Molecular Immunology 67 (2015) 193–205

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# Identification of peptidic inhibitors of the alternative complement pathway based on *Staphylococcus aureus* SCIN proteins

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ARTICLE INFO

*Article history:*
Received 4 February 2015
Received in revised form 11 May 2015
Accepted 13 May 2015
Available online 4 June 2015

*Keywords:*
Complement
Inhibitors
Alternative pathway C3 convertase
Peptides
Surface plasmon resonance

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.


## 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 toward 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

*Abbreviations:* AP, alternative pathway; CP, classical pathway; fB, factor B; fD, factor D; fI, factor I; LP, lectin pathway; MA, membrane attack complex; NMR, nuclear magnetic resonance; RCA, regulator of complement activation; SCIN, staphylococcal complement inhibitor; SPR, surface plasmon resonance.

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http://dx.doi.org/10.1016/j.molimm.2015.05.012
0161-5890/

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 et al., 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 C3b 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 (Jongerius et al., 2007; Rooijakkers et al., 2005). Although SCIN function is multi-faceted, a hallmark of SCIN activity is stabilization of an inactive form of the AP C3 convertase via the formation of a ternary C3bBb/SCIN complex (Jongerius 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., 2013, 2012b, 2010; Jongerius et al., 2010; Rooijakkers et al., 2009, 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 CRIg and CR1, both of which are found on the surface of phagocytic cells (Garcia et al., 2013; Jongerius 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 (Jongerius 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. >5500 $Å^2$ across 6 fH domains) (Kajander et al., 2011; Wu et al., 2009), the primary C3b-binding site for SCINs buries less than 800 $Å^2$ (Garcia et al., 2010, 2012b). This feature of a relatively small target area on C3b combined with the importance of the functional "hot-spot" 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 *S. 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 20 mM HEPES (7.3), 150 mM NaCl (HBS), passed through a 0.45 μM 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 to C3b was 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 μL $min^{-1}$ 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