Structural basis for inhibition of complement C5 by the SSL7 protein from Staphylococcus aureus

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Edited* by Pamela J. Bjorkman, California Institute of Technology, Pasadena, CA, and approved January 11, 2010 (received for review September 21, 2009)

Staphylococcus aureus secretes the SSL7 protein as part of its immune evasion strategy. The protein binds both complement C5 and IgA, yet it is unclear whether SSL7 cross-links these two proteins and, if so, what purpose this serves the pathogen. We have isolated a stable IgA-SSL7-C5 complex, and our crystal structure of the C5-SSL7 complex confirms that binding to C5 occurs exclusively through the C-terminal β-grasp domain of SSL7 leaving the OB domain free to interact with IgA. SSL7 interacts with C5 > 70 Å from the C5a cleavage site without inducing significant conformational changes in C5, and efficient inhibition of convertase cleavage of C5 is shown to be IgA dependent. Inhibition of C5a production and bacteriolysis are all shown to require C5 and IgA binding while inhibition of hemolysis is achieved by the C5 binding SSL7 β-grasp domain alone. These results provide a conceptual and structural basis for the development of a highly specific complement inhibitor preventing only the formation of the lytic membrane attack complex without affecting the important signaling functions of C5a.

The complement system comprises >30 plasma and membrane proteins and is an essential component of innate immunity against Gram-positive bacteria such as Staphylococcus aureus. Complement proteins interact directly with surface molecules unique to microorganisms or alternatively to the effector region of bound antibodies. The proteolytic activation of complement results in dramatically enhanced phagocytosis (1), clearance of immune complexes and apoptotic cells (2), and mediation of inflammation (3). Complement activation also promotes adaptive immune responses by serving as a natural adjuvant, enhancing and directing responses by lymphoid cells (4, 5).

Complement activation triggers cleavage of the three homologous 185- to 200-kDa proteins C3, C4, and C5. Three activation pathways converge on C3 cleavage to C3b (6). Activation by the alternative pathway (AP) results from spontaneous hydrolysis of an internal thioester bond in C3 or by deposition of properdin on an appropriate surface (7), whereas the classical pathway (CP) and lectin-mediated pathways are activated by surface-bound immune complexes or mannan binding lectins/ficolins, respectively. Activation of the CP or the lectin pathway generates the surface-bound C3 convertase (a proteolytic enzyme cleaving C3) C4b2a, whereas the AP generates the C3 convertase C3bBb. Both may recruit an additional C3b molecule to form the CP C5 convertase C4b2a3b or the AP C5 convertase C3bBb3b (8, 9), which cleaves C5 to generate the large fragment C5b and the anaphylatoxin C5a. This binds with high affinity to the C5a receptor (CD88) on myeloid cells triggering G protein (Gq/11 and Go13)-mediated cell activation, chemotaxis, respiratory burst, and release of proinflammatory mediators (10). C5b quickly associates with C6, C7, C8, and multiple molecules of C9 to form the membrane attack complex (MAC) that results in rapid cell lysis (11). Elevated levels of C5a are implicated in a wide range of inflammatory disorders, such as rheumatoid arthritis, ischemia/reperfusion injury, sepsis, and fibrotic conditions (12). MAC deposition through C5b on erythrocytes results in destruction of these cells in the hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH) (13). In addition, excessive MAC formation is linked with the pathophysiology of conditions such as antibody-mediated transplant rejection (14), inflammatory neuropathies (15) and multiple sclerosis (16).

Given its importance to innate immune clearance, pathogens have developed many strategies to prevent complement activation (17). The Staphylococcal Superantigen-Like protein 7 (SSL7) binds to C5 to inhibit complement-mediated hemolytic and bactericidal activity (18). SSL7 is bifunctional and binds to C5 and IgA Fc, thereby blocking both C5 cleavage and recognition of IgA by FcεRI (18, 19). To decipher the molecular mechanism of complement inhibition by SSL7 and to understand the functional consequences of its dual binding, we have determined the crystal structure of the C5-SSL7 complex at 3.6 Å resolution. We show that SSL7 interacts with both C5 and the cleaved C5b, and that inhibition of C5 cleavage requires the presence of IgA Fc bound simultaneously to SSL7. Our structure allows us to rationalize these results, and a detailed molecular model for the recognition of C5 by the C5 convertase is presented.

Results

Overall Structure. We determined the structure of human C5 in complex with SSL7 cloned from strain 12598 (residues 40–230 traced, 3.6 Å resolution; Table S1 and Figs. S1 and S2) or the C-terminal β-grasp domain of SSL7 (SSL7c) derived from strain 4227 (residues 129–230 traced, 4.2 Å resolution). Because the protein sequences of SSL7 and SSL7c show variations in the C5 interaction region (Fig. S2), it was of interest to examine complexes with C5 of both versions of SSL7. The structures show that the C-terminal β-grasp domain in SSL7 is almost exclusively responsible for the interaction with C5 (Fig. 1 A and B), with the structures of the two complexes being essentially identical except for the absence of the OB domain in the C5-SSL7c complex. In the C5-SSL7 complex a single electrostatic interaction is formed between E53 in the C5a sequence and D41 in SSL7c, whereas in the C5-SSL7 complex a single electrostatic interaction is formed between E53 in the C5a sequence and D41 in SSL7c.

The authors declare no conflict of interest.

*This Direct Submission article had a prerearranged editor.

FJL and G.R.A. designed research; N.S.L., N.G., L.S.-J., J.D.F., and G.R.A. wrote the paper.


www.pnas.org/cgi/doi/10.1073/pnas.0910565107

PNAS | February 23, 2010 | vol. 107 | no. 8 | 3681–3686
Formation of the C5-SSL7 complex buries 1,919 Å² of interface (Fig. S4). The structure of SSL7 compared to the structure of the SSL7-IgA Fc complex (19) or the structure of isolated SSL7 (20). A local conformational change occurs in the loop V141-D145 within the CUB domain (21). The interaction of C5 with SSL7 is mediated mainly by the MG1 and MG5 domains with minor contributions from the MG2 and MG6 domains (Figs. 1B and 24, and Fig. S4). Formation of the C5-SSL7 complex buries 1,919 Å² of surface area, which is even larger than the area of 1,568 Å² buried in the SSL7-IgA Fc interaction. However, the two sets of interactions are of rather different nature. In agreement with our isothermal calorimetry (ITC) measurements (Table S2 and Fig. S5), the C5-SSL7 interface (Fig. 24 and Fig. S3) is dominated by hydrophobic bonds and electrostatic interactions, and relatively few hydrophobic interactions (PISA $\Delta G = -2.8$ kcal/mol), whereas the SSL7-Fc interface (19) is more hydrophobic (PISA $\Delta G = -5.7$ kcal/mol).

**IgA-SSL7-C5 Complex.** Gel-filtration analysis of combinations of pure C5, SSL7, and IgA Fc (Fig. 1C) show that SSL7 can bind C5 and IgA simultaneously, and strongly suggests that an Fc-SSL7-C5 pentameric complex (predicted molecular weight, 476 kDa) is formed in solution, along with the partially saturated complex Fc-SSL7-C5 (286 kDa). To further examine the relationship between C5 and IgA binding, mutations were introduced to disrupt either or both binding sites. The SSL7 C5* mutant contained a single charge change D147K in the $\beta$-grasp domain. The SSL7 IgA* mutant contained three mutations (N68T.L109A.P112A) in the OB-domain that were necessary to significantly deplete IgA binding and the SSL7 C5* IgA* mutant combined all four mutations (N68T.L109A.P112A.D147K) (Fig. S6 A and B). The potential of SSL7 to form complexes with IgA and C5 in blood was examined by incubating SSL7*, SSL7 IgA*, or SSL7 C5* with human serum followed immediately by gel filtration (Fig. 1D). Incubation with SSL7* resulted in a predominant C5 containing peak eluting at $\approx 600$ kDa consistent in size with an IgA-SSL7-C5 pentameric complex (predicted molecular weight, 590 kDa). Incubation with the SSL7 IgA* mutant produced a single C5 containing peak eluting at $\approx 200$ kDa consistent in size with the SSL7-C5 heterodimer (molecular weight, 213 kDa). The SSL7 C5* mutant formed no detectable C5 containing complexes. The saturated pentameric complex Fc-SSL7-C5 was modeled (Fig. 1E) by superposition of two copies of the C5-SSL7 complex onto the Fc-SSL7-C5 structure (19). This model predicts that a pentameric structure is entirely feasible with no clashes between the five molecules. The molar concentrations of IgA and C5 in blood are on the order of 10 and 0.4 μM, respectively, and so the limiting factor in the formation of this complex would be the concentration of SSL7. Notably, in the Fc-SSL7-C5 model, neither SSL7 nor IgA Fc are within 70 Å of the cleavage site at R751 in C5, suggesting that IgA forms a scaffold for SSL7 to recruit C5 in such a way as to mask a convertase binding site distant from the C5a domain.

**C5-SSL7 Interface.** The core of the C5-SSL7 interaction is the antiparallel pairing of the SSL7 $\beta$-strand E144-I150 and the C5 $\beta$-strand H511$^*$-E516$^*$ with five hydrogen bonds formed between main chain atoms in the two strands (Fig. 24 and Figs. S2-S4). Their pairing leads to formation of a large continuous $\beta$-sheet with five strands (28 kDa) from the inhibitor and four strands from C5. SSL7 D147K further stabilizes the interaction by electrostatic interactions with C5 R515* and by engaging in a hydrogen bond with the side chain of Y501*. SSL7 is known to strongly bind human, chimpanzee, baboon, pig, sheep, goat, and rabbit C5, but not bovine, rat, or horse C5 (18). Sequence alignment (Fig. S4) suggests that C5 residues 511–516 are important, because this region is strictly conserved in C5 molecules that bind SSL7, whereas there are 1–3 residues with sequence variation in nonbinding C5. Hence, although this stretch forms main chain $\beta$-sheet hydrogen bonds with SSL7, even small perturbations to the C5 structure in this area can apparently lead to a significant drop in SSL7 sensitivity.
Functional and Biophysical Studies of SSL7 Mutants. The structures were validated by introducing mutations in SSL7 at residues suggested by the structures to function in C5 binding. In isothermal calorimetry (ITC) measurements (Fig. S5), wild type SSL7 formed a 1:1 complex with C5, \( K_d = 5.5 \text{ nM} \), in good agreement with SPR measurements (18). Except for the D144A mutant all other substitutions caused weaker SSL7 affinity for C5 with the most severe being the DI47A and S149A mutations resulting in \( K_d \) values of 62 and 37 nM, respectively (Table S2). The same mutants were assayed for their ability to inhibit formation of soluble MAC complexes (sMAC) (22) at a fixed concentration of 0.4 \( \mu \text{M} \). Full-length SSL7 inhibited sMAC formation by 63%, and all mutations resulted in weaker inhibition with substitution at DI47 and S149 resulting in the largest losses (Fig. 2B). SSL7 DI47A displayed almost no inhibition of sMAC formation, consistent with ITC results. Variations in the activities of naturally occurring alleles are also consistent with this. SSL7 from the strain US6301 was consistently a weaker inhibitor of hemolytic activity compared to four other natural alleles tested (Fig. 2C). SSL7 (US6301) has a histidine at position 147, whereas all other alleles have aspartic acid (Fig. S2). The sMAC assay also showed that SSL7c was not significantly less inhibitory than full-length SSL7, indicating that IgA binding through the N-terminal OB-domain was not required to inhibit sMAC formation. SSL7, SSL7 IgA', SSL7 C5', and SSL7c were tested by three functional assays for their ability to inhibit three complement C5-mediated functions in serum. The first assay examined the ability of SSL7 to inhibit red blood cell (RBC) hemolytic activity in 20% human or rabbit serum using alternative pathway complement activation (Fig. 3 A and B). Native SSL7 strongly inhibited both human (IC\(_{50} = 0.05 \mu \text{M}\)) and rabbit (IC\(_{50} = 0.5 \mu \text{M}\)) serum hemolytic activity. The 10-fold difference between species in part reflects the significantly higher concentration of C5 in rabbit serum. A mutation at DI47A substantially reduced SSL7 inhibition and a charge change to lysine in SSL7 C5' resulted in complete loss of inhibition in both species (Fig. 3 A and B). Notably, inhibition profiles for both SSL7 IgA' and SSL7c were essentially superimposed on native SSL7 for both species clearly indicating that IgA binding was not required to inhibit hemolysis. The second assay examined the ability of SSL7 to inhibit cell-free serum killing of a sensitive Gram-negative bacteria (Fig. 3C). The addition of 5% human serum to Escherichia coli (K12) resulted in 99.9% killing and this was strongly inhibited by the addition of native SSL7. Bacteria killing was clearly mediated by C5 because SSL7 mutants defective in C5 binding (SSL7 DI47A, SSL7 C5' and SSL7 C5' IgA') displayed a complete loss of function in this assay. Surprisingly, SSL7 IgA' and SSL7c displayed almost no inhibition (7.5% and 15% of native SSL7 inhibition, respectively) at 1 \( \mu \text{M}\) (Fig. 3C). Remarkably, even a single P112A mutation in the IgA binding site had a substantial effect (18% the level of native SSL7 at 1 \( \mu \text{M}\)) (Fig. 3C and Fig. S6C). This mutant has a 30-fold lower affinity for IgA (1–35 \( \text{nm}\)) (19). Thus, in contrast to inhibition of hemolysis and sMAC formation, inhibition of bacteriolysis by SSL7 was exquisitely dependent on high-affinity binding of IgA through the OB-domain.

In the third functional assay, SSL7 and mutants were tested for their ability to inhibit C5a production in response to heat-killed S. aureus in 5% human serum. The calculated IC\(_{50}\) for SSL7 in 5% serum was 0.02 \( \mu \text{M}\), and a maximal inhibition of 90% was achieved above 0.06 \( \mu \text{M}\), consistent with the predicted concentration of C5 in 5% human serum of 0.02 \( \mu \text{M}\) (Fig. 3D). No significant inhibition was observed for SSL7 C5' and SSL7 C5' IgA' at any concentration. The SSL7 IgA' and SSL7c mutants displayed significantly reduced inhibition (40% and 50% of maximum levels, respectively) above 0.06 \( \mu \text{M}\), confirming that IgA binding contributes strongly to the blocking of C5 cleavage by convertase bound to S. aureus. The IC\(_{50}\) for native SSL7 (allele 4427) in 100% human serum for the hemolytic assay and C5a production was calculated at 0.2–0.5 \( \mu \text{M}\) and did not vary significantly among a number of normal donors tested (Table S3). This is consistent with the known concentration for C5 of 0.4 \( \mu \text{M}\) (7.5 ng/mL) and suggests that the levels of naturally occurring anti-SSL7 IgG antibodies that we have identified in most individuals do not substantially affect the ability of SSL7 to bind C5 and IgA.

Steric Hindrance and Immune Evasion. To further analyze how IgA binding to SSL7 might influence C5 cleavage, we formed a minimal and soluble C5 convertase with cobra venom factor (CVF) in complex with Bb. CVF most likely mimics the role of C3b in the AP C5 convertase and C4b in the CP C5 convertase. A high molar surplus of SSL7 in the absence of IgA decreased CVF-Bb-mediated cleavage of C5 from 74% (no SSL7) to 59% (with SSL7) after 4 h, whereas the addition of both IgA Fc and SSL7 reduced cleavage to 9% after 4 h (Fig. 4A).

Based on the recent structure of C3bBb-SCIN, a model was proposed for how the C3bBb and C4b2a C3 convertases recognize the C3 substrate (23). We have extrapolated this model to suggest how C5 might also be bound by the convertase without any steric clashes between the substrate C5 and the C5 convertase C3b/C4b via dimerization of the MG4-MG5 domains (Fig. 4 B and C, and Fig. S7). This model offers an explanation as to why IgA Fc is
molecules captured in the complex. Completely prevent convertase binding to not one but both C5 and a second C5 molecule bound to the second SSL7 would produce large complexes with multiple large abundant host proteins (24). Several other complement inhibitor proteins from pathogens bind to SSL7 as well as other SSL7-complexes with multiple large abundant host proteins. Host antibodies may apply to other pathogen proteins that form complexes with SSL7 (25).

Inhibition by 1 μM SSL7 and SSL7 mutants against killing of E. coli by 5% human serum. Bacterial survival was enumerated by colony plating in triplicate for each dilution. Results are representative of three repeat experiments. (D) Inhibition by varying concentrations of SSL7 and SSL7 mutants of complement C5a production in response to the addition of 10^5 heat-killed S. aureus to 10% cell-free human serum. C5a was quantified by sandwich ELISA, using a commercial C5a as a concentration standard. The results are representative of two separate experiments from a single donor.

Discussion

C5 Convertase. There are now two known binding sites for pathogen proteins inhibiting cleavage of C5. OmiC1 appears to lock the conformation of C5 and in particular the C345C domain (21), whereas SSL7 binds at the opposite end of the molecule between the distal MG1 and MG5 domains. Both inhibitors appear to block C5 cleavage by interfering with convertase recognition far from C5a. Convertase binding seems to require surface regions distributed along the entire length of the C5. In both the AP and CP C5 convertases, an additional molecule of C3b is present in addition to the C3b/C4b and Bb/C2a. This switches the specificity from C3 to C5 by decreasing the Km for the C5 substrate (27). This additional C3b molecule required to convert the C3 convertase to a C5 convertase is apparently not necessary for the SSL7-IgA-mediated inhibition according to our proteolysis results with the minimal CVF-Bb C5 convertase. Our model of C5 recognition by C3b/C4b is based on an interaction similar to that proposed for C3 with C3b/C4b (23), and the model explains the importance of IgA Fc in SSL7-mediated inhibition of CVF-Bb C5 cleavage. In addition, it agrees with results showing that residues centered around R462 (mature C4 numbering) in the MG5 domain of human C4b are directly involved in C5 recognition by the CP C5 convertase (28).

Inhibition Modes of SSL7. Our model of the IgA-SSL7-C52 complex (Fig. 1E) does not predict any direct IgA-C5 interactions. But IgA-mediated steric hindrance appears to be the most likely needed to efficiently inhibit cleavage of the C5-SSL7 complex. One SSL7 molecule bound directly to C5 does not overlap with C3b in the convertase, whereas IgA Fc bound to this SSL7 overlaps with the C3b at a single loop. A second SSL7 bound to Fc is predicted to significantly constrain the binding of C3b (Fig. 4B and C) and a second C5 molecule bound to the second SSL7 would completely prevent convertase binding to not one but both C5 molecules captured in the complex.

We have noted another potential consequence of SSL7 cross-linking C5 and IgA. Only a single edge of the relatively flat SSL7 molecules appears to be accessible to antibody recognition once the C5-SSL7-IgA complex is formed (Fig. S8). On top of this, the affinity for C5 and IgA is so high that the concentration of neutralizing SSL7 specific IgG will have to be similarly high to achieve neutralization. A similar mechanism for evading aggregation by host antibodies may apply to other pathogen proteins that form large complexes with multiple large abundant host proteins. Several other complement inhibitor proteins from pathogens bind to SSL7 molecules to efficiently inhibit C5 substrate (27). This additional C3b molecule required to convert the C3 convertase to a C5 convertase is apparently not necessary for the SSL7-IgA-mediated inhibition according to our proteolysis results with the minimal CVF-Bb C5 convertase. Our model of C5 recognition by C3b/C4b is based on an interaction similar to that proposed for C3 with C3b/C4b (23), and the model explains the importance of IgA Fc in SSL7-mediated inhibition of CVF-Bb C5 cleavage. In addition, it agrees with results showing that residues centered around R462 (mature C4 numbering) in the MG5 domain of human C4b are directly involved in C5 recognition by the CP C5 convertase (28).

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A striking outcome of the functional assays is that SSL7 distinguishes two different mechanisms of C5-mediated lysis, one for red blood cells where the β-grasp domain of SSL7 inhibits as effectively as full-length SSL7 and another for E. coli bacteriolysis where even the slightest drop in IgA binding reduced SSL7 inhibition. We interpret this to mean that SSL7 inhibits at two distinct steps in the end-stage of the complement response. The first step is to block the recognition and cleavage by capturing C5 in a large complex with IgA preventing access by surface bound C5 convertase or the soluble CVF-Bb complex. The second step is blocking downstream formation of MAC complex once C5b has been formed, which is in agreement with the ability of SSL7 to bind preformed C5b and the inhibition of RBC lysis and SMAC formation by the β-grasp domain in SSL7 c. From the perspective of the Gram positive S. aureus which is impervious to MAC, the most likely role of SSL7 is to limit C5a production because of its critical regulatory role in the activation of phagocytosis, the main mechanism of clearance of this bacterium.

In contrast to RBC lysis by MAC, inhibition of bactericidal activity and C5a production are exquisitely dependent on IgA binding. This suggests that blocking MAC formation on its own is not sufficient to inhibit the killing of bacteria further suggesting that MAC assembly may occur differently on RBC compared to E. coli cells. A clue to the differential inhibition of hemolysis and bacteriolysis is found in a comparison of the three IC50 values. 50% inhibition of hemolysis and C5a production in whole serum (here performed in 20% and 10% serum, respectively) was 0.5 μM which is close to the serum concentration of C5 (Table S3). However the IC50 for bacteriolysis (performed here in 5% serum) was 10-times higher at 8 μM for complete serum. One explanation is that inhibition of the alternative pathway C5 convertase C3bBb3b (regulating hemolysis) does not require the pentameric IgA-SSL7-c-C52 complex, whereas the classical pathway C5 convertase C4b2a3b (regulating bactericidal activity) is less sensitive to SSL7 and thus demands higher concentrations of SSL7 to saturate the available IgA, thereby favoring the pentameric complex.

Why then did the β-grasp domain show partial inhibition of C5a production when IgA was completely absent from the complex? The answer may lie in the presence of naturally occurring anti-SSL7 IgG that bind epitopes in the β-grasp domain that are exposed in the absence of IgA. These may form trimeric complexes with C5-SSL7 that block convertase access similar to C5-SSL7-IgA complexes. Interestingly, the inhibition of C5a production by both SSL7 c and SSL7-IgA− did not increase above a concentration of 0.06 μM (Fig. 3D), suggesting that the anti-SSL7 IgG that substitutes for IgA had been saturated and that, above this concentration, only dimeric C5-SSL7 c was generated that blocks MAC formation but not C5 activation.

Implications for Development of Novel C5 Inhibitors. Blocking of C5 cleavage or the downstream functions of C5a or C5b represents a strategy for treating conditions uniquely associated with end-stage complement activation while preserving the essential immunoregulatory and immunoprotective roles of proximal complement activation. Eculizumab, a humanized monoclonal antibody for the treatment of PNH that prevents C5 cleavage, is the first complement inhibitor approved by the FDA and EMEA (13). SSL7, a small and easy to produce protein, may offer an alternative to expensive humanized antibodies. Moreover, the small C-terminal domain of SSL7 offers the potential to selectively block the lytic effects of MAC without affecting the essential role of C5a in regulating innate immunity. Whether or not the native SSL7 of Naja naja siamensis venom by gel filtration.

Materials and Methods

Protein Expression and Purification. Human C5 was purified as described in ref. 21. The SSL7 and SSL7 c proteins and their mutants were produced as recombinant proteins in E. coli and purified with affinity chromatography. Before crystallization, the C5-SSL7c-SSL7 complex was purified by gel filtration. Human IgA1-Fc (C242 to P455; IgA1 myeloma Bur numbering) was expressed using a derivative of pAPEX-3p in HEK293EBNA cells as described in ref. 19. CVF was purified from lyophilized Naja naja siamensis venom by gel filtration.
Crysatallization and Structure Determination. Crystals were obtained through vapor diffusion by mixing of 6 mg/mL protein in a 1:1 ratio with the reservoir buffer containing 1 M MgCl2, 100 mM Tris/150 mM NaCl/10 mM MgCl2 (pH 8.0) at 37 °C for 1 h. Before cleavage, CSSL and CSSL7 mutants were formed by mixing CVF, fB, and fD (Complement Technology) in a molar ratio of 1:1:1, respectively. Diffraction data were obtained from ice-cold 150 mM NaCl in 96-well, flat-bottom tissue culture plates (Falcon), and the absorbance at 412 nm was measured using an uQuant plate reader (BioTek).

Cell-Free Bactericidal Assay. Cells were incubated for 5,000 x g for 5 min before use. Cell-free serum bactericidal activity was assessed using a fresh overnight culture of E. coli K12 strain D585. SSL7 protein was preincubated with 5% normal human serum diluted in Hank's buffered saline solution (HBSS) (Sigma Aldrich) for 30 min at 37 °C in borosilicate glass tubes and then incubated with 1 x 10^9 colony-forming units (CFU) of D585 cells for 90 min at 37 °C. Tubes were plated in ice to stop reactions, and then a dilution series of each was prepared in HBSS and then plated in triplicate on LB agar and then incubated overnight at 37 °C. Colony-forming units (CFU) were enumerated the following day on a Baccount (BioTek) and expressed as mean CFU ± SD.

Detection of Serum C5a Production. One hundred-microliter samples of 25% serum were mixed with 50 μL of serially diluted SSL7 or SSL7 mutants before the addition of 100 μL of PBS-BSA containing 10% heat-killed S. aureus. Plates were incubated at 37 °C for 30 min and then centrifuged for 5 min at 1,250 x g to pellet bacteria, and the amount of C5a formed was quantified with a C5a mAb-based sandwich ELISA.

Biological Measurements. The ITC experiments were performed at 30 °C in a VP-ITC instrument from MicroCal and data were analyzed using the Origin software package.

Inhibition of sMAC (C5b-9) Formation. SSL7 and SSL7 mutants (20 μM) were mixed with human and rabbit serum, respectively. The RBCs from human or sheep (Invitrogen; Alsevers solution) were combined with human and rabbit serum, respectively. The generation of SSL7 or SSL7 + IgA-Fc was incubated at 37 °C for 1 h before mixing with the preformed convertases and digestion took place at 37 °C for up to 4 h. SSL7 pull-down experiments with CS or CSSL7 were done by incubating N-Tris-HCl-agarose with each CSSL7 or CSSL7SSL7. The intensities of the α- and α′-chains in C5CSSL7 were quantified with ImageJ.

Materials and Methods


Acknowledgments. We thank L. Kristensen, G. Hartzigsen, D. Wulff, H. Trist, S. Ling, and F. Clow for excellent technical assistance; the staff at the MAX-lab, European Synchrotron Radiation Facility, and Swiss Light Source beamlines for help with data collection; and K. Poulsen for S. aureus DNA. G. R.A. was supported by the Danish Science Research Council, Danscatt, the Vilhelm Petersen Foundation, and a Hallas-Møller stipend from the Novo Nordisk Foundation. J.D.F. was supported by the Health Research Council of New Zealand.

www.pnas.org/cgi/doi/10.1073/pnas.0910565107

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Supporting Information

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SI Materials and Methods

**Protein Expression and Purification.** The SSL7 gene from *S. aureus* strain ATCC 12598 was amplified by PCR and cloned into the pET-32 Eκ/LIC vector (Novagen) with primers containing a TEV protease site. Mutants of strain ATCC 12598 were generated by the use of the QuikChange Lightning Kit (Stratagene). Protein was expressed in BL21 (DE3) cells overnight in LB media. SSL7 was purified by Ni-affinity chromatography on a HiTrap HP column (GE Healthcare) followed by cleavage with TEV protease. Subsequently, the tag was removed by chromatography on a HiTrap HP column and SSL7 was dialyzed against 10 mM Hepes/100 mM NaCl (pH 7.4) and stored at −20 °C. The SSL7 was generated from strain 4427 (a local clinical isolate obtained from Greenlane Hospital, Auckland) as described in ref. 1. The C-terminal domain SSL7c (residues S129–I231) was generated by PCR from the full-length gene, and SSL7 mutants were produced by the technique of overlap PCR as described in ref. 2. SSL7 protein and its mutated proteins were produced from the vector pET-32a-3C in the *E. coli* K12 strain AD494(DE3)pLysS. Thiodorexin fusion proteins were purified from bacterial lysates by metal chelate chromatography, cleaved with 3C protease, and further purified by cation exchange chromatography as described in ref. 1. SSL7′ possesses and extra G-P-G at the N terminus due to the requirements of protease 3-C cleavage. Before crystallization, C5 and SSL7 or SSL7c were mixed in a 1:2 ratio and purified on a Superdex 200 10/300 column (GE Healthcare) equilibrated in 20 mM Hepes/100 mM NaCl (pH 7.4). For purification of CVF, lyophilized venom (0.2 g) from *Naja naja siamensis* (Miami Serpentarium) was dissolved in 4 mL of running buffer (50 mM Tris-HCl/150 mM NaCl pH 7.5). Subsequently, the venom solution was stirred for 12 h at room temperature, centrifuged for 10 min at 16,000 g, and filtered using a 0.2-μm filter. The filtrate was applied to a Superdex 200 26/60 (GE Healthcare) gel-filtration column equilibrated with running buffer at 4 °C at a linear flow rate of 1.5 mL/min. Fractions (3 mL) containing purified CVF as assessed by 7.5% SDS/PAGE under nonreducing conditions were pooled and quantified on the basis of SDS/PAGE gels or using the BCA protein assay.

**Structure Determination.** Before data collection, crystals were transferred to a cryo-protection buffer containing 50 mM MgAc2, 50 mM Mes (pH 6.2), 37.5% (wt/vol) PEG400, and 10 μM CdCl2. The structure of C5-SSL7 was determined by molecular replacement using PHASER (3) with C5 (residue 22–1514) and SSL7 (RCSB entry 1V10) as independent search models. However, direct positioning of SSL7 by PHASER was unsuccessful, and SSL7 was entry 1V10) as independent search models. However, direct positioning of SSL7 by PHASER was unsuccessful, and SSL7 was

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C5b-SSL7 Pull-Down. C5b was generated by mixing C5:CVF:βb:DF in the molar ratio 10:0.5:0.1:1,000 for 14 h, resulting in C5 cleavage being at least 95% complete as measured by comparison of the intensities for the C5 α-chain and the C5b α-chain. C5 or C5b were incubated with 20 μL Ni2+-NTA agarose (Qiagen) washed in 20 mM Tris-HCl/100 mM NaCl (pH 7.8) with either (i) 10 μg of C5 + 30 μg of SSL7-His, (ii) 20 μg of C5b + 30 μg of SSL7-His, (iii) 10 μg of C5, or (iv) 20 μg of C5b for 30 min at room temperature. Each sample of Ni2+-beads was first washed with 1 mL of 20 mM Tris/100 mM NaCl (pH 7.8) and then eluted with 30 μL of 20 mM Tris/100 mM NaCl/300 mM imidazole (pH 7.8) by incubation for 30 min at room temperature. The eluted supernatants were analyzed by 4–20% SDS/PAGE. The SSL7-His, for these experiments was cloned from strain 12598, expressed in pET32 Eκ/LIC, and therefore contains an N-terminal Trx-tag, a 6His-tag, and an S-tag.

**Gel-Filtration Analysis of Reconstituted C5-SSL7 and C5-SSL7-IgA Fc Complexes.** To generate the C5-SSL7 complex, C5 was mixed with a 4-fold molar excess of SSL7 in a total volume of 450 μL and incubated for 20 min at 22 °C. For analysis of the C5-SSL7-IgA complexes, C5 was incubated with a 2-fold molar excess of SSL7 and IgA-Fc for 20 min at 22 °C in a total volume of 450 μL. The complexes were separately analyzed by gel filtration on a 24-mL Superose 6 10/300 GL column equilibrated in 20 mM Hepes pH 7.4, 100 mM NaCl. The elution profile was examined by 12% SDS/PAGE.

**Gel-Filtration Analysis of IgA-SSL7-C5 Complexes Isolated from Serum.** Ninety-six-well MaxiSorp immunoassay plates (Nunc) were precoated overnight at 4 °C with 1 μg/mL Laffinity purified mouse anti-SSL7 in 15 mM Na2CO3, 35 mM NaHCO3, 0.01% NaN3 and blocked with 1% BSA/PBS (pH 7.4) for 1 h at room temperature, then washed extensively with PBS-Tween. 50 μL aliquots of fresh human serum were incubated for 30 min at room temperature with 10 μg each of SSL7, SSL7-IgA+, or SSL7-C5 protein. Samples were individually resolved on a Superdex 200 5/150 GL analytical column (GE Healthcare). The 50-μL eluted fractions were diluted to 2 mL with PBS and 50 μL added to the anti-SSL7 coated plates and incubated for 1 h at room temperature before washing and blocking as above. Complement C5 was detected with a 1:5,000 dilution of a rabbit anti-human C5 antibody (Dako) followed by 1:2,000 dilution of HRP-conjugated goat anti-rabbit. Peroxidase activity was detected with 0.5 mg/mL o-phenylenediamine dihydrochloride in 50 mM citric acid/100 mM Na2HPO4/0.012% H2O2. Reactions were stopped with 10% HCl, and the absorbance was measured at OD at 490 nm.

**Biophysical Measurements.** All experiments were conducted with SSL7 from the 12598 strain or its mutants. Before ITC, the C5 and SSL7 preparations were dialyzed overnight against 10 mM Hepes/100 mM NaCl (pH 7.5) overnight at 4 °C and degassed before analysis. The concentration of C5 was 1.88 ± 0.23 μM, determined using Α280 = 1.08 for a 1 mg/mL solution and a molecular weight of 190 kDa. The concentrations of wild-type SSL7 and its variants were 46–111 μM, determined using Α280 = 0.52 for 1 mg/mL and a molecular weight of 23 kDa. Isothermal titration calorimetry was performed in a MicroCal VP-ITC instrument operated according to the manufacturer’s instructions at 30 °C. Titration data were analyzed using the Origin software package. Correction for heat of mixing was performed by determining the average value of the
deflections after saturation was reached and subtracting this value from the data points.

**Serum Hemolytic Assay.** RBCs from either human or sheep (Invitrogen; Alsevers sheep blood) were combined with human and rabbit serum, respectively. The optimal concentration of serum was first determined by dilution assay and for human serum. This was normally ~20% but varied slightly between donors. Five milliliters of RBCs was added to 45 mL of GHB [150 mM NaCl/5mM Hepes/0.11 (wt/vol) Gelatin from bovine skin type B] with 2 mM MgCl$_2$ and 0.2 mM EGTA and incubated at 37 °C for 15 min to lyse any unstable RBCs. Cells were centrifuged at 1,250 × g for 10 min at 4 °C, the supernatant was removed, and the cells were resuspended in 50 mL of ice-cold GHB/MgEGTA buffer. This was repeated until the supernatant was clear following centrifugation. The RBCs were standardized to 2 × 10$^9$ cells per mL. SSL7 protein was added to a 96 well U-bottom tissue culture plates (Falcon) to give a 2-fold dilution series. One hundred microliters of serum diluted with GHB/MgEGTA was added. Fifty microliters of RBCs was added and the plate was incubated for 1 h at 37 °C with periodic shaking. The cells were pelleted by centrifugation at 1,250 × g for 5 min at 4 °C. One hundred microliters of the supernatant was added to 150 μL of ice-cold GHB in 96-well, flat-bottom tissue culture plates (Falcon), and the absorbance measured at 412 nm was measured using a uQuant plate reader (BioTek).

**Serum Cell-Free Bactericidal Assay.** Sera from normal volunteers were allowed to coagulate for 1 h at room temperature. Serum was centrifuged at 5,000 × g for 5 min before use to remove any particulate or cells. Cell-free serum bactericidal activity was assessed using a fresh overnight culture of *E. coli* K12 strain DH5α. SSL7 protein was preincubated with 5% normal human serum diluted in Hanks’s buffered saline solution (HBSS) (Sigma Aldrich) for 30 min at 37 °C in 5-mL borosilicate glass tubes and then incubated with ~1 × 10$^7$ stationary-phase (∼400 ~ 0.15) DH5α for 90 min at 37 °C. Five percent normal human serum was typically 99.99% bactericidal. Tubes were placed in ice to stop reactions and then a dilution series of each was prepared in HBSS, plated in triplicate on LB agar, and incubated O/N at 37 °C. Colony forming units (CFU) were enumerated the following day on a BacCount (BioTek) and expressed as mean CFU ± SD. Bacteria incubated with buffer alone were included as a no lysis control.

**Detection of Serum C5a Production.** Blood from healthy donors was clotted at room temperature for 1 h, centrifuged for 5 min at 1,250 × g, and diluted to 25% with 0.5% BSA, PBS (pH 7.3) (PBS-B). Duplicate 100-μL samples of 25% serum were mixed with 50 μL of serially diluted SSL7 or SSL7 mutant proteins before the addition of 100 μL of PBS-B containing 10$^7$ heat-killed *S. aureus* (Wood 46 strain). Plates were incubated at 37 °C for 30 min and then centrifuged for 5 min at 1,250 × g to pellet bacteria. The supernatants were further diluted to 1% with PBS-B, and 100 μL was added to the C5a sandwich ELISA. The anti-human C5a mAb (MAB2037; R&D Systems) was adsorbed at 2 μg/mL to 96-well MaxiSorp immunoassay plates (Nunc) overnight at 4 °C in 15 mM Na$_2$CO$_3$/35 mM NaHCO$_3$0.01% NaN$_3$. Unbound antibody was removed, and the plates washed four times with PBS-T (0.5% vol/vol Tween-20/PBS, pH 7.4). Wells were blocked with 1% BSA/PBS (pH 7.4) for 1 h at room temperature and then washed extensively with PBS-T and stored at 4 °C until required. Diluted serum samples (100 μL) were incubated for 1 h at room temperature in the ELISA plates before extensive washing with PBS-T followed by 1:5,000 dilution of anti-C5a rabbit serum pAB (Calbiochem) in PBS-B for 1 h at room temperature and a 1:2,000 dilution of HRP-conjugated goat anti-rabbit. Peroxidase activity was detected with 0.5 mg/mL o-phenylenediamine dihydrochloride (OPD) in 50 mM citric acid/100 mM Na$_2$HPO$_4$/0.01% H$_2$O$_2$. Reactions were stopped with 10% HCl, and the absorbance was measured at OD of 490 nm.

Fig. S1. Electron density of the complex and comparison with other structures. (A) Stereoview of a 2Fo−DFc electron density around the interaction between the β-sheets in SSL7 (green carbon atoms) and CS (gray carbon atoms, residues marked with *). The unit cell parameters of crystals of both the CS-SSL7 and the CS-SSL7c complexes are almost identical to those found for crystals of CS [Fredslund F, et al. (2008) Nat Immunol 9:753–760], and they also contain the same pseudo-crystallographic twofold axis with one copy of the C345C domain located directly on this axis. All atoms shown were omitted for map calculation, and the resulting map was then averaged over the twofold noncrystallographic axis. The map is contoured at 1σ. (B) CS from the SSL7 complex (gray) superimposed on free CS (magenta, RCSB entry 3CU7) with the C5a domain colored red/yellow (SSL7 bound/free CS). Least square superposition yields a root mean square deviation (rmsd) of 1.22 Å over residues 22–1510 between the structure of CS and the structure of CS bound to SSL7. In particular, the rmsd for residues 673–760, which includes C5a and N terminus of the C5 α′-chain is 0.44 Å, suggesting that C5a is essentially unaffected by the presence of SSL7. (C) Comparison of SSL7 from the CS complex (OB domain blue, β-grasp domain green), isolated SSL7 (gray, RCSB entry 1V1O), and SSL7 in complex with IgA Fc (red, RCSB entry 2QEJ). The rmsd between 190 Cα atoms in SSL7 bound to either CS or IgA Fc is 1.41 Å, and the rmsd between 190 Cα atoms in free SSL7 and CS-bound SSL7 is 1.29 Å.
Fig. S2. Sequence alignment of selected SSL7 sequences with secondary structure elements derived from the C5-SSL7 structure shown above. The label for the 12589 SSL7 and the 4427 SSL7 sequences are colored red or green. Helices and β-strands are shown as rectangles and arrows, respectively. Secondary structure elements in the OB-domain are blue, and those in the β-domain are colored green. Red triangles indicate residues forming hydrogen bonds or electrostatic interactions with CS; residues with black triangles only form van der Waal interactions.

Fig. S3. Selected atomic interactions between CS (gray carbon atoms, * after residue number) and SSL7 (green carbon atoms). The view is identical to that in Fig. 2A showing the CS-SSL7 interface. Intermolecular hydrogen bonds and electrostatic interactions are shown with dotted lines.

Fig. S4 (Continued)
Fig. S4 (Continued)
Fig. S4 (Continued)
Fig. S4. C5 sequence alignment of the MG1-MG6 domains. Names of species where C5 binds SSL7 are colored red; species where C5 does not bind SSL7 are shown in blue. Red triangles indicate residues forming hydrogen bonds or electrostatic interactions with SSL7; residues with black triangles only form van der Waal interactions. Residues marked by blue triangles are within 5 Å of C3b in a modeled C5-C5 convertase complex. Residue numbers for human C5 is shown below the secondary structure elements, where arrows and cylinders indicate β-strands and α-helices, respectively.
Fig. S5 (Continued)
Fig. S5. ITC thermograms obtained upon titration of CS with wild-type or mutant SSL7. The graphs were produced using the “ITC final” option of the Origin program. In two cases (E131A and N133A) the differential power used for maintaining temperature equilibration between the cells attained a value outside the range recommended by the manufacturer, and the instrument became unstable as revealed by oscillating power recordings. The corresponding data points were deleted.
Fig. 56. (A) Position of mutations in SSL7. Mutations D147K (magenta sphere) in the β-grasp domain (green) (SSL7′ C5−) and N68T.L109A.P112A (red spheres) in the OB domain (blue). (B) Purified mutants immobilized to Sepharose fail to bind either IgA, C5, or both from human serum. (C) Inhibition of SSL7′ and SSL7′-P112A on the killing of E. coli cells in the presence of 5% cell free human serum in triplicate. In the absence of SSL7, 99.99% killing was achieved within 30 min of incubation at 37 °C. Addition of SSL7 to 1 μM resulted in ~20% survival. Note that the single mutation in the IgA binding site significantly reduces the inhibition of bacterial killing. Results are representative of three separate experiments with separate donors.
Fig. S7. Cs interactions with the convertase. (A) Model of complex between Cs (in surface representation, domains colored individually) and C3b-Bb (cartoon representation, C3b colored magenta, Bb von Willebrand domain wheat colored, and the Bb serine proteinase colored gray) taken from RCSB entry 2WIN [Rooijakkers SH, et al. (2009) Nat Immunol 10:721–727]. The model was created by superimposing the MG4 domain of Cs with the MG4 domain of C3b in the (C3b-SCIN-Bb)2 complex. Notice that the position of the C345C domain may be a result of crystal packing in the structure of free Cs [Fredslund F, et al. (2008) Nat Immunol 9:753–760]. The Cs region 1628–33 (colored green), is likely to come in direct contact with the convertase [Sandoval A, et al. (2000) J Immunol 165:1066–1073]. (B) Two overall views of the modeled complex. (Upper) Related to A by rotation around a horizontal axis. (Lower) Related to B Upper by a rotation around a vertical axis.
Fig. S8. Shielding of SSL7 epitopes. A model of an SSL7 molecule (green and blue ribbon) bound between a C5 molecule (gray surface, only MG1-MG6 domains and linker region is shown) and Fc (red surface, carbohydrate not included) is compared with a Fab fragment (orange cartoon). Large areas of SSL7 not in direct contact with C5 or IgA would not be accessible to a host antibody due to steric hindrance.

Fig. S9. Interaction of SSL7 with C5b. (A) A model of C5b (gray cartoon) based on the structure of C3b [Janssen BJ, et al. (2006) Nature 444:213–216] suggests that C5b can interact with SSL7 (green and blue cartoon) in the presence of IgA Fc (red cartoon). (B) Experimental verification of the C5b-SSL7 interaction with histidine-tagged SSL7 bound to Ni²⁺-charged beads.
Table S1. Statistics for data collection and refinement

<table>
<thead>
<tr>
<th>Data collection</th>
<th>CS-SSL7</th>
<th>CS-SSL7c</th>
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<tr>
<td>Beamline</td>
<td>MAX-lab 911-2</td>
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<tr>
<td>λ, Å</td>
<td>1.0379</td>
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<tr>
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<td>P31</td>
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<td></td>
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<td>Resolution, Å</td>
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<td>50–4.2 (4.4–4.2)</td>
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<tr>
<td>Completeness, %</td>
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<td>99.8 (100)</td>
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<tr>
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<td>5.3 (5.5)</td>
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<tr>
<td>Mean I/σ(I)</td>
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<td>Reflections with I/σ(I) &gt; 3, %</td>
<td>64 (23)</td>
<td>61 (21)</td>
</tr>
</tbody>
</table>

Refinement

| Reflections: work set/test set | 61,125/3,205 | 39,763/2,029 |
| No. of atoms                  | 27,684       | 24,804       |
| rms deviation                 |              |              |
| Bond lengths, Å               | 0.012        | 0.011        |
| Bond angles, °                | 1.651        | 1.549        |
| R-factor/|Rfree-factor| 19.6/26.2 | 22.7/29.6 |
| Ramachandran plot: most favored/allowed/disallowed, % | 71.0/28.2/0.8 | 67.0/32.4/0.6 |
|                               |              |              |

*R_sym = (Σᵢ∑₇|h|I(h)ᵢ − <|I(h)|>)/∑ᵢ∑₇|h|I(h)ᵢ)| for the intensity of reflection h measured N times. Values in parentheses are for outer resolution shell.

*|R-factor = (Σᵢ|Fₒᵢ| − k|Fᵢ|)/Σᵢ|Fᵢ|, where Fₒ and Fᵢ are the observed and calculated structure factor, respectively, and k is a scaling factor.

|R_free-factor is identical to the R-factor on a subset of test reflections not used in refinement.

Table S2. Thermodynamic parameters for the dissociation equilibrium C5·SSL7 = C5 + SSL7 derived by isothermal titration calorimetry of human C5 with SSL7

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<th>SSL7</th>
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<th>Kₒ, M</th>
<th>ΔH, kcal/mol</th>
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<td>wt</td>
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<td>E144A</td>
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Table S3. IC₅₀ comparison of SSL7 inhibition across three C5-mediated functions

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<th>Serum IC₅₀, μM</th>
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<td>Bacteriolysis</td>
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<td>C5a production</td>
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