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Identification of Complin, a Novel Complement Inhibitor that Targets Complement Proteins Factor B and C2

Archana P. Kadam and Arvind Sahu

Complement factor B (fB) is a key constituent of the alternative pathway (AP). Its central role in causing inflammation and tissue injury through activation of the AP urges the need for its therapeutic targeting. In the current study, we have screened phage-displayed random peptide libraries against fB and identified a novel cyclic hendecapeptide that inhibits activation of fB and the AP. Structure-activity studies revealed that: 1) the cysteine-constrained structure of the peptide is essential for its activity; 2) Ile^{5}, Arg^{6}, Leu^{7}, and Tyr^{8} contribute significantly to its inhibitory activity; and 3) retro-inverso modification of the peptide results in loss of its activity. Binding studies performed using surface plasmon resonance suggested that the peptide has two binding sites on fB, which are located on the Ba and Bb fragments. Studies on the mechanism of inhibition revealed that the peptide does not block the interaction of fB with the activated form of C3, thereby suggesting that the peptide inhibits fB activation primarily by inhibiting its cleavage by factor D. The peptide showed a weak effect on preformed C3 and C5 convertases. Like inhibition of fB cleavage, the peptide also inhibited C2 cleavage by activated C1s and activation of the classical as well as lectin pathways. Based on its inhibitory activities, we named the peptide Complin. The Journal of Immunology, 2010, 184: 7116–7124.

The complement system is one of the strong bulwarks of the host immune system against a wide repertoire of pathogens (1, 2). During the complement system surveillance, pathogens are marked as foreign by covalent tethering of activated C3 on their surface and then killed by various mechanisms (3–5). Although the system is designed to target pathogens, its excessive activation as well as inappropriate intrinsic regulation leads to destruction of the host tissues (6–8). Over the years, a wealth of knowledge has been accumulated revealing the hazardous effects of complement during various diseases and pathological conditions, which include diseases linked to deficiencies, mutations, and polymorphisms in complement regulatory proteins and/or its components, such as paroxysmal nocturnal hemoglobinuria, hereditary angioedema, age-related macular degeneration, atypical hemolytic uremic syndrome, and membranoproliferative glomerulonephritis type II (9–12). Thus, recent times have seen a wide research focus on therapeutic targeting of the complement system (7, 9, 13).

The complement system is activated by three major pathways: classical (CP), alternative (AP), and lectin (LP). Consequently, the damage mediated by complement can occur as a result of activation of any of the three pathways. It is, however, important to point out in this paper that even if tethering of activated C3 (C3b) onto the host tissues is initiated by the CP or LP, this results in activation of the amplification loop of the AP (11, 12, 14). It is therefore not surprising that in many of the above-mentioned diseases, complement-mediated pathologies are galvanized by the amplification loop of the AP (11). Recently, it has been reported that under in vitro conditions, as much as 80% of the total complement activation initiated by the CP and LP is contributed by the AP alone (12). Together, these data clearly point out a need to target the AP for therapeutic benefit.

The complement component factor B (fB), which circulates in blood as a proenzyme, is the key protease of the AP. It forms a three-lobed structure: one corresponds to three complement control protein domains, the second represents a von Willebrand factor type A domain, and the third corresponds to a serine protease domain (15, 16). Association of fB with fluid phase C3(H2O) formed by the process of tick over, or C3b attached to the activating surface, and its cleavage by factor D (fD) leads to activation of fB and formation of the initial [C3(H2O),Bb] or amplification C3 convertase (C3b,Bb) of the AP, respectively (17–19). The C3b,Bb formed on the activating surface triggers the amplification loop of the AP, leading to the attachment of millions of C3b molecules on the target surface within minutes and activation of the terminal complement cascade (20, 21). Thus, blocking fB activation would lead to inhibition of generation of C3a, C5a, and the membrane attack complex, which are responsible for causing tissue injuries (6, 11, 22). Previous efforts to identify small molecule inhibitors against fB led to the identification of a substrate-based peptide inhibitor (Ac-SHLGLAR-H) that showed inhibition of fB-mediated cleavage of C3 with an IC_{50} of 19 μM at nonphysiological alkaline pH (23). In addition, complement C2 receptor inhibitor tripanning-17 peptide (HEVKIKHFSPY) that inhibits C2 activation also showed inhibition of fB activation, but only at a millimolar concentration (24).

In the current study, we have screened phage-displayed random peptide libraries to identify a small peptide inhibitor of fB. Our efforts led to the identification of a novel cyclic hendecapeptide that inhibits activation of fB (IC_{50} = 9.5 μM). The peptide also showed inhibition of C2 activation (IC_{50} = 11.3 μM), suggesting that it has the potential to inhibit pathologies mediated by all the three pathways.
Materials and Methods

Reagents and buffers

Polyclonal Ab against human complement C2 was purchased from Calbiochem (La Jolla, CA). Ab-coated sheep erythrocytes (EAs) were made by incubating sheep erythrocytes with anti-sheep erythrocyte Ab procured from ICN Biomedicals (Irvine, CA). Veronal-buffered saline (VBS) containing 5 mM barbital, 145 mM NaCl, and 0.02% sodium azide (pH 7.4). VBS** was made by adding 0.5 mM MgCl₂ and 0.15 mM CaCl₂ to VBS. Gelatin, Veronal buffer (GVB) was VBS containing 0.1% gelatin, GVB** was GVB containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂. GVB-EDTA was GVB with 10 mM EDTA. MgEGTA contained 0.1 M MgCl₂ and 0.1 M EGTA, and PBS contained 10 mM sodium phosphate and 145 mM NaCl (pH 7.4). TBS was 50 mM Tris-HCl and 150 mM NaCl (pH 7.5).

Complement proteins and their proteolytically cleaved fragments

The human complement proteins C3 (25) and fB (26) were purified from plasma as described earlier. Human complement factor H and D were kindly provided by Prof. Michael K. Pangburn (University of Texas Health Center, Tyler, TX), and C2 and Cls were purchased from Calbiochem. The proteolytically cleaved fragments of fB (Ba and Bb) were generated by incubating fB with C3b, fD, and factor H and purified on a Mono Q column (Amersham Biosciences, Uppsala, Sweden) (27). C3b, the proteolytically activated form of C3, was generated by limited tryptic digestion of C3 and purified on a Mono Q column (28). Native C3 and C3(H₂O) were separated by running the sample on a Mono S column (Amersham Biosciences) (29). Coba venom factor (CVF) was purified from Naja naja kaouthia venom as previously described (30).

Synthetic peptides

All of the peptides used in this study were synthesized by GenoMechanix (Gainesville, FL). The peptides were purified by the supplier on a reversed phase C18 column, and purity was verified by analytical HPLC and mass spectral analysis. The purity of all of the peptides was >95%. For cyclic peptides, cyclization was confirmed by Ellman’s reagent by the supplier.

Biopanning of the phage libraries

Affinity selection for isolation of fB-binding phage clones was performed as described earlier (31) with minor modifications. In brief, microtiter wells were coated with fB (20 µg/well) in PBS by incubating the plate overnight at 4°C. The wells were then blocked with 1% BSA for 30 min at 22°C. After washing the wells once with PBS containing 0.1% Tween 20 (v/v), 2 × 10⁻⁶ M PFUs of each of the M13 libraries (AC-X₇-CGGGS or X₁₂GGGS, where X = any amino acid; New England Biolabs, Ipswich, MA) was added to the wells and incubated overnight at 4°C. The next day, the wells were washed five times with washing buffer (PBS with 0.1% BSA and 0.1% Tween 20 (v/v)). Bound phages were then eluted with 100 mM glycine-HCl (pH 2.7) for 5 min and neutralized immediately with an equal volume of Tris-HCl (pH 8.5). The recovered phage particles were amplified in ER2738 (Biacore, Uppsala, Sweden). All of the binding measurements were performed in PBS containing 0.05% Tween 20 and 5 mg/ml CM Dextran (Fluka, Buchs, Switzerland) at 25°C. These additions to PBS blocked the nonspecific adsorption of the analytes to the sensor chip. To measure binding, ~2000 response units (RU) of fB or its fragments (Ba or Bb) was immobilized on a test flow cell of a CM5 chip using amine-coupling chemistry; nonimmobilized (blank) flow cell served as a control flow cell. Various concentrations of the analyte were then injected for 120 s at a flow rate of 50 µl/min, and dissociation was followed for 180 s. The chip was regenerated by injecting brief alternate pulses of 4 M MgCl₂ and 0.2 M sodium carbonate (pH 9.5). The biosensor data obtained for the control flow cell were subtracted from those obtained for test flow cell and evaluated using BIACore evaluation software version 4.1 using global fitting (Biacore).

ELISA for measurement of binding to fB and C2 cleavage by peptides

The effect of the peptides on fB activity was studied by utilizing a fluid-phase assay (31). Briefly, various concentrations of the peptides were preincubated with fB (2 µg/l) for 15 min at 37°C. A mixture of 2.5 µg C3b and 50 ng fD in a total volume of 20 µl PBS containing 5 mM MgEGTA was then added to each reaction mixture and further incubated at 37°C for 45 min. The inhibition of fB cleavage was visualized by running the reaction mixtures on an 8.5% SDS-PAGE gel under reducing conditions and staining the gel with Coomassie blue. The percentage of fB cleaved was quantitated by densitometric analysis using the VersaDoc XRS system (Bio-Rad, Segrate, Italy). The data obtained were normalized as percent inhibition.

Effect of Complin on C2 cleavage, indicated concentrations of the peptide were preincubated with C2 (150 ng) in 3.5 µl VBS** for 15 min at 37°C and 100 ng activated Cls was then added to each reaction mixture, volume was adjusted to 10 µl by adding VBS**, and reactions were further incubated for 1 h at 37°C. Inhibition of C2 cleavage by the peptide was then determined by running the reaction mixtures on an 8.5% SDS-PAGE gel under reducing conditions followed by Western blot analysis using anti-C2 Ab and anti-goat HRP conjugate (Bio-Rad). The blot was developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and subjected to densitometric analysis to determine the percentage of C2 cleaved. The data obtained were normalized as percent inhibition.

ELISA for measurement of effect on CP, AP, and LP

The effect of Complin on the various complement pathways was also measured using commercially available Wiscal complement system reagent ELISA kit (Euro-Diagnostica, Malmo, Sweden), wherein individual pathways are activated by pathway-specific activators. In brief, suggested dilutions of normal human serum were mixed with graded concentrations of Complin and incubated at 22°C for 15 min. These reaction mixtures were then added to microtiter wells precoated with IgM (for CP), mannan (for LP), or LPS (for AP), incubated at 37°C for 1 h, and washed three times before addition of the conjugate (alkaline phosphatase-labeled anti-C3b). The plate was then incubated at 22°C for 30 min and washed, and the color was developed by adding the substrate. The absorbance was read at 405 nm, and data were normalized as percent inhibition.

Surface plasmon resonance measurements

The kinetics of binding of Complin to fB and its fragments (Ba and Bb) was measured using the surface plasmon resonance (SPR)-based biosensor Biacore 2000 (Biacore, Uppsala, Sweden). All of the binding measurements were performed in PBS containing 0.05% Tween 20 and 5 mg/ml CM Dextran (Fluka, Buchs, Switzerland) at 25°C. These additions to PBS blocked the nonspecific adsorption of the analytes to the sensor chip. To measure binding, ~2000 response units (RU) of fB or its fragments (Ba or Bb) was immobilized on a test flow cell of a CM5 chip using amine-coupling chemistry; nonimmobilized (blank) flow cell served as a control flow cell. Various concentrations of the analyte were then injected for 120 s at a flow rate of 50 µl/min, and dissociation was followed for 180 s. The chip was regenerated by injecting brief alternate pulses of 4 M MgCl₂ and 0.2 M sodium carbonate (pH 9.5). The biosensor data obtained for the control flow cell were subtracted from those obtained for test flow cell and evaluated using BiACore software version 4.1 using global fitting (Biacore).

Measurement of effect on the AP C3 convertase

The effect of Complin on the preformed AP C3 convertase was measured as described below. The AP C3-cleaving enzyme [C3(H₂O),Bb] was formed by incubating 1 µM C3(H₂O) with 2 µg fB and 40 ng fD in 7 µl PBS containing 5 mM MgEGTA for 5 min at 22°C. The reaction was stopped by adding 10 mM EDTA. Immediately thereafter, 3 µg C3 was added along with indicated concentrations of the peptide in a total volume of 20 µl PBS and incubated at 37°C for 45 min. The amount of C3 cleaved (indicated by the generation of α'-chain) in the presence/absence of peptide was determined by running the reaction mixtures on a 6% SDS-PAGE gel under reducing conditions and staining with Coomassie blue.

The effect of Complin on C3 convertase was also evaluated by forming CVF (fB). To form CVF, 1.5 µg CVF was incubated with 2 µg fB and 80 ng fD in 7 µl PBS containing 5 mM MgEGTA for 15 min at 22°C. After stopping the reaction by adding 10 mM EDTA, C3 (3 µg) along with the indicated concentrations of the peptide in 20 µl PBS was
mixtures were then subjected to SDS-PAGE analysis to determine the inhibition of fB and AP activation by peptides. **FIGURE 2.**

As described above, and then it was incubated in a total volume of 20 μl with indicated amount of peptide and C5 (2 μg) for 1 h at 37°C. The reaction mixtures were then subjected to SDS-PAGE analysis to determine the amount of C5 cleaved. The C5 cleavage was indicated by the generation of α’-chain of C5.

**Results**

**Isolation and characterization of fB-binding phages**

By virtue of its rich structural diversity, the phage-displayed peptide libraries have emerged as a powerful tool for isolating ligands against various proteins. In view of this, we screened 12-mer linear and 7-mer cysteine-constrained phage-displayed peptide libraries for isolating ligands against human fB. The affinity selection of clones was performed by biopanning against fB coated on microtiter plates. Both of the libraries were subjected to three rounds of biopanning against fB, and at the end of the third round, isolated phages were screened by ELISA. The screening of ~500 clones from the cysteine-constrained library yielded 25 positive clones. Further DNA sequencing revealed that all of the clones had identical sequences. Concurrent screening of ~1000 clones from the linear library yielded 51 positive clones. Sequencing of these clones confirmed the presence of four unique clones. Binding of these five unique clones displaying constrained and linear peptides to fB is depicted in Fig. 1.

**Effect of phase-displayed peptides on activation of fB**

We next determined whether binding of these phase-displayed peptides to fB inhibits its activation. During AP activation, fB binds to C3b and is then cleaved by fD into Bb and Ba fragments. The cleavage of fB results in its activation. Thus, to examine the effect of peptides on fB activation, we synthesized five peptides corresponding to the peptide sequences displayed on the fB-binding clones and tested them in a fluid-phase assay wherein purified C3b, fB, and fD were incubated with or without peptides in the presence of MgEGTA, and then fB cleavage was monitored by SDS-PAGE analysis. In this assay, inhibition of fB cleavage into Ba and Bb indicated the inhibition of fB activation.

Of the five synthetic peptides examined, only peptide 1 corresponding to the clone L14 displayed inhibitory activity. The IC50 of this linear peptide for inhibition of fB activation was 56 μM (Fig. 2, Table I). Thus, 50% inhibition of fB activation required ~52-fold molar excess of this peptide.

**FIGURE 1.** Binding of phages isolated from phage-displayed random peptide libraries to fB and sequence alignment of the phage-displayed peptides. **Top panel,** ELISA depicting binding of the phage clones isolated from phage-displayed peptide libraries to fB. Microtiter wells were coated with fB and blocked with BSA, and phage clones were allowed to bind. Binding of phages was detected by anti-M13 HRP Ab. Phage clone numbers starting with L and C denote clones isolated from linear and constrained libraries, respectively. Clone L15 served as a negative control. **Bottom panel,** alignment of peptide sequences displayed on the fB-binding phages using DIALIGN 2.2.1.

**TABLE 1.** Peptide length, phage displayed amino acid sequence, and percentage of fB cleavage inhibition is represented graphically against concentration. The intensities of fB in gels were quantitated densitometrically, and percentage of fB cleavage inhibition is represented graphically against concentration. **Bottom middle panel,** graph showing increase in the inhibitory activity of Complin over a period of time on storage on ice. **Bottom right panel,** inhibition of AP-mediated lysis of rabbit erythrocytes by Complin. Inhibition of C3b associated fB cleavage by peptide 1 (○); inhibition of C3b-associated fB cleavage by Complin (●); inhibition of CVF-associated fB cleavage by Complin (○); inhibition of AP-mediated lysis of erythrocytes by Complin (●).
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*aInhibition assays were performed after keeping the peptide solutions for seven days on ice. Asterisk indicate oxidized cysteines.

*bInhibition of Fb cleavage was measured using a fluid phase assay (see Materials and Methods).

*cInhibition of AP activity was measured by hemolytic assay (see Materials and Methods).

dMaximum concentration of the peptide that could be tested in the assay due to solubility limitations.
Design and characterization of motif-based cyclic peptides and identification of Complin

In an effort to design a peptide with enhanced inhibitory activity, we examined the phage-displayed peptide sequences for the presence of a motif. Alignment of five phage-displayed peptide sequences using DIALIGN (33), which constructs multiple alignments by comparing whole segments of the sequences but does not use gap penalty, showed the presence of eight conserved residues with a highly conserved four-residue core (Fig. 1). Next, based on the motif, we synthesized 15 different disulfide-constrained peptides. Although all the phage-displayed peptides except peptide 5 (corresponding to the phage clone C5) were linear, we designed motif-based peptides as cyclic peptides, as they are likely to be structured and have increased $t_{1/2}$ in serum as compared with the linear peptides (34, 35). To optimize the structure of the peptide, we primarily varied the ring size and residues outside the four-residue core. Functional characterization of these peptides for inhibition of fB activation demonstrated that only 4 of the 15 peptides (peptides 9, 15, 18, and 19) showed increased inhibitory activity compared with peptide 1 (Fig. 2, Table I). The most active peptide was peptide 19 with an IC$_{50}$ of 9.5 $\mu$M, indicating that ~9-fold molar excess concentration of this peptide was sufficient to inhibit 50% of fB activation. As expected, peptide 19 also inhibited AP-mediated lysis of rabbit erythrocytes (IC$_{50}$ = 33 $\mu$M; Fig. 2, Table I). Based on its activities (described here and below), we have named this peptide Complin (“compl”ement “in”hibitor).

Apart from its association with C3b, fB activation also occurs when it is associated with C3b-like molecule CVF followed by its cleavage by fD. Moreover, recently it has been demonstrated that CVF-bound fB show a difference in conformation compared with C3b-bound fB (36). Thus, we also sought to determine if Complin has the ability to inhibit the activation of CVF-associated fB. Data presented in Fig. 2 (top right panel) clearly show that Complin is equally efficient in inhibiting the activation of CVF-associated fB (IC$_{50}$ = 12 $\mu$M).

An intriguing observation we made during the course of this study was that Complin showed a gain in activity when stored on ice over 7 d (Fig. 2). We suspected that the peptide might be forming multimers upon storage. To verify this, we performed MALDI-mass spectrometry (MALDI-MS) as well as electrospray ionization-MS (ESI-MS) analyses of a 1-mo-old sample stored on ice. The stored sample did not show the presence of multimers by MALDI-MS, but did confirm their presence (2–6M species) when analyzed by ESI-MS (data not shown).

Identification of structural features important for inhibitory activity of Complin

During designing the motif-based constrained peptides, we made an assumption that cyclization would result in stabilization of peptide structure. Thus, to validate our premise, we synthesized a linear analog of Complin wherein cysteines were replaced with Ala (peptide 21). This analog did not show any inhibitory activity even at 500 $\mu$M concentration (Table I), suggesting that, indeed, the disulfide bond plays an important role in the maintenance of a preferred structure of the peptide required for its inhibitory activity.

To determine the importance of amino acid residues of Complin between Cys 2 and Cys 10 in its inhibitory activity, we performed an Ala scan analysis. We synthesized a set of six Ala analogs (peptides 22–27; Table I) and examined their effect on fB activation using the fluid-phase assay described above. Substitutions of each of the residues between Cys 2 and Cys 10 with Ala showed that substitutions at all the positions except at positions 4 and 9 drastically hampered its activity (Table I). These data therefore suggested that Ile$^2$, Arg$^6$, Leu$^7$, and Tyr$^8$ play a critical role in maintaining the functional activity of the peptide.

Retro-inverso analogs are considered important tools in dissecting the relative role of the main-chain and side-chain interactions in ligand-protein recognition because in such analogs, the conformation of the side-chains are same as that in the l-isof orm, but conformation of the main-chain atoms is reversed (37). Synthesis and functional analysis of a retro-inverso analog of Complin (peptide 28) showed that it was completely inactive (Table I). From these results, it can be inferred that in addition to the side chains, the main-chain atoms of Complin also contribute in its interactions with fB.

Kinetic analysis of interaction of Complin with fB and its fragments Ba and Bb

To examine the binding kinetics of Complin with fB, we undertook Biacore studies. We first synthesized biotinylated peptides for orientation onto the streptavidin (SA) chip. Addition of biotin at the C terminus of Complin resulted in loss of activity of Complin (peptide 29; Table I). We therefore reversed the setup and coupled fB or its fragments onto CM5 chips by amine coupling chemistry and flowed Complin; the control flow cell was either left blank or immobilized with C3b. Complin bound to fB in a dose-dependent manner. Its linear analog (peptide 21), however, showed negligible binding to fB, suggesting that the cyclic nature of the peptide is important for binding to fB (Fig. 3). To analyze the binding affinity and the nature of interaction, the association and dissociation data were fitted to various models. Data showed a good fit to a bivalent analyte model ($\chi^2 = 1.58$); the apparent equilibrium rate constants ($K_i$ and $K_{ij}$) were 3.05 $\mu$M and 4.16 $\mu$M, respectively (Fig. 3, Table II).

Because binding data fit well to a bivalent analyte model, we asked whether binding sites are localized only in one of the subfragments of fB or on both Ba and Bb. We generated Ba and Bb by cleaving C3b-associated fB by fD, purified them on a Mono Q
column (Amersham Biosciences), and coupled them onto different flow cells of a CM5 chip. When Complin was flown over Ba and Bb, binding was observed to both the fragments, suggesting that binding sites are located in Ba as well as Bb fragments (Fig. 3). As expected, binding data for both Ba and Bb fit well to the 1:1 Langmuir binding model (Fig. 3, Table II).

**Complin does not inhibit binding of fB to C3b**

Inhibition of fB activation by Complin could occur as a result of blocking of fB binding to C3b. To delineate this, we designed the following experiment. We coupled C3b onto an SA chip and incubated either fB or fB preincubated with Complin or its linear analog (peptide 21). A positive control was formed by injecting fB preincubated with anti-fB mAb NCCS 34.78, which binds to Ba and inhibits fB activation. fB showed good binding response, but preincubation of fB with either 10- or 30-fold molar excess of Complin compared with fB did not result in a decrease in fB binding response (Fig. 4). No effect on the binding response was also observed when fB preincubated with 30-fold molar excess of the linear analog (peptide 21) was injected onto the chip. As expected (19), preincubation of mAb resulted in a significant decrease in fB binding response (Fig. 4). It is therefore apparent that Complin does not inhibit interaction of fB with C3b.

**Effect of Complin on the AP C3 and C5 convertase activities**

Our SPR data indicated that Complin binds to Bb as well as Ba fragments of fB (Fig. 3). The Bb fragment provides the catalytic subunit to the AP C3 and C5 convertases. It was therefore likely that in addition to inhibition of fB activation, the observed inhibitory effect of Complin on activation of the AP (Fig. 2) could also be in part due to its effect on the C3 and C5 convertases. To verify this premise, we studied the effect of Complin on preformed C3 and C5 convertases. The fluid-phase C3 convertases C3(H2O), Bb and CVF, Bb were formed by incubating C3(H2O) or CVF with fB and fD in the presence of Mg2+. These were then incubated with native C3 in the presence of increasing concentrations of Complin. The peptide had very weak effect on C3(H2O), Bb-mediated cleavage of C3 even at 100 μM concentration, which was ~10 times higher than that required for 50% inhibition of fB activation (Fig. 5). It, however, showed ~30% inhibition of CVF, Bb-mediated C3 cleavage at 30% inhibition of CVF, Bb-mediated C3 cleavage at

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>k_{d1}/k_{a1} (1/Ms)</th>
<th>SE (k_{d1}/k_{a1})</th>
<th>K_{11} (μM)</th>
<th>k_{d2}/k_{a2} (1/Ms)</th>
<th>SE (k_{d2}/k_{a2})</th>
<th>K_{12} (μM)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>fB</td>
<td>Complin</td>
<td>1.58 × 10^{-3}/518</td>
<td>5.08 × 10^{-3}/525</td>
<td>3.05</td>
<td>0.0529/1.27 × 10^{-5}</td>
<td>2.77 × 10^{-3}/817</td>
<td>4.16</td>
<td>1.58</td>
</tr>
<tr>
<td>Ba</td>
<td>Complin</td>
<td>2.42 × 10^{-3}/2.3 × 10^{-5}</td>
<td>3.59 × 10^{-3}/50.8</td>
<td>1.05</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.299</td>
</tr>
<tr>
<td>Bb</td>
<td>Complin</td>
<td>2.04 × 10^{-3}/1.3 × 10^{-3}</td>
<td>3.09 × 10^{-3}/21.5</td>
<td>1.57</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.889</td>
</tr>
</tbody>
</table>

* k_{d2}/k_{a2} (1/Ms) = k_{d2}/k_{a2} × 100 × (m.w. of the ligand).
* Data were calculated by global fitting to a bivalent analyte model (BIAevaluation 4.1).
* Data were calculated by global fitting to a 1:1 Langmuir binding model (BIAevaluation 4.1).

NA, not applicable.

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**FIGURE 4.** Effect of Complin on binding of fB to C3b. fB (100 nM) preincubated with Complin (1 μM or 3 μM), linear analog of Complin (peptide 21; 3 μM), or a control anti-fB mAb (400 nM; it binds to Ba fragment and inhibits fB activation) at 25°C for 15 min was injected on an SA chip coated with C3b, and binding and dissociation were measured. The C3b-coated flow cell was generated by immobilizing biotinylated C3b (189 RU) on an SA chip followed by deposition of additional C3b molecules (~4000 RU) by forming AP C3-convertase onto the flow cell (26, 41).

**FIGURE 5.** Effect of Complin on C3 and C5 convertases. Top and middle panels, effect of Complin on C3 convertases. The C3 convertases C3(H2O), Bb (top left panel) and CVF, Bb (middle left panel) were formed by incubating fB, fD, and C3(H2O) or CVF in the presence of MgEGTA at 25°C for 5 min or 15 min, respectively. The formation of the convertases was stopped by adding 10 mM EDTA. Native C3 and Complin or the linear analog of Complin (peptide 21) were then added to the preformed C3 convertases [C3(H2O), Bb or CVF, Bb]. The reaction mixtures were analyzed for C3 cleavage (revealed by the generation of α’-chain) on a 6% SDS-PAGE gel under reducing conditions. Bottom left panel, effect of Complin on C5 convertase. CVF, Bb was formed as described above and incubated with C5 and Complin or the linear analog (peptide 21). The reaction mixtures were then analyzed for C5 cleavage (revealed by the generation of α’-chain) on a 6% SDS-PAGE gel under reducing conditions. All of the gels were stained by Coomassie blue to visualize the cleavage products and scanned for densitometric analysis. Results of the respective densitometry are presented in the right panels.
100 μM concentration (Fig. 5). The CVF-derived convertase CVF,Bb is also known to cleave C5. Therefore, to study the effect of Complin on C5-convertase activity, we measured inhibition of C5 cleavage by CVF,Bb. The peptide displayed a weak effect up to 10 μM concentration (14% inhibition), but showed ~70% inhibition of C5 cleavage at 100 μM concentration (Fig. 5).

**Effect of Complin on activation of C2 and CP and LP**

Human fB is homologous in structure and function to the complement protein C2. We therefore also sought to examine whether Complin inhibits C2 activation. To measure the effect of Complin on C2 activation, purified C2 was incubated with activated C1s in the presence of various concentrations of Complin and C2 cleavage was quantitated. The results showed that like fB, Complin also inhibited C2 activation in a dose-dependent manner. The IC50 for inhibition of C2 cleavage was 11.3 μM (Fig. 5).

Because Complin inhibited C2 activation, we next examined its effect on the CP using hemolytic assay. To selectively activate the CP, EAs were incubated with fB-depleted sera, and the degree of lysis was measured. As expected, Complin inhibited the CP-mediated lysis of erythrocytes in a concentration-dependent manner. Its IC50 for inhibition of C2 activation was 11.3 μM (Fig. 6).

Inhibition of fB and C2 activation by Complin (Figs. 2, 6) clearly suggested that Complin has an ability to inhibit all the three pathways of complement activation. We thus measured its effect on the LP and compared it with that on the CP and AP using a commercially available ELISA-based functional assay kit (Wieslab kit, Euro-Diagnostica) (Fig. 7). Complin showed inhibition of all the pathways with IC50 of 3.1 μM for the CP and 6.1 μM and 31.5 μM for the CP and AP, respectively.

**Discussion**

Therapeutic targeting of the complement system has garnered much attention in the past decade due to its involvement in a plethora of pathologies (7, 10–13). Because the AP has been implicated in mediating tissue damage in many human diseases (12, 38), we sought to identify a small molecule inhibitor against the AP component fB. Our efforts led to the identification of an 11-mer cyclic peptide that showed inhibition of fB and C2 activation. The peptide has been named Complin.

In the current study, we screened random peptide phage-display libraries against fB as a means to identify small molecule inhibitors against fB because this technique has been successful in identifying peptide ligands against various protein targets (39, 40), including complement protein C3 (31). It is believed that this method is more successful in identifying ligands against various proteins because the peptide ligands favor binding to recesses or cavities in the target proteins by displacing water molecules, and typically, such sites are biologically important (39). Our screening led to the identification of five peptide ligands against fB, but only one showed inhibition of fB activation at a high micromolar concentration (IC50 = 56 μM) (Fig. 1, Table I). The same peptide (peptide 1) also inhibited AP-mediated lysis of erythrocytes, but only at a millimolar concentration (IC50 = 2.7 mM) (Table I), presumably because of proteolysis of the peptide by serum enzymes. Thus, to design analogs with increased efficacy and 11/2, in serum, we first searched for the presence of a motif in these fB-binding peptides. Interestingly, sequence alignment showed the presence of eight conserved residues with a highly conserved four-residue core (Fig. 1). Consequently, we synthesized a series of constrained peptides (peptides 6–20; Table I) based on this
motif with variations in ring size and residues flanking the four-residue core. This exercise resulted in the identification of Complin, which was ~6-fold more potent in inhibiting fB activation and 82-fold more potent in inhibiting the AP (Fig. 2, Table I) than the parent peptide.

Human fB is known to interact with C3b, the proteolytically activated form of C3, and CVF, the C3 homolog present in Indian cobra venom. We therefore examined whether there is a sequence similarity between Complin and any part of C3b/CFVF. We, however, did not observe any obvious sequence similarity between Complin and these proteins. Further, there was also no sequence similarity between Complin and any of the known complement regulators, indicating Complin is a novel peptide inhibitor of fB.

Structure-activity relationship studies in Complin showed that oxidation of cysteine residues is important for its binding to fB as well as its functional activity (Fig. 3, Table I). These data therefore indicate that cyclization is a prerequisite for maintaining the preferred structure of Complin for its binding and inhibitory function. Ala scan analysis of the peptide revealed that residues Ile6, Arg7, Leu8, and Tyr9 are critical for the functional activity of the peptide (Table I). Because the peptide binds to both the Ba and Bb fragments of fB (Fig. 3), it is likely that these residues are part of the binding sites for Ba and Bb. Binding of Complin to fB can involve interactions between side-chain as well as main-chain atoms. Data presented in Table I show that a retro-inverso peptidomimetic was completely inactive. It is therefore logical to presume that apart from side-chain interactions, main-chain atoms are also critical for binding of Complin to fB.

Activation of fB entails two steps: 1) its association with fluid-phase C3(H2O) or target-bound C3b; and 2) its cleavage at Arg234-Lys235 by fD into Ba and Bb fragments. Thus, to understand the mechanistic basis of Complin-mediated inhibition of fB activation, we asked whether Complin inhibits binding of fB to C3b. We designed an SPR assay wherein we measured binding of fB or fB preincubated with Complin to C3b immobilized on a sensor chip. No inhibition of fB binding to C3b was observed in this assay (Fig. 4), suggesting that inhibition of fB activation by Complin must be due to inhibition of fB cleavage by fD. During binding measurements, we observed that Complin binds to both Ba and Bb fragments of fB (Fig. 3). We therefore hypothesize that by binding to Ba and Bb fragments, Complin locks the conformation of fB in its proenzyme state (16) in which the scissile bond (Arg234-Lys235) remains occluded and thereby inhibits its cleavage. Interestingly, we observed that activity of Complin increases with time (Fig. 2). ESI-MS analysis of the stored sample indicated that Complin multimerizes after storage. Thus, we speculate that multimerization increases the steric hindrance for access to fD, which results in an increase in its activity.

Because Complin showed binding to Bb, we also examined whether it has the ability to inhibit the activities of preformed convertases C3(H2O)Bb and CVF:Bb. It is clear from data presented in Fig. 5 that it has only a partial effect, if any, at a high concentration on these enzymes. Thus, the peptide primarily inhibits the AP by inhibiting activation of fB. The complement components fB and C2 are structural homologs. We therefore looked whether Complin inhibits C2 activation. Interestingly, the peptide did show inhibition of C2 activation and also the CP and LP (Figs. 6, 7). The fact that Complin inhibits C2 cleavage by activated Cls also supports our hypothesis that Complin inhibits the second step of fB activation (i.e., its cleavage by fD).

In summary, we have identified a novel small cyclic peptide that inhibits activation of fB and C2 and thus has the potential to block complement-activated pathways resulting from activation of any of the three pathways. Because small m.w. inhibitors form an attractive option for developing therapeutics, Complin could serve as a lead molecule to design analogs with therapeutic potential. In addition, Complin could also serve as an important tool to study fB and C2 activation process, the molecular basis of which is still poorly understood.

Disclosures
The authors have no financial conflicts of interest.

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