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Identification of Peptidic Inhibitors of the Alternative Complement Pathway Based on *Staphylococcus aureus* SCIN Proteins

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Abstract

The complement system plays a central role in a number of human inflammatory diseases, and there is a significant need for development of complement-directed therapies. The discovery of an arsenal of anti-complement proteins secreted by the pathogen *Staphylococcus aureus* brought with it the potential for harnessing the powerful inhibitory properties of these molecules. One such family of inhibitors, the SCINs, interact with a functional “hot-spot” on the surface of C3b. SCINs not only stabilize an inactive form of the alternative pathway (AP) C3 convertase (C3bBb), but also overlap the C3b binding site of complement factors B and H. Here we determined that a conserved Arg residue in SCINs is critical for function of full-length SCIN proteins. Despite this, we also found SCIN-specific differences in the contributions of other residues found at the C3b contact site, which suggested that a more diverse repertoire of residues might be able to recognize this region of C3b. To investigate this possibility, we conducted a phage display screen aimed at identifying SCIN-competitive 12-mer peptides. In total, seven unique sequences were identified and all exhibited direct C3b binding. A subset of these specifically inhibited the AP in assays of complement function. The mechanism of AP inhibition by these peptides was probed through surface plasmon resonance approaches, which revealed that six of the seven peptides disrupted C3bBb formation by interfering with factor B/C3b binding. To our knowledge this study has identified the first small molecules that retain inhibitory properties of larger staphylococcal immune evasion proteins.

Graphical Abstract

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Keywords

Complement; Inhibitors; Alternative Pathway C3 Convertase; Peptides; Surface Plasmon Resonance

1. Introduction

The complement system is an evolutionarily ancient network of serum and cell surface-associated proteins that serves as an essential defense against infiltrating microorganisms. Along with this quintessential innate immune function, complement is also an important surveillance system capable of recognizing and marking unhealthy host cells and debris for elimination and targeting immune complexes for clearance. Thus, under normal physiological conditions, complement makes a fundamental contribution to homeostasis (Ricklin et al., 2010). When the tightly regulated control of complement is disrupted, however, the destructive nature of the cascade can become directed towards healthy host tissues. Indeed, an ever growing list of pathologies associated with either acute or chronic complement activation is now known (Asgari et al., 2010; Ekdahl et al., 2011; Lachmann and Smith, 2009; Nilsson et al., 2010; Ricklin et al., 2010; Ricklin and Lambris, 2013a). Despite this, few therapeutic interventions have proven effective in clinical trials, and even less have received regulatory approval for patient use in the United States (Ricklin and Lambris, 2013b; Thurman, 2014). Thus, there remains a great need for new concepts toward development of economical, complement-targeted drugs for treating prevalent diseases and conditions such as ischemia/reperfusion injury, systemic inflammatory response syndrome, and sepsis (Ricklin and Lambris, 2013a).

In principle, the complement system becomes activated by one of three pathways termed “classical” (CP), “lectin” (LP), or “alternative” (AP) which can be generally defined by mode of recognition (antibody/antigen, carbohydrate/lectin, or spontaneous “tick-over”, respectively). Regardless of the triggering event, all pathways lead to the proteolytic cleavage of complement component C3 into its opsonin (C3b) and chemotactic fragments (C3a) by multi-subunit enzymes called C3 convertases. Following activation, C3b becomes covalently attached to nearby biomaterial via exposure of a highly reactive thioester bond. Surface immobilized C3b participates in a powerful self-amplification reaction by virtue of its ability to interact with complement factor B (fB), which is then cleaved by factor D (fD) to form the central enzymatic complex of the complement system known as the AP C3

convertase (C3bBb). C3bBb rapidly activates large amounts of C3 to C3b, and is responsible for over 80% of downstream complement products (Harboe, 2004). Surface-associated C3b further serves as a molecular platform for assembly of C5 convertases that mediate downstream proteolytic activation of C5. It is cleavage of C5 into its C5b and C5a fragments which results in formation of the lytic membrane attack complex (MAC), as well as inflammatory cell recruitment to the site of complement activation.

To protect themselves from the deleterious effects of complement activation, host cells express several complement regulatory proteins that exact exquisite control over various steps of the cascade (Zipfel and Skerka, 2009). One of these “regulators of complement activation” (RCAs), complement factor H (fH), binds to and dissociates the AP C3 convertase and additionally functions to degrade C3b by acting as a cofactor for the protease factor I (fI). As a negative regulator of the C3b self-amplification loop, fH greatly diminishes complement activity on the surface of host cells. Microbes, however, lack these RCAs and thus are typically subjected to the full force of complement attack. Indeed, many human pathogens have evolved what appear to be effective strategies for defending themselves against complement attack (Lambris et al., 2008; Zipfel et al., 2013). Chief amongst these so-called immune evasion mechanisms is the direct recruitment of endogenous RCAs to the microbial surface (Blom et al., 2009).

While the Gram-positive bacterium *Staphylococcus aureus* has been reported to recruit RCAs such as fH to its surface (Amdahl et al., 2013; Sharp and Cunnion, 2011), *S. aureus* is also known to secrete several classes of complement inhibitory proteins [e.g. (Garcia et al., 2012a; Kang et al., 2013; Woehl et al., 2014)]. Among these factors are the staphylococcal complement inhibitors SCIN-A and SCIN-B/C (Jongerijs et al., 2007; Rooijakkers et al., 2005). Although SCIN function is multi-faceted, a hallmark of SCIN activity is the stabilization of an inactive form of the AP C3 convertase via the formation of a ternary C3bBb/SCIN complex (Jongerijs et al., 2007; Rooijakkers et al., 2005). More recently, structure/function studies have shed light on the molecular basis for complement inhibition by the SCIN family (Garcia et al., 2010; Garcia et al., 2013; Garcia et al., 2012b; Jongerijs et al., 2010; Rooijakkers et al., 2009; Rooijakkers et al., 2007). These studies revealed that SCINs also participate in large, multi-protein complexes (e.g. (C3bBb/SCIN)₂ and C3bBb/C3/SCIN) mediated by a conformationally-flexible, N-terminal region of the SCIN proteins. Interestingly, naturally occurring sequence variation for residues found in this secondary C3b contact site on SCINs augmented both the C3b-binding properties and overall function of SCIN proteins. Furthermore, it was shown that these multi-protein complexes are capable of directly blocking phagocytosis by masking the complement receptors CR1g and CR1, both of which are found on the surface of phagocytic cells (Garcia et al., 2013; Jongerijs et al., 2010). Thus, by targeting a vulnerable site on C3b, SCINs simultaneously block the key amplification step in the complement cascade and impede C3b-mediated phagocytosis.

Although these activities make SCINs potent complement inhibitors, their immunogenicity almost certainly precludes direct use of these proteins as a therapy for complement-mediated diseases (Jongerijs et al., 2007). One potential way to overcome this barrier is through the development of small molecule inhibitors which are specifically designed to mimic critical

interactions necessary for SCIN/C3b binding. Unlike many endogenous C3b ligands, such as fH, that engage C3b via multi-domain contacts and bury large surface areas (e.g. $> 5,500 \text{ \AA}^2$ across 6 fH domains) (Kajander et al., 2011; Wu et al., 2009), the primary C3b-binding site for SCINs buries less than $800 \text{ \AA}^2$ (Garcia et al., 2010; Garcia et al., 2012b). This feature of a relatively small target area on C3b combined with the importance of the functional “hotspot” associated with the SCIN/C3b interface makes a smaller molecule approach particularly promising. In the work presented here, we began building toward this approach by better defining the functionally critical residues in full-length SCIN proteins. We expanded upon these results by carrying out a phage display screen that successfully identified SCIN-competitive 12-mer peptides. Finally, we demonstrated that a subset of these peptides are indeed SCIN peptidomimetics that retain complement inhibition activity. Together, our results provide a valuable proof-of-concept that smaller molecules capable of binding the SCIN site on C3b exist, and that they also retain complement inhibitory activities.

2. Materials and Methods

2.1 Proteins and Peptides

DNA fragments encoding SCIN-A and SCIN-B were amplified from *Staphylococcus aureus* (strain Mu50 / ATCC700699) and sub-cloned into the prokaryotic over-expression vector pT7HMT (Geisbrecht et al., 2006). Overlapping primer extension PCR was used to produce single and double point SCIN mutations and all SCIN proteins were expressed and purified as previously described (Garcia et al., 2009). All peptides were commercially synthesized (GenScript, Piscataway, NJ) to $> 95\%$ purity. The 12-mer peptide YHPNGMNPYTKA was identified in an unrelated site-specific phage display screen and was used as a negative control. Peptides were resuspended in 20mM HEPES (7.3), 150mM NaCl (HBS), passed through a $0.45\mu\text{m}$ pore filter, and further purified over a PD MiniTrap G-10 column (GE Healthcare). Peptide concentrations were obtained by measuring absorbance at 205 nm as described (Anthis and Clore, 2013). The Compstatin derivative Cp40 was a gift from Dr. John D. Lambris (University of Pennsylvania School of Medicine). Purified C3, C3b, fB, fH, and fD were obtained from Complement Technology (Tyler, TX). Site-specifically biotinylated C3b was prepared as previously described (Garcia et al., 2012b; Ricklin et al., 2009).

2.2 Protein-protein and protein-peptide binding assays

AlphaScreen microbead technology was used to compare binding of various SCIN proteins or peptides using experimental protocols identical to those published previously for myc-tagged SCIN-A (Acceptor) and biotinylated-C3b (Donor) (Garcia et al., 2012b). Data were fit to a four-parameter (variable slope) dose-response curve using GraphPad Prism5 software (GraphPad, La Jolla, CA). Direct binding of SCIN proteins or peptides were measured by surface plasmon resonance (SPR) using a Biacore 3000 instrument (GE Healthcare) at 25°C . HBS-T (20 mM HEPES (pH 7.3), 150 mM NaCl, 0.005% (v/v) Tween 20) was used as the running buffer and a flowrate of $20 \mu\text{l min}^{-1}$ was maintained across all experiments. Site-specifically biotinylated C3b was immobilized on a streptavidin sensor chip (GE Healthcare) at a density of 4400 RU (SCIN injections) or 5100 RU (peptide injections). A concentration

series of each SCIN protein was injected for 2 min followed by 3 min of dissociation at which time baseline regeneration was achieved. Maximal response ($R_{\max}$) was treated as steady-state for each injection and was determined by averaging the response for 20 s just prior to injection stop. Four-parameter variable slope non-linear regression analysis was used to assess steady-state affinities. BIAevaluation software (GE Healthcare) was used to perform kinetic analyses using a 1:1 Langmuir model of interaction. For peptide ranking experiments, peptides were diluted to 1 mM in HBS-T and injected for 2 min followed by 2 min of dissociation phase with no baseline regeneration required. All experiments were performed in duplicate. An empty streptavidin flow cell and an ensemble of buffer blank injections were used to perform double referencing of sample responses. BIAevaluation software was used to process and analyze the resulting sensorgrams and a ranking phage-derived peptides was made by correcting for the molecular weight of each species.

2.3 Complement hemolytic assays

The ability of site-directed SCIN mutants to inhibit the activity of the alternative pathway (AP) was assessed using a modified AP hemolytic assay (APH50). For this purpose 5×10^8 cells mL^{-1} rabbit erythrocytes (Er) (Complement Tech) were washed once by centrifugation for 3 min at $500 \times g$ and 4°C . Cells were then resuspended in a GHBS $^\circ$ buffer (20 mM HEPES (pH 7.5), 140 mM NaCl, and 0.1% (w/v) gelatin). Reactions consisted of 100 μL and began by diluting 5 μL of 0.1 M MgEGTA into 30 μL GHBS $^\circ$, followed by 20 μL of $5 \times$ SCIN proteins at various concentrations, followed by 20 μL of pooled complement human serum (NHS) (Innovative Research). Finally, 25 μL of Er were added to the mixture and the reactions were allowed to incubate at 37°C for 30 min with intermittent agitation. Reactions were spun down (3 min, $500 \times g$) and diluted 1:10 with GHBS $^\circ$ in a 96-well flat-bottom half-area clear microplate (Greiner Bio-One Inc., Monroe, NC). Absorbance was measured at 412 nm using a VersaMax microplate reader (Molecular Devices). A well using buffer in place of SCIN proteins was treated as 100% while background was measured by replacing serum with buffer. Percent lysis versus control was computed by subtracting background readings from each well and comparing each reading to 100% controls. All experiments were performed in duplicate. The ability of peptides to inhibit AP mediated hemolysis of Er cells was measured in an identical way with the following modifications. Peptides were present at a final fixed concentration of 500 μM and NHS was used at either a fixed 6.5% (v/v) or in a concentration series ranging from 0 to 20% (v/v). To assess the role of properdin/factor P (fP) identical reaction conditions were used with the following changes: i) the source of serum was fP-depleted (Complement Tech), and ii) the incubation time was increased to 60 min.

The ability of peptides to inhibit the classical pathway (CP) mediated hemolysis was performed as follows. Sheep erythrocytes sensitized with human IgM (Ea) (Complement Tech) at 5×10^8 cells mL^{-1} were washed once by centrifugation at $500 \times g$ for 3 min and resuspended in GHB $^{++}$ buffer (20 mM HEPES (pH 7.3), 140 mM NaCl, 0.1% gelatin (w/v), 0.15 mM CaCl_2 , and 0.5 mM MgCl_2). 100 μL reactions were made by mixing 35 μL GHB $^{++}$ with 20 μL of peptide (500 μM final concentration), 20 μL NHS (1% (v/v) final concentration), and 25 μL Ea. Reactions were incubated at 37°C for 60 min with intermittent

agitation. After centrifugation and transfer, absorbance was read at 541 nm and % lysis versus control was computed as described for the AP hemolysis assays above.

2.4 Phage display screen

SCIN competitive peptides were identified by employing a modified solution-phase biopanning procedure using a commercially available 12-mer Ph.D. Phage Display library (New England Biolabs, Ipswich, MA). The library consisting of 10^9 individual peptides fused to the N-terminus of the M13-phage protein pIII was incubated with site-specifically biotinylated C3b. Following washes of increasing stringency, bound phage were eluted using 100 μ M SCIN-A to specifically select for SCIN-competitive, C3b-binding sequences. Three rounds of panning and enrichment were carried out followed by phage recovery, amplification, and sequencing of the variable insert regions.

2.5 Nuclear magnetic resonance (NMR) spectroscopy

1D proton NMR was used to assess the secondary structures of the peptides alone in solution. To better observe the H α signals, which largely overlap with those of H $_2$ O, we used D $_2$ O as solvent. Under this condition, backbone amide protons were no longer observed due to exchange with D $_2$ O. Peptides were dissolved in 20 mM sodium phosphate buffer (pH 6.0). The peptide solutions were then lyophilized followed by addition of D $_2$ O to achieve final peptide concentrations of 0.5–1.1 mM for NMR experiments. All NMR experiments were performed at 25°C on a Varian Inova 600 MHz spectrometer. The compound 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate was used for chemical shift referencing (Nowick et al., 2003). NMR data were processed using NMRPipe (Delaglio, 1995).

2.6 Circular dichroism (CD)

Far-UV CD was used to assess the secondary structure of peptides in solution. Samples were dissolved in 20 mM HEPES (pH 7.3), 150 mM NaCl at a concentration of 125 μ M (peptides) or 50 μ M (SCIN-A). A buffer control was also collected. Spectra were collected across a 190–260 nm range, at 50 nm min $^{-1}$, using 0.5 nm pitch, 1 s response, and 1 nm bandwidth. All data was collected on a Jasco J-815 instrument using a cylindrical small volume quartz cuvette (1 mm path length) (Starna Cells, Inc., Atascadero, CA).

2.7 Alternative pathway complement activity on an artificial surface

Functional activity of the AP and CP were determined as previously described (Roos et al., 2003). Briefly, Costar EIA/RIA plates (Fisher Scientific) were coated overnight with 25 μ g ml $^{-1}$ *Salmonella enteritidis* LPS (AP) (Sigma Aldrich) or 3 μ g mL $^{-1}$ human IgM (CP) (Athens Research & Technology, Athens, GA) in coating buffer (100 mM Na $_2$ CO $_3$ /NaHCO $_3$, pH 9.6). All subsequent steps were preceded by washing the plates with TBS-T (50 mM Tris (pH 8.0) 150 mM NaCl, 0.05% (v/v) Tween 20) three times and all reaction volumes were 100 μ L. Plates were blocked with 1% (w/v) BSA in PBS with 0.05% (v/v) Tween 20. 20% (v/v) (AP) or 1% (v/v) (CP) final concentration NHS was mixed with or without various concentrations of peptide in an AP buffer containing 20 mM HEPES (pH 7.5), 0.1% (w/v) gelatin, 140 mM NaCl, 5 mM MgCl $_2$ and 10 mM EGTA. Buffer for CP

experiments was composed of 20 mM HEPES (pH 7.3), 0.1% (w/v) gelatin, 140 mM NaCl, 2 mM CaCl₂, 0.5 mM MgCl₂. Serum/peptide mixtures were then added to the wells and incubated at 37 °C for 1 h. C3b deposition was detected by a 1:300 dilution of anti-C3d monoclonal antibody (030-08, Santa Cruz Biotechnology) followed by incubation with a 1:5000 dilution of goat anti-mouse HRP secondary antibody (Thermo Scientific). HRP-labeled antibody was detected by incubation of SureBlue TMB Microwell peroxidase Substrate (KPL, Gaithersburg, MD) for 10 min. The reaction was stopped by addition of 0.16 M sulfuric acid and the absorbance at 450 nm was measured using a VersaMax microplate reader (Molecular Devices).

2.8 Alternative pathway convertase formation SPR assay

The ability of peptides to inhibit the formation of the AP C3 convertase (C3bBb) was monitored in real time using a SPR based approach. Experimental conditions were identical to those described above with the exceptions of a 10 $\mu\text{L min}^{-1}$ flowrate and the HBS-T running buffer was supplemented with 5 mM MgCl₂. Each experiment began by a 1 min control injection of 1 mM peptide alone which was allowed to dissociate to baseline. Next the AP C3 convertase was formed on the C3b chip surface by injecting a mixture of 100 nM fB and 100 nM fD for 1 min. C3bBb was then allowed to dissociate for 2 min and regenerated to baseline by a series of 1 min injections of 100 nM fH, 2 M NaCl, and 0.2 M sodium bicarbonate (pH 9.0). Next the convertase was formed in the presence of 1 mM peptide followed by dissociation for 2 min and baseline regeneration. To ensure integrity of the chip surface and reliability of the regeneration protocol, the convertase was formed again in the absence of the peptide followed by surface regeneration. The average response of 10 seconds just prior to regeneration of C3bBb was calculated for convertase formation in the absence of peptide and was compared to the response observed in the presence of 1 mM peptide. Inhibition percentage of the convertase was computed by setting the fB/fD alone injection to 100% response and comparing to the fB/fD/peptide injection.

2.9 fB competition SPR assay

The ability of peptides to compete with the fB binding site on C3b was likewise evaluated using SPR. A constant concentration of fB (150 nM) was injected for 1 min at 10 $\mu\text{L min}^{-1}$ in a running buffer of HBS-T supplemented with 5 mM MgCl₂ and allowed to dissociate to baseline. Next a mixture of fB and 1 mM peptide was injected and the response just prior to injection stop was evaluated. An injection of pure peptide was subtracted from the corresponding fB/peptide injection. Corrected signals were then compared to the fB alone injection, which was treated as 100% response.

2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 5.0 (La Jolla, CA).

3. Results

3.1. A conserved arginine residue on the 2nd α -helix of “full-length” SCIN proteins is critical for C3b binding

Residues found on the 2nd α -helix of SCIN-A and SCIN-B make direct contributions to the complement inhibitory properties of SCINs (Garcia et al., 2010; Garcia et al., 2012b; Jongerius et al., 2007). In particular, considerable interactions have been observed for a pair of homologous residue positions (Arg-42/Gln-49 in SCIN-A and Arg-44/Tyr-51 in SCIN-B) as judged by published co-crystal structures of SCIN proteins bound to the C3 degradation product C3c (Garcia et al., 2010; Garcia et al., 2012b). When individual or pairwise mutations to these residues were made in N-terminally truncated SCIN proteins, C3b-binding activity was diminished or abolished altogether (Garcia et al., 2012b). This resulted in a corresponding loss of AP C3 convertase inhibitory properties in these SCIN mutants (Garcia et al., 2012b).

To investigate their relative contributions to wild-type protein function (which more accurately reflects the physiological situation), we introduced both individual and pairwise alanine mutations to these amino acid positions in full-length SCIN-A and SCIN-B. Whereas a 103–190-fold reduction in apparent affinity was observed for the SCIN-A double mutant ($K_D^{\text{Alpha}} = 14 \mu\text{M}$, $K_D^{\text{SPR,ss}} = 15 \mu\text{M}$) when compared to its wild-type counterpart ($K_D^{\text{Alpha}} = 74 \text{ nM}$, $K_D^{\text{SPR,ss}} = 145 \text{ nM}$), only a 6.4 to 11-fold loss was observed for the SCIN-B double mutant ($K_D^{\text{Alpha}} = 3.5 \mu\text{M}$, $K_D^{\text{SPR,ss}} = 2.5 \mu\text{M}$) when compared to SCIN-B ($K_D^{\text{Alpha}} = 310 \text{ nM}$, $K_D^{\text{SPR,ss}} = 390 \text{ nM}$) (Fig. 1 A–D and Table 1). Curiously, mutation of SCIN-A Arg-42 alone yielded a protein with a similar binding profile ($K_D^{\text{Alpha}} = 14 \mu\text{M}$, $K_D^{\text{SPR,ss}} = 8.5 \mu\text{M}$) to that found for the SCIN-A double mutant, which suggested that SCIN-A Gln-49 provides only a very minor contribution to the SCIN-A/C3b interaction. This is supported by data obtained for the SCIN-A Gln-49 single mutant which binds C3b with affinity very near wild-type ($K_D^{\text{Alpha}} = 120 \text{ nM}$, $K_D^{\text{SPR,ss}} = 220 \text{ nM}$). This stood in contrast to the situation for SCIN-B, where both Arg-44 ($K_D^{\text{Alpha}} = 1.9 \mu\text{M}$, $K_D^{\text{SPR,ss}} = 2.5 \mu\text{M}$) and Tyr-49 ($K_D^{\text{Alpha}} = 900 \text{ nM}$, $K_D^{\text{SPR,ss}} = 810 \text{ nM}$) clearly contributed to C3b binding even though loss of Arg-44 was slightly more detrimental (Fig. 1 A–D, Table 1). Despite these differences, mutations to these residues consistently resulted in less potent inhibition of AP-mediated hemolytic activity by both SCIN-A and SCIN-B (Fig. 1 E and F). Taken together, these studies revealed that the conserved position represented by SCIN-A Arg-42 and the homologous SCIN-B Arg-44 fulfills the most dominant role in both C3b binding and complement inhibitory function in full-length, wild-type SCIN proteins.

3.2. Identification of SCIN-competitive peptides through randomized phage displacement biopanning

The SCIN-A and SCIN-B binding site on C3b is also shared in part by fB and fH (Garcia et al., 2010; Garcia et al., 2012b). Thus, this site constitutes a functional hotspot that serves an essential role in regulating C3b stability as well as formation and dynamics of the AP C3 convertase. Although there are conserved determinants between all SCINs that allow for recognition of this site (e.g. the arginine residue described above), we also noted important differences in both the sequence and nature of the interactions present at the SCIN-A

(Garcia et al., 2010) and SCIN-B (Garcia et al., 2012b) interfaces. This raised the possibility that additional sequences, perhaps not related to SCINs, might also be able to recognize this functional hotspot and thereby retain complement inhibitory activities.

To investigate this directly, we designed and carried-out a phage-display screen to identify 12-mer peptides whose interaction with C3b could be disrupted by saturating concentrations of SCIN-A. A library consisting of approximately 10^9 individual peptides fused to the amino terminus of M13-phage minor coat protein pIII was incubated with biotinylated human C3b (Garcia et al., 2012b), washed several times under increasing stringency, and specifically bound phage were eluted through the addition of 100 μ M SCIN-A. Following three rounds of panning and enrichment, the recovered phage were amplified and their variable insert regions were sequenced. Seven unique sequences were obtained from a total of 19 independent phage clones, and were designated 12.1–12.7, respectively. Of these, peptides 12.1, 12.2, and 12.3 were the most highly represented, and were encoded by 11 of the 19 (~58%) individual sequences (Fig. 2A).

When considered as a whole, the peptide sequences identified had little in common with one another. The lone exception to this was that peptides 12.1 and 12.6 share the sequence Pro-His-Ser-Asn-Arg-Lys-Lys at their respective amino termini. Multiple sequence alignment analysis, however, uncovered interesting relationships between a subset of the peptides and SCIN-A, and also with the human complement protein fH. In total three of the seven peptides could be aligned to regions of SCIN-A with varying degrees of conservation (Fig. S1A). 12.1 and to a stronger extent 12.6, align to the N-terminal region of SCIN-A, while peptide 12.2 aligns to a region corresponding to the 2nd α -helix and primary C3b-binding site of SCIN-A (Fig. S1A). No significant homology to SCINs could be found for peptides 12.3, 12.4, 12.5, or 12.7, however, the C-terminal residues of 12.3 are homologous to a region of the 14th Sushi domain of fH (Fig. S1B). The percentage of hydrophobic residues in each sequence varied widely from a low of 0 in 12.1 to a high of 58% (7/12) in peptide 12.2. Significant differences were also observed in terms of the representation of the various amino acids. For example, there was a nearly uniform bias against acidic residues (only 3 instances in 84 positions total), and Cys, Gly, and Ile were not found in any of the peptides. On the other hand, there was a notable presence of basic residues (23 instances in 84 positions total). This corresponded well with the binding studies described above (Fig. 1 and Table 1) and structural analyses of SCIN complexes with C3c, both of which indicated that inclusion of positive charge on the α 2 helix is a fundamental feature that drives SCIN binding to C3c/C3b (Garcia et al., 2012b) (Fig. 2B).

3.3. SCIN-competitive peptides are unstructured, but reversibly bind C3b

The peptides described above were discovered based on the ability of SCIN-A to displace them from the surface of C3b. While this strongly suggested that these peptides' binding sites must at least partly overlap with SCINs, their significant sequence diversity and unknown structural properties raised questions regarding the nature of their interactions with C3b. Furthermore, it was unclear whether these peptides maintained any of the AP inhibitory attributes of the SCIN proteins themselves (Garcia et al., 2012b; Jongerius et al., 2010; Ricklin et al., 2009; Rooijakkers et al., 2005; Rooijakkers et al., 2007). In light of

these considerations, we used synthetic peptides to characterize the structure, C3b-binding properties and functional attributes of each representative sequence identified in greater detail.

An initial evaluation of peptide secondary structure was performed using CD spectropolarimetry (Fig. 3A). These data indicate that these peptides lack the α -helical structure which is a hallmark of full-length SCIN proteins. To further evaluate the conformations of the peptides in solution, we next acquired 1D ^1H spectra of all 7 peptides (Fig. 3B). While no attempt was made to assign the peaks, the chemical shift values of H_α are highly dependent on secondary structure and their distributions still reveal the overall secondary structures of the peptides (Wang and Jardetzky, 2002; Wishart et al., 1991). Using the statistical average chemical shift values of a certain residue in either α -helical, β -strand or random coil structure, we estimated the H_α chemical shift distributions of the peptides in these conformations. These estimates appear as horizontal bars of different colors in Fig. 3B. Almost no peaks were observed at the estimated β -strand region. The H_α peaks of all peptides mostly fell into the random coil region, although some peaks were found within the estimated α -helical region. It is worth noting that the chemical shift values of some side chain protons such as Pro H_δ , Thr H_β , Ser H_β , His H_β , Arg H_δ , as well as some aromatic H_β s also reside in the 3.5 – 4.2 ppm region. While we could not completely rule out residual helical structure in some of the peptides without more detailed analyses, these spectra were consistent with largely unfolded conformations for the unbound form of these peptides in solution. Thus, while SCIN proteins bind C3b largely through the contribution of residues found on their second α -helix, the phage-derived peptides we identified based on their ability to compete with SCINs for C3b binding appear to be unstructured in the absence of ligand.

One limitation of unstructured peptide inhibitors is that their apparent affinities are often relatively weak when compared to folded protein ligands. This low affinity binding arises from the entropic penalty of adopting an ordered structure in the presence of ligand when no such conformational restriction exists in the unbound state. To obtain insight into the C3b-binding affinities of the peptides identified above, we initially used an AlphaAssay where a label-free ligand displaces the interaction between epitope-tagged SCIN-A and biotinylated C3b in a dose-dependent manner (Garcia et al., 2012b). Aside from peptide 12.4 (which could not be dissolved in buffer alone to concentrations great enough to obtain data at the high end of the range examined), each of the peptides displayed saturable binding to C3b (Fig. 4A). All peptides exhibited similar apparent affinities, and displayed K_D values in the 0.2–1.0 mM range; the lone exception was peptide 12.2, however, which bound more tightly to C3b with an apparent K_D of approximately 50 μM .

Given the relatively low affinities we observed in the AlphaAssay, we decided to use SPR to provide complementary information on these peptides' C3b-binding properties. To this end, site specifically-biotinylated C3b was immobilized on a streptavidin derivatized sensor chip and each peptide was injected in triplicate at a fixed concentration of 1 mM. The reference-subtracted response for each peptide was averaged and corrected for its molecular weight to arrive at an indicator of direct binding ranking under these experimental conditions (Fig. 4B). Consistent with the previous AlphaAssay studies, peptide 12.2 bound C3b most tightly.

Despite these minor differences, both methods demonstrated that the peptides bind C3b specifically and reversibly, and compete for the SCIN binding site, but do so with relatively weak apparent affinities.

3.4. SCIN-competitive peptides inhibit activity of the alternative complement pathway by impairing the rate of AP C3 convertase formation

The SCIN-binding site on C3b is shared with several other proteins, including fB and fH, which play crucial roles in defining the activity of the AP. Thus, we wondered if any of the C3b-binding peptides identified above were capable of affecting AP function similarly to SCINs. To test this hypothesis, we utilized an ELISA-based method to detect the level of C3b deposition following activation of normal human serum by *Salmonella* lipopolysaccharide (LPS), either in the absence or presence of a fixed concentration of synthetic peptide. Significant inhibition of the AP was observed for peptides 12.2, 12.4, 12.5, and 12.7 when used at a fixed concentration of 500 μ M (Fig. 5A). Of these, peptides 12.2 and 12.4 inhibited the AP by nearly 75% and 50%, respectively, while peptides 12.5 and 12.7 displayed inhibitory activities of less than 30% under these assay conditions. When a modified AP hemolytic assay (APH50) was employed, however, only peptide 12.2 and 12.7 significantly protected rabbit erythrocytes (Er) from AP-mediated lysis (Fig. 5B and C). Interestingly, when 12.2 and 12.7 were mixed a synergistic effect is observed suggesting the C3b-binding site for each peptide is non-identical (Fig. 5C). To understand if the inhibitory activities of the peptides were specific for the AP we performed both ELISA-based and hemolytic assays under conditions selective for CP activation (Fig. S2). Curiously, all peptides except 12.7 had some inhibitory effect in the ELISA assay, however, no protective effect could be measured for any peptide in the CP hemolytic assay. Taken together these results suggest that peptide inhibition is selective for the AP with 12.2 and 12.7 possessing the strongest inhibitory activities

Though SCINs primary mode of AP inhibition arises from their ability to stabilize the AP C3 convertase in an inactive state, they also inhibit the rate of AP C3 convertase formation, albeit to a lesser extent (Ricklin et al., 2009). Since it seemed unlikely that 12-mer peptides could recapitulate the long-range contacts necessary to stabilize and inactivate the AP C3 convertase through either higher-order oligomerization or steric hindrance of convertase access to its C3 substrate as is the case for SCIN-A (Rooijakkers et al., 2009), we hypothesized that the AP inhibitory peptides identified here may instead act by disrupting convertase formation. To test this prediction directly, we employed an SPR-based method whereby the rate of AP C3 convertase formation on site specifically-immobilized C3b was monitored in real time, either in the presence or absence of 1 mM peptide (Garcia et al., 2013; Ricklin et al., 2009). In this assay, six of the seven peptides significantly inhibited AP C3 convertase formation *in vitro* when compared to an unrelated control peptide (Fig. 6 and Fig. S3). Among those peptides which displayed such activity, the magnitude of inhibition ranged from a low of 5% for peptide 12.6 to a high of 15% for peptide 12.4 (Fig. 6I). Furthermore, three of the four peptides that inhibited AP C3 convertase formation (12.4, 12.5, and 12.7) also significantly inhibited deposition of C3b via the AP (Fig. 5A).

The ability of SCINs to impair AP C3 convertase formation arises from the fact that their binding site partly overlaps with fB (Garcia et al., 2010; Garcia et al., 2012b; Ricklin et al., 2009). Thus, it seemed plausible that the peptides which recognize this same site on C3b might likewise compete with fB for C3b binding. We examined this possibility through an SPR-based method where the ability of fB to bind C3b was monitored, either in the presence or absence of 1 mM peptide. In this assay, six of the seven peptides diminished fB binding when co-injected at 1 mM final concentration as compared to an unrelated control peptide (Fig. 7). In contrast to the convertase formation assay described above (Fig. 5), the ability of these peptides to disrupt fB binding was relatively consistent and ranged between 10–15% of the total signal (Fig. 7I). Nevertheless, three peptides, namely 12.4, 12.5, and 12.7, impaired fB binding (Fig. 7I), diminished the rate of AP C3 convertase formation (Fig. 6I), and inhibited the activity of the AP, while of this group, 12.7 alone was also able to block AP activity in the hemolysis assays (Fig. 5B and C).

4. Discussion

The complement system has been identified as an aggravating factor in a wide range of local and systemic human inflammatory diseases, including age-related macular degeneration, ischemia and reperfusion injury, as well as various hematological disorders such as atypical Hemolytic Uremic Syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH), to name only a few (Ricklin and Lambris, 2013a). Most of these inflammatory diseases have few viable treatment options, and thus there has been considerable interest in developing complement targeted therapeutics toward providing a tangible clinical benefit to those affected. The last decade has witnessed a great acceleration in the pace of research in this area, with two biologics (eculizumab and C1-inhibitor) having received regulatory approval for human use in the US and several others on the way (Ricklin and Lambris, 2013b). Despite these successes, the number of available drugs to target the complement system is comparatively small, while the list of potential indications continues to expand.

We believe that a detailed structure/function understanding of staphylococcal complement inhibitor proteins may provide crucial information to facilitate development of novel complement-targeted therapeutics. Among these, the SCIN family of proteins have been perhaps the most extensively studied at both the structural (Garcia et al., 2010; Garcia et al., 2012b; Rooijackers et al., 2009; Rooijackers et al., 2007) and mechanistic levels (Garcia et al., 2013; Jongerius et al., 2007; Ricklin et al., 2009; Rooijackers et al., 2005). Nevertheless, one issue that remained prior to the present study was assignment of functional contribution to the various C3b-contacting residues in full-length SCIN-A and SCIN-B. Although they displayed a significant reduction in affinity, we found here that the double mutants of both full-length SCIN-A and SCIN-B still retained a measure of C3b binding and inhibitory activity (Fig. 1 **and** Table 1). This result was somewhat unexpected, as the identical double mutants completely ablate C3b binding and AP C3 convertase inhibition in N-terminally truncated forms of SCIN-A and SCIN-B (Garcia et al., 2012b). Additionally, the Arg residue found at the equivalent position 42 in SCIN-A and 44 in SCIN-B did not seem to be equally important in these two proteins, despite its conservation (Fig. 1 **and** Table 1). We believe there are at least three potential reasons for this observation. First, it could simply be that other residues at the SCIN-B/C3b interface make subtle, yet important contributions to

ligand binding for which standard quantitative metrics such as buried surface area and hydrogen bonds do not account. Second, and similarly, it is possible that C3b-binding by SCIN-B is more complicated than its co-crystal initially suggested, and that processes such as protein dynamics that are difficult to assess by crystallography alone might be playing a role. Finally, it could be that N-terminal residues in full-length SCIN-B can somehow compensate for loss-of-function at the main C3b binding site (Garcia et al., 2010; Garcia et al., 2012b). In this regard, we have previously described structural plasticity within the N-terminal region of SCINs, and we and others have shown that this region of the protein makes important contributions to SCINs function (Garcia et al., 2013; Jongerius et al., 2010). This raises the possibility that some level of internal functional redundancy vis-à-vis C3b binding and complement inhibition might be an important attribute of SCIN proteins.

While the SCIN proteins are indeed potent complement inhibitors, they are also highly antigenic and have been shown to elicit strong antibody responses in even healthy volunteers (Jongerius et al., 2007). Because of this, SCIN proteins themselves cannot be used directly as complement-targeted therapeutics. Still, the fact that the SCIN binding site on C3b represents a functional and inhibitory hotspot within this central complement component suggests that smaller molecules which target this site could likewise manifest complement inhibitory properties. In the current study, we investigated this possibility by conducting a site-specific phage display screen to identify 12-mer peptides that compete with SCIN-A for C3b binding. Seven distinct peptides were identified and characterized in terms of their structure, C3b binding affinity, and effects on the AP and CP. Four of the seven peptides inhibited C3b deposition via the AP (Fig. 5A), and three of these (12.3, 12.5, and 12.7) partially blocked formation of the AP C3 convertase (Fig. 6). Despite this, only peptides 12.2 and 12.7 inhibited hemolysis in the APH50 assays (Fig. 5B and C). Importantly, neither 12.2 nor 12.7 elicit an inhibitory effect in hemolytic assays for the CP (Fig. S2B). Thus, these two peptides appear to selectively inhibit the AP, which is a property consistent with the activity of full length SCIN molecules (Ricklin et al., 2009).

Interestingly, these two peptides appear to be mechanistically divergent. While 12.7 likely acts by inhibiting fB binding to C3b (Fig. 7), 12.2 has no effect on fB/C3b binding or the formation of AP C3 convertases or (Fig. 6 and 7). Furthermore, when a mixture of 12.2 and 12.7 are used in an APH50 assay, a greater inhibitory effect can be measured (Fig. 5C). This synergism is consistent with the idea that the mode of inhibition by 12.2 and 12.7 are at least in part different. To the best of our knowledge, these peptides are the first small molecules that retain desirable functional activities of a larger staphylococcal immune evasion protein. They therefore represent a valuable proof of concept that small-molecule targeting of a critical site which has been evolutionarily selected by millennia of host/pathogen interaction is practical, and one that merits further investigation.

Not all of the peptides identified in this way yielded readily interpretable functional data, however. For example, peptide 12.4 significantly affects C3b deposition via both the AP and CP in the ELISA assays, has no effect on hemolysis via CP, and yet appears to enhance hemolysis in an APH50 assay. While the reasons for these apparent discrepancies are unclear at the present time, we offer three potential explanations. First, and most simply, it is unlikely that any linear peptide could make the precise types of intermolecular contacts

which recapitulate fully the behavior of a ~10 kDa α -helical bundle protein such as SCIN-A. Second, although all of the peptides identified here compete with SCINA for C3b binding, the structural basis for their interactions with C3b remain unknown. Because the SCIN-A binding site occupies greater than 800 Å² of C3b surface area, it is possible that the conformation of the C3b-bound peptide is not ideal to inhibit all of the events which culminate in activation/cleavage of C3 via the AP C3 convertase. Finally, while the C3b binding, convertase formation, and fB competition assays were carried out *in vitro* using highly purified complement proteins, while the AP/CP ELISA-based and hemolytic activity assays used normal human serum as a source of complement components. Thus the presence of additional proteins (e.g. proteases), whose actions could have either diminished or augmented the effects of these particular synthetic peptides, must be taken into consideration.

Perhaps the most enigmatic molecule we discovered in this study is peptide 12.2. This peptide bound C3b with the highest apparent affinity (Fig.4) and was the most potent inhibitor of the AP (Fig. 5), yet failed to block either convertase formation (Fig. 6) or fB binding to C3b (Fig. 7). The lack of these last two properties strongly suggests that the mechanism of 12.2 action is distinct from peptides 12.4, 12.5, and 12.7, which share key biochemical features with SCINs as described above. We reasoned that “SCIN-competitive” phage sequences may arise from: i) sequences related to SCINs themselves; ii) sequences related to C3b binding molecules that perturb the SCIN binding site (*i.e.* fB or fH); or iii) sequences that have no known relationship to C3b binding proteins but nonetheless bind C3b near the SCIN site. To determine if any of the peptide sequences may be related to host C3b binding molecules we initially looked sequence homology between the peptides and fB or fH. Although no homology could be detected between any of the peptides and fB, a BLASTp search revealed that peptide 12.3 aligns to a region of fH (Fig. S1B). A surprising and potentially important observation regarding 12.2 function was made when we extended this methodology to other known C3b-binding proteins. The 12.2 sequence shows a striking level of homology (42% identical and 75% similar) to the carboxy terminal-most thrombospondin-1 repeat (*i.e.* TSP6) of the endogenous complement factor, properdin (fP) (Fig. S4A). Intriguingly, a disease-associated properdin mutant has been previously described wherein the tyrosine residue shared with peptide 12.2 is changed to an aspartate (Fredrikson et al., 1996). While patients harboring this mutation produce normal levels of properdin, this variant fails to stabilize the AP C3 convertase and therefore does not stimulate AP activity as does the wild-type protein. Recent structural characterization of a properdin/convertase complex by electron microscopy suggests that the TSP6 region of properdin makes direct contact with the fully assembled convertase (Alcorlo et al., 2013). Thus, it seems plausible that C3b binding by this discrete region of properdin TSP6 might be competitively inhibited by peptide 12.2.

To understand if 12.2 was in fact acting as a competitive inhibitor of fP we conducted an AP hemolytic assay using fP-depleted serum (Fig. S4B). We noted similarity in cell lysis apparent for fP-depleted (fP-dpl) serum with no peptide and that seen when 12.2 is incubated in NHS at 5% (v/v) serum. However, 12.2 inhibitory activity is overall significantly enhanced in fP-dpl serum and this peptide is nearly completely inhibitory in

5% (v/v) fP-dpl serum. We conclude from these experiments that although clear effects are measured in fP-dpl serum when 12.2 is used, there must also be properdin-independent contribution to 12.2 inhibition. These experiments raise the interesting possibility that full-length SCIN proteins have a previously uncharacterized functional interdependence with properdin. In any case, the relatively strong binding and activity profile of 12.2 makes it an attractive target for further investigation.

When considering this study as a whole, it is clear that the greatest limitation to further development of these peptides is their low apparent affinity. Indeed, characterization of these molecules by NMR spectroscopy strongly suggested that they are unstructured in the absence of ligand, and likely subject to a significant entropic penalty when binding C3b. Any future work on optimization of the most promising candidates (e.g. 12.2 and/or 12.7) should explore synthetic approaches such as cyclization, which greatly restricts conformational flexibility of peptides and thereby mitigates entropic cost. Alternatively, it may be possible to enhance the apparent affinity of these linear molecules through oligomerization in the form of a peptide dendrimer. Coupling multiple copies of a low affinity ligand into a single structure would be expected to increase apparent affinity by taking advantage of avidity effects. It is worth noting that the phage particles which harbored each of these peptides during their initial discovery were polyvalent, and contained five copies of each peptide in the capsid assembly (Russel et al., 1997); thus, there is no reason to expect oligomerization to preclude C3b binding, or activity, *a priori*. While their affinities are weak in their current state, tractable strategies to address this important limitation do exist. In the long term, this may provide a path for taking advantage of these peptides' valuable functional properties. Underscoring the validity of this approach, it is worth noting that the peptidic complement-directed therapeutic Compstatin, was originally discovered using phage display methodology (Sahu et al., 1996). Nearly two decades of intensive optimization efforts has resulted in identification of high affinity Compstatin derivatives that are considered to be among the most promising candidate drugs for treating a wide range of complement-mediated diseases (Ricklin and Lambris, 2008). Unlike Compstatin that acts at the level of C3, however, drugs based on SCINs are expected to specifically target the AP C3 convertase. This is a particularly important distinction in light of the diverse pathological mechanisms that underlie many complement diseases. Indeed current thought within the field has suggested that a 'one size fits all' approach is unlikely to be either practical or successful when it comes to therapeutic complement inhibition (Ricklin and Lambris, 2013b). As a consequence, the need to discover novel complement inhibitory strategies remains great.

5. Conclusions

The number of complement-mediated immune and inflammatory disorders where therapeutic intervention could provide benefit continues to grow (Ricklin and Lambris, 2013a). Traditional approaches based on large proteins have been limited by expensive production costs or issues related to immunogenicity of the molecules (Thurman, 2014). The complement cascade itself is predicated upon interactions that result in large multi-protein complexes encompassing enormous protein-protein contact areas. Because of this, it was initially speculated that small molecules would be unlikely to successfully disrupt these

interactions. However, recent advances for small molecule inhibitors such as Compstatin have made a striking case for the therapeutic value of small anti-complement drugs (Ricklin and Lambris, 2008; Ricklin and Lambris, 2013b). This approach carries with it the critical benefit of tractable large-scale production that in the case of Compstatin could yield costs as low as several dollars per dose (Risitano et al., 2014). In the same light, evidence that targeting relatively small areas of key complement components can result in potent inhibition can be found through the study of the human pathogen *S. aureus*. Advances in the structural and mechanistic details of complement inhibition by *S. aureus* proteins, in particular the SCIN family, have provided insight into how these small proteins are able to act as anti-complement molecules. A significant challenge remains to translate this knowledge into compounds suitable for clinical intervention. Identification of small peptides that retain SCIN inhibitory properties as described here represents a step forward in our ability to exploit these evolutionarily optimized bacterial inhibitors for the purpose of developing new complement-directed therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	alternative pathway
CP	classical pathway
fB	factor B
fD	factor D
fI	factor I
LP	lectin pathway
MAC	membrane attack complex
NMR	nuclear magnetic resonance
RCA	regulator of complement activity
SCIN	Staphylococcal complement inhibitor
SPR	surface plasmon resonance

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Highlights

- A conserved arginine residue is required for maximal C3b binding by SCIN proteins
- A site-specific phage display screen identified seven peptides that share features of SCINs
- A subset of these peptides inhibit alternative complement pathway activity in functional assays
- These are the first small molecules that retain inhibitory activities of a staphylococcal immune evasion protein

Fig. 1. A conserved Arg residue contributes significantly to C3b binding in full length SCIN proteins

A and B, AlphaScreen competition assay for SCIN-A and SCIN-B single and double mutants. *C and D*, Dose-response curves obtained from SPR experiments were conducted by injecting various concentrations of full-length SCIN proteins over site-specifically immobilized C3b. Steady-state affinity analysis ($K_{D,ss}$) was performed by analyzing the response just before injection stop (R_{max}). Kinetic analysis was conducted in parallel and values for dissociation constants ($K_{D,kin}$) and rate constants (k_{on}/k_{off}) are reported in Table 1. SCIN-A and SCIN-B double mutants show significantly reduced affinity for C3b. Single mutants reveal that a single conserved arginine position in full-length SCIN-A proteins is important for direct interaction with C3b. In contrast, SCIN-B single mutants indicate that while R44 is critical for C3b interaction, Y51 also contributes significantly to C3b-binding. *E and F*, A dose-response curve for the inhibition of Er cell hemolysis under conditions that promote AP activation. All SCIN-A and SCIN-B proteins containing a R42/R44 mutation position are less potent inhibitors. In contrast, SCIN-B.Y51A shows impaired inhibitory

properties unlike the homologous SCIN-A.Q49A mutant that inhibits identically to wild-type SCIN-A.

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Fig. 2. Sequence identification and characterization of peptides identified by a SCIN-based randomized phage displacement biopanning strategy

A, Identity of the seven unique sequences following three rounds of panning using an experimental protocol designed for enrichment of SCIN-competitive sequences. All peptides had basic character but were otherwise dissimilar with the exception of 12.1 and 12.6 exhibiting seven identical N-terminal residues. **B**, A fundamental feature of C3b recognition by SCIN proteins is the presence of a positively charged surface corresponding to the 2nd α -helix on SCIN. This compares favorably to the overwhelmingly basic nature of the

identified peptides (23 basic residues vs. 3 acidic residues in a total of 84 positions). Electrostatic surface potentials were calculated for SCIN-A (PDBID: 3L30, chain M) and SCIN-B (PDBID: 3T4A, chain G) using the Adaptive Poisson-Boltzmann Solver (APBS) software package (Baker et al., 2001). Figures were generated using PyMOL (Version 1.2r3pre, Schrödinger, LLC.)

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Fig. 3. Secondary structure analysis of peptides in solution

A, Far-UV CD spectra for SCIN-A and all peptides. **B**, Overlay of 1D proton NMR spectra of the peptides. Estimated chemical shift ranges of H α in different secondary structures are indicated in colored horizontal bars for each peptide (α -helical: green; β -strand: blue; random coil: black).

A AlphaAssay: C3b/SCIN-A competition w/ SCIN-like Peptides

B SPR: Phage peptide/C3b-binding 1 mM ranking

Fig 4. C3b/peptide binding assays

A, AlphaAssay microbead technology was used in a competition format where the ability of each peptide to compete with SCIN-A for C3b binding was assessed. Apparent affinities for each peptide were in the $K_D = 0.2\text{--}1.0$ mM range with the exception of 12.2 which bound slightly more tightly ($K_D = 50$ μM). B, Direct binding of peptides was measured using SPR by injecting 1 mM of each peptide over site-specifically immobilized C3b. A control peptide exhibits minimal response at this concentration. Signals were corrected for the molecular weight of each species.

Fig. 5. Inhibition of AP by peptides

The ability of the phage derived peptides to inhibit complement via the AP was assessed using an ELISA-based assay. 20% (v/v) normal human serum was incubated in wells previously coated with *Salmonella enteritidis* LPS in a buffer selective for AP activation (20mM HEPES (pH 7.5), 140 mM NaCl, 0.1% (w/v) gelatin, 5 mM MgCl₂ and 10mM EGTA). 1 μ M SCIN-A was used as a positive control. *B*, The ability of the peptides to prevent AP-mediated hemolysis of Er was assessed using a fixed concentration of peptide (500 μ M) and NHS (6.5% (v/v)). Data is presented as the amount of lysis in the presence of

peptide compared to NHS. C, The ability of peptides 12.2 and 12.7 (500 μ M either alone, or mixed) to inhibit Er lysis across a range of concentrations was measured. Measures of statistical significance were determined by an unpaired student *t*-test of each experimental series versus buffer control and compared to values obtained for an unrelated negative control peptide. **** $p < 0.001$, * $p < 0.05$, † $p = 0.07$.

Fig. 6. AP convertase formation measured by SPR

The ability of peptides to disrupt the formation of the AP C3 convertase was monitored in real time using an SPR based assay. A–H, C3bBb was formed on the surface of a site-specifically immobilized C3b biosensor by injecting an equimolar (100 nM) mixture of fB and fD. Following regeneration to baseline, a mixture containing fB, fD, and 1 mM peptide was injected over the surface. To ensure proper surface regeneration and integrity of the biosensor a duplicate injection of fB and fD alone was then performed. An injection of 1mM peptide alone was also carried out. Each injection series were performed in at least

duplicate. *I*, The average response observed for a 10 s window surrounding 2 min following injection stop of the fB/fD injections alone were averaged and considered 100% AP C3 convertase formation. Values of the identical time points for injections including peptides were also averaged and the percent inhibition of AP C3 convertase formation was calculated.

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Fig. 7. Competition of fB/C3b binding by peptides

The ability of peptides to compete for the fB binding site on C3b was measured by SPR. A–H, 150 nM fB was injected alone or in the presence of 1 mM peptide. The response of a 1 mM peptide alone injection was subtracted from the fB/peptide response. All injections were performed in triplicate. I, Signal just prior to injection stop for the fB alone injection was considered as 100% response and was compared to the corrected signal for the fB/peptide injection to calculate percent competition.

Table 1

C3b-binding Parameters for SCIN-A and SCIN-B Mutants

Competitor	K_D^{Alpha} (nM) ^a	95% CI K_D^{Alpha} (nM) ^a	$K_D^{SPR,ss}$ (nM) ^a	$K_D^{SPR,kin}$ (nM) ^a	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)
SCIN-A	74 ^b	48 to 110	145 ± 56	96 ± 16	(5.5 ± 0.15) × 10 ⁵	(5.1 ± 0.52) × 10 ⁻²
SCIN-A.R42A	14,000	7,100 to 27,000	8,500 ± 120	6,600 ± 620	(8.9 ± 0.15) × 10 ⁴	(5.8 ± 0.45) × 10 ⁻¹
SCIN-A.Q49A	120	78 to 180	220 ± 98	69 ± 8.3	(7.6 ± 2.0) × 10 ⁵	(5.1 ± 0.73) × 10 ⁻²
SCIN-A.R42A.Q49A	14,000	9,100 to 21,000	15,000 ± 2,500	NA	NA	NA
SCIN-B	310 ^b	200 to 470	390 ± 95	202 ± 27	(5.2 ± 0.62) × 10 ⁵	(1.0 ± 0.13) × 10 ⁻²
SCIN-B.R44A	1,900	1,200 to 3,200	2,500 ± 48	2,600 ± 880	(1.4 ± 0.33) × 10 ⁵	(3.5 ± 0.40) × 10 ⁻¹
SCIN-B.Y51A	900	620 to 1,300	1,000 ± 640	810 ± 310	(2.8 ± 1.9) × 10 ⁵	(2.0 ± 0.67) × 10 ⁻¹
SCIN-B.R44A.Y51A	3,500	2,400 to 5,100	2,500 ± 810	5,100 ± 740	(1.1 ± 0.43) × 10 ⁵	(5.1 ± 0.14) × 10 ⁻¹

^a K_D^{Alpha} refers to apparent affinity obtained from Alpha competition assays, $K_D^{SPR,ss}$ refers to affinity obtained from steady-state analysis of SPR data, and $K_D^{SPR,kin}$ is the affinity obtained from kinetic analysis of the SPR experiments.

^b AlphaAssay data are presented for reference and have been published previously (Garcia et al., 2013).

^c Parameters for data analysis and curve fitting are found in Experimental Procedures.