High-resolution mapping of epitopes on the C2 domain of factor VIII by analysis of point mutants using surface plasmon resonance

Phuong-Cac T. Nguyen¹, Kenneth B. Lewis¹, Ruth A. Ettinger¹, Jason T. Schuman², Jasper C. Lin¹, John F. Healey³, Shannon L. Meeks³, Pete Lollar³ and Kathleen P. Pratt¹,4,5

¹Puget Sound Blood Center Research Institute, Seattle, WA; ²GE Health Sciences, Portland, OR; ³Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University, Atlanta, GA; ⁴Division of Hematology, University of Washington, Seattle, WA; ⁵Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

Correspondence to: Kathleen P. Pratt, Ph.D., Department of Medicine (MED) A3075, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. Email: kathleen.pratt@usuhs.edu. PH: 301-295-3607. FAX: 301-295-3557.

Running title: B-cell epitopes on the factor VIII C2 domain
Key points:

1. Amino acid residues comprising B-cell epitopes recognized by neutralizing anti-factor VIII antibodies (inhibitors) have been identified.

2. Amino acids contributing significant antigen-antibody binding avidity are candidates for mutagenesis in the design of less antigenic proteins.

ABSTRACT

Neutralizing anti-factor VIII (FVIII) antibodies that develop in hemophilia A patients and in murine hemophilia A models, clinically termed “inhibitors”, bind to several distinct surfaces on the FVIII-C2 domain. In order to map these epitopes at high resolution, 60 recombinant FVIII-C2 proteins were generated, each having a single surface-exposed residue mutated to alanine or a conservative substitution. Binding kinetics of these muteins to eleven monoclonal, inhibitory anti-FVIII-C2 antibodies were evaluated by surface plasmon resonance (SPR) and results compared to those obtained for wild-type FVIII-C2. Clusters of residues with significantly altered binding kinetics identified “functional” B-cell epitopes, defined as those residues contributing appreciable antigen-antibody avidity. These antibodies were previously shown to neutralize FVIII activity by interfering with proteolytic activation of FVIII by thrombin or factor Xa, or with its binding to phospholipid surfaces, von Willebrand factor, or other components of the intrinsic tenase complex. Fine mapping of epitopes by SPR also indicated surfaces through which FVIII interacts with proteins and phospholipids as it participates in coagulation. Mutations that significantly altered the dissociation times/half-lives identified functionally important interactions within antigen-antibody interfaces and suggested specific sequence modifications to generate novel, less antigenic FVIII proteins with possible therapeutic potential for treatment of inhibitor patients.
INTRODUCTION

The development of neutralizing anti-factor VIII (FVIII) antibodies is a serious complication that may be encountered when FVIII replacement therapy is administered to hemophilia A (HA) patients, affecting 25-30% of the treated HA population with a peak occurrence following ~14 FVIII infusions\(^1-^3\). Autoimmune responses to FVIII can also occur\(^4\), and although this happens only rarely, the resulting bleeding phenotype can be severe. Inhibitors can be difficult and extremely expensive to manage clinically. Interestingly, porcine FVIII has been used effectively in the clinic as a “bypass” therapy, i.e. a therapeutic protein that can evade neutralization by anti-FVIII antibodies in many allo- and autoimmune inhibitor patients\(^5-^7\). However, some patients have or could develop antibodies that neutralize porcine FVIII as well\(^8\), due to antigenic cross-reactivity\(^9\) or because regions in which the porcine sequence differs from the human FVIII sequence stimulate effector T cells, leading to antibody production. Identification of the binding sites (B-cell epitopes) on FVIII that are recognized by inhibitors would allow rational design of novel therapeutic FVIII proteins that are more similar to human FVIII and hence likely to be less immunogenic.

The most common epitopes recognized by hemophilic inhibitors are on the FVIII A2 and C2 domains\(^10,^11\). The FVIII C2 domain (FVIII-C2) mediates numerous functions that are essential for the full procoagulant cofactor activity of FVIII, including membrane binding and assembly of the intrinsic tenase complex\(^12\). The goal of the present study is to identify B-cell epitopes on FVIII-C2 that are recognized by neutralizing anti-FVIII antibodies. In an earlier study\(^13\), competition ELISA assays were employed to characterize 56 murine monoclonal antibodies (mAbs) that bound to FVIII-C2 and blocked FVIII procoagulant activity. Results of these assays indicated there were three distinct epitopes on this domain, types A, B and C, and inhibitory antibodies also bound to partially overlapping epitopes AB and BC. A, B and AB antibodies, termed “classical” anti-C2 antibodies, inhibit the assembly of the intrinsic tenase complex on negatively-charged phospholipid membranes. C and BC antibodies, termed “non-
classical” anti-C2 antibodies, inhibit the proteolytic activation of FVIII to FVIIla by thrombin and/or by activated factor X (FXa). In order to identify the specific amino acid residues comprising these five types of epitopes, 60 recombinant FVIII-C2 mutant proteins (muteins) plus the wild-type (WT) protein (WT-FVIII-C2) were generated using an *E. coli* expression system, 59 with an alanine substitution at a surface-exposed amino acid side chain plus the conservative substitution R2307Q. (The “legacy” numbering for FVIII residues is employed in this study, for consistency with the earlier study13). Surface plasmon resonance (SPR) experiments were carried out to measure binding kinetics of WT-FVIII-C2 and FVIII-C2 muteins to 10 representative mAbs from the series characterized earlier by competition ELISA and functional assays, as well as to the human-derived monoclonal anti-FVIII antibody BO2C1114.

**METHODS**

**Antibodies:** Ten murine mAbs were selected from 56 mAbs characterized earlier using ELISA assays13 as representative of Type A, AB, B, BC and C inhibitors. Murine anti-FVIII C2 domain mAbs ESH4 and ESH8 were from American Diagnostica (Stamford, CT), while mAbs 3E6 (GMA-8013), I54, I109, 1B5 (GMA-8008), 3D12, 3G6 (GMA-8014), 2-77 (GMA-8006) and 2-117 (GMA-8003) were prepared as described previously13 or were kindly provided by Dr. William Church (Green Mountain Antibodies, Burlington, VT). The human anti-FVIII mAb BO2C11 was kindly provided by Dr. Marc Jacquemin. Goat anti-mouse IgG, Fc-γ (115-005-071) was from Jackson ImmunoResearch (Westgrove, PA).

**FVIII-C2 proteins and SPR measurements:** FVIII-C2 proteins were expressed in *E. coli*, purified and analyzed by SPR as described in Supplemental Data. Briefly, SPR measurements were carried out on a Biacore T100 instrument (GE Healthcare Life Sciences) under standard conditions (25°C and 1 atm). Goat anti-mouse IgG specifically directed towards the Fc-gamma fragment was immobilized covalently on all channels of a CM5 chip by amine derivatization. Murine anti-FVIII mAb stock solutions were
injected over the sensor in three flow channels, while the fourth channel served as a reference. BO2C11-Fab was immobilized covalently by amine derivatization. Single-cycle kinetics experiments were carried out in which wild-type or mutant FVIII-C2 proteins were injected serially over the biosensor surfaces at increasing concentrations, without regenerating the biosensor surface after each injection, followed by a 30-60 minute buffer injection to measure dissociation rates. The association (k_a) and dissociation (k_d) rate constants for binding of WT-FVIII-C2 were measured during each set of SPR runs and the resulting k_d values used to compute the k_d(mutein)/k_d(WT) ratios for that set of muteins. FVIII-C2 muteins with a k_d > 2.0X the k_d for WT-FVIII-C2 were considered candidates for B-cell epitope residues. For each of the mAbs, the rate constants for the binding of WT-FVIII-C2 were determined by averaging the results obtained from at least three SPR runs. Dissociation rate constants (k_d), rather than affinities, were chosen as the most relevant metric for identifying “functional B-cell epitopes” because the residence time (1/k_d for a bimolecular interaction) of an antibody-antigen complex indicates its maximum potential lifetime in the circulation. Analysis of residence times is widely utilized in lead optimization studies of potential inhibitory drug targets.

**Visualization of B-cell epitopes:** After the SPR data were collected the crystal structures of FVIII-C2 and B-domain-deleted FVIII were visualized using the graphics program PyMOL to localize the sites showing altered binding kinetics to the mAbs analyzed herein. The cutoff k_d value was chosen empirically, to minimize the number of potential epitope residues located distal from the primary clusters of candidate residues for this series of mAbs.

**RESULTS**

**FVIII-C2 proteins:** WT-FVIII-C2 and 60 FVIII-C2 muteins were purified to >95% homogeneity (Supplemental Figure 1). Six additional FVIII-C2 muteins (S2193A, K2227A, V2232A, K2236A, K2279A and K2281A) were not expressed in a soluble form and were therefore not analyzed. Dynamic light scattering analyses of the purified proteins, carried out for aliquots of each protein preparation both before and after freezing at -80°C, showed a single peak at the expected size for monomeric FVIII-C2.
(not shown). Some of the mutant protein preparations showed evidence of higher molecular weight aggregates; these were not analyzed further and were discarded. Multiple aliquots of each well-behaved FVIII-C2 protein preparation were stored frozen to avoid multiple freeze-thaw cycles that could endanger protein structural integrity.

**Altered binding kinetics due to amino acid substitutions in FVIII-C2:** The amino acid substitutions affected $k_d$ values (relative to the values for WT-FVIII-C2) more than $k_a$ values in almost all cases. Therefore, a cutoff value based on the ratio of measured $k_d$ values of the mutant versus WT protein was chosen to indicate whether the wild-type residues at these positions should be considered as potential contributors to functional B-cell epitopes recognized by the corresponding antibodies. Setting this cutoff value at $k_d > 2.0 \times$ the measured $k_d$ for WT-FVIII-C2 resulted in the identification of 5-18 residues as candidates for the B-cell epitopes recognized by the 10 murine and one human (BO2C11) mAbs. Because all of the mAbs were attached on the biosensor surface, the antigen-antibody binding interactions could be modeled as 1:1 interactions (with each Fab region available to bind a single FVIII-C2 protein).

Representative sensorgrams are shown in Figure 1. Almost all of the FVIII-C2 muteins showed binding kinetics to some or all of the mAbs that were highly similar to the binding of WT-FVIII-C2 (see summary of results in Table 1 and kinetic constants in Supplemental Table 2), indicating the substitutions did not cause global protein misfolding. Thirty-eight of the 60 muteins tested showed altered binding kinetics restricted to a subset of the mAbs. FVIII-C2-F2200A showed altered kinetics in binding to type AB and type B mAbs (I109, BO2C11, 1B5 and 3D12) with a $k_d > 2.0 \times$ that of WT-FVIII-C2. FVIII-C2-R2220A showed altered binding to all mAbs except type A antibodies 3E6 and I54 and type BC antibody 3G6. The ratio $[k_d(\text{mutein})/k_d(\text{WT})]$ for binding to the third type A mAb, ESH4, was 2.7, and with a more stringent cutoff value for this ratio R2220 would not be identified as part of the epitope recognized by this antibody. Removal of the mostly-buried R2220 side chain would be expected to
destabilize the membrane-binding region of FVIII-C2. Therefore its effect on binding kinetics to ESH4 was judged to be a conformational effect and it was not assigned to the epitope for this mAb. The kinetic data from analyzing several preparations of FVIII-C2-F2200A and FVIII-C2-R2220A were more variable and of poorer quality than that of WT-FVIII-C2 and the other muteins, indicating these substitutions altered the protein stability. Nevertheless, the fact that neighboring residues were also pinpointed as parts of epitopes recognized by type AB and B antibodies supported their assignment to these epitopes. Qualitatively, several other FVIII-C2 muteins showed increased antibody-antigen dissociation rates relative to WT-FVIII-C2 although accurate kinetic constants could not be obtained. These results are indicated in Table 1 as “QFD” for “Qualitative Fast Dissociation”.

**Identification of B-cell epitopes:** The residues identified by the cutoff criterion of $k_d$mutein) > 2.0X $k_d$(WT), when visualized using PyMOL, formed distinct clusters indicating the FVIII-C2 surfaces recognized by each mAb (Figure 2). Once a cluster was localized to a specific surface region, the full set of FVIII-C2 muteins was not analyzed by SPR as it was considered sufficient to concentrate on residues adjacent to the initial surface thus identified. Substitutions causing altered kinetics at positions that were conformationally non-contiguous with the SPR-identified clusters of potential B-cell epitope residues are noted below as “outliers”. Specific characteristics of the binding interactions in each antibody-antigen complex are described below, with the inhibitory mAbs identified as Type A, AB, B, BC or C according to the criteria of Meeks et al.13

**Type A Inhibitors:** Three type A inhibitors (ESH4, 3E6 and I54) were evaluated. SPR assays identified the following residues as possibly interacting with all three of these mAbs: D2187, K2207, H2211, L2212 and Q2213. These three epitopes identified kinetically were similar but not identical. Experiments with ESH4 also identified residues E2181, T2202, S2206 and R2220 as possibly contributing to this epitope, but R2220 was excluded subsequently as an outlier, as described above. Experiments with I54 also identified residues E2181 and S2206. These Type A epitopes are
immediately adjacent to the beta turn at FVIII residues 2198-2201, which is one of the two “greasy feet” hydrophobic regions of FVIII that bind to phospholipid membranes.²⁰,²⁵

**Type AB Inhibitors:** Two type AB inhibitors (I109 and BO2C11) were evaluated. SPR assays identified one cluster of residues as possibly interacting with both of these mAbs: F2196, N2198, F2200 and R2220. I109 also possibly interacted with T2202, S2250, L2251, L2252, T2253, S2254 and H2315, and BO2C11 also possibly interacted with E2181 (outlier), M2199, R2215, Q2270 (outlier) and Q2316. Thus the type AB inhibitors were seen to bind either or both of these hydrophobic beta turns.

**Type B Inhibitors:** Two type B inhibitors (1B5 and 3D12) were evaluated. SPR assays identified the following residues as possibly interacting with both of them: F2196, N2198, F2200, T2202, R2220, N2225, E2228, L2252, S2254 and Q2316. Ala substitutions at residues T2197, Q2222, K2239 (outlier) and H2315 also affected binding to 1B5, while Ala substitutions at residues Y2195, M2199, N2224, K2249, S2250, L2251, T2253 and H2309 affected binding to 3D12. The type B epitopes include the two hydrophobic beta turns, as well as migrating further up the “back face” of the molecule to include the loop from N2224-E2228 and H2309. Being of type AB, the epitope of BO2C11 overlaps with the type B inhibitors, 1B5 and 3D12, at one of the hydrophobic beta turns.

**Type BC Inhibitors:** Two type BC inhibitors (3G6 and 2-77) were evaluated. SPR indicated the following residues as possibly interacting with both of them: N2225, E2228, L2273, R2307 and H2309. Ala substitutions at residues T2197 (outlier) and Q2270 affected binding only to 3G6, while Ala substitutions at R2220 (outlier), K2239 (outlier), H2269 and V2280 affected binding only to 2-77.

**Type C Inhibitors:** Two type C inhibitors (2-117 and ESH8) were evaluated. SPR indicated the following residues as possibly interacting with both 2-117 and ESH8: R2220 (outlier), T2272, L2273, V2282 and H2309. Ala substitutions at residues H2269 and Q2270 affected binding only to 2-117 while
Ala substitutions at residues V2280 and Q2311 affected only ESH8 binding. The conservative substitution R2307Q affected binding to mAb 2-117 but not to ESH8.

**Modeling of epitopes in the FVIII structure:** Figure 3A shows the BC and C epitope residues that comprise “non-classical” inhibitor antibodies, e.g. antibodies that prevent FVIII activation by thrombin and/or FXa. Figure 3B shows the location of the epitopes recognized by Type A, AB, B, BC and C antibodies.

**DISCUSSION**

The SPR experiments identified three distinct clusters of surface-exposed side chains on FVIII-C2 that contributed significant binding avidity for Type A, B and C FVIII-neutralizing antibodies, plus two clusters containing residues that comprised overlap regions for mAb types AB and BC, respectively. SPR experiments were carried out for three type A and two each of types AB, B, BC and C mAbs. The resulting assignments of epitope residues were consistent within each mAb type and were also consistent with the competition ELISA experiments13, with peptide-based epitope mapping of FVIII-C226,27, as well as with ELISA assays evaluating binding of the mAbs to the following FVIII muteins: F2196L, K2227E, M2199I/F2200L, V2223A/K2227E and M2199I/F2200L/L2251V/L2252F13. A recent analysis of antibodies purified from a subject with an autoimmune response to FVIII (acquired hemophilia A), indicated that these included antibodies with epitopes similar to those recognized by ESH4 (Type A) and ESH8 (Type C)28. The bleeding phenotype of acquired hemophilia A is often more severe than that of congenital severe hemophilia A, possibly because these antibodies bind to a somewhat different set of immunodominant B-cell epitopes and block FVIII functionality more effectively.

The strategy chosen to identify specific residues as members of a B-cell epitope by SPR was to compare the experimental dissociation rate constant $k_d$ for a given FVIII-C2 mutein with the $k_d$ for WT-FVIII-C2 dissociating from the same antibody, noting which substitutions increased the $k_d$ to >2.0X that
of WT-FVIII-C2. Once one or more muteins with this property were identified, the FVIII-C2 structure was visualized using PyMOL\textsuperscript{23}. Care was taken to analyze the FVIII-C2 muteins with substitutions in close proximity to the initial cluster of surface-exposed residues identified by their altered $k_d$ values, but it was not considered essential to analyze the entire series of muteins for each antibody. The precise mapping of this series of B-cell epitopes, combined with the earlier analysis of the biochemical events (intrinsic tenase assembly, proteolytic activation of FVIII, etc.) that were blocked by each type of inhibitor\textsuperscript{13}, pinpointed specific surfaces on the FVIII protein that interact with its partners in promoting blood coagulation. The lists of residues comprising these epitopes (Table 1) are not comprehensive, because not all surface residues were mutated, and several substitutions affected protein stability so were not analyzed further. However, the coverage of the protein surface was sufficient to definitively identify distinct clusters of residues contributing to specific antigen-antibody binding avidities.

The type A, AB and B inhibitors interfere with FVIII or FVIIIa binding to phosphatidylinerine-containing phospholipid membranes\textsuperscript{13}. As expected, the epitopes recognized by the AB and B mAbs included the hydrophobic beta hairpin turns as well as residues H2315-Q2316, which are known to participate in membrane binding\textsuperscript{25,29}. ELISA experiments reported earlier\textsuperscript{13} showed that the epitope recognized by Type AB inhibitor I109 includes residues M2199 and F2200, and the SPR results confirm that this mAb also binds to the second hairpin turn containing L2251 and L2252. Interestingly, the epitopes recognized by the Type A inhibitors ESH4, I54 and 3E6 are poised just above these projecting hairpin turns, and their inclusion of charged residues (E2181, D2187, K2206, K2207) indicates that these inhibitors block electrostatic interactions that could otherwise form between the positively charged FVIII side chains and negatively charged membrane surfaces. The kinetics of FVIII neutralization by inhibitory antibodies have long been classified as Type I or Type II\textsuperscript{30,31}. Type I inhibitors completely block FVIII activity at saturating concentrations, whereas Type II inhibitors do not completely inhibit clotting, even at saturating levels. Mabs I54 and 3E6 are Type I inhibitors, while ESH4 is a type II inhibitor. The three Type A epitopes identified by SPR are highly similar (Figure 2). The different
inhibition kinetics might be a result of binding to FVIII with different affinities/avidities or with a slightly
different orientation that does not completely preclude FVIIIa assembly into the intrinsic tenase
complex. The patient-derived inhibitory mAb BO2C11 is a Type AB antibody that buries a large surface
area on the FVIII C2 domain. A crystal structure of a BO2C11-Fab fragment to FVIII-C2 identified
15 FVIII side chains that contacted the antibody. However, SPR-based analysis of BO2C11 binding to
a series of FVIII-C2 muteins has shown that fewer than half of these side chains contributed sufficient
binding avidity to be considered part of this “functional” B-cell epitope. Similarly, we expect that the B-
cell epitope residues identified herein comprise a subset of the actual contact areas between each
inhibitory antibody and FVIII. This expectation has been confirmed, in part, by recent crystallographic
studies illustrating the epitope recognized by another Type BC inhibitor (mAb G99), and by an
NMR/mass spectrometry study that identified several FVIII-C2 surfaces occluded by binding of four
neutralizing mAbs. SPR-based assignments of epitope residues presented herein are consistent with
the regions identified using the same or similar mAbs and these other techniques. The advantage of the
mutagenesis-plus-SPR methodology is that it accurately pinpoints specific amino acid side chains that
contribute significant binding avidity, distinguishing them from “bystander” residues at the protein-
protein interface and thus identifying promising targets for B-cell epitope modification.

Type C and BC inhibitors including ESH8, 2-117, 2-77 and 3G6 do not prevent FVIII binding to
phospholipids or to von Willebrand factor (vWF). ESH8 slows the release of thrombin-activated FVIII
from vWF, and a similar mode of inhibition has been reported for IgG from an inhibitor subject,
demonstrating the physiological relevance of this inhibitory mechanism. ESH8 is inhibitory only in the
presence of vWF. The epitope for ESH8 has been localized by immunoblotting to FVIII residues 2248-
2285. ESH8 blocks FVIII activation by FXa and patient-derived antibodies with a similar immunoblot
profile have been shown to block FVIII activation by thrombin. Low molecular weight peptide decoys
that mimic ESH8 epitopes have been used to map this antibody’s epitope, identifying the FVIII regions
2231-2240 and 2267-2270. Recently, peptide array analysis localized this epitope to residues 2265-
The SPR results reported herein confirm that residues T2272, L2273, V2280 and V2282 comprise part of this epitope. The ESH8 epitope also includes residues H2309 and Q2311, which are distal in the linear amino acid sequence of FVIII but adjacent to the other epitope residues on the protein surface. This surface is on the opposite side of the C2 domain from the Type A epitopes (Figures 2 and 3). The epitope recognized by the other type C mAb, 2-117, maps to a similar surface on FVIII-C2. Unlike ESH8, however, it includes residues H2269 and Q2270, which are on a loop adjacent to the ESH8 epitope, and R2307. Unlike ESH8, 2-117 is only weakly inhibitory. Possibly its binding to this loop precludes stabilization of the FVIII-vWF complex and/or the facilitated removal of thrombin-cleaved FVIIIa that has been hypothesized for the other Type C inhibitor, ESH8. The substitution R2220A affected binding to type C mAbs 2-117 and ESH8, increasing their $k_d$ constants by 3.0X and 4.5X, respectively. R2220 is not conformationally contiguous with the other residues identified by SPR, therefore it was identified as an “outlier” and not included as part of these functional epitopes. The epitopes recognized by Type BC mAbs 2-77 and 3G6 overlap the Type C epitopes but also include N2225 and E2228, which are part of Type B epitopes and are further down the “back” face of FVIII-C2 towards the membrane-binding surface. Type C and BC antibodies inhibit FVIII activation by thrombin and/or FXa, but most of those analyzed to date did not compete effectively for FVIII binding to vWF. These antibodies are considered “non-classical” inhibitors because their identification pointed to a previously under-appreciated and important role for the FVIII-C2 domain in proteolytic activation of FVIII. Their localization at a surface distinct from the other types of epitopes, and also on an outer surface of the FVIII protein (i.e. not at an interdomain interface) is shown in Figures 3A and 3B.

The mapping of this series of FVIII-C2 domain epitopes will facilitate additional studies to model the domain orientations in FVIII (which may well differ among the solution, vWF-bound and membrane-bound FVIII structures), as well as the interactions between FVIIIa and the other components of the intrinsic tenase complex. The high-resolution definition of these physiologically relevant and medically important epitopes also suggests specific sites at which the FVIII sequence could be modified.
to generate less antigenic FVIII variants. We propose that next-generation therapeutic FVIII proteins could include rationally designed variants of human FVIII that, similar to porcine FVIII, could provide at least short-term hemostatic support in patients with high-titer inhibitors. The C2 domain of porcine FVIII contains 32 residues that differ from the human FVIII sequence. Soluble FVIII-C2 proteins with alanine substitutions at 20 of these sites were characterized by SPR. The human residues at 14 of these sites were identified as contributing to B-cell epitopes recognized by neutralizing anti-FVIII antibodies (Figure 3C).

Both antigen-antibody affinities and residence times (which reflect avidity of binding) of these complexes are of interest in characterizing inhibitor responses. Amino acid substitutions at the antigen-antibody interfaces decreased some of the binding affinities ($\Delta \Delta G$ values) by 0-18 kJ/mol (Supplemental Table 2). Because $k_d$ values are directly related to residence times and dissociation half-lives (half-life = $\ln 2/k_d$), and hence the expected duration of FVIII neutralization by inhibitor antibodies, functional B-cell epitopes were identified on the basis of the effects of amino acid substitutions on $k_d$ constants. The results presented herein may be used to target functional B-cell epitopes, including critical residues in antigenic loops in the FVIII A2 domain and in other regions of FVIII, in designing novel FVIII muteins that could provide useful bypass therapy options for inhibitor patients. Because their sequences would be closer to that of the FVIII used to treat the original bleeding disorder, the risk of provoking new T-cell responses and subsequent new inhibitors to such rationally designed therapeutic FVIII muteins might also be lowered, in comparison with porcine FVIII used as bypass therapy. We expect that sequence modifications to neutralize immunodominant B-cell and T-cell epitopes will eventually be a feature of therapeutic FVIII proteins targeted to patients with refractory inhibitor responses, as well as to patients with poor prognostic factors such as high-risk $F8$ gene mutations or a family history of inhibitors.
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AUTHORSHIP CONTRIBUTIONS

PCN, KBL, RAE, JTS and JCL carried out the experiments; KBL, PCN, JTS, RAE, JCL and KPP designed the experiments; JFH, SLM and PL performed monoclonal antibody isolation and characterization; PCN, KBL, RAE, JTS, JCL and KPP analyzed the data and wrote the manuscript that was reviewed and approved by all authors.

DISCLAIMER

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

DISCLOSURE OF CONFLICTS OF INTEREST

KPP and KBL are inventors on a patent (International Patent Application No. PCT/US2012/61/553,660) that describes characterization of B-cell epitopes using SPR. JTS is an employee of GE Healthcare. KPP has received unrestricted research funding from Bayer, Pfizer and CSL Behring and honoraria from Bayer, Pfizer and Novo Nordisk.
DEDICATION

This paper is dedicated with gratitude and respect to Dr. Dorothea Scandella, who forged paths we continue to follow and extend in hemophilia research.
REFERENCES


Table 1. Criteria for assigning residues to B-cell epitopes: (1) FD = Fast Dissociation. The $k_d$(mutein) was >2.0X the $k_d$ for WT-FVIII-C2 binding to this mAb. (2) QFD = Qualitative Fast Dissociation. In these cases, the $k_d$ could be estimated (by visual inspection) as >2.0X the $k_d$ for WT-FVIII-C2, but the quality of the sensorgram was insufficient to fit the data to theoretical binding curves as required to determine accurate kinetic constants for

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these interactions. (3) NB = Non-Binding. These amino acid substitutions completely abrogated binding to the antibody. Shaded cells: These alanine substitutions resulted in $k_d$(mutein) $>2.0X$ the $k_d$ for WT-FVIII-C2 binding to this mAb, but these residues were not conformationally contiguous with the other surface-exposed candidate residues identified using the same kinetic criterion. Therefore, these “outlier” residues were not assigned to the corresponding epitopes. These substitutions likely had a localized effect on the stability/conformation of the protein, rather than disrupting specific interactions between the native FVIII side chains and the mAb. Twenty-two of the 60 FVIII-C2 muteins tested did not show altered binding kinetics to any of the mAbs (Supplemental Table 2) and therefore were not assigned to any of the functional B-cell epitopes.
FIGURE LEGENDS

Figure 1. Representative superimposed sensorgrams showing single-cycle kinetics experiments in which WT-FVIII-C2 and FVIII-C2 muteins were injected at five increasing concentrations over biosensor flow channels with captured murine anti-FVIII mAbs, as indicated. Residues were flagged as potential contributors to the epitope if the $k_d$ for the FVIII-C2 mutein was $>2.0X$ the $k_d$ for the wild-type protein. A. Alanine substitutions at residues E2228, L2252, S2254, H2315 and Q2316 met this criterion in this SPR run with mAb 1B5 (which is a type B inhibitor). Separate SPR runs identified residues F2196, T2197, N2198, F2200, T2202, R2220, Q2222, N2225 and K2239 (subsequently identified as an outlier) as also possibly contributing to the epitope recognized by mAb IB5. B. WT-FVIII-C2 and FVIII-C2 muteins Y2195A, Q2213A, N2225A, Q2270A and T2272A were injected over the flow channel containing mAb I54 (which is a type A inhibitor). The substitution Q2213A abrogated binding to this mAb, indicating that Q2213 forms part of the epitope recognized by I54. C. WT-FVIII-C2 and FVIII-C2 muteins Y2195A, Q2213A, N2225A, Q2270A and T2272A were injected over the flow channel in a second SPR run to analyze interactions of FVIII-C2 muteins with mAb 1B5. Altered binding kinetics indicated that residue N2225 forms part of the epitope recognized by 1B5. D. WT-FVIII-C2 and FVIII-C2 muteins Y2195A, Q2213A, N2225A, Q2270A and T2272A were injected over the flow channel containing mAb 2-117 (which is a type C inhibitor). Altered binding kinetics indicated that residues Q2270 and T2272 form part of the epitope recognized by 2-117.

Figure 2. The B-cell epitopes indicated by the SPR experiments are visualized using space-filling depictions of the FVIII-C2 domain crystal structure in standard orientation, with the membrane-interacting loops pointing downwards. The FVIII-C2 structure is also shown rotated 180 degrees about the vertical axis for Type AB and Type B mAbs, in order to visualize both sides of the molecule. The B-cell epitopes identified on the basis of altered binding kinetics are color-coded according to FVIII inhibitor type, i.e. A (red/salmon), AB (orange/yellow), B (dark/light green), BC (dark/light blue) and C
(dark/light magenta). The darker colors indicate residues for which amino acid substitutions increased the residence time by at least 10X compared to that for WT-FVIII-C2 binding to this mAb. Substitutions abrogating binding were also colored darker. Substitutions for which accurate $k_d$ values could not be obtained were not colored darker, because their effects on kinetics may have been due in part to effects on protein stability. Several “outlier” residues identified as candidates using the cutoff criterion of $k_d$(mutant) >2.0 $k_d$(WT) are not shown, as they were eliminated following visualization of the FVIII-C2 crystal structure.

Figure 3. Visualization of FVIII-C2 epitopes in the B-domain-deleted FVIII crystal structure\textsuperscript{21}. With the exception of Type A inhibitors, the neutralizing mAbs analyzed herein bound to an outside-facing surface of FVIII, where they would not be expected to interfere with the packing or orientation of FVIII domains. \textbf{A.} The type BC and C epitopes recognized by non-classical inhibitors are shown as space-filling purple spheres in the FVIII structure\textsuperscript{21}. The protein is oriented with the membrane binding residues M2199, F2200, L2251, L2252, K2092 and F2093 pointing down. Also indicated by space filling spheres are residues known to be at the interface between FVIIIa and activated factor IX (FIXa) in the intrinsic tenase complex: region i (FVIII residues 558-565) is colored light blue; region ii consists of residues near residue 712, which is colored gray; region iii (residues 1811-1818) is colored salmon. Note that the Type BC epitope, which corresponds to a docking site for activated thrombin, is on the opposite side of FVIII to the FVIIIa-FIXa interface. \textbf{B.} All of the residues identified as contributing to each of the five types of epitopes are shown as space-filling spheres in the FVIII crystal structure\textsuperscript{21}. \textbf{C.} Venn diagram depictions of specific amino acid residues localized to the B-cell epitopes A, AB, B, BC and C. Amino acid side chains that SPR assays, followed by visual inspection of the FVIII-C2 crystal structure, indicated contribute to functional B-cell epitopes in human FVIII. Amino acid residues in porcine FVIII that differ from the human FVIII sequence at positions corresponding to functional B-cell epitopes identified in this study are indicated in the second Venn diagram.
Figure 1

A. Mab 1B5 (Type B)

B. Mab 154 (Type A)

C. Mab 1B5 (Type B)

D. Mab 2-117 (Type C)
High-resolution mapping of epitopes on the C2 domain of factor VIII by analysis of point mutants using surface plasmon resonance