A Conserved Element in the Serine Protease Domain of Complement Factor B*

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Factor B and C2 are serine proteases that carry the catalytic sites of the complement C3 and C5 convertases. Their protease domains are activated by conformational changes that occur during convertase assembly and are deactivated upon convertase dissociation. Factor B and C2 share an 8-amino acid conserved sequence near their serine protease termini that is not seen in other serine proteases. To determine its importance, 24 factor B mutants were generated, each with a single amino acid substitution in this region. Whereas most mutants were functionally neutral, all five different substitutions of aspartic acid 715 and one phenylalanine 716 substitution severely reduced hemolytic activity. Several aspartic acid 715 mutants permitted the steps of convertase assembly including C3b-dependent factor D-mediated cleavage and activation of the high affinity C3b-binding site, but the resulting complexes did not cleave C3. Given that factor B and C2 share the same biological substrates and that part of the trypsin-like substrate specificity region is not apparent in either protein, we propose that the conserved region plays a critical role in the conformational regulation of the catalytic site and could offer a highly specific target for the therapeutic inhibition of complement.

The complement system consists of about 30 proteins that participate in both innate and acquired immunity (1, 2). The complement response is initiated through the activation of several distinct pathways (3). Each pathway converges in the assembly of the C3 and C5 convertases, multicomponent serine proteases that mark targets for immune clearance or cell lysis (3) and direct antigen selection by B lymphocytes (4). Factor B is a zymogen that carries the catalytic site of the complement alternative pathway (AP)1 convertases (5). Assembly of the AP C3 convertase begins with the association of factor B with C3b in the presence of Mg2+. This association permits factor B to be cleaved at a single site by factor D, which produces the Ba and Bb fragments. Ba dissociates from the complex, whereas Bb and Mg2+ remain bound to C3b. C3bBb, the active AP C3 convertase, is capable of catalyzing C3 cleavage. Association of C3bBb with additional C3b yields the AP C5 convertase, C3bC3bBb, which cleaves C5. Both the C3 and C5 convertases can be further stabilized by association with properdin. Dissociation of Bb from either complex results in irreversible loss in C3b-binding capacity and C5 protease activity.

Factor B is a 90-kDa single-chain mosaic glycoprotein composed of three different types of protein modules (6). 1) The amino-terminal region, which constitutes most of Ba, features three complement control protein modules, found in tandem arrays in many complement proteins and frequently carrying binding sites for C3b and closely related C4b (7); 2) the middle region is a von Willebrand factor type A module that also occurs in several members of integrin family, often serving as a ligand-binding site and a metal-binding site (8); and 3) the carboxyl terminus is a serine protease (SP) domain similar to that of trypsin (9). C2, the complement classical pathway analog of factor B, is similar in structure to factor B (10).

Whereas the factor B and C2 SP domains closely resemble trypsin in primary structure, their regulation features an unusual interplay between the SP domain and the adjoining type A domain. Although both factor B and Bb are capable of cleaving small molecule substrate analogs (11), the capacity to cleave C3 is acquired only through C3 convertase assembly (to C3bBb) and is sustained by continuous association with C3b. Moreover, association with additional C3b (C3bC3bBb) is required for C5 convertase activity. This general activation scheme, which also occurs in the case of C2, is in contrast with that utilized by typical serine proteases in which the amino terminus generated by cleavage of the zymogen translocates to the protein interior, rearranging the specificity pocket (12, 13). The highly conserved amino-terminal sequence that mediates the typical activation mechanism does not occur in factor B or C2 (see discussion in Ref. 5).

Electron microscopic examination of Bb and C3bBb have indicated that Bb is a dumbbell-shaped structure, with only one lobe binding to C3b (14, 15). Mutational studies have strongly implicated the type A domain in binding to C3b (16, 17), and genetic or chemical modification of the C2 (18, 19) and factor B2 type A region increases convertase stability. Thus it is likely in C3bBb that the factor B type A domain is in contact with C3b whereas the SP domain is not, and SP activation is regulated by a conformational signal that propagates from the type A ligand-binding site to the SP catalytic site. In this report, a highly conserved sequence in the factor B and C2 SP catalytic region, not observed in other trypsin-like SP, is partially characterized, and its possible role in interdomain signaling is discussed.

EXPERIMENTAL PROCEDURES

Expression of Mutant and Wild Type Recombinant Factor B—As described previously (16), COS-7 cells were transfected with factor B A14 cDNA cloned in expression vector pShG3 (Stratagene). Cells were biosynthetically labeled, and the supernatants were analyzed by immu-

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‡ The abbreviations used are: AP, alternative pathway; SP, serine protease; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CVF, cobra venom factor.

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**RESULTS**

In an effort to understand the structural basis for the regulation of the factor B and C2 SP domains, we compared the amino acid sequences of factor B and C2 to those of other trypsin-like serine proteases. In the case of trypsin and chymotrypsin, important determinants of substrate specificity are clustered near the COOH terminus (21, 22). In factor B and C2, at least one of the typical trypsin-like specificity determinants is absent (Fig. 1), and its relative importance is only demonstrated by the presence of eight identical amino acids (RDFHINLF) in the SP region of Factor B, which is translated into the peptide sequence HMRLSQWPLLKP. Computer analysis employing the GeneWorks program (Intelligenetics, Mountain View, CA) revealed one homologous site in the human factor B sequence.

Based on its level of conservation, we hypothesized that RDFHINLF plays an important role in the function of the factor B/C2 serine protease. To test this hypothesis, each of the eight conserved residues of human factor B was replaced by site-directed mutagenesis, and the resulting mutant proteins were examined (Fig. 2). Whereas substitutions were permitted at most sites, the substitution of Asp-715 severely reduced hemolytic activity in all five cases. Activity was also affected in three of the Phe-716 substitutions: P716Y was reduced to 23% that of wild type. Activity was observed at 27% that of wild type, and hemolytic activity of F716A was nearly abrogated.

Three Asp-715 substitutions were selected for further examination: D715E was chosen because it retained the wild type negative charge, D715N because it retained some of the wild

**FIG. 1. Comparison of a portion of the serine protease region of human factor B with homologous segments of human C2, bovine trypsin, and bovine chymotrypsin.** Alignment of trypsin and chymotrypsin and delineation of the S1 binding pocket are as in Ref. 21. Key specificity determinants in the trypsin-like consensus are underlined and numbered using the chymotrypsin positions. Sequences are derived from the following: bovine chymotrypsin (28); bovine trypsin (29); human factor B (6); human C2 (10).
type side-chain structure, and D715A because it removed most of the wild type side-chain. Factor B forms were treated with factor D and C3b in the presence of Mg\(^{2+}\) in a fluid phase reaction, and the results were analyzed by Western blot (Fig. 3A). All of the mutants underwent C3b-dependent cleavage, the first indication that the earliest steps of convertase assembly, the low affinity interaction between factor B and C3b and subsequent recognition and cleavage by factor D, were proceeding normally. Mutant F716A, which lacked hemolytic activity, was also examined by this method, and C3b-dependent cleavage was also observed (Fig. 3B).

The cleavage of factor B by factor D results in a higher affinity between ligand and the Bb fragment. This is indicated by a factor D-dependent increase in factor B binding to immobilized ligand (17). Wild type factor B bound C3b in the presence of properdin, which stabilizes C3bBb (3), with binding increasing severalfold when factor D was also supplied (Fig. 4). Asp-715 mutants also exhibited factor D-dependent increases in binding activity (Fig. 4), a strong indication that both factor D cleavage and high affinity bond formation occurred normally. C3b-binding was not detected with F716A (Fig. 4).

Once factor D-mediated cleavage occurs and the high-affinity complex is established, assembly of the C3 convertase is complete, and the serine protease domain is capable of catalyzing the cleavage of C3. To determine whether the Asp-715 mutants are capable of C3 cleavage, a fluid phase assay was employed. CVF, an analog of human C3b, was used as ligand because the resulting C3 convertases are 50-fold more stable than those that incorporate C3b (23), and the 715D mutants undergo factor D-dependent CVF-binding at the same rate as wild type factor B (Fig. 5). Biotinylated C3 was used as substrate, and the C3b product was resolved from C3 by gel electrophoresis. As seen in Fig. 6, C3b was formed with wild type recombinant factor B, with the P716A substitution.

Several Asp-715 and Phe-716 substitutions were screened for recognition by the Bb-specific mAb. Mutant proteins were cleaved by factor D, Bb generation was assessed by Western blot, and Bb samples were analyzed using a commercially available Bb-specific ELISA that utilizes the anti-human Bb mAb. Of six mutant Bb forms, the five hemolytically defective mutants were recognized at lower efficiency than wild type, and the one hemolytically normal mutant was recognized at the wild type level (Fig. 7B). Most affected was Bb\(^{F716A}\), which was recognized about 25% as effectively as wild type Bb.

**DISCUSSION**

Factor B and C2 share an 8-amino acid conserved sequence (RDFHINLF) near the SP carboxyl terminus that is not seen in other trypsin-like serine proteases. A panel of factor B mutants was assembled, each with a single amino acid substitution in this region. Whereas most substitutions were functionally neutral, all five different substitutions of Asp-715 as well as one Phe-716 substitution exhibited severely reduced hemolytic activity.

The initial association between factor B and C3b is a low affinity interaction. The C3b-dependent cleavage of factor B by factor D produces a high affinity ligand-binding site in the type A domain. All of the Asp-715 mutations examined permitted C3b-dependent factor D cleavage. In vitro binding studies indicated that the high affinity ligand-binding conformation occurred in at least several of these cases. High affinity binding...
was dependent on factor D activity, and the formation of high affinity complexes proceeded at rates similar to those of wild type factor B. Nevertheless, whereas C3 convertases appear to assemble, C3 cleavage was not detected with any of the Asp-715 substitutions examined. This shortcoming is likely the primary defect of the Asp-715 mutations. It is not clear from the present study whether the Asp-715 mutations would also interfere with C5 cleavage directly because assembly of the C5 convertase depends on a functioning C3 convertase. The F716A mutant also permitted C3b-dependent cleavage, but high affinity C3b-binding was abolished. Thus, F716A is defective in convertase assembly.

The conserved SP sequence, RDFHINLF, distinguishes the

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\text{FIG. 5.} \quad \text{Factor D-dependent binding of factor B forms to immobilized C3b in the presence of properdin.}
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\text{FIG. 6.} \quad \text{Factor D-dependent binding of factor B forms to immobilized CVF.}
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\text{FIG. 4.} \quad \text{C3 convertase activity of wild type and mutant factor B.}
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In the complete reactions (even-numbered lanes) factor B forms (indicated above the lanes) were incubated with CVF, factor D, and biotinylated C3 substrate. CVF was withheld from the odd-numbered samples. For the lanes marked No B, control transfection supernatant was utilized. Samples were resolved by gel electrophoresis under denaturing conditions. The single C3 cleavage site lies in the \(a\) chain (113 kDa), producing 104- and 9 kDa products. The 113-kDa substrate chain and the 104-kDa product chain, marked C3 and C3b, respectively, indicate relative convertase activity. The unmodified C3 \(b\) chain (75 kDa) is present in each lane although the 9-kDa product migrated out of the gel. A, D715E and D715A substitutions; B, D715N substitution.

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\text{FIG. 7.} \quad \text{Correlation of the conserved SP sequence with an anti-Bb mAb neo-epitope.}
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A, comparison of an anti-Bb neo-epitope sequence derived by peptide display methods to a portion of the factor B serine protease domain. The conserved SP region is underlined. B, recognition of Bb forms by a Bb-specific mAb. Values are given as percent of wild type recombinant Bb with standard deviation. Un-cleaved recombinant factor B was recognized at the 13 ± 3% level.

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\text{FIG. 8.} \quad \text{Conservation of the conserved SP sequence in the factor B and C2 primary sequences of other species.}
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Sequences were derived from the following references: human factor B (6), mouse factor B (30), human C2 (10), mouse C2 (30), Xenopus factor B (31), zebrafish factor B (32), medaka fish factor B (33), and lamprey factor B (34).

was dependent on factor D activity, and the formation of high affinity complexes proceeded at rates similar to those of wild type factor B. Nevertheless, whereas C3 convertases appear to assemble, C3 cleavage was not detected with any of the Asp-715 substitutions examined. This shortcoming is likely the primary defect of the Asp-715 mutations. It is not clear from the present study whether the Asp-715 mutations would also interfere with C5 cleavage directly because assembly of the C5 convertase depends on a functioning C3 convertase. The F716A mutant also permitted C3b-dependent cleavage, but high affinity C3b-binding was abolished. Thus, F716A is defective in convertase assembly.

The conserved SP sequence, RDFHINLF, distinguishes the
human complement proteases factor B and C2 from other serine proteases. It is encoded by a single exon (24) that encodes the translational stop codon as well as 214-SWG, found in the S1 binding pocket of trypsin and chymotrypsin (Fig. 1). Several non-human factor B and C2 forms were examined for the presence of the conserved sequence. Remarkably, the sequence was found in all reported factor B/C2 forms of mammals, amphibians, and fish, in several cases without substitution (Fig. 8). Preserved since the evolution of bony fish, RDFHINLF is likely involved in a fundamental role that has little tolerance for subtle structural changes, especially in the first three positions. Given that factor B and C2 share the same biological substrates, C3 and C5, and that parts of the trypsin-like S1 pocket and/or its neighboring loop are not apparent in factor B and C2 amino acid sequences, we propose that the conserved region has a critical role in substrate specificity. Residue Asp-715 could be directly involved in substrate recognition.

Residue Phe-716 appears to be of structural significance: Its replacement with alanine inhibits mAb recognition at a proximal site and high-affinity ligand binding at a distal site. Interestingly, substitution of Phe-716 with aromatic amino acids Tyr and Trp permitted at least partial functional activity, which suggests the possibility of its participation in cation-π interactions (25).

Although it is unclear why factor B/C2 might have evolved with a different specificity determinant, this novel element may facilitate the unusual conformational regulation of SP function. By this model, RDFHINLF is a key part of a conformationally regulated SP specificity determinant that is controlled by ligand interactions in the type A domain. Its absence in lamprey factor (Fig. 8) suggests its approximate evolutionary origins and indicates that it is likely unique to factor B and C2. In any case, the conserved region could offer a highly specific target for the therapeutic inhibition of complement.

Several SP mutations reduced Bb-specific mAb recognition, with the most extreme effects observed with the F716A mutation. The corresponding neoeptope, partially characterized using phage display methods, overlaps the SP conserved sequence. Two simple interpretations are possible: 1) Expression of the mAb recognition site and the possible activation of the conserved sequence requires conformational changes that occur upon factor B cleavage and the release of the Ba region; and 2) Ba could physically block an already existing mAb recognition site and possibly the site of the conserved sequence. Given the likely close proximity between the mAb neoeptope, the conserved sequence, and the SP active site, either explanation could also account for the impact that factor B cleavage and Ba release has on SP catalytic activity.

Recently, Tuckwell et al. (17) described a factor B type A domain mutant that is unable to make or sustain the high affinity Bb conformation, although capable of ligand-dependent factor D-mediated cleavage. That mutant, Ch1, is a sequence substitution of a putative Mg²⁺-binding loop located distal to the type A/S/SP connection (26, 27). Similar functional behavior is reported here in the case of the SP mutant F716A. The cleavage of C3bB by factor D precipitates a conformational change from a low affinity ligand-binding catalytically inactive state to a high affinity ligand-binding catalytically active state. Both the Ch1 and F716A mutations appear to block this transition. We propose that these two physically distant mutants indicate an inter-domain signaling mechanism that alters the structure of the SP specificity determinants in response to the ligand-binding state of the type A domain.

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