A Structurally Dynamic N-terminal Helix Is a Key Functional Determinant in Staphylococcal Complement Inhibitor (SCIN) Proteins

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Background: SCIN proteins from S. aureus mediate immune evasion by binding to C3b. The N terminus is a critical functional determinant in multiple SCINs. This work provides a new model for understanding SCIN structure and function.

Results: A structurally dynamic region exists within N termini of SCIN proteins and affects their C3b binding and inhibitory properties.

Significance: The N terminus is a critical functional determinant in multiple SCINs.

Complement is a network of interacting circulatory and cell surface proteins that recognizes, marks, and facilitates clearance of microbial invaders. To evade complement attack, the pathogenic organism Staphylococcus aureus expresses a number of secreted proteins that interfere with activation and regulation of the complement cascade. Staphylococcal complement inhibitors (SCINs) are one important class of these immunomodulators and consist of three active members (SCIN-A/-B/-C). SCINs inhibit a critical enzymatic complex, the alternative pathway C3 convertase, by targeting a functional “hot spot” on the central opsonin of complement, C3b. Although N-terminal truncation mutants of SCINs retain complement inhibitory properties, they are significantly weaker binders of C3b. To provide a structural basis for this observation, we undertook a series of crystallographic and NMR dynamics studies on full-length SCINs. This work reveals that N-terminal SCIN domains are characterized by a conformationally dynamic helical motif. C3b binding and functional experiments further demonstrate that this sequence-divergent N-terminal region of SCINs is both functionally important and context-dependent. Finally, surface plasmon resonance data provide evidence for the formation of inhibitor-enzyme-substrate complexes ((SCIN-C3bBb)-C3). Similar to the (SCIN-C3bBb)2 pseudodimeric complexes, (SCIN-C3bBb)-C3 interferes with the interaction of complement receptors and C3b. This activity provides an additional mechanism by which SCIN couples convertase inhibition to direct blocking of phagocytosis. Together, these data suggest that tethering multi-host protein complexes by small modular bacterial inhibitors may be a global strategy of immune evasion used by S. aureus. The work presented here provides detailed structure-activity relationships and improves our understanding of how S. aureus circumvents human innate immunity.

The complement system is a self-amplifying network of proteolytic reactions and molecular recognition events that serves a central role in host defense, immune development, and homeostasis. Although it fulfills many functions, one hallmark of complement activity lies in the opsonization of invading bacteria. Complement is mainly initiated by either antibody/antigen (classical pathway) or lectin/carbohydrate (lectin pathway) interactions at the bacterial surface, both of which intersect at the conversion of complement component C3 (185 kDa) into its bioactive fragments C3a (9 kDa) and C3b (175 kDa). The proteolytic activation of C3 is accompanied by enormous conformational changes; this exposes a reactive thioester group that allows C3b to covalently opsonize nearby bacterial structures. Once bound to the bacterial surface, C3b can form a Mg2+-dependent complex with circulating complement factor B (fB). This surface-bound C3bB complex is a substrate for a separate protease, called factor D (fD), which specifically cleaves C3b-bound fB and generates the fully functional C3 convertase, C3bBb. C3bBb is capable of proteolytically activating large quantities of C3 into C3b and is the driving force behind complement self-amplification that is typically known as the alternative pathway (AP).

It is now understood that the AP may be responsible for more than 80% of the downstream products (e.g. C5a, membrane attack complex, etc.) generated via various routes of comple-

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The abbreviations used are: fB, factor B; fD, factor D; fH, factor H; AP, alternative pathway; SCIN, staphylococcal complement inhibitor; CR, complement receptor; CR1gl, Ig repeat region of human complement receptor of the immunoglobulin superfamily; HBSS, Hanks’ balanced salt solution; NHS, N-hydroxysuccinimide; Efb, extracellular fibrinogen-binding protein; SSL-7, staphylococcal superantigen-like protein 7; C5, complement component 5.
ment activation (4). Because of this, self-amplification that results from AP C3 convertase function in vivo must be tightly regulated to mitigate potentially undesirable effects of complement activity against host cells and tissues. Indeed, a number of negative regulators of complement activity have been described and characterized (5). Several of these regulators, including freely circulating factor H (fH) and membrane-bound CD35 and CD55, function at the level of the AP C3 convertase. Although they differ somewhat in their mechanistic details, these proteins share in common the ability to bind C3b and accelerate the irreversible dissociation of the AP C3 convertase.

Like many successful pathogens, the Gram-positive bacterium Staphylococcus aureus has evolved a web of intricate strategies to evade complement-mediated immunity (6, 7). A body of work over the last decade has demonstrated that many of these small (∼10–15-kDa), secreted proteins also bind directly to C3b and thereby alter rates of formation, decay, and/or catalytic activity of the AP C3 convertase (8–18). Among these convertase-targeted inhibitors, the so-called staphylococcal complement inhibitor (SCIN)-A is of particular interest because it stabilizes the convertase in a catalytically inactive form (9–11, 15). SCIN-A also appears to promote formation of inhibited convertase dimers (i.e. (SCIN-C3bBb)2) that sterically mask the C3b binding sites of complement receptors (CRs) expressed on the surface of lymphoid and phagocytic cells (10, 19). As a consequence, SCIN-A interferes with opsonophagocytosis not only by inhibiting additional C3b deposition but also by directly blocking essential C3b/CR interactions.

Recent biochemical and structural studies have shed light on several mechanistic aspects of SCIN protein function (13–15). SCIN-A-containing co-crystal structures revealed that SCIN-A interacts directly with C3b in the absence of the Bb fragment and that the SCIN-A/C3b binding site is identical to the SCIN-A/C3c site (10, 13). Subsequent studies using a protease-stable form of the SCIN-B protein showed that, like SCIN-A, SCIN-B binds C3b near the MG7 domain (14). Together, these structure/function studies identified residues on the second α-helix of SCINs that are required for high affinity interaction with C3b (14). Furthermore, comparison of SCIN co-crystal structures with the C3b/fH(1–4) structure reveals that SCIN binding of C3b sterically occludes the fH binding site on C3b (13, 14, 20). In doing so, SCIN proteins render the catalytically inactive convertase insensitive to the decay accelerating properties of fH (15). By targeting a functional “hot spot” on C3b, SCIN proteins entrap convertases in a stable but inactive state (SCIN-C3bBb), and they also promote formation of dimeric inhibited convertases ((SCIN-C3bBb)2) that directly block C3b-dependent opsonophagocytosis (18, 19).

Genome sequencing efforts have revealed the presence of three additional SCIN-A-like proteins across S. aureus strains. Although the most distantly related of these, denoted ORF-D or SCIN-D, does not inhibit complement activity and is modestly structurally divergent from SCIN-A (11, 14), the other two, termed SCIN-B and SCIN-C, both inhibit generation of C3b via the AP (11). Interestingly, the genes that encode SCIN-B and SCIN-C are mutually exclusive (21), which suggests that these two proteins are allelic variants of one another. Although SCIN-B and SCIN-C share roughly 73% identity overall, all of the residues that make C3c contact in the SCIN-B(18–85)-C3c crystal structure appear to be conserved in SCIN-C (14). This high level of conservation raises questions about the N-terminal region of SCIN proteins that harbors considerably more sequence diversity than the helical bundle domain, and which contributes to formation of dimeric C3b complexes by SCIN-A (10, 13, 15). Unfortunately, there is limited structural information concerning this region of SCINs, and this precludes a detailed understanding of its precise contributions to SCIN function.

Toward addressing this need, we undertook a structure/function analysis of the N-terminal region of SCIN proteins. A combination of crystallographic and solution NMR data reveals that SCIN proteins contain an intrinsically dynamic helical region in their respective N termini. Curiously, the function of SCIN N termini is context-dependent and impacts both the C3b binding and convertase inhibitory properties of SCIN chimeras. As was seen for SCIN-A, the N terminus of other SCINs is required for forming higher order complexes of C3b that block phagocytosis by neutrophils. Surface plasmon resonance studies presented here also indicate that this region of SCINs participates in the formation of inhibitor-enzyme-substrate (i.e. (SCIN-C3bBb)-C3) complexes. Together, these studies further our understanding of SCIN structure and expand our knowledge of the multifaceted immune evasion mechanism of SCIN proteins.

EXPERIMENTAL PROCEDURES

Proteins—Purified C3, C3b, fB, fH, and fD were obtained from Complement Technology (Tyler, TX), whereas site-specifically biotinylated C3b was prepared according to a published protocol (14). DNA fragments encoding SCIN-A and SCIN-B and fragments thereof were amplified from S. aureus (strain Mu50) genomic DNA, whereas SCIN chimeras and SCIN-C DNA fragments were made by overlapping primer extension PCR and subcloned into the prokaryotic overexpression vector pT7HMT as described previously (22). Individual clones were confirmed by DNA sequencing. All SCIN proteins were expressed and purified according to a previously published protocol (23) with the exception that 15N and 15N/13C isotopically enriched samples were prepared using the appropriate minimal medium as described in detail previously (24). Chimeric proteins were analyzed for structural integrity by comparative circular dichroism spectropolarimetry with their respective wild-type counterparts. The Ig repeat region of human complement receptor of the immunoglobulin superfamily (CR1g) was prepared as described (25).

Crystallization, X-ray Diffraction Data Collection, Structure Solution, and Refinement—Crystallization experiments were carried out in a fashion identical to experiments described previously (14). Briefly, crystallization was achieved using vapor diffusion of hanging drops followed by cryoprotection and flash cooling in liquid nitrogen. X-ray diffraction data were collected at beamline 22-ID or 22-BM of the Advanced Photon Source (Argonne National Laboratory). Diffraction data were indexed, integrated, and scaled using HKL2000 (26). Structure solution and refinement were carried out by individual programs as implemented within the PHENIX software package (27). Final
models were obtained after iterative manual building in Coot (28) followed by refinement using PHENIX.REFINE. Temperature factor analysis was conducted using the program BAVER-AGE from the CCP4 program suite (29).

SCIN-B crystals grew in 5–7 days at 4 °C using a precipitant solution of 0.2 M ammonium sulfate and 30% (w/v) PEG 8,000. Drops consisted of 1 μl of protein sample at 16 mg/ml in 10 mM Tris (pH 7.4) and 50 mM NaCl and 1 μl of precipitant solution that had previously been diluted with 1 volume of double distilled H2O. Crystals were cryoprotected using a precipitant solution containing 5% (v/v) glycerol. A single copy of SCIN-B(18–85) (Protein Data Bank code 3T49) was used as a search model to solve this structure by molecular replacement.

SCIN-B(4–85) crystals grew in 1–3 days at 4 °C using a precipitant solution of 0.1 M Tris (pH 8.5) and 8% (w/v) PEG 8,000. Drops consisted of 1 μl of protein sample at 18 mg/ml in 10 mM Tris (7.4) and 50 mM NaCl and 1 μl of precipitant solution that had previously been diluted with 1 volume of double distilled H2O. Crystals were cryoprotected using a precipitant solution containing 30% (w/v) glycerol. A single copy of SCIN-B(18–85) (Protein Data Bank code 3T49) was used as a search model to solve this structure by molecular replacement.

Nuclear Magnetic Resonance Spectroscopy—NMR sample preparation and the general experimental protocol were performed as described previously (24). Briefly, isotopically 15N- and 13C-labeled SCIN-A and SCIN-B samples were dissolved in argon-saturated d13-MES (pH 6.9) with 5% (v/v) D2O, 0.2 mM 3-(trimethylsilyl)-1-propane-sulfonic acid, and 1 mM NaN3 at a final protein concentration of 800 μM. All NMR data were collected at 30 °C from a 14.1 Tesla Varian Inova spectrometer (599.7 MHz for 1H). Standard double and triple resonance spectra were used to assign the backbone 1H, 15N, 13C, and 13C resonances. Specifically, 1H, 15N heteronuclear single quantum correlation, HNCACB, CBCA(CO)NH, HNCA, and (HCA)CO(CA)NH spectra were recorded using water flip-back methods and/or 15N gradient coherence selection with sensitivity enhancement for solvent suppression (30–34). All NMR data processing and analysis were carried out with the NMRpipe and NMRView/NMRView software packages, respectively (35, 36). SCIN-A and SCIN-B resonance assignments were made by correlating intra- (i) and inter-residue (i−1) 13CO, 13CB, and 13CO resonances to the corresponding backbone 1H, 15N, 13C resonances through scalar coupling for each non-proline amino acid sequentially in the protein (37). Sequence-dependent backbone 13CO and 13Ca random coil chemical shift values were obtained using randomcoilshift.sh (38). 15N longitudinal (R1) and transverse (R2) relaxation time constants were measured by the steady-state, inversion-recovery, and Carr-Purcell-Meiboom-Gill methods, respectively (39–41). The R1 and R2 spectra were collected as follows: SCIN-A: R1 delays, 0.03, 0.05, 0.07, 0.12, 0.15, 0.23, 0.35, 0.80, 1.00, 1.20, 1.50, and 1.80 s and R2, 0.03, 0.05, 0.07, 0.13, 0.15, 0.17, 0.19, 0.21, and 0.23 s; SCIN-B: R1 delays, 0.03, 0.05, 0.07, 0.12, 0.15, 0.23, 0.29, 0.35, 0.50, 0.65, 0.80, 1.20, and 1.60 s and R2, 0.03, 0.05, 0.07, 0.09, 0.11, 0.13, 0.17, 0.19, and 0.23 s. These spectra included one or more R1 and R2 data sets that were used to estimate the precision of the peak intensities. Similarly, at least five interleaved steady-state 15N-1H NOE experiments were collected with and without 1H saturation to allow for estimation of experimental uncertainty. The R1 and R2 time constants were calculated as two-parameter exponential decay curves described by I(t) = I0e−t/τ where I(t) is the measured peak intensity as a function of the known relaxation delay time, t, I0 is the fitted initial peak intensity at t = 0, and R is the calculated 15N relaxation time constant (R1 or R2). All exponential curves were calculated using the Rate Analysis feature in NMRViewJ (36).

Protein-Protein Binding Assays—Assays for binding between various SCIN proteins and C3b were conducted using a luminescent microbead AlphaScreen technology. The experimental setup was identical to that previously described for myc-tagged SCIN-A and biotinylated C3b (14). The interaction between various SCIN proteins and C3b was also assessed in real time using a surface plasmon resonance ranking assay, which has likewise been described in a previous publication (14).

Alternative Pathway C3 Convertase Assay—An assay for the formation, activity, and inhibition of a fluid-phase alternative pathway C3 convertase was carried out using a SDS-PAGE-based method according to a previously published protocol (14). The effect of SCIN proteins on AP C3 convertase activity was assessed over a concentration series that reflected the C3b binding affinities observed for each individual SCIN variant.

Cross-linking Assay—All reactions were buffered in 10 mM HEPES (pH 7.4) and 150 mM NaCl. Biotinylated C3b at 0.1 mg/ml was incubated with 12.5 μM SCIN protein for 20 min at room temperature. 300 μM bis(sulfosuccinimidyl)suberate cross-linking reagent (Thermo Scientific) was then added to each solution, and this was followed by an additional 20 min incubation time. Reactions were quenched with 100 mM Tris (pH 6.8) for 15 min, and the presence of pseudodimers was monitored by silver-stained SDS-PAGE.

Phagocytosis Inhibition Assays—S. aureus strain Mu50 cells were grown to saturation overnight in tryptic soy broth, washed twice in Hanks’ balanced salt solution (HBSS), and resuspended in isotonic 50 mM borate buffer (pH 8.3). Cells at an A600 of 5 were labeled with 10 μg of DyLight488-NHS ester (Thermo Scientific) for 30 min at room temperature and washed twice with HBSS. Citrate-stabilized human serum freshly prepared from a donor was diluted to 25% (v/v) in HBSS supplemented with 5 mM EGTA and 10 mM MgCl2. Labeled cells at a milli-A600 of 50 were incubated with 12.5 μl of diluted serum and the indicated SCIN protein for 2 min at room temperature to allow deposition of C3b and any associated complexes. Decorated bacteria were separated from excess serum using a 20% (w/v) sucrose and HBSS cushion. Autologous neutrophils were simultaneously prepared by density gradient purification (Accurate Chemicals), and 1 × 106 cells were added to each bacterial condition. Phagocytosis was allowed to proceed for 30 min at 37 °C prior to addition of FACS buffer (1× HBSS, 0.5% (w/v) BSA, 1 mM EDTA, 0.02% (w/v) NaN3, and 50 ng/ml 7-aminocinachromycin D). Neutrophil phagocytosis was measured on a FACSCalibur cytometer by gating on the neutrophil population and excluding the 7-aminoactinomycin D-positive population and excluding the 7-aminoactinomycin D-positive population and excluding the 7-aminoactinomycin D-positive fraction. Data were analyzed for percentage of gated cells positive for bacterial uptake as well as mean fluorescence intensity using FlowJo software. All human samples were obtained and

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processed in accordance with an Institutional Review Board-approved protocol.

C3b Deposition Assay—S. aureus strain Mu50 cells were grown as outlined above with the exception of DyLight488-NHS labeling. Serum was prepared as described above and incubated in the presence of a 2-fold dilution series of SCIN proteins for 5 min followed by washing. DyLight488-conjugated F(ab’)2 anti-human C3 (Protos Immunoresearch) was prepared according to the manufacturer’s instructions and used to detect C3b deposition by flow cytometry.

Surface Plasmon Resonance Convertase Assay—All injections were performed on a Biacore X instrument (GE Healthcare) at a flow rate of 10 μl/min in a running buffer of 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, and 0.005% (v/v) Tween 20. A 100 nM equimolar solution of fB and fD was injected for 2 min followed by a 1 min dissociation phase. Next 1 μM SCIN-A or SCIN-C “MR252” was injected for 1 min. This was followed by an injection of 500 nM C3 or C3b for 1 min. Finally, 500 nM CRIg was injected for 1 min. Regeneration was done in a running buffer of HEPES (pH 7.4), 150 mM NaCl, 10 mM EGTA, and 10 mM EDTA. Regeneration to base line was achieved by three injection cycles of 100 nM fH, 2 μM NaCl, and 0.2 μM sodium carbonate (pH 9.0).

Miscellaneous—All plots were made using GraphPad Prism5 software (GraphPad, La Jolla, CA). All structural representations were made using VMD.

RESULTS

Crystal Structures of SCIN-B Reveal a Flexible N-terminal Region—Although N-terminal truncations in either SCIN-A or SCIN-B retain AP C3 convertase inhibitor activity, the affinities of their respective complexes with C3b are reduced by nearly 2 orders of magnitude (14). This strongly suggests that interactions formed by the N termini of SCIN proteins are required for maximal C3b binding and anticomplement function. However, the precise role of these N-terminal residues in SCIN function remains unknown due in large part to a lack of detailed structural information for this region.

To begin addressing this limitation, we determined crystallographic structures for full-length SCIN-B and a short N-terminal truncation mutant that removes the first three amino acids, termed SCIN-B(4–85) (Fig. 1, A and B, and Table 1). These structures are typified by a compact, three-helix bundle motif that is preserved across all structures of SCIN-B (Fig. 1C) (14). Overall, only minor changes, such as variations in sidechain rotamer conformation, are observed in this core region upon comparison of these structures with each other and with that of SCIN-B(18–85) (14). Outside of the core region, a well formed α-helix is found in the N terminus of the SCIN-B(4–85) structure, and this closely matches the secondary structure observed for this area of SCIN-A (supplemental Fig. S1) (11). Surprisingly, the N-terminal region of full-length SCIN-B deviated greatly from that of the SCIN-B(4–85) crystal structure. Here, non-classical secondary structure is found beginning near Gln-19 and persists to the most N-terminal residue observed in the SCIN-B crystal, Gln-9.

Crystallographic temperature factors (B-factors) provide quantitative measurement of the motion of protein atoms in the crystalline state (42). An examination of main-chain B-factors for the SCIN-B crystal structures indicates a high degree of atomic displacement for backbone atoms in the N terminus relative to the core part of the structure (supplemental Fig. S2). The presence of high B-factors is not surprising for the residues modeled as coil in the full-length SCIN-B structure because residues in loops often display higher relative temperature factors (43). However, subsequent analysis of the SCIN-B(4–85) structure likewise reveals a significant increase in B-factors for the same N-terminal residues (supplemental Fig. S2). This finding is unexpected as residues constrained by hydrogen-bonded secondary structure typically have smaller fluctuations in atomic displacement than those found in coil or loop regions (43).

TABLE 1

| Data collection | SCIN-B | SCIN-B(4–85) |
|-----------------|--------|-------------|
| Beams line | 22-BM | 22-ID |
| Wavelength (Å) | 1.00 | 0.972 |
| Space group | C222 | P2₁ |
| Cell dimensions (Å) | 66.54, 115.25, 79.57 | 84.38, 84.38, 72.92 |
| Resolution Limits (Å) | 50.3–31.1 (3.2–3.1) | 50.2–2.5 (2.6–2.5) |
| Unique reflections | 5,149 | 17,612 |
| Completeness (%) | 93.2 (61.4) | 87.6 (45.8) |
| Rmerge (%) | 7.1 (27.2) | 10.8 (41.3) |
| Redundancy | 4.6 (4.3) | 7.5 (2.6) |
| I/σ | 18.6 (3.4) | 18.6 (2.1) |

Rmerge = Σ ||I(hk) − F(hk)||/Σ |F(hk)|, where |F(hk)| is the ith measurement of reflection h and F(hk) is a weighted mean of all measurements of h. Rmerge was calculated from the working and test reflection sets, respectively. The test set constituted 5% of the total reflections not used in refinement.
SCIN-B and SCIN-B(4–85) crystals grew in different space groups (Table 1) and thus are expected to make different crystal contacts. Analysis of contacts made by molecules in each respective asymmetric unit was carried out using the European Bioinformatics Institute PISA server. N-terminal residues in SCIN-B(4–85) are involved in nearly twice as many crystallographic contacts as is seen for the same residues in SCIN-B. As crystal contacts can influence protein backbone structure, the unique local environment experienced by N-terminal residues in each crystal form may have stabilized helix formation in SCIN-B(4–85) yet favored an N-terminal coil conformation in the SCIN-B crystal (44). When considered as a whole, these two structures suggest that the N-terminal region of SCIN-B exhibits backbone flexibility and raise the possibility that SCIN-B might adopt alternate conformations within its N terminus.

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The N-terminal Regions of SCIN Proteins Are Flexible in Solution—In light of the differences between the SCIN-B structures described above, it was important to determine whether full-length SCIN-B has helical secondary structure at the N terminus in solution. To test this and to understand whether backbone flexibility is unique to SCIN-B, we conducted $^{15}$N relaxation experiments on full-length SCIN-A and SCIN-B. NMR spin relaxation can provide atomic resolution information on local backbone structure and extent of ensemble structural fluctuations through measurements of residue-specific dynamic changes that occur on the ns-ps time scale, including motions of the protein backbone (45). $^1$H-$^{15}$N heteronuclear single quantum correlation spectra were initially recorded for both SCIN-A and SCIN-B at the N-terminal positions. Uncertainties were calculated as detailed in “Experimental Methods.”

To assess the secondary structure of SCIN proteins in solution, we first compared observed $^{13}$CO and $^{13}$Ca chemical shifts with those determined previously for sequence-dependent random coil peptides (46, 47). Specifically, because residues in $\alpha$-helices experience a pronounced upfield shift for resonances corresponding to their $^{13}$Ca atoms (38, 48), residue positions with $\Delta^{\alpha\beta}<0.50$ ppm $^{13}$Ca chemical shift difference relative to random coil ($\Delta^{\alpha\beta}_{rc}$) were mapped onto the respective crystal structures of SCIN-A and SCIN-B(4–85) (Fig. 2, A and B). In accord with the SCIN-A crystal structure, NMR chemical shift data indicate the beginning of an N-terminal $\alpha$-helix near residue Asn-8, continuing until a break at Glu-25 and then resuming until a 3-residue loop that starts at Gly-34. The first definitive and contiguous N-terminal $\alpha$-helix for SCIN-B

![Figure 2. NMR data analysis for SCIN-A and SCIN-B.](image-url)
appears to begin at His-13 and continues until a kink at Lys-27; however, \( \alpha \)-helical character for SCIN-B is detectable as early as Glu-9. The presence of low \( ^{13} \text{C} \Delta \delta_{ \text{C} } \) values in the positions connecting \( \alpha \)-helix 1 to 2 and \( \alpha \)-helix 2 to 3 as well as the disordered C-terminal residues is consistent with loops as modeled in both crystal structures. An unexpected deviation from the predicted structures appears for a 2-residue stretch in the third \( \alpha \)-helix of SCIN-A at Ile-73/Tyr-74 and for SCIN-B at Ile-74/Tyr-75; however, the importance of this deviation from the predicted structure is unclear at present. Chemical shift differences for SCIN-A and SCIN-B are consistent with variable ensemble average \( \alpha \)-helical secondary structure in solution of SCIN proteins that is progressively greater proceeding from the N terminus inward into the first \( \alpha \)-helix.

We next selected residues with well resolved \( ^{1} \text{H}-^{15} \text{N} \) resonances for SCIN-A (63 of 85 residues) and SCIN-B (64 of 85 residues) for \( ^{15} \text{N} \) backbone relaxation analysis (supplemental Table S1). A summary of \( ^{15} \text{N} \) relaxation parameters for SCIN-A and SCIN-B is presented (Fig. 2, C and D). Whereas internally rigid proteins display homogenous longitudinal relaxation \( (R_\text{l}) \), transverse relaxation \( (R_\text{t}) \), and \( ^{15} \text{N}^{-1} \text{H} \) NOE values, those sites undergoing fast internal dynamics (backbone flexibility) are expected to have values that deviate from the average of the overall molecule (45). In this regard, the average \( ^{15} \text{N}^{-1} \text{H} \) NOE values are similar between SCIN-A \( (0.69 \pm 0.02) \) and SCIN-B \( (0.66 \pm 0.01) \). Interestingly, a dramatic reduction in \( ^{15} \text{N}^{-1} \text{H} \) NOE values is observed for N-terminal residues of both SCIN-A \( (0.33 \pm 0.08) \) and SCIN-B \( (0.32 \pm 0.01) \). Furthermore, although the \( R_\text{t} \) values are relatively homogenous throughout SCIN-A \( (1.48 \pm 0.25 \text{ Hz}) \) and SCIN-B \( (1.40 \pm 0.01 \text{ Hz}) \) (supplemental Table S1), the \( R_\text{t} \) and thus \( R_\text{t}/R_\text{l} \) ratios for N-terminal residues in both SCINs are substantially lower when compared with the entire proteins. This is significant because \( R_\text{t}/R_\text{l} \) ratios are directly impacted by rotational diffusion motions and are typically elevated in areas of secondary structure (24). As a consequence, a decrease in these ratios for N-terminal SCIN residues is consistent with ensemble averaged protein flexibility at these sites and suggestive of dynamic sequential order/disorder exchange in the N-terminal region of the helix.

In summary, the \( ^{15} \text{N} \) relaxation data we present here show that SCIN proteins are characterized in solution by a stable helical core with a flexible N-terminal \( \alpha \)-helix. Although this area of conformational flexibility extends further into the structure of SCIN-B, it is nevertheless evident in SCIN-A as well. When considered along with the crystallographic data presented above, these solution NMR studies highlight the structurally dynamic nature of SCIN proteins, particularly within their N termini.

The Function of the N-terminal SCIN Sequence Is Context-dependent—One peculiar aspect of increased dynamics at the N termini of both SCIN-A and SCIN-B is that these proteins share less amino acid sequence identity across these residues (14). This raises the possibility that sequence-dependent, functional differences might exist between the N termini of SCIN-A and SCIN-B. To address this question, we constructed and characterized two N-terminally swapped SCIN chimera proteins (supplemental Fig. S4A) and investigated their properties in a series of binding and convertase inhibition assays.

Whereas SCIN-B_AN (mostly SCIN-B, N-terminal SCIN-A) shows considerably impaired binding to C3b \( (K_\text{D} = 13 \text{ mM}) \) relative to wild-type proteins in an AlphaScreen competition assay (Fig. 3A and Table 2), SCIN-A_BN (mostly SCIN-A, N-terminal SCIN-B) binds C3b nearly 17-fold tighter \( (K_\text{D} = 4.4 \text{ nm}) \) than does wild-type SCIN-A (Fig. 3A and Table 2). Qualitatively similar results are also observed in a surface plasmon resonance ranking assay wherein binding of a constant concentration of various SCIN proteins to immobilized C3b was measured (Fig. 3B). Analysis of these same sensograms suggests that the SCIN-A_BN chimera has a much slower relative off-rate from C3b when compared with either SCIN-A or SCIN-B, which may explain the apparent increase in affinity (Fig. 3B).

Recent work from our group established a direct correlation between the affinity of the SCIN-C3b complex and inhibition of the AP C3 convertase (C3bBb) \textit{in vitro} (14). Thus, we hypothesized that the same general trend was likely to hold for SCIN chimeras. As expected, the weaker binding SCIN-B_AN chimera is a weaker inhibitor of the convertase (Fig. 3C and supplemental Fig. S5). Surprisingly, however, the inhibitory potency of SCIN-A_BN is intermediate between those of wild-type SCIN-A and SCIN-B (Fig. 3C). To our knowledge, this is the first example of a SCIN-like protein whose affinity for C3b does not directly correlate with its ability to inhibit the C3bBb convertase (8, 14, 15).

The studies on the SCIN chimeras described above strongly suggest that functional contributions of SCIN N termini are context-dependent. One implication of this predicts that changes in sequence within the N-terminal region of SCINs will produce proteins with different C3b binding properties and potentially altered functional activities. With this in mind, we examined the sequences of available \textit{S. aureus} genomes to determine whether sequence variability exists in the N termini of SCIN proteins. Interestingly, we found that SCIN-A sequence is nearly absolutely conserved (only a single Gln-49 to Leu-49 variant is identified), and the N-terminal region of SCIN-A is 100% identical in all available sequences (as judged by BLASTP). For analysis of SCIN-B and SCIN-C, we began by aligning SCIN-C (strain MRSA252) with SCIN-B (Mu50 strain). This alignment reveals that nearly half of the N-terminal residues contain amino acid substitutions (supplemental Fig. S4C). Likewise, we uncovered a surprising number of SCIN-C variants that are overall 77–97% identical to SCIN-C “MRSA252.” For example, SCIN-C from \textit{S. aureus} strain “H19” is 89% identical to SCIN-C MR252 yet harbors 8 of the 9 amino acid substitutions within their N-terminal 15 residues (supplemental Fig. S4, B and C). This analysis suggests that SCIN-B and SCIN-C are a spectrum of variants that are closely related to one another but are more distantly related to SCIN-A. We tested two representative members of SCIN-C molecules (MRSA252 and H19) for their ability to bind C3b as well as to inhibit the AP C3 convertase. Remarkably, both SCIN-C proteins bind C3b with higher affinity (SCIN-C MR252 \( K_\text{D} = 12 \text{ nm} \) and SCIN-C H19 \( K_\text{D} = 21 \text{ nm} \)) than does either SCIN-A or SCIN-B (Fig. 3A and Table 2), and each exhibits a slower relative off-rate when compared with SCIN-A or SCIN-B (Fig. 3B).
and supplemental Fig. S6). Despite this, the most tightly binding SCIN-C (SCIN-C MR252) still is not a more potent inhibitor of C3bBb activity (Fig. 3C). As a whole, the results obtained for both SCIN-C proteins compellingly mirror those obtained for the SCIN-A_BN chimera.

We also obtained a relative comparison of AP C3 convertase inhibitor activity by using a fixed concentration of various SCIN proteins (12.5 μM) in the solution-phase C3 cleavage assay (Fig. 3D and Fig. S7B). Although all SCIN-A-derived proteins are strong inhibitors of C3 cleavage, only wild-type SCIN-B effectively blocked the enzymatic activity of C3bBb. One potential explanation for this may simply be the amount of convertase bound under these experimental conditions due to the low affinity of several inhibitors for C3b (e.g. SCIN-B(18–85)KD/11005 μM and SCIN-B_AN KD/11005 μM) (14). However, the SCIN-A(11–85) complex has a dissociation constant similar to that of both SCIN-B(18–85) and SCIN-B_AN (SCIN-A(11–85)KD/11005 μM) (14), yet this N-terminally truncated form of SCIN-A blocks the convertase equally well as wild-type proteins in this assay format (Fig. 3D). Although the N terminus of SCIN proteins is positioned far from the complement inhibition binding site on C3b (as judged by available SCIN C3b fragment co-crystal structures (13)), this structurally dynamic region clearly contributes to inhibition of the AP C3 convertase in a subset of SCIN proteins.

The N Terminus of SCIN Molecules Impacts the Efficiency of C3b-dependent Phagocytosis—In addition to their stabilizing an inhibited form of the C3bBb convertase, SCINs also contribute to S. aureus immune evasion by blocking C3b-dependent phagocytosis (9, 19, 49). This activity is believed to arise...
through formation of SCIN-C3b-containing multiprotein complexes at the surface of the bacterium (11, 19). For example, SCIN-A promotes assembly of (SCIN-A-C3bBb)2 structures in solution and presumably on the bacterial surface when it engages an adjacent C3b molecule through a second lower affinity binding site (19); this pseudodimeric configuration masks C3b binding to complement receptors and therefore blocks phagocytic uptake of complement-opsonized bacteria. However, it is unknown whether other wild-type SCIN molecules can form such pseudodimers, and the role of the N-terminal SCIN residues on downstream phagocytic processes is not clearly established.

To address these questions, we investigated the impact of N-terminally truncated, chimeric, and wild-type SCIN molecules on both C3b dimerization and phagocytosis. A cross-linking assay was initially used to assess the ability of various SCIN proteins to form (SCIN-C3b)2 structures (Fig. 4A and Fig. S7A). These results show that all wild-type SCIN proteins and the SCIN-A_BN chimera form pseudodimeric structures, whereas SCIN-B_AN and both N-terminally truncated mutants do not. Because previous work has shown that formation of these pseudodimeric complexes strongly correlates with the antiphagocytic properties of SCIN-A, the efficiency of freshly isolated neutrophils to phagocytose complement-opsonized S. aureus Mu50 cells in the presence of various SCIN molecules was assessed using flow cytometry. Data represent mean ± S.E. (error bars) of three separate experiments. ***p < 0.0001 versus buffer; †††p < 0.001 versus buffer; ††p < 0.01 versus SCIN-A(11–85); ns, not significant.

SCIN contacts are buried within the large non-covalent interface between the α- and β-chains in uncleaved C3 (9, 13–15). Interestingly, those C3b residues that participate in the secondary and lower affinity SCIN-A binding site lie in what appears to be a preformed binding surface on native C3 (13). For these reasons, we hypothesized that a quaternary structure may form between SCIN-inhibited convertases (SCIN-C3bBb) and native C3. To test this directly, we developed a convertase interaction assay using a surface plasmon resonance approach (Fig. 5 and supplemental Fig. S8). First, we generated a SCIN-inhibited convertase on the surface of a C3b biosensor by injection of an equimolar solution of Fb and Fd followed by injection of SCIN-A (Fig. 5A) (15). Subsequently, a solution consisting of either 500 nM C3 or C3b was injected, and the presence of a corresponding binding event was monitored (Fig. 5, A and B). Whereas no detectable binding is observed during the C3b injection, a clear response is measured upon C3 injection onto the SCIN-A-inhibited convertase (Fig. 5, A and B, and supplemental Fig. S9A). As the injection solution lacks SCIN-A, the absence of signal following C3b injection is interpreted as evidence for lack of 1:2:1 (SCIN-A-C3bBb)-C3b complex formation. Previous work has proposed that inhibited convertase pseudodimers (SCIN-A-C3bBb)2 stericly occlude CR1 and CR1g binding sites on C3b and thereby account for the antiphagocytic properties of SCIN-A (19). To test whether the (SCIN-A-C3bBb)-C3 complexes identified above can also disrupt CR binding, we then injected a solution of 500 nM CR1g (Fig. 5, A and C). Indeed, the relative CR1g signal is reduced post-C3 injection when compared with post-C3b injection (Fig. 5, A and C, and supplemental Fig. S9B).

As a final test of the role of N-terminal SCIN residues in SCIN-C3bBb)-C3 formation, we repeated the experimental protocol described above using SCIN-C MR252 instead of SCIN-A (Fig. 5D). This SCIN-C was chosen on the basis of its unique C3b binding properties that originate from its divergent N-terminal sequence and that impart greater kinetic stability to its C3b complex when compared with either SCIN-A or SCIN-B (Fig. 3, supplemental Figs. S4 and S6, and Table 2). In summary, whereas this SCIN-C-bound convertase also fails to bind C3b (Fig. 5D), it displays higher relative C3 signal and...
lower relative CRIg signal than does the SCIN-A-bound convertase (Fig. 5, D–F). When considered together, these data indicate that inhibited convertase-C3 complexes not only form readily on surfaces but do so in a manner that interferes with CRIg recognition of C3b.

DISCUSSION

Since they were identified some years ago, the SCIN family of \textit{S. aureus} complement inhibitors has traditionally been characterized by studies on its prototypical member, SCIN-A (9, 11, 13, 15, 19, 23, 49). Because of this, our recent co-crystal structure of an N-terminal truncation mutant of SCIN-B bound to C3c provided the first detailed molecular basis for comparing how C3b/C3c is recognized in multiple SCIN proteins (14). These studies identified key residues on the second \( \alpha \)-helix of SCIN proteins that were critical in mediating direct C3b interaction by SCINs (13, 14). Here, we have expanded upon these studies by constructing chimeric SCIN proteins and demonstrating that the function of N-terminal SCIN residues is both sequence- and context-dependent (Figs. 3–5). We initially found that increased affinity for C3b by the SCIN-A_BN chimera did not result in a better inhibitor of the convertase when compared with wild-type SCIN-A (Fig. 3 and Table 2). Subsequent analysis led to the discovery that SCIN-C proteins bind C3b with nearly 25-fold higher affinity than does the closely related SCIN-B protein (Fig. 3 and Table 2). But despite this increase in affinity, we observed that SCIN-C fails to more potently inhibit convertase activity than does SCIN-A (Fig. 3, C and D). Together, our findings raise the possibility that although C3b affinity contributes to complement inhibition (14) there may be yet another role for high affinity C3b binding by SCINs. One potential explanation for fine-tuning of this interaction could be that optimum SCIN inhibition occurs through multiprotein interaction of the convertase and its substrate (C3) or in the formation of SCIN-inhibited pseudodimeric convertases. In any case, additional structure/function studies will be required to provide answers to such questions.

To better understand the functional role of SCIN N-terminal residues, we solved crystal structures of two closely related forms of SCIN-B. These structures deviate in secondary structure near the N-terminal portion of the protein and provide evidence for a helix-to-coil conformational transition (Figs. 1 and 2). The solution NMR dynamics study of SCIN-A and SCIN-B presented here reveals that a dynamic helical region is present at the N termini of both proteins. Thus, it now appears that dynamic secondary structure elements are an important feature of multiple SCINs (Fig. 2). Dynamic \( \alpha \)-helices and in particular helix-to-coil transitions have been described for a number of protein regions that make significant impacts on macromolecular function (50). In this regard, the ability to act as a tether or scaffold for assembly of multiprotein complexes has been proposed as a generalized role for intrinsically disordered protein motifs (51). This possibility is especially intriguing as an emerging theme for all classes of complement-targeted, staphylococcal immune evasion proteins is their ability to disrupt conceptually distinct host processes through bundling multiple host proteins into a larger complex. For example,
the disordered N-terminal region of Efb binds fibrinogen, whereas the C-terminal domain binds the thioester-containing domain found in C3/C3b/iC3b/C3d (17). In doing so, Efb simultaneously slows the rate of convertase formation (12), down-regulates anaphylatoxin production (17, 49), blocks CR2-dependent phagocytosis and engagement of adaptive immunity (52), and impedes neutrophil adherence (53). Separately, the staphylococcal superantigen-like protein 7 (SSL-7) has been shown to bind both complement component 5 (C5) and the Fc region of IgA (54, 55). Although SSL-7 inhibits both binding of FcαRI to IgA and activation of C5 to C5b, the latter activity is dependent upon IgA binding by SSL-7 (56). This intriguing result has been attributed to IgA-mediated steric masking of C5 binding to C5 convertases (56). Because the complement system is predicated upon a network of proteolytic and molecular recognition events, many of which occur between rather large proteins (>100 kDa), it seems to be particularly susceptible to steric disruptions at key enzymatic or regulatory steps. Indeed, we now propose that this is a focal point of the overall S. aureus immune evasion strategy.

Like Efb and SSL-7, the SCIN family members participate in a multiprotein complex that functions to inhibit the central enzymatic complex of the alternative pathway (C3bBb) and block opsonophagocytosis (9, 49). In light of the work described here, we feel that two changes may now be included in a refined model of SCIN function (Fig. 6A). This updated model highlights the importance of a flexible region near the N terminus of SCINs. In concert with residues on the second α-helix of SCINs, this N-terminal structure aids in initial binding of C3b and the formation of SCIN-inhibited convertases. Targeting of a functional hot spot on C3b by SCINs locks the convertase in a kinetically stabilized but inactive state through interaction with the proteolytic Bb fragment (13–15). The SCIN-bound convertase is further stabilized due to direct competition with the fH binding site on C3b, thus slowing decay acceleration (13, 15). SCIN-C3bBb can form SCIN-inhibited pseudodimeric convertases (SCIN-C3bBb)_2 or engage native C3, which futilely binds to and is tethered partly through direct interaction with N-terminal SCIN residues. These enzymatically inactive convertase complexes sterically occlude the C3b binding site for complement receptors, such as CR1g, that are present on the surface of circulating phagocytes (25). In this way, the structurally dynamic N terminus acts a “molecular bridge” that couples separate but related mechanisms of immune evasion.

A model of the (SCIN-C3bBb)-C3 complex can be generated by superimposition of the native C3 crystal structure onto one copy of C3b in the (SCIN-A-C3bBb)_2 crystal structure (Fig. 6B) (10, 57). This model has three important features that are consistent with the activities of SCIN-A and C3bBb. First, the position of C3 is such that the scissile loop of C3 is near the active site on Bb (10). Second, the second “C3b” binding site for SCIN-A is preformed in C3, and the N-terminal residues of SCIN-A involved in this site lie in close proximity to these C3 residues (13). Finally, a large interface is formed between C3 and C3b that masks the residues involved in the CR1g/C3b interaction (25).

The biological significance of a multiprotein (SCIN-C3bBb)-C3 complex is supported by a number of independent observations. It is true that simply preventing C3 binding to the convertase would provide an effective means for SCIN inhibition of C3bBb. However, by tethering C3 to an inactive surface-bound convertase rather than blocking C3 binding alone, SCINs not only prevent C3 activation but also form a protective structure that inhibits phagocytic uptake of C3b-opsonized S. aureus. The abundance of C3 in human plasma (~5 μM) coupled with its status as a natural substrate for C3bBb likely provides a favorable environment for the formation of (SCIN-C3bBb)-C3 (58).

In conclusion, we have identified several specific structure-activity relationships across all SCIN family members. Our data suggest that these findings may be relevant to a larger group of proteins that together make up the S. aureus complement inhibitory arsenal. Overall, this work highlights the modular nature of SCIN structure/function and provides new insight into the detailed mechanisms of innate immune evasion at work in S. aureus.
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