Analysis of the Human Factor VIII A2 Inhibitor Epitope by Alanine Scanning Mutagenesis*

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Antibodies directed to the A2 domain of factor VIII (fVIII) are usually an important component of the polyclonal response in patients who have clinically significant inhibitory antibodies to fVIII. A major determinant of the A2 epitope has been located by homolog scanning mutagenesis using recombinant hybrid human/porcine fVIII molecules to a sequence bounded by Arg484–Ile508 (Healey, J. F., Lubin, I. M., Nakai, H., Saenko, E. L., Hoyer, L. W., Scandella, D., and Lollar, P. (1995) J. Biol. Chem. 270, 14505–14509). Within this region, human residues Arg484, Pro485, Tyr487, Ser488, Arg489, Pro492, Val495, Phe501, and Ile508 differ from porcine fVIII. We stably expressed in mammalian cells nine active B-domainless human fVIII molecules containing single alanine substitutions at these sites. Their inhibition by a murine anti-A2 monoclonal antibody, monoclonal antibody (mAb) 413, and by three A2-specific alloimmune and two A2-specific autoimmune human inhibitor plasmas was measured by the Bethesda assay. The inhibition of Arg484→Ala, Tyr487→Ala, Arg489→Ala, and Arg492→Ala by mAb413 was reduced by greater than 90% compared with wild-type, B-domainless human fVIII. mAb413 inhibited the most severely affected mutant, Arg489→Ala, 0.01% as well as wild-type fVIII. For all five patient plasmas, the Tyr487→Ala mutant displayed the greatest reduction in inhibition. The inhibition of the Tyr487→Ala mutant by these antibodies ranged from 10% to 20% of that of wild-type fVIII. The inhibition of the Ser488→Ala, Arg489→Ala, Pro492→Ala, Val495→Ala, Ala, Phe501→Ala, and Ile508→Ala mutants by most of the plasmas also was significantly reduced. In contrast, the Arg484→Ala and Pro485→Ala mutants were relatively unaffected. Thus, although mAb413 binds to the same region as human A2 inhibitors, it recognizes a different set of amino acid side chains. The side chains recognized by human A2 inhibitors appear to be similar, despite the differing immune settings that give rise to fVIII alloantibodies and autoantibodies.

Factor VIII (fVIII)† is the large (M, ~300,000) plasma glycoprotein that is missing or deficient in hemophilia A. It contains a sequence of domains designated NH2-A1-A2-B-A3-C1-C2-COOH (1). Inhibitory antibodies (inhibitors) to fVIII can arise as alloantibodies in patients with hemophilia A transfused with fVIII or as autoantibodies in non-hemophiliacs (2). They present a serious challenge in the management of bleeding disorders. Most fVIII inhibitor plasmas contain antibodies that bind to epitopes located in the A2, C2, and/or A3-C1 domains of fVIII (3–8). Porcine fVIII usually exhibits limited cross-reactivity with anti-human fVIII antibodies (9–11), which forms the basis for porcine fVIII therapy of fVIII inhibitor patients (10, 12–16).

We have used recombinant hybrid human/porcine fVIII molecules to map a major inhibitor epitope determinant to a 25-residue segment bounded by residues Arg484→Ile508 in the A2 domain (17). In this region, there are nine amino acid differences between human and porcine fVIII (18). In the present study, we constructed human fVIII cDNAs that encode single alanine substitutions at positions where the human and porcine fVIII sequences differ to identify specific amino acid residues that contribute to the A2 inhibitor epitope. These constructs were stably expressed as active fVIII molecules in mammalian cells, and their inhibition by mAb413 (a model murine monoclonal A2 inhibitor) and by a panel of A2-specific patient antibodies was measured.

EXPERIMENTAL PROCEDURES

Materials—Citrated hemophilia A plasma and normal pooled human plasmas were purchased from George King Biomedical, Inc. (Overland Park, KS). Immunoadfinity-purified, albumin-free recombinant human fVIII was a generous gift from Baxter Biotech. mAb413 IgG was prepared as described previously (6). Heparin-Sepharose was purchased from Sigma. DNA modification enzymes were obtained from New England Biolabs (Beverly, MA) or Promega Corp. (Madison, WI). BlueScript II KS+ phagemid was purchased from Stratagene (La Jolla, CA). Oligonucleotide PCR primers, fetal bovine serum, AIM-V expression medium, geneticin, penicillin, and streptomycin were purchased from Life Technologies, Inc. Deep Vent DNA polymerase was used for all PCR reactions and was purchased from New England Biolabs. cDNAs corresponding to human B-domainless (B–) fVIII (fVIIIdes1–1648), and hybrid human/porcine B–fVIII molecules HP9, HP12, and HP13 were prepared as described previously (17).

Construction of Human B–fVIII Alanine Mutant cDNAs—Residues Arg484→Ile508 in the human fVIII A2 domain are encoded by nucleotides 1507–1581 in the human fVIII cDNA (19). To facilitate construction of the alanine mutants, synonymous MluI and SacII sites at nucleotides 1369 and 1633, respectively, flanking this region were introduced into the human B–fVIII cDNA. Because it does not contain MluI or SacII restriction sites, human B–fVIII cDNA in Bluescript II KS+ was initially used as an intermediate step to introduce the sites prior to construction of MluI/SacII-modified human B–fVIII in ReNeo.

The mutations were made in human fVIII B–fDNA in Bluescript II KS+ by splicing-by-overlap extension (SOE) mutagenesis (20) using conditions as described previously (17). SOE mutagenesis consists of two rounds of PCR using outer primer pairs a (sense) and d (antisense), that are common to all reactions, and inner primer pairs b (sense) and c (antisense), that define the splice site. The a and d primers were 5′-GGG ATG GAA GCT TAT GTC AAA G-3′ and 5′-CTT CCT TGG TCT ACA GAT TCT TTG-3′, respectively. The 5′ residues of a and d correspond to nucleotides 1012 and 1722, respectively, in human fVIII. To
introduce the MluI site, and b and c primers were 5'-GAA ACC TTT AAG ACG CGT GAA GCT A-3' and 5'-TAG CCT CAC GGC TCT TAA AGG TTT C-3', respectively. To introduce the SacII site, b and c primers were 5'-AAA TCA GAT CGG CGG TGC CTG TTA AGG TTT C-3' and 5'-AAT ACA AAG GGG GGA CAT TAC G-3', for Pro455 -> Ala, 5'-CTG ATG TCT CTT CCT ATT C-3' and 5'-GAA TAC AAG GCA CGG ACA TCA G-3' for Tyr479 -> Ala, 5'-CGT CCT TGT GCC TCA AGA AGA T-3' and 5'-AAT CTC CTT GAG GCC AAA GGA CG-3', for Ser484 -> Ala, 5'-CGT CCT TGT TAT GAC ACC AGA AGT C-3' and 5'-GTA ATC TCC TGG CAT ACA AAG GAC G-3', for Arg484 -> Ala, 5'-AAT ACA AAG GGG GGA CAT TAC G-3' and 5'-AAT CAC CTA TCC TCT CTT CGT GAT CCT GCT GAA TAC A-3'; for Pro492 -> Ala, 5'-CGT CCT TTG GCC TCA AGA AGA T-3' and 5'-CTT CAC ATG TGC ACC ACC TGG T-3'; for Phe470 -> Ala, 5'-CAT TTG AGG GAT GCC GCA ATT CT-3' and 5'-AGA ATT GGC GCA TCC TTC AA-3'; and for Ile493 -> Ala, 5'-GCC AGG AGC AGC CAT CAA ATA ATC-3' and 5'-CGT CAG TAT TGG TCT AAG GAG TCT GCT GGC-3', respectively. SOE PCR products were cleaved with MluI and SacII and ligated into the expression vector ReNeo. Following construction, all sequences generated by PCR were verified using an automated ABI 373A sequencer.

Characterization of fVIII Alanine Mutants—Nine B-domainless VIII A2 alanine mutants corresponding to the nine sites where human and porcine sequences differ in the region bound by Arg484-Arg505 were prepared as described under “Experimental Procedures” (Fig. 1). The mutant cDNAs were stably expressed in baby hamster kidney cells, and mutant proteins were partially purified from conditioned medium. Coagulant activity was measured in a one-stage clotting assay, and VIII antigen levels were measured by ELISA to determine the specific coagulant activity as described under “Experimental Procedures.”

When expressed in 10% fetal bovine serum, the specific coagulant activity of the Arg484 -> Ala, Pro485 -> Ala, Ser486 -> Ala, Pro492 -> Ala, Val495 -> Ala, Phe501 -> Ala, and Ile505 -> Ala mutants were significantly lower (28% and 36% of human B fVIII) than wild-type human B fVIII. The specific activity of the Tyr487 -> Ala and Arg488 -> Ala mutants were indistinguishable from human B fVIII. The specific activities of the Tyr487 and Arg488 -> Ala and Arg489 -> Ala mutants were significantly lower (28% and 36% of human B fVIII, respectively). However, when expressed in serum-free medium, the specific activity of the Arg489 -> Ala mutant was not significantly lower than human B fVIII, whose specific activity was unchanged when expressed in serum-free medium. The specific activity of the Tyr487 -> Ala mutant was 72% of human B fVIII, which, although statistically significant, represents a minor reduction in specific activity. The reduction in specific activity of the Tyr487 -> Ala and Arg489 -> Ala mutants may represent proteolysis due to components in fetal bovine serum, which is not observed with human B fVIII or the other mutants.

Inhibition of VIII Alanine Mutants by Murine Monoclonal A2 Inhibitor, mAb413—Previous studies have shown that mAb413 binds to a similar or the same epitope as human A2 inhibitors (6). The inhibition of the VIII mutants by mAb413 was measured using the Bethesda assay and compared with human B fVIII (Fig. 2). The inhibition of four of the mutants, Arg484 -> Ala, Tyr487 -> Ala, Arg489 -> Ala, and Pro492 -> Ala was decreased by greater than 90%. The Bethesda titer of mAb413 toward the most severely affected mutant, Arg489 -> Ala, was only 0.01% of human B fVIII. These results are consistent with our earlier conclusion that residues on each side of the Ser488-Arg489 bond in the region bounded by Arg484-Ile505 contribute to inhibition by mAb413 (17).

Inhibition of Hybrid Human/Porcine B fVIII Molecules by A2-specific Patient Inhibitors—Five patient inhibitor plasmas,
CHA, JM, NS, RC, and WD (Table I), were identified that contain predominantly A2 inhibitory antibodies (“Experimental Procedures”). To further evaluate the A2 specificity of the plasmas, their ability to inhibit three hybrid human/porcine B\(^{2}fVIII\) A2 substitution mutants, HP9 (porcine substitution for Arg\(^{484}–\)Ile\(^{508}\)), HP12 (porcine substitution for Arg\(^{489}–\)Ile\(^{508}\)), and HP13 (porcine substitution for Arg\(^{484}–\)Ser\(^{488}\)) was measured in the Bethesda assay. Purified JM, NS, and RC IgG had previously been evaluated and found to have reduced inhibition of HP9, HP12, and HP13 (17). Fig. 3 shows that inhibition of HP9 by all the plasmas except WD was reduced by greater than 90%. The inhibition by WD of HP9 was 75% of human B\(^{2}fVIII\), which is most likely due to the presence of inhibitory non-A2 antibodies (see “Experimental Procedures”). All the plasmas inhibited HP13 better than HP9 or HP12, suggesting that the antibodies bind human residues within the Arg\(^{484}–\)Ile\(^{508}\) segment, outside the HP13 substitution region (Arg\(^{484}–\)Ser\(^{488}\)). Overall, the data show that A2 binding by the antibodies in the plasmas is directed to the Arg\(^{484}–\)Ile\(^{508}\) determinant.

### Inhibition of fVIII Alanine Mutants by A2-specific Patient Inhibitors

Inhibition of fVIII Alanine Mutants by A2-specific Patient Inhibitors—The inhibition of the nine alanine mutants by CHA, JM, NS, RC, and WD patient inhibitor plasmas was measured by the Bethesda assay (Fig. 4). For all of the plasmas, the Tyr\(^{487}\) → Ala mutant was the least inhibited, suggesting that Tyr\(^{487}\) is important for antibody binding. The Pro\(^{485}\) → Ala mutant was inhibited well by all of the plasmas, suggesting that Pro\(^{485}\) is not antigenically important. RC and WD inhibited Pro\(^{485}\) → Ala significantly better than human B\(^{2}fVIII\), suggesting that Pro\(^{485}\) may contribute unfavorably to binding antibodies in these plasmas. The Arg\(^{484}\) → Ala mutant was inhibited similarly to human B\(^{2}fVIII\) by all inhibitors except NS and JM. All of the plasmas inhibited Ser\(^{486}\) → Ala, Arg\(^{489}\) → Ala, Pro\(^{492}\) → Ala, Val\(^{495}\) → Ala, Phe\(^{501}\) → Ala, and Ile\(^{508}\) → Ala less well than human B\(^{2}fVIII\), except WD, which inhibited Pro\(^{492}\) → Ala and Ile\(^{508}\) → Ala the same as human B\(^{2}fVIII\). Generally, the patient plasmas were superior to mAb413 as A2 inhibitors (cf. Figs. 2 and 4). In particular, Arg\(^{489}\) → Ala, which is the most important energetically in the interaction of fVIII with mAb413, was inhibited relatively well by the patient plasmas.

### DISCUSSION

Alanine scanning mutagenesis has been used widely to study binding sites on proteins (25, 26). Protein-protein interactions are dominated by electrostatic effects, hydrogen bonding, and hydrophobic interactions between large, nonpolar side chains. Alanine is used as a substitute because it is uncharged and has the smallest amino acid side chain except glycine, which is not

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**Table I**

| Inhibitor | Antibody type | Bethesda titert
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<thead>
<tr>
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<tbody>
<tr>
<td>413</td>
<td>Murine mAb</td>
<td>9430 units/mg</td>
</tr>
<tr>
<td>JM</td>
<td>Autoantibody</td>
<td>180 units/ml</td>
</tr>
<tr>
<td>RC</td>
<td>Alloantibody</td>
<td>520 units/ml</td>
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<tr>
<td>NS</td>
<td>Autoantibody</td>
<td>4200 units/ml</td>
</tr>
<tr>
<td>WD</td>
<td>Alloantibody</td>
<td>31 units/ml</td>
</tr>
<tr>
<td>CHA</td>
<td>Alloantibody</td>
<td>59 units/ml</td>
</tr>
</tbody>
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a Titer obtained against human B\(^{2}fVIII\).

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**Fig. 2.** Inhibition of human B\(^{2}\) fVIII and fVIII A2 alanine mutants by murine anti-human A2 mAb413. The Bethesda titers for human B\(^{2}fVIII\) and the mutants were measured and compared as described under “Experimental Procedures.” The inhibition of human B\(^{2}fVIII\) is defined as 100%.

**Fig. 3.** Inhibition of A2-substituted hybrid human/porcine B\(^{2}fVIII\) molecules, HP9, HP12, and HP13 by human inhibitor plasmas JM, RC, NS, WD, and CHA. fVIII inhibition was determined as described in Fig. 2.
The Human Factor VIII A2 Inhibitor Epitope

**FIG. 4.** Inhibition of fVIII mutants Arg484 → Ala, Pro485 → Ala, Tyr487 → Ala, Ser488 → Ala, Arg489 → Ala, Pro492 → Ala, Val495 → Ala, Phe501 → Ala, and Ile508 → Ala by human inhibitor plasmas JM, RC, NS, WD, and CHA. fVIII inhibition was determined as described in Fig. 2.

preferred because it can change the main chain conformation of the protein.

Analysis of protein-protein complexes by x-ray crystallography shows that typically 20–30 amino acid side chains of a protein make contact with its binding partner (27). Interestingly, alanine scanning mutagenesis studies typically reveal that only a few of these residues contribute significantly to the binding energy (28–31). Thus, the structural epitope of a protein, which is defined as the side chains buried at the binding interface, is larger than the functional epitope, which is defined as the side chains that contribute to the binding energy (32). Additionally, the sum of the changes in binding energy of individual alanine mutants typically exceeds the total binding energy for the interaction of the wild-type protein because of side chain interactions (26). Therefore, although alanine scanning mutagenesis can be used to identify amino acid side chains that contribute significantly to binding, it is not possible to determine quantitatively the binding energies associated with individual amino acid side chains.

Although the structural epitope for fVIII A2 inhibitors has not been determined by x-ray crystallography, homolog scanning mutagenesis using recombinant human/porcine hybrid fVIII molecules has located a major determinant for human Arg484–Ile508 segment where human and porcine fVIII A1, A2, and A3 domains based on the ceruloplasmin and plasmin A domain is composed of two homologous subdomains that form a plastocyanin-type β-barrel. Homology models of the fVIII A1, A2, and A3 domains based on the ceruloplasmin and nitrite reductase structures suggest that residues Val483–Glu507 lie in an exposed loop between β-sheets bounded by D482 and Ile508 (34, 35) in close agreement with the region identified by homolog scanning mutagenesis.

In this study, we made nine alanine substitutions within the human Arg484–Ile508 segment where human and porcine fVIII differ. The results with mAb413 are typical of an alanine scanning mutagenesis study. Four of the nine mutants showed greater than 90% decreases in antibody inhibition in the Bethesda assay, and the remainder showed only mild decreases in antibody inhibition (Fig. 2). Arg489 appears to be the most important residue that contributes to inhibition by mAb413, since the inhibition of the Arg489 → Ala mutant was reduced by over 10-fold compared with the other mutants.

Several findings have indicated that mAb413 and patient A2 inhibitors bind to the same structural region in fVIII. mAb413 competes with human A2 inhibitors for binding to fVIII (6). Both inhibit fVIIIa in the intrinsic pathway factor X activation complex by a noncompetitive mechanism (36). Patient A2 inhibitors and mAb413 are poor inhibitors of HP9, which contains substitution of the human Arg484–Ile508 by the homologous porcine segment (17). However, the pattern of inhibition of the patient antibodies toward the alanine mutants is different from mAb413 (Figs. 2 and 4). Thus, although patient inhibitors appear to recognize the same antibody binding loop as mAb413, the residues that contribute to the binding energy differ. mAb413 binding is dominated by Arg489 > Tyr487 > Arg484 > Pro492. Patient antibody binding is dominated by Tyr487 and to a lesser extent by Ser488 → Ala, Arg489 → Ala, Pro492 → Ala, Val495 → Ala, Phe501 → Ala, and Ile508 → Ala. The difference between mAb413 and patient inhibitors is consistent with behavior observed for the binding of antibody D1.3 to hen egg white lysozyme and to an anti-D1.3 antibody (31). X-ray structural analysis shows that, whereas D1.3 binds lysozyme and anti-D1.3 using essentially the same set of combining site residues, the functionally important residues of D1.3 are different for the two interactions.

Another difference between patient antibodies and mAb413 is that patient antibodies inhibit mutants more effectively than mAb413 does. The polyclonal nature of the patient antibodies may account for this behavior. Most likely, A2 antibodies in a given plasma are a population of high affinity antibodies that compete for binding to fVIII because they recognize different functional epitopes within a structural epitope. Single alanine substitutions could greatly reduce the inhibition by a given monoclonal antibody within this population with little change in the inhibition by the entire population. Alternatively, residues within the region bounded by Arg484–Ile508 that are identical in human and porcine fVIII may contribute significantly to antibody binding. These residues were not mutated in this study. This hypothesis must be considered together with the finding that insertion of the porcine Arg484–Ile508 sequence into human B → fVIII decreases the Bethesda titer by greater than 90% in four of the five patient antibodies tested (Fig. 3). It is possible that some porcine replacements do not remove a favorable interaction between antibody and a human residue but instead substitute a residue that is highly unfavorable for antibody binding. If so, substitution of alanine at this position could have little effect on antibody binding. Further analysis of the A2 epitope will require additional mutagenesis and the
testing of A2 monoclonal antibodies isolated from the human A2 inhibitor population.

Alanine substitutions at critical positions produce novel recombinant FVIII molecules that retain procoagulant function and have reduced inhibition by A2 antibodies. Extension of this approach to other FVIII epitopes could lead to the design of a recombinant FVIII molecule that is useful in the treatment of inhibitor patients. However, the reduction in antigenicity toward patient A2 inhibitors achieved by single alanine substitutions is less than that seen with hybrid human/porcine FVIII molecules. Multiple alanine substitutions at inhibitor epitopes may further reduce antigenicity. Alternatively, replacement of the common inhibitor epitopes with porcine sequences may be superior to alanine mutagenesis for therapeutic purposes.

A more general problem is to decrease immunogenicity of FVIII, i.e., to decrease the occurrence of clinically significant inhibitors, in hemophilia A patients who are undergoing FVIII replacement therapy. The regulation of the immune response to FVIII is poorly understood. In particular, it is not clear why only two or three epitopes dominate the FVIII inhibitor response. Structural studies indicate that the entire surface of a protein is immunogenic, at least with selected experimental protein antigens under certain conditions of antigenic stimulus, such as the use of adjuvants (28, 37, 38). An epitope could be immunodominant, by virtue of the concentration or affinity of its cognate antibodies, because of T cell regulation of antibody producing B cells and be independent of the structure of the epitope. Alternatively, the structure of the epitope could contribute intrinsically to immunodominance. In the latter instance, amino acid side chains within the epitope would partly determine the degree to which the immune system can make high affinity antibodies.

All of the patient antibodies in this study appear to recognize a similar epitope (Fig. 4). The consistent pattern of antibody recognition at the amino acid level is remarkable, especially considering that the antibodies come from patients in two very different immunological settings (i.e. alloimmune and autoimmune, Table I). If the A2 epitope is intrinsically immunogenic, then it is possible that mutagenesis of immunodominant residues could yield a less immunogenic FVIII molecule.

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REFERENCES

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