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Construction and Characterization of an Active Factor VIII Variant Lacking the Central One-Third of the Molecule

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ABSTRACT: The primary structure of factor VIII consists of 2332 amino acids that exhibit 3 distinct structural domains, including a triplicated region (A domains), a unique region of 909 amino acids (B domain), and a carboxy-terminal duplicated region (C domains), that are arranged in the order A1-A2-B-A3-C1-C2. The B domain (residues 741–1648) of factor VIII is lost when factor VIII is activated by thrombin, which proteolytically processes factor VIII to active subunits of M_r 50000 (domain A1), 43000 (domain A2), and 73000 (domains A3-C1-C2). To determine if the B domain is required for factor VIII coagulant activity, a variant was constructed by using recombinant DNA techniques in which residues 797–1562 were eliminated. This shortened the B domain from 909 to 142 amino acids. This variant factor $VIII_{des\text{-}797\text{–}1652}$ was expressed in mammalian cells and was found to be functional. The factor $VIII_{des\text{-}797\text{–}1562}$ protein was purified and shown to be processed by thrombin in the same manner as full-length factor VIII. The factor $VIII_{des\text{-}797\text{–}1562}$ variant also bound to von Willebrand factor (vWF) immobilized on Sepharose. These results indicate that most of the highly glycosylated B domain of factor VIII is not required for the expression of factor VIII coagulant activity and its interaction with vWF.

Factor VIII functions in the middle of the coagulation cascade as a cofactor for the activation of factor X by factor IXa (Jackson & Nemerson, 1980). Complete cDNA clones for human factor VIII have now been obtained and, along with protein characterization studies, have elucidated the structure of factor VIII (Wood et al., 1984; Toole et al., 1984; Fass et al., 1982; Fulcher & Zimmerman, 1982; Vehar & Davie, 1980; Rotblat et al., 1985; Eaton et al., 1986a). The factor VIII gene codes for a single-chain protein (M_r ~300000) consisting of 2332 amino acids including 25 potential asparagine-linked glycosylation sites (Vehar et al., 1984). Analysis of the factor VIII sequence revealed 3 distinct domains, including a triplicated domain of ~330 amino acids (A domains), a unique region of 909 amino acids containing 19 asparagine-linked glycosylation sites (the B domain), and a carboxy-terminal duplicated domain of ~150 amino acids, which are arranged in the order A1-A2-B-A3-C1-C2 (Figure 1; Vehar et al., 1984). The single-chain form of factor VIII is readily proteolyzed in vitro and in vivo to multiple polypeptides having molecular weights ranging from 80000 to 210000 (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Weinstein et al., 1983; Eaton et al., 1986a; Figure 1). Amino-terminal sequence analysis shows that the M_r 210000 and 80000 proteins represent the amino- and carboxy-terminal regions of factor VIII, respectively (Vehar et al., 1984; Eaton et al., 1986a; Figure 1). Proteolysis within the carboxy-terminal region of the M_r 210000 protein yields a series of proteins of M_r 90000–180000 (Toole et al., 1984; Vehar et al., 1984; Eaton et al., 1986a,b). The M_r 80000 protein appears to form a metal (perhaps Ca^{2+}) linked complex with each of the M_r 90000–210000 proteins. Neither the M_r 80000 protein nor the M_r 90000–210000 proteins have coagulant activity when separated (Eaton et al., 1986b).

The single-chain form and also the above-mentioned multiple-polypeptide form of factor VIII are proteolytically processed by thrombin to an active form (Fass et al., 1982; Fulcher et al., 1983; Eaton et al., 1986a) consisting of subunits with M_r 50000 (domain A1), 43000 (domain A2), and 73000 (domains A3-C1-C2) (Fass et al., 1982; Eaton et al., 1986a; Figure 1). Between these functional regions of factor VIII is the highly glycosylated B domain (residues 742–1649) that appears to be proteolytically removed when factor VIII is activated. This suggests that this domain may not be required for factor VIII coagulant activity. To test this hypothesis, we

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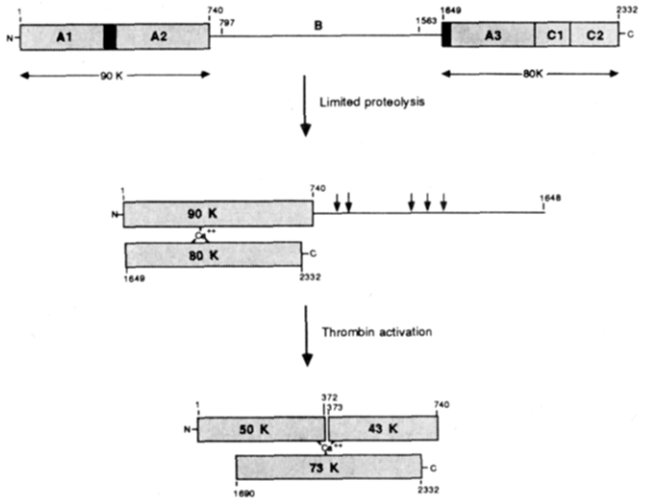

FIGURE 1: Proteolytic processing of factor VIII. Cleavage sites shown are taken from Eaton et al. (1986a) and Toole et al. (1984). Residues 797–1562 are removed in factor $VIII_{des\text{-}797\text{-}1562}$.

used recombinant DNA techniques to construct a factor VIII variant (factor $VIII_{des\text{-}797\text{-}1562}$ in which most of the B domain is removed. When this variant is expressed in mammalian cells, factor VIII activity is found in the culture medium, demonstrating that the highly glycosylated B domain is not needed for activity. Purification and characterization of the variant show that it is functionally similar to full-length factor VIII.

Materials and Methods

Factor VIII deficient and normal human plasmas were from George King Biomedical; platelin was from General Diagnostics; factor VIII chromogenic Coatest assay was from Helena; human α-thrombin was from Sigma Chemical Co. The plasmid pUC8 containing the CMV enhancer, promoter, and splice donor was obtained from Dr. B. Fleckenstein (Nurnberg, West Germany). DMEM and Hams F12 media were obtained from Flow Laboratories; Geneticin was from Gibco; Affigel-10 was from Bio-Rad.

Factor $VIII_{des\text{-}797\text{-}1562}$ Fusion Expression Plasmid. A deletion of the B domain of factor VIII cDNA (Wood et al., 1984) sequence was made by fusing the *Tth*111 I site at amino acid 796 to the *Bam*HI site at amino acid 1563 after filling both sites with DNA polymerase I. The expected junction was confirmed by DNA sequence analysis. This fusion construction deletes amino acids 797–1562 of the B domain. This factor VIII variant cDNA was expressed in a pML (Lusky & Botchan, 1981) vector containing transcriptional control region of the human cytomegalovirus (CMV). This 5′ control region includes the CMV enhancer, promoter, and splice donor sequence (Boshart et al., 1985; Thomsen et al., 1984; Sternberg et al., 1984). To complete the intron and provide a splice acceptor site, we synthesized a 99 base pair (bp) oligomer containing the sequence of the splice acceptor of the Ig variable region (Bothwell et al., 1981). The variant cDNA is followed by the poly(A) addition site and the transcription terminator of the early region of SV40 (Fiers et al., 1978). This plasmid is designated pF8CIS9080.

Transfection and Cell Culture. Mammalian kidney cells were cotransfected with pF8CIS9080 and pRSVneo (Gorman et al., 1983) by the calcium phosphate precipitation method (Graham & van der Eb, 1973). Two 60-mm dishes of cells were transfected with 1 μg of pF8CIS9080, 0.5 μg of pRSVneo, and 3.5 μg of salmon sperm DNA each. Forty eight hours later, cells were either assayed for transient expression

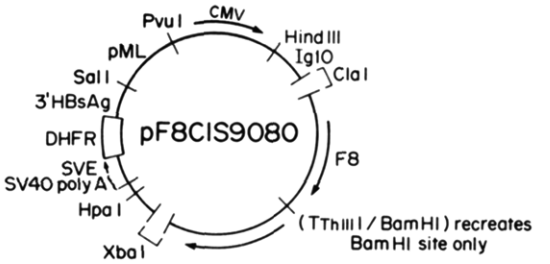

FIGURE 2: pF8CIS9080 expression plasmid. The vector diagram includes the Amp^R gene and procaryotic origin of replication from pML; the immediate early gene enhancer, promoter, and splice donor from the human cytomegalovirus; and an immunoglobin splice acceptor and the sequence for factor $VIII_{des\text{-}797\text{-}1562}$ flanked by the SV40 poly(A) addition site. Also included in this plasmid is the SV40-dhfr transcription unit (Kaufman & Sharp, 1982; Simonsen & Levinson, 1983).

by immunoperoxidase staining using a factor VIII monoclonal antibody (Wood et al., 1984; Gorman et al., 1985) or subcultured into 400 μg/mL Geneticin. Clones were subsequently pooled to give a mass population of cells. Expression of factor VIII from this new cell line, 90/80 cells, was determined by immunostaining of cells and assay of culture medium for factor VIII coagulant activity. Cells were cultured in an F12/DMEM mix containing 7% fetal calf serum. To obtain serum-free medium containing the factor $VIII_{des\text{-}797\text{-}1562}$ variant, the cells were cultured in F12/DMEM medium without serum for 48 h.

Purification and Characterization of the Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. The factor VIII variant was purified by using DEAE-Sepharose and a factor VIII monoclonal antibody column as described for recombinant full-length factor VIII (Eaton et al., 1986b). The antibody (C7F7) reacts with the M_r 80000 protein of factor VIII (Wood et al., 1984). Factor VIII activity was assayed by coagulation analysis and by the factor VIII Coatest assay (Wood et al., 1984). Protein determinations were done by the method of Bradford (1976). For amino-terminal sequencing, the factor $VIII_{des\text{-}797\text{-}1562}$ variant (0.2–0.5 mg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (6–12%) (Laemmli, 1970), and proteins were detected by staining with Coomassie blue. Proteins were then gel-eluted (Hunkapiller et al., 1983) and sequenced by using an Applied Biosystems vapor phase sequencer (Hewick et al., 1982).

Results

Expression of the Functional Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. The eukaryotic expression vector used to express factor $VIII_{des\text{-}797\text{-}1562}$, as described under Materials and Methods, is shown in Figure 2. The protein coding region lacks amino acid residues 797–1562 of the factor VIII protein (Figure 1). This expreession plasmid was used to transfect mammalian kidney cells, and factor VIII expression was monitored by immunoperoxidase staining (Gorman et al., 1985) and by coagulation analysis. As shown in Table I, serum-free medium obtained from 90/80 cells shortens the coagulation time of hemophilic plasma, while medium from the parent cell line did not. This activity was inhibited by a polyclonal antibody raised against purified plasma-derived factor VIII. Factor VIII was also detected by immunostaining of 90/80 cells using a factor VIII monoclonal antibody (C7F7) Wood et al., 1984) (data not shown).

Characterization of the Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. Analysis of purified factor $VIII_{des\text{-}797\text{-}1562}$ by SDS–PAGE shows that it consists primarily of two bands of M_r 80000 and 115000 (Figure 3). A small amount of the single-chain fusion having an M_r of ~200000 was also observed by SDS–PAGE.

Table I: Coagulant Activity of 90/80 Medium[a]

sample	clot time (s)
90/80 medium	58.9
90/80 medium preincubated with factor VIII polyclonal antibody	101.1
buffer	109.2
parent cell line medium	102.3

[a] 90/80 cells or the parent cell line was cultured in serum-free medium for 48 h, at which time it was harvested. The medium was diluted with 0.05 M Tris, pH 7.4, containing 0.01% BSA and assayed by coagulation analysis. For antibody neutralization, 90/80 medium (1 mL) was preincubated with 10 μg of a factor VIII polyclonal antibody for 45 min at 37 °C and subsequently assayed.

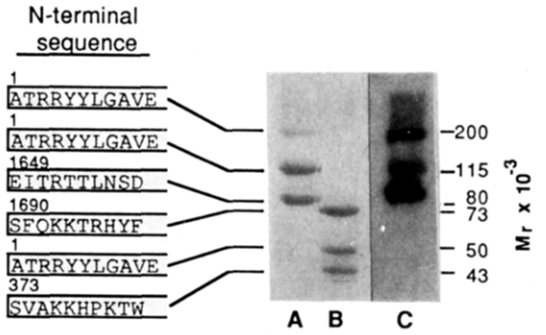

FIGURE 3: SDS–PAGE and Western blot analysis of factor $VIII_{des\text{-}797\text{-}1562}$. 15 μg of the variant (A) and 15 μg of the variant pretreated with 0.15 unit (50 ng) of thrombin (B) were resolved on a 6–12% SDS–polyacrylamide gel, and proteins were detected by staining with Coomassie blue. After SDS–PAGE, factor $VIII_{des\text{-}797\text{-}1562}$ was also transferred to nitrocellulose for Western blot analysis (Towbin et al., 1979) (C). A polyclonal antibody against plasma-derived factor VIII was used to detect proteins transferred to nitrocellulose. For amino-terminal sequencing, 0.5 mg of factor $VIII_{des\text{-}797\text{-}1562}$ or 0.5 mg of factor $VIII_{des\text{-}797\text{-}1562}$ incubated with 5 units (1.7 μg) of thrombin for 10 min at 37 °C and proteins were isolated and sequenced as described under Materials and Methods. The amino acid numbering shown is that for full-length factor VIII (Eaton et al., 1986a).

The M_r 200 000, 115 000, and 80 000 proteins were all detected by a factor VIII polyclonal antibody by Western blot analysis (Figure 3). Amino-terminal sequencing shows that the M_r 115 000 and 80 000 proteins represent the amino- and carboxy-terminal regions of the M_r 200 000 single-chain protein, respectively (Figures 1 and 3). The amino-terminal sequence obtained for the M_r 200 000 single-chain protein is identical with the DNA-predicted amino-terminal sequence of factor VIII (Wood et al., 1984). The sequence obtained from the M_r 80 000 protein is also identical with the amino-terminal sequence obtained from the M_r 80 000 protein of plasma-derived factor VIII (Figure 3; Eaton et al., 1986a).

The specific activity of purified factor $VIII_{des\text{-}797\text{-}1562}$ was found to be 4000–6000 units/mg. This is comparable to the specific activity of unactivated plasma-derived factor VIII (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Eaton et al., 1986a). Treatment of factor $VIII_{des\text{-}797\text{-}1562}$ with thrombin resulted in a 15–20-fold activation of coagulant activity (Figure 4). This activation was correlated with the cleavage of the M_r 200 000, 115 000, and 80 000 proteins to subunits with M_r 50 000, 43 000, and 73 000 (Figures 3 and 4). Initially, the M_r 200 000 protein appears to be cleaved to the M_r 115 000, 90 000, and 80 000 proteins. Proteolysis within the carboxy terminus of the M_r 115 000 protein removes the shortened B domain, generating the M_r 90 000 protein, which is subsequently cleaved at arginine-372 to generate the M_r 50 000 and 43 000 subunits (Figures 3 and 4). Concomitantly, proteolysis of the M_r 80 000 protein generates the M_r 73 000 subunit. Amino-terminal amino acid sequencing confirms that

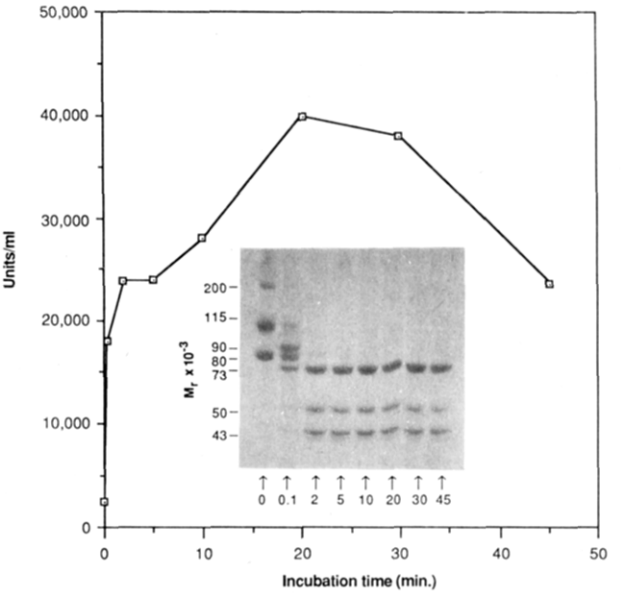

FIGURE 4: Thrombin activation of factor $VIII_{des\text{-}797\text{-}1562}$. 18 μg (450 μg/mL) of factor $VIII_{des\text{-}797\text{-}1562}$ in 0.05 M tris(hydroxymethyl)-aminomethane (Tris), pH 7.4, 0.15 M NaCl, 2.5 mM $CaCl_2$, and 5% glycerol was incubated with 60 ng (7.2 units/mL) of thrombin for 0.1–60 min at 37 °C. At the times indicated, an aliquot was removed and diluted (1/10 000)–(1/20 000) into 0.05 M Tris, pH 7.4, containing 0.01% bovine serum albumin (BSA) and assayed for coagulant activity. The remainder of the sample was brought to 0.5% SDS and heated at 90 °C for 5 min. Subsequently, the samples were resolved by SDS–PAGE (6–12%).

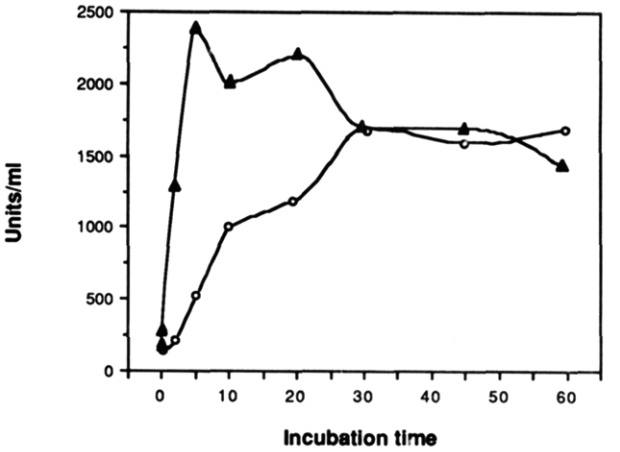

FIGURE 5: Comparison of factor $VIII_{des\text{-}797\text{-}1562}$ and full-length factor VIII activation. 2.4 μg (60 μg/mL) of either factor $VIII_{des\text{-}797\text{-}1562}$ (▲) or full-length factor VIII (○) was incubated with 4.8 ng (0.35 unit/mL) of thrombin for 0–60 min at 37 °C. At the times indicated, an aliquot was removed and diluted (1/1000)–(1/2000) into 0.05 M Tris, pH 7.4, containing 0.01% BSA and assayed for coagulant activity. Full-length factor VIII used in this experiment was purified from factor VIII concentrates as previously described (Eaton et al., 1986a).

the M_r 50 000, 43 000, and 73 000 subunits of the activated variant have the same amino-terminal sequence as their counterparts derived from plasma-derived factor VIII (Figure 3; Eaton et al., 1986a). This shows that factor $VIII_{des\text{-}797\text{-}1562}$ is processed by thrombin in a manner very similar to full-length factor VIII. By SDS–PAGE and staining with Coomassie blue, we could not detect the shortened B domain after thrombin activation. This may be the result of proteolysis to smaller peptides not resolved by the gel system used. Interestingly, we found that factor $VIII_{des\text{-}797\text{-}1562}$ was activated by thrombin at a significantly faster rate than full-length factor VIII. As shown in Figure 5, factor $VIII_{des\text{-}797\text{-}1562}$ was fully activated by thrombin (1/500 thrombin/factor VIII) in ~5

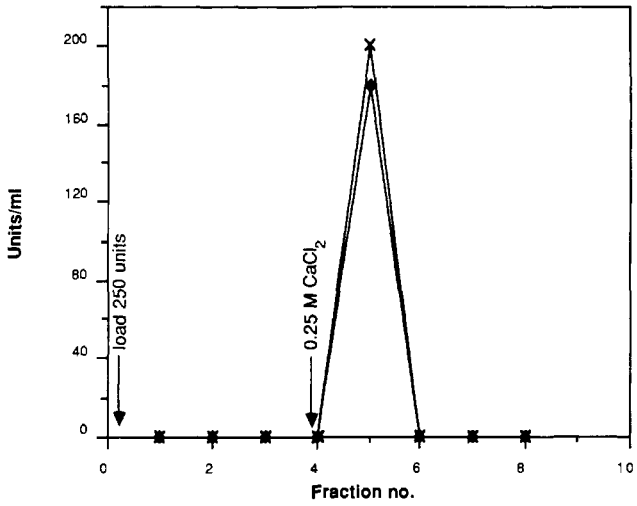

FIGURE 6: Interaction of factor $VIII_{des\text{-}797\text{-}1562}$ with von Willebrand factor. vWF was purified and coupled to Affigel-10 as previously described (Wood et al., 1984). The vWF column was equilibrated in 0.05 M Tris, pH 7.4, 0.15 M NaCl, and 2.5 mM $CaCl_2$. Factor $VIII_{des\text{-}797\text{-}1562}$ (×) or natural factor VIII (♦) (250 units of each) was passed through the column, which was subsequently washed with 3 column volumes of the above buffer. Factor $VIII_{des\text{-}797\text{-}1562}$ or natural factor VIII was eluted from the column with the above buffer containing 0.25 M $CaCl_2$, and 2-mL fractions were collected. Factor VIII coagulant activity was determined by coagulation analysis.

min while activation of full-length factor VIII by thrombin (1/500) took ~30 min.

In plasma, factor VIII circulates bound to von Willebrand factor (vWF) (Hoyer, 1981). In the absence of circulating vWF, such as the case in patients with vWF disease, factor VIII levels are depressed (Hoyer, 1981; Zimmerman et al., 1983). This is in part due to the lability and shortened half-life of factor VIII in the absence of circulating vWF (Brinkhous et al., 1985). When purified factor $VIII_{des\text{-}797\text{-}1562}$ was chromatographed on a vWF–Sepharose column, it bound to the resin (Figure 6). Subsequently, the variant was eluted with 0.25 M $CaCl_2$, which is known to dissociate vWF–Factor VIII complexes (Hoyer, 1981). The same result was obtained when full-length factor VIII was chromatographed on this column (Figure 6).

Discussion

On the basis of the observation that the B domain of factor VIII is proteolytically removed after activation of factor VIII by thrombin (Eaton et al., 1986a,b), we constructed a factor VIII variant in which 767 amino acids of the B domain were removed. This shortened the B domain from 909 amino acids containing 19 asparagine-linked glycosylation sites to 142 amino acids containing only 2 asparagine-linked glycosylation sites. This factor VIII variant was expressed and secreted from mammalian cells and found to be functional. While this paper was in preparation, the expression of an active factor VIII variant in which the B domain was shortened to 28 amino acids was reported (Toole et al., 1986). This factor VIII variant has not been purified or characterized as yet.

Factor $VIII_{des\text{-}797\text{-}1562}$ purified from 90/80 cell culture supernatants showed two major bands on SDS–PAGE of M_r 115000 and 80000 (Figure 3). These proteins probably form a metal-linked complex since ethylenediaminetetraacetic acid (EDTA) inactivated the variant (D. Eaton, unpublished observation). A small amount of the single-chain factor $VIII_{des\text{-}797\text{-}1562}$ of M_r 200000 was also observed by SDS–PAGE. The specific activity of factor $VIII_{des\text{-}797\text{-}1562}$ was found to be 4000–6000 units/mg, which is similar to the specific activity of full-length human factor VIII (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Eaton et al., 1986a). This is not unexpected since the specific activity of the isolated M_r 90000–80000 complex has the sample specific activity as the M_r 210000–80000 complex (D. Eaton, unpublished observations; Fay et al., 1986). This observation itself suggests that the presence of the highly glycosylated B domain has little effect on factor VIII coagulant activity.

Treatment of factor $VIII_{des\text{-}797\text{-}1562}$ with thrombin resulted in a 10–20-fold activation that was correlated with the generation of subunits with M_r 50000, 43000, and 73000 (Figure 4). This shows that factor $VIII_{des\text{-}767\text{-}1562}$ is activated and processed by thrombin in the same manner as full-length factor VIII. Factor $VIII_{des\text{-}797\text{-}1562}$ was also found to interact with vWF–Sepharose in a manner similar to that of full-length factor VIII. This indicates that the B domain may not mediate the binding of factor VIII to vWF, supporting recent findings that factor VIII binding to vWF is mediated by the carboxy-terminal region of factor VIII (Hamer et al., 1985).

Demonstration that factor $VIII_{des\text{-}797\text{-}1562}$ is active shows one more way in which factor VIII and factor V are functionally and structurally similar proteins (Church et al., 1984). Like factor VIII, factor V is initially synthesized as a large single-chain precursor of M_r 330000 that is proteolytically processed by thrombin to an active form (Mann et al., 1981). Activated factor V is a Ca^{2+}-linked complex consisting of two subunits with M_r 90000 (from the amino terminus) and 74000 (from the carboxy terminus) (Esmon, 1979). These subunits have been purified and are fully functional when reconstituted (Esmon, 1979). In the single-ohain form, these subunits are separated by a large highly glycosylated region that is proteolytically removed during activation (Mann et al., 1981; Esmon, 1979). Thus, the precursors of both factor V and factor VIII contain a large (~100 kilodaltons), highly glycosylated domain separating the functional domains of these proteins.

We find that the rate of thrombin activation of factor $VIII_{des\text{-}797\text{-}1562}$ was faster than that for full-length factor VIII, indicating that lower concentrations of thrombin are necessary to activate factor $VIII_{des\text{-}797\text{-}1562}$ when compared to full-length VIII (Figure 5). Whether this difference is enough to alter the functional properties of factor $VIII_{des\text{-}797\text{-}1562}$ (compared to full-length) in vivo is as yet undetermined. In vitro experiments, however, suggest that factor $VIII_{des\text{-}797\text{-}1562}$ may be more susceptible to proteolytic attack. This makes it tempting to speculate that the B domain may function to protect the functional domains of factor VIII from proteolysis that may occur as the result of low amounts of circulating active protease, perhaps thrombin. This would ensure that factor VIII is activated only at the site of vascular injury where local protease activity would be high. While the physiological function of this region is as yet undetermined, the results of this study show that this region in factor VIII is not required either for the expression of factor VIII coagulant activity or for its interaction with vWF in vitro.

Acknowledgments

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Registry No. Thrombin, 9002-04-4; blood coagulation factor VIII, 9001-27-8.

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