The Role of Properdin in the Assembly of the Alternative Pathway C3 Convertases of Complement*

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Complement is a powerful host defense system that contributes to both innate and acquired immunity. There are three pathways of complement activation, the classical pathway, lectin pathway, and alternative pathway. Each generates a C3 convertase, a serine protease that cleaves the central complement protein, C3. Nearly all the biological consequences of complement are dependent on the resulting cleavage products. Properdin is a positive regulator of complement activation that stabilizes the alternative pathway convertases (C3bBb). Properdin is composed of multiple identical protein subunits, with each subunit carrying a separate ligand-binding site. Previous reports suggest that properdin function depends on multiple interactions between its subunits with its ligands. In this study I used surface plasmon resonance assays to examine properdin interactions with C3b and factor B. I demonstrated that properdin promotes the association of C3b with factor B and provides a focal point for the assembly of C3bBb on a surface. I also observed that properdin binds to preformed alternative pathway C3 convertases. These findings support a model in which properdin, bound to a target surface via C3b, C3b, or other ligands, can use its unoccupied C3b-binding sites as receptors for nascent C3b, bystander C3b, or other ligands, and can occupy its unoccupied focal point for the assembly of C3bBb on a surface. I also found that properdin increases the stability of the AP convertases 10-fold on target surfaces. The full stabilizing effect of properdin on C3bBb would be attained as properdin binds more than one ligand at a time, forming a lattice of properdin:ligand interactions bound to a surface scaffold.

The complement system contributes to both innate and acquired immunity (1). Recent reports indicate that complement has additional biological roles, participating in tissue regeneration (2) and in sperm: egg recognition (3). There are three pathways of complement activation (4), the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP). Although each pathway is induced by a different set of activators, all three pathways lead to the enzymatic cleavage of C3. That step is catalyzed by the C3 convertases. Two homologous C3 convertases occur, one generated by the AP and the other by the CP and LP. Both convertases are serine proteases that cleave C3 at a single site, generating C3a and C3b. Nascent C3b covalently binds to macromolecules, immune complexes, and cell surfaces, where it promotes immune adherence and phagocytosis or membrane lysis, and C3a directs proinflammatory and vasoactive reactions.

The AP presents a unique role in the complement activation cascade. Because it is activated by C3b, its own product, it functions as a positive feedback loop, producing a rapid and vigorous local response. Through C3b, the AP also amplifies activation initially triggered by the CP and LP. The first step in the assembly of the alternative pathway C3 convertase is the association of factor D (a zymogen) with C3b in the presence of Mn⁴⁺ (4). Once bound to C3b, factor B is cleaved by the serine protease factor D, producing the Bα and Bβ fragments. Bβ dissociates from the complex while Bα and the cation remain bound to C3b, forming the active AP C3 convertase, C3bBb. The C3 convertases are serine proteases that cleave only C3. Nascent C3b (C3b’) generated by convertase activity binds to a target surface or water molecule via reaction of its metastable thioester moiety. That reaction takes place within ~60 μs of cleavage, and during that time C3b’ can diffuse up to ~30 nm before it becomes covalently bound to a target (5). C3b can also bind the C3 convertase, forming the C5 convertase, C3bBbC3b. C5 convertase activity leads to the formation of the membrane attack complex.

The C3 convertases are deactivated upon spontaneous dissociation or by facilitated dissociation (decay acceleration) mediated by the regulators of complement activation proteins decay acceleration factor (DAF), complement receptor one (CR1), C4b-binding protein, and factor H (6, 7). Convertase assembly is suppressed by the proteolytic cleavage of C3b (and C4b) as mediated by factor I in the presence of membrane cofactor protein (MCP, CD46), C4b-binding protein, CR1, or factor H (6, 7). These negative control processes are essential for the protection of self-tissue from C-mediated damage and for the prevention of unregulated C3 turnover and depletion.

Properdin is the only known naturally occurring positive regulator of complement activation (8). Individuals deficient for properdin are prone to lethal pyogenic (especially neisserial) infections (9–11). Properdin accelerates the formation of C3 convertases 10-fold and the assembly of C5 convertases, thereby amplifying AP activation (8). Individuals deficient for properdin are prone to lethal pyogenic (especially neisserial) infections (9–11). Properdin increases the stability of the AP convertases 10-fold on target surfaces and immune complexes (12), and inhibits the factor H-mediated cleavage of C3b by factor I (13). We want to understand how properdin influences the AP convertase, and in particular, how it directs AP activation to surfaces. Native properdin occurs in head-to-tail dimers, trimers, and tetramers in the fixed ratio of 22:52:28 (14). The higher oligomers have greater C-regulatory activity (14). Previously, Farries et al. (15) showed that properdin has a greater affinity for cell-bound C3Bb and cell-bound C3bBb than for cell-bound C3b. Moreover, they offered a model for properdin; convertase interactions based on the multivalent nature of properdin:ligand associations (15).

In this study, I used surface plasmon resonance-based assays to examine properdin interactions with C3b and factor B. I demonstrated that properdin accelerates the association of C3b with factor B and provides a focal point for the assembly of C3bBb on a surface. I also observed that properdin binds preformed AP C3 convertases. These findings support a model in which properdin, bound to a target surface via C3b, C3b, or...
other ligands, can use its unoccupied C3b-binding sites as receptors for nascent C3b, bystander C3b, or preformed C3bB and C3bBb complexes. Further C3bBb is formed from the new C3bP and C3bBP intermediates. This model accounts for the capacity of properdin to direct C activation to surface targets.

**MATERIALS AND METHODS**

**Surface Plasmon Resonance Analysis**—Surface plasmon resonance (SPR) methodology provides a sensitive real time measure of protein:protein interactions (16). SPR analysis was conducted with a BIAcore 2000 instrument and the manufacturer’s software package (BIAcore, Piscataway, NJ). CM5 sensor chips (BIAcore catalog number BR-1000-14) were used throughout with Mg2+ dissolved 10 mM HEPES, 150 mM NaCl, 2 mM MgCl2, 0.005% Tween 20, pH 7.4. All experiments were carried out at 25 °C. Kinetic values were obtained using the manufacturer’s software.

BIAcore flow paths were activated with a fresh 1:1 mixture of 0.05 M N-hydroxysuccinimide and 0.2 M ethyl diethylaminopropyl carbodiimide injected at 5 ml/min for 7 min. Complement proteins were then covalently attached to the activated surface. Properdin, C3b, or iC3b (10 μg/ml in 10 mM citrate buffer, pH 4.8) was injected in the experimental flow path, at 10 μl/min for 3–7 min followed by 1 ml ethanolamine, pH 8.5, at 5 μl/min for 7 min. A control flow path was produced as above but without protein in the buffer.

Combinations of purified complement proteins in running buffer (or running buffer alone to observe protein dissociation) were injected at 30 μl/min for varying times. Output was expressed in resonance units (RU). Changes in RU, indicative of changes in mass bound to the biosensor surface, were expressed as the difference between the experiment and control flow paths. Because RUs are linear units, quantitation of ligand binding can be calculated directly from changes in RUs. Non-covalently bound protein was stripped from the properdin-coated surface with a 20-s pulse of 5 M NaCl. N-Hydroxysuccinimide, ethyl diethylaminopropyl carbodiimide, and ethanolamine solutions were purchased from BIAcore and stored at −20 °C.

Proteins—Native human complement proteins (properdin, C3b, iC3b, factor B, Bb, factor H, and factor D) were obtained from Advanced Research Technologies, San Diego, CA. Soluble DAF, CPPs 2–4, which carries the active site of DAF, was a gift of Dr. Feng Lin of Case Western University.

**RESULTS**

**Impact of Properdin on the Association of Factor B with C3b-coated Biosensor Surfaces**—The impact of properdin on C3bBb stability has long been appreciated (12). C3bBb has a half-life of ~2–3 min at 37 °C. Properdin extends that half-life 10-fold. Previous biochemical studies also suggest that properdin can promote C3bBb association (17).

To explore this possibility further, we examined the association of factor B with surface-bound C3b in the presence or absence of properdin. Each SPR biosensor is composed of a series of up to four activated surfaces. Injected fluids flow over each surface in rapid succession. C3b was covalently attached to two flow path surfaces. Subsequently, properdin was bound to one of the two surfaces via non-covalent association with C3b. The properdin:C3b association was relatively stable (~1/12 of 23 min) and likely involved concomitant attachment of polyclonal properdin to multiple C3b ligands. The C3b-coated and the C3bP-coated surfaces were simultaneously treated with fluid phase factor B, and the generation of C3bB and C3bBbP was examined. As shown in Fig. 1, 5-fold more C3bB than C3bBb accumulated during the treatment period. These results suggest that C3bP could be an important intermediate in the formation of AP convertases.
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A biosensor surface was coated with iC3b, another with iC3b and properdin, and a third was coated with C3b. Complement components were injected simultaneously over these three surfaces and the control surface (Fig. 5). When factor B and factor D were added together, as expected, a complex formed on the C3b-coated surface (20). The complex was shown to be C3bBb because it was sensitive to DAF. No complex formed on the iC3b and the iC3b/properdin biosensors, because neither carried the necessary C3b subunit. In contrast, when a mixture of C3b, factor B, and factor D was injected together, a complex was formed on the iC3bP surface. Again, the complex was determined to be C3bBb, as it was sensitive to DAF. A complex was not formed on the iC3b-coated surface, because properdin was not present. These experiments demonstrate that properdin bound to a surface by one ligand-binding site promotes convertase assembly at ligand-binding sites of adjoining subunits.

**Discussion**

Properdin is a positive regulator of the alternative pathway convertases. Its capacity to stabilize AP has been long known (12). In this report I used SPR technology to demonstrate that properdin can also accelerate convertase assembly by several mechanisms. 1) Properdin bound to C3b promotes subsequent association of C3b with factor B. 2) Properdin bound to a C3b-coated target surface through one subunit can provide, through its other subunits, receptors for nascent and bystander C3b, preformed C3bB, and preformed C3bBb. C3bP and C3bBP intermediates can then fully assemble to C3bBb in situ. 3) Properdin bound to an iC3b-coated target surface can provide receptor sites for C3bBb formation.

**Figure 4.** Binding of preformed C3bBb to a properdin-coated biosensor surface. In one case, a properdin-coated biosensor surface (2189 RUs) was treated for 180 s with a mixture of C3b (20 μg/ml), factor B (20 μg/ml), and factor D (2 μg/ml) that had been preincubated in Mg²⁺-HEPES buffer for 3 min prior to injection. In another case, indicated above, a mAb to the Ba portion of factor B (50 μg/ml) known to block C3bBb assembly (18), was added to the protein mixture after the 3-min preincubation and just prior to injection. In a third case, indicated above, factor B was incubated with the anti-Ba mAb for 3 min prior to preincubation with C3b and factor D and subsequent injection. The complexes made in the first two cases were sensitive to factor H at 50 μg/ml for 180 s (not shown). It takes ∼135 s after injection for the protein mix to reach the biosensor surface.

**Figure 2.** Assembly and decay acceleration of C3bBb on a properdin-coated biosensor surface. A, properdin (1652 RUs) was covalently attached to a biosensor surface (see “Materials and Methods”). At t = 0, the biosensor surface was treated for 180 s with a mixture of C3b (20 μg/ml), factor B (fB, 20 μg/ml), and factor D (fD, 2 μg/ml) or with a mixture of C3b (20 μg/ml) and factor B (20 μg/ml) without factor D. The C3bBb peak represents complexes formed with ∼3% of the surface-bound properdin monomers, and the C3bBb-P peak represents complexes formed with ∼3% of the surface-bound properdin monomers. B, C3bBb-P was assembled from C3b, factor B, and factor D on a properdin-coated biosensor surface (1652 RUs) (see A) and then treated with either decay acceleration factor (DAF) at 10 μg/ml for 180 s or factor H (fH) at 50 μg/ml for 180 s. No binding was observed with factor B, factor D, or Bb alone, and less than 10 RUs was seen with C3b alone (not shown).

**Figure 3.** In situ assembly of C3bBbP on a properdin-coated biosensor surface. Properdin (2645 RUs) was covalently attached to a biosensor surface. A, at t = 0, the biosensor surface was treated sequentially with C3b (50 μg/ml) at 20 μl/min for 2 min, a mixture of factor B (fB, 50 μg/ml) and factor D (fD, 5 μg/ml) for 2 min and DAF (10 μg/ml) for 2 min. B, at t = 0, the biosensor surface was treated sequentially with C3b (50 μg/ml) at 20 μl/min for 2 min, factor B (50 μg/ml) for 2 min, and DAF (10 μg/ml) for 2 min.

preincubated together in Mg²⁺-HEPES buffer for 3 min to allow for C3bBb assembly and treated with anti-Ba mAb just prior to injection into the biosensor. In a second case, factor B was first treated in buffer with mAb for 3 min, and C3b and factor D were added to the mixture just prior to injection. In the third case, C3b, factor B and factor D were mixed together in buffer and preincubated for 3 min prior to injection (no mAb). It takes ∼2 additional min for the mixtures to reach the flow cells after injection. The results (Fig. 4) show that anti-Ba mAb of factor B treatment prior to the preincubation period suppresses the formation of properdin C3bBb complexes. However, after the 3-min incubation,
Previous models have proposed that properdin binds to a pre-existing cluster of surface-bound C3b (15). Our findings support a new model in which properdin first binds to a surface ligand (C3b, iC3b, C3bB, or C3bBb) via one of its subunits and then promotes the assembly of a new C3bBB complex at the ligand-binding sites of its adjoining subunits (Fig. 6). The full stabilizing effect of properdin on C3bBB would be attained when properdin binds more than one ligand at a time, with some of those ligands (i.e. C3b) covalently attached to the surface. A lattice of properdin:ligand interactions supported by C3b- or iC3b-dependent covalent bonds to a surface scaffold is more strongly stabilized than each isolated C3bP interaction. Consistent with the model, although not directly supported by the experiments in this study, nascent C3b bound to a target via properdin might be advantageously positioned to form a covalent bond with the target surface.

Using surface-bound C3b, I determined that factor B binds C3bP better than it binds C3b (Fig. 1). Using surface-bound properdin, I found that properdin binds C3bB and C3bBb better than it binds C3b (Fig. 2A).

Two simple explanations could account for these observations. 1) The C3bBP and C3bBb complexes are more stable than C3bP, C3bB, or C3bBb because in the trimolecular complexes, properdin binds directly to factor B and Bb in addition to C3b (21), and 2) C3b may take on two different conformations, one conformation that binds both factor B and properdin with relatively high affinity and a second conformation that binds both proteins with relatively low affinity. By this view, association of properdin or factor B with C3b would partially stabilize its high affinity binding conformation, thus promoting association of C3bBB with properdin or of C3bBP with factor B.

The results of this study have several other interesting implications. First, properdin is a potent complement activator that binds to surface ligands, creating new sites for C3bBB generation. This process is possible because the multiple properdin subunits work together. Although it is known that properdin binds to C3b and iC3b (19), products of an ongoing complement response, properdin is also known to bind sulfatides (sulfated glycosphingolipids (22)). These or other ligands could be the basis for the antibody-independent initiation of the AP on certain target surfaces (see Ref. 23).

Second, properdin could be useful in the immunotherapeutic targeting of complement activation to the surfaces of harmful agents. Thus, a bifunctional agent composed of a targeting element and a properdin sequence could bind a cell or viral surface site and activate complement at that site. Vuagnat et al. (24) demonstrate that a cell surface that expresses a transmembrane form of properdin can activate the AP. Their result suggests that a properdin-dependent targeting strategy may be feasible.

Third, iC3b is a degradative product of C3b that interacts with complement receptor 3 (CR3) and complement receptor 4 (CR4) on macrophages during phagocytosis (25). Previously, it was thought that C activation ceased once C3b was converted to iC3b. The results presented here indicate that iC3b can have a continued role in complement activation, through its affinity for properdin. With C3b, this activity would occur on a surface. This model would make the stability of surface-bound iC3b a critical parameter for complement activation/regulation. iC3b is degraded to C3dg and C3c by factor I in the presence of the regulator CR1. The resulting surface-bound C3dg fragments do not bind properdin and so cannot promote further complement activation. If iC3b is not processed to C3dg efficiently, the accumulation of iC3b on a cell surface could limit cell lifespan or contribute to pathogenesis.

Recent reports demonstrate certain disease states including atypical hemolytic uremic syndrome (26–28) and age-related macular degeneration (29–31) are associated with genetic defects in regulators of the AP. The role of properdin and its ligands in these and other cases should be reevaluated.

In summary, I used SPR to examine properdin interactions with C3b and factor B. I demonstrated that properdin accelerates the association
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of C3b with factor B and provides a focal point for the assembly of C3bBb on a C3b- or iC3b-coated surface. I presented a model in which properdin, bound to a target surface via C3b, iC3b, or other ligands, employs its unoccupied binding sites on its remaining subunits as receptors for nascent C3b, bystander C3b, or preformed C3bB and C3bBb complexes. The new C3bP and C3bBP act as intermediates in the generation of C3bBbP. Thus, properdin amplifies complement AP activation on a target surface through an organized assembly process.

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