Molecular Immunology 50 (2012) 253–261

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

Complement regulator C4BP binds to *Staphylococcus aureus* and decreases opsonization

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form 24 January 2012
Accepted 26 January 2012
Available online 12 February 2012

Keywords:
Staphylococcus aureus
Complement
C4b-binding protein
Classical pathway
C3-convertase

ABSTRACT

Staphylococcus aureus is the major cause of human skin and soft-tissue infections as well as invasive infections like post-operative wound infections, septic arthritis, and osteomyelitis. The complement system plays an important role in the immunological control of many bacteria, but can be inhibited by a variety of strategies including recruitment of complement regulatory proteins like C4b-binding protein (C4BP). These experiments demonstrate that *S. aureus* opsonization with C4b occurs rapidly in serum and is predominantly initiated by anti-staphylococcal antibodies. Much of the *S. aureus*-bound C4b is quickly cleaved to the inactive forms iC4b and C4d. Clinical *S. aureus* strains rapidly bind significant amounts of the complement regulator C4BP from serum. *S. aureus* also binds purified C4BP. *S. aureus*-bound C4BP functions as a cofactor for factor I-mediated C4b cleavage to iC4b and C4d. In the absence of factor I, C4BP decreases classical pathway-mediated deposition of C3b on the *S. aureus* surface by inhibiting the classical pathway C3-convertase. In summary, C4BP is recruited to the *S. aureus* surface where it functions to inhibit C4 complement effectors, suggesting a previously undescribed immune evasion strategy for this pathogen.

1. Introduction

Staphylococcus aureus is the major cause of skin and soft-tissue infections (Dryden, 2009) as well as post-operative infections (Fry and Barie, 2011), osteomyelitis (Calhoun et al., 2009), and septic arthritis (Young et al., 2011). Antibiotic resistance among clinical *S. aureus* isolates continues to grow including hospital- and community associated MRSA (Gould et al., 2010), as well as isolates with decreased susceptibility to vancomycin (van Hal and Paterson, 2011). In this increasingly challenging setting, understanding the mechanisms *S. aureus* employs to escape immunological control is imperative to the future development of prevention and treatment strategies.

To date, investigations have highlighted the roles of secreted *S. aureus* proteins Efb and SCIN in preventing C3b deposition on the staphylococcal surface (Jongerius et al., 2007; Rooijakkers et al., 2005, 2006; van Wamel et al., 2006). However, the role of *S. aureus* cell-wall components in modulating complement responses on the bacterial surface is beginning to be elucidated. Understanding humoral immune interactions at the bacterial surface is critical because that is where opsonins bind and complement activation occurs. Previous work from our laboratory has identified *S. aureus* surface-expressed clumping factor A (ClfA) binding the serum complement regulator factor I enhancing factor I cleavage of C3b on the bacterial surface with resultant inhibition of phagocytosis (Hair et al., 2008, 2010). More recent work from our laboratory has shown that *S. aureus* efficiently binds serum complement regulator factor H resulting in displacement of Bb and degradation of the alternative pathway C3-convertase (Sharp and Cunnion, 2011). Of the three major serum complement regulators, factor H, factor I, and C4b binding protein (C4BP), only the later has yet to be evaluated in terms of a potential functional role on the *S. aureus* surface.

Antibody binding to a microbial surface initiates classical pathway activation via C1 which then activates C4 leading to C4b binding covalently to the microbe. C4b is an opsonin and also activates C2 yielding the classical pathway C3-convertase, C4bC2a.

Abbreviations: NHS, normal human serum; C4BP, C4b-binding protein; MRSA, methicillin-resistant *Staphylococcus aureus*; GVBS, gelatin veronal buffered saline; CVF, cobra venom factor.

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doi:10.1016/j.molimm.2012.01.010

C4bC2a will then activate C3 leading to binding of the opsonin C3b and then generation of C5-convertases yielding the potent anaphylatoxin C5a. C4BP is a serum complement regulator that can work in concert with factor I to cleave C4b to iC4b and then C4d, neither of which have known immunological functions (Fujita et al., 1978). Additionally, C4BP can displace C2a degrading the convertase complex and inhibiting the formation of new convertase complexes (Daha and van Es, 1980; Gigli et al., 1979). The unique structural aspects of this large (500 kDa) spider-like multimeric protein are discussed elsewhere (Blom et al., 2004).

Multiple pathogenic bacteria have been shown to acquire C4BP to their surface with resulting inhibition of complement-mediated effectors. Among Gram-positive bacterial pathogens, *Streptococcus pyogenes* binds C4BP via the surface-expressed M protein (Andre et al., 2006; Jenkins et al., 2006) and *Streptococcus pneumoniae* binds C4BP via PspC (Dieudonne-Vatran et al., 2009). Several pathogenic Gram-negative organisms bind C4BP including *Borrelia recurrentis* (Grosskinsky et al., 2010), *Borrelia burgdorferi* (Pietikainen et al., 2010), *Neisseria gonorrhoeae* (Blom and Ram, 2008), *Neisseria meningitidis* (Jarva et al., 2005), *Porphyromonas gingivalis* (Potempa et al., 2008), *Yersinia enterocolitica* (Kirjavainen et al., 2008), *Haemophilus influenzae* (Hallstrom et al., 2007), *Escherichia coli* (Wooster et al., 2006), *Moraxella catarrhalis* (Nordstrom et al., 2004), and *Bordetella pertussis* (Berggard et al., 2001). Leptospira spirochetes also bind C4BP (Barbosa et al., 2009).

Although we have previously reported classical complement pathway activation by *S. aureus* (Cunnion et al., 2001), to our knowledge, C4b opsonization of *S. aureus* has not been described in detail. We will also elucidate C4BP binding to the staphylococcal surface and evaluate the extent to which this mediates complement effectors.

2. Materials and methods

2.1. Bacteria and growth conditions

S. aureus strains used include the reference strains Reynolds and JL022, which is an isogenic capsule-deficient mutant of Reynolds described elsewhere (Portoles et al., 2001). Clinical methicillin-resistant MRSA isolates were obtained as deidentified discarded cultures from a hospital microbiology laboratory under an IRB-approved protocol (Eastern Virginia Medical School IRB protocol #06-04-WC-0040), as described elsewhere (Hair et al., 2010). The isolates were typed by pulse field gel electrophoresis and found to be non-identical. Unless otherwise noted, *S. aureus* cultures were grown to mid-logarithmic phase in Columbia 2% NaCl media at 37 °C, as described elsewhere (Cunnion et al., 2001).

2.2. Buffers

Binding experiments were performed with $GVBS^{++}$ buffer (VBS with 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 ml mM $MgCl_2$), or EDTA-$GVBS^{--}$ buffer (VBS with 0.1% gelatin and 0.01 M EDTA).

2.3. Human serum and purified complement proteins

Normal human serum (NHS) was prepared from the blood of four healthy donors and pooled, as previously described (Cunnion et al., 2004). Blood was obtained with consent under an IRB-approved protocol (Eastern Virginia Medical School IRB 02-06-EX-0216). Purified C1, purified C4, purified C4b, purified C4 binding protein (C4BP), purified factor I, and cobra venom factor (CVF) were obtained commercially (CompTech). C4d was generated by incubating 10 μg of C4b with 0.25 μg of factor I and 1.25 μg of C4BP. Serum depleted of anti-staphylococcal antibodies was accomplished via repeated adsorption in an ice-water bath, as previously described (Cunnion et al., 2001). Preserved complement activity was confirmed by CH50 assay. Purified pooled human IgG was obtained commercially (Gammagard; Baxter). C4BP-depleted serum was prepared by using monoclonal anti-C4BP (Quidel) antibody-coated cyanogen bromide activated Separose (Sigma–Aldrich). C4BP depletion was confirmed by quantitative C4BP Dot Blot. C4BP-depleted serum was repleted with purified C4BP (0.2 mg/ml), and purified C1q (0.07 mg/ml), to physiological conditions according to previously described methods (Potempa et al., 2008). Additionally we added purified C4 (0.4 mg/ml), to compensate for any potential depletion of C4 during adsorption.

2.4. Serum opsonization of S. aureus

Unless otherwise noted, mid-log or stationary phase *S. aureus* cultures were washed and their concentration adjusted to 1×10^9 bacteria per ml. For NHS or IgG-depleted serum experiments, 1×10^8 bacteria were added to the indicated amount of serum in $GVBS^{++}$ for the desired amount of time at 37 °C. For C3 and C4 measurements, opsonized bacteria were washed twice in $GVBS^{--}$, incubated in 25 mM methylamine for 1 h, and samples were then spun to collect the supernatant. For C4BP measurements, opsonized bacteria were washed, boiled in 2% SDS in 0.06 M Tris buffer for 5 min, and then spun to collect the supernatant. Supernatants were then analyzed as described below.

2.5. C4 detection

Methylamine supernatants of surface-bound C4 fragments were analyzed by Western blot analysis performed with a monoclonal anti-human C4d antibody (Quidel Corp.) and a horseradish peroxidase-labeled goat anti-mouse antibody (Sigma–Aldrich).

Total C4-fragments were measured by ELISA. Flat-bottom Immulon-2 plates were coated with 50 μl of a 1:5000 dilution of goat anti-human C4 (CompTech) in carbonate buffer overnight at 4 °C. Wells were washed with PBS-Tween and blocked with 3% bovine serum albumin (BSA) for 2 h before being incubated with the samples (1 h at room temperature) and washed again. The primary probe was a chicken polyclonal anti-C4 antibody (Abcam) used at 1:1000 for 1 h at room temperature, and the secondary probe was a horse radish peroxidase-labeled goat anti-chicken antibody (GenWay), also used at 1:1000 for 1 h at room temperature.

2.6. C4b binding protein (C4BP) detection

Surface-bound C4BP was collected by stripping cell pellets with 2% SDS in Tris buffer, boiling for 5 min, and collecting the supernatant. These samples were visualized by Western blot with a monoclonal anti-C4BP antibody (Quidel Corp.) and HRP-labeled goat anti-mouse antibody. Total C4PB was analyzed by a quantitative Dot Blot assay. Pure C4BP was titrated onto PVDF membrane, along with the samples, via a Dot Blot apparatus. The membrane was blocked with 3% BSA, probed with a monoclonal mouse anti-C4BP primary antibody, and then a HRP-labeled goat anti-mouse secondary antibody. Bound antibody was detected by enhanced chemiluminescence. Using the Quantity One software (Bio-Rad), grey scale values were assigned to the pure C4BP titration and the samples so that a linear regression could be used to quantify the amount of C4BP in each sample. Total protein stained SDS-PAGE gels were stained with SYPRO-Ruby (Invitrogen).

2.7. C4b cleavage and C3-fragment opsonization

C4b cleavage due to surface bound C4BP was performed by pre-incubating *S. aureus* with either pure C4BP (20 μg/ml) in $GVBS^{++}$ or 10% NHS in $GVBS^{--}$ for 30 min at 37 °C. Following washing the