Short communication

Compstatin inhibits complement activation by binding to the β-chain of complement factor 3

Athena M. Soulika, M. Claire H. Holland,
Georgia Sfyroera, Arvind Sahu [1], John D. Lambris [*]

*Protein Chemistry Laboratory, Department of Pathology and Laboratory Medicine, 402 Stellar Chance, 422 Curie Blvd.,
University of Pennsylvania, Philadelphia, PA 19104, United States*

Abstract

Compstatin is a peptidic complement inhibitor that prevents the cleavage of complement factor 3 (C3) by C3 convertase. Compstatin differs from other C3-regulatory proteins, such as complement receptor (CR) 1 and decay-accelerating factor (DAF), in that it binds native as well as activated C3 fragments and acts through mechanisms that do not involve the destabilization of the C3 convertase or the accelerated degradation of C3b. Compstatin's activity most likely relies on its affinity for native C3 and the conformational change that results upon binding with C3. Although the intermolecular interactions between compstatin and C3 have been studied, the identity of the targeted region on C3 is still elusive. To address this issue, we synthesized a photo-crosslinking compstatin analog and used it to probe C3 for sites of interaction. We identified a 40-kDa region at the C-terminus of the β-chain of C3 that included the binding site of the compstatin analog. The specificity of the binding was confirmed by inhibition studies, which showed reduced crosslinking signal after pre-incubation of C3 with compstatin but not with various inactive analogs. Binding studies performed with a recombinant homolog of the 40-kDa region confirmed these findings. Five smaller recombinant proteins corresponding to various overlapping regions of the 40-kDa fragment did not bind compstatin, suggesting that a proper protein conformation, only found in larger fragments, is required for compstatin binding. The identified region on the β-chain has, thus far, not been implicated in C3 cleavage or interactions with other proteins. Therefore, further research on this part of the C3 molecule may have implications for studies on the regulation of C3 cleavage, as well as for complement-based drug design.
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1. Introduction

Uncontrolled complement activation has been associated with over 25 immuno-inflammatory conditions, including psoriasis, glomerulonephritis, rheumatoid arthritis, myocardial infarction, asthma, and hemolytic anemia (Morgan and Harris, 2003; Lambris and Holers, 2000). The search for an efficacious and safe complement inhibitor has yielded various candidates, none of which are yet clinically available. These compounds range from small molecules that block the actions of the C5a anaphylatoxin to large complex proteins targeting the enzymatic complexes that regulate C3 activity (Holland et al., 2004; Morgan and Harris, 2003). Several other compounds that hold promise for further development are still in the discovery phase. One such compound is compstatin, a cyclic 13-amino acid peptide that blocks complement activation by binding to C3 (Sahu et al., 1996).

Compstatin was originally identified after screening a phage-display library for C3b-binding peptides. Since then, it has been shown to be an effective inhibitor of complement activation in various clinically relevant experimental settings (reviewed in Holland et al., 2004), such as an in vitro whole human blood tubing loop model of biopolymer-induced complement activation and an in vivo non-human primate model of heparin–protamine complex-associated inflammation (Nilsson et al., 1998; Soulika et al., 2000). By targeting C3, compstatin is able to block acti-

* Corresponding author. Tel.: +1 215 746 5762/5765; fax: +1 215 573 8738.
  E-mail address: lambris@mail.med.upenn.edu (J.D. Lambris).
[1] Present address: National Centre for Cell Science, Pune University Campus, Ganeshkhind, Pune 411007, India.

vation via all three complement activation pathways, the classical (CP), lectin (LP) and alternative (AP) pathways, and it inhibits all downstream complement activation events, including the generation of the C3a and C5a anaphylatoxins and C3b(i) opsonins and the formation of the membrane attack complex (MAC; C5b-9).

In recent years, considerable progress has been made in resolving the structure–activity relationships of compstatin (Klepeis et al., 2003; Morikis et al., 2002; Morikis and Lambris, 2002). Using computational and rational design approaches and compstatin's three-dimensional structure, two residues, Val[4] and His[9], were shown to be amenable to substitution, leading to the development of six more potent analogs, including the 45-fold more active V4W/H9A analog and the 99-fold more potent V4(2Nal)/H9A compound (Katragadda et al., 2004; Mallik et al., 2005).

The use of surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) has led to the identification of some of the molecular interactions that govern the binding between compstatin and C3. These studies have demonstrated that compstatin binds to C3b with a 1:1 stoichiometry (Sahu et al., 2000; Soulika et al., 2003). In addition, three of the most potent analogs displayed enhanced affinity for native C3 by allowing additional enthalpically favorable non-covalent interactions (Katragadda et al., 2004).

Despite the wealth of information that has been obtained regarding compstatin's structural and physicochemical properties, the binding site of compstatin on C3 has not yet been identified. Therefore, in the present study we have synthesized a photo-crosslinking compstatin analog and used it to probe C3 for potential sites of interaction. Identification of these sites is crucial for our understanding of compstatin's activity and may reveal novel regulatory regions in C3.

2. Materials and methods

2.1. Purified proteins

Human complement protein C3 was purified from normal human plasma as previously described (Becherer and Lambris, 1988). C3c was generated by elastase treatment of C3 and purified on a Mono Q column (Amersham Biosciences, Piscataway, NJ). Trypsinized C3c was obtained by treating purified C3c overnight with 5% trypsin. The reaction was stopped by adding 10% soybean trypsin inhibitor (Becherer and Lambris, 1988).

2.2. Peptide synthesis, purification, and characterization

Compstatin (Ac-Ile-Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys-Thr-NH$_2$) was chemically synthesized using solid-phase peptide synthesis employing F-moc chemistry as previously described (Sahu et al., 1996, 2000). The peptide was purified on a C-18 reversed-phase high performance liquid chromatography column (Waters, Milford, MA) to 95% purity and cyclized (Angeletti et al., 1996). Subsequently, the peptide's N-terminus was acetylated. The purity and identity of

the peptide was critically monitored by analytical chromatography on a reversed-phase C-18 column and by laser desorption mass spectrometry (Moore, 1997). Formation of the disulfide bond was confirmed by mass spectrometry using a mass shift assay (Angeletti et al., 1996). For the crosslinking studies, a compstatin analog was essentially synthesized as described above for native compstatin, but with the addition of the photo-crosslinking amino acid, *p*-benzoyl-L-phenylalanine (Bpa), followed by a FLAG sequence (Asp, Tyr, Lys, Asp, Asp, Asp, Asp, Lys). The FLAG tag was added to facilitate the detection of the peptide by Western blotting, using the anti-FLAG M2 antibody (Sigma, St. Louis, MO). The extra nine residues were added to the C-terminus of the peptide because this region, in contrast to the N-terminus, is believed to be non-critical for C3-compstatin binding interactions (Sahu et al., 1996, 2000).

2.3. Cloning and bacterial expression of a 40-kDa C3 fragment

A 40-kDa fragment corresponding to the C-terminus of the β-chain of human C3 (see Fig. 4) was amplified by PCR using the forward primer: GAC AGC AGC AAG ATG CAG GAT GGC GAA CAG AG and reverse primer: GAG GAG GAA CCC GGT TCG GCG GGC TGG CT. The 1269-base fragment was cloned into the pET-30LIC vector (EMD Biosciences, Madison, WI) according to the manufacturer's instructions. The pET-30LIC vector added a cleavable six-amino acid His-Tag to the N- and C-termini of the protein. The recombinant plasmid was transformed into the *E. coli* host strain BL21(DE3), and protein expression was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to the growing cell culture. The cells were pelleted and resuspended in 0.1 M NaH$_2$PO$_4$, 0.01 M Tris–HCl, 0.3 M NaCl, 8 M urea, and 20 mM imidazole, pH 8.0. The cleared lysate was mixed with Ni-NTA agarose (Qiagen, Valencia, CA) for 1–2 h and loaded onto a disposable column. The column was washed with 0.1 M NaH$_2$PO$_4$, 0.01 M Tris–HCl, 0.3 M NaCl, and 20 mM imidazole, pH 8.0, followed by 0.1 M NaH$_2$PO$_4$, 0.01 M Tris–HCl, 0.15 M NaCl, and 50 mM imidazole, pH 8.0. The recombinant protein was eluted with 0.1 M NaH$_2$PO$_4$, 0.01 M Tris–HCl, 0.15 M NaCl, and 250 mM imidazole, pH 8.0, and dialyzed overnight in PBS. The protein solution was aliquotted and frozen at −70 °C. The 39.5-kDa C-terminal fragment of the α chain of C3c was produced similarly and was used as a control protein for the compstatin binding studies. Binding studies were also carried out with the β-chain fragment refolded overnight in refolding buffer (0.1 M Tris–HCl containing 2 mM reduced glutathione, 0.2 mM glutathione, and 0.005% Tween 80). However, since no differences in binding could be observed when compared to the first protein preparation, the data obtained with the additionally refolded protein preparation are not presented.

Seven overlapping fragments (10–12 kDa each) within the 40-kDa protein were expressed separately as described above (see Fig. 4). Five of the constructs yielded soluble protein, and only these five proteins were used for additional phage binding studies.

2.4. Crosslinking studies and detection of the compstatin-Bpa-FLAG peptide

To identify the compstatin binding site on C3, purified human C3, C3c, trypsinized C3c, and a 40-kDa recombinant C3 fragment were incubated individually with the compstatin-Bpa-FLAG peptide in PBS at a molar ratio of 1:5 for 2 h at room temperature. The mixture was exposed to UV radiation (350 nm) for 1 h, then subjected to electrophoresis on a 7.5% SDS-PAGE gel under reducing conditions. For Western blot analyses, the proteins were transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk and immunoblotted with an anti-FLAG M2 antibody (1:1000; Stratagene, La Jolla, CA), followed by an anti-mouse HRP-conjugated antibody (Bio-Rad, Hercules, CA). ECL was used as the substrate to identify the compstatin-Bpa-FLAG peptide. After Western blotting, membranes were stained with Amido black for visualization of the transferred protein.

For the inhibition experiments, C3, C3c, trypsinized C3c, and the 40-kDa fragment were individually incubated with the compstatin-Bpa-FLAG peptide in the presence of a 100–1000 molar excess of compstatin, linear compstatin (which is inactive), or an inactive control peptide (Ile-Pro-Pro-Ser-Leu-Arg-Tyr-Leu-Glu-Asp-Asn-Glu-Glu-Arg-Met-Ser-Arg-Leu-Ser-Lys). The samples were further processed as described above.

2.5. Compstatin-phage binding assay

Microtiter wells (Nunc, Rochester, NY) were coated with 50 μl of 10 μg/ml C3, the 40-kDa recombinant C3 β-chain fragment, or a 39.5-kDa fragment from the α chain of C3c (control protein), in PBS for 2 h at room temperature. The wells were blocked with 1% nonfat milk in half-ionic strength PBS (7 mS/cm, pH 7.4) for 1 h, and 25 μl of an in blocking solution serially diluted compstatin-phage or control phage solu-
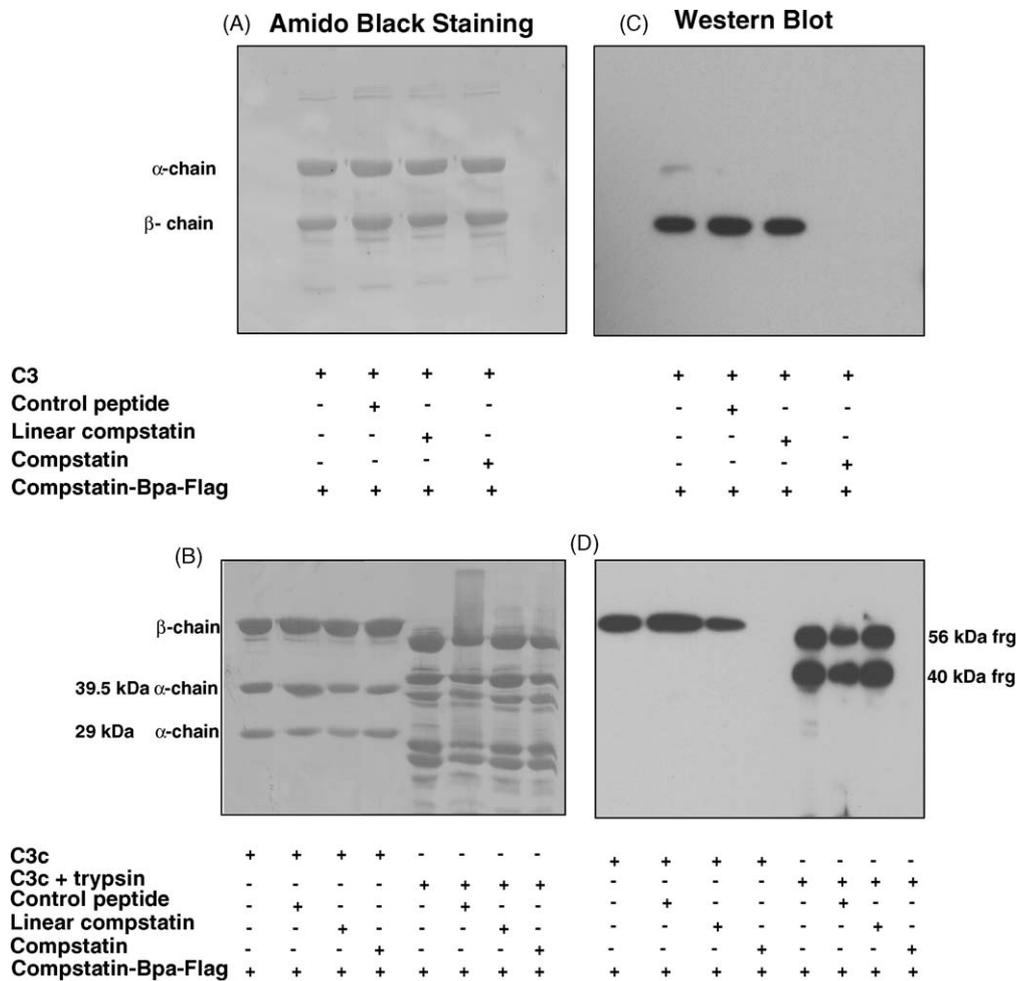

Fig. 1. Binding of compstatin with the β-chain of C3 and C3c. A photo-crosslinking compstatin analog (compstatin-Bpa-FLAG) was allowed to bind to purified human C3, C3c and trypsinized C3c and exposed to UV light. The crosslinked protein mixture was run on a 7.5% SDS-PAGE gel under reducing conditions, followed by Western blotting. (A) Staining of the membrane with Amido black after Western blot analysis revealed the location of the α- and β-chains of C3. (B) Amido black staining of C3c revealed the the β-chain and the 29 kDa N-terminal and 39.5 kDa C-terminal α-chain fragments. Trypsinization of C3c resulted in the fragmentation of all three proteins. (C, D) Western blot analysis and an anti-FLAG antibody were used to detect the location of the compstatin-Bpa-FLAG analog. Compstatin immunoreactivity co-localized with the β-chain of C3 and C3c and two β-chain fragments (56 and 40 kDa) of trypsinized C3c. Compstatin-Bpa-FLAG binding to C3, C3c and trypsinized C3c was competitively inhibited by intact compstatin, but not by two inactive analogs (linear and a control peptide).

tion (Soulika, 2003; Sahu, 1996) was added directly to each well. The plate was incubated for 30 min at room temperature. After two washes with 200 µl PBS containing 0.002% Tween (PBST), 50 µl of 1:1000 diluted anti-M13 mAb conjugated to HRP (Amersham Biosciences, Piscataway, NJ) was added to each well. After a 40-min incubation, the wells were washed three times with 200 µl PBST, and the binding was detected by addition of the HRP substrate (0.05% ABTS in 0.1 M sodium citrate, pH 4.2). The plates were read at 405 nm.

3. Results

3.1. Compstatin binds to the β-chain of C3

To identify the compstatin binding site on C3, a photo-crosslinking compstatin analog (compstatin-Bpa-FLAG) was synthesized. This analog was found to possess a complement inhibitory activity equal to that of compstatin, based on a complement-mediated hemolysis assay (data not shown). Exposure of a pre-incubated mixture of purified C3 and compstatin-Bpa-FLAG to UV light, which results in the formation of a covalent bond between the Bpa residue of compstatin and neighboring residues on the target C3 molecule, followed by anti-FLAG immunoblotting, led to the identification of the peptide binding region on C3 molecule. The anti-FLAG immunoreactivity we observed co-localized with the β-chain of C3 (Fig. 1). Similar results were obtained with C3c, the smallest C3 fragment that still binds compstatin (Fig. 1). To ensure that the crosslinking represented a true interaction with the binding site of compstatin, and not merely a non-specific binding interaction between the analog peptide and C3, we conducted a series of inhibition experiments. For this purpose, we incubated C3 and C3c with the compstatin analog in the presence of excess amounts of compstatin or one of two inactive control peptides (linear compstatin and a scrambled peptide). The anti-FLAG immunoreactivity was completely lost in the presence of intact compstatin, but not when C3/C3c was incubated with either one of the inactive peptides (Fig. 1). This finding strongly suggests

that the compstatin-Bpa-FLAG analog binds to the same region as compstatin.

3.2. Compstatin binds to a 40-kDa fragment of the C3 β-chain

To further localize the compstatin binding region, purified C3c was trypsinized, causing fragmentation of the molecule, and the trypsinized protein was incubated with the compstatin-Bpa-FLAG analog. Immunoblotting of the trypsinized C3c preparation showed two fragments displaying anti-FLAG immunoreactivity, a 56-kDa and a 40-kDa fragment (Fig. 1). Their sizes implied a β-chain origin. This hypothesis was confirmed by Edman sequencing of the excised protein bands, which revealed that both fragments were derived from the C-terminal region of the β-chain, the 40-kDa being a fragment of the 56-kDa protein. More extensive digestion of C3c with trypsin or elastase did not result in additional, lower molecular weight immunoreactive fragments (data not shown).

Although Bpa crosslinking is a very sensitive method for detecting protein/peptide binding sites, even those that display low binding affinity, its specificity is arguable. The C3 residues that mediate the covalent bond with Bpa may be in close proximity to compstatin's binding site in the three-dimensional molecule but be further removed in terms of the primary structure. To confirm that the 40-kDa fragment of the β-chain of C3 contains the binding site for compstatin, the fragment was cloned into an expression vector and expressed in *E. coli*. The expressed fragment was subsequently incubated with the compstatin-Bpa-FLAG analog. Western blotting of the UV-exposed protein mixture showed intense anti-FLAG immunoreactivity that could be inhibited by compstatin but not by either one of the inactive peptides (Fig. 2).

3.3. Compstatin phages bind to the recombinant 40-kDa protein, but not to smaller β-chain fragments.

Binding of compstatin to the recombinant 40-kDa fragment was also tested in a more quantifiable compstatin-phage bind-

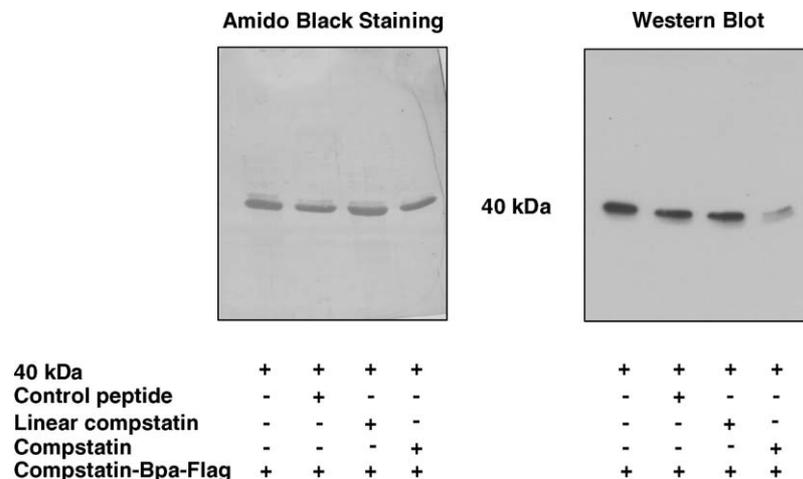|	**Amido Black Staining**					**Western Blot**			
40 kDa	+	+	+	+		+	+	+	+
Control peptide	-	+	-	-		-	+	-	-
Linear compstatin	-	-	+	-		-	-	+	-
Compstatin	-	-	-	+		-	-	-	+
Compstatin-Bpa-Flag	+	+	+	+		+	+	+	+

Fig. 2. Binding of a photo-crosslinking compstatin analog to a recombinant 40-kDa fragment of the β-chain of C3. The binding could be inhibited by compstatin but not by two inactive analogs (linear and a control peptide). Conditions were the same as in Fig. 1.
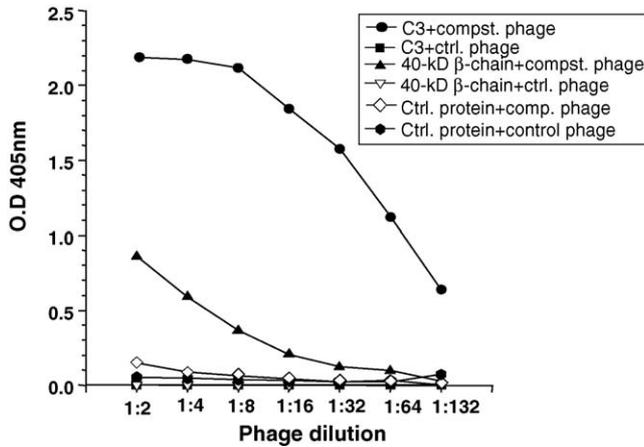
Fig. 3. Direct binding of a compstatin phage and a control clone to immobilized C3, recombinant 40-kDa β-chain fragment, and control protein (39.5-kDa α-chain fragment). Microtiter wells were coated with 500 ng protein, saturated with 1% milk, washed, and incubated with various dilutions of phage clones. The starting dilution for both phages was 108 PFU/μl. Binding of the phages was detected by HRP-conjugated anti-M13 Ab.

ing assay: phages expressing the compstatin sequence bound to immobilized C3 and the 40-kDa β-chain fragment, but not to the recombinant 39.5-kDa fragment of C3c's α chain, which served as a control protein (Fig. 3). Control phage clones did not bind C3 or any of the C3 fragments. Compstatin phages displayed greater binding to C3 than to the 40-kDa β-chain fragment. This reduced binding could have been caused by improper folding of the recombinant 40-kDa protein, since it displayed relatively low solubility in aqueous solutions. Attempts to purify the fragment from trypsinized C3c, which would overcome the folding issue,

did not yield sufficient homogeneous quantities of the protein to allow us to perform the binding experiments.

Seven smaller fragments corresponding to various overlapping regions of the 40-kDa β-chain fragment were also produced (Fig. 4). Only five of these were sufficiently soluble to be used for binding studies. However, none of these proteins, which ranged in size from 10 to12 kDa, were found to bind compstatin (data not shown).

4. Discussion

In the present study, we have identified a 40-kDa region of the β-chain of C3 that contains the binding site of the complement inhibitor compstatin. This region is distinct from those involved in the binding of other complement regulatory molecules, such as CR1, CR2, factor B, and factor H, all of which are found on the α chain (Lambris et al., 1996). We have previously shown that compstatin does not block the binding of factor B to C3b, nor its cleavage into Bb (Sahu et al., 1996). In addition, compstatin does not interfere with the binding site of properdin, a protein essential for the stabilization of the C3bBb complex. Also, compstatin does not hinder access to the C3a/C3b cleavage site, nor does it prevent the interaction between factor H and C3b (Sahu et al., 1996). Taken together, these results strongly suggest that compstatin prevents C3 cleavage by binding to a new, yet to be identified, site on the C3 molecule. The present study has demonstrated that this region is contained within the 40-kDa C-terminal region of the β-chain of C3.

Thus far, the β-chain has not been directly implicated in any crucial interactions with other proteins, and the present study
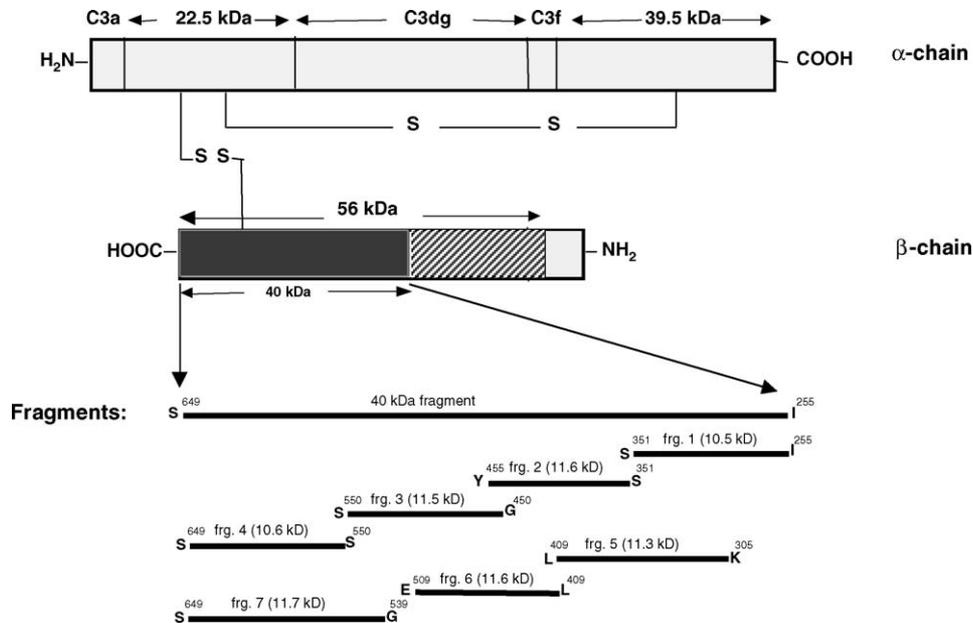

Fig. 4. Schematic representation of C3 showing the 40-kDa β-chain fragment and the various overlapping fragments produced in the present study. The N- and C-terminal residues are shown for each fragment. Compstatin-Bpa-FLAG immunoreactivity was detected on the 56- and 40-kDa C-terminal fragments of the β-chain. Fragments 1, 2, 3, 5 and 6 were used for phage binding studies, but no compstatin binding was detected with any of these five fragments. Fragments 4 and 7 were insoluble and therefore not tested.

may bring renewed attention to this part of the C3 molecule. Attempts to identify a fragment smaller than the 40-kDa region have been unsuccessful, as additional crosslinking studies with various enzymatically digested C3 preparations did not yield any active segments smaller than the 40-kDa fragment identified in this study. In addition, various recombinantly expressed, overlapping regions failed to bind compstatin or its analog. This finding suggests that a bigger fragment, such as the 40-kDa protein, is necessary to produce the proper conformational structure required for compstatin binding.

Although compstatin binds the C3bBb complex as well as native C3, its affinity for the native form is believed to be largely responsible for its complement inhibiting activity (Furlong et al., 2000; Sahu et al., 2000). Compstatin differs in this binding property from other regulatory proteins, which bind C3b and other C3 cleavage products but not the native form (Alsenz et al., 1992; Becherer et al., 1989). The fact that compstatin binds native C3 and prevents its cleavage suggests two possible modi operandi: compstatin can either competitively inhibit binding of the convertases to C3 or alter its access to the molecule by changing the conformation of C3. Although the exact sites of interaction with both convertases have not yet been elucidated, various candidate regions have been suggested, all of which are located on the α chain (Mathias et al., 1992; Ogata and Low, 1997). Therefore, the binding of compstatin to C3 probably results in a structural change that prevents further association with the convertases. This hypothesis is further supported by SPR studies which have shown that compstatin's binding to native C3 and $C3(H_2O)$ is associated with a conformational change, while its binding to C3b and C3c appears to follow the 1:1 Langmuir model (Sahu et al., 2000; Soulika et al., 2003).

It is particularly interesting that compstatin has been shown to possess a greater inhibitory activity in the AP than the CP, suggesting that it affects the interactions between C3 and C3bBb to a greater extent than those involving C4bC2a. The reason for this lower potency in the CP remains to be determined. However, recent structural improvements in compstatin have led to the production of analogs displaying greatly enhanced affinities for C3, as well as up to 99-fold greater inhibitory activity than that of the original compound (Katragadda et al., 2004; Mallik et al., 2005). Recently, an analog has been produced that displays a 264-fold increased activity over the original molecule (Lambris et al., unpublished results). Most of these improvements have affected the inhibitory activity of compstatin in the CP, and some of the novel analogs possess equal or even greater inhibitory activity in the CP than in the AP (Lambris et al., unpublished data). How the enhanced affinity of the new analogs influences the intermolecular interactions between C3 and the C4bC2a and C3bBb complexes is an interesting question that deserves further pursuit.

In conclusion, the present study revealed the binding site of compstatin to be located within the 40-kDa C-terminal region of the β-chain of C3. Further studies will aim to identify the residues that are critical for compstatin's binding. Identification of these residues will provide important information regarding the regulation of complement activity and may have implications for the development of new complement inhibitors.

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