Mapping of a Putative Surface-binding Site of Human Coagulation Factor XII*

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We have localized the binding epitope(s) of two murine monoclonal antibodies (B7C9 and P5-2-1) that were shown previously to inhibit the activation of human coagulation factor XII by negatively charged surfaces. A factor XII cDNA expression library in λgt11 was screened with antibody B7C9, and 16 immunoreactive bacteriophage were isolated. Fusion proteins from each of the recombinant phage were reactive with both monoclonal antibodies. Two of the phage cDNA inserts were found to code for amino acid residues 6–31 and 1–4–47 of factor XII, respectively, thereby defining the limits of the antigenic peptide to amino acids +1–+31. Each of the remaining 14 recombinant phage contained longer factor XII cDNA inserts that included sequences coding for the amino-terminal 31 amino acid residues. These results were confirmed by direct binding of antibody B7C9 to synthetic peptides containing amino acids 1–14 and 1–28 of factor XII. Further experiments with a set of nested peptides also indicated that amino acid residues 1–4 were essential but not sufficient for binding of B7C9 to the peptides. Hydrophobicity analysis of the amino-terminal region of plasma factor XII revealed a highly hydrophilic region between amino acid residues 5 and 15 that contained positively charged lysine residues at positions 8, 11, and 13. We conclude that a major epitope(s) recognized by monoclonal antibodies B7C9 and P5-2-1 is present in the amino-terminal 28 amino acids of factor XII. It is proposed that binding of these antibodies to factor XII blocks interaction of the positively charged region between residues 5 and 15 with negatively charged surfaces, thereby inhibiting activation.

Human Hageman factor, or coagulation factor XII, circulates in plasma as a single glycopolyptide chain of M, 80,000

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MATERIALS AND METHODS

Enzymes and Chemicals—T4 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, Klonef fragment of DNA polymerase I, EcoRI linkers (octamers), deoxy- and dideoxyribonucleotide triphosphates were purchased from Pharmacia LKB Biotechnology Inc. Restriction endonucleases were also purchased from Pharmacia LKB Biotechnology Inc., except EcoRI which was from Bethesda Research Laboratories. Alkaline phosphatase-conjugated goat anti-mouse IgG, nitro blue tetrazolium (NBT), and bromochloroindolyl phosphate (BCIP), bromochloroindolyl phosphate; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunoabsorbent assay.

1 The abbreviations used are: NBT, nitro blue tetrazolium; BCIP, bromochloroindolyl phosphate; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunoabsorbent assay.

(1) In vitro, the enzymatically inert zymogen form of factor XII is activated following contact with a negatively charged surface such as glass in the presence of the plasma constituents high molecular weight kininogen, kallikrein, and factor XII to form an active serine protease, thus initiating the intrinsic coagulation pathway and blood clot formation (2).

Recently, the organization of the human factor XII gene has been determined (3), confirming the previously published cDNA sequence (4–6) and the amino acid sequence of plasma factor XII (7, 8). Several notable similarities to other mammalian proteins occur within the factor XII polypeptide. These include areas resembling both the type I and type II homologies found in fibronectin, two epidermal growth factor-like regions, a kringle homology found also in prothrombin, plasminogen, and the plasminogen activators, a unique proline-rich region, and the carboxyl-terminal catalytic portion of factor XII that is homologous to other serine proteases (4–8). Other than the catalytic region, the functional significance of these areas has not yet been determined.

Despite our knowledge of the DNA and protein structure of factor XII, its physiological role remains unclear. Those rare individuals who are deficient in factor XII do not have prolonged bleeding times, thereby raising doubts as to the significance of factor XII in normal hemostasis (9). Alternative theories for the function of this abundant plasma protein include roles in inflammation and neutrophil activation (9, 10). Progress in these areas of factor XII biology has been hindered by the absence of a plausible physiological activator of factor XII (2). As an initial step towards the elucidation of this rate-limiting function, we sought to assign the surface-binding site to one of the structural domains in factor XII. Recently, two murine monoclonal antibodies have been shown to inhibit the activation of factor XII zymogen by negatively charged surfaces (11, 12). We have used these antibodies as probes for the identification of the corresponding factor XII epitope(s) by expressing regions of the factor XII molecule as fusion proteins in Escherichia coli (13, 14). The localization of the epitope was confirmed by using synthetic peptides.
Factor XII Epitope

(BCIP) were manufactured by Promega Biotec. λgt11 arms and packaging extracts were from Stratagene. All 5′-containing radioisotopes were supplied by Amersham.

Monoclonal Antibodies—Both hybridoma cell lines were grown in mouse ascites fluid. The monoclonal antibody RT7C9 had been purified by chromatography on a column of protein A linked to Sepharose as described (17). The monoclonal antibody B7C9 had been purified by affinity chromatography on a column (0.2 X 30 cm) of Bio-Gel A-50m, 100-200 mesh, in 0.1 M acetic acid, followed by dialysis against phosphate-buffered saline and chromatography with protein A-Sepharose (15).

Preparation of Factor XII cDNA Library in λgt11—E. coli containing plasmid pCH1501 (4) was grown in LB broth containing tetracycline (12.5 μg/ml), and plasmid DNA was purified by the alkaline lysis method with cesium chloride density centrifugation (16). Plasmid DNA was subsequently dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and precipitated with ethanol, and its concentration was estimated by absorbance at 260 nm. Approximately 100 μg of plasmid DNA was sheared in a Branson sonic power cell disruptor five times for 5-s intervals, and the sonicated fragments were rendered blunt-ended by using T4 DNA polymerase (17). The blunt-ended DNA fragments were fractionated by electrophoresis on a 5% polyacrylamide gel (16) using Hifi-distilled pBR322 as a molecular weight standard. DNA fragments in the 200-300-base pair range were excised from the polyacrylamide gel and electroeluted into 0.4 ml of TBE buffer (0.09 M Tris base, 0.09 M boric acid, 0.0025 M EDTA; pH 8.3) (16) in a 5 × 20-mm piece of dialysis tubing. DNA fragments were collected by ethanol precipitation and recovered by ethanol precipitation. Quantitation of the DNA was done by the ethidium bromide-agarose spot method (16). EcoRI linkers were end-labeled using [γ−32P]ATP and T4 polynucleotide kinase (16) and ligated to the blunt-ended factor XII cDNA with T4 DNA ligase polymor by the alkaline phosphatase conjugated goat anti-mouse IgG and the enzyme substrate p-nitrophenyl phosphate (Sigma).

RESULTS

Screening the Factor XII cDNA Library with the B7C9 Monoclonal Antibody—Approximately 1 × 108 bacteriophage from the factor XII 200-300-base pair λgt11 cDNA library were plated on E. coli 1088r−, and fusion proteins were in duced with isopropyl-1-thio-β-D-galactopyranoside (16). After transfer to nitrocellulose, the plaques were incubated with the monoclonal antibody B7C9 followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG and the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and/or nitroblue tetrazolium (NBT) as described above.

Competitive ELISA of factor XII peptides was performed by incubation of 10 ng of B7C9 antibody in 200 μl of TBS buffer containing 0.05% Tween 20 and 0.025% gelatin with 1-2000 nmol of peptide in an equal volume of the above buffer for 1 h at room temperature, followed by 1 h at 4°C. The antigen/antibody mixtures (100 μl) were then added in triplicate to Immulon-2 (Dynatech, Fisher) microtiter wells onto which 100 ng of factor XII peptide 1-28 had been allowed to bind by incubation overnight at 4°C. B7C9 antibody binding to the factor XII peptide 1-28 was detected by colorimetric assay using alkaline phosphatase-conjugated goat anti-mouse IgG followed by the enzyme substrate p-nitrophenyl phosphate (Sigma).

Characterization of the Immunoreactive Phage—The factor XII cDNA insert was released from the vector DNA by digestion with EcoRI followed by purification of the insert DNA by electrophoresis on a 5% polyacrylamide gel. After subcloning into the EcoRI site of bacteriophage M13mp18, the factor XII cDNA sequences were determined by the chain termination method (20). The cDNA sequences of 16 of the 20 positive phage were determined. Each insert contained DNA coding for a region of factor XII (Table I). Many of the factor XII fragments began at a common point (amino acid 6 of the hydrophobic leader sequence) resulting from shearing within the poly(G-C) region from the cloning procedure (4). However, only clones 6 and 17 of the 16 factor XII insert DNAs isolated were identical, attesting to the random nature of the factor XII plasmid DNA fragments generated by sonication. The two shortest factor XII inserts, contained in phage clones 9 (amino acids 6-31) and 16 (amino acids 74-467), limit the B7C9 epitope to the amino-terminal 31 amino acids of factor XII (summarized in Fig. 1). Each of the other 14

20 strongly positive and 26 weakly positive B7C9 immunoreactive plaques were identified on eight separate filters. Only the strongly positive plaques were examined further. Each of the 20 positive phage populations were rescreened until a single immunoreactive phage population was obtained. DNA was then isolated from each of the positive phage.
factor XII insert DNAs also contained DNA coding for this peptide (Table I). The DNA sequences of the four remaining phage inserts were not analyzed because their longer lengths made it unlikely that new information would result. Because the factor XII plasmid pHXXI501 was originally isolated from a human liver cDNA library in the plasmid pKT218 (24) generated by using the G:C tailing method, seven of the 16 factor XII insert DNAs contained a poly(G-C) tail at one end (Table I). In some instances, this created a problem in reading the DNA sequence due to stalling of the Klenow fragment of DNA polymerase I during the chain termination reactions. This technical difficulty was overcome by determining the DNA sequence of the complementary strand of insert DNA. This technical difficulty was overcome by determining the DNA sequence of the complementary strand of insert DNA by immunoscreening of the phage to that originally chosen (25).

All of the data generated using λ phage clones selected with antibody B7C9 were consistent in identifying the region containing amino acids 1-31 as the B7C9 immunoreactive region of factor XII (Fig. 1). A second monoclonal antibody P5-2-1 (12) has also been described that inhibits the surface activation of factor XII. To determine if the two antibodies recognized the same region in factor XII, we tested 14 of the 16 phage clones for which we had determined the complete factor XII insert DNA sequence by immunoscreening of the phage with monoclonal antibody P5-2-1 under conditions identical to those used for B7C9. In every phage tested, the plaques were strongly reactive with P5-2-1 (Table I). We conclude that the independently derived anti-factor XII monoclonal antibodies B7C9 and P5-2-1 recognize identical or contiguous antigenic determinants in the amino-terminal 31 amino acids of factor XII.

Expression of the Factor XII Fusion Protein in E. coli—To characterize the factor XII fusion proteins further and to allow their expression in E. coli in biochemically useful amounts, the recombinant λgt1 clone B2-2B (containing the 83 amino-terminal amino acids of factor XII (Table I)) was lysogenized into the protease-deficient lysis-defective E. coli host 1089r- (Promega Biotec). After selection of lysogenic colonies and induction of fusion protein synthesis, the cells were harvested and lysed, and the soluble proteins were analyzed by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions. The left panel of Fig. 2 illustrates the expected absence of the β-galactosidase subunit (M, 116,000) in E. coli 1089r- (lane 1), its presence in the E. coli lysogen of wild-type λgt11 (lane 2), and the appearance of a unique high molecular weight band of M, 125,000 representing roughly 1% of the protein in the E. coli λB2-2B lysogen (lane 3). Partial purification of the putative factor XII fusion protein was achieved by using a B7C9 anti-factor XII Affi-Gel 10 immunoadfinity column (11) (lane 4). Confirmation that the M, 125,000 protein was a factor XII-galactosidase fusion product was achieved by Western blot analysis (26) of duplicate samples onto nitrocellulose followed by immunoblotting with B7C9 monoclonal antibody (lanes 5-8). The slight difference in mobility of the M, 125,000 band in lanes 3 and 4 is probably caused by distortion due to the heavy loading of protein in lane 3.

**Table I**

| Phage clone | Factor XII amino acids encoded | Antibody reactivity |
|-------------|--------------------------------|---------------------|
| B2-2B       | 5-78                           | B7C9, P5-2-1        |
| 1           | -5 to +78                       | +                   |
| 2           | -5 to +58                       | +, NT               |
| 3           | -5 to +56                       | +                   |
| 4           | -5 to +57                       | +                   |
| 5           | -5 to +65                       | +                   |
| 6-8         | +                               |                     |
| 7           | -5 to +64                       | +                   |
| 9-11        | -5 to +31                       | +                   |
| 12          | -5 to +52                       | +                   |
| 13          | -5 to +55                       | +                   |
| 14          | -5 to +74                       | +                   |
| 15          | -5 to +69                       | +                   |
| 16'         | +1 to +47                       | +                   |
| 17          | -5 to +71                       | +                   |
| 20          | -4 to +73                       | +                   |

*G:C tails in DNA insert.

NT Not tested.

*a* Defines limits of epitope.

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![Molecular mapping of the putative surface-binding epitope of human factor XII using recombinant DNA techniques](chart.png)

The polypeptide chain of prefactor XII (amino acid residues -19 to 690) is indicated by the line from the amino terminus (N) to the carboxyl terminus (C). Regions of homology (4) are indicated below the polypeptide chain and include a signal peptide (S), a fibronectin type II homology (II), two epidermal growth factor-like regions (EGF), a fibronectin type I homology (I), a kringle (K), a proline-rich region (Pro), and the protease region (PROTEASE). The region from residues -19 to 80 is expanded below the polypeptide chain. The regions of factor XII encoded by the three recombinant phage clones λ12, λ16, and λ9 are indicated together with the common region encoded by all 16 immunoreactive phage (amino acids 1-31). Also shown are the positions of the two immunoreactive peptides 1-14 and 1-28.
Factor XII Epitope

Binding of Anti-factor XII Monoclonal Antibodies to Synthetic Peptides—To establish the reactivity of the B7C9 monoclonal antibody with the amino-terminal region of factor XII and to define the immunoreactive epitope(s) implicated in the surface-mediated binding of factor XII, a set of four peptides was synthesized that included amino acids 1-28, 5-28, 9-28, and 14-28 of factor XII. A control peptide containing residues 1-17 of interleukin-3 (22) was also synthesized. The binding of the B7C9 antibody to the peptides was first tested by slot blot analysis of the individual peptides in 10-μg amounts on nitrocellulose paper followed by incubation with B7C9 and the alkaline phosphatase-conjugated second antibody as described for the phage screens. The B7C9 antibody reacted only with the peptide containing amino acids 1-28 of factor XII (Fig. 4). To test the specificity of this binding, a

Hydropathy Plot of the Amino-terminal Region of Factor XII—In view of the predicted antigenicity of hydrophilic regions of proteins (27), we were interested in determining the hydrophilicity of the region of factor XII that appeared to contain the epitope for the two monoclonal antibodies B7C9 and P5-2-1. Fig. 3 shows a computer-generated hydropathy plot (28) of the amino-terminal region of factor XII. Scanning of the entire factor XII polypeptide indicated that the region between amino acids 5 and 15 included one of the most hydrophilic areas of the molecule. Particularly notable was the presence of three positively charged lysine residues at positions 8, 11, and 13 that could potentially interact electrostatically with negatively charged surfaces.

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Factor XII Epitope

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duplicate blot of peptides was incubated with an anti-factor XII monoclonal antibody (KOK-5 obtained from Dr. Erik Hack, Central Red Cross Laboratory, Amsterdam) that does not inhibit the surface-mediated activation of factor XII. This antibody bound to none of the peptides (data not shown). Together, these results confirmed the previous data that the B7C9 epitope(s) resides in the first 31 amino acids of factor XII but suggested that amino acids 1–4 are critical for binding of the antibody. Because different peptides may not bind quantitatively to nitrocellulose, an ELISA was established to test the ability of B7C9 antibody to bind to peptides 1–28, 5–28, 9–28, and 14–28 that had been bound to the microtiter dish wells. Again, only peptide 1–28 bound to the antibody (data not shown), confirming the results of the nitrocellullose slot blot assay.

To map the epitope more precisely, a competitive ELISA was established to test the ability of various peptides to block the binding of B7C9 antibody to 33 nmol of peptide 1–28 that had been immobilized on the microtiter dish wells. When peptides 1–28, 5–28, 9–28, and 14–28 were tested, only peptide 1–28 competed for the antibody binding (Fig. 5), confirming the previous ELISA results. In addition, peptide 1–14 competed with peptide 1–28, although a 10-fold higher concentration of peptide 1–14 was required to obtain the same degree of competition as peptide 1–28 competing for binding with itself (Fig. 5). Interestingly, peptide 4–14 did not compete at all (Fig. 5).

Because these results suggested that the region from 1 to 4 was important for binding of the B7C9 antibody, a series of short peptides (1–4, 1–5, 1–6, 2–7, 3–8, and 4–9) were synthesized and tested in the competitive ELISA, but none of them affected the binding (Fig. 5). Taken together, these results suggest that residues 1–4 of factor XII are essential but not sufficient for binding of B7C9 antibody. Moreover, although peptide 1–14 was sufficient to compete for binding, it was not as effective as peptide 1–28, suggesting that the epitope may also involve some as yet undefined secondary structure (29). These results are summarized in Fig. 1.

DISCUSSION

In this study, screening of a factor XII cDNA expression library with two murine monoclonal antibodies known to inhibit the activation of factor XII by negatively charged surfaces (11, 12) resulted in the isolation of 16 independent bacteriophage clones, all of which contained DNA encoding the first 31 amino acids of factor XII (Table I, Fig. 1). Hydrophilic analysis of this region of the factor XII polypeptide (Fig. 3) revealed a highly hydrophilic cluster between amino acids 1 and 28 of factor XII and tested their reactivity with the B7C9 antibody used in both studies. These experiments strongly implicated that the first 14 amino acids of factor XII in antibody binding (Fig. 4), as peptides 1–28 and 1–14, are easily detectable by the B7C9 antibody at peptide concentrations two orders of magnitude below those employed for peptide 134–153 (11). However, it is possible that the B7C9 antibody recognizes two noncontiguous sequences of factor XII as has been reported for human von Willebrand’s factor (30) where the binding of a monoclonal antibody to the protein was blocked by two peptides that were separated by 220 amino acid residues of linear sequence. We plan to investigate this further by functional analysis of deletion mutants of recombinant factor XII expressed in eukaryotic cells.

The first 19 amino acids of the amino-terminal region of factor XII are encoded by the second exon in factor XII genomic DNA (3). In contrast to the sequence homologies between factor XII and other plasma proteins (Fig. 1; Ref. 3), the exon 5-encoded region of factor XII is unique within the blood-clotting factors (31). A (168 amino acid residues) of the first and second exons of factor XII, tissue-type plasminogen activator and urokinase-type plasminogen activator, has been noted (3), but there is no sequence identity among these three proteins in exon 2. Interestingly, four out of 15 amino acids encoded by the second exon of tissue-type plasminogen activator are arginine residues in close proximity to one another. This constitutes a positively charged region reminiscent of the lysine cluster between amino acids 5 and 15 in factor XII. More interesting, however, is the presence of a common tetrapeptide in factor XII and bovine (31) and human (32) high molecular weight kininogens. These positively charged peptide fragments have been implicated in the binding of high molecular weight kininogens to negatively charged surfaces (2). Bovine and human high molecular weight kini

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