Mapping of Monoclonal Antibodies to Human Factor IX

By Dan Frazier, Kenneth J. Smith, Wing-Fai Cheung, Jerry Ware, Shu-Wha Lin, A.R. Thompson, Howard Reisner, S. Paul Bajaj, and Darrel W. Stafford

We used recombinant DNA techniques to map a panel of six monoclonal antibodies (MoAbs) to regions of the human factor IX molecule. A-2 maps to 17 amino acids at the amino terminus of the heavy chain of IXa; 2D5, an inhibitor of clotting, is defined to 36 amino acids of the first EGF-like domain of human factor IX. A-4, A-5, C10D, and FXCO08 all map to a region of the heavy chain containing amino acids 180 through 310, suggesting an immunodominant site. FXCO08 has been reported to interfere with binding of factor IXa to factor VIIIa.

FUNCTIONAL FACTOR IX is essential for normal hemostasis. A defective factor IX molecule results in hemophilia B, an X-linked hereditary bleeding disorder. Human factor IX has a molecular weight (mol wt) of ~57,000 daltons and contains ~17% carbohydrate.1,2 The first 12 amino terminal glutamic acid residues of the zymogen are modified to ε-carboxyglutamic acids (Gla) in a posttranslational vitamin K-dependent reaction.3-5 These Gla residues are required for function and are responsible for most of the calcium-dependent binding of factor IX to phospholipid.6 In addition to the Gla domain, the light chain of factor IX, like many of the other vitamin K-dependent blood-clotting factors, contains two epidermal growth factor-like domains of unknown function. Factor IX circulates as a zymogen and can be activated by factor VIIa-tissue factor or XIa and calcium,2,8 generating an activation peptide of ~11,000 daltons and activated factor IX (factor IXa). Factor IXa is a serine protease consisting of light and heavy chains joined by a disulfide bond, which, together with its cofactors (factor VIIIa, phospholipid and calcium), activates factor X to factor Xa.9,10

Monoclonal antibodies (MoAbs), which have been used to determine the relationship of protein structure to function,11 are also proving valuable for localizing defects in the abnormal molecules present in some hemophilia B patients.12,13 For these reasons, it is useful to know, as accurately as possible, the specific amino acids that constitute an epitope reactive with a MoAb.

We spliced small DNase I fragments of a human factor IX cDNA into Agt11 to express a nested set of factor IX polypeptides fused to β-galactosidase. Lifts of these recombinant λ phage on filters were probed with specific MoAbs to identify recombinant phage that expressed the epitope (or a portion of the epitope) of interest. Sequencing overlapping DNA fragments from recombinant phage makes it possible to determine a binding site on the factor IX molecule recognized by a particular antibody. Previously,14 we described the mapping of MoAb IX-30 to residues 111 through 132 of the factor IX zymogen. We now report mapping of six additional MoAbs specific for the human factor IX molecule. Included is a summary of the factor IX antibodies that have been mapped to date in our laboratory.

MATERIALS AND METHODS

Expression of fusion proteins. The 1,300 base pair (bp) HaeIII cDNA fragment which codes for amino acids ~3 through 415 of factor IX14 was digested with Dnase I to generate small, random DNA fragments. The HaeIII fragment (100 μg/mL) was incubated at 25°C with Dnase I (10 ng/mL; Worthington, Malvern, PA) in the presence of 1.5 mmol/L MnCl₂.14 The size distribution at each time point of digestion was analyzed by ethidium bromide fluorescence in agarose gels. A 90-minute digestion generated a peak which migrated between 100 and 500 bp. Fragments were fractionated by agarose electrophoresis and the desired size class of DNA was recovered by electroelution. The recovered DNA was phenol-extracted, ethanol-precipitated, and redissolved in 10 mmol/L Tris, pH 8, 1 mmol/L EDTA (TE). Termini of the recovered DNA fragments were rendered coterminal with T4 DNA polymerase (New England Biolabs, Beverly, MA) in the presence of all four dNTPs and ligated to phosphorylated EcoRI linkers (5'-CGCGAATTCGCG-3'). After ligation, the material was digested with EcoRI and gel-purified in 4% NuSieve agarose (FMC, Rockland, ME). Fragments ranging in size from 50 to 500 bp were again eluted, purified, and ligated to phosphatase-treated Agt11 arms (Promega Biotech, Madison, WI). The ligated material was packaged15 and used for screening without amplification. Approximately 90% of the plaques contained fragments of the factor IX cDNA as estimated by in situ plaque hybridization.16

MoAbs. Production and purification of mouse monoclonal antibodies, A-1, A-2, A-4, A-5, A-7, C10D,19 and FXCO0818 have been reported. Antibody A-7, which binds to a metal dependent epitope contained within residues 1 through 42 of the light chain of factor IX (KJ Smith, unpublished observations, June 1987), could not be mapped with fusion protein fragments because its reactivity depends on the modification of glutamic acid to ε-carboxyglutamic acid residues. Description of the properties of 2DS is in preparation. For enzyme-linked immunosorbent assay (ELISA) studies, MoAbs were dialyzed overnight in 100 mmol/L NaHCO₃ (pH 8.2) before biotinylation. Protein concentration was determined by absorbance at 280 nm, assuming an extinction coefficient of 1.4 for a 1-mg/mL solution.

From the Department of Biology and Center for Thrombosis and Hemostasis, and Department of Pathology and Center for Thrombosis and Hemostasis, University of North Carolina, Chapel Hill; Departments of Pathology and Medicine, University of New Mexico, Albuquerque; BCR8 Division of Experimental Hemostasis, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, CA; Department of Medicine, University of Washington and Puget Sound Blood Center, Seattle; and Section of Biochemistry, St Louis University School of Medicine.

Submitted September 1, 1988; accepted April 7, 1989.

Supported by Grants No. HL06350-26 and HL07255-01 (D.W.S.) from the National Heart, Lung and Blood Institute, Bethesda, MD.

Address reprint requests to Darrel Stafford, PhD, Department of Biology, Coker Hall 010/A/CB#3280, UNC, Chapel Hill, NC 27599-3280.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

0006-4971/89/7403-0002$3.00/0
solution. One hundred sixty micrograms of the N-hydroxysuccinimide ester of biotin (Calbiochem, La Jolla, CA) in dimethyl sulfoxide (DMSO) was added per milligram protein, and the reaction was allowed to proceed for two hours at room temperature. After overnight dialysis against MoAb buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl pH 7.2), IgG concentration was determined by immunoelectrophoresis25 using a MoAb standard and 0.5% rabbit antiserum to mouse IgG (Cappel, Malvern, PA). Before immunoelectrophoresis, antibodies were carbamylated by mixing with equal volumes of 2 mol/L KOCN for one hour at 4°C. Antibodies were compared in ELISA assays using human and bovine factor IX as the target antigens. Plates (Falcon 3915, Becton Dickinson, Oxnard, CA) were coated with 50 μL of a 1 g/mL solution of human or bovine factor IX in MoAb buffer and left overnight at 4°C. The plates were washed three times with washing buffer (MoAb buffer containing 5 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.1% Tween-20) and then blocked with 3% ovalbumin. After overnight incubation at 4°C, the plates were washed and the bound antibody was detected with streptavidin-peroxidase as described above. Immunoradiometric assays were conducted as described21 with MoAbs 2D5, IX-30, and C10D on plasma and urine samples from hemophilia B patients.

Screening recombinant antigens with MoAb. Approximately 10,000 plaque-forming units were plated on a 150-mm dish containing a lawn of Escherichia coli Y109023 and incubated at 42°C. The factor IX fusion peptides were induced as soon as the plaques became visible by overlaying the plate with a nitrocellulose filter impregnated with 10 mmol/L isopropyl β-D-thiogalactoside

F IX Fragments

A-2,36
A-2,37
A-2,33
A-2,32
A-2,27
A-2,31
A-2,5
A-2,26
A-2,28
pF IX 4j
pF IX 4b

Gly 183
Thr 172
Arg 180
Gly 218
Asn 199
Val 201
Arg 180
Gln 171
Asp 145
Lys 265
Lys 200
Arg 180
Trp 310
Thr 415

Fig 1. Map of A-2 epitope (gly183 - asn199). Eleven immunoreactive FIX peptides define a region sufficient to bind MoAb A-2 (Gly 183 through Asn 199). The first nine FIX fragments (designated A-2,36; A-2,37; etc.) were derived from λgt11 picked from a library containing small random FIX fragments. Two of these clones (A-2,36 and A-2,37) generate the smallest overlap; this localizes the binding region (shaded area). pFIX 4j and pFIX 4b are plasmids which make partial FIX proteins fused to the phage T7 gene 10 product. The resulting T7 gene 10 to FIX fusion proteins were immunologically detected using western blotting techniques. Inset: Immunoreactive screening (1′) of a λgt11 library containing small random fragments of FIX yields several positives. Subsequent secondary screening (2′) of clones A-2,36 and A-2,37 confirmed that they are genuinely reactive with A-2. DNA sequence analysis of the inserts from these clones yields the amino acid sequence of the FIX fragments.
(IPTG, Sigma, St Louis). After overnight incubation at 37°C, the filter was marked for reference and removed from the plate. Each filter was washed for ten minutes in 50 mmol/L Tris pH 7.5, 150 mmol/L NaCl (TBS) and blocked for two hours in TBS-gel (0.5% gelatin; BioRad, Richmond, CA). About 5 µg of each MoAb was used for 10 mL TBS-gel. MoAbs were preadsorbed in TBS-gel for 10 hours at 25°C in an E coli Y1090 lysate. After preadsorption, the lysate was centrifuged (10,000 g, ten minutes), and the supernatant was added directly to the nitrocellulose filter suspended in 20 mL TBS-gel. After shaking two hours at 8°C, the unbound antibody was removed by washing for ten minutes in TBS-gel, ten minutes in TBS-NP40 (0.1% nonidet P-40, Sigma), and ten minutes in TBS-gel. A second antibody, affinity-purified rabbit anti-mouse IgG (1 µm/mL, Jackson ImmunoResearch, Avondale, PA), was incubated at 25°C in TBS-gel with the filter for two hours and washed as described above. The filter was marked for reference and removed from the plate. Each filter was washed for ten minutes in 50 mmol/L Tris pH 7.5, 0.01 mol/L sodium phosphate, 0.15 mol/L sodium chloride, pH 7.2 (PBS). The blots were incubated in PBS, 0.5% Tween-20, and 125I-Protein A (1 µCi/30 mL) for 1.5 hours, washed in the same buffer without isotope, air-dried, and autoradiographed.

**DNA sequence analysis.** Bacteriophage DNA was prepared from plaque-pure immunoreactive phage by the diethylaminoethyl (DEAE) extraction procedure of Yanisch et al. The purified DNA was restricted with EcoRI and ligated to the sequencing vector M13mp18. The DNA sequence of each insert was determined for both strands with [α-32P]dATP (Amersham, Arlington, IL) and dideoxy chain termination.

**RESULTS**

Figure 1 shows the results obtained by sequencing DNA fragments from nine independent recombinant phage and two plasmid constructions that reacted with MoAb A-2. The minimal overlap for these sequences comprises the 17 amino acids 183 through 199 of the factor IX zymogen. Figure 2 shows the results for antibody 2D5 (H. Reisner et al; manuscript in preparation). Three reactive λgt11 clones were found whose DNA fragments defined the epitope to residues 28 to 93 of the zymogen. Western blots with pFlX 4f (residues 50 through 111) and pFlX 4j (residues 3 through 180) fusion proteins further restricted the reactive area to residues 50 through 93. These observations were confirmed and extended by results obtained from three hemophilia B

**Fig 2.** Map of 2D5 epitope (gln⁵⁰ – leu⁸⁴). Six FIX fragments define a region sufficient to bind MoAb 2D5 (Gln 50 through Leu 84). Initially, three λgt11 clones were picked and sequenced (9.7; 2D5.2; 2D5.17). Western blotting yielded positive results with plasmids pFlX 4f and pFlX 4j. Finally, FIX Seattle₂, a patient with a known deletion, was discovered to bind A-2 as well. The overlap between FIX Seattle₂ and pFlX 4j localizes the binding region (shaded area). Inset a: Autoradiograph shows primary and secondary screening for two λ clones. Inset b: Western blot showed that 2D5 binds to the fusion proteins produced from pFlX 4f and pFlX 4j; 2D5 also binds the FIX control and does not bind any products from the plasmid vector without an insert.
patients. Factor IX Seattle, has a DNA deletion which includes the DNA coding for amino acids 85 through 195. Seattle, has an adenine deleted, which results in residue 85 being converted from aspartic acid to valine while codon 86 is converted to a termination codon. The plasma and urine of each of these patients have an indistinguishable amino terminal fragment of factor IX that reacts with a calcium-dependent polyclonal antibody to factor IX. Factor IX fragments from each of these two patients react with 2D5 but not with IX-30 or C10D in immunoradiometric assays. These results further restrict the epitope of 2D5 to residues 50 through 84 of the zymogen. As expected from the assignment of the epitope to residues 50 through 84, 2D5 fails to react with factor IX Strasbourg, a variant which lacks the first EGF-like domain of human factor IX.

Only one λgt11 clone was found that reacts with MoAbs A-4, A-5, FXCO08, and C10D. The only immunoreactive DNA fragment that we were able to isolate coded for amino acids 147 through 310 of the zymogen. We were able further to define the epitope by isolating the EcoRI fragment coding for residues 147 through 310 of factor IX and removing the sequences coding for amino acids 147 through 179 by cleavage with the restriction endonuclease Aval. The shortened fragment was then recloned into λgt11 and screened with the four antibodies. All four antibodies maintained their reactivity with the translation products of the

---

**Fig 3.** ELISA determinations with human and bovine factor IX. Binding of biotinylated antibodies to solid-phase factor IX was detected using streptavidin-peroxidase as described in the Materials and Methods section. Antibodies shown are A-7 ( ), A-1 ( ), A-2 ( ), A-4 ( ), A-5 ( ), C10D ( ), and FXCO08 ( ). Binding of human factor IX is shown. Inset: Bovine factor IX. C10D is the only tested antibody that shows demonstrable binding to bovine factor IX in the ELISA assay. Autoradiograph showed that A-4, A-5, C10D, and FXCO08 react with λgt11 clone 28M, which codes for FIX residues 180 through 310. (B) Antibody competition for human factor IX. Antibodies at final concentrations of 100 nmol/L were mixed with biotinylated antibodies at 1 nmol/L and added to microtiter plates coated with factor IX. The competing MoAb is shown on the x-axis. Connected lines show groups of unmodified antibodies which behave similarly in competition with biotinylated antibodies. The percentage of binding, shown on the y-axis, was determined by dividing ELISA values obtained with buffer by those obtained with the competing antibody added. A-7 ( ), A-1 ( ), A-2 ( ), A-4 ( ), A-5 ( ), C10D ( ), and FXCO08 ( ).
shortened fragment containing amino acids 180 through 310 (Fig. 3A, inset). In an attempt to define the epitope further, we reisolated the fragment 180 through 310 and digested it with NsiI to remove nucleotides coding for amino terminal sequences 180 through 204 or XmnI to remove nucleotides coding for carboxy terminal sequences 298 through 310. The fragments were recovered, their ends were rendered coterminal, and they were religated into Agt11. Removal of these amino acids from either end resulted in the loss of antigenicity for all four antibodies. Sequencing data confirmed that these three constructions were correct. In addition, the constructions were still in the correct reading frame because the XmnI shortened fragment still reacted with antibody A-2 and the NsiI fragment was reactive with a polyclonal factor IX-specific antibody.

The relative affinities of these antibodies are shown in Fig 3A. All but FXC008 were tested with bovine factor IX; only C10D showed any reaction (Fig 3A, inset). The competition between each of the six biotinylated MoAbs and a 100-fold excess of each of the other antibodies are shown in Fig 3B. A-4, A-5, FXC008, and C10D all compete with one another. By observing that A-4 and A-5 are more effective competitors of A-2 for binding to factor IX than are FXC008 and C10D, one may postulate that A-4 and A-5 lie closer to the amino terminus of the heavy chain than do FXC008 and C10D. The mapping of MoAbs A-1,33 IX-30,14 and 9.914 has been described previously but is included in Fig 4 to present all factor IX antibodies mapped to date in our laboratory.

**DISCUSSION**

The results obtained from mapping nine MoAbs suggest that a limited number of immunodominant regions exist on the surface of the factor IX molecule. Two independent MoAbs, A-1 and 9.9, react with residues 147 through 153 near the first cleavage site for factor IX activation.33,34 Four
independent MoAbs (A-4, A-5, FXCO08, and C10D) were mapped to the region from 180 through 310. All compete strongly with each other and to a lesser extent with A-2.

Although A-4, A-5, FXCO08, and C10D bind to the same region of the factor IX molecule, ELISA assays (Fig 3A) demonstrate differences between A-4, FXCO08, and C10D (A-5 cannot be distinguished from A-4). In addition, C10D cross-reacts with bovine factor IX (Fig 3A, inset), whereas A-4 and A-5 fail to react. Although the affinity of C10D for bovine factor IX was reduced about threefold relative to its affinity to human factor IX, the reaction was readily discernible on nitrocellulose filters spotted with the antigen and in ELISA testing. A-2 competes very weakly with either C10D or FXCO08, suggesting that the epitopes for these antibodies may be further restricted to residues 197 through 310. However, A-2 shows more competition with A-4 and A-5.

Because the fragment that reacts with MoAbs A-4, A-5, C10D, and FXCO08 cannot be appreciably shortened without loss of antigenicity, the four MoAbs are probably conformation-dependent antibodies, which require longer stretches to form a reactive epitope. The conclusion that conformational epitopes can be detected by this system was also reached by Reinach et al, who screened a chicken ß-gal 1 library with antibodies to chicken myosin light chain 2 and demonstrated that certain MoAbs reacted only with large fragments of the myosin light chain.

Because it completely inhibits coagulation (H. Reisner et al., manuscript in preparation), 2D5 is an interesting antibody. It binds to the first EGF-like domain of human factor IX. This domain has been modeled on the 2D NMR structure of human EGF. It binds to the first EGF-like domain of human factor IX. According to this model, residues 52 through 76 another turn. It is generally agreed that immunogenic regions of a protein are found on its surface and that most amino acid substitutions that fail to affect function are found on the surface of the molecule. By comparing the first EGF-like domain of human, bovine, mouse, and canine factor IX, we predict, by these criteria, that residues 59 through 61, 74 to 75, and 80 are on the surface of the factor IX molecule (residue 61 is different in each species). Reisner et al showed that 2D5 fails to bind to a factor IX from a patient with mild hemophilia whose defect is a Gly to Ser conversion at residue 60. Thus, the epitope for 2D5 probably includes residues 59 through 61 of human factor IX.

Because of the high cost of synthetic peptides and the technical difficulties in making long peptides, random fragments expressed as fusion proteins in ß-gal 1 offer a powerful supplemental, or alternative, method for defining epitopes. For example, sets of 2,326 and 409 overlapping hexapeptides would be required to analyze human factor VIII and factor IX in a manner similar to the analysis of myohemerythrin. Because of reading frame and orientation, one needs six times more recombinant phage than synthetic peptides. However, 15,000 to 40,000 recombinants can be screened easily on a single plate. One can, moreover, make larger recombinant peptides, which are more likely to assume a conformation suitable for the detection of conformational epitopes.

Knowing the locus of binding of an antibody to an enzyme is useful in assigning specific functions to definite regions of the factor IX molecule. For example, Bajaj et al. reported that FXCO08 inhibited the binding of factor IXa to factor VIIIa. Their observation, together with the data we reported, suggests that a portion of the heavy chain of factor IX is involved in the binding of factor VIIIa.

Another example of use of MoAb epitope specificity is that 2D5 has been used to localize the region of mutation in a patient with hemophilia B. This same antibody has allowed the defect from additional patients, not yet characterized in detail, to be localized to a small region of the light chain of human factor IX. Almost nothing is understood about the role of the epidermal growth factor domains in factor IX. Knowing that 2D5 binds to this region should allow us to design experiments to answer our questions about this factor. Similarly, the antibody C10D has been used to screen patients to find mutations that lie in the mapped region. A-1 and 9.9 have also proved useful for mapping the naturally occurring dimorphism at residue 148. Characterizing additional antibodies for factor IX and other clotting factors should facilitate development of experimental approaches to defining functional domains and should also permit more rapid characterization of mutant factor IX molecules.

REFERENCES

27. Biggin MD, Gibson TJ, Hong GF: Buffer gradient gels and 35S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80:3963, 1983
41. Wu SM, Dafoe DW, Ware J: Sequence of the cDNA for mouse factor IX. Manuscript in preparation, 1989
Mapping of monoclonal antibodies to human factor IX

D Frazier, KJ Smith, WF Cheung, J Ware, SW Lin, AR Thompson, H Reisner, SP Bajaj and DW Stafford