed that the factor VIII-heparin cofactor hybrid had normal cofactor activity (data not shown). Also the amount of factor VIII antigen in the conditioned medium was similar for both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII (data not shown). Inspection of the procoagulant activity as measured in a one-stage clotting assay showed that limited amounts of des-(868–1562)-factor VIII-HCII sufficed to effectively reduce the clotting time of plasma deficient of factor VIII (Fig. 1B). This observation indicates that amino acid sequence Ile65–Leu680 of heparin cofactor II endows factor VIII with improved functional properties.

To determine the molecular basis of the enhanced procoagulant activity of des-(868–1562)-factor VIII-HCII, we have immunopurified and characterized this hybrid protein. First, the subunit composition of the purified material was analyzed by SDS-PAGE followed by immunoblotting (Fig. 2). Monoclonal antibody CLB-CAg 69 directed against amino acid sequence Lys1673–Arg1689 of the factor VIII light chain (30), reacted with the 200- and 80-kDa species of both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII while monoclonal antibody MAS530 directed against the heavy chain of factor VIII reacted with the 200- and 120-kDa species of these proteins (Fig. 2). These data indicate that in addition to a small amount of single chain protein both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-HCII consist predominantly of a heterodimer composed of a light chain of 80 kDa and a heavy chain of 120 kDa, which contains a residual portion of the B-domain. Monoclonal antibody CLB-Cag 9 did not react with des-(868–1562)-factor VIII-HCII, since amino acid sequence Asp713–Ala736, which constitutes the epitope of this monoclonal antibody (30), has been replaced by amino acid sequence Ile65–Leu680 of heparin cofactor II (Fig. 2).

Previous studies from our laboratory have shown that factor VIII-del(713–1637) lacks procoagulant activity in a one-stage clotting assay and is fully activated only in the presence of relatively high concentrations of thrombin (23). The increased procoagulant activity of des-(868–1562)-factor VIII-HCII in a one-stage clotting assay suggests that this molecule is more...
readily activated by thrombin than des-(868–1562)-factor VIII. To study this issue in more detail the sensitivity of both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII toward thrombin was determined. Activation of des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII by thrombin was monitored by probing the ability of the activated factor VIII to function as a cofactor in the factor IXa-dependent conversion of factor X into Xa (27). Addition of various amounts of thrombin allows for determination of the thrombin sensitivity of both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII (Fig. 3). Inspection of the pattern obtained when 0.1 nM thrombin was used for activation of both proteins revealed that des-(868–1562)-factor VIII-HCII is more readily activated then des-(868–1562)-factor VIII at this particular concentration of thrombin (Fig. 3). A similar pattern is observed when 0.5 and 1.0 nM thrombin were used to activate the two different proteins (Fig. 3). These results provide evidence for an increased thrombin sensitivity of des-(868–1562)-factor VIII-HCII compared with des-(868–1562)-factor VIII.

In order to define the increased thrombin sensitivity in a quantitative manner, we have determined the second order rate constant of activation by thrombin for both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII using a previously established method (24). Factor Xa generation curves obtained at different concentrations of thrombin were used to calculate the first order rate constant of activation for both des-(868–1562)-factor VIII-HCII and des-(868–1562)-factor VIII. Secondary plots of the first order rate constants against the concentration of thrombin yielded a second order rate constant of activation for des-(868–1562)-factor VIII-HCII (12.0 ± 0.48 × 10^6 M^-1 s^-1) and des-(868–1562)-factor VIII (1.77 ± 0.21 × 10^6 M^-1 s^-1) (Fig. 4). The data obtained show that des-(868–1562)-factor VIII-HCII is approximately 7-fold more readily activated by thrombin than des-(868–1562)-factor VIII. Our results suggest that des-(868–1562)-factor VIII-HCII may more efficiently compete for the limited amounts of thrombin generated at a site of vascular injury than des-(868–1562)-factor VIII. This property may render des-(868–1562)-factor VIII more effective in treatment of patients with hemophilia A than currently available factor VIII preparations. Our study implies that exchange of acidic regions of other substrates of thrombin may be utilized to selectively modulate the action of this serine protease under physiological and pathophysiological conditions.

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