Antigenic Determinant in Human Coagulation Factor IX: Immunological Screening and DNA Sequence Analysis of Recombinant Phage Map a Monoclonal Antibody to Residues 111 Through 132 of the Zymogen

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As an approach to the study of structure–function relationships in the normal and defective forms of human coagulation factor IX, we have begun to develop a series of monoclonal antibodies against specific sites on the protein. Zymogen and activated forms of normal factor IX were used initially as antigens for the preparation of monoclonal antibodies. Recombinant phage were prepared by cloning small (50- to 500-nucleotide) random DNA fragments from the coding region of a factor IX cDNA clone into the expression vector λ gt11. Immunological screening of these recombinants with mixtures of monoclonal antibodies identified several immunoreactive phage. Further analysis showed that the monoclonal antibody designated IX-30 was generating the positive signals at a frequency of ~1/2,500 recombinants. Subcloning and sequence analysis of the inserted DNA in the immunoreactive phage revealed overlapping in-frame insertions, from which it could be inferred that the site in factor IX recognized by IX-30 is confined to residues 111 through 132 in the light chain. Similar mapping with other monoclonal antibodies should provide additional probes for the protein structure of human factor IX.

**MATERIALS AND METHODS**

**Monoclonal antibodies.** Balb/c mice were immunized intraperitoneally with a mixture of zymogen and activated forms of human factor IX (with adjuvants) over a six-month period at triweekly intervals. After four to six months of rest, they were boosted twice, with the last injection being given five days prior to fusion. Splenocytes were fused with P3X63-Ag8.653 cells. Fusion methodology and screening protocols were similar to those previously described.

For direct enzyme-linked immunosorbent assays (ELISAs), the wells of polystyrene microtiter plates were coated with 75 μL of factor IX or factor IXa solutions containing 25 μg/mL of protein. Plates were blocked with an excess of bovine serum albumin (BSA) prior to use. Assays to determine the influence of diluent cations were done with either 5 mmol/L of Ca²⁺ or 10 mmol/L of EDTA at all steps. Monoclonality was achieved by cloning at limiting dilution and was verified by isoelectric focusing of the antibody preparations.

Nine of these monoclonal reagents, showing ELISA titers for factor IX between 1 × 10⁶ and 1 × 10⁷, were used for screening the recombinant phage library. Four of these showed >1,000-fold enhancement of binding in the presence of Ca²⁺, whereas three showed significant reactivity in the absence of divalent cations.

**Inhibition assays and immunoblots with monoclonal reagent IX-30.** ELISA-based inhibition assays were done as above except that a 1.0 μg/mL solution of factor IX was used to coat the plates. Dilutions (0.5 × 10² or 1.0 × 10²) of IX-30 ascites fluid were preincubated with dilutions of test protein or buffer for two hours at 37°C and were then added to the assay plates. Test proteins included factor IX, factor IXa, and factor X (provided by Drs D.M. Monroe and H.R. Roberts), bovine factor IX (provided by Dr A. Thompson), and human protein C (provided by Dr W. Kisiel).
Samples of the test proteins (10 μg) were analyzed by electrophoresis on discontinuous 10% sodium dodecyl sulfate (SDS) gels as described by Laemmli. The proteins were electrophoretically transferred to cellulose nitrate sheets (Bio-Rad, Richmond, Calif) according to manufacturer's recommendations. The sheets were incubated with a 1/100 dilution of IX-30 ascites, followed by peroxidase-labeled goat anti-mouse immunoglobulin (Jackson Laboratories, Avondale, Penn). Horseradish peroxidase (HRP) reagent (Bio-Rad) was then used to visualize immunoreactive bands. Proteins in gels were also directly visualized by staining with Coomassie brilliant blue.

**Recombinant phage.** Most DNA manipulations were done by established methods. The starting material was a 1.3-kilobase (kb) HaeIII restriction fragment from the cloned human factor IX cDNA. This fragment contains all of the coding sequences for the prothrombin fragment of factor IX. As a positive control for the immunological screening, this fragment was cloned directly into the EcoRI site of the phage vector λgt11 using 12-mer EcoRI linkers. Inserted with the appropriate orientation, this would give a correct reading frame for expression. The 1.3-kb fragment was also used to prepare a library of random, small insertions in λgt11. This was done essentially as described by Nunberg and co-workers, with minor variations. The cDNA fragment (~3 μg) was partially degraded with DNase I (deoxyribonuclease I) to give fragments predominantly in the size range of 50 to 500 base pairs (bp). The ends of these fragments were made blunt with T4 DNA Polymerase. EcoRI linkers were added with T4 DNA ligase, and, following digestion with EcoRI, this material was fractionated by electrophoresis in a 1.5% gel of low-melting-point agarose. A gel slice containing fragments in the desired size range was excised and melted. The DNA was then purified by repeated phenol extractions and ethanol precipitation.

Vector DNA was purified from liquid culture by conventional means. This DNA was then self-ligated, cleaved with EcoRI, and treated with calf intestinal alkaline phosphatase to prevent religation of the arms. The random factor IX fragments were then ligated with vector and packaged into phage particles using extracts prepared according to Grosveld et al.

**Immunological screening.** The recombinant phage were screened for factor IX antigens essentially as described by Nunberg and co-workers. In brief, the following steps were involved: (a) phage were plated at a density of several thousand plaques per 150-mm dish; (b) a few hours after plating, the phage were covered with isopropyl β-D-thiogalactoside (IPTG)-impregnated nitrocellulose filters, and incubation was continued overnight (IPTG is a galactose analog and a potent inducer of bacterial β-galactosidase gene expression); (c) the filters were removed and rinsed, and nonspecific binding was blocked with bovine serum albumin (BSA); (d) filters were rinsed, incubated with monoclonal antibodies overnight at 4 °C; (e) filters were washed, incubated with affinity-purified rabbit anti-mouse serum; (f) filters were washed, incubated with 125I-labeled Protein A, and washed again; and (g) filters were autoradiographed. Positive phage were picked, replated at lower density, and rescreened until pure.

**Sequence analysis.** Positive phage were grown in 100-mL liquid cultures to obtain sufficient DNA for analysis of the inserted factor IX segments. Phage DNA was cut with EcoRI and fractionated by agarose (0.7%) gel electrophoresis. Insert fragments were electrophoresed, purified, and recloned into the EcoRI site of M13 mp8 for sequence analysis. These recloned segments were then sequenced by the dideoxy method, as modified by McGraw, using end-labeled universal primer as the source of radiolabel.

The relevant protein sequences were inferred from DNA sequence. The hydrophilicity/hydrophathy profile was generated by a computer program based on the method of Kyte and Doolittle.

**RESULTS**

**Immunological screening.** As shown in Fig 1a, ~50% of the plaques resulting from ligation of the 1.3-kb factor IX cDNA segment into λgt11 gave positive signals. This was consistent with the expectation that only half of the insertions would be oriented appropriately for expression. This result indicated that our screening procedure was correct, and that at least one of the monoclonals (three were used as a mixture in this experiment) was capable of recognizing the bacterially synthesized factor IX polypeptide.

Subsequent screening with individual monoclonal sera (data not shown) indicated that only one of the antibodies, designated IX-30, gave positive signals. The eight others gave negative results both with the control clone and with the fragment library. When IX-30 was used to screen the fragment library, positive plaques were identified at a frequency of ~1/2,500 recombinants. A positive signal obtained on primary screening of the fragment library is shown in Figure 1b. Three such clones were plaque-purified and analyzed further.

**Sequence analysis.** The first immunoreactive recombinant to be fully characterized had the designation Gor9. The inserted fragment in this phage was 169 nucleotides in length and was shown by DNA sequencing to code for the 37
residues from Pro through Cys in the light-chain region of factor IX. Sequence patterns were obtained from both ends of the insert by recloning the isolated fragment into the sequencing vector M13mp8. Each end had an EcoRI half-linker as expected from the cloning regimen, and the end representing the amino-terminus of the encoded peptide had the appropriate reading frame for expression when placed in the EcoRI site of λgt11.

Two other positive clones from the fragment library were characterized similarly. The results are summarized in Fig 2. The clone designated Gorl contained a 222-nucleotide fragment encoding the 74 residues from Cys to Gly. This sequence covers 35 residues of the light chain, the entire activation peptide region (also 35 residues), and 4 residues of the heavy chain. Because this sequence overlaps one end of the sequence in Gor9, it could be inferred that the antigenic determinant recognized by IX-30 lies in the region of overlap. The overlapping region is confined to the light chain, and as shown in Fig 2, includes the 22 residues extending from Cys through Cys.

The third positive clone, designated Gor7, was found to encode 196 factor-IX amino acid residues, including four residues of the leader peptide, all of the light chain, the activation peptide, and 12 residues of the heavy chain (Arg-Phe). This clone spans all sequences of both Gor9 and Gorl. As such, it imposes no further restrictions on the extent of the antigenic site.

Hydrophilicity profile. Consistent with the prediction of Hopp and Woods, the peptide sequence mapped with IX-30 represents a region of local hydrophilicity in the factor IX protein. The result of a computer analysis demonstrating this feature of the antigenic region is presented in Fig 3. Because the seven residues at the carboxyl end of the mapped region are clearly hydrophilic in character, it seems likely that the essential antigenic determinant may be contained entirely within the preceding 10 or 12 residues.

Immunoreactivity of IX-30. The monoclonal antibody IX-30 appeared to have a limited reactivity with the human factor X preparations as judged by ELISA. Inhibition assays also suggested that both the factor X and protein C preparations were able to inhibit the binding of IX-30 to human factor IX slightly. The extent of this inhibition appeared greater with factor X than with protein C, but was equivalent to no more than a few percent of the factor IX reactivity in either case (-3% and 1%, respectively). These results are shown in Fig 4. Also shown are results obtained with bovine factor IX and activated human factor IX, both of which proved to be highly effective competitors for the binding of IX-30 to human factor IX.

The immunoreactivity of IX-30 was further assessed by immunoblotting. Lanes 1 through 4 in Fig 5 show protein (Coomassie blue) staining of the factor IX, factor IXa, factor X, and protein C preparations. Lanes 5 through 8 show corresponding results obtained by immunological staining with IX-30. The factor X (lane 1) appears to migrate as a single prominent band, as do factor X (lane 3) and protein C (lane 4). The two prominent bands in lane 2 (factor IXa) are due to incomplete activation of the zymogen.

The immunological staining with IX-30 reveals additional bands in the factor IX and IXa preparations (lanes 5 and 6). These bands correspond to specific degradation products lacking a carboxy-terminal segment of the heavy chain. Although barely visible by protein staining, their prominence in the immunoblot is due to the extreme sensitivity of detection. No immunoreactivity was detected in the protein C preparation (lane 8). The factor X preparation contained a small amount of immunoreactive material (lane 7) with an electrophoretic mobility matching that of factor IX. We now interpret this to mean that there is a trace factor IX contaminant in the factor X preparation rather than a limited cross-reactivity of IX-30 with factor X as suggested previously. No immunoreactivity was observed to correspond to the major band of factor X protein. Reduced factor IX and IXa were also reactive in immunoblots. A low molecular weight band tentatively identified as the light
Fig 5. Immunoblotting. Lanes 1 through 4 show the Coomassie blue staining of human factor IX, human factor IXa, human factor X, and human protein C, respectively. Lanes 5 through 8 show the immunological staining with IX-30 of a similar gel.

chain of factor IXa was detected in the activated sample (data not shown).

DISCUSSION

IX-30 epitope. We used the recombinant DNA approach first described by Nunberg and co-workers to map an antigenic site in human factor IX. We showed that the epitope recognized by monoclonal antibody IX-30 lies within a region of 22 amino acid residues in the light chain, extending from Cys to Cys. These results were obtained with the most potent of the nine tested monoclonal antibodies. (IX-30 has an ELISA titer of 1 x 10⁶ even in the absence of calcium ions). It may be simply for this reason that only IX-30 gave positive results, but rescreening with the other monoclonals at lesser dilution yielded no additional clones.

The ability of IX-30 to recognize factor IX and factor IXa in immunoblots on a charged solid-phase matrix suggests that the epitope is primarily sequential in nature. It also is clear from the results presented here that the antigenic site detected by IX-30 can be recognized in various peptide contexts. The peptide sequence mapped with IX-30 represents a region of local hydrophilicity in the factor IX protein, consistent with the analysis of Hopp and Woods, which predicts that such sites will frequently act as antigenic determinants.

Although IX-30 reacts only with factor IX, the region mapped with this antibody has interesting homologies with several other proteins, as has been noted previously by others (Table 1). There is a homology with a consensus sequence for a group of proteins that includes not only the clotting factors, but several growth factors, the LDL receptor protein, and possibly a Vaccinia viral protein. The presence of sites analogous to the IX-30 epitope in other proteins could be taken as evidence of a conserved structural role for this domain. If the region has functional significance, one might imagine that it is involved with a cell-surface receptor. A recent report of a coagulation pathway dependent on factor IX binding to perturbed endothelial cells may lend support to this hypothesis. However, any such evidence will have to be reconciled with the observation that IX-30 does not interfere with coagulation in vitro, even at high concentrations (data not shown).

Generality of the method. Further studies will obviously be required to determine the overall usefulness of this method. Several factors are important for its success. On a purely random basis, only one in six recombinants will be expected to generate a factor IX-specific peptide. This is because an inserted fragment must have both the appropriate orientation (one of two possibilities) and the correct reading frame (one of three) in order to be expressed. In practice, this is not a severe limitation, however, since it is possible to generate and screen very large populations of recombinants.

A more important consideration is that the experimental system requires expression of the antigen as part of a “fusion protein” consisting primarily of bacterial β-galactosidase. Many antigenic sites on the original immunizing protein may not be regenerated in this context. Some epitopes may involve residues that are juxtaposed in the folded protein but distant in terms of the primary amino acid (or DNA) sequence. Others may involve chemical moieties of post-translational origin. These could include any of the carbohydrate additions, β-hydroxylation at Asp, or the well-documented γ-carboxylation of glutamic acid residues in the amino-terminal region.

It may be, therefore, that only a small percentage of monoclonal antibodies raised against the factor IX protein will recognize short “primary-sequence” epitopes such as the one described in this report. However, when one already has a large number of monoclonals, it will probably be worthwhile to attempt this approach. The primary screening can be done with a mixture of many monoclonals, and useful information will be obtained even if only a few yield positive clones. As an alternative means to the same end, one might choose to generate region-specific probes by raising antisera against synthetic peptides.

Table 1. Homologies With Other Proteins

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Regions corresponding to the IX-30 epitope are given in single-letter amino acid code: (a) Human factor IX, (b) Bovine factor IX, (c) Human factor X, (d) Human protein C, (e) Consensus sequence for homologous regions in various other proteins. Residues matching the consensus are underlined.
REFERENCES


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