

Epitope mapping factor VIII A2 domain by affinity-directed mass spectrometry: residues 497–510 and 584–593 comprise a discontinuous epitope for the monoclonal antibody R8B12

C. ANSONG, S. M. MILES and P. J. FAY

Department of Biochemistry and Biophysics, University of Rochester, School of Medicine, Rochester, NY, USA

To cite this article: Ansong C, Miles SM, Fay PJ. Epitope mapping factor VIII A2 domain by affinity-directed mass spectrometry: residues 497–510 and 584–593 comprise a discontinuous epitope for the monoclonal antibody R8B12. *J Thromb Haemost* 2006; 4: 842–7.

Summary. The murine monoclonal antibody R8B12 recognizes the C-terminal region (residues 563–740) of the A2 subunit of factor VIIIa [*J Biol Chem* 266: 1991; p. 20139], as judged by Western blotting. However, the location of the epitope within this region is not known. In the present study, we used affinity-directed mass spectrometry to map the epitope. A2 subunit was digested with trypsin or chymotrypsin and then subjected to immunoprecipitation (IP) using R8B12 IgG. Masses of the affinity-selected peptides were determined directly from the immune complexes by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Proteolysis of A2 with the two proteases generated a pre-IP peptide fingerprint that covered ~70% of the A2 domain sequence. Analysis of the post-IP tryptic peptide fingerprint showed two masses, 1309 and 1653 Da representing residues 584–593 and 497–510, respectively, determined from a theoretical database search and confirmed by direct sequencing. Results using a chymotryptic digest yielded a single, weakly reactive fragment consistent with residues 577–586, suggesting the importance of residues Ser⁵⁸⁴-Tyr⁵⁸⁶ in forming the epitope. A synthetic peptide to residues 584–593 was immunoprecipitated by the IgG and blocked R8B12-directed blotting to A2 subunit. The 497–510 and 584–593 segments were observed to be adjacent and surface exposed in the A2 domain model, and together with the above results suggest that A2 domain residues 497–510 and 584–593 represent a discontinuous epitope for R8B12. Furthermore, based upon blotting specificity, we speculate that residues 584–593 make a substantially greater contribution to the binding energy for this interaction.

Correspondence: Philip J. Fay, Department of Biochemistry and Biophysics, University of Rochester Medical Center, PO Box 712, 601 Elmwood Avenue, Rochester, NY 14642, USA.

Tel.: +1 585 275 6576; fax: +1 585 473 4314; e-mail: philip_fay@urmc.rochester.edu

Received 26 October 2005, accepted 19 December 2005

Keywords: affinity-directed mass spectrometry, epitope, factor VIIIa.

Introduction

The hereditary bleeding disorder hemophilia A results from a defect or deficiency in the plasma protein factor VIII (FVIII). The activated form of FVIII (factor VIIIa) assembles with the serine protease factor IXa (FIXa) on an anionic phospholipid surface to constitute the intrinsic factor Xase complex. This complex catalyzes the conversion of zymogen factor X (FX) to the serine protease FXa, a reaction critical for the propagation phase of the coagulation cascade [1–3]. Factor VIIIa serves as a cofactor in the intrinsic Xase, where it increases the catalytic efficiency of FIXa toward FX by several orders of magnitude (see [4] for review). Factor VIIIa is a heterotrimer composed of A1 and A2 subunits derived from cleavage of the FVIII heavy chain and the A3C1C2 subunit derived from the light chain. Following the cleavage, the A1 and A3C1C2 subunits retain a stable association by metal ion-dependent and -independent interactions [5,6], while association of the A2 subunit occurs via relatively weak electrostatic interactions [7,8].

Numerous studies have employed antibodies to FVIII in order to identify structure/function correlates. Historically, this approach derived in large part from the trace nature of FVIII in plasma as well as the propensity for a significant fraction of (severe) hemophilic patients to develop inhibitor antibodies in response to replacement therapy. Implicit in this approach is knowledge of the binding site or epitope for the antibody. For antibodies obtained from the intact protein, including inhibitor antibodies from patient plasmas, mapping epitopes have involved cumbersome techniques including competition or direct binding assays with recombinant or proteolytically derived fragments, peptide competition studies, phage display and/or mutational approaches such as alanine-scanning mutagenesis [9–11].

Affinity-directed mass spectrometry (MS) is a technique developed to rapidly and accurately map protein–protein interactive sites. Zhao *et al.* [12] have used it to study paratope–epitope interactions, defining the epitope of a human growth

factor for a monoclonal antibody. This approach has also been employed to identify new antigenic regions of streptokinase [13]. In the present study, we used affinity-directed MS to fine-point map the epitope of the anti-FVIII A2 domain monoclonal antibody R8B12. Prior results grossly localized the epitope to residues 563–740 following the Western blotting using an activated protein C-cleaved A2 subunit [14]. We now show that the R8B12 IgG binds to a discontinuous epitope in the A2 domain contained within residues 497–510 and 584–593, and suggest that the latter sequence, in particular residues Ser⁵⁸⁴-Tyr⁵⁸⁶, contributes the majority of the binding energy for this interaction.

Materials and methods

Reagents

Recombinant FVIII (KogenateTM; Bayer Corp., Berkeley, CA, USA) was a gift from Dr Lisa Regan. A synthetic peptide representing FVIIIa A2 subunit residues 584–593 (SWYLTE-NIQR) was obtained from Quality Controlled Biochemicals (Hopkinton, MA, USA) and was >90% pure as judged by high performance liquid chromatography (HPLC). The anti-FVIII A2 domain monoclonal antibody R8B12 [8] was obtained from Green Mountain Antibodies (Burlington, VT, USA). The Protein G PLUS-agarose suspension (Calbiochem, La Jolla, CA, USA), the serine protease inhibitor Pefabloc SC (Roche, Indianapolis, IN, USA), MS grade trypsin (Promega, Madison, WI, USA) and chymotrypsin (Sigma, St Louis, MO, USA) were purchased from the indicated vendors.

Isolation of FVIIIa A2 subunit

Isolation of FVIIIa A2 subunit was performed as previously described [15] with minor modifications. The subunit was dialyzed into buffer containing 20 mM HEPES, 100 mM NaCl, 0.01% Tween 20, and 0.1 mM EDTA, pH 7.2, and stored at –80 °C.

Proteolysis of A2 subunit

Factor VIIIa A2 subunit (10–20 µg) was digested with trypsin or chymotrypsin (20:1 wt/wt) under non-denaturing conditions (50 mM ammonium bicarbonate buffer, pH 8.0) for 5–10 min at 37 °C. Digestions were terminated by addition of one-tenth volume of 10 mM Pefabloc SC solution, followed by heating at 100 °C for 5 min.

Immunoprecipitation

Immunoprecipitation (IP) protocols used a modification of that described by Zhao *et al.* [12]. The tryptic or chymotryptic digest was mixed with R8B12 IgG (10 µg) in 60 µL of TSO solution (75 mM Tris-HCl/200 mM NaCl/0.5% *N*-octyl glucoside, pH 8.0) and incubated for 2 h at 4 °C with gentle agitation. A Protein G PLUS-agarose suspension (60 µL) was

added to the solution and incubated for another 1 h at 4 °C with gentle agitation. The solution was then centrifuged for 2 min at 13 000 × *g* and the supernatant was removed by aspiration. The beads were washed three times with 200 µL TSO buffer and then three times with 200 µL TMSK buffer (10 mM Tris-HCl/200 mM NaCl/5 mM 2-mercaptoethanol, pH 8.0). An equal volume of matrix solution [α -cyano-hydroxycinnamic acid (Sigma), 15 mg mL⁻¹ in 1:1 acetonitrile/1% TFA] was mixed with the washed agarose beads. The matrix/agarose mixture (1 µL) was spotted on a matrix-assisted laser desorption/ionization (MALDI) plate and air-dried.

Mass spectrometry

MALDI-time of flight spectra were recorded in reflector mode (positive ion) on a Voyager-DE STR mass spectrometer. Mass spectra were externally calibrated using a set of five MS peptide standards (New England Biolabs, Ipswich, MA, USA). Identification of the peptide masses was performed using the University of California, San Francisco (UCSF) Protein Prospector suite of proteomic tools (<http://prospector.ucsf.edu>; accessed 24 January 2006) and the MASCOT program (<http://www.matrixscience.com>; accessed 24 January 2006) to match the experimental masses to the FVIII sequence (accession number: P00451). Peptide sequence determination was performed using a tandem LC/MS/MS system (Micromass Q-TOF 2, Waters Corporation, Milford, MA, USA) at the Center for Functional Genomics at the University at Albany. The tandem mass spectra were processed using the MASCOT MS/MS Ion Search tool.

Results and discussion

A2 subunit sequence coverage

Treatment of the A2 subunit with trypsin under non-denaturing conditions produced a set of peptide fragments that represented ~40% of the A2 sequence (Fig. 1). These conditions were employed to yield a limit digest focusing on surface exposed regions of the subunit. The sequence coverage identified residues from the first ~270 residues of this 368-residue subunit. Failure to identify fragments from the C-terminal region of A2 likely resulted from the lower concentration of Lys and Arg residues and/or the potential for this region to be less surface exposed, as suggested by the higher relative concentration of hydrophobic residues. The A2 subunit was also treated with chymotrypsin in an attempt to increase the overall sequence coverage, especially within the region comprising the last 100 residues. This digest produced a set of fragments that included peptides, which overlapped with the tryptic peptide fragments providing additional coverage within the first ~270 residues of the A2 subunit as well as identifying several sequence stretches in the C-terminal region.

The use of both proteases resulted in an overall coverage of ~70% of the A2 subunit. This coverage identified peptide fragments representing regions of significant structural

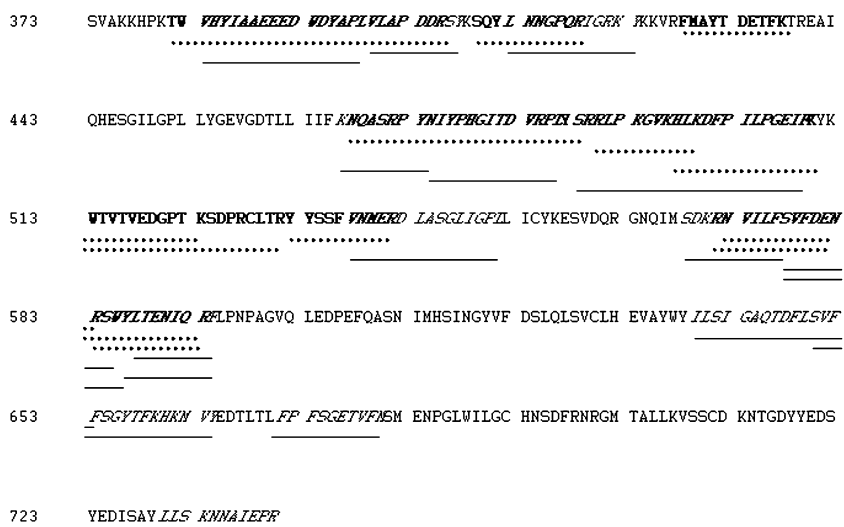


Fig. 1. Sequence coverage of the A2 subunit following the enzymatic digest. Peptides identified are represented by underlined sequences using trypsin (broken lines and bold font) and chymotrypsin (solid lines and italics font). Peptides overlapping both tryptic and chymotryptic digests are represented in bold italic font. The coverage is ~70% of the A2 subunit sequence.

importance in FVIII including residues 373–385 and 418–428 proposed as interactive sites for the A1/A3C1C2 dimer [16]; residues 558–565 that represent an FIXa-binding region [17] and contain the activated protein C cleavage site at Arg⁵⁶² [8]; and residues 484–509 that contribute to factor Xase-catalyzed FXa generation [18] possibly by an electrostatic steering mechanism [19], and comprise the binding site for the potent inhibitory monoclonal antibody 413 [20].

Affinity-directed MS of trypsin-digested A2 subunit

The tryptic digest of A2 subunit was analyzed by MALDI-MS as described in Materials and methods (Fig. 2A). Each peak in the spectrum, spanning m/z of 1176 to 2975, corresponds to a peptide fragment, and those peaks considered to be of significant abundance are labeled. Greater than 90% of the component peptides in the set of peptide fragments generated by the enzymatic digest were identified using sequence matching. The tryptic digest was then incubated with an immobilized R8B12 IgG. Resulting immune complexes comprised of the immobilized IgG and affinity-selected peptides were washed, mixed with matrix and directly analyzed by MALDI-mass spectrometry (MS) (Fig. 2B). Two peptides affinity-selected by the R8B12 IgG exhibited m/z values of 1309 and 1653 that correspond to the A2 subunit fragments 584–593 and 497–510, respectively, as predicted from a theoretical trypsin digest (Table 1). The two peptides were subjected to tandem mass spectrometric analysis in order to confirm their identity (Fig. 2C,D). The *de novo* sequence of both peptides accurately matched to the theoretically predicted A2 domain sequences. The most abundant product ions from the tandem MS spectra of each peptide fragment were submitted to the MASCOT database. Only human FVIII was identified as the protein sequence containing both the m/z 1309 and 1653 peptide sequences. The above results indicate that the FVIII A2

domain residues 497–510 and 584–593 contain portions of the R8B12-binding site thereby indicating that the epitope for this antibody is discontinuous rather than a single linear sequence.

In a previous study, Fay *et al.* [14] demonstrated that the R8B12 antibody recognizes the C-terminal region (residues 563–740) of the A2 subunit of FVIIIa as judged by Western blotting of an activated protein C-cleaved subunit. This result is consistent with the observation that residues 584–593 contribute to the R8B12 antibody epitope. However, residues 497–510 lie outside of this C-terminal region recognized by R8B12 antibody. We speculate that this latter sequence makes a lesser contribution to the antigen–antibody-binding energy and this property dictates the specificity observed in Western blotting.

Affinity-directed MS of chymotrypsin-digested A2 subunit

Similar IP analyses were performed using a chymotryptic digest of the A2 subunit. This digest was employed to extend coverage of the A2 sequences into the C-terminal region of the domain as well as to generate fragments that would overlap with the tryptic digest in an attempt to verify the epitope. The chymotryptic digest produced only one peptide fragment that was recovered in a relatively low abundance following the IP protocol (data not shown). This observation suggested treatment with chymotrypsin may have cleaved within the epitope, reducing antibody affinity. The peptide fragment recovered was identified as A2 domain residues 577–586, predicted from a theoretical chymotrypsin digest. This fragment partially overlaps with the tryptic peptide fragment A2 domain residues 584–593, which were recovered in high relative yield from the tryptic digest following the IP protocol. Together, these results suggest that the tripeptide sequence corresponding to residues 584–586 (Ser-Trp-Tyr) contributes to the epitope along with other residues C-terminal to this sequence.

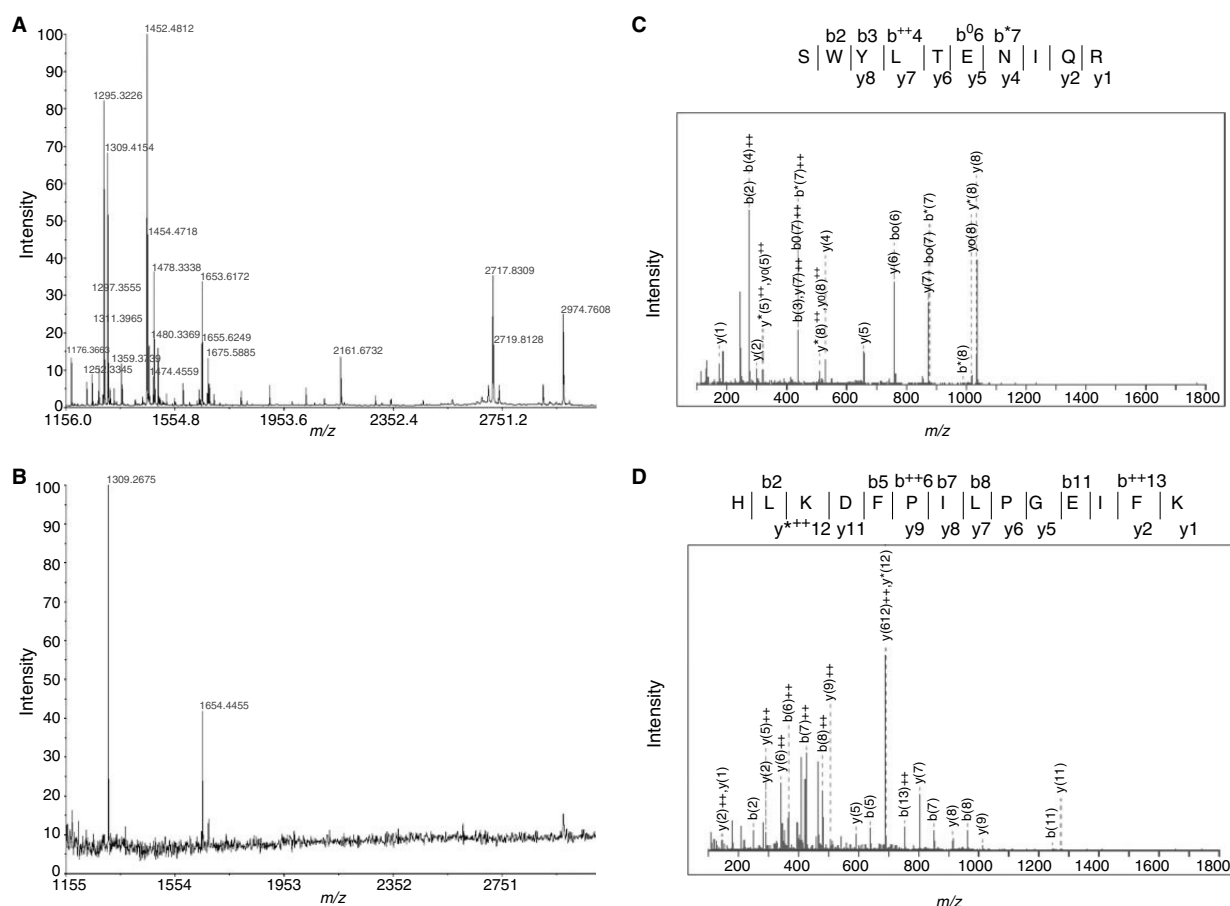


Fig. 2. Mass spectrum of peptide fragments produced by enzymatic digest of the A2 subunit. Spectra were acquired from the tryptic digest of the factor VIIIa (FVIIIa) A2 subunit before (A) and after (B) immunoprecipitation (IP) with monoclonal antibody R8B12. The MS/MS spectrum of the ions at m/z 1309 and m/z 1653 is shown in (C) and (D) respectively. The b ions represent residues sequenced from the N-terminus and the y ions represent residues sequenced from the C-terminus of the peptide ion. Doubly charged ions are denoted as b^{++} and y^{++} . Ions that have lost ammonia (-17 Da) are denoted as b^* and y^* , and ions that have lost water (-18 Da) are denoted as b^0 and y^0 .

Table 1 Match of affinity-selected A2 subunit peptide sequences to theoretically predicted A2 subunit peptide sequences

| No. | m/z submitted | m/z matched | Error (Da) | Length | Position | Sequence |
|-----|-----------------|---------------|------------|--------|----------|----------------|
| 1 | 1309.6357 | 1309.4154 | -0.22 | 10 | 584–593 | SWYLTENIQR |
| 2 | 1653.9184 | 1653.6163 | -0.30 | 14 | 497–510 | HLKDFPILPGEIFK |

Matching of experimental m/z values to theoretically predicted m/z values was performed using the University of California, San Francisco (UCSF) Protein Prospector suite of proteomic tools (<http://prospector.ucsf.edu>; accessed 24 January 2006) and the MASCOT program (<http://www.matrixscience.com>; accessed 24 January 2006).

Binding of a synthetic peptide 584–593 to R8B12 IgG

The above data demonstrate that residues 584–593 make a primary contribution to the R8B12 epitope. To verify this observation, we prepared a synthetic peptide to A2 subunit residues 584–593 and subjected it to the affinity-directed MS assay. As shown in Fig. 3, the synthetic peptide bound the IgG as judged by its detection following the IP assay. Furthermore, when R8B12 IgG (16 nM) was treated with the peptide (1 μ M) prior to use in Western blotting, the resultant density observed on the blot for the A2 subunit was reduced by $>80\%$ compared with that using untreated

IgG (data not shown). These results confirm an important role for residues 584–593 in comprising the epitope.

Visualizing the R8B12 epitope

The above results indicate a discontinuous epitope for the R8B12. The 497–510 and 584–593 segments comprising this binding site were visualized using the FVIII A domain homology model [21]. Both peptide fragments map to regions of the A2 domain that appear to be surface exposed and in close proximity to each other, consistent with these sequences comprising a single interactive site in the folded protein (Fig. 4).

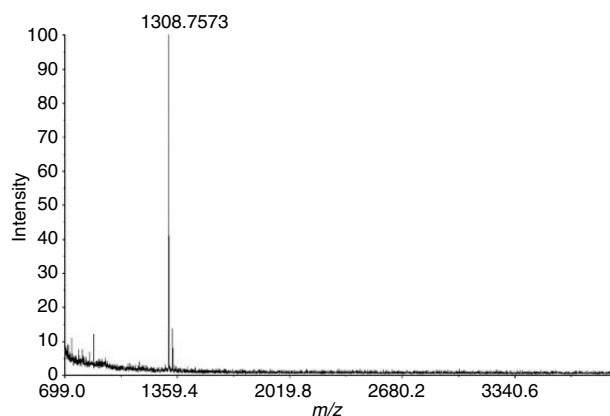


Fig. 3. Binding of a synthetic peptide 584–593 to R8B12 IgG. Mass spectrum shows an aliquot of the immune complex analyzed by MALDI-MS after IP of the synthetic peptide (1 μ M) with the anti-FVIII antibody R8B12 (16 nM).

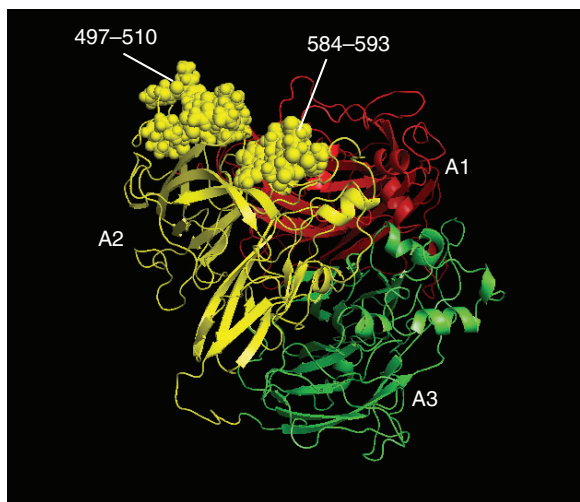


Fig. 4. Visualizing the R8B12 epitope. The ribbon diagram illustrates the factor VIII A1 domain (red), A2 domain (yellow), and A3 domain (green). A2 domain sequences 497–510 and 584–593 are drawn in space-filling format.

Earlier results assessing effects of the R8B12 IgG on cofactor activity showed it to be marginally inhibitory, yielding \sim 80% residual FVIII activity at saturating levels of antibody [22]. We speculate this inhibitory activity derives from binding within the 497–510 segment of the epitope. This region comprises a portion of the sequence (residues 484–510) that maps to the binding site for the potent inhibitory monoclonal antibody 413 [20]. Importantly, this sequence represents an epitope for a number of anti-A2 domain-specific human inhibitor antibodies [9]. Thus, we speculate the weak inhibitory activity of R8B12 may be derived either from its (relatively) lower affinity for the N-terminal portion of its epitope, its binding to only a portion of this segment, and/or steric effects in blocking macromolecular interactions involving this site necessary for cofactor-dependent activation of FX.

Conclusions

In this study, we identify a highly defined epitope for the anti-A2-domain-specific monoclonal antibody, R8B12 using this technique. Fragmentation using two different proteases yielded coverage over \sim 70% of the A2 domain in an MS-based assay. Analyses of the IP data indicated that sequences 584–593 and 497–510 represented a discontinuous epitope, and based upon blotting specificity, suggested that the former segment contributed the majority of the binding energy for this interaction. Furthermore, comparison of the tryptic and chymotryptic data supported a role for residues Ser⁵⁸⁴-Tyr⁵⁸⁶ as directly contributing to the epitope. Thus, the methods described in this report have utility in fine point mapping epitopes for other antibodies to FVIII. This would include monoclonals that demonstrate unique effects on FVIII activity in order to localize functionally important sites in the cofactor, as well as assessing potentially more complex interactions of FVIII with human inhibitors to identify highly immunogenic and/or antigenic regions in the protein.

Acknowledgements

We thank Dr Qishan Lin of the University at Albany Proteomics Facility in the Center for Functional Genomics for the tandem MS analysis. We also thank Dr Lisa Regan of Bayer Corp. for providing recombinant FVIII and Dr Bill Church of Green Mountain Antibodies for providing the R8B12 IgG. This work was supported by Grants HL76213 and HL38199 from the National Institutes of Health. C. A. acknowledges support from an American Heart Association Predoctoral Fellowship. S. M. M. is supported by NIH Postdoctoral Training grant T32-HL07152.

References

- 1 Mann KG, Jenny RJ, Krishnaswamy S. Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu Rev Biochem* 1988; **57**: 915–56.
- 2 Lollar P. Structure and function of Factor VIII. *Adv Exp Med Biol* 1995; **386**: 3–17.
- 3 Fay PJ. Regulation of factor VIIIa in the intrinsic factor Xase. *Thromb Haemost* 1999; **82**: 193–200.
- 4 Fay PJ. Activation of factor VIII and mechanisms of cofactor action. *Blood Rev* 2004; **18**: 1–15.
- 5 Wakabayashi H, Koszelak ME, Mastro M, Fay PJ. Metal ion-independent association of factor VIII subunits and the roles of calcium and copper ions for cofactor activity and inter-subunit affinity. *Biochemistry* 2001; **40**: 10293–300.
- 6 Ansong C, Fay PJ. Factor VIII A3 domain residues 1954–1961 represent an A1 domain-interactive site. *Biochemistry* 2005; **44**: 8850–7.
- 7 Lollar P, Parker CG. Subunit structure of thrombin-activated porcine factor VIII. *Biochemistry* 1989; **28**: 666–74.
- 8 Fay PJ, Haidaris PJ, Smudzin TM. Human factor VIIIa subunit structure. Reconstitution of factor VIIIa from isolated A1/A3-C1-C2 dimer and A2 subunit. *J Biol Chem* 1991; **266**: 8957–62.
- 9 Scandella DH, Nakai H, Felch M, Mondorf W, Scharrer I, Hoyer LW, Saenko EL. In hemophilia A and autoantibody inhibitor patients: the factor VIII A2 domain and light chain are most immunogenic. *Thromb Res* 2001; **101**: 377–85.

- 10 Saenko EL, Ananyeva NM, Kouliavskaya DV, Khrenov AV, Anderson JA, Shima M, Qian J, Scott D. Haemophilia A: effects of inhibitory antibodies on factor VIII functional interactions and approaches to prevent their action. *Haemophilia* 2002; **8**: 1–11.
- 11 Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *Thromb Haemost* 2004; **2**: 1082–95.
- 12 Zhao Y, Muir TW, Kent SBH, Tischer E, Scardina JM, Chait BT. Mapping protein–protein interactions by affinity-directed mass spectrometry. *Proc Natl Acad Sci USA* 1996; **93**: 4020–4.
- 13 Coffey JA, Jennings KR, Dalton H. New antigenic regions of streptokinase are identified by affinity-directed mass spectrometry. *Eur J Biochem* 2001; **268**: 5215–21.
- 14 Fay PJ, Smudzin TM, Walker FJ. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. *J Biol Chem* 1991; **266**: 20139–45.
- 15 Nogami K, Wakabayashi H, Schmidt K, Fay PJ. Altered interactions between the A1 and A2 subunits of factor VIIIa following cleavage of A1 subunit by factor Xa. *J Biol Chem* 2003; **278**: 1634–41.
- 16 Koszelak ME, Huggins CF, Fay PJ. Sites in the A2 subunit involved in the interfactor VIIIa interaction. *J Biol Chem* 2000; **275**: 27137–44.
- 17 Fay PJ, Beattie T, Huggins CF, Regan LM. Factor VIIIa A2 subunit residues 558–565 represent a factor IXa interactive site. *J Biol Chem* 1994; **269**: 20522–7.
- 18 Lollar P, Parker ET, Curtis JE, Helgerson SL, Hoyer LW, Scott ME, Scandella D. Inhibition of human factor VIIIa by anti-A2 subunit antibodies. *J Clin Invest* 1994; **93**: 2497–504.
- 19 Jenkins PV, Dill JL, Zhou Q, Fay PJ. Clustered basic residues within segment 484–510 of the factor VIIIa A2 subunit contribute to the catalytic efficiency for factor Xa generation. *Thromb Haemost* 2004; **2**: 452–8.
- 20 Lubin IM, Healey JF, Barrow RT, Scandella D, Lollar P. Analysis of the human factor VIII A2 inhibitor epitope by alanine scanning mutagenesis. *J Biol Chem* 1997; **272**: 30191–5.
- 21 Pemberton S, Lindley P, Zaitsev V, Card G, Tuddenham EGD, Kembell-Cook G. A molecular model for the triplicated A domains of human factor VIII based on the crystal structure of human ceruloplasmin. *Blood* 1997; **89**: 2413–21.
- 22 Fay PJ, Scandella D. Human inhibitor antibodies specific for the factor VIII A2 domain disrupt the interaction between the subunit and factor IXa. *J Biol Chem* 1999; **274**: 29826–30.