Characterization of des-(741–1668)-factor VIII, a single-chain factor VIII variant with a fusion site susceptible to proteolysis by thrombin and factor Xa

Marie-José S. H. DONATH,* Rozalia T. M. DE LAAF,* Pieter T. M. BIESSELS,† Peter J. LENTING,* Jan-Willem VAN DE LOO,* Jan A. VAN MOURIK,* Jan VOORBERG* and Koen MERTENS*‡

Departments of *Blood Coagulation and †Transfusion Technology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

A factor VIII variant has been characterized in which the heavy chain is directly fused to the light chain. Des-(741–1668)-factor VIII lacks the processing site at Arg¹⁶⁴⁸, as Arg⁷⁴⁰ of the heavy chain is fused to Ser¹⁶⁶⁹ of the light chain. The sequence of the fusion site is similar to that of other cleavage sites in factor VIII. The fusion site of des-(741–1668)-factor VIII was readily cleaved by both thrombin and factor Xa, and the same result was obtained for heavy chain cleavage. In contrast, des-(741–1668)-factor VIII cleavage by thrombin at position Arg¹⁶⁸⁹ proceeded at a lower rate than the analogous cleavage by factor Xa, which presumably takes place at position Arg¹⁷²¹. The rate of cleavage at position Arg¹⁶⁸⁹ by thrombin was also lower than that at the other processing sites. When des-(741–1668)-factor VIII was activated by thrombin, initial rates of factor Xa formation were similar to the rates obtained when plasma-derived factor VIII was activated by thrombin or factor Xa. Remarkably, activation of des-(741–1668)-factor VIII proceeded at a higher rate by factor Xa than by thrombin. These results indicate that factor VIII activation is strongly associated with cleavage at position

Arg¹⁶⁸⁹ or Arg¹⁷²¹. For the interaction between des-(741–1668)-factor VIII and von Willebrand factor, a K_d value of $(0.8 \pm 0.3) \times 10^{-10}$ M was determined, which is similar to that of heterodimeric factor VIII. The affinity of single-chain des-(741–1668)-factor VIII for factor IXa was found to be 27 ± 6 nM. The *in vivo* recovery and half-life of des-(741–1668)-factor VIII were assessed in guinea pigs. Upon infusion of des-(741–1668)-factor VIII at a dosage of 50 units/kg body weight, a rise of 1.0 ± 0.3 unit/ml in factor VIII activity was obtained. The same recovery was determined for wild-type factor VIII. The half-life of des-(741–1668)-factor VIII was found to be 3 ± 1 h, compared with 4 ± 2 h for heterodimeric recombinant factor VIII. In conclusion, des-(741–1668)-factor VIII displays normal activity, is readily cleaved by thrombin and factor Xa at its fusion site, binds with high affinity to von Willebrand factor and factor IXa, and behaves like heterodimeric recombinant factor VIII in guinea pigs. By virtue of these properties, des-(741–1668)-factor VIII may prove useful for the treatment of bleeding episodes in patients with haemophilia A.

INTRODUCTION

Dysfunction of blood coagulation factor VIII is associated with the bleeding disorder haemophilia A. In the intrinsic pathway of blood coagulation, factor VIII functions as cofactor for factor IXa and accelerates activation of factor X by 500-fold [1,2]. Factor VIII is synthesized as a single-chain protein consisting of 2332 amino acids organized in domains with the following sequence: A1-A2-B-A3-C1-C2 [3,4]. Although in plasma some factor VIII has been found to be single-chain [5], most of the pro-cofactor circulates as a heterodimeric species due to cleavage at position Arg¹⁶⁴⁸ by an as yet unidentified enzyme. The factor VIII heterodimer consists of a light chain (A3-C1-C2) and a variably sized heavy chain (A1-A2-B). The latter is heterogeneous due to limited proteolysis of the B domain [6–8].

In order to function as cofactor, the inactive pro-cofactor has to be dissociated from its physiological carrier protein, von Willebrand factor, and activated by thrombin or factor Xa [9,10]. These processes involve limited proteolysis in the heavy and light chains of factor VIII at specific cleavage sites [7,11]. Factor VIII light chain is cleaved by thrombin at position Arg¹⁶⁸⁹, which results in the release of 41 residues of its N-

terminus concomitant with loss of the high-affinity interaction of factor VIII with von Willebrand factor [12,13]. Proteolysis of factor VIII heavy chain occurs at positions Arg³⁷² and Arg⁷⁴⁰ [7,14]. Fully activated factor VIII consists of a heterotrimer of A1, A2 and A3-C1-C2 subunits. The B domain seems to be dispensable for factor VIII haemostatic function, as *in vivo* no difference was observed in activity between plasma factor VIII and recombinant factor VIII lacking major parts of the B domain [15,16].

Haemophilia A patients are treated by administration of factor VIII preparations derived from plasma or with recombinant factor VIII. In an attempt to improve the therapy, the development of alternative recombinant factor VIII species has gained increased attention. Deletion of the B domain was previously found to result in increased levels of production of recombinant factor VIII [17,18]. However, such a deletion immediately raises the question of the position at which the constitutive units of factor VIII are to be fused. A fusion site comprising a non-physiological amino acid sequence was found to induce immunogenicity in rabbits [19]. We evaluated a B-domainless factor VIII variant in which the fusion site represents the amino acid sequence Pro⁷³⁹-Arg⁷⁴⁰-Ser¹⁶⁶⁹-Val¹⁶⁷⁰. This site

Abbreviation used: HSA, human serum albumin.

‡ To whom correspondence should be addressed.

displays a strong resemblance to the sequence of various factor VIII activation cleavage sites. In this way, a single-chain species has been obtained that is not able to dissociate into separate components as previously observed for the relatively labile heterodimeric factor VIII [20,21]. We investigated des-(741–1668)-factor VIII *in vitro* with respect to proteolytic activation, interaction with its carrier protein von Willebrand factor, and binding to factor IXa. The recovery and half-life of des-(741–1668)-factor VIII were tested *in vivo* employing a guinea pig model.

MATERIALS AND METHODS

Production of des-(741–1668)-factor VIII

Construction of a bovine papilloma virus-derived expression plasmid containing cDNA encoding des-(741–1668)-factor VIII was described previously [factor VIII-del(741–1668) [21a]] [15]. DNA was co-transfected with a plasmid containing a hygromycin selection marker into C127 cells. Selection was at 200 µg/ml hygromycin B (Boehringer, Mannheim, Germany). Cells were maintained in a 4 litre fermenter (Celligen; New Brunswick Scientific, Nijmegen, The Netherlands) on Cytodex 2 micro-carriers (Pharmacia Bioprocess, Uppsala, Sweden). RPMI 1640 medium was supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml amphotericin B (Fungizone). These reagents were obtained from Gibco BRL, Breda, The Netherlands. Medium was harvested every day, filtered over a 0.2 µm hollow fibre cartridge (Plasmaflux P1; Fresenius, Bad Homburg, Germany) to remove cell debris, and concentrated approx. 10-fold employing a hollow fibre cartridge (Hemoflow F5; Fresenius). Benzamidine was added to a final concentration of 10 mM prior to freezing of the material in liquid nitrogen. Concentrate was stored at –20 °C.

Purification of des-(741–1668)-factor VIII

Des-(741–1668)-factor VIII was purified from concentrated medium by immuno-affinity chromatography employing anti-(factor VIII light chain) antibody CLB-CAG 117 [15]. The factor VIII variant was further purified by Q-Sepharose FF chromatography as described [22]. Des-(741–1668)-factor VIII was stored at –20 °C in 0.15 M NaCl, 10 mM CaCl₂, 55% (v/v) glycerol, 20 mM Tris (pH 7.5). Plasma factor VIII was purified from concentrate according to the same procedure. The specific activity of purified factor VIII ranged from 2 to 4 units/µg.

Characterization of factor VIII

Factor VIII activity was determined either by employing bovine coagulation factors and a synthetic substrate for factor Xa in a spectrophotometric assay (Coatest Factor VIII; Chromogenix, Mölndal, Sweden) or in a one-stage coagulation assay as described [23]. Proteins on immunoblots were visualized by a chemiluminescence technique (ECL; Amersham International). Proteolysis of factor VIII was measured using various combinations of monoclonal antibodies in enzyme-linked immunosorbent assays. These antibodies included the anti-(factor VIII light chain) antibodies CLB-CAG 12, CLB-CAG 69 and CLB-CAG 117, and the anti-(factor VIII heavy chain) antibody CLB-CAG 9 [24,25]. The polyclonal anti-(factor VIII heavy chain) antibody p53 was generously donated by Dr. D. S. Pepper (Scottish National Blood Transfusion Service, Edinburgh, Scotland, U.K.).

All enzyme-linked immunosorbent assays were performed according to the following procedure unless stated otherwise.

Antibody was coated on to microtitre plates (5 µg/ml; 100 µl/well) in 50 mM NaHCO₃ (pH 9.5) for at least 16 h at 4 °C. Wells were washed with 0.1 M NaCl, 0.1% (v/v) Tween, 50 mM Tris (pH 7.5) and remaining binding sites were blocked with wash buffer containing 1% (w/v) human serum albumin (HSA) for 2 h at 37 °C. Samples were diluted in 0.15 M NaCl, 0.1% (v/v) Tween, 1% (w/v) HSA, 50 mM Tris (pH 7.5) and incubated with the immobilized antibody for 2 h at 37 °C. The detection antibody was diluted in washing buffer and incubated in the wells for 1 h at 37 °C.

In order to determine cleavage of factor VIII heavy chain, CLB-CAG 9 was immobilized at a concentration of 5 µg/well. Samples and the detection antibody p53 were diluted in 0.4 M NaCl, 0.1% (v/v) Tween, 1% (w/v) HSA, 50 mM Tris (pH 7.5). A polyclonal anti-chicken antibody conjugated to peroxidase (Sigma, St. Louis, MO, U.S.A.) was used to detect the bound antibody. Factor VIII light chain cleavage was determined by immobilizing CLB-CAG 12 and employing peroxidase-conjugated CLB-CAG 69 to detect bound factor VIII light chain. Cleavage at the fusion site of des-(741–1668)-factor VIII was determined employing CLB-CAG 9 and peroxidase-conjugated CLB-CAG 69. The decay in factor VIII antigen was calculated relative to the concentration of factor VIII at $t = 0$.

Initial rates of factor Xa formation

Factor Xa formation by factor IXa in the presence of factor VIII, Ca²⁺ and phospholipids was determined as described previously [21,26]. Factor VIII was activated by addition of thrombin or factor Xa to the incubation mixture at $t = 0$. To prevent additional activation of factor VIII by generated factor Xa, acetylated factor X was used as substrate [27]. Subsamples of the incubation mixture were analysed for factor Xa concentration employing the synthetic substrate S2337 (Chromogenix AB, Mölndal, Sweden). Initial rates of factor Xa formation were calculated from at least three measurements between 0.5 and 3 min of incubation, during which period factor Xa formation was linear with time. Either thrombin or factor Xa was used to activate factor VIII. To prevent substrate hydrolysis by thrombin, this enzyme was inhibited by hirudin (1 unit per pmol of thrombin).

Factor VIII–factor IXa interaction

The affinity of des-(741–1668)-factor VIII for factor IXa was determined in an equilibrium binding assay essentially as described previously [22]. Briefly, anti-(factor VIII light chain) antibody CLB-CAG 12 was immobilized to microtitre plates (1 µg/well). Des-(741–1668)-factor VIII was added at a concentration of 14 nM. The concentration of bound factor VIII was found to be 7 ± 1 nM, as calculated from total and non-bound factor VIII quantified in the factor VIII light chain assay. To obtain factor IXa, factor IX was activated with factor XIa [28]. L-Glutamyl-glycyl-L-arginine chloromethyl ketone-inactivated factor IXa was incubated with immobilized factor VIII for 4 h at 37 °C. The amount of bound factor IXa was determined by employing a polyclonal antibody against factor IX, and dissociation constants were calculated assuming one single class of binding sites [22].

Von Willebrand factor binding studies

The interaction between factor VIII and recombinant von Willebrand factor was studied by employing a method essentially as described previously [15,29]. For these experiments, recombinant von Willebrand factor was produced and purified [22].

Briefly, wells of a microtitre plate were coated with 0.5 μ g of CLB-RAg 20 instead of CLB-RAg 41. Recombinant von Willebrand factor was added (0.1 μ g/well) and subsequently dilutions of purified des-(741–1668)-factor VIII were incubated. Bound factor VIII and von Willebrand factor were quantified and dissociation constants were determined. For calculation of the stoichiometry of von Willebrand factor monomers and des-(741–1668)-factor VIII, we assumed plasma concentrations of 10 μ g/ml and 0.1 μ g/ml, and molecular masses of 250 kDa and 160 kDa, respectively.

Animal studies

Guinea pigs weighing 300–350 g were anaesthetized with 0.5 ml/kg body weight Hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands) (10 mg of fluanison and 0.2 mg of fentanyl) and 10 mg/kg Nembutal (Sanofi, Maassluis, The Netherlands) intraperitoneally. Anaesthesia was maintained for the duration of the experiment by injections of Nembutal at 10 mg/kg when needed. Body temperature was maintained by placing the animals on a heating pad at 37 °C. A cannula was inserted into the carotid artery and access was maintained by infusion of heparin (5 units/ml) at a rate of 0.2 ml/h. After a period of approx. 10 min the compound of interest was injected as a bolus, and the cannula was flushed with saline. Blood samples of 250–300 μ l were withdrawn at desired time intervals using a two-syringe technique. The first 0.5 ml of blood was discarded and samples were collected into 25 μ l of 3.8 % (w/v) trisodium citrate solution. Blood samples were centrifuged for 2 min in an Eppendorf Microfuge; plasma was collected, frozen in liquid nitrogen and stored at –70 °C.

The recovery and half-life of des-(741–1668)-factor VIII were determined in guinea pigs. For this purpose, des-(741–1668)-factor VIII or control factor VIII (Recombinant; Baxter Hyland, Glendale, CA, U.S.A.) was injected at a dosage of 50 units/kg body weight. Blood samples were withdrawn before infusion and 5, 10, 15, 20, 30, 60, 120, 180, 270 and 360 min after infusion. Human heterodimeric factor VIII was detected in guinea pig plasma samples by employing an assay which discriminates between human and guinea pig factor VIII. This enzyme-linked immunosorbent assay for the detection of human heterodimer was performed as described above using CLB-CAG 9 and peroxidase-conjugated CLB-CAG 117.

RESULTS

Purification of des-(741–1668)-factor VIII

A single-chain recombinant factor VIII species has been constructed with a junction between the heavy and light chains of factor VIII by fusion of Arg⁷⁴⁰ to Ser¹⁶⁶⁹. The amino acid sequence of this site displays a strong resemblance to those of two other cleavage sites at positions Arg³⁷² and Arg¹⁶⁸⁹ that are important in factor VIII activation. The amino acid sequences of the cleavage sites are depicted in Figure 1. In des-(741–1668)-factor VIII the fusion of Arg⁷⁴⁰ to Ser¹⁶⁶⁹ results in replacement of two cleavage sites at positions Arg⁷⁴⁰ and Arg¹⁶⁴⁸ by one thrombin cleavage site.

Des-(741–1668)-factor VIII was immunopurified using an anti-(factor VIII light chain) monoclonal antibody. One protein band of expected mass (160 000 Da) was observed on SDS/PAGE (Figure 1, lane A). The single-chain nature of the factor VIII variant was confirmed by immunoblotting with a number of antibodies directed against either the heavy or the light chain (Figure 1, lanes B–E). No fragment of 80 000 Da, representing factor VIII light chain, was observed upon purification with the

anti-(light chain) antibody. Thus des-(741–1668)-factor VIII is isolated as a single-chain species. The recovery of des-(741–1668)-factor VIII was between 50 and 60 %, which is approx. 3-fold greater than that of plasma factor VIII employing the same immuno-affinity procedure (results not shown). This may be associated with the inherent improvement of stability of a single-chain protein over a heterodimeric species, which may dissociate during purification. The specific activity of purified des-(741–1668)-factor VIII was 2–4 units/ μ g. The cofactor activity of purified des-(741–1668)-factor VIII, as determined in a spectrophotometric assay, was similar to the procoagulant activity measured in a one-stage coagulation assay. These results are in agreement with those obtained previously for non-purified des-(741–1668)-factor VIII [15]. Thus des-(741–1668)-factor VIII is similar to plasma factor VIII in its ability to correct the clotting defect of haemophilic plasma.

Limited proteolysis of des-(741–1668)-factor VIII

Activation of factor VIII involves limited proteolysis at specific sites by either thrombin or factor Xa. These cleavages were investigated by employing defined combinations of antibodies with epitopes at appropriate positions. The epitopes are indicated by bars below the des-(741–1668)-factor VIII model in Figure 1. Cleavage rates of heavy and light chains, and at the fusion site of des-(741–1668)-factor VIII, were determined (see the Materials and methods section). Approx. 70 % of the heavy chain in des-(741–1668)-factor VIII was cleaved by thrombin after 2 min of incubation, whereas at least the same amount was cleaved by factor Xa (Figure 2a). Similar results were obtained with respect to cleavage of the fusion site by either thrombin or factor Xa (Figure 2b). One striking observation is that, with respect to the light chain of des-(741–1668)-factor VIII, thrombin and factor Xa display different cleavage rates (Figure 2c) (see also the Discussion section). The rate of light chain cleavage by thrombin was lower than that of both heavy chain and fusion site cleavage (Figure 2). This observation indicates that light chain cleavage is rate limiting when thrombin is used as activator. The finding that the cleavage rates of the light chain were dependent on the enzyme used raises the question of whether proteolysis of des-(741–1668)-factor VIII by thrombin or factor Xa results in differentially activated species. Furthermore, activation of des-(741–1668)-factor VIII has to be compared with that of plasma factor VIII to elucidate whether activation of the mutant by thrombin and by factor Xa is similar to the activation of plasma factor VIII.

Des-(741–1668)-factor VIII activation *in vitro*

Activation of des-(741–1668)-factor VIII was determined by measuring factor X activation in the presence of factor IXa, phospholipids and bivalent metal ions. Initial rates of factor X activation are dependent on the amount of activated factor VIII. Factor VIII was activated by adding various concentrations of thrombin and factor Xa at $t = 0$. When thrombin was used as activator, equal initial rates of factor Xa formation were obtained for des-(741–1668)-factor VIII and plasma factor VIII (Figure 3). Apparently, fusion of Arg⁷⁴⁰ to Ser¹⁶⁶⁹ does not affect the activation of factor VIII by thrombin. In contrast, rates of factor Xa formation were 3–10 times higher when factor Xa rather than thrombin was used as the activator of des-(741–1668)-factor VIII, indicating that des-(741–1668)-factor VIII is more readily activated by factor Xa than by thrombin. No difference was observed between the rates of activation of plasma factor VIII by thrombin and factor Xa. In all experiments initial rates of factor Xa formation did not increase further at activator concentrations

Figure 1 Design of des-(741–1668)-factor VIII

Des-(741–1668)-factor VIII is compared with plasma factor VIII in a bar model. Amino acid sequences surrounding thrombin cleavage sites are indicated in single-letter code. The numbering of the cleavage sites refers to the arginine (R) residues. The fusion site of des-(741–1668)-factor VIII comprises the amino acids P, R, S and V, which correspond to positions 739, 740, 1669 and 1670 respectively in plasma factor VIII. After purification, des-(741–1668)-factor VIII was subjected to SDS/PAGE followed by silver staining (lane A) or immunoblotting. Single-chain factor VIII was detected employing anti-(heavy chain) antibodies p53 (lane B) and CLB-CaG 9 (lane C) and a monoclonal antibody, CLB-CaG 69 (lane D), or a polyclonal antibody (lane E) against factor VIII light chain (see the Materials and methods section). Epitopes of antibodies which were employed to determine cleavage kinetics (see Figure 2) are indicated by thin bars. CLB-CaG 12 recognizes the factor VIII light chain between Ala¹⁷²² and Tyr²³³².

Figure 2 Proteolysis of des-(741–1668)-factor VIII by thrombin and factor Xa

Des-(741–1668)-factor VIII (0.4 nM) was incubated with phospholipid vesicles (100 μ M) and thrombin (2 nM) (●) or factor Xa (2 nM) (○) in 150 mM NaCl, 10 mM CaCl₂, 50 mM Tris (pH 7.5), and cleavage was analysed at the indicated time points. Factor VIII heavy chain (a) was detected by employing CLB-CaG 9 and p53 in an enzyme-linked immunosorbent assay. Proteolysis at the fusion site (b) was analysed similarly by employing CLB-CaG 9 and CLB-CaG 69, and cleavage in the light chain (c) was assessed using CLB-CaG 12 and CLB-CaG 69. The epitopes of these antibodies are indicated in Figure 1 and the immunological assays are described in the Materials and methods section. Data represent the means ($\pm$ S.D.) of three experiments.

Figure 3 Initial rates of factor X activation

Factor Xa formation was determined by employing purified human coagulation factors in 10 mM CaCl_2 , 150 mM NaCl, 5% (v/v) glycerin and 50 mM Tris (pH 7.5). Phospholipids (100 μM) were incubated with Ca^{2+} ions in 60% of the final volume prior to the addition of coagulation factors in the following order: factor IXa (0.3 nM), des-(741–1668)-factor VIII (●, ○) or plasma factor VIII (▲, △) (0.4 nM), acetylated factor X (0.2 μM), and various concentrations of thrombin (●, ▲) or factor Xa (○, △). Subsamples were drawn every 30 s and the factor Xa concentration was determined with the substrate S2337. Initial rates of factor Xa formation were calculated from at least three subsequent measurements of factor Xa. Data represent the means ($\pm$ S.D.) of at least three experiments.

above 5–10 nM (Figure 3). Furthermore, the total concentration of factor Xa obtained in factor Xa formation experiments was equal under all conditions (results not shown). This indicates that des-(741–1668)-factor VIII when complexed with factor IXa is effective in factor Xa formation independent of the enzyme used to activate factor VIII. In conclusion, des-(741–1668)-factor VIII differs from plasma factor VIII with respect to the increased rate of activation by factor Xa. This acceleration of des-(741–1668)-factor VIII activation is correlated with the increased rate of light chain cleavage by factor Xa.

Interaction of des-(741–1668)-factor VIII with factor IXa

Prior to the assessment of des-(741–1668)-factor VIII function *in vivo*, we investigated some characteristics that are important for factor VIII function, such as binding to the physiological carrier protein von Willebrand factor and to the enzyme in intrinsic factor Xa formation, i.e. factor IXa. The affinity of des-(741–1668)-factor VIII for factor IXa was determined in an equilibrium binding assay (see the Materials and methods section). Factor IXa was added at various concentrations to immobilized des-(741–1668)-factor VIII and after 4 h of incubation the amount of bound factor IXa was determined. From these experiments, we obtained a K_d value of 27 ± 6 nM (mean $\pm$ S.D. of three experiments) for the interaction between des-(741–1668)-factor VIII and factor IXa. This affinity is close to that obtained previously for the interaction between factor IXa and the light chain of factor VIII (15 ± 3 nM) [22].

Interaction of des-(741–1668)-factor VIII with von Willebrand factor

The acidic region at the N-terminus of factor VIII light chain is important in maintaining a high-affinity interaction of factor VIII with von Willebrand factor. In des-(741–1668)-factor VIII, the N-terminal 20 amino acid residues of the light chain have been deleted, and the heavy and light chains are directly fused,

Figure 4 Interaction of des-(741–1668)-factor VIII with von Willebrand factor

Recombinant von Willebrand factor (0.1 μg /well) was immobilized to the monoclonal antibody CLB-Rag 20 (0.5 μg /well). The amount of bound von Willebrand factor was determined to be 24 m-units/well. Various concentrations of des-(741–1668)-factor VIII were incubated and the amount of bound factor VIII was determined as described in the Materials and methods section. The results of one typical experiment are shown. The inset shows the Scatchard analysis of the presented data. For this experiment a stoichiometry of 1 mol of factor VIII per 40 mol of von Willebrand factor monomers was calculated (see the Materials and methods section). A K_d value of $(0.8 \pm 0.3) \times 10^{-10}$ M was derived from three individual experiments for the interaction between von Willebrand factor and des-(741–1668)-factor VIII.

which might affect the interaction with von Willebrand factor. Figure 4 shows the results of an equilibrium binding assay employing recombinant von Willebrand factor and des-(741–1668)-factor VIII. A K_d value of $(0.8 \pm 0.3) \times 10^{-10}$ M was obtained for this interaction. This value is identical to those of both plasma factor VIII and heterodimeric factor VIII mutants [15]. In the experiment of Figure 4, the amount of bound von Willebrand factor was determined to be 24 m-units/well. The maximal amount of des-(741–1668)-factor VIII bound (4.2 m-units/well) was obtained from the intercept of the x-axis of the Scatchard plot. The stoichiometry was calculated to be approx. 1:40 for the des-(741–1668)-factor VIII–von Willebrand factor interaction, which is similar to that for the interaction between plasma factor VIII and recombinant von Willebrand factor (see [29]). We therefore conclude that the affinity of factor VIII for von Willebrand factor is not affected by deleting half of the acidic region of the factor VIII light chain, and thereby fusing the heavy and light chains.

Pharmacokinetic studies

In order to assess the pharmacokinetic parameters of des-(741–1668)-factor VIII, a number of laboratory animals were investigated. Various monoclonal antibodies were tested for their species-specificity. We could detect human factor VIII heterodimer and light chain antigen without interference by the endogenous factor VIII from the plasma of guinea pigs. Employing the guinea pig as animal model, des-(741–1668)-factor VIII and wild-type factor VIII were investigated with respect to *in vivo* recovery and survival. Des-(741–1668)-factor VIII was infused at a dosage of 50 units/kg body weight, which resulted in an increase in factor VIII activity of 1.0 ± 0.3 unit/ml (Table 1). Equal values were obtained for the recovery of des-(741–1668)-factor VIII and recombinant heterodimeric factor VIII. Subse-

Table 1 Recovery and half-life of des-(741–1668)-factor VIII in the guinea pig

Recoveries were calculated from the increase in factor VIII activity 5 min after infusion of either des-(741–1668)-factor VIII or wild-type factor VIII at a dosage of 50 units/kg body weight. Factor VIII activity was measured in a spectrophotometric assay. The half-life of factor VIII antigen was estimated by regression analysis of a semi-logarithmic plot of factor VIII antigen levels between 1 and 6 h after infusion. Antigen was measured as factor VIII heterodimer as described in the Materials and methods section. All values are means $\pm$ S.D. of three experiments.

	Recovery (activity rise) (units/ml)	Half-life (h)
Des-(741–1668)-factor VIII	1.0 $\pm$ 0.3	3 $\pm$ 1
Recombinant wild-type factor VIII	1.1 $\pm$ 0.4	4 $\pm$ 2

quently the decay of human factor VIII antigen was determined, and the half-life of des-(741–1668)-factor VIII was found to be 3 ± 1 h. A similar half-life was determined for a heterodimeric recombinant factor VIII species (Recombinant), which served as control (Table 1). This indicates that des-(741–1668)-factor VIII is similar to wild-type factor VIII with respect to both recovery and half-life.

DISCUSSION

In the present study we have analysed the functional characteristics of des-(741–1668)-factor VIII *in vitro* and *in vivo*. In this single-chain factor VIII species residue Arg⁷⁴⁰ of the heavy chain was fused to Ser¹⁶⁶⁹ of the light chain. By constructing a single-chain species lacking the natural endoproteolytic cleavage site at Arg¹⁶⁴⁸-Glu¹⁶⁴⁹, dissociation of factor VIII subunits is prevented. The B-domain of factor VIII was deleted as it is dispensable for factor VIII function *in vitro* [17,18,30] and *in vivo* [15,16]. The target residue for fusion of the light chain to the heavy chain of des-(741–1668)-factor VIII was chosen as amino acid Ser¹⁶⁶⁹ for various reasons, as follows. (1) This residue is located between two hydrophilic segments, both containing a sulphated tyrosine residue (1664 and 1680). Fusion at this position therefore ensures that one sulphated-tyrosine-containing segment remains intact in des-(741–1668)-factor VIII. (2) The presence of the sulphated tyrosine at position 1680 is required for the high-affinity interaction with von Willebrand factor [29]. Therefore this residue has to be present in the factor VIII variant. (3) The fusion site of des-(741–1668)-factor VIII displays an amino acid sequence which is very similar to that of other activation cleavage sites in factor VIII (Figure 1). Thus it is highly unlikely that endoproteolytic processing will occur at this fusion site. Cleavage of des-(741–1668)-factor VIII was found to proceed efficiently in its heavy chain and light chain, and at the fusion site (Figure 2). Consequently, proteolytic activation of des-(741–1668)-factor VIII does not result in the formation of fragments that contain the intact fusion site sequence as a potential source of immunological response [19].

Light chain cleavage of factor VIII appears to be associated with factor VIII activation, since the difference in the rates of cleavage of the light chain of des-(741–1668)-factor VIII by thrombin and factor Xa (Figure 2) is reflected by a similar difference in factor X activation rates (Figure 3). Thus light chain cleavage is associated with factor VIII activation. This finding may seem to be in conflict with previous studies suggesting that heavy chain cleavage is sufficient for factor VIII activation [31]. The apparent discrepancy may be explained by the observation

that cleavage of the heavy chain alone results in a factor VIII species with reduced activity compared with thrombin-activated, completely cleaved factor VIII [31]. Furthermore, cleavage of the light chain alone is found to partially activate factor VIII [21]. In plasma-derived factor VIII, the position of light chain cleavage by thrombin, Arg¹⁶⁸⁹, appears to be different from that by factor Xa, Arg¹⁷²¹ [21]. Although we did not verify the precise positions of cleavage in des-(741–1668)-factor VIII, it seems reasonable to suppose that thrombin and factor Xa cleave at different positions. Therefore one explanation for the observed higher rate of light chain cleavage of des-(741–1668)-factor VIII by factor Xa might be that the cleavage site at Arg¹⁷²¹ is better exposed in des-(741–1668)-factor VIII than in plasma factor VIII. This might be due to attachment of factor VIII heavy chain to light chain, or to deletion of the region Glu¹⁶⁴⁹-Ile¹⁶⁶⁸. Previously, sulphation of specific tyrosine residues has been reported to be required for optimal factor VIII activity [32]. The deleted region Glu¹⁶⁴⁹-Ile¹⁶⁶⁸ in des-(741–1668)-factor VIII contains such a residue at position 1664. Our data indicate that this tyrosine residue is dispensable for proteolytic activation of factor VIII (Figures 2 and 3).

Sulphation of the tyrosine residue at position 1680 is required for the high-affinity interaction of factor VIII with von Willebrand factor. The dissociation constant determined for the interaction between des-(741–1668)-factor VIII and recombinant von Willebrand factor was indistinguishable from that obtained with heterodimeric factor VIII (Figure 4). This observation is important for the interpretation of des-(741–1668)-factor VIII function *in vivo*, as the half-life of factor VIII is dependent on its interaction with von Willebrand factor [15,33]. Furthermore, von Willebrand factor is known to interfere in the factor VIII light chain-factor IXa interaction [22]. A K_d value of approx. 15 nM has been derived for the affinities of both the factor VIII light chain and the factor VIII heterodimer for factor IXa [22]. In plasma, factor VIII is associated with von Willebrand factor with a 100-fold higher affinity than that for factor IXa. Thus cleavage of the factor VIII light chain is required to release von Willebrand factor from factor VIII and for the exposure of the factor IXa binding site within the A3 domain. The interaction between des-(741–1668)-factor VIII and factor IXa is characterized by a K_d value of 27 nM, which is slightly higher than that of the factor VIII light chain-factor IXa interaction. In plasma this minor difference is insignificant, since des-(741–1668)-factor VIII binds with high affinity to von Willebrand factor (Figure 4). Consequently, cleavage at position Arg¹⁶⁸⁹ or Arg¹⁷²¹ is required for the exposure of the factor IXa binding site. Moreover, the resulting factor IXa binding fragments of des-(741–1668)-factor VIII are identical to those of plasma-derived factor VIII, since the affinity of the cleaved factor VIII light chain fragments is equal to that of intact light chain [21]. In agreement with this notion, we observed that des-(741–1668)-factor VIII functions similarly to wild-type factor VIII in factor X activation studies at approximately stoichiometric concentrations of factor VIII and factor IXa (Figure 3). These results indicate that the interaction between activated des-(741–1668)-factor VIII and factor IXa is indeed normal. Therefore *in vitro* data suggest that des-(741–1668)-factor VIII should function appropriately *in vivo*.

In the present study, factor VIII function has been investigated *in vivo* in a guinea pig model which was previously used to investigate the pharmacokinetics of Protein C and Protein S [34]. The results of our studies indicate that the recovery and half-lives of both des-(741–1668)-factor VIII and heterodimeric recombinant factor VIII are similar (Table 1). The half-life of factor VIII in guinea pigs is 3 h, which is shorter than that observed in humans (approx. 14 h) [35] or in haemophilic dogs (8–14 h) [15,16,33]. The same difference was found for Protein C and

Protein S in guinea pigs (2 and 4 h respectively) [34] compared with humans (8–10 h) [36] and for factor IX in mice (2.5 h) [37] compared with humans (20 h) [38]. We conclude that the relatively short half-life of des-(741–1668)-factor VIII in guinea pigs is to be explained by the experimental model itself.

Various recombinant factor VIII species have previously been investigated employing haemophilic dogs [15,16,39,40]. However, some major differences exist among these factor VIII species. Three of the previously described variants involve mainly heterodimeric species [15,16,40], in contrast to the single-chain des-(741–1668)-factor VIII. A previous study describes a single-chain species, factor VIII-ΔII [39], which contains non-physiological sequences that are potential sources of an immunological response [19]. The fusion site of factor VIII-ΔII comprises the sequence Pro⁷⁷¹–Asp¹⁶⁶⁶, whereas in des-(741–1668)-factor VIII Arg⁷⁴⁰ is fused to Ser¹⁶⁶⁹ resulting in a naturally occurring factor VIII sequence that is processed appropriately upon activation. One functional difference exists between these single-chain species in that des-(741–1668)-factor VIII displays an increased sensitivity to activation by factor Xa compared with wild-type factor VIII (Figure 3). Whether or not this characteristic of des-(741–1668)-factor VIII may be advantageous remains to be investigated. In conclusion, the single-chain des-(741–1668)-factor VIII seems to represent a promising agent for therapeutic application.

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REFERENCES

- van Dieijen, G., Tans, G., Rosing, J. and Hemker, H. C. (1981) *J. Biol. Chem.* **256**, 3433–3442
- Mertens, K., Van Wijngaarden, A. and Bertina, R. M. (1985) *Thromb. Haemostasis* **54**, 654–660
- Toole, J. J., Knopf, J. L., Wozney, J. M. et al. (1984) *Nature (London)* **312**, 342–347
- Vehar, G. A., Keyt, B., Eaton, D. et al. (1984) *Nature (London)* **312**, 337–342
- Rotblat, F., O'Brien, D. P., O'Brien, F. J., Goodall, A. and Tuddenham, E. G. D. (1985) *Biochemistry* **24**, 4294–4300
- Andersson, L. O., Forsman, N., Huang, K. et al. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2979–2983
- Eaton, D., Rodriguez, H. and Vehar, G. A. (1986) *Biochemistry* **25**, 505–512
- Fay, P. J., Anderson, M. T., Chavin, S. I. and Marder, V. J. (1986) *Biochim. Biophys. Acta* **871**, 268–278
- Kaufman, R. J. (1992) *Annu. Rev. Med.* **43**, 325–339
- Fay, P. J. (1993) *Thromb. Haemostasis* **70**, 63–70
- Pittman, D. D. and Kaufman, R. J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2429–2433
- Lollar, P., Hill-Eubanks, D. C. and Parker, C. G. (1988) *J. Biol. Chem.* **263**, 10451–10455
- Leyte, A., Verbeet, M. P., Brodniewicz-Proba, T., van Mourik, J. A. and Mertens, K. (1989) *Biochem. J.* **257**, 679–683
- Lollar, P. and Parker, C. G. (1989) *Biochemistry* **28**, 666–674
- Mertens, K., Donath, M.-J. S. H., Van Leen, R. W. et al. (1993) *Br. J. Haematol.* **85**, 133–142
- Pittman, D. D., Alderman, E. M., Tomkinson, K. N., Wang, J. H., Giles, A. R. and Kaufman, R. J. (1993) *Blood* **81**, 2925–2935
- Toole, J. J., Pittman, D. D., Orr, E. C., Murtha, P., Wasley, L. C. and Kaufman, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5939–5942
- Eaton, D. L., Wood, W. I., Eaton, D. et al. (1986) *Biochemistry* **25**, 8343–8347
- Esmon, P. C., Harg, S. K. and Fournel, M. A. (1990) *Blood* **76**, 1593–1600
- Kaufman, R. J., Wasley, L. C. and Dorner, A. J. (1988) *J. Biol. Chem.* **263**, 6352–6362
- Donath, M.-J. S. H., Lenting, P. J., van Mourik, J. A. and Mertens, K. (1995) *J. Biol. Chem.* **270**, 3648–3655
- Pannekoek, H., Lijnen, H. R. and Loskutoff, D. J. (1990) *Thromb. Haemostasis* **64**, 600–603
- Lenting, P. J., Donath, M.-J. S. H., van Mourik, J. A. and Mertens, K. (1994) *J. Biol. Chem.* **269**, 7150–7155
- Veltkamp, J. J., Orion, E. F. and Loelliger, E. A. (1968) *Thromb. Diath. Haemorrh.* **19**, 279–303
- Stel, H. V., Sakariassen, K. S., Scholte, B. J. et al. (1984) *Blood* **63**, 1408–1415
- Leyte, A., Mertens, K., Distel, B. et al. (1989) *Biochem. J.* **263**, 187–194
- Mertens, K. and Bertina, R. M. (1984) *Biochem. J.* **223**, 607–615
- Neuenschwander, P. and Jesty, J. (1990) *Anal. Biochem.* **184**, 347–352
- Mertens, K. and Bertina, R. M. (1982) *Thromb. Haemostasis* **47**, 96–100
- Leyte, A., Voorberg, J., Van Schijndel, H. B., Duim, B., Pannekoek, H. and van Mourik, J. A. (1991) *Biochem. J.* **274**, 257–261
- Salver, N., Ricca, G. A., Link, J., Nathan, M. H., Newman, J. and Drohan, W. N. (1987) *DNA* **6**, 553–564
- Hill-Eubanks, D. C., Parker, C. G. and Lollar, P. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6508–6512
- Michnick, D. A., Pittman, D. D., Wise, R. J. and Kaufman, R. J. (1994) *J. Biol. Chem.* **269**, 20095–20102
- Brinkhous, K. M., Sandberg, H., Garriss, J. B., Mattsson, C., Palm, M., Griggs, T. and Read, M. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8752–8756
- Berger, J. R. H., Kirstein, C. G. and Orthner, C. L. (1991) *Blood* **77**, 2174–2184
- Harrison, J. F. M., Bloom, A. L. and Abildgaard, C. F. (1991) *Semin. Hematol.* **28**, suppl. 1, 29–35
- Okajima, K., Koga, S., Kaji, M. et al. (1990) *Thromb. Haemostasis* **63**, 48–53
- Fuchs, H. E., Trapp, H. G., Griffith, M. J., Roberts, H. R. and Pizzo, S. V. (1984) *J. Clin. Invest.* **73**, 1696–1703
- Comp, P. C. (1990) In *Hematology* (Williams, W. J., Beutler, E., Erslev, A. J. and Lichtman, M. A., eds.), pp. 1290–1294, McGraw-Hill, New York
- Meulien, P., Faure, T., Mischler, F. et al. (1988) *Protein Eng.* **2**, 301–306
- Brinkhous, K. M., Widlund, L., Read, M. S., Sigman, J. L., Oswaldsson, U., Brandt, J. and Sandberg, H. (1993) *Thromb. Haemostasis* **69**, 852 (abstr. 1112)