Novel forms of B-domain-deleted recombinant factor VIII molecules
Construction and biochemical characterization

Peter LIND, Kerstin LARSSON, Jack SPIRA, Mona SYDOW-BÄCKMAN, Annelie ALMSTEDT, Eva GRAY and Helena SANDBERG
Pharmacia AB, Biopharmaceuticals, Stockholm, Sweden

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Recombinant molecules similar to the smallest active plasma-derived factor VIII molecule, a complex of an 80-kDa and a 90-kDa polypeptide chain lacking the B domain, have been produced using various factor VIII cDNA constructs in order to obtain primary translation products which were efficiently processed into the 80+90-kDa complex. Three types of single-chain cDNAs encoding B-domain-deleted derivatives factor VIII were designed, taking account of sites at Arg740 and Glu1649, assumed to be important for processing factor VIII.

In the type 1 constructs, either Arg747, Arg752, or Arg776 in the N-terminal region of factor VIII B domain was fused to the N-terminus (Glu1649) of the 80-kDa subunit. In the type 2 construct r-VIII SQ, Ser743 was fused to Gln1638, creating a link of 14 amino acids between the C-terminus (Arg740) of the 90-kDa chain and N-terminus of the 80-kDa chain, whereas in type 2 r-VIII RH, Arg747 was fused to His1646. In the type 3 constructs, the B-domain was completely removed or replaced with 1–4 Arg residues.

After expression in Chinese hamster ovary cells, the type 1 derivatives and the type 3 derivatives with 0–2 Arg residues inserted were found to be only partially processed and contained a large amount of the 170-kDa primary translation product. In contrast, most of the type 2 derivatives r-VIII SQ and r-VIII RH and the type 3 derivatives r-VIII R4 and r-VIII R5 containing three or four extra Arg residues preceding the N-terminus of the 80-kDa chain were processed into the desired 80+90-kDa chain complexes. The feature common to the most efficiently processed factor VIII deletion derivatives was that they contained the recognition motif for proteolytic cleavage by the membrane-bound subtilisin-like protease furin, which is expressed in most types of cells; that is, basic amino acid residues at positions −1 and −4 relative to the cleavage site at Gln1649. Biochemical studies of r-VIII SQ and r-VIII R5, two of the most effectively processed factor VIII derivatives, showed that both proteins had a normal factor VIII cofactor function, and had N- and C-termini of the 80-kDa and 90-kDa chains corresponding to those found in plasma-derived factor VIII.

Keywords: recombinant; factor VIII; B-domain-deletion; processing; characterization.

Factor VIII is a protein which functions as an essential cofactor in the activation of factor X in the intrinsic blood coagulation system. Human factor VIII is synthesized as a single-chain molecule of approximately 300 kDa and consists of the structural domains A1-A2-B-A3-C1-C2 (Gitschier et al., 1984; Wood et al., 1984; Vehar et al., 1984; Toole et al., 1984). In newly drawn plasma prepared in the presence of protease inhibitors, factor VIII appears as a complex of two polypeptide chains of 200 and 80 kDa (Andersson et al., 1986). The different forms of factor VIII, having molecular masses between 170–280 kDa were shown to consist of one heavy chain, between 90–200 kDa, in combination with one 80-kDa light chain. In all variants, the N-terminal region of the heavy chain was identical to that of the single-chain factor VIII protein, as deduced from the nucleotide sequence data of the factor VIII cDNA obtained by Toole et al. (1984), Vehar et al. (1984), Wood et al. (1984); likewise, the 80-kDa light chains were shown to have a common N-terminal sequence. The heavy and light chains in the different molecular forms could be dissociated by incubation with EDTA, indicating that the two chains are held together by metal ions.

The smallest active form of factor VIII, with a molecular mass of 170 kDa, consisting of one 90-kDa and one 80-kDa chain, lacks the entire B domain residing between amino acids Arg740 and Glu1649. It can be activated with thrombin to the same extent as the forms of higher molecular mass, and thus represents a non-activated form (Andersson et al., 1986). It has sensitive to proteolytic attack by serine proteases. Such proteolysis occurs readily during storage and purification of the protein. More or less proteolytically degraded forms of factor VIII have been found as active molecules in factor VIII material purified from therapeutic factor VIII concentrates (Andersson et al., 1986). The different forms of factor VIII, having molecular masses between 170–280 kDa were shown to consist of one heavy chain, between 90–200 kDa, in combination with one 80-kDa light chain. In all variants, the N-terminal region of the heavy chain was identical to that of the single-chain factor VIII protein, as deduced from the nucleotide sequence data of the factor VIII cDNA obtained by Toole et al. (1984), Vehar et al. (1984), Wood et al. (1984); likewise, the 80-kDa light chains were shown to have a common N-terminal sequence. The heavy and light chains in the different molecular forms could be dissociated by incubation with EDTA, indicating that the two chains are held together by metal ions.

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also been shown to be hemostatically effective in vivo as tested in hemophilic dogs using a toe-nail bleeding time test (Brinkhaus et al., 1985).

The fact that the B domain does not seem to be necessary for biological activity has prompted us to produce derivatives of recombinant factor VIII lacking this region.

Other authors have reported the construction and expression of recombinant factor VIII lacking most or all of the B domain (Eaton et al., 1986; Toole et al., 1986; Sarver et al., 1987; Lagner et al., 1988; Meulien et al., 1988; Bihoreau et al., 1991; Pittman et al., 1993). The products of the single cDNA gene constructs described either contained the light and heavy chains and, in addition, the single peptide chain representing the non-processed primary translation product as the main species or the latter peptide chain as most dominating component.

In order to obtain expressed proteins with factor VIII activity and consisting solely of the 80-kDa and 90-kDa chains, novel forms of cDNA encoding factor VIII were designed, taking account of sites assumed to be important for the processing of the factor VIII polypeptide chain. One such site is located at the C-terminus of Arg1648, where cleavage gives rise to the 200-kDa plus the 80-kDa form. Another processing site exists at Arg740, where cleavage of the 200-kDa chain produces a 90-kDa chain, creating the 90-kDa plus 80-kDa form of factor VIII. Three types of cDNA were designed. In two of these one or both of the natural processing sites were retained. In type 1, Arg747, Arg752, or Arg776 in the N-terminal region of the factor VIII B domain was fused to Glu1649 (the N-terminus of the 80-kDa chain). In type 2, the N- and C-terminal regions of the B domain were fused in two ways. In recombinant factor VIII SQ (r-VIII SQ), Ser743 was fused to Glu1638, creating a link of 14 amino acids between the C-terminus of the 90-kDa chain (Arg740) and the N-terminus of the 80-kDa chain, and in r-VIII RH, Arg747 was fused to His1646. In the type 3 constructs another approach was used. The B domain was completely removed and the result of insertion of one to four extra arginine residues between the 80-kDa and 90-kDa subunits was studied.

MATERIALS AND METHODS

Biochemicals. Gels used for gel filtration, ion-exchange chromatography and covalent coupling of antibodies were obtained from Pharmacia.

Human α-thrombin (2400 NIH U/mg) was prepared by the method of Miller and Copeland (1962) and further purified by ion-exchange chromatography on SP-Septadex. Human von Willebrand factor (vWF) was prepared from a high-purity thrombin preparation (Pharmacia, Sweden). The resulting conjugate was used for gel filtration on Sepharose 6B.

Biochemical analysis. Factor VIII activity was measured by a chromogenic substrate assay (Rosén, 1984) using the Coatest factor VIII kit (Chromogenix AB, Malmö, Sweden) or by a one-stage clotting assay (Mikaelsson and Oswaldsson, 1984).

Interaction with thrombin was studied after dilution of the factor VIII samples to a concentration corresponding to a factor VIII activity of approximately 100 IU/ml in the reaction mixture. Dilution buffer was 50 mM Tris pH 7.4 containing 0.15 M NaCl and 4 mM CaCl₂. Human α-thrombin was added to factor VIII samples in plastic tubes at a final concentration of 0.01 NIH U/II VIII in a water bath at 37°C. The VIII activity changes were followed for 20 min by use of the one-stage clotting assay method.

Levels of factor VIII antigen (VIII Ag) were determined by an enzyme-linked immunosorbent assay (ELISA) utilizing three different monoclonal antibodies. One of these was directed against a sequence present between Asp1658 and Glu1671 (from the International Immunology Corporation, IIC, California) and was conjugated to horseradish peroxidase. The other two were directed against sequences following Glu1671 in the factor VIII 80-kDa chain and were used as coating antibodies.

vWF antigen (vWF Ag) was determined by ELISA using a test kit Asserachrom vWF from Diagnostica Stago (Asnières, France).

SDS/PAGE was performed according to Laemmli (1970), with a gel thickness of 0.75 mm, a total polyacrylamide concentration of 4% in the stacking gel and 7.5% in the separating gel, and cross-linkage of 3.3%. Samples (0.7 μg) were reduced with 5% (by vol.) 2-mercaptoethanol in 2% (mass/vol.) SDS and heated for 5 min at 90°C. The PAGE was run at 20 mA/gel for 4 h. The electrophoresis was followed either by silver staining of the gels (Johansson and Skoog, 1987) or by Western blot analysis.

Western blot analysis was performed using polyclonal antibodies against plasma factor VIII as described previously (Andersson et al., 1986). Three primary anti-(factor VIII) antibodies raised in New Zealand White rabbits were used. One was raised against the whole factor VIII molecule by immunization with plasma-derived factor VIII as described previously (Andersson et al., 1986). A second was directed against the C-terminus of the 90-kDa chain of plasma factor VIII. A peptide conjugate containing a peptide identical with the amino acid sequence Tyr729 to Arg740 was produced biosynthetically in Escherichia coli expressing the peptide attached to the ZZ segment of protein A from Staphylococcus aureus (Löwenadler et al., 1987). The peptide conjugate was purified by IgG-affinity chromatography prior to immunization. The third antibody was directed against the N-terminus of the 80-kDa chain of plasma-derived factor VIII. A synthetic peptide identical with the sequence Glu1649 to Ser1657 containing an extra cysteine residue at the N-terminus was obtained from Multiple Peptide Systems (San Diego, USA). The peptide was coupled to the carrier protein keyhole limpet hemocyanin using maleimido-benzoyl-N-hydroxysuccinimide ester (Liu et al., 1980). The resulting conjugate was used for immunization.

For N-terminal sequence analysis, the peptide chains of factor VIII samples were separated in reduced SDS/PAGE and subsequently electrophoretically transferred to polyvinylidene difluoride membranes (ProBlott from Applied BioSystems, Foster City, CA). Discrete Comassie-stained protein bands were cut from the membranes and sequence analysis was performed by Edman degradation (Edman, 1950) on a fully automated ABI 477A protein sequencer (Applied BioSystems, Foster City, CA). The cut out pieces of the membranes were applied either in the standard reaction cartridge between two pieces of teflon (Zites) seal or in the specially designed Blott Cartridge. Analysis of the phenylthiocarbamidate derivatives was performed on-line by reverse-phase HPLC using an ABI 120A analyzer.

Construction of expression vectors encoding factor VIII deletion derivatives. For the construction of factor VIII cDNA molecules encoding the factor VIII deletion derivatives r-VIII TRE and r-VIII HRE, a 627-bp KpnI–PstI restriction fragment obtained from the cDNA of the deletion derivative r-VIII RE (obtained from M. Pasek, Biogen, Boston), encoding the amino acids Leu587–Ala1702, was introduced into M13mp19 (Ya-
nisch-Perron et al., 1985). 

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**Fig. 1. Plasmid vectors used for expression of recombinant factor VIII derivatives.** Left: expression vector pKGE448 used for derivation of factor-VIII-producing CHO cells. cDNAs encoding various deletion derivatives of factor VIII are inserted downstream of the human cytomegalovirus enhancer/promoter (Boshart et al., 1985) using the SV40 t-antigen splice and polyadenylation sequences (Mulligan and Berg, 1991). Right: the mouse dhfr-encoding vector pKGE327 used for selection of stably expressing CHO cell lines. cDNA-encoding mouse dhfr (Chang et al., 1978) is under the transcriptional control of the mouse mammary tumor virus LTR (Fasel et al., 1982).
Performing after immunoadsorption of the factor VIII product onto Sepharose 2B with coupled monoclonal antibodies directed against the factor VIII molecule (Vehar et al., 1984) and a general outline of the various unprocessed B-domain-deleted factor VIII molecules. The lower part summarizes the amino acid sequences linking the carboxy-terminus of the 90-kDa chain (Arg740) and the amino-terminus of the 80-kDa chain (Glu1649) in the three different types of deletion derivatives.

The various factor VIII deletion derivatives were subject to gene amplification by culturing in stepwise increasing concentrations of methotrexate up to 500 nM. Pools of resistant cells were obtained that expressed between 0.5 -1.5 IU VIII/ml. These pools of gene-amplified CHO cells expressing the different factor VIII deletion derivatives were routinely cultivated in roller bottles in the presence of 3% heat-inactivated fetal calf serum. The recombinant proteins were purified from the conditioned media.

**Purification of factor VIII.** Initial studies on the post-translational cleavage of the recombinant factor VIII products were performed after immunoadsorption of the factor VIII product onto Sepharose 2B with coupled monoclonal antibodies directed against the A2 domain of the 90-kDa peptide chain, prepared from plasma factor VIII. Following a washing procedure, the concentrated recombinant factor VIII protein was eluted with a buffer containing 50 mM Tris, 0.15 M NaCl, 5 mM CaCl₂ and 2% SDS, pH 7.4, and then subjected to SDS/PAGE and Western blot analysis.

Where further biochemical characterization was performed, partial purification of the recombinant factor VIII proteins from the culture medium was accomplished by immunoaffinity chromatography using the same type of immunoaffinity resin as described above. The factor VIII material was eluted with a buffer containing 20 mM Tris, 50 mM CaCl₂, 0.02% polysorbate and 50% ethylene glycol pH 6.8, and then submitted to ion-exchange chromatography on Q-Sepharose. Elution of material having VIII activity was obtained by passing 50 mM histidine, 0.6 M NaCl, 4 mM CaCl₂, pH 6.8, over the resin.

Plasma-derived factor VIII was purified as described by Andersson et al. (1986).

**RESULTS**

The expressed recombinant factor VIII constructs. Three different groups of factor VIII molecules with a deleted B domain were designed for the study of expression and processing in CHO cells (Fig. 2).

In type 1 constructs, 7, 12 or 36 amino acids derived from the N-terminus of the B domain are retained after Arg740. These fragments, which end at the first (Arg740), second (Arg752), and third (Arg776) arginine residue in the B domain following Arg740, are fused directly to the Glu1649 constituting the N-terminus of the 80-kDa chain. These derivatives were named recombinant factors VIII SRE (r-VIII SRE), VIII TRE (r-VIII TRE), and VIII HRE (r-VIII HRE), respectively.

In type 2 constructs, fusion between the N- and C-terminal amino acids of the B domain was created in two different ways. In recombinant factor VIII SQ (r-VIII SQ), Ser743 was fused to Glu1638, and in recombinant factor VIII RH (r-VIII RH), Arg747 was fused to His1646, retaining either three N-terminal and eleven C-terminal amino acids, or seven N-terminal and three C-terminal amino acids of the B domain.

In type 3 constructs, the B domain was totally deleted or replaced with one, two, three or four Arg residues. The constructs were named recombinant factors VIII RE (r-VIII RE), VIII RRE (r-VIII RRE) VIII R3 (r-VIII R3), VIII R4 (r-VIII R4) and VIII R5 (r-VIII R5), respectively.

Expression and characterization of different factor VIII deletion derivatives. To characterize the products encoded by the various cDNA constructs described above, the different recombinant factor VIII proteins were produced from cultures of CHO cells that consisted of pools of selected and gene-amplified cells. After selection of dhfr-positive cell pools in 500 nM methotrexate, typical expression levels were between 0.5 and 1.5 IU VIII/ml as measured by the chromogenic substrate assay method.

The products were initially studied after purification and concentration using monoclonal anti-(factor VIII) antibodies. SDS/PAGE followed by Western blot analysis with use of monoclonal antibodies directed against the whole factor VIII molecule was used to determine the cleavage patterns obtained.

Fig. 3a shows the results obtained with the type 1 constructs containing various lengths of the N-terminus of the B domain. All three constructs exhibited electrophoresis bands at 90-kDa, in the same position as the 90-kDa band seen for plasma factor
the same position as those of plasma factor VIII, and the 80-kDa component migrated as a tight quartet of bands corresponding to containing part of the N- and C-termini of the B domain, seemed to be processed to 80-kDa and 90-kDa chains with high efficacy teases not involved in the normal processing of factor VIII. processed factor VIII proteins or such proteins degraded by pro-
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the 90-kDa and 80-kDa peptide chains directly connected to 
sequences of the 90-kDa and 80-kDa peptide chains (r-VIII RE, 
90-kDa chain in r-VIII R4 and r-VIII R5, however, produced

80-kDa chain. However, a large part of the total material was obviously not processed as a heavily stained band could be seen at 170 kDa, probably representing the primary translation product in all three constructs. There were also some other bands in r-VIII TRE and r-VIII HRE which may represent incorrectly processed factor VIII proteins or such proteins degraded by pro-

In contrast, the type 2 derivatives r-VIII SQ and r-VIII RH, containing part of the N- and C-termini of the B domain, seemed to be processed to 80-kDa and 90-kDa chains with high efficacy as shown in Fig. 3b. The bands at 80 and 90 kDa were seen at the same position as those of plasma factor VIII, and the 80-kDa component migrated as a tight quartet of bands corresponding to those of plasma factor VIII. Only very thin bands were observed at 170 kDa. Of the two constructs, r-VIII RH seemed to be slightly less processed than r-VIII SQ.

The two derivatives r-VIII SQ and r-VIII R5, as well as in the plasma factor VIII, were also present in the 80-kDa chain in r-VIII R4 and r-VIII R5, however, produced

Analysis of the peptide sequences adjacent to the processing site in the primary translation products of r-VIII SQ and r-VIII R5. The two derivatives r-VIII SQ and r-VIII R5 were further characterized after a two-step purification yielding partially purified proteins with a specific factor VIII activity of about 4000 IU/A mg and a ratio of VIII activity/VIII Ag close to 1. Antibodies derived after immunization with peptides corre-
sponding to the C-terminus of the 90-kDa chain (Tyr729–Arg740) and to the N-terminus of the 80-kDa chain of plasma factor VIII (Glu1649–Ser1657) were used to analyse the purified proteins in Western blotting. Fig. 5 shows that the anti-

very efficient processing to 80-kDa and 90-kDa chains, similar to the results with the type 2 derivatives. Thus, heavily stained bands were present at 80 and 90 kDa, with the same position and pattern as those of plasma factor VIII, and only faint bands were visible at 170 kDa (Fig. 3c). The bands in the region of 40–45 kDa present in various amounts in the lanes of all type 3 variants might be due to a partial degradation of the proteins in the cell or in the cell culture medium.

Analysis of the function of r-VIII SQ and r-VIII R5. Partial analysis of the function of r-VIII SQ and r-VIII R5 was performed. Trace amounts of thrombin are known to enhance the activity of factor VIII. This activation is necessary before inter-
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Fig. 5. Western blot analysis of the factor VIII deletion derivatives r-VIII SQ and r-VIII R5, using polyclonal antibodies specific to the C-terminus of the factor VIII 90-kDa chain and the N-terminus of the factor VIII 80-kDa chain. Left: use of anti-(90-kDa C-terminus) antibodies. Lane 1, r-VIII SQ; lane 2, plasma factor VIII; lane 3, r-VIII R5. Middle: use of anti-(80-kDa N-terminus) antibodies. Lane 4, r-VIII SQ; lane 5, plasma factor VIII; lane 6, r-VIII R5. Right: control. Use of anti-factor VIII antibodies raised against whole factor VIII. The sample applied was plasma factor VIII. All heavy chains of 90–200 kDa and the light chain of 80 kDa are visualized.

Table 1. Sequence of N-terminal amino acid residues of the 80-kDa peptide chains of r-VIII SQ, r-VIII R5 and plasma factor VIII (p-VIII).

<table>
<thead>
<tr>
<th>Chain</th>
<th>N-terminal amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>r-VIII SQ sequence 1:</td>
<td>EITRTTLQSDQEEIDYDD...</td>
</tr>
<tr>
<td></td>
<td>1649</td>
</tr>
<tr>
<td></td>
<td>sequence 2:</td>
</tr>
<tr>
<td></td>
<td>1658</td>
</tr>
<tr>
<td>r-VIII R5 sequence 1:</td>
<td>EITRTTLQSDQEEIDYDD...</td>
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<td></td>
<td>1649</td>
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<tr>
<td></td>
<td>sequence 2:</td>
</tr>
<tr>
<td></td>
<td>1658</td>
</tr>
<tr>
<td>p-VIII sequence 1:</td>
<td>EITRTTLQSDQEEIDYDD...</td>
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<tr>
<td></td>
<td>1649</td>
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<td>sequence 3:</td>
</tr>
<tr>
<td></td>
<td>1658</td>
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Table: Sequence of N-terminal amino acid residues of the 80-kDa peptide chains of r-VIII SQ, r-VIII R5 and plasma factor VIII (p-VIII).

> after incubation of r-VIII SQ and r-VIII R5 with thrombin (0.01 NIH U thrombin/IU VIII corresponding to 0.3 mol thrombin/mol r-VIII SQ or r-VIII R5) are shown in Fig. 6. A one-stage clotting assay was used for immediate assay of samples from the incubation mixture. With both products an approximately 30-fold activation was obtained within 2 min followed by rapid inactivation. This result was consistent with that obtained using plasma-derived factor VIII containing forms with molecular masses of 170–280 kDa (Fig. 6).

r-VIII SQ and r-VIII R5 were also tested for their capacity to bind to human vWF, a high-molecular-mass plasma protein consisting of multimers of 1 MDa to over 12 MDa. vWF exerts an important stabilizing effect on the factor VIII molecule in that it protects the molecule from degradation by proteases (Tuddenham et al., 1982; Brinkhous et al., 1985). Both recombinant molecules were found to bind normally to vWF in gel filtration experiments using a Superose 12 HR column. In the presence of vWF, all factor VIII activity was recovered in the void volume of the column along with the vWF analysed as vWF Ag, while in the absence of vWF all VIII activity was found in the inner volume of the column at the expected position considering the molecular mass.

DISCUSSION

In order to investigate the structural requirements for producing recombinant factor VIII molecules with a deleted B domain which could be processed into proteins consisting of two non-covalently associated polypeptide chains of 90 and 80 kDa, several factor VIII cDNA molecules were created by site-directed mutagenesis. In particular, the structural requirements for liberation of an authentic N-terminus of the 80-kDa chain were studied.

In the type 1 derivatives, where amino-terminal fragments of the B domain ending with Arg were fused to the N-terminus of the 80-kDa subunit, only partial processing occurred, leaving most of the 170-kDa secreted primary translation product intact. Large amounts of the 170-kDa component were also present in the type 3 derivatives in which the N-terminus of the 80-kDa subunit was preceded by one, two or three Arg residues, including Arg740 of the 90-kDa chain. A 130-kDa peptide, probably derived from the 170-kDa peptidie chain, was also a dominating species with an N-terminal sequence starting at Asp356. The type of protease responsible for the cleavage of the 170-kDa peptide chain at Met355-Asp356 as well as its cellular site of action is unknown. In contrast, the type 2 derivatives, in which a few amino acid residues from the N- and C-terminal regions of
the B domain were fused to each other, gave primary translation products that were effectively secreted and processed into double-chain proteins of 90 and 80 kDa polypeptides. Similarly, in the type 3 derivatives, where the N-terminus of the 80-kDa chain is preceded by four or five Arg residues, efficient processing into double-chain molecules occurred.

As has been shown for recombinant full-length factor VIII, processing of the single polypeptide chain precursor product into polypeptide chains of 200 and 80 kDa takes place in the Golgi compartment (Kaufman et al., 1988; Kaufman, 1992). Several proteins are synthesized as precursors that are proteolytically processed during passage through the secretory pathway of animal cells (Docherty and Steiner, 1982; Burgess and Kelly, 1987). These proteins include a diverse variety of plasma proteins, polypeptide hormones, growth factors, cell surface receptors, and viral glycoproteins (Hutton, 1990; Barr, 1991; Hosaka et al., 1991). It has been noticed that precursors that are being processed constitutively upon secretion have Arg at positions −1 and −4 relative to the cleavage site. Also, a basic residue (Lys or Arg) is preferred at position −2, and basic residues frequently occur at positions −3 and −5 relative to the processing site (Hosaka et al., 1991). In the factor VIII B domain this cleavage motif can be found at Arg 1313 and Arg 1648, the latter of which seems to be preferentially cleaved during intracellular processing of factor VIII (Kaufman et al., 1989). The enzyme(s) involved in the proteolytic cleavage of the factor VIII single-chain precursor at Arg1648 is not precisely known. However, it seems likely that one or more of the recently described membrane-bound subtilisin-like proteases furin, PACE4, or PC6A, which seem to be expressed in most cell types, might be responsible for this processing (Van den Ouweland et al., 1990; Barr et al., 1991; Barr 1991, Van de Ven et al., 1993; Kiefer et al., 1991; Nakagawa et al., 1993).

In the factor VIII type 2 deletion derivatives r-VIII SQ and r-VIII RH, the sequence Arg-His-Glu-Arg, which normally occurs at the C-terminus of the B domain, is preserved and constitutes a cleavage site similar to the one in full-length factor VIII. Comparing the polypeptide pattern of r-VIII SQ and r-VIII RH, the presence of Lys in position −5 relative to the processing site does not seem to have any significant importance for cleavage to occur. In the type 1 deletion derivatives r-VIII SRE, r-VIII TRE and r-VIII HRE, there is only a single basic amino acid (Arg) preceding Glu1649, which does not result in high-efficiency cleavage of the 170-kDa polypeptide chain. The enzymes responsible for the partial processing into the 90-kDa and 80-kDa polypeptide chains that occurs with the r-VIII TRE deletion derivative probably have a different kind of cleavage specificity than the above-mentioned conversion endoproteases. These enzymes may be related to those responsible for the creation of the slight heterogeneity of the N-terminus in secreted r-VIII SQ as shown above. The nature of the proteases or aminopeptidases responsible for this is not known. A similar situation seems to exist for the type 3 derivatives r-VIII RE, r-VIII RRE and r-VIII R3, where one, two and three Arg residues precede Glu1649. Although the latter two derivatives contain paired basic residues that frequently occur in precursor polypeptide chains undergoing proteolytic processing, this is apparently not sufficient for efficient cleavage in CHO cells. In contrast, the other two type 3 derivatives r-VIII R4 and r-VIII R5, in which four and five Arg residues precede Glu1649, undergo efficient precursor cleavage into 90-kDa and 80-kDa polypeptide chains. In these two derivatives the recognition motif for cleavage by the mammalian conversion endoprotease furin is satisfied in the sense that there are Arg residues present at both positions −1 and −4 relative to the cleavage site, and that a basic residue (Arg) is also present at position −3 (Hosaka et al., 1991). A slight heterogeneity at the N-terminus of the 80-kDa subunit, similar to r-VIII SQ, was also observed for r-VIII R5, indicating an identical mode of processing.

Two of the most effectively processed factor VIII deletion derivatives, r-VIII SQ and r-VIII R5, were selected for further biochemical characterization. Analysis of the peptide sequences adjacent to the processing site showed a conformity in the N-terminus of the 80-kDa chain for the two products with that of plasma factor VIII. Two sequences were obtained in the two products starting with Glu1649 and Asp1658. In both these cases the important cleavage site for thrombin at Arg1689 (Vehar et al., 1984) has been retained. A similar heterogeneity in the N-terminus of the 80-kDa chain was present in plasma factor VIII and has also been reported by other authors (Ezban et al., 1993).

Furthermore, a binding domain for vWF is located at the N-terminus of the 80-kDa chain, most probably between residues 1677 or 1675 and 1684 (Foster et al., 1990; Shima et al., 1992). Thus the amino acid sequence involved in vWF binding is also present in both molecules. The results from the structural analysis, indicating that r-VIII SQ and r-VIII R5 are normal factor VIII molecules, were further supported by the results of the functional analysis. Thus, both molecules exhibited a ratio of factor VIII activity to factor VIII antigen which was close to 1, and a normal interaction with both thrombin and vWF was found.

Biochemical characterization of the products of two other recombinant deletion derivatives of factor VIII lacking the sequences Pro771–Asp1666 and Thr760–Asn1639 has been published previously (Bihoreau et al., 1991; Pittman et al., 1993). Both these products were shown to have factor VIII cofactor activity. In the first case, however, the dominating species obtained were 120-kDa and 165-kDa peptide chains, and in the second case the prominent species were 80-kDa, 90-kDa and 170-kDa peptide chains. The data were in agreement with preliminary studies on the function of r-VIII RE in which it was found that it could be activated by thrombin and bound to von Willebrand factor similarly to plasma factor VIII (not shown). In the case of r-VIII SQ and r-VIII R5, the molecules also have factor VIII cofactor activity and, in addition, seem to be more authentic molecules as regards peptide chain composition. Future studies may show whether the latter fact confers a biological advantage.

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