Mutations of the Type A Domain of Complement Factor B That Promote High-Affinity C3b-Binding

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Mutations of the Type A Domain of Complement Factor B That Promote High-Affinity C3b-Binding

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Factor B is a zymogen that carries the catalytic site of the complement alternative pathway convertases. During C3 convertase assembly, factor B associates with C3b and is cleaved at a single site by factor D. The Ba fragment is released, leaving the active complex, C3bBb. During the course of this process, the protease domain becomes activated. The type A domain of factor B, also part of Bb, is similar in structure to the type A domain of the complement receptor and integrin, CR3. Previously, mutations in the factor B type A domain were described that impair C3b-binding. This report describes “gain of function” mutations obtained by substituting factor B type A domain amino acids with homologous ones derived from the type A domain of CR3. Replacement of the βA-α1 Mg²⁺ binding loop residue D254 with smaller amino acids, especially glycine, increased hemolytic activity and C3bBb stability. The removal of the oligosaccharide at position 260, near the Mg²⁺ binding cleft, when combined with the D254G substitution, resulted in increased affinity for C3b and iC3b, a C3b derivative. These findings offer strong evidence for the direct involvement of the type A domain in C3b binding, and are suggestive that steric effects of the D254 sidechain and the N260-linked oligosaccharide may contribute to the regulation of ligand binding.


Materials and Methods

Recombinant factor B forms

The transient expression of mutant and wild-type recombinant factor B forms was conducted in COS-7 cells transfected with factor B cDNA subcloned into the expression vector pSG5 (Stratagene, La Jolla, CA) using serum-free medium (see Ref. 11). Supernatants were dialyzed and stored in phosphate buffer (PB; 11 mM Na₂HPO₄ and 1.8 mM NaH₂PO₄ (pH 7.4)) supplemented with 25 mM NaCl. Factor B content was assayed by ELISA using immobilized mouse anti-human Ba mAb (Quidel, San Diego, CA) for capture and goat anti-human factor B polyclonal Ab followed by rabbit anti-goat IgG polyclonal Ab for detection (11). Mutant factor B forms were initially compared with wild-type factor B by either immunoprecipitation of biosynthetically-labeled protein followed by PAGE (11) or by Western blot analysis of unlabeled full-length and Bb forms (24). Mutations were introduced into the factor B/pSG5 construct using three different methods.

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2 Abbreviations used in this paper: AP, alternative pathway; PB, phosphate buffer; DGVB, dextrose veronal-buffered saline containing gelatin and cations; SP, serine protease; P, properdin.
the transformer site-directed mutagenesis method (25) (Clontech Laboratories, Palo Alto, CA), the QuiikChange site-directed mutagenesis method (Stratagene), and the double-take double-stranded site-directed mutagenesis method (Stratagene), following the manufacturers’ instructions.

**Hemolysis assays**

Cells were prepared by first assembling classical pathway C3 convertases on sheep erythrocytes and then using them to coat the cell surface with C3b. Ab-sensitized sheep erythrocytes (EA cells, 5 ml, 5 × 10^7/ml) obtained from Advanced Research Technology (San Diego, CA) were washed twice and resuspended in 5 ml of dextrose veronal-buffered saline containing gelatin and cations (DGVB2) (26), mixed with 37.5 μg of human C1 in 5 ml of DGVB2, and incubated for 15 min at 30°C. The resulting cells (EAC1) were washed twice and resuspended in 5 ml of DGVB2, mixed with 50 μg of human C4 suspended in 5 ml of DGVB2, and incubated for 15 min at 30°C. These cells (EAC1, 4) were washed twice and suspended in 5 ml of DGVB2, mixed with 250 μg of human C3 and 5 μg of human C2 suspended in 5 ml of DGVB2, and incubated for 30 min at 30°C. The resultting cells (EAC1, 4, 2, 3) were washed and suspended in 5 ml of 10 mM EDTA buffer (26) and incubated at 37°C for 2 h to allow dissociation of the active classical pathway convertases. The resulting C3b-coated cells were washed twice in 5 ml 10 mM MgCl₂ buffer, twice in 5 ml of 10 mM MgCl₂, 5 mM NiCl₂, or 20 mM EDTA buffer (26), and resuspended in 10 mM MgCl₂, EGTA buffer to a final concentration of 1 × 10^7/ml. All purified complement proteins in this study were obtained from Advanced Research Technologies.

For each determination, duplicate samples were made from 100 μl of prepared C3b-coated sheep erythrocytes, 50 μl of purified factor D (5 ng in MgCl₂, EGTA buffer), 50 μl of properdin (P; 45 ng in MgCl₂, EGTA buffer), and 50 μl of factor B source or standard (typically 0.1–0.5 ng) were mixed together and incubated at 30°C for 30 min. In some cases, the P addition was replaced by 50 μl of MgCl₂, EGTA buffer. The negative control substituted 50 μl of DGVB2 buffer for the factor B source. AP C3 convertase sites were developed with 300 μl of a 1/40 dilution of guinea pig serum (Colorado Serum, Denver, CO) in 40 mM EDTA buffer (26) at 37°C for 60 min. Additional controls included complete cell lysis by 450 μl of distilled water and a negative control in which cells were mixed with 450 μl of DGVB2 buffer only. These samples were also incubated at 37°C for 60 min. All samples were then centrifuged and the OD₄₁₄ of the supernatants were obtained from Advanced Research Technologies. 

**Measurement of convertase stability**

AP convertases were assembled on sheep erythrocytes using 0.15–0.25 ng of factor B. After the 30 min incubation of duplicate samples of C3b-coated cells with factor B, factor D, and P, 250 μl of 40 mM EDTA buffer was added to prevent the assembly of new convertases, and incubation was continued at 30°C to allow decay. At various times, 50 μl of 1/8 guinea pig serum diluted in 40 mM EDTA buffer was added, and samples were incubated at 37°C for 1 h. Hemolysis proceeded during this period. The remaining cells were centrifuged, the OD₄₁₄ of the supernatants determined, and Z values were calculated. t₁/₂ was calculated for each experiment by linear regression analysis of log Z vs t. t₁/₂ was expressed as the average number of lytic sites per cell (26).

**C3b binding and iC3b binding**

Factor B in PB supplemented to 75 mM NaCl, 4% BSA, 0.05% Tween 20, and 10 mM MgCl₂, 5 mM NiCl₂, or 20 mM EDTA was incubated in duplicate C3b-coated microtiter wells at 37°C for 30–120 min, the wells washed in PB supplemented with 25 mM NaCl, 4% BSA, and 0.05% Tween 20, and detected by ELISA using goat anti-human factor B polyclonal Abs followed by peroxidase-conjugated rabbit anti-goat IgG polyclonal Abs (11). For iC3b binding, microtiter wells were prepared as with C3b, but in the factor B incubation step NaCl was 25 mM.

**Generation of Bb**

Mutant or wild-type recombinant factor B (500 ng/ml) was treated with factor D (200 ng/ml) and C3b (2000 ng/ml) in PB supplemented with 10 mM MgCl₂ and 25 mM NaCl for 30 min at 37°C, and the factor B cleavage was confirmed by Western blot analysis (24). Control uncleaved factor B was prepared similarly except C3b was omitted from the reaction. In those cases, the Western blot analysis confirmed that factor B-mediated cleavage did not occur. The Bb fraction retained <3% of the hemolytic activity of the full-length factor B fraction.

**Results**

**Factor B type A mutants that increase C3b-binding affinity**

Complement proteins factor B, C2, and CR3 each feature a type A domain that is a major ligand-binding site. To explore the basis for their ligand-specificity we began to substitute factor B type A amino acids with homologous residues of C2 and CR3. In the course of this process a hybrid protein of particular interest was generated.

The Bαα-loop notation (of Lee et al. (22)), which carries three Mg²⁺-coordination residues (Fig. 1, A and B) (22, 23), was subjected to site-directed mutagenesis. Five factor B amino acids were replaced with CR3 residues (254-DSIQGASN² to 254-GSNIPHD, where N* indicates an N-linked oligosaccharide). The Mg²⁺-coordination residues were not altered. The resulting mutant form, Bmut31 (Fig. 1C) exhibited a 2-fold to 3-fold increase in hemolytic activity over wild type (Table I).

Several observations indicated that the Bmut31 substitution increases C3b-binding affinity: 1) Bmut31 convertase was relatively stable over a 2-h period at 30°C, while wild-type convertase decayed with a half-life of 53 ± 6 min (n = 4) (Fig. 2). Moreover, Bmut31 convertases assembled in the absence of P decayed with a half-life of 17 ± 4 min (n = 3), while equivalent wild-type convertases were not detected; 2) Bmut31 attained 30–40% maximal hemolytic activity without P, an agent that stabilizes AP convertases (1), while wild-type factor B was dependent on P for activity.
slightly increased C3b binding (Fig. 4A). The N*260D single mutation was similar to wild-type factor B by both of these criterion (Figs. 3 and 4A). Combining D254G with N*260D resulted in a mutant factor B form that was very similar to Bmut31 in both hemolytic activity and C3b-binding capacity (Fig. 4A).

High-affinity C3b binding was dependent on Mg\(^{2+}\), although a wide range of Mg\(^{2+}\) concentrations would suffice (Fig. 4B). C3b binding was abolished by EDTA (not shown) and by the replacement of putative Mg\(^{2+}\) coordination residue D364 with alanine in the D254G, N*260D, D364A triple mutant (Fig. 5A), and the Bmut31, D364A double mutant (not shown). Both EDTA treatment (not shown) and D364A substitution (Table I) also abrogated hemolytic activity. In contrast, high-affinity C3b binding did not require an intact factor D-mediated cleavage site. When the factor D cleavage site, K233RK\(\rightarrow\)AAA, was disrupted by triple Ala substitution (K233RK\(\rightarrow\)AAA), ligand binding was not impaired (Fig. 5A), although hemolytic activity was abolished (Table I) and fluid phase C3b-dependent factor D-mediated cleavage was abrogated (not shown). High-affinity binding was also observed in the D254G, N*260A double mutant (Fig. 5B), suggesting that C3b binding was accentuated by the loss of the glycosylated asparagine at this position rather than the gain of a specific amino acid sidechain. In general, as the residue at position 254 became smaller (D,N\(\rightarrow\)A\rightarrow G), the observed ligand-binding activity and hemolytic activity became greater. The D254G, N*260D double mutant bound C3b better than the D254A, N*260D double mutant that bound C3b better than the D254A, N*260D double mutant (Fig. 5B). Bb derived from the D254G, N*260D mutant did not bind C3b (Fig. 6) and was hemolytically inactive.

The Bmut31-related mutants bind iC3b

The Bmut31 substitution was derived from the type A domain of CR3, a domain that binds iC3b, a cleavage product of C3b but not a factor B ligand. Given that the Bmut31 substitution greatly accentuated C3b binding, it seemed plausible that it might also affect iC3b binding. Thus, iC3b was immobilized to microtiter wells and treated with mutant and wild-type factor B forms. N\(^2\), which enhances convertase assembly and stability (17), was used in addition to Mg\(^{2+}\) as divalent cation. The main results of these experiments were: 1) The Bmut31 derivative, D254G, N*260D, bound iC3b in the presence of Ni\(^2\), although at lower affinity than C3b (Fig. 7, A and B); 2) The binding of D254G, N*260D to iC3b was distinguished from C3b binding by its strong Ni\(^2\)-dependence (Fig. 7, A and B) and its salt sensitivity (Fig. 7D). In addition, iC3b binding required both D254G and N*260D substitutions, to be sensitive to EDTA, and occurred when the factor D

Table I. Hemolytic activity of factor B mutant forms

<table>
<thead>
<tr>
<th>Mutant</th>
<th>% of Wild Type (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βA-α1 loop mutations, Mg(^{2+})-coordination residues conserved</td>
<td></td>
</tr>
<tr>
<td>Bmut31</td>
<td>246 (3)</td>
</tr>
<tr>
<td>D254G</td>
<td>264 (3)</td>
</tr>
<tr>
<td>D254A</td>
<td>256 (3)</td>
</tr>
<tr>
<td>D254N</td>
<td>148 (3)</td>
</tr>
<tr>
<td>N*260D</td>
<td>91 (4)</td>
</tr>
<tr>
<td>D254G, N*260D</td>
<td>285 (3)</td>
</tr>
<tr>
<td>D254A, N*260D</td>
<td>183 (3)</td>
</tr>
<tr>
<td>D254N, N*260D</td>
<td>41 (3)</td>
</tr>
<tr>
<td>Mg(^{2+})-coordination residue mutations</td>
<td></td>
</tr>
<tr>
<td>D364A</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Bmut31, D364A</td>
<td>≤2 (2)</td>
</tr>
<tr>
<td>D254G, D364A</td>
<td>≤2 (2)</td>
</tr>
<tr>
<td>N*260D, D364A</td>
<td>≤2 (2)</td>
</tr>
<tr>
<td>D254G, N*260D, D364A</td>
<td>≤2 (4)</td>
</tr>
<tr>
<td>Factor D cleavage site mutations</td>
<td></td>
</tr>
<tr>
<td>K233RK(\rightarrow)AAA</td>
<td>≤2 (3)</td>
</tr>
<tr>
<td>D254G, K233RK(\rightarrow)AAA</td>
<td>≤3 (3)</td>
</tr>
<tr>
<td>D254G, N*260D, K233RK(\rightarrow)AAA</td>
<td>≤2 (3)</td>
</tr>
</tbody>
</table>
cleavage site was disrupted (data not shown). No iC3b-binding was observed with the wild-type factor B form (Fig. 7C).

Discussion

Factor B is a mosaic protein consisting of three different types of protein modules: three short consensus repeats, one type A domain, and one SP domain. Activity of the SP catalytic site is strictly regulated by an assembly process that culminates in a C3bBb complex, the active C3 convertase. The factor B type A domain is believed to mediate C3b binding in C3bBb, based on the negative effects of several site-directed type A mutations on C3b binding, C3bBb assembly, and/or C3bBb stability. However, in these cases it is difficult to rule out indirect negative effects on either Mg2+-binding or C3b-binding elements in the SP domain (14).

In this report we describe factor B “gain of function” mutations altered in a type A domain $\beta$A-$\alpha_1$ loop, a region previously implicated directly in Mg2+-binding or C3b-binding elements in the SP domain (14).

Two amino acid substitutions together accounted for the novel properties of Bmut31: replacing D254, positioned directly between two metal-coordination residues on the $\beta$A-$\alpha_1$ loop, with glycine resulted in enhanced hemolytic activity. Combining the D254G mutation with mutations that remove an N-linked oligosaccharide located near the Mg2+-binding cleft (22, 23) resulted in factor D-independent high-affinity C3b binding. High-affinity C3b binding required divalent cation but did not require factor D-mediated cleavage. It was EDTA-sensitive and abolished by mutations that
disrupt Mg\(^{2+}\) binding, but not by mutations that disrupt the factor D cleavage site. High-affinity binding derivatives also bound the CR3 ligand, iC3b. Together, these findings present a compelling case for the positive role of the type A domain in C3b-binding affinity, C3bBb stability, and ligand-binding specificity.

The assembly of the AP convertases involves critical changes in the Bb region, including the formation of a C3b/Bb connection and the activation of the serine protease domain. We have observed that D254G alone is sufficient for P-independent hemolytic activity, but removal of the oligosaccharide at position 260 is also required for enhanced factor D-independent C3b binding. Thus, the D254G substitution may directly alter the C3b-binding site, while removal of the oligosaccharide at N*260 may allow access of the modified C3b-binding site to C3b without factor D-mediated cleavage and Ba release. By this model, the transition from C3bB to C3bBb would involve at least two important events: 1) improved access of the type A ligand-binding region to C3b by a conformational shift of the N260-linked oligosaccharide, and 2) formation of a new type A/C3b interaction at the Mg\(^{2+}\) site. Both of these events would be normally driven by factor D-mediated cleavage and/or Ba release.

The type A domain serves as a ligand-binding site in several integrins, and in some of those cases, amino acids that are structurally homologous to D254 and N*260 of factor B have been implicated in ligand binding. In LFA-1, the M140Q and E146D substitutions reduced ICAM-1 binding by 35% and 50%, respectively (29), while in CR3, binding to neutrophil inhibitory factor (22). While it is unclear from our results whether the cation derivative from C3b is irreversible. The biochemical basis for this effect is not understood. Previous findings indicate that the Ba region is critically involved in the assembly of C3bBb, possibly contributing an essential C3b-binding site or providing a scaffold necessary for type A/C3b interactions (11–13). Thus, loss of Ba during convertase assembly could be a negative controlling factor. Alternatively, it is also possible that upon dissociation of convertase, the ligand-binding site on Bb is itself deactivated.
In summary, this report describes "gain of function" mutations of the complement factor B type A domain that increase hemolytic activity, convertase stability, and affinity for the natural ligand C3b, as well as iC3b, the C3b derivative. Two amino acid changes account for these new properties. D254 lies in the βA-α1 Mg$^{2+}$-binding loop but does not coordinate the divalent cation. Its replacement with the smaller amino acids increases hemolytic activity and C3bBb stability. Removal of the oligosaccharide at position 260, near the Mg$^{2+}$-binding cleft, when combined with D254G, results in high affinity ligand binding without factor D-mediated cleavage. These findings offer strong evidence for the direct involvement of the type A domain in C3b binding and are suggestive that steric effects of the D254 residue and the nearby N-linked oligosaccharide may contribute to the regulation of the type A C3b-binding site.

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