Cutting Edge: Members of the *Staphylococcus aureus* Extracellular Fibrinogen-Binding Protein Family Inhibit the Interaction of C3d with Complement Receptor 2[1]

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Staphylococcus aureus expresses a highly diversified arsenal of immune evasion proteins, many of which target the complement system. The extracellular fibrinogen-binding protein (Efb) and the Efb homologous protein (Ehp) have previously been demonstrated to bind to C3 and inhibit complement activation and amplification. In this study we present the first evidence that Efb and Ehp are also capable of inhibiting the interaction of C3d with complement receptor 2 (CR2), which plays an important role in B cell activation and maturation. The C-terminal domain of Efb efficiently blocked this interaction both in surface plasmon resonance-based competition studies and cellular assays and prevented the CR2-mediated stimulation of B cells. Furthermore, analyses of the available structural data were consistent with a molecular mechanism that reflects both steric and electrostatic effects on the C3d-CR2 interaction. Our study therefore suggests that *S. aureus* may disrupt both the innate and adaptive immune responses with a single protein module. *The Journal of Immunology,* 2008, 181: 7463–7467.

The ability to evade attack of the immune system is a key determinant in the survival of microbial pathogens inside the human body. Although the expression of tailored evasion proteins is a common strategy of many bacteria, *Staphylococcus aureus* has developed a particularly versatile arsenal of such inhibitors (1). In light of the increasingly threatening infections caused by methicillin-resistant *S. aureus* strains, a thorough understanding about these escape mechanisms is essential. Owing to its central role in innate immunity (2) and its bridging function to adaptive immune responses (3), the complement system is an important target for evasion strategies of *S. aureus* (4). In particular, several proteins have been found to inhibit the central activation step of complement component C3, which simultaneously blocks the initiation of the alternative pathway, the amplification of the complement response, and the induction of downstream effector functions. The extracellular fibrinogen-binding protein (Efb)[3] C-terminal domain (Efb-C) and the Efb homologous protein (Ehp) have been described to bind to native C3 and some of its activation fragments (i.e., C3b, C3d) and to inhibit complement action via the formation of stable complexes and the induction of conformational changes in C3 and C3b (5–8). To date, these complement-targeted activities of the Efb family of proteins have primarily been attributed to C3b deposition and the generation of anaphylatoxins via the C3 and C5 convertases (8).

However, the C3d fragment, which shows a particularly high affinity for Efb-C, also mediates important biological functions via binding to complement receptor 2 (CR2/CD21). CR2 is primarily expressed on B cells, follicular dendritic cells, and immature T lymphocytes (3, 9). As part of the B cell coreceptor complex (together with CD19 and CD81), CR2 lowers the threshold for B cell activation. Binding of C3d-tagged pathogens to CR2 is therefore regarded as an essential link between the innate and the adaptive immune response (3). The tight binding of both Efb-C and Ehp at a C3d region that has been previously connected to the binding of CR2 (7, 10) led us to the hypothesis that these bacterial complement inhibitors may also affect the interaction of C3d with its major receptor. By disabling C3d from binding to CR2, *S. aureus* may have therefore evolved an efficient strategy to attenuate the initiation of downstream immune responses.

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[3] Abbreviations used in this paper: Efb, extracellular fibrinogen-binding protein; CR2, complement receptor type 2; Efb-C, C-terminal domain of Efb; Ehp, Efb homologous protein; RU, resonance units; pC3, polymeric glutaraldehyde-aggregated C3; RA/NA, R131A/N138A mutations; SCR, short consensus repeat domain; SPR, surface plasmon resonance.

In this study we investigated the effect of Efb-C and Ehp on the C3d:CR2 interaction using both biophysical and cellular assays and on the CR2-mediated stimulation of B cells. We show for the first time that the spectrum of anticomplement activities of Efb and Ehp may be far more diverse than originally anticipated and that it may also interfere with adaptive immune responses. We further project our findings to the structures of the C3d:Efb-C/Ehp and C3d:CR2 complexes and suggest potential molecular mechanisms of interference between these ligands of C3d.

Materials and Methods

Proteins and cells

Efb-C, its R131A/N138A mutant (RA/NA), Ehp, and C3d were expressed and purified as described previously (6, 7). Soluble CR2 and biotinylated C3d were prepared according to published methods (11). Polymeric C3 (pC3) was produced by glutaraldehyde treatment of C3 as described previously (12) and labeled with FITC. Blocking (OKB-7; Ortho Diagnostics) and nonblocking (HB-5; American Type Culture Collection (ATCC)) mAbs against CR2 were used for specificity determinations. Anti-mouse IgM mAb b-7-6 was kindly provided by Dr. J. Cambier, University of Colorado, Denver, CO and was labeled using sulfo-NHS-LC-biotin (Pierce). Raji cells (ATCC) were grown in RPMI 1640 containing 10% FBS and penicillin/streptomycin.

Surface plasmon resonance (SPR) studies

Direct binding studies were performed on a Biacore 2000 at 25°C in PBS (pH 7.4) containing 0.025% Tween 20. In a surface competition assay, biotinylated C3d (30 μg/ml) was captured on a streptavidin-coated sensor chip (Biacore biotin capture kit; prototype) to a protein density of 800 resonance units (RU), and CR2 was injected for 1 min at a concentration of 1 μM and a flow rate of 20 μl/min. After dissociation of CR2, either Efb-C or Ehp were injected over C3d to form a stable complex, and CR2 was injected as described above. SPR signals were processed using Scrubber (version 2.0; BioLogic) with a plain streptavidin surface subtracted as a reference. The signal intensities of CR2 before and after on-chip formation of the inhibitor complex with C3d were compared. Efb-C RA/NA was included as a control.

In a solution competition experiment, 9,000–10,000 RU of mAb HB-5 was immobilized on a CM5 chip by amine coupling and CR2 was captured to a density of ~2,000 RU. C3d (1 μM) was mixed with increasing concentrations (0.125 - 8 μM) of either wild-type Efb-C or Efb-C RA/NA and injected for 1 min at 20 μl/min. To compensate for the decay of CR2 from HB-5, a constant C3d concentration was injected at the end of each cycle and the competition signals were normalized to 100% of initial activity. The corrected signals were plotted against the molar ratio of C3d:Efb-C, and the inhibitory ratio was estimated from the drop in signal intensity.

FACS analysis

The binding specificity of pC3 to CR2 on Raji cells was tested by incubating 5×10^5 cells in 100 μl of PBS with either a blocking (OKB-7) or nonblocking (HB-5) mAb against CR2 (0.1 mg/ml) for 30 min on ice before adding 10 μl of pC3 (0.1 mg/ml in PBS). For the Efb-C competition experiments, either wild-type Efb-C or Efb-C RA/NA were premixed with pC3 at an equimolar ratio (based on the concentration of "free" C3) and incubated for 30 min at room temperature. Ten microliters of the individual mixtures were added to 5×10^5 Raji cells in 100 μl of PBS and stored on ice until analysis. All samples were measured on a FACSCanto cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Calcium mobilization assay

CR2-mediated calcium mobilization was analyzed based on a protocol by Henson et al. (13). Splenocytes were isolated from B57BL/6 mice and loaded with Indo-1 acetoxymethylester (Invitrogen). Biotinylated forms of C3d (4 μg) and anti-IgM (0.4 μg) were cross-linked using streptavidin (0.8 μg) and injected to 6×10^6 cells in 1.5 ml HBSS. A 10-fold molar excess of Efb-C (or its RA/NA mutant) was added to an aliquot of the cross-linked sample for testing inhibitory effects. Calcium mobilization was detected as a change in Indo-1 fluorescence using a Hitachi F-2500 spectrofluorometer at room temperature.
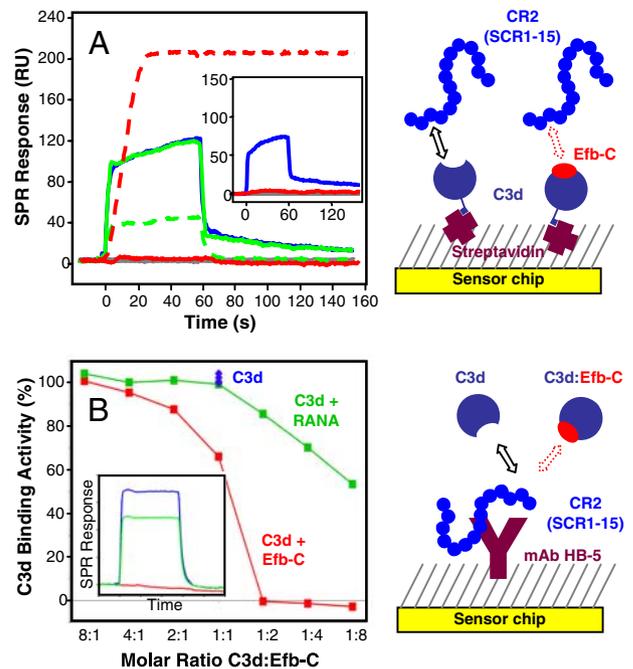

FIGURE 1. Efb-C efficiently inhibits the C3d-CR2 interaction in two competition assays. *A*, CR2 bound to site-specifically immobilized C3d in a biphasic manner (blue signal). Whereas Efb-C forms a stable complex with C3d (dashed red signal) and completely inhibits binding of CR2 (solid red signal), its impaired mutant RA/NA only shows weak binding (dashed green line) and does not affect subsequent binding of CR2 (solid green line). As expected, Ehp showed the same inhibitory activity as Efb-C (*inset*). *B*, In a solution competition assay, CR2 was captured via the nonblocking mAb HB-5 and mixtures of C3d (1 μM; blue) with increasing amounts (125 nM to 8 μM) of either Efb-C wild type (red) or RA/NA (green) were injected. Again, wild-type Efb-C blocked binding of C3d at slight molar excess, whereas much higher concentrations were required in case of the mutant. Typical SPR responses for C3d alone (blue) and C3d in competition with Efb-C wild type (red) and RA/NA (green) are shown in the *inset*.

Results and Discussion

Direct binding of C3d to CR2 is completely inhibited by Efb-C and Ehp

During pathogen-induced complement activation, the foreign cell surface is opsonized by C3b via a highly active thioester moiety. This leads to localized amplification of the complement response and to the induction of downstream effector functions. Surface-bound C3b is degraded by factor I, essentially leaving a C3d tag on the surface that plays a key role in B cell activation and maturation via binding to CR2. To closely mimic this physiological scenario in a direct binding assay, we immobilized C3d via its thioester on a SPR chip surface and observed the binding of the extracellular CR2 domains (short consensus repeat domains (SCR) 1–15) to C3d in the presence or absence of Efb-C. Injection of CR2 on immobilized C3d resulted in a slightly biphasic binding signal (Fig. 1*A*) as was observed in previous SPR studies (11). As expected, Efb-C readily formed a saturated complex with C3d, which did not significantly decay during the course of the experiment. In agreement with our hypothesis, subsequent injection of CR2 across the C3d:Efb-C complex did not show any binding signal, indicating that Efb-C indeed inhibited the interaction. Mutation of two key residues (R131, N138) in Efb-C to alanine (i.e., Efb-C RA/NA; Ref. 7) impaired complex formation with C3d and showed therefore no

inhibitory effect. In agreement with the previously described structural and functional similarity to Efb-C (6), Ehp also inhibited the interaction with CR2 (Fig. 1A, inset).

To estimate the molar ratio of Efb-C needed for this inhibition, CR2 was captured on the sensor chip by the nonblocking mAb HB-5 and mixtures of C3d with increasing concentrations of Efb-C (molar ratios from 8:1 to 1:8) were injected over the receptor. In good agreement with the surface competition assay, Efb-C effectively inhibited the binding of C3d to CR2 even at a slight molar excess. In contrast, much higher concentrations were needed for observing at least a partial inhibition in case of the RA/NA mutant (Fig. 1B). No direct binding could be detected for wild-type or mutant Efb-C to captured CR2 (data not shown), therefore indicating that the observed effect was again mediated through complex formation with C3d. In summary, both SPR-based competition studies demonstrate that the binding of Efb-C/Ehp to C3d interferes with the ability of the complement fragment to interact with its receptor and that only a slight excess of these inhibitors is sufficient for this effect.

Efb-C prevents binding and stimulation of CR2 by polymeric C3/C3d

To further explore the SPR-based observations, we performed an independent competition experiment in a cellular assay with functional receptors. The Raji Burkitt's lymphoma cell line is an ideal model for CR2 binding studies because these cells are known to express high amounts of CR2 on their surfaces (14). Because monovalent C3d exhibits a fast dissociation rate from the receptor (11), we used glutaraldehyde-aggregated pC3 as a multivalent equivalent that has been previously described to bind to CR2-bearing cell lines (12, 15). In addition, binding of pC3 to Efb-C was confirmed by SPR before the cell experiments (data not shown).

Addition of FITC-labeled pC3 to Raji cells led to a large increase in fluorescence and indicated binding to the receptor (Fig. 2A). Preincubation with mAb OKB-7 but not mAb HB-5 decreased fluorescence intensity (Fig. 2B). Because both Abs have been described to be specific for CR2 but only OKB-7 has blocking activity (16), this experiment confirmed that the observed shift in fluorescence is caused by specific binding of pC3 to CR2. In good agreement with our SPR experiments, the addition of an equimolar amount of Efb-C to the pC3 solution impaired the interaction and led to a significant decrease in fluorescence. Again, the RA/NA mutant showed only minor inhibitory activity (Fig. 2B). This confirmed our previous observation that formation of a stable 1:1 complex with C3d is sufficient for inhibition.

Cross-linking of substimulatory amounts of anti-IgM mAb with C3d has previously been shown to trigger the CR2-mediated costimulation of the B cell response as measured by calcium mobilization (13). When murine splenocytes were incubated with anti-IgM/C3d tetramers, a significant increase in the intracellular Ca^{2+} mobilization was detected. This costimulation was completely inhibited by Efb-C but not by the RA/NA mutant (Fig. 2C). As a consequence, Efb and Ehp are likely to have an impact on the complement-mediated activation of the adaptive immune response.

Efb-C and Ehp occupy an area of C3d that is potentially important for CR2 binding

Previous structural studies on the C3d complexes with Efb-C (7), Ehp (6), or CR2 (SCR1–2) (17, 18) allow us to
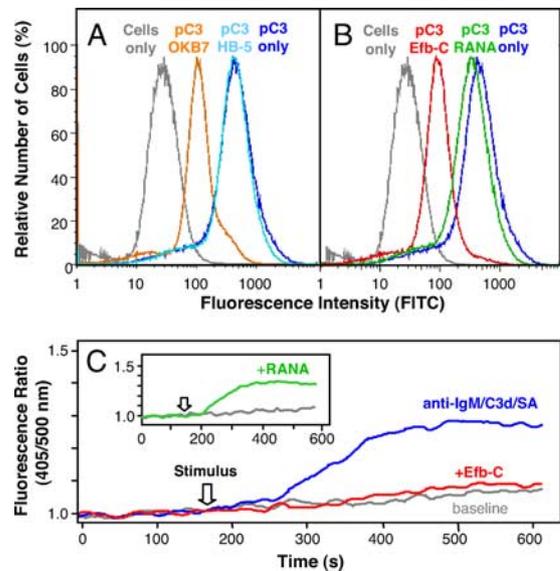

FIGURE 2. Efb-C blocks the binding and function of polymeric C3/C3d on CR2. A, Binding of pC3 to Raji cells (gray) caused a shift in fluorescence intensity (blue) that was only inhibited by the blocking anti-CR2 mAb OKB-7 (orange) but not by the nonblocking mAb HB-5 (cyan), indicating specific binding of pC3 to CR2. B, Although preincubation of pC3 with Efb-C (red) led to strong inhibition of the pC3-CR2 binding, an equal amount of the RA/NA mutant caused only a minor shift in intensity (green). This demonstrates specific inhibition of pC3 to Raji cell-bound CR2 by Efb-C. C, Mouse splenocytes were stimulated with mixed tetramers of anti-IgM and C3d on streptavidin (SA) as detected by calcium mobilization (blue). Excess of Efb-C inhibited the stimulation completely (red), whereas no inhibition was observed for the RA/NA mutant (green; inset).

hypothesize on the molecular basis for the inhibition of C3d/CR2 binding by these bacterial proteins. It should be noted, however, that despite a wealth of structural, biochemical, biophysical, and computational data, the exact location of the CR2 binding site on C3d is still discussed controversially (19). The publication of the C3d crystal structure (20) led to the identification of an acidic pocket as a possible CR2 binding site. Indeed, mutation of several residues on both sides of this pocket completely impaired binding of CR2 to C3d, which strongly supported this hypothesis (10). Strikingly, the mutated residues are in very close proximity to the contact residues in the C3d:Efb-C and C3d:Ehp cocrystal structure (Fig. 3A and data not shown). This finding clearly supports the idea of a common binding interface for Efb-C/Ehp and CR2 on the C3d fragment. In contrast, the cocrystal structure of CR2 SCR1–2 with C3d (17) suggests a binding area that is more distant from the Efb-C site (Fig. 3B, inset). However, subsequent studies by the same authors demonstrated that the cocrystal does not reflect all aspects of the interaction in solution (18, 21). Most importantly, they showed by small-angle x-ray scattering, analytical ultracentrifugation, and molecular modeling that both SCR1 and SCR2 interact with C3d (18), whereas the cocrystal only showed direct contacts for SCR2 (17). In the top-ranked binding models of this study (as seen in Protein Data Bank code 1W2S), SCR1 interacts in proximity to the Efb-C/Ehp-binding site on C3d (Fig. 3B). Even more, the crystal structure of C3 revealed that the proposed SCR2 binding site is fully exposed in C3 (19), even though native C3 has been shown not to interact with CR2. This suggests that the
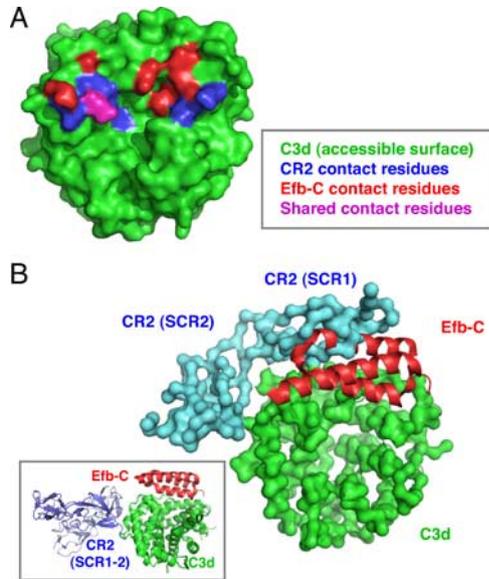
FIGURE 3. The binding sites of Efb-C and CR2 are likely to overlap on C3d. *A,* Mutational analysis of the acidic cleft on C3d (10) previously identified several key residues in this area (marked blue on C3d in green). The C3d-contact residues for Efb-C (red), as derived from the cocrystal structure (Protein Data Bank code 2GOX; Ref. 7), largely overlap with this region, with residue D1156 (magenta) shared across both binding sites. *B,* Small-angle x-ray scattering studies of CR2 SCR1–2 in interaction with C3d (Protein Data Bank code 1W2S; Ref. 18) further support this finding, because alignment with the C3d:Efb-C cocrystal reveals that SCR1 of CR2 (blue) and Efb-C (red) bind to similar sites on C3d (green). Although the currently available cocrystal between C3d and CR2 SCR1–2 (PDB: 1GHQ; Ref. 17) only shows contacts of C3d with SCR2 (*inset*), subsequent studies have suggested that this does not reflect the binding in solution and that the binding of CR2 SCR1 is pivotal for correct recognition and function. Analogous studies with the structurally similar C3d: Ehp complex (Protein Data Bank code 2NOJ; Ref. 6) revealed the same results and are therefore omitted. Structural alignment and visualization was done using PyMOL (DeLano Scientific).

SCR1 site in proximity of the Efb-C/Ehp contact area may be predominant in determining the target specificity.

Although these structural comparisons render a steric hindrance of the CR2-C3d interaction by Efb-C and Ehp highly plausible, the inhibition may also be induced by electrostatic interference. Both experimental data and computational analyses suggest that a short- or long-range electrostatic steering between the electronegative acidic pocket on C3d and a positively charged patch of amino acids on one side of CR2 SCR1 are highly important for the interaction (10, 11, 21, 22). In this respect, binding of the strongly electropositive Efb-C molecule (isoelectric point ~ 10) has recently been shown to change the overall charge pattern of C3d (23) and is therefore likely to interfere with the encounter of the CR2-C3d complex. Together, the available structural information on the complexes between C3d and these two ligands clearly supports the current experimental findings and provides a molecular basis for the inhibitory effect that could be mediated by both steric hindrance and electrostatic shielding.

Although a similar three helix bundle motif as that of Efb-C/ Ehp has also been found in staphylococcal protein A-related proteins (4), no CR2-targeted functions have been described to date for the structurally similar SCIN (staphylococcal complement inhibitor) protein family nor for staphylococcal protein A

itself. Interestingly, recent studies indicate a significant, yet weaker inhibitory activity against the C3d-CR2 interaction for the *Staphylococcus* binder of immunoglobulins, Sbi (24). Although this protein was originally found to impair Ab-mediated complement activation by binding to the Fc part of IgG, its C-terminal domains have been shown to exhibit a similar binding specificity toward C3 fragments as that of Efb-C/Ehp (24). However, the complex formation of C3d with Sbi is far less stable than with Efb-C or Ehp, and the binding site for C3d has not been described yet. It will therefore be interesting to explore whether these these proteins share a common binding site and exert similar physiological effects.

The multiplicity and redundancy of *S. aureus* proteins in regard to inhibiting complement activation may appear confusing at a first view. However, closer analysis of the specificities and known activities of these inhibitors draws a fascinating picture of a highly tailored regulatory network of agents. Several members of the Efb (Efb, Ehp) and SCIN families of immunomodulatory effectors directly inhibit the alternative pathway at the level of C3 or the C3 convertase and therefore reduce the opsonization of the bacterial surface with C3b and, consequently, with C3d (4, 7, 8). However, due to only partial inhibition of the alternative pathway and C3 convertase-independent activation, *S. aureus* particles could still become tagged by C3d and may induce the adaptive immune response via binding to CR2. Therefore, blocking this crucial bridge between the branches of the immune system would add another layer of security and efficacy to the already impressive immune evasion arsenal of this human pathogen. In this respect the identification of a family of evasion proteins with inhibitory activity against targets in both the innate and adaptive branches of the immune system is an important finding.

Acknowledgments

Disclosures

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