Inhibition of human coagulation Factor VIII by monoclonal antibodies

Mapping of functional epitopes with the use of recombinant Factor VIII fragments

Anja LEYTE, Koen MERTENS, Ben DISTEL, Ron F. EVERS, May J. M. DE KEYZER-NELLEN, Monique M. C. L. GROENEN-VAN DOOREN, Jeanine DE BRUIN, Hans PANNEKOEK, Jan A. VAN MOURIK* and Martin Ph. VERBEET

Department of Blood Coagulation and Department of Molecular Biology,
Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, 1006 AK Amsterdam, The Netherlands

The epitopes of four monoclonal antibodies against coagulation Factor VIII were mapped with the use of recombinant DNA techniques. Full-length Factor VIII cDNA and parts thereof were inserted into the vector pSP64, permitting transcription *in vitro* with the use of a promoter specific for SP6 RNA polymerase. Factor VIII DNA inserts were truncated from their 3'-ends by selective restriction-enzyme digestion and used as templates for 'run-off' mRNA synthesis. Translation *in vitro* with rabbit reticulocyte lysate provided defined radiolabelled Factor VIII fragments for immunoprecipitation studies. Two antibodies are shown to be directed against epitopes on the 90 kDa chain of Factor VIII, between residues 712 and 741. The 80 kDa chain appeared to contain the epitopes of the other two antibodies, within the sequences 1649–1778 and 1779–1840 respectively. The effect of antibody binding to these sequences was evaluated at two distinct levels within the coagulation cascade. Both Factor VIII procoagulant activity and Factor VIII cofactor function in Factor Xa generation were neutralized upon binding to the region 1779–1840. The antibodies recognizing the region 713–740 or 1649–1778, though interfering with Factor VIII procoagulant activity, did not inhibit in Factor Xa generation. These findings demonstrate that antibodies that virtually inhibit Factor VIII in coagulation *in vitro* are not necessarily directed against epitopes involved in Factor VIII cofactor function. Inhibition of procoagulant activity rather than of cofactor function itself may be explained by interference in proteolytic activation of Factor VIII. This hypothesis is in agreement with the localization of the epitopes in the proximity of thrombin-cleavage or Factor Xa-cleavage sites.

INTRODUCTION

Factor VIII is a plasma glycoprotein that plays an essential role in the intrinsic pathway of coagulation, as a cofactor in the proteolytic activation of Factor X by Factor IXa [1–3]. With the construction of the full-length Factor VIII cDNA and the establishment of the entire nucleotide sequence [4,5], the primary structure of the protein has been completely resolved. The precursor consists of 2332 amino acid residues, preceded by a signal peptide of 19 amino acid residues. It is composed of three homologous A domains, two homologous C domains and a unique B domain, arranged in the following order: A₁–A₂–B–A₃–C₁–C₂ [6] (cf. Fig. 1). In plasma, Factor VIII circulates in complex with a carrier protein, von Willebrand Factor [7,8].

The structure–function relationship of Factor VIII is still largely unknown. The protein displays sequence homology with Factor V, another major cofactor in the coagulation system [1]. As a consequence of limited proteolysis, Factor VIII circulates as heterodimers of a C-terminal light chain (*M_r* 80000, containing domains A₃, C₁ and C₂) and variable derivatives of the N-terminal heavy chain (*M_r* 50000–180000, A₁ plus parts of A₂ and B) [9–11]. Expression of Factor VIII activity is modulated through proteolytic cleavage by thrombin,

Factor Xa and activated protein C [1,10,12–15]. Activation by thrombin or Factor Xa involves removal of the B domain, a region that appears not to be required for activity of recombinant Factor VIII *in vitro* [16–18]. Fully activated Factor VIII possibly consists of the 90 kDa fragment of the heavy chain and the 80 kDa chain [9,13], resulting from proteolytic cleavage at Arg-740 and Arg-1648 respectively [10]. In addition, both chains contain other putatively activating cleavage sites: the 90 kDa chain at position 372 and the 80 kDa chain at 1689; cleavage at these positions results in conversion of the 90 kDa chain into fragments of 50 kDa and 43 kDa, and of the 80 kDa chain into a fragment of 73 kDa [10,15]. Proteolytic inactivation of Factor VIII has been proposed to occur through cleavage by Factor Xa or activated protein C at Arg-336 [10].

As an approach for the localization of functionally important domains, the mapping of epitopes of human antibodies and mouse monoclonal antibodies to Factor VIII together with studies on the inhibitory properties of these antibodies in coagulation *in vitro* has proven informative. Thus immunoblotting studies of plasma Factor VIII and its thrombin digestion fragments have shown that both the 90 kDa chain and the 73 kDa chain, but not the B domain, harbour epitopes that may serve an essential role in the expression of Factor VIII activity

* To whom correspondence should be addressed: c/o Publication Secretariat, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, P.O. Box 9406, 1006 AK Amsterdam, The Netherlands.

[9,19,20]. A more precise location of antibody-binding sites with this method is restricted by the availability of suitable proteinase-cleavage sites on the Factor VIII protein.

In the present study we describe an epitope mapping strategy that makes use of appropriate restriction sites on the Factor VIII cDNA. This approach provided defined truncated Factor VIII fragments that could be used for immunoprecipitation studies. The epitopes of four monoclonal antibodies that were located by using this method were investigated with regard to their involvement in Factor VIII function.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.) or from Boehringer Mannheim (Mannheim, Germany). SP6 RNA polymerase, RNasin and rabbit reticulocyte lysate were purchased from Promega (Madison, WI, U.S.A.). [³⁵S]Methionine (1100 Ci/mmol; 10 mCi/ml) was from Amersham International (Amersham, Bucks., U.K.). Oligonucleotides were synthesized with an automated DNA synthesizer (type 381A; Applied Biosystems, Foster City, CA, U.S.A.). L-Amino acids were obtained from Merck (Darmstadt, Germany). Human serum albumin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CNBr-activated Sepharose 4B, Protein A-Sepharose and the RNA cap structure analogue m⁷G(5')ppp(5')G were from Pharmacia (Uppsala, Sweden). Nitrocellulose was from Schleicher und Schüll (Dassel, Germany).

Cloning of a full-length Factor VIII cDNA

cDNA encoding human Factor VIII was obtained by means of primer-directed cDNA synthesis essentially as reported by Toole *et al.* [5], with human liver polyadenylated RNA as template. The ten utmost 5' base-pairs, from the ATG initiation codon (A = 1) to the *Sac*I site at position 10 (numbering according to Wood *et al.* [4]), were provided by the chemical synthesis of both strands of a DNA fragment of 21 nucleotide residues (5'-GTCGACATGCAAATAGAGCTC-3', 'upper' strand sequence). The design of this synthetic DNA fragment creates a *Sal*I site, not present in the Factor VIII cDNA, at the 5' side of the ATG initiation codon. The full-length Factor VIII cDNA from the *Sal*I site to the *Hpa*I site at position 7433 was inserted into a vector digested with *Sal*I and *Hpa*I. The Factor VIII cDNA insert of 7436 bp was sequenced by the dideoxy chain termination procedure [21] and was found to be identical with the one published by Wood *et al.* [4], except for one silent substitution of a T by a C residue at position 2976.

Design of 'in-vitro-expression' plasmids and mRNA fragments

Fig. 1 illustrates the 'in-vitro-expression' plasmids designed for this study.

pSP/F8-FL (Fig. 1; I). The full length 7436 bp *Sal*I-*Hpa*I Factor VIII cDNA fragment was inserted into the vector pSP64 [22] digested with *Sal*I and *Sma*I.

pSP/F8-80k1 (Fig. 1; II). The construction of this plasmid, which encodes the Factor VIII 80 kDa chain fused to the signal peptide of human tissue-type plasmin-

Fig. 1. Structure of the Factor VIII protein, the Factor VIII cDNA and the 'in-vitro-expression' plasmids

(a) The vertical arrows indicate the cleavage sites of thrombin (Th), Factor Xa (Xa) and activated protein C (APC) on the Factor VIII protein [10]. The signal peptide (SP) and the domain structure (A₁, A₂, B, A₃, C₁ and C₂) are indicated; the numbers below the bar refer to the domain borders [4]. (b) The Factor VIII cDNA and the position of restriction sites that are relevant for the construction of the 'in-vitro-expression' plasmids. The numbers refer to the cDNA nucleotide sequence reported by Wood *et al.* [4], where the A residue of the ATG initiation codon is denoted 1. (c) The 'in-vitro-expression' plasmids. Continuous line, Factor VIII cDNA; black block, Factor VIII signal sequence; white block, tissue-type plasminogen-activator signal sequence; stippled block, SP6 promoter. The construction of the expression plasmids containing different Factor VIII cDNA fragments is described in detail in the Materials and methods section.

ogen activator [23], involved three steps as follows. (a) A 2690 bp *Bam*HI (4743)–*Hpa*I (7433) Factor VIII cDNA fragment was inserted into the vector pUC19 [24] digested with *Bam*HI and *Hinc*II, resulting in the plasmid pUC80a. (b) A 868 bp *Eco*RI fragment from pSV2tPA [25] encompassing the tissue-type plasminogen-activator signal sequence was incubated with DNA polymerase I (Klenow fragment) to fill in the sticky ends and was inserted into pUC80a, digested with *Xma*I and incubated with DNA polymerase I (Klenow fragment) as well; the resulting plasmid was designated pUC80b. (c) A 2750 bp *Hind*III fragment of pUC80b, containing the in-frame fusion of the tissue-type plasminogen-activator signal sequence and the Factor VIII light-chain cDNA was inserted into pSP64 digested with *Hind*III. The desired orientation, having the tissue-type plasminogen-activator signal-peptide cDNA sequence directly downstream of the SP6 promoter, was verified by mapping of characteristic restriction sites.

pSP/F8-80k2 (Fig. 1; III). The construction of this plasmid, which encodes the Factor VIII 80 kDa chain fused to the Factor VIII signal peptide, involved three steps, as follows. (a) A 668 bp *Apa*I (6194)–*Ban*I (5526) fragment was isolated from pSP/F8-FL, as well as a 634 bp *Hgi*AI fragment, extending from position 4962 to 5596; the latter fragment was incubated with T4 DNA polymerase to generate blunt ends and subsequently digested with *Ban*I at position 5526; the resulting 565 bp blunt-*Ban*I fragment was isolated. (b) pSP/F8-FL was digested with *Mbo*II and subsequently a 474 bp fragment was isolated, containing cDNA encoding the Factor VIII signal peptide; after incubation with T4 DNA polymerase to generate blunt ends, this fragment was digested with *Sal*I and the 71 bp cDNA fragment encoding the Factor VIII signal peptide was isolated. (c) The fragments obtained in steps (a) and (b) were ligated with the vector-containing *Sal*I–*Apa*I fragment of pSP/F8-FL.

pSP/F8-d90K (Fig. 1; IV). This plasmid was generated from pSP/F8-FL by deleting the 2287 bp between the *Hind*III site of the vector and the *Hind*III site of the Factor VIII cDNA at position 2277.

pSP/F8-dB925 (Fig. 1; V). The construction of this plasmid, which encodes a fusion product of the Factor VIII 90 kDa and 80 kDa chains, involved the following steps. (a) pSP/F8-FL was digested with *Kpn*I (1811) and *Tth*III.I (2241) to generate a 630 bp fragment; this fragment was purified and digested with *Mae*III (2189); the *Mae*III cohesive end was filled in with DNA polymerase I (Klenow fragment) and the resulting 383 bp *Kpn*I–*Mae*III fragment was isolated. (b) and (c) As steps (a) and (b) for the construction of pSP/F8-80k2 respectively. (d) pSP/F8-FL was digested with *Apa*I (6194) and *Kpn*I (1811), generating a 5.9 kb fragment containing the pSP64 vector sequences. (e) The fragments obtained in steps (a)–(d) were ligated in the presence of a 10-fold molar excess of an *Mlu*I linker (5'-CCACGCGTGG-3'). Plasmid pSP/F8-dB925 thus contains an *Mlu*I linker between the *Mae*III and *Hgi*AI sites within the Factor VIII cDNA. The *Mlu*I linker introduces four additional amino acid residues (Pro-Arg-Val-Ala), abridging the deleted segment between amino acid residues 712 and 1638.

Choice of restriction sites. To define the 3'-termini of mRNAs, synthesized via 'run-off' transcription, the

following restriction sites were chosen on the Factor VIII sequences of the 'in-vitro-expression' plasmids: 1038 (*Pvu*II), 1869 (*Bam*HI), 2149 (*Mlu*I), 2441 (*Tth*III.I), 4743 (*Bam*HI), 5390 (*Stu*I), 5578 (*Hinc*II), 6056 (*Bgl*II), 6945 (*Xba*I) and past 7433 (*Sma*I).

Transcription and translation in vitro

Transcription reactions were performed in 30 µl containing 4.5 µg of linearized template DNA, 0.5 mM each of UTP, CTP and ATP, 0.02 mM-GTP, 1 mM-m⁷G(5')ppp(5')G to provide mRNAs with a capped 5'-terminus, and 1 unit of SP6 RNA polymerase/µl. After incubation for 1 h at 40 °C, nucleic acids were extracted with phenol/chloroform (1:1, v/v) and precipitated with ethanol. The pellet was dissolved in 30 µl of distilled water.

Translation reactions were performed in 50 µl containing 6.5 µl of DNA/RNA solution, 35 µl of nuclease-treated rabbit reticulocyte lysate, 0.02 mM each of the amino acids (except methionine) and 1 µCi of [³⁵S]methionine/µl. Incubation was for 1 h at 30 °C.

Immunoprecipitation

Immunoprecipitation of Factor VIII polypeptides synthesized *in vitro* was performed as follows. Translation reaction mixtures were diluted 30-fold with 10 mM-Tris/HCl buffer, pH 7.8, containing 150 mM-NaCl, 10 mM-EDTA, 1% (v/v) Nonidet P40, 1 mM-phenylmethanesulphonyl fluoride, 0.02 mg of soya-bean trypsin inhibitor/ml, 10 mM-benzamidine and 5 mM-N-ethylmaleimide. Monoclonal antibodies were immobilized by attachment to Protein A-Sepharose according to the manufacturer's instructions. Preclearing, specific incubations and washing were essentially as described elsewhere [26]. After washing, the beads were boiled in 10% (w/v) SDS for 5 min and centrifuged. The supernatant was analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions by the method of Laemmli [27], followed by autoradiography.

Monoclonal antibodies

Mouse monoclonal antibodies to human Factor VIII, namely CLB-CAG 117, CLB-CAG A, CLB-CAG 69 and CLB-CAG 9, were produced and purified as has been described previously [28]. Monoclonal antibody 1B3 was a gift from Dr. R. M. Bertina (Leiden University Hospital, Leiden, The Netherlands) and was described previously [29]. As a control, a monoclonal antibody to grass pollen (4B1, denoted here 'anti-pollen antibody') that was developed at our institute [30] was used.

Purified Factor VIII

Human Factor VIII was purified from Factor VIII concentrate (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service). Approx. 10000 units of Factor VIII were dissolved in 50 mM-Tris/HCl buffer, pH 7.4, containing 2.5 mM-CaCl₂ and 10 mM-benzamidine to a final volume of 1 litre. After centrifugation of the mixture at 2750 g for 5 min, CaCl₂ was added to the supernatant to a final concentration of 250 mM in order to dissociate the Factor VIII–von Willebrand Factor complex. Moreover, 200 ml of 10% (w/v) sucrose containing the stabilizing amino acids L-threonine (4.5%, w/v), L-alanine (1.5%, w/v) and L-valine (1.5%, w/v) were added. Subsequently, a monoclonal antibody to Factor VIII (CLB-CAG 117)

coupled to CNBr-activated Sepharose-4B was added up to a ratio of 1 mg of antibody per 2000 units of Factor VIII. The total volume was adjusted to 2 litres with 50 mM-Tris/HCl buffer, pH 7.4, containing 250 mM-CaCl₂ and 10 mM-benzamidine, and the mixture was stirred gently for 6 h at room temperature. Afterwards, the Sepharose was collected into a column (1 cm × 10 cm) at 4 °C. The column was washed with 160 ml of 10 mM-Tris/HCl buffer, pH 7.4, containing 2.5 mM-CaCl₂ and 150 mM-NaCl and subsequently with 2 column volumes of 10 mM-Tris/HCl buffer, pH 7.4, containing 2.5 mM-CaCl₂ and 1.5 M-NaCl, at a flow rate of 10 ml/h. Factor VIII was eluted with a solution containing 2.5 mM-CaCl₂, 150 mM-NaCl, 10 mM-benzamidine, 50 % (v/v) ethylene glycol and 25 mM-lysine, pH 11. Fractions (1 ml) were collected, immediately neutralized with 10 % (v/v) of 1.5 M-imidazole, pH 7.0, and stored at -20 °C. Factor VIII purified by this method has a specific activity of 2500–3000 units/mg and a Factor VIII activity/antigen ratio of 0.6–0.9:1.

Immunoblotting procedure

Samples were subjected to SDS/7.5 %-(w/v)-polyacrylamide-gel electrophoresis under reducing conditions [27]. After electrophoresis, the separated proteins were transferred electrophoretically to nitrocellulose at 0.2 A for 16 h at 4 °C. The transfer buffer contained 25 mM-Tris, 192 mM-glycine and 20 % (v/v) methanol. Subsequently, the protein blots were treated with monoclonal antibody, as follows. Ascites was diluted 1:100 in phosphate-buffered saline (150 mM-NaCl/10.5 mM-sodium phosphate buffer, pH 7.4) containing 0.1 % (w/v) Tween 20, and the nitrocellulose was incubated with 1 ml of this solution per lane (0.4 cm width) for 2 h at room temperature. The membrane was then washed three times with phosphate-buffered saline containing 0.1 % (w/v) Tween 20 and incubated with 1 ml/lane of goat anti-(mouse IgG) antibody horse-radish-peroxidase-conjugated (25 µg/ml) (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) in the same buffer for 2 h at room temperature. Finally, after three more washes, the immune complexes were stained with 100 ml of phosphate-buffered saline containing 0.1 mg of 4-chloro-1-naphthol/ml and 0.015 % (v/v) H₂O₂. The reaction was stopped by rinsing the membrane with water.

Factor VIII assays

Total protein concentration was determined by the method of Bradford [31], with human serum albumin as standard. Factor VIII activity was determined either spectrophotometrically or in a one-stage clotting assay. The former method employs a chromogenic substrate and purified bovine coagulation factors (Coatest Factor VIII; KabiVitrum, Stockholm, Sweden) and was performed according to the manufacturer's instructions. The one-stage clotting assay was performed with congenitally Factor VIII-deficient plasma as the substrate [32]. Factor VIII antigen was measured with an e.l.i.s.a. assay based on a previously described method [33]. Factor VIII concentrations are expressed in units/ml; 1 unit/ml represents the concentration of Factor VIII in pooled normal human plasma. In order to evaluate various functional aspects of Factor VIII, we distinguish between the following terms. (a) Factor VIII cofactor activity is defined as the effect of Factor VIII as a

cofactor of Factor IXa in the activation of Factor X. This relates to the Factor VIII activity that is measured in the spectrophotometric assay. (b) Factor VIII procoagulant activity is defined as the activity that corrects the coagulation defect of plasma of a severe haemophilia A patient in a one-stage clotting assay.

RESULTS

Immunoblotting

As a first step in the mapping of the epitopes of the monoclonal antibodies, we used immunoblotting of purified plasma Factor VIII. As is shown in Fig. 2, this preparation consisted of multiple polypeptides with apparent molecular masses concurring with those of the C-terminal 80 kDa chain and the N-terminal 90 kDa chain plus parts of the central B domain [9,11,19]. The antibodies 1B3 and CLB-CAG 9 recognized all polypeptides that contain the 90 kDa fragment, whereas CLB-CAG 69 reacted with the 80 kDa chain. CLB-CAG A and CLB-CAG 117, the antibody used for the Factor VIII purification, did not bind to Factor VIII under the reducing conditions in the immunoblotting experiments. However, it has previously been reported that these antibodies recognize the isolated 80 kDa chain [34]. On the basis of this preliminary localization of the antibody-binding sites we concluded that the fine mapping strategy should be focused on the 90 kDa and 80 kDa chains rather than on the B domain.

Fine mapping strategy

Our approach for a more detailed localization of the antibody-binding sites included immunoprecipitation of truncated recombinant fragments of the 90 kDa and

Fig. 2. Monoclonal-antibody reactivities with purified human Factor VIII

Lanes 1 and 2, SDS/7.5 %-(w/v)-polyacrylamide-gel electrophoresis of reduced Factor VIII (25 µg); proteins were detected by Coomassie Brilliant Blue (lane 1) and silver staining (lane 2). Molecular masses of Factor VIII polypeptides are indicated at the left. Lanes 3, 4 and 5, immunoblot of the same Factor VIII preparation (2.5 µg/lane). Individual lanes were incubated with monoclonal antibodies: CLB-CAG 9 (lane 3); 1B3 (lane 4); CLB-CAG 69 (lane 5). The mobilities of molecular-mass markers are indicated at the right.

Fig. 3. Immunoprecipitations of Factor VIII fragments synthesized *in vitro*

The derivation of the Factor VIII fragments is outlined in the text (see also Fig. 4). The fragments were radiolabelled during translation *in vitro* by using [^{35} S]methionine. Translation products (lanes 13–16) and immune complexes (lanes 1–12) were analysed with SDS/5–15%-(w/v)-polyacrylamide-gradient-gel electrophoresis followed by autoradiography. Lanes 1–3, Factor VIII fragments containing amino acid residues 1562 to 2332, immunoprecipitated with CLB-CAg A (lane 1), CLB-CAg 69 (lane 2) and the control anti-pollen antibody (lane 3). Lanes 4–6, Factor VIII fragments containing amino acid residues 1562 to 2001, immunoprecipitated with CLB-CAg A (lane 4), CLB-CAg 69 (lane 5) and anti-pollen antibody (lane 6). Lanes 7–9, Factor VIII fragments containing amino acid residues 1562 to 1778, immunoprecipitated with CLB-CAg A (lane 7), CLB-CAg 69 (lane 8) and anti-pollen antibody (lane 9). Lanes 10–12, Factor VIII fragments containing amino acid residues 1638 to 1778, immunoprecipitated with CLB-CAg A (lane 10), CLB-CAg 69 (lane 11) and anti-pollen antibody (lane 12). Lanes 13–16, Factor VIII fragments containing amino acid residues 1562 to 2332 (lane 13), 1562 to 2001 (lane 14), 1562 to 1778 (lane 15) and 1638 to 1778 (lane 16). The mobilities of molecular-mass markers are indicated at the right.

80 kDa chains. For this purpose, restriction sites were selected that are evenly distributed over the Factor VIII cDNA encoding these two chains. The chosen restriction sites (see the Materials and methods section) were utilized to define the 3'-termini of mRNAs, synthesized via 'run-off' transcription with Factor VIII 'in-vitro-expression' plasmids providing various 5'-termini, as templates (Fig. 1). Translation of these mRNAs in rabbit reticulocyte lysate provided polypeptides with *N*-termini corresponding to Factor VIII residue number (apart from the added signal peptides) – 19, 779, 1562 or 1638, and *C*-termini at position 327, 604, 712, 795, 1562, 1778, 1840, 2001, 2297 or 2332. Such radiolabelled Factor VIII fragments were used for immunoprecipitation studies.

Immunoprecipitation

Fig. 3 illustrates the experimental approach for two monoclonal antibodies directed against the 80 kDa chain. Factor VIII fragments were obtained via digestion of the plasmids pSP/F8-80k1 and pSP/F8-80k2 (cf. Fig. 1) at several of the restriction sites mentioned above. This resulted in *C*-terminally truncated forms of the 80 kDa chain, with molecular masses between 90 kDa and 18 kDa (Fig. 3, lanes 13–16). These values correspond to the sum of the molecular masses of the respective fragments and the fused signal peptides required for appropriate sites for translation starts. In the case of the longer polypeptides smaller fragments were also observed. These probably arise as a result of incorrect starts or stops during translation, since intactness of mRNAs had been confirmed by Northern blotting (results not shown). Immunoprecipitation studies revealed that the antibody CLB-CAg 69 recognized all four 80 kDa-chain-derived fragments, whereas CLB-CAg A recognized

only the two longer peptides (Fig. 3, lanes 1–12). This illustrates that our method provides a straightforward approach for detecting immunoreactive sequences. Accordingly, immunoprecipitation studies were performed with other fragments and monoclonal antibodies. The results are summarized in Fig. 4. It appeared that the region between amino acid residues 712 and 779 is involved in the binding of both the antibody 1B3 and CLB-CAg 9. Since these antibodies are known to recognize the 90 kDa chain (Fig. 2) with Arg-740 as *C*-terminal residue [10], it is concluded that the sequence from residue 713 to 740 contains at least part of the epitopes of 1B3 and CLB-CAg 9. The smallest common sequence of the peptides binding to the antibody CLB-CAg 69 extends from amino acid residue 1638 to 1778. Together with the knowledge that CLB-CAg 69 recognizes the 80 kDa chain (Fig. 2) with Glu-1649 as *N*-terminal residue [10], this provides evidence that the epitope of this antibody lies between residues 1648 and 1779. Fig. 4 further summarizes that CLB-CAg A recognized the polypeptides extending from amino acid residue 1562 to 2332, 2001 or 1840, but not from residue 1562 to 1778. Consequently, the epitope or part of the epitope of CLB-CAg A must be situated between amino acid residues 1778 and 1841. The third anti-(80 kDa chain) antibody included in these studies, CLB-CAg 117, displayed cross-reactivity with non-overlapping sequences. Therefore the apparently more complex epitope of this antibody could not be mapped precisely.

Inhibition studies

The antibody-binding regions that evolved from the mapping studies (Fig. 4) were investigated with regard to their involvement in Factor VIII function. For this

Fig. 4. Summary of immunoprecipitation studies with recombinant Factor VIII fragments

Protein fragments synthesized *in vitro* were immunoprecipitated with the monoclonal antibodies 1B3, CLB-CAG 9, CLB-CAG 69 and CLB-CAG A. The left-hand panel shows the Factor VIII fragments. The Roman numerals refer to the 'in-vitro-expression' plasmids (see Fig. 1). The N-terminal and C-terminal amino acid residues are indicated. The right-hand panel shows the reactivity of fragments with antibodies scored as present (+) or absent (-). Abbreviation: N.D., not determined.

Fig. 5. Inhibition of Factor VIII by monoclonal antibodies

Plasma was incubated with an equal volume of IgG (0–100 $\mu\text{g/ml}$ in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.8) for 2 h at 37 °C. Subsequently, Factor VIII activity was measured either spectrophotometrically (a) or in a clotting assay (b) as described in the Materials and methods section. The monoclonal antibodies were 1B3 (x), CLB-CAG 9 (O), CLB-CAG 69 (■) and CLB-CAG A (●).

purpose the monoclonal antibodies were tested for inhibition in two distinct functional Factor VIII assays. Fig. 5 shows the results of these studies. When, upon incubation of plasma with each of the antibodies, residual Factor VIII was determined spectrophotometrically (Fig. 5a), CLB-CAG A appeared to be an effective inhibitor of Factor VIII cofactor activity. However, CLB-CAG 69, 1B3 and CLB-CAG 9 did not inhibit Factor VIII up to the highest concentration tested (100 $\mu\text{g/ml}$), suggesting that these antibodies are non-inhibitory. Interestingly, this is in marked contrast with the results obtained when residual Factor VIII activity was quantified by use of the clotting assay (Fig. 5b). CLB-CAG 69 was found to be a weak inhibitor of Factor VIII procoagulant activity. Both 1B3 and CLB-CAG 9 appeared to inhibit Factor VIII up to about 50%. At antibody concentrations exceeding 5 $\mu\text{g/ml}$ inhibition was no longer dose-

dependent, indicating that Factor VIII was saturated with antibody. It was concluded that inhibition of Factor VIII procoagulant activity is not necessarily identical with inhibition of Factor VIII cofactor function.

DISCUSSION

The mapping of epitopes of both inhibitory and non-inhibitory antibodies has proven a valuable tool in studying the structure-function relationship of coagulation Factor VIII. Most studies reported so far [9,13,19,20,35] have been performed with purified plasma Factor VIII and fragments obtained by thrombin digestion. This approach, however, is restricted by the location of the specific sites susceptible to limited proteolysis. In the present study we used a mapping strategy based on the selection of appropriate restriction sites on

Fig. 6. Epitope map of the monoclonal antibodies to Factor VIII described in the present paper

The epitopes of the various antibodies are given below the bar, which represents the Factor VIII protein. The domain structure and the position of the cleavage sites are indicated as in Fig. 1. The epitope of CLB-CaG 69 was recently demonstrated to be encompassed by a synthetic peptide representing the sequence Lys-1673-Arg-1689 [41].

the Factor VIII cDNA. Factor VIII 'in-vitro-expression' plasmids were derived from pSP64, a vector containing the bacteriophage SP6 promoter that allows specific 'run-off' transcription of inserted cDNA sequences [22]. This permits the synthesis of precisely defined radiolabelled Factor VIII fragments that can be used directly in immunoprecipitation studies (Figs. 3 and 4). This method provides multiple protein fragments from a limited number of template-containing plasmids. As such, it has some technical advantages over techniques requiring the construction of separate expression plasmids for each individual fragment [36]. Similar strategies have been applied successfully to the mouse transformation-associated protein p53 [37] and the rat glyocorticoid receptor [38]. This approach avoids the need for sequencing multiple randomly generated cDNA fragments encoding immunoreactive polypeptides, as is required when employing a recombinant DNA epitope library [39,40]. The method is particularly relevant to linear epitopes not containing post-translational modifications.

Fig. 6 summarizes the results of the mapping studies of four monoclonal antibodies to Factor VIII. Two antibodies (CLB-CaG 9 and 1B3) were both directed against the C-terminal region of 28 amino acid residues of the 90 kDa chain. Of two antibodies (CLB-CaG A and CLB-CaG 69) recognizing the 80 kDa chain of 684 amino acid residues the epitope-containing regions were confined to sequences spanning 62 and 130 amino acid residues respectively. A more precise localization may be obtained by further truncation of the Factor VIII cDNA within the regions encoding these amino acid sequences. Alternatively, the present data may serve as a basis for an approach utilizing synthetic peptides. For instance, the epitope of the antibody CLB-CaG 69 was mapped in more detail on a peptide extending from Lys-1673 to Arg-1689 [41]. Our studies on the localization of the epitopes of CLB-CaG A and 1B3 extend previous observations that these antibodies recognize the 80 kDa chain and 90 kDa chain respectively [34]. On the other hand, 1B3 has previously been suggested to bind the 80 kDa chain on the basis of monoclonal-antibody cross-reactivity studies [42]. However, we were not able to confirm this using our direct mapping technique.

In order to establish the involvement of the antibody-binding regions in Factor VIII function, the inhibitory characteristics of the antibodies were studied. Until now, such studies have been limited to one-stage clotting assays [9,19,20,43]. In the present study we also characterized the inhibitory properties of anti-(Factor VIII) antibodies at the level of Factor Xa formation, which is the Factor-VIII-dependent step in the coagulation cascade. This dual approach revealed differences that may have major implications for the interpretation of functional studies with monoclonal antibodies as inhibitory probes: whereas all four antibodies studied were inhibitory in the clotting assay, three of them were non-inhibitory in the spectrophotometric assay (see Fig. 5). This finding suggests that these antibodies interfere in a process that is rate-limiting in clot formation, but not in Factor Xa generation. Previous studies have shown that Factor VIII supports Factor Xa generation only after a short lag period, during which feedback activation of Factor VIII by Factor Xa or thrombin takes place [44,45]. Studies on Factor X activation in plasma have demonstrated that coagulation already occurs as an early event, within the lag period, in Factor Xa formation [45-48]. Accordingly, the activation of Factor VIII that precedes the Factor VIIIa-dependent Factor Xa generation has been suggested to be rate-limiting for coagulation *in vitro*, without affecting the final extent of Factor Xa generated [45]. Therefore the inhibition by the three monoclonal antibodies in the clotting assay may be explained by interference in Factor VIII activation rather than inhibition of Factor VIII cofactor activity itself. This explanation would be in agreement with an earlier proposal concerning the antibody 1B3 [29]. Our suggestion for the inhibition mechanism is supported by the location of the epitopes of the antibodies displaying this inhibitory effect (Fig. 6): all three antibodies are directed against sequences in the close proximity of cleavage sites involved in the proteolytic activation of Factor VIII [10,15].

Several other investigators have described monoclonal antibodies directed against epitopes close to thrombin-cleavage or Factor Xa-cleavage sites, both on the C-terminal region of the 90 kDa chain [9,49] and on the N-terminal region of the 80 kDa chain [19,50]. Although these antibodies do inhibit Factor VIII procoagulant activity, no data are available from inhibition studies on the level of Factor X activation. Therefore it is uncertain whether, as in our studies, Factor Xa generation is unaffected by these antibodies. For structure-function studies based on the epitope mapping of 'inhibitory' antibodies, it should be established whether the antibodies in question actually inhibit Factor VIII cofactor function. Antibodies of this type, such as CLB-CaG A, may be the subject of further studies on the assembly of the Factor X-activating complex. The antibodies binding in the vicinity of cleavage sites may prove of interest for studies on the proteolytic activation of Factor VIII. With regard to the physiological relevance of such studies, it may be noted that the antibody CLB-CaG 69 binds to a sequence that is involved in the high-affinity interaction of Factor VIII with von Willebrand Factor [41]. The interference of this antibody in Factor VIII activation raises the question whether von Willebrand Factor may function as a modulator in the proteolytic control of Factor VIII in plasma.

We thank Dr. R. M. Bertina for kindly providing the monoclonal antibody 1B3, Dr. A. J. J. van Ooyen for sequencing the Factor VIII cDNA, Mrs. P. van de Brink-Zantingh for assistance in the inhibition studies, and Miss E. T. M. van Rijn and Mrs. J. Gerritsen for assistance in preparing the typescript.

REFERENCES

- Kane, W. H. & Davie, E. W. (1988) *Blood* **71**, 539–555
- Van Dieijen, G., Tans, G., Rosing, J. & Hemker, H. C. (1981) *J. Biol. Chem.* **256**, 3433–3442
- Mertens, K., Van Wijngaarden, A. & Bertina, R. M. (1985) *Thromb. Haemostasis* **54**, 654–660
- Wood, W. I., Capon, D. J., Simonsen, C. G., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hollingshead, P., Wion, K. L., Delwart, E., Tuddenham, E. G. D., Vehar, G. A. & Lawn, R. M. (1984) *Nature (London)* **312**, 330–337
- Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Bueker, J. L., Pittman, D. D., Kaufman, R. D., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N. & Hewick, R. M. (1984) *Nature (London)* **312**, 342–347
- Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M. & Capon, D. J. (1984) *Nature (London)* **312**, 337–342
- Sadler, J. E. & Davie, E. W. (1987) in *The Molecular Basis of Blood Diseases* (Stamatoyannopoulos, G., Nienhuis, A., Leder, P. & Majerus, P., eds.), pp. 575–629, W. B. Saunders Co., Philadelphia
- Brinkhous, K. M., Sandberg, H., Garriss, J. B., Mattsson, C., Palm, M., Griggs, T. & Read, M. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8752–8756
- Rotblat, F., O'Brien, S. D. P., O'Brien, F. J., Goodall, A. H. & Tuddenham, E. G. D. (1985) *Biochemistry* **24**, 4294–4300
- Eaton, D., Rodriguez, H. & Vehar, G. A. (1986) *Biochemistry* **25**, 505–512
- Andersson, L. O., Forsman, N., Huang, K., Larsen, K., Lundin, A., Pavlu, B., Sandberg, H., Sewerin, K. & Smart, J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2979–2983
- Vehar, G. A. & Davie, E. W. (1980) *Biochemistry* **19**, 401–410
- Fulcher, C. A., Roberts, J. R. & Zimmermann, T. S. (1983) *Blood* **61**, 807–811
- Lollar, P., Knutson, G. J. & Fass, D. N. (1985) *Biochemistry* **24**, 8056–8064
- Fay, P. J., Anderson, M. T., Chavin, S. I. & Marder, V. J. (1986) *Biochim. Biophys. Acta* **871**, 268–278
- Toole, J. J., Pittman, D. D., Orr, E. C., Murtha, P., Wasley, L. C. & Kaufman, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5939–5942
- Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vehar, G. A. & Gorman, C. (1986) *Biochemistry* **25**, 8343–8347
- Burke, R. L., Pachl, C., Quiroga, M., Rosenberg, S., Haigwood N., Nordfang, O. & Ezban, M. (1986) *J. Biol. Chem.* **261**, 12574–12578
- Fulcher, C. A., Roberts, J. R., Holland, L. Z. & Zimmerman, T. S. (1985) *J. Clin. Invest.* **76**, 117–124
- Fulcher, C. A., De Graaf Mahoney, S., Roberts, J. R., Kasper, C. K. & Zimmerman, T. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7728–7732
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1988) *Nucleic Acids Res.* **12**, 7035–7056
- Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennet, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L. & Goeddel, D. V. (1983) *Nature (London)* **301**, 214–220
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33**, 103–119
- Van Zonneveld, A. J., Veerman, H. & Pannekoek, H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4670–4674
- Tetteroo, P. A. T., Lansdorp, P. M., Leeksa, O. C. & Von dem Borne, A. E. G. Kr. (1983) *Br. J. Haematol.* **55**, 509–522
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Stel, H. V., Sakariassen, K. S., Scholte, B. J., Veerman, E. C. I., Van der Kwast, Th. H., De Groot, P. G. & Van Mourik, J. A. (1984) *Blood* **63**, 1408–1415
- Muller, H. P., Van Tilburg, N. H., Derks, J., Klein-Breteler, E. & Bertina, R. M. (1981) *Blood* **58**, 1000–1006
- Van Ree, R., Clemens, J. G. J., Aalbers, M., Stapel, S. O. & Aalberse, R. C. (1989) *J. Allergy Clin. Immunol.* **83**, 144–151
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Veltkamp, J. J., Drion, E. F. & Loeliger, E. A. (1968) *Thromb. Diath. Haemorrh.* **19**, 279–303
- Veerman, E. C. I., Stel, H. V., Huisman, J. G. & Van Mourik, J. A. (1983) *Thromb. Res.* **33**, 89–93
- Hamer, R. J., Koedam, J. A., Beeser-Visser, N. H., Bertina, R. M., Van Mourik, J. A. & Sixma, J. J. (1987) *Eur. J. Biochem.* **166**, 37–43
- Fulcher, C. A., De Graaf Mahoney, S. & Zimmerman, T. S. (1987) *Blood* **69**, 1475–1480
- Scandella, D., De Graaf Mahoney, S., Mattingly, M., Roeder, D., Timmons, L. & Fulcher, C. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6152–6156
- Wade-Evans, A. & Jenkins, J. R. (1985) *EMBO J.* **4**, 669–706
- Rusconi, S. & Yamamoto, K. R. (1987) *EMBO J.* **6**, 1309–1315
- Nunberg, J. H., Rodgers, G., Gilbert, J. H. & Snead, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3675–3679
- Ware, J., Toomey, J. R. & Stafford, D. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3165–3169
- Leyte, A., Verbeet, M. Ph., Brodniewicz-Proba, T., Van Mourik, J. A. & Mertens, K. (1989) *Biochem. J.* **257**, 679–683
- Tiarks, C., Ghalili, K., Humphreys, R. E., Goodall, A. H., Tuddenham, E. G. D. & Peckett, L. (1987) *Thromb. Res.* **45**, 527–537
- Foster, P. A., Fulcher, C. A., Houghten, R. A., De Graaf Mahoney, S. & Zimmerman, T. S. (1988) *J. Clin. Invest.* **82**, 123–128
- Hultin, M. B. (1982) *J. Clin. Invest.* **69**, 950–958
- Mertens, K. & Bertina, R. M. (1982) *Thromb. Haemostasis* **47**, 96–100
- Jesty, J. & Silverberg, S. A. (1979) *J. Biol. Chem.* **254**, 12337–12345
- Osterud, B. & Rapaport, S. I. (1980) *Scand. J. Haematol.* **24**, 213–226
- Marlar, R. A. & Griffin, J. H. (1981) *Ann. N.Y. Acad. Sci.* **370**, 325–335
- Ware, J., Toomey, J., Fulcher, C. & Zimmerman, T. S. (1987) *Blood* **70**, 396a
- Foster, P. A., Fulcher, C. A., Houghten, R. A. & Zimmerman, T. S. (1988) *J. Biol. Chem.* **263**, 5230–5234