Collagen-binding Microbial Surface Components Recognizing Adhesive Matrix Molecule (MSCRAMM) of Gram-positive Bacteria Inhibit Complement Activation via the Classical Pathway*

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Background: Collagen-binding MSCRAMMs from Gram-positive bacteria are adhesins and are virulence factors in several infectious diseases models.

Results: Cna and related collagen-binding MSCRAMMs bind C1q and block activation of the classical complement pathway.

Conclusion: Collagen-binding MSCRAMMs are novel classical complement pathway inhibitors.

Significance: The novel function of the Cna-like collagen-binding MSCRAMMs represents an immune evasion strategy potentially used by numerous Gram-positive pathogens.

Members of a family of collagen-binding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) from Gram-positive bacteria are established virulence factors in several infectious diseases models. Here, we report that these adhesins also can bind C1q and act as inhibitors of the classical complement pathway. Molecular analyses of Cna from Staphylococcus aureus suggested that this prototype MSCRAMM bound to the collagenous domain of C1q and interfered with the interactions of C1r with C1q. As a result, C1r2C1s2 was displaced from C1q, and the C1 complex was deactivated. This novel function of the Cna-like MSCRAMMs represents a potential immune evasion strategy that could be used by numerous Gram-positive pathogens.

The complement system is a critical part of the host’s defense against microbial infection. It acts through three distinct pathways as follows: the classical pathway (CP), the mannose-binding lectin pathway, and the alternative pathway (AP). These pathways differ in their mode of molecule recognition but converge at the generation of C3 convertase, which subsequently cleaves the central complement component C3. Activation of these complement pathways will result in C3b opsonization of the pathogen and associated enhanced phagocytosis, recruitment of phagocytes via chemotaxis factors such as the generated C3a and C5a, as well as lysis of the pathogen by formation of the membrane attack complex on the pathogen surface.

Activation of the CP is initiated via recognition of pathogen-bound antibodies by the 766-kDa C1 complex, which is formed by association of a recognition protein C1q with the proteases C1r and C1s. Binding of the C1 complex to an immune complex is mediated by C1q. This interaction is thought to induce self-activation of C1r, which subsequently converts the proenzyme C1s into an active serine protease that ultimately cleaves C4 and C2, thereby activating the CP (1). C1q is composed of six identical heterotrimeric proteins, each dominated by a collagen-like domain forming a characteristic triple helix structure. C1q has an overall bouquet-like structure. The six collagen-like triple helices associate to form an N-terminal “stalk” and then diverge to form six individual triple helix “stems,” each terminating in a heterotrimeric globular “head” (2). The globular head region contains the antibody recognition function of C1q. The C1r and C1s associate to form the heterotetramer (C1r3C1s2), which binds to the individual collagenous stems of C1q (3). Subversion of the CP provides a benefit to pathogen survival and helps in establishing infection (4, 5). Individuals with deficiency in the CP component, including C1q, C1r, C1s, and C4, have increased risk for bacterial infections such as Staphylococcus aureus bacteremia, S. aureus liver abscess, and adenitis (6). In addition, these individuals are prone to develop systemic lupus erythematosus-like symptoms due to insufficient clearance of immune complexes and apoptotic cells (7).

In light of the threat that the complement systems pose to invading microbes, it is not surprising that successful pathogens have evolved strategies to counter the attack of the complement system. However, the complement-evading microbial molecules often target the alternative pathway (8, 9) and are usually specific to microbial species (8–10). We report here that a family of structurally related collagen-binding MSCRAMMs from Gram-positive bacteria, including the prototype Cna of S. aureus, binds C1q and blocks activation of the CP. Cna contains a collagen-binding “A
region,” followed by several repeated sequences called “B repeats” and C-terminal cell wall attachment elements (Fig. 1). The N1 and N2 subdomains located within the A region are involved in collagen binding via the so-called “collagen hug” binding mechanism (11), a multistep process where the rope-like triple helical structure of monomeric collagen is critical for recognition (11).

Members of the Cna-like family of collagen-binding MSCRAMMs are structurally related (Fig. 1) and are found in many Gram-positive bacterial species, e.g. Ace in Enterococcus faecalis (12), Acm in Enterococcus faecium (13), Cne in Streptococcus equi (14), and Cnm in Streptococcus mutans (15). These collagen-binding MSCRAMMs are virulence factors in several animal models of infectious diseases (16–22) and can function as adhesins and mediate bacterial attachment to collagen-rich tissues.

In this communication, we report that the Cna-like family of collagen-binding MSCRAMMs also bind C1q and inhibit the complement classical pathway activation. The molecular bases for this inhibition are dissected and described.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Polyclonal goat anti-human C1q and goat anti-human C4 antibodies were purchased from Complement Technology (Tyler, TX). Monoclonal anti-C1q antibody was purchased from Quidel (San Diego). Goat anti-human C1r and sheep anti-human C1s polyclonal antibodies were products of R&D Systems (Minneapolis, MN). Rabbit anti-goat and rabbit anti-sheep polyclonal antibodies conjugated with HRP were from Invitrogen. Monoclonal anti-poly-His conjugated with HRP antibody was supplied by Alpha Diagnostics (San Antonio, TX), and human IgM was ordered from EMD Chemical (Philadelphia).

Purified human C1q, C1r, C1s, normal human serum (NHS), C1q-depleted serum (C1q-dpl), factor B-depleted serum (fB-dpl), and antibody-sensitized sheep erythrocytes (EAs) were obtained from Complement Technology. Recombinant human type I collagen was from FibroGen (San Francisco), and o-phenylenediamine dihydrochloride (SigmaFast) was purchased from Sigma.

Expression and Purification of Recombinant Proteins—Expression plasmids were generated as described previously (23, 24). Briefly, the DNAs encoding the N1N2 domains of collagen-binding MSCRAMMs were subjected to PCR and ligated into pQE30. The Cna mutants harboring the mutations in N1N2 domains were generated by using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Escherichia coli Top3 (Stratagene) containing the pQE30-derived plasmids were grown overnight at 37 °C in LB containing 100 µg/ml ampicillin. The overnight cultures were diluted 1:50 into fresh LB medium, and expression of the recombinant proteins was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside for 5 h at 37 °C. Bacteria were harvested by centrifugation and lysed using a French press (SLM Aminco). Soluble Hisε-tagged recombinant proteins were purified through a HisTrap HP column and a HiTrap Q HP column (GE Healthcare) according to the manufacturer’s manual. The purified proteins were analyzed by SDS-PAGE, and appropriate fractions were dialyzed into TBS and stored at −20 °C.

ELISA-type Binding Assays—Wells on Immulon 4BH plates (Thermo Scientific) were coated overnight at 4 °C with 1 µg of recombinant human collagen or purified human C1q. After blocking with 2% BSA in TBST (0.05% Tween 20, TBS) at RT for 2 h, recombinant proteins were added to the wells and incubated for 1 h at RT. Following incubation with HRP-conjugated anti-His antibodies (10,000 × dilution) at RT for 1 h, the substrate o-phenylenediamine dihydrochloride was added. Bound proteins were quantified after measuring the absorbance at 450 nm in a microtiter plate reader (Thermomax), and data were analyzed by GraphPad Prism software.
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The competition assay used a protocol similar to that described above, with the following alterations. First, recombinant MSCRAMM was preincubated with C1 complex or C1q (10 nM) in the HBS++ buffer (20 mM HEPES, 140 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, and 0.025% bovine serum albumin (BSA), pH 7.3) at RT for 1 h. The mixture was subsequently added to microtiter plates previously coated overnight at 4 °C with purified IgM (0.5 µg/well) and C1r or C1s (0.5 µg/well). The following primary antibodies were used where applicable: goat anti-human C1q antibody (5000× dilution), goat anti-human C1r (2000× dilution), and sheep anti-human C1s (2000× dilution). Secondary rabbit anti-goat and rabbit anti-sheep antibodies (3000× dilution) were added for detection and visualization of the bound protein.

In the case of determining C1 complex binding to human IgM or Cna, microtiter wells were coated with 0.5 µg/well of human IgM or of rCna (31–344), and the bound C1 complex was detected using goat anti-human C1q (5000× dilution), goat anti-human C1r (2000× dilution), and sheep anti-human C1s (2000× dilution), followed by HRP-conjugated rabbit anti-goat or rabbit anti-sheep antibodies (3000× dilution).

Surface Plasmon Resonance (SPR)-based BIAcore Analysis—The interactions between C1q and Cna (31–344) were characterized using a BIAcore 3000 (GE Healthcare) at 25 °C. Sensor chip C1 and amine-coupling kits were obtained from the same company and used to covalently attach ligands onto the sensor surface using the amine coupling procedure as recommended by the manufacturer. During immobilization, HEPES-buffered saline (HBST: 10 mM HEPES, pH 7.3, 150 mM NaCl, and 0.005% Tween 20) was used as running buffer at a flow rate of 5 µl/min. Following activation of sensor surface, 5 µg/ml ligand solution (collagen or Cna (31–344) in 10 mM sodium acetate, pH 5.5, C1q in 100 mM sodium phosphate buffer, pH 7.0) was injected and coupled to the flow cell on a C1 chip. A reference flow cell was prepared for each sensor chip with activation and deactivation steps where no protein was coupled. Binding was performed at a flow rate of 30 µl/min with HBST (25 mM Tris, pH 7.4, 3 mM KCl, 140 mM NaCl, and 0.01% Tween 20) as running buffer. To regenerate the sensor surface, bound Cna proteins were removed from the collagen surface by injecting 10 µM glycine, pH 1.5, for 30 s; bound C1q was removed with 0.5% SDS from the Cna (31–344) surface.

Base-line corrected SPR response curves (with buffer blank run further subtracted) were used for affinity determination. For steady-state analysis, equilibrium response (R_eq) of each injection was collected and plotted against the concentration of injected protein. A binding isotherm was fitted to the data (GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA) to obtain the equilibrium dissociation constant (K_d).

Non-equilibrium data were globally fit to a two-state model using BIAevaluation software (version 4.1). Association and dissociation rate constants (k_1, k_2) for the binding state and forward and backward rate constants (k_a, k_d) for the conformational change state were obtained from the fitting, and apparent dissociation constant (K_app) was calculated from the rate constants: K_app = 1/((k_a/k_d)/(1 + k_d/k_a)).

Bacterial Adherence Assay—Adherence assay was performed as described previously (17) with modification. S. aureus strain Phillips and correspondence Cna deletion mutant strain (PH100) (25) were grown in BHI media overnight, and serial dilutions of bacteria were made. Subsequently, bacteria were incubated in the wells of microtiter plates coated with C1q (1 µg/well). Adhered bacteria were fixed with 4% formaldehyde and stained with 0.5% crystal violet. After washing, 50 µl of 10% acetic acid was added, and the absorbance at 590 nm was measured.

Hemolysis Assays—Complement-mediated hemolysis of EAs was performed as described previously (26) with slight modifications. Briefly, NHS, C1q-dpl, or Bb-dpl (final concentration 0.5%) was preincubated with parent and mutant collagen-binding MSCRAMMs at room temperature (RT) for 1 h in a final volume of 260 µl. Subsequently, 40 µl of EAs (5 × 10^6/ml) were added, and the mixture was incubated at 37 °C for 45 min. The clear supernatant was transferred to a 96-well PVC flat-bottom plate (BD Biosciences) after centrifugation (2000 rpm, 10 min). Lysis of EAs was quantified by measuring the absorbance at 405 nm using a microtiter plate reader (Thermomax, Molecular Devices). Data were present as percentages of lysis by dividing with the absorbance value obtained from 100% EAs lysis. The data represent the mean ± S.D. of three independent experiments performed in triplicate (GraphPad Prism). All sera were diluted in BHEPES buffer (20 mM HEPES, 140 mM NaCl, and 0.025% BSA, pH 7.3) containing 0.15 mM CaCl2, 0.5 mM MgCl2.

C4b Deposition on ELISA—C4b deposition was performed as described (27) with slight modification. 96-Well Immulon 4BHX microtiter plates (Thermo Scientific) were coated overnight at 4 °C with 0.1 µg of human IgM. Diluted serum (in HBS++) was preincubated with Cna (0–40 µM) or other collagen-binding MSCRAMMs (20 µM) at RT for 1 h. After blocking the wells with 2% BSA in TBST (0.05% Tween 20, TBS) at RT for 2 h, the serum/protein mixtures were added, and the plates were incubated for 1 h at RT. Bound-C4b was detected by goat anti-human C4 antibody (10,000× dilution), followed by horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibody (10,000× dilution) for 1 h each and quantified as described above.

Pulldown Immunoassays—Monoclonal anti-human C1q antibodies were captured with protein G beads (Thermo Scientific) at RT for 2 h. Subsequently, a mixture of the C1 complex (35 nM) and recombinant MSCRAMMs (80 µM) previously incubated at RT for 1–2 h in HBS++ buffer was added to protein G beads bearing anti-C1q antibodies and allowed to incubate for 2 h at 37 °C. After washing the beads with HBS++ containing 0.1% Triton X-100 (Sigma), 50 µl of nonreducing buffer was added, and proteins were denatured at 95 °C for 10 min. After centrifugation at 2500 × g for 5 min, 10 µl of supernatant was subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad). The component of the C1 complex was detected with polyclonal goat anti-human C1q (10,000× dilution), goat anti-human C1r (8000× dilution), or sheep anti-human C1s (8000× dilution) and followed by HRP-conjugated rabbit anti-goat antibody (10,000× dilution). Chemiluminescent substrate (SuperSignal West Pico from Thermo Scientific) was used to visualize cross-reacting material.
Statistical Analysis—Statistics were analyzed with Student’s $t$ test; $p$ values less than 0.05 were considered significant.

RESULTS

Cna Binds to C1q—A recombinant form of Cna, Cna(31–344), bound to C1q or collagen type I coated in microtiter wells in processes that were dose-dependent and exhibited saturation kinetics (Fig. 2A). SPR analyses using C1q and collagen type I immobilized on BIAcore chips corroborated our ELISA results and further indicated that the Cna(31–344) interaction with C1q involves a lower affinity compared with that with collagen type I (calculated $K_D$ of 12 $\mu$M and 91 nM, respectively) (Fig. 2, C and B). However, when the system was reversed and Cna(31–344) was immobilized, C1q bound to Cna(31–344) with a much higher affinity ($K_D$ of 13 nM) (Fig. 2D). This phenomenon is consistent with what we observed for collagen binding to Cna-coated chips, in which a significantly higher affinity is also observed (calculated $K_D = 0.4$ pM) (28) compared with when Cna binds to collagen immobilized on the chip (calculated $K_D = 91$ nM). The molecular explanation for this difference is presently unclear; however, the potential for a triple helix collagen monomer and a hexameric C1q to simultaneously bind multiple immobilized Cna(31–344) molecules likely contributes to the observed differences.

The ability of Cna to interact with C1q in the presence of serum was also evaluated in an ELISA in which Cna-coated wells were incubated with NHS or C1q-dpl serum, and bound C1q was detected by polyclonal anti-C1q antibody. The results showed that Cna indeed bound to C1q and/or the C1 complex in NHS (data not shown). This observation demonstrated that Cna was able to interact with C1q in its native condition.

The S. aureus clinical strain Phillips that expresses full-length native Cna on the bacterial surface and a correspondence Cna deletion mutant strain (PH100) (25) were evaluated...
for their ability to bind to the C1q-coated surface. Only the wild-type Phillips strain that expresses full-length Cna bound to C1q, whereas the Cna deletion strain PH100 did not (Fig. 2E). This result demonstrated that the bacterial surface-anchored native Cna protein interacts with C1q.

Our previous studies have demonstrated that Tyr-175 in the N2 domain of Cna is important for collagen interaction (24), and the linkers between N1N2 domain harboring insertions or deletions affect Cna binding to type 1 collagen (28). In addition, the latch segment found in the C-terminal extension of N2 is likely important for the interaction (11, 29). Thus, we have generated a panel of Cna mutants, including a point mutation in the N2 domain resulting in loss of collagen binding (Y175K), extending or truncating the linker between the N1 and N2 domains in Cna (QIE, ΔEAG, and ΔEAGTSS) (28) and a deletion of the latch segment (ΔLatch) (Fig. 3A and Table 1). These mutants have altered collagen binding activities compared with that of the wild-type Cna protein (Fig. 3, B and D). The ability of these mutants to bind to C1q as compared with type 1 collagen was evaluated by ELISA (B and C) and BIACore analyses (D and E). Sensograms are shown for comparing the interaction of 10 μM Cna proteins with immobilized collagen (6800 RU) (D) and C1q (7400 RU) (E) on a CM5 chip.

**FIGURE 3. Cna likely binds to the collagenous domain in C1q.** A, crystal structure of the CnaN1N2 domains with specific mutations indicated. B–E, binding of Cna(31–344) (red), QIE (purple), ΔLatch (green), ΔEAG (orange), Y175K (blue), and ΔEAGTSS (black) to collagen (B and D) and to C1q (C and E) by ELISA (B and C) and BIACore analyses (D and E). Sensograms are shown for comparing the interaction of 10 μM Cna proteins with immobilized collagen (6800 RU) (D) and C1q (7400 RU) (E) on a CM5 chip.

These results strongly suggest that Cna recognizes C1q and collagen...
TABLE 1
Proteins used in this study

| Name | Description | Ref. |
|------|-------------|------|
| Cna(31–344) | Recombinant protein containing N1N2 domains of Cna of S. aureus with an N-terminal tail of six histidine residues | 9 |
| Cna Y175K | Cna (31–344) with Y175K substitution | 20 |
| Cna Δlatch | Cna (31–344) without C-terminal “latch” residual | This study |
| Cna QIE | Cna(31–344) with insertion of three amino acids (QIE) from Ace linker to linker region (168–169) | 27 |
| Cna ΔEAG | Cna(31–344) with deletion of three amino acids (EAG) from linker region (166–168) | 27 |
| Cna ΔEAGTSS | Cna(31–344) with deletion of six amino acids (EAGTSS) from linker region (168–171) | This study |
| Cne(28–323) | Recombinant protein containing N1N2 domains of Cne of S. equi with an N-terminal tail of six histidine residues | This study |
| Cnm(32–323) | Recombinant protein containing N1N2 domains of Cnm of S. mutans with an N-terminal tail of six histidine residues | This study |
| Acm(29–320) | Recombinant protein containing N1N2 domains of Acm of E. faecium with an N-terminal tail of six histidine residues | This study |
| Ace(32–367) | Recombinant protein containing N1N2 domains of Ace of E. faecalis with an N-terminal tail of six histidine residues | 10 |

type I in similar ways and indicate that Cna likely binds to the collagenous domains in C1q.

Cna Inhibits Activation of the Classical Complement Pathway—The observed binding of Cna(31–344) to C1q raised the possibility that the MSCRAMP could modulate complement activation. To explore this possibility, we used functional readouts for activation of the CP, including a hemolytic assay and an ELISA where we quantitated the deposition of C4b on antibody-coated surfaces. Hemolysis of antibody-sensitized sheep erythrocytes (EAs) was effective when the EAs were incubated with NHS but not when the EAs were incubated with C1q-dpl serum (Fig. 4A). Supplementing C1q-dpl serum with C1q restored full hemolytic activity (Fig. 4A), demonstrating that under the conditions used the hemolysis of EAs is primarily the consequence of CP activation and that the presence of C1q is required for the activity. Factor B is a key protease in the activation of the AP. EA hemolysis in fB-dpl serum is indistinguishable from that in NHS (Fig. 4A), suggesting that the AP has a minor (if any) effect on hemolysis under these experimental conditions. However, generation of C3b convertase (C4bC2a) as the result of CP activation may possibly initiate the AP. To exclude any involvement of the AP, we used fB-dpl serum in some experiments.

Addition of purified recombinant His-tagged Cna(31–344) to NHS or to fB-dpl serum prior to the addition of sensitized erythrocytes inhibited hemolysis of EAs in a dose-dependent manner (Fig. 4B), demonstrating that Cna(31–344) blocks the formation of the membrane attack complex on the erythrocytes surface. The inhibitory effect of Cna(31–344) was somewhat more pronounced when fB-dpl serum was used compared with NHS, supporting the hypothesis that Cna inhibits the CP. Cna(31–344) also inhibited hemolysis in C1q-dpl serum complemented with C1q (Fig. 4C).

Next, we determined the relative amounts of C4b generated and deposited on a microtiter plate coated with IgM as a read-out for CP activation. C4b deposition was increased with increasing concentrations of NHS. At a fixed concentration of NHS, preincubation of the serum with Cna(31–344) decreased C4b deposition, and the effect depended on the amount of added Cna (Fig. 4D). In contrast to the IgM-coated surface, we did not observe C4b deposition on the Cna-coated surface (data not shown), suggesting that Cna did not by itself activate the CP. Thus the observed CP inhibition (Fig. 4, C and D) is not due to a consumption of complement components.

We next evaluated the ability of the Cna mutants to inhibit activation of the CP. Compared with wild-type Cna, Cna mutants (QIE, Δlatch, Y175K, and ΔEAGTSS) that had a reduced affinity for C1q showed a diminished ability to inhibit EAs hemolysis. The percentage of erythrocytes lysed in the presence of wild-type Cna (26%) was increased to 44, 50, 58, and 56% in the presence of mutants QIE, Δlatch, Y175K, and ΔEAGTSS, respectively (Fig. 4E). The ability of these mutants to inhibit C4b generation and deposition was reduced in a similar manner (Fig. 4F). These results correlated the degree of inhibition of CP activation with the relative C1q-binding affinities (Figs. 3, C and E, and 4, E and F).

Taken together, our data strongly suggest that Cna(31–344) interferes with activation of the classical complement pathway, and the relative inhibitory activity on Cna mutants correlates with their relative collagen binding activity.

Other Gram-positive Collagen-binding MSCRAMPs Bind C1q and Block the Classical Pathway—A number of Gram-positive bacteria express collagen-binding MSCRAMPs that have similar structural organizations (Fig. 1) and show a high degree of sequence and structural similarities within the collagen-binding segments. Cna from S. aureus is the prototype member of this family of collagen-binding MSCRAMPs that include Ace from E. faecalis (12), Acm from E. faecium (13), Cne from S. equi (14), and Cnm from S. mutans (15) and others (30). To determine whether these collagen-binding MSCRAMPs bind C1q and function as complement inhibitors, we first compared the collagen and C1q binding activities of purified recombinant proteins containing N1N2 domains of these MSCRAMPs (Table 1). These proteins all bound to collagen and C1q in a dose-dependent manner, although they exhibited different binding capacities in ELISA (Fig. 5, A and B). Among them, Cna(31–344) bound to collagen with the highest affinity, with half-maximum binding observed at a concentration of 0.02 μM, whereas Ace(32–367) showed minimal binding activity under the conditions used (Fig. 5A). Binding of these proteins to C1q largely followed the relative binding activities for collagen type I (Fig. 5B). However, there were exceptions; Cne(28–323) bound somewhat more effectively to C1q than Cna(31–344) (Fig.
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5B), whereas the reverse was seen when collagen type I was targeted (Fig. 5A). Ace(32–367) showed noticeable binding to C1q (Fig. 5B), whereas Acm(29–320) bound weaker to C1q (Fig. 5B) than to collagen type 1 (Fig. 5A) and was the worst C1q binder of the MSCRAMMs tested (Fig. 5B).

We next determined the effect of these collagen-binding MSCRAMMs on hemolysis and C4b deposition. Consistent with our observation for Cna(31–344), all MSCRAMMs inhibited EA hemolysis (Fig. 5C) and C4b deposition (Fig. 5D), and the extent of inhibition was directly related to their relative C1q-binding affinities, in which Cne(28–323) showed the strongest and Acm(29–320) showed the weakest inhibition (Fig. 5, C and D). In summary, our results demonstrated that collagen-binding MSCRAMMs from Gram-pos-
positive bacteria are also inhibitors of the classical complement pathway.

Molecular Bases for Cna-dependent Inhibition of the Classical Pathway—Formation of the C1 complex is a prerequisite for activation of the CP. The C1r₂C1s₂ tetramers bind to the collagenous regions in C1q to form the C1 complex (3). Our results indicated that Cna(31–344) also binds to the collagenous domain in C1q. We therefore asked if Cna(31–344) could interfere with the interactions between C1r₂C1s₂ and C1q. Cna(31–344) inhibited C1q from binding to C1r in a competition ELISA where wells coated with C1r were incubated with a constant amount of C1q and increasing concentrations of Cna(31–344) (Fig. 6A). Cna(31–344) had a marginal inhibitory effect on C1q binding to immobilized C1s (Fig. 6B). The Y175K mutant, which lost its affinity for C1q, did not affect the C1q-C1r or the C1q-C1s interactions (Fig. 6, A and B). These results suggested that Cna interferes with the C1r-C1q interaction by binding to C1q.

The majority of C1q in normal serum is present in the C1 complex (31). We therefore investigated if Cna(31–344) could disassemble a preformed C1 complex. Pulldown assays were conducted using specific antibodies against C1r or C1s. Strikingly, addition of soluble Cna(31–344) to the C1 complex significantly decreased the amount of C1r and C1s that was retained in the C1 complex, whereas the amount of C1q pulled down was similar to that in the control group (Fig. 6C), suggesting that Cna displaced C1r and C1s from the C1 complex. Preincubation of the C1 complex with the Y175K mutant did not affect the amount of C1r and C1s in the pulled down complex (Fig. 6C).

We also investigated if surface-bound Cna(31–344) is capable of displacing C1r and C1s from the C1 complex. Microtiter wells were coated with either IgM (Fig. 6D) or Cna(31–344) (Fig. 6E) and incubated with increasing concentrations of the C1 complex. The presence of C1q, C1r, and C1s within the bound complex was detected with specific antibodies. In IgM-coated wells, all three C1 components were successfully detected, and the amount of C1q, C1r, and C1s detected increased in parallel with the amount of complex added (Fig. 6D). Isolated C1r and C1s had negligible IgM binding activity (data not shown), indicating that the detected C1r and C1s is

![Figure 5: Structurally related collagen-binding MSCRAMMs bind to C1q and inhibit the CP activation. A and B, ELISAs for binding of recombinant N1N2 domain of collagen-binding MSCRAMMs to immobilized collagen (A) and C1q (B). C, classical pathway-mediated hemolysis of EAs in 0.5% Fb-dpl serum with 20 μM collagen-binding MSCRAMMs. Data represent mean ± S.D. of three separate experiments. D, C4b deposition in various concentrations of NHS with 20 μM collagen-binding MSCRAMMs. Control, buffer.](image-url)
part of an IgM-bound C1 complex. Intriguingly, C1q was the only C1 complex subunit that could be detected on the Cna(31–344)-coated surface, demonstrating that surface-bound Cna(31–344) also displaced C1r2C1s2 from the C1 complex (Fig. 6E). Collectively, these data demonstrate that Cna(31–344) binding to C1q interferes with the C1q interaction with C1r and results in dissociation of the C1 complex.

Recognition and binding to surface-bound antibodies by the C1 complex is required for initiation of the CP. We next investigated if Cna(31–344) also affects the recognition of surface-bound antibodies by the C1 complex. This hypothesis was tested in ELISA-type binding experiments using IgM-coated microtiter wells. Increasing concentrations of Cna(31–344) inhibited the C1 complex binding to the IgM-coated surface (Fig. 6F). In contrast, Y175K, which does not bind C1q, did not interfere with the C1 complex-IgM interactions (Fig. 6F). Consistent with this finding, dose-dependent binding of the C1 complex to the IgM-coated surface was significantly attenuated by addition of Cna(31–344) (50 nM) (Fig. 6G). The half-maximum binding concentration increased from 6 nM (control, in the absence of Cna(31–344)) to 94 nM (in the presence of Cna(31–344)), indicating a nearly 17-fold decrease in binding affinity. Addition of Y175K did not alter the binding kinetics of the C1 complex to IgM, and the kinetics were indistinguishable from those of the control (Fig. 6G). These results demonstrated that Cna inhibits the C1 complex binding to the immune complex and that the activity is determined by the C1q-binding affinity. Interestingly, the influence of Cna on binding of isolated C1q to IgM was insignificant (Fig. 6H). The fact that the C1 complex exhibits a higher affinity for immune complexes than does isolated C1q (data not shown) supports the idea that two distinct conformational states with different binding properties exist for C1q in these two conditions (32), and our results indicate that Cna binding to the C1q subunit may stabilize the conformation of C1q that has a low affinity for immune complexes.

In summary, we demonstrated that Cna (and presumably other members of the Cna-like family of collagen-binding MSCRAMMs) inhibits activation of the CP by binding to C1q and interfering with the C1q-C1r interaction, thereby disassembling and deactivating the C1 complex.

**DISCUSSION**

The Cna-related collagen-binding MSCRAMMs represent a growing family that currently includes almost 20 members from a variety of human and animal Gram-positive (33–37) and Gram-negative pathogens (30, 38–40). We report that at least five members of this MSCRAMM family bind C1q and inhibit the CP of the complement system. Cna(31–344) appears to bind to the triple helix collagenous domains of C1q, and a set of Cna mutants showed similar relative affinities for collagen and C1q. Cna interferes with the interaction of C1r with the collagenous stems of C1q. As a result, the C1r2C1s2 tetramer cannot dock with C1q, or if Cna encounters a preformed C1 complex, C1s and C1r are displaced resulting in a single isolated hexameric nonactive C1q. It is likely that all members of the Cna family of collagen-binding MSCRAMMs inhibit the CP by the same molecular mechanism, and there is a direct correla-
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The N1N2 domains to encompass the triple helix and “hug” the subdomains in the binding region of the MSCRAMM allowing partial interaction of Cna with collagen induces a redirection of the inflammatory mediators (C3a and C5a) during the process of complement activation is also blocked by the presence of these MSCRAMMs and subsequently neutrophil recruitment and phagocytosis could also be possibly inhibited. Future studies will address these questions.

Cna binds collagen by the collagen-hug mechanism. An initial interaction of Cna with collagen induces a redirection of the subdomains in the binding region of the MSCRAMM allowing the N1N2 domains to encompass the triple helix and “hug” the collagen monomer (11). The diameter of the “hole” formed by the N1N2 domains of Cna when complexed to a collagen triple helix peptide is about 15 Å, as calculated from the crystal structure (11). This space can accommodate only one monomeric collagen triple helix structure (diameter ~15 Å) (43, 44). Thus Cna can only bind to collagen triple helix monomers but not to collagen fibers or fibrils. This property has recently been taken advantage of when investigators have used a fluorescently tagged form of Cna to detect monomeric collagen product during fibrosis (45). This specificity also limits the type of collagenous tissue that can be targeted in Cna-dependent adhesion because collagen is primarily found in higher orders of fibrous super structures in healthy mature tissues. However, the collagenous stems of C1q are always available as monomeric triple helix structures. Hence, the ability of the Cna-like MSCRAMMs to bind to C1q and inactivate the CP may be an important component of the virulence potential of these molecules.

Surface-bound Cna interacts with C1q in solution with high affinity (Fig. 2D), which may be due to the ability of one C1q molecule to interact with multiple MSCRAMMs. This represents the most direct and effective way in which the Cna-like MSCRAMMs encounter and inactivate the CP. However, surface-anchored MSCRAMMs such as Ace can be cleaved by protease (46–48), and generated MSCRAMM fragments released into the environment may retain their ligand binding activity. This observation raises the possibility that cell wall-anchored MSCRAMMs may not only function as surface-bound virulence factors but also as a reservoir for the release of biologically active compounds. Thus, the release of C1q-binding fragments of Cna-like MSCRAMMs would allow these fragments to inhibit the CP away from the bacterial colony and could result in a more systemic inhibition of the complement system.

Earlier SPR analyses of Cna binding to type 1 collagen demonstrated that the collagen molecule contains multiple classes of binding sites with different affinities for the MSCRAMM (49). Thus, the high affinity Cna-binding sites in type 1 collagen are likely composed of different sequences than the Cna-binding sites in C1q, which exhibit moderate affinity for the MSCRAMM. Whereas Cna binds with much higher affinity to type I collagen compared with C1q, some other members of the Cna-like MSCRAMM family studied do not differ in their binding to collagen and C1q, for example Cne. These observations suggest that, although the different members of the Cna-like family of MSCRAMMs have similar sequences and structural organization, they have evolved to target different triple helix motifs with different affinities.

Previous studies suggest that C1q is a flexible molecule that undergoes conformational changes upon binding to the C1r2C1s2 tetramer and antibodies on the bacterial surface (32, 50). Our data revealed that the C1 complex and isolated C1q differ in their affinities for immobilized IgM (data not shown), supporting the existence of two distinct C1q conformations (50). This result also demonstrates that the C1 complex form of C1q is in a state that is favorable for antibody binding, whereas the isolated C1q has a relatively unfavorable binding conformation. The ability of Cna(31–344) to displace C1r2C1s2 from the C1 complex (Fig. 6, C–E) and to inhibit C1 complex binding to surface-bound antibodies (Fig. 6, F and G) suggests that binding of Cna(31–344) to C1q stabilizes the low affinity conformation of the complement component. The observation that Cna(31–344) did not affect the interaction of isolated C1q with antibodies supports this hypothesis.

In summary, our data provide evidence for the first time that Cna-like collagen-binding MSCRAMMs are not only adhesins but also are potential complement inhibitors. The dual functions of MSCRAMMs illustrate the elaborate strategies evolved by pathogenic microorganisms to adhere to and colonize host tissues and evade the host defense systems. The molecular mechanism uncovered in this study of how Cna-like MSCRAMMs interact with C1q and disrupt the complement activation may represent a global mechanism used by collagen-binding MSCRAMMs from different bacterial species.

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