



Compstatin, a peptide inhibitor of complement, exhibits species-specific binding to complement component C3

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Abstract

Although activation of complement protein C3 is essential for the generation of normal inflammatory responses against pathogens, its unregulated activation during various pathological conditions leads to host cell damage. Previously we have identified a 13-residue cyclic peptide, Compstatin, that inhibits C3 activation. In this study, we have examined the species-specificity of Compstatin. Bimolecular interaction analysis using a real-time surface plasmon resonance-based assay showed that Compstatin exhibits exclusive specificity for primate C3s and does not bind either to C3s from lower mammalian species or to two structural homologs of C3, human C4 and C5. Furthermore, it showed that although the kinetics of binding of Compstatin to non-human primate C3s were distinctly different from those to human C3, like human C3 its mechanism of binding to non-human primate C3 was biphasic and did not follow a simple 1:1 interaction, suggesting that this binding mechanism could be important for its inhibitory activity. Analysis of Ala substitution analogs of Compstatin for their inhibitory activities against mouse and rat complement suggested that the lack of binding of Compstatin to mouse and rat C3s was not a result of sterically hindered access to the binding pocket due to individual bulky side chains or the presence of charge on the Compstatin molecule. These results suggest that Compstatin's exclusive specificity for primate C3s could be exploited for the development of species-specific complement inhibitors.

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1. Introduction

Complement protein C3 is an integral participant in the complement system whose activation is essential to the activation of all three pathways of the complement system (the classical, alternative, and lectin pathways). Proteolytic cleavage of C3 by the classical/lectin (C4b,2a) or alternative pathway (C3b,Bb) leads to covalent attachment of C3b

(the proteolytically activated form of C3) to activating target molecules, tagging them as foreign and susceptible to lysis, phagocytosis, and immune clearance. Attachment of C3b also helps in the selection and enhancement of the organism's antibody repertoire and probably in the elimination of self-reactive B cells (Carroll, 1998; Sahu and Lambris, 2001; Marsh et al., 2001).

Although activation of the complement system is the key to the development of normal immune and inflammatory responses against foreign pathogens, its unregulated activation leads to host cell damage in a variety of pathological conditions, including Alzheimer's disease (Rogers et al., 1992), asthma (Regal et al., 1993), adult respiratory distress syndrome (Robbins et al., 1987), experimental allergic encephalomyelitis (Davoust et al., 1999), experimental allergic neuritis (Vriesendorp et al., 1995), glomerulonephritis (Couser et al., 1985), ischemia/reperfusion injuries (Kilgore et al., 1994; Weiser et al., 1996), myasthenia gravis (Piddlesden et al., 1996), psoriasis (Rosenberg et al., 1990), rheumatoid arthritis (Wang et al., 1995), stroke

Abbreviations: C3, third component of complement; C3b, the proteolytically activated form of C3; C3c, the 135,300M_r fragment of C3 generated using elastase; Er, rabbit erythrocytes; EA, sheep erythrocytes coated with antibody; EGTA, ethylene bis(oxyethylenitrilo) tetraacetic acid; GVB, gelatin veronal-buffered saline; MALDI-MS, matrix-assisted laser desorption mass spectrometry; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RCA, regulators of complement activation; RU, response units; SPR, surface plasmon resonance; VBS, veronal-buffered saline

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(Huang et al., 1999), allotransplantation (Pruitt et al., 1991), xenotransplantation (Dalmasso, 1992), and many other conditions (Sahu and Lambris, 2000). Thus, there is a critical need for the development of complement inhibitors.

Complement-related tissue injury is mediated directly by a cytotoxin, C5b–C9, and indirectly by the specific activation fragments C3a, C4a, and C5a through their effect on neutrophils, eosinophils, basophils, and mast cells (Wetsel, 1995; Ember et al., 1998). In humans, complement activation is controlled at the cell membrane and in body fluids by a family of structurally and functionally related proteins named regulators of complement activation (RCA), which target C3 convertase. This family of inhibitors includes plasma proteins (factor H and C4 binding protein) as well as membrane proteins (complement receptor Type 1, decay-accelerating factor and membrane cofactor protein). In addition to these RCA proteins, control is also achieved through the C1 inhibitor, carboxypeptidase N, and CD59. Although some of these proteins are being developed as complement inhibitors (Lambris and Holers, 2000), the current emphasis is on identifying small molecular weight inhibitors for use in developing oral drugs against complement.

Since C3 participates in the activation of all three pathways of complement activation, we targeted this complement component for the development of a small-molecule complement inhibitor and identified a 13-residue C3-binding cyclic peptide, Compstatin (ICVVQDWGHRCT-NH₂) (Sahu et al., 1996). This peptide binds to native C3 and inhibits its activation (Sahu et al., 1996). These results have more recently been confirmed by others (Furlong et al., 2000). A three-dimensional structural analysis of a major conformer of Compstatin in solution, performed using two-dimensional NMR and a hybrid distance geometry-restrained simulated annealing methodology, revealed that the backbone forms a Type I β -turn comprising residues Gln⁵–Asp⁶–Trp⁷–Gly⁸ (Morikis et al., 1998). It further indicated that side-chain interactions among Val³, Val⁴, and Trp⁷ and Type I β -turn residues (Gln⁵–Gly⁸) are important for the preservation of the conformational stability of the peptide (Morikis et al., 1998). Recently, we have also shown that side-chain interactions exist between some of the Type I β -turn residues and C3 and that, apart from side-chain interactions, correctly oriented main-chain atoms also contribute to C3 binding (Sahu et al., 2000a).

Compstatin has been tested in three different clinically relevant models. Its effect on discordant xenograft hyperacute rejection was evaluated *ex vivo* in a porcine-to-human perfusion model. In this model Compstatin significantly prolonged the survival of the kidneys (Fiane et al., 1999). Its effect has also been tested in models of extracorporeal circulation (Nilsson et al., 1998), where it effectively inhibited the generation of C3a and sC5b-9 and the binding of C3/C3 fragments to a polymer surface. As a result of the inhibition of complement activation, the activation of polymorphonuclear leukocytes (assessed by the expression of CD11b) and the binding of these cells (CD16+) to the polymer surface

were almost completely lost (Nilsson et al., 1998). In order to determine whether the peptide inhibitor is effective and safe *in vivo* it has been tested in primates to examine its effect on complement activation induced by a heparin–protamine complex. The data indicated that Compstatin is both safe and effective in inhibiting the activation of the complement system *in vivo* (Souluka et al., 2000).

Since a number of animal models of disease are available in mice and rats, we tested the inhibitory effect of Compstatin on the mouse and rat complement system. Surprisingly, Compstatin failed to inhibit the complement-mediated hemolytic activity of mouse and rat sera. This observation suggested that Compstatin might act in a species-specific manner. We therefore have designed the present study to investigate whether Compstatin does indeed exhibit species-specificity. Here, we show that Compstatin exhibits exclusive specificity for primate C3s and does not bind either to C3s from lower mammalian species or to the structural homologs of C3, C4 and C5. Furthermore, using surface plasmon resonance analysis we have shown that the mechanism of binding of Compstatin to non-human primate C3 resembles that to human C3. We have also investigated whether the lack of binding of Compstatin to mouse and rat C3s is a result of steric hindrance caused by the presence of bulky side chains (if the size of the binding pocket is restricted as compared to human C3) or charged residues (if the same charge is present in the binding pocket of lower mammalian species) on the Compstatin molecule that are not involved in preservation of structural stability or maintaining the functional activity of Compstatin. Our data suggest that individual bulky side chains or charges on Compstatin are not responsible for the lack of binding of Compstatin to C3s of lower mammalian species.

2. Materials and methods

2.1. Chemicals and buffers

Peptide synthesis chemicals and reagents used for this study were purchased from Applied Biosystems (Foster City, CA), with the exception of Fmoc amino acids, which were obtained from Nova Biochem (San Diego, CA). Veronal-buffered saline (VBS), pH 7.4, contained 5 mM barbital and 145 mM NaCl. Gelatin veronal-buffered saline (GVB) was VBS containing 0.1% gelatin; GVB⁺⁺ was GVB containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂; and GVBE was GVB with 10 mM EDTA. MgEGTA contained 0.1 M MgCl₂ and 0.1 M EGTA. Phosphate-buffered saline (PBS) contained 10 mM phosphate, 145 mM NaCl, pH 7.4. PBS-T was PBS containing 0.05% Tween-20.

2.2. Animal sera and purified proteins

Human serum was obtained from the blood bank of the Hospital of the University of Pennsylvania; monkey

plasmas, except baboon plasma, were a generous gift from Dr. Patsy Giclas (Department of Pediatrics, University of Colorado, Denver); rabbit, guinea pig, rat, and mouse sera were purchased from Cocalico Biological (Reamstown, PA). C3-deficient serum was obtained by bleeding C3 knockout mice that had been kindly provided by Dr. Rick Wetsel (University of Texas Health Science Center, Houston, TX).

Human complement proteins C3, C4, and C5 were purified from normal human plasma as described (Hammer et al., 1981; Hessing et al., 1993). Baboon C3 was purified as follows: 20 parts of baboon plasma (kindly provided by Dr. L. Henry Edmunds Jr., Harrison Department of Surgery, University of Pennsylvania, Philadelphia) were treated with one part of inhibitor solution containing 1 M KH_2PO_4 , 0.2 M Na_4EDTA , 0.2 M benzamidine, and 1 mM PMSF. Plasma was precipitated first with 5% polyethylene glycol (PEG) and then with 16% PEG at 0 °C. The pellet containing C3 was dissolved in 10 mM Na_2HPO_4 , pH 7.9, and passed through a Mono Q column (Pharmacia, Piscataway, NJ). Fractions containing C3 were pooled, concentrated, and passed through Superose 12 (1.6 cm \times 60 cm, Pharmacia) in PBS. Similar protocol was followed to purify mouse and rabbit C3s.

2.3. Peptide synthesis, purification, and characterization

Compstatin and its analogs were synthesized in an Applied Biosystems peptide synthesizer (model 431A) using Fmoc amide resin (4-(2',4'-dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin) as previously described (Sahu et al., 1996; Morikis et al., 1998) and purified using a reversed-phase C-18 column (Waters, Milford, MA). Disulfide oxidation of peptides was performed by stirring a 0.15 mM solution of the peptide in 0.1 M ammonium bicarbonate, pH 8.0, and bubbling with oxygen at 22 °C for 48 h. On-resin biotinylation of the peptides was performed as follows: The Dde group on the Lys side-chain was removed by treating the peptide-resin with three short treatments (3 min each) of 2% hydrazine in dimethylformamide (Bycroft et al., 1993). The peptide was then placed on the synthesizer and washed with *N*-methylpyrrolidone. Biotin (1 mmol) was dissolved in equal volumes (2.9 ml) of dimethylsulfoxide and *N*-methylpyrrolidone, placed in an amino acid cartridge, and activated according to Applied Biosystems User Bulletin #35. The purity and identity of Compstatin and its analogs were critically monitored by analytical chromatography on a reversed-phase C-18 column and by laser desorption mass spectrometry (Moore, 1997). Formation of a disulfide bond in each cyclic peptide was confirmed by mass spectrometry using a mass shift assay that involves reaction of thiols with *p*-hydroxy mercuribenzoic acid (Angeletti et al., 1996).

2.4. Surface plasmon resonance (SPR) measurement

Binding of Compstatin to human C3, C4, and C5 and to C3s from other mammalian species was studied using an

SPR-based biosensor BIACORE X (Biacore AB, Piscataway, NJ) at 25 °C. All binding experiments were performed in physiologic ionic strength buffer (PBS-T) using a streptavidin chip (Sensor Chip SA, Biacore AB). Addition of 0.05% Tween to PBS blocked the non-specific binding of analytes to the sensor chip. In this assay, Compstatin was biotinylated at a specific residue to orient it on the sensor chip surface; this approach provided a homogeneous ligand surface. In brief, Compstatin containing a C-terminal spacer and biotin ($\text{I}^*\text{CVVQDWGHHRC}^*\text{TAGHMANLTSHASAK-biotin}$) was immobilized (approximately 100–200 response units, RU) on the test flow cell, and the control flow cell was immobilized with equivalent RUs of the linear analog ($\text{IAVVQDWGHHRRATAGHMANLTSHASAK-biotin}$). Addition of a spacer (extra residues) at the C-terminus was based on the sequence of the parent peptide originally isolated from the phage (Sahu et al., 1996). Binding was measured at $30 \mu\text{l min}^{-1}$ by injecting various concentrations of analyte for 120 s, and dissociation was followed for an additional 120 s. The binding data obtained for the control flow cell were subtracted from the data for the test flow cell. The sensor chip was regenerated by brief pulses of 0.2 M sodium carbonate, pH 9.5. Since the biosensor data obtained for baboon C3 did not fit a simple 1:1 model, the data were evaluated by linear transformation analysis as previously described for human C3 (Casasnovas and Springer, 1995; Morton et al., 1994; Sahu et al., 2000a).

2.5. Hemolytic assays

The effect of Compstatin on serum complement activity of various animal species, with the exception of rabbit, was studied by measuring the effect on the alternative pathway. Inhibition of alternative pathway complement activation was determined by measuring the lysis of rabbit erythrocytes (Er) in serum from various animal species as previously described (Sahu and Pangburn, 1996). Various concentrations of peptides were mixed with an appropriate amount of serum (that gave 60–70% lysis), 5 μl of 0.1 M MgEGTA, and 10 μl of Er ($1 \times 10^9 \text{ ml}^{-1}$), and the final volume was adjusted to 100 μl with GVB. The reaction mixture was incubated at 37 °C for 20 min and stopped by adding 200 μl of GVBE. After centrifugation, lysis of Er was determined by measuring the optical density of supernatants at 405 nm. To determine the effect of Compstatin on rabbit complement, various concentrations of Compstatin were mixed with 3.7 μl of rabbit serum and 5 μl of EA ($1 \times 10^9 \text{ ml}^{-1}$), and GVB⁺⁺ was added to give a total volume of 250 μl . The reaction mixture was incubated at 37 °C for 1 h and centrifuged. The percentage of lysis was determined by measuring the optical density of the supernatant at 414 nm. The percentage of lysis obtained was normalized by considering 100% lysis to be equal to the lysis occurring in the absence of Compstatin. The concentration of Compstatin causing 50% inhibition of hemolytic activity was taken as the IC_{50} .

2.6. Biotransformation of Compstatin

To determine whether Compstatin is cleaved in mouse or rat serum, 1 ml of fresh heparinized serum was mixed with 0.3 mg of Compstatin and incubated at 22 °C. Two hundred microlitres of sample were removed at various time intervals, mixed with an equal volume of 0.1% TFA in H₂O, and centrifuge-filtered through Ultrafree-MC filters (Millipore Corporation, Bedford, MA) with a molecular weight cut-off of 5000 Da. The filtrate was injected onto an HPLC apparatus (Waters LC 600, Milford, MA) connected to a reversed-phase C-18 column (Vydac, Heperia, CA), and the peaks obtained were analyzed by matrix-assisted laser desorption mass spectrometry (MALDI-MS) using a time-of-flight mass spectrometer (MicroMass TofSpec, formerly Fisons Instruments, Beverly, MA) and Edman degradation.

3. Results

3.1. Effect of Compstatin on complement activities of various animal species

In our previous studies, we have shown that Compstatin inhibits human complement by interacting with human C3 and inhibiting its cleavage by C3 convertase (Sahu et al., 1996, 2000a; Nilsson et al., 1998). Since a number of animal models of disease are available in mice and rats, we sought to determine whether Compstatin inhibits mouse and rat complement. When we measured the effect of Compstatin on the complement-mediated lysis of Er by mouse or rat complement, we were intrigued to discover that Compstatin failed to inhibit either mouse or rat complement (Table 1). Since the lack of inhibition could have been due to proteolytic cleav-

Table 1

Complement inhibitory activities of Compstatin in different animal species

Species	Inhibition of complement ^a IC ₅₀ (μM)
Human	12
Rhesus	5
Baboon	8
Cynomolgus	10
Marmoset	45
Squirrel monkey	60
Aotus	71
Pig	>600 ^b
Rabbit	>600 ^c
Guinea pig	>600
Rat	>600
Mouse	>600

^a Complement activities were determined by measuring alternative pathway-mediated lysis of Er unless indicated.

^b Maximum amount of Compstatin that could be used in the assay because of solubility limitations.

^c Complement activity was determined by measuring classical pathway-mediated lysis of EA.

age of Compstatin in mouse and rat serum, we incubated Compstatin with mouse or rat sera for 1 h at room temperature and then separated the products by membrane filtration, injected the filtrates onto a reversed-phase C-18 column attached to a high-performance liquid chromatography apparatus, and characterized the peaks by MALDI-MS and Edman degradation. About 20% of the peptide was cleaved at the N-terminus at the peptide bond between Ile¹ and Cys², resulting in the generation of Compstatin (2–13) (data not shown). However, this cleavage did not account for the inability of Compstatin to inhibit mouse and rat complement, because the rate of peptide cleavage was very slow, and even the cleaved peptide effectively inhibited the activity of human complement (Table 2). These results suggest that Compstatin inhibits complement in a species-specific manner.

Table 2

Complement inhibitory activities of Compstatin and its analogs in humans, mice and rats

Peptide	Amino acid sequence ^a	Mass spectral analysis		Inhibition of complement ^b IC ₅₀ (μM)		
		Expected	Observed	Human ^c	Mouse	Rat
Compstatin	I*CVVQDWGHHRC*T-NH ₂	1552	1551	12	>750 ^d	>750
Comp-[2–13]	*CVVQDWGHHRC*T-NH ₂	1438	1439	25	>600	>600
Comp-[2–12]	*CVVQDWGHHRC*-NH ₂	1340	1339	33	>750	>750
Comp-[2–12,3Ala]	*CAVQDWGHHRC*-NH ₂	1311	1309	120	>960	>960
Comp-[2–12,4Ala]	*CVAQDWGHHRC*-NH ₂	1311	1309	67	>960	>960
Comp-[2–12,5Ala]	*CVVADWGHHRC*-NH ₂	1282	1281	910	>960	>960
Comp-[2–12,6Ala]	*CVVQAWGHHRC*-NH ₂	1296	1297	257	>650	>650
Comp-[2–12,7Ala]	*CVVQDAGHHRC*-NH ₂	1224	1223	182	>600	>600
Comp-[2–12,8Ala]	*CVVQDWAHHRC*-NH ₂	1354	1352	>1200	>960	>960
Comp-[2–12,9Ala]	*CVVQDWGAHRC*-NH ₂	1273	1272	15	>650	>650
Comp-[2–12,10Ala]	*CVVQDWGHARC*-NH ₂	1273	1272	74	>650	>650
Comp-[2–12,11Ala]	*CVVQDWGHHAC*-NH ₂	1254	1255	70	>650	>650

^a Asterisks in superscript denote oxidized cysteines.

^b Complement activities were determined by measuring alternative pathway-mediated lysis of Er.

^c Data is taken from Morikis et al. (1998).

^d Maximum amount of peptide that could be used in the assay because of solubility limitations.

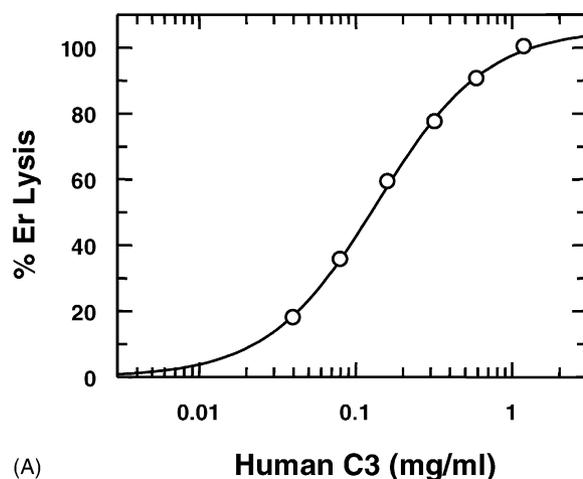
Since Compstatin inhibited the activity of human but not mouse or rat complement, we speculated that the peptide binding site might have been conserved only in higher mammals. Thus, we extended our study to various mammals and New and Old World monkeys. The peptide inhibited monkey complement but failed to inhibit rabbit, guinea pig, or pig complement. The IC_{50} values for the various monkey complements varied by more than 12-fold (Table 1). Complement inhibition in Rhesus, cynomolgus monkeys, and baboons was comparable to that in humans.

3.2. Does Compstatin bind specifically to primate C3 and inhibit complement activation?

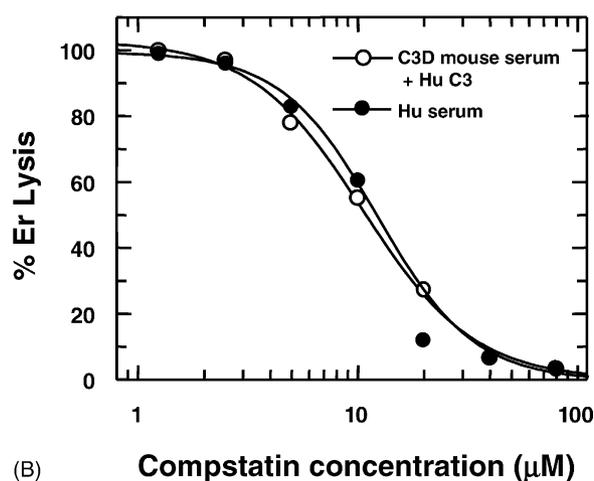
The data presented above indicate that Compstatin inhibits primate complement, but they do not completely rule out the possibility that the lack of inhibition of murine complement by Compstatin is due to an artifact of the heterologous system or to the high concentrations of serum required for hemolysis. Thus, to rule out these possibilities and confirm that the inhibition of complement is primate C3-specific, we tried to reconstitute the hemolytic activity of C3-deficient mouse serum by adding human C3. It is clear from the data presented in Fig. 1A that addition of human C3 to C3-deficient mouse serum reconstitutes the hemolytic activity of the mouse serum and suggests that human C3 can interact with mouse factor B and form the alternative pathway C3 convertase C3b,Bb. Using this system, we ascertained whether Compstatin could inhibit the hemolytic activity of the reconstituted serum. The peptide inhibited the hemolytic activity of the reconstituted serum at concentrations similar to those seen for human serum (Fig. 1B). These results indicate that Compstatin's inhibitory activity is specific to human C3.

In order to demonstrate the physical interaction between Compstatin and various C3s, we utilized the SPR-based assay used in our previous study (Sahu et al., 2000a). In this assay, biotinylated Compstatin was immobilized on a test flow cell, and a linear biotinylated analog immobilized on a control flow cell served as a control. When purified C3s from human, baboon, mouse, and rabbit were allowed to flow over the sensor chip, only the human and baboon C3s bound to Compstatin (Fig. 2A and B). These results, together with the hemolytic data, indicate that the failure of Compstatin to inhibit mouse complement is related to a lack of physical interaction between Compstatin and mouse C3.

The complement components C3, C4, and C5 are structural homologs; thus, it is possible that Compstatin may also interact with human C4 and C5, and its effect on the human complement system could be a result of its interaction with these three proteins. When we examined the binding of human C4 and C5 to Compstatin immobilized on the sensor chip, we found that Compstatin did not bind to human C4 or C5 (Fig. 2B).



(A)



(B)

Fig. 1. Analysis of inhibition of complement-mediated lysis of erythrocytes by Compstatin. (A) Reconstitution of alternative pathway hemolytic activity in C3-deficient mouse serum by adding human C3. C3-deficient mouse serum was mixed with various amounts of human C3 and incubated for 20 min with Er in the presence of MgEGTA. The amount of lysis was determined by centrifuging the cells and measuring the absorbance of the supernatant at 405 nm. The data were normalized by considering 100% lysis to be equal to the lysis occurring after addition of 1.2 mg ml^{-1} of C3. (B) Comparison of Compstatin-mediated inhibition of erythrocyte lysis mediated by normal human serum and C3-deficient mouse serum reconstituted with human C3. Inhibition of complement activity by Compstatin was studied by measuring its effect on alternative pathway-mediated lysis of Er.

3.3. Binding kinetics of Compstatin–baboon C3 interaction

We next examined the interaction of Compstatin with baboon C3 by SPR technology in order to (1) measure the affinity of Compstatin for baboon C3 and (2) understand the mechanism of binding of Compstatin to baboon C3.

The sensograms obtained for Compstatin–C3 interaction showed that the binding reaction was dose-dependent and saturable (Fig. 3). Global fitting analysis of sensograms using a 1:1 model showed that the data could not be fitted to

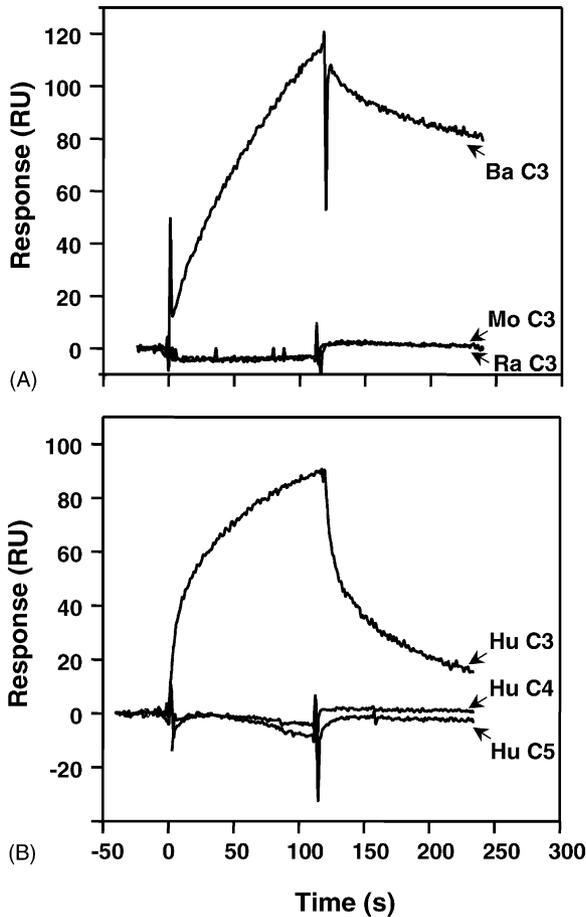


Fig. 2. Binding of Compstatin to C3 from various species and its structural homologs C4 and C5. Binding was determined by SPR. Sensograms were generated by injecting various analytes over a streptavidin sensor chip containing biotinylated Compstatin immobilized on flow cell-1 (Fc-1) and a linear analog (control peptide) immobilized on flow cell-2 (Fc-2). (A) Ba C3, baboon C3 (270 nM); Mo C3, mouse C3 (3.5 μ M); Ra C3, rabbit C3 (5 μ M). (B) Hu C3, human C3 (270 nM); Hu C4, human C4 (3.7 μ M); Hu C5, human C5 (5 μ M).

this model ($\chi^2 = 14$). Analysis of the binding data using linear transformation produced non-linear plots (Fig. 3B), indicating that the binding follows complex models. Thus, in order to calculate an association constant, $d(\text{RU})/dt$ plots were divided into fast and slow components as previously described (Casasnovas and Springer, 1995; Morton et al., 1994). The k_s values for the fast component obtained from the slopes of the $d(\text{RU})/dt$ plots were plotted against the C3 concentrations to calculate k_{on} values. To calculate the dissociation constant (k_{off}), data from the dissociation phase of the highest concentration were plotted as $\ln(\text{response at time zero of dissociation}/\text{response at time } n)$, and the dissociation rate constant was calculated from the slope of the line defined by the initial data points of this plot. When we compared the k_{on} and k_{off} values for the Compstatin–baboon C3 interaction with those for the Compstatin–human C3 interaction, we found that the kinetics were distinctly different (Table 3). There was a 7-fold decrease in k_{on} and 10-fold

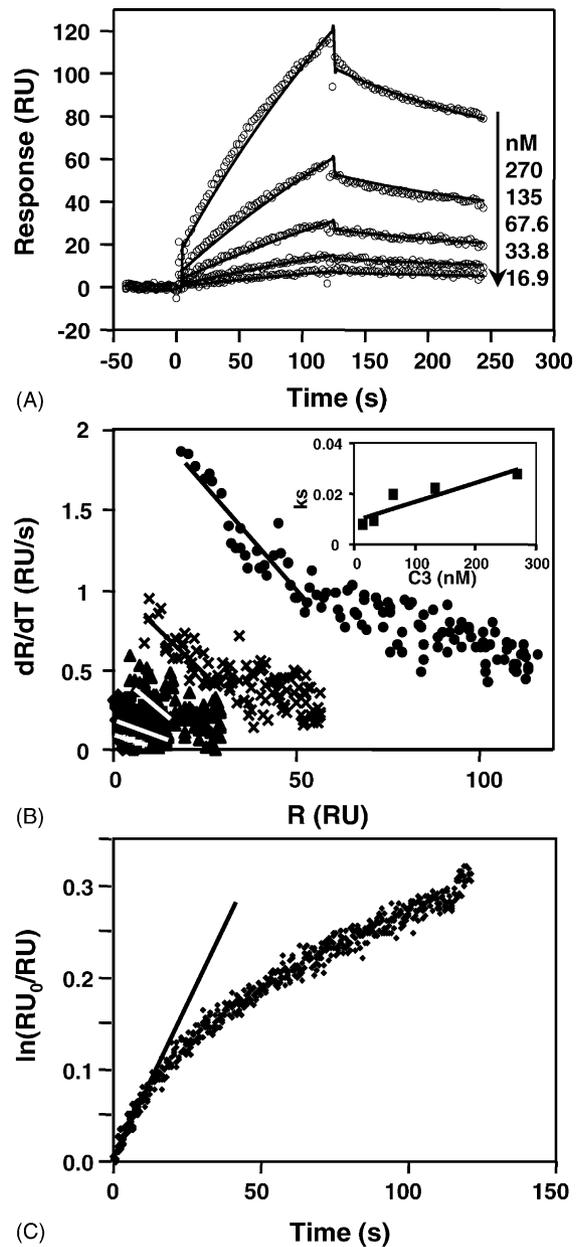


Fig. 3. Analysis of the binding of Compstatin to baboon C3 by SPR. (A) Sensogram overlays for interactions between immobilized biotinylated Compstatin and baboon C3. The concentration of analyte injected is indicated at the right of each sensogram. Solid lines correspond to the global fitting of the data simultaneously. Data were fit to a two-step conformational change model ($A + B \rightarrow AB \rightarrow AB^*$, Bievaluation 3.0). (B) Linear transformations of the association phase data for the sensogram data shown in the (A). The straight lines are linear least squares fits to the data. Inset shows k_s values (determined from the slope of the fits) replotted against analyte concentrations. (C) Linear transformation of the dissociation phase data for the highest concentration of baboon C3 (270 nM). The slope of the straight line provided the off-rate.

decrease in k_{off} for the Compstatin–baboon C3 interaction as compared to the Compstatin–human C3 interaction. Since both the on-rate and off-rate were decreased, this change did not result in a decrease in the affinity (K_D) of Compstatin for baboon C3. This conclusion is further supported by the

Table 3
Kinetic and affinity data for the Compstatin–C3 interaction

	Human C3	Baboon C3
$k_{\text{on1}}^{\text{a}}$ ($\times 10^5 \text{ s}^{-1} \text{ M}^{-1}$)	5.0	0.7
$k_{\text{off}}^{\text{a}}$ ($\times 10^{-2} \text{ s}^{-1}$)	6.6	0.65
K_{D1}^{b} ($\times 10^{-6} \text{ M}$)	0.13	0.09
Relative affinity	1	0.69

^a k_{on} and k_{off} were calculated by linear transformation of the respective data.

^b K_{D} was calculated from $k_{\text{off}}/k_{\text{on}}$.

fact that the IC_{50} values of Compstatin were comparable for human and baboon complement (Table 1).

3.4. Effect of Ala substitution analogs of Compstatin on mouse and rat complement

The data presented above clearly demonstrate that Compstatin specifically binds to primate C3s and inhibits complement activation in these species. The lack of binding of Compstatin to C3s from lower mammalian species could have either been due to the absence of Compstatin binding sites on the C3s of these species or to an inaccessibility of the binding site resulting from the presence of complementary features in the molecule (e.g. opposite charge or bulky side chains). In the hope of elucidating whether side chains of Compstatin other than those that are important for binding and conformational stability would interfere with the binding to C3s of lower species, we tested the activity of Ala substitution analogs against mouse and rat complement. In each of these analogs, a different residue between Cys² and Cys¹² was replaced by Ala. Previously we had established that residues involved in the formation of the Type I β -turn (Gln⁵–Asp⁶–Trp⁷–Gly⁸) and Val³ are important for conformational stability, and some of the turn residues are involved in inter-molecular recognition (Morikis et al., 1998; Sahu et al., 2000a). Table 2 shows that analogs in which residues with bulky side chains (Trp⁷, His⁹, His¹⁰, and Arg¹¹) or charged side chains (Asp⁶, Arg¹¹, or His⁹ and His¹⁰ in the event of altered pK_{a}) were replaced by Ala were inactive against rat and mouse complement, indicating that the individual side chains on Compstatin are not responsible for interfering with the binding of Compstatin to mouse and rat C3s.

4. Discussion

In theory, any clinical condition in which complement activation occurs and activation fragments are detected could serve as a candidate for therapeutic intervention. Recent data on animal models of diseases generated using complement-deficient, knockout, and transgenic animals have produced substantial evidence of complement-mediated pathology in a wide range of diseases (Lambris and Holers, 2000; Sahu and Lambris, 2000). Complement inhibitors

that are in clinical trials include soluble complement receptor 1 (sCR1) and anti-C5 mAb. Studies of these inhibitors have provided evidence that complement inhibition can not only prevent disease progression but can also ameliorate established disease (Sahu et al., 2000b; Wang et al., 1995; Huang et al., 1999).

Although these recombinant proteins will be useful in reducing the clinical morbidity in several diseases, current emphasis is being given to the development of small-molecule inhibitors that would allow for better tissue penetration and development of oral drugs. Our efforts in this direction have led to the identification of Compstatin, a 13-residue peptide inhibitor of complement that blocks C3 activation (Sahu et al., 1996). Previously, we have identified its solution structure, studied its structure–activity relationship, characterized its mechanism of binding to human C3, and identified its clinical potential (Morikis et al., 1998; Sahu et al., 2000a; Nilsson et al., 1998; Fiame et al., 1999; Soulika et al., 2000). In the present study, we have characterized the species-specificity of Compstatin.

Compstatin was identified by screening a phage-displayed random peptide library against human C3. Therefore, in our initial studies we characterized its inhibitory activity against human C3 (Sahu et al., 1996, 2000a; Morikis et al., 1998). In order to examine the efficacy of this peptide in vivo, we have recently tested its activity in a baboon model of heparin/protamine-induced complement activation (Soulika et al., 2000). Furthermore, since a large number of animal models of disease are available in mice and rats, we also tested its activity against mouse and rat complement. It is clear from the data presented in Table 1 that Compstatin does not inhibit murine complement. These results could have been a simple result of proteolytic cleavage of the peptide in serum or of the high concentration of serum used in hemolytic experiments performed in lower mammalian species. However, we have ruled out both these possibilities: (1) We found only limited cleavage of the first residue of Compstatin in mouse and rat serum (generating Comp-[2–13], Table 2), and the cleaved product was still active against human complement (Table 2). (2) We found that Compstatin could inhibit the hemolytic activity of C3-deficient serum reconstituted with human C3 (Fig. 1) at a concentration similar to that seen for human complement. These data, along with the failure to inhibit complement-mediated hemolytic activities of sera from various non-primate mammalian species (Table 1) and the lack of direct binding to C3s of these species (Fig. 2) indicate that Compstatin exhibits an exclusive species-specificity for primate C3s.

Sequence analysis of C3s from various mammalian species has shown that their sequence similarity varies from 85 to 94% (Lambris et al., 1993, 1998; Zarkadis et al., 2001). Considering this high sequence homology, it is surprising that Compstatin binds only to primate C3s. Nevertheless, since the binding site of Compstatin is so specific, analysis of the three-dimensional structure of the

subdomain of the C3 molecule that binds Compstatin may reveal one or more significant control points on human C3 that will be of pharmacological importance. Our preliminary efforts in this direction have localized the binding site within the 40 kDa C-terminal half of the β -chain of human C3 (Soulaka et al., 1998).

Previous studies have revealed that Compstatin inhibits complement activation by binding to native C3 and inhibiting its cleavage by C3 convertase (Sahu et al., 1996; Nilsson et al., 1998); this inhibition is not the result of sterically hindered access to the C3a/C3b cleavage site (Sahu et al., 1996). The peptide also has no effect on the formation of the C3 convertase C3b,Bb, or on the stabilization of C3b,Bb by properdin (Sahu et al., 1996; Nilsson et al., 1998). These findings indicate that Compstatin inhibits complement activation by inhibiting C3 activation. However, they do not rule out the possibility that Compstatin may also inhibit complement activation by acting at multiple steps in the activation pathways. Since human C3, C4, and C5 are structural homologs, we asked whether Compstatin might bind to human C4 and C5 and exert its effect by acting on these proteins. Using a real-time SPR-based assay, we determined that Compstatin does not bind either to human C4 or to C5 (Fig. 2B). Thus, the inhibitory action of Compstatin on complement activation is solely due to its effect on C3.

Peptides and small molecules are known to undergo conformational changes upon binding to other molecules. Important examples include the cyclophilin–cyclosporine complex (Wüthrich et al., 1991), the anti-apoptotic protein Bcl-x_L complexed to the Bak peptide (Sattler et al., 1997), and the immunosuppressive organic molecule FK506 bound to its receptor, FKB (Jorgensen, 1991; Schreiber, 1991). In the case of the 11-residue cyclic peptide cyclosporine complexed to cyclophilin, it was found that an inversion occurred in the orientation of the dominant hydrophobic cluster and the peptide backbone, together with a *cis*–*trans* peptide bond isomerization, which provided the proper orientation for recognition and binding through the formation of inter-molecular hydrogen bonding with cyclophilin (Wüthrich et al., 1991). Similarly, in the case of Bcl-x_L–Bak interaction it has been suggested that the Bcl-x_L receptor undergoes a structural reorientation to expose a hydrophobic segment upon binding of the Bak peptide (Sattler et al., 1997), and in the case of FK506–FKBP, the immunosuppressive FK506 undergoes conformational changes involving *cis*–*trans* isomerization and alteration in the orientation of specific rings (Jorgensen, 1991; Schreiber, 1991).

In our earlier study, we examined the binding mechanism of Compstatin with human C3 using SPR analyses (Sahu et al., 2000a). Our real-time kinetic binding data for C3 fitted well to a two-state conformational change model ($A + B \leftrightarrow AB \leftrightarrow AB^*$); however, binding to C3b and C3c followed the 1:1 Langmuir binding model (Sahu et al., 2000a). These data suggested that Compstatin or/and C3 change conformation upon binding. Since binding to human C3, but not to C3b or C3c, followed the conformational change model, we

speculated that reorientation of the peptide or/and C3 might be essential to the functional activity of Compstatin.

To test our premise, we examined the binding of Compstatin to baboon C3, since Compstatin inhibits baboon and human complement at approximately equimolar concentrations (Table 1). The data clearly showed that binding of Compstatin to baboon C3 does not follow a simple 1:1 interaction and, like human C3, it fits well with a two-state conformational change model (Fig. 3A), a finding which suggests a binding mechanism similar to that for human C3. Since binding to C3, but not C3b and C3c, is biphasic in nature, it is possible that a portion of C3a (e.g. loops or elements of secondary structure) is necessary for the formation and closure of the binding site. To test our conclusion that conformational changes occur upon binding of Compstatin to C3 and to identify the type of changes involved, the structures of free C3 and the C3–Compstatin complex are needed, in addition to the known structure of free Compstatin (Morikis et al., 1998; Klepeis et al., 1999).

Given that there is very high sequence similarity (>85%) between C3 and C3s from lower mammalian species (and therefore a high structural similarity among C3s of various mammals), it is unlikely that the lack of binding of Compstatin to C3s from lower mammalian species is due to the absence of a Compstatin binding pocket on these C3s. The most plausible explanation is that the presence of complementary structural features makes the Compstatin–lower mammalian C3 interaction unfavorable. These complementary features could include: (1) bulky side chains of Compstatin that could sterically hinder entry of Compstatin into the binding pocket of the C3s of lower mammalian species, if the size of the binding pocket is restricted; (2) the presence of the same charge on Compstatin and the binding pocket, generating an unfavorable electrostatic interaction; (3) disruption of hydrophobicity (e.g. if a charged or polar residue is present in the binding pocket of C3 from lower mammalian species where a hydrophobic residue is present in primate C3); we have previously shown that disruption of the hydrophobic clustering by mutating Val⁴ results in a loss of inhibition (Morikis et al., 1998); (4) hydrogen bond formation (e.g. involving Trp⁷) in which one inter-molecular partner, donor or acceptor, is missing; and (5) a combination of conditions (1)–(4).

Given our hypothesis of a loss of complementary structural features in rat and mouse C3s, we have prepared and tested the activity of Ala scan Compstatin analogs, searching for the possibility of compensatory effects when steric, charge, hydrophobic, or hydrogen bond-forming side chains are removed from Compstatin. If such a compensatory effect were present, a corresponding analog would demonstrate increased inhibitory activity. Compstatin contains four residues with bulky side chains (Trp⁷, His⁹, His¹⁰ and Arg¹¹). Of the four, Trp⁷ is involved in the formation of the Type I β -turn and is important for the preservation of the conformational stability of Compstatin; His⁹, His¹⁰, and Arg¹¹ do not appear to participate directly in either the

conformational stability or functional activity of the peptide (Morikis et al., 1998). Replacement of any of these residues with Ala did not impart functional activity to Compstatin against mouse and rat complement (Table 2). With regard to the possibility of specific charge–charge interactions, the obvious candidates are Asp⁶ and Arg¹¹, and also His⁹ and His¹⁰, if the pK_a of His is altered upon binding as a result of favorable coulombic interactions with acidic residue(s). Among these residues, Asp⁶ is the only one that participates in the formation of the Type I β-turn and is important for the functional activity of the peptide. It is clear from the data that substitution of even these residues with Ala did not impart functional activity to Compstatin against the complement of lower mammalian species (Table 2). These results suggest that the individual bulky side chains or charge on Compstatin are not responsible for the lack of binding of Compstatin to the C3s of lower mammalian species. Obtaining the structure of Compstatin complexed with primate C3 is needed to provide detailed insight into this issue of species-specificity.

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