Streptococcus pyogenes Employs Strain-dependent Mechanisms of C3b Inactivation to Inhibit Phagocytosis and Killing of Bacteria*

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Evasion of complement-mediated opsonophagocytosis enables group A Streptococcus pyogenes (GAS) to establish infection. Different strain-dependent mechanisms are employed by the host to accomplish this goal. In general, GAS inhibits the amplification of the complement cascade on its cell surface by facilitating the degradation of C3b, an opsonin, to an inactive product, inactivated C3b (iC3b), in a step catalyzed by factor I (FI) and its cofactor, factor H (FH), with or without the participation of human host plasmin (hPm). GAS recruits FH to its cell surface via FH receptors, which are transcriptionally controlled by the two-component cluster of virulence responder-sensor system. The manner in which FI-FH and hPm function together on GAS cells is unknown. Using GAS strain AP53, which strongly binds host human plasminogen/plasmin (hPg/hPm) directly via an hPg/hPm surface receptor (PAM), we show that both FI-FH and hPm sequentially cleave C3b. Whereas FI-FH proteolytically cleaves C3b into iC3b, PAM-bound hPm catalyzes cleavage of iC3b into multiple smaller peptides. Unlike AP53, GAS strain M23ND weakly binds FH and recruits hPg/hPm to its cell surface indirectly via fibrinogen bound to M-protein, M23. In this case, FH-FI cleaves C3b into iC3b, with negligible degradation of iC3b by hPm that is bound to fibrinogen on the cells. AP53 and M23ND display similar resistance to human neutrophil-mediated phagocytosis, which results in a corresponding high lethality in mice after injection of these cells. These results suggest that GAS utilizes diverse mechanisms to degrade C3b and thus to protect bacterial cells from the complement response of the host.

Group A Streptococcus pyogenes (GAS) is a spherical, Gram-positive bacterium that is responsible for numerous diseases with diverse clinical manifestations, with high specificity for humans. GAS can cause common illnesses, such as impetigo, pharyngitis, and scarlet fever, and can also cause life-threatening sequelae, such as necrotizing fasciitis, streptococcal toxic shock syndrome, and acute post-streptococcal glomerulonephritis (1, 2). GAS remains a leading infectious cause of global mortality and morbidity. Of the estimated 700 million infections annually, 18 million prevalent cases are found to be severe, with nearly 2 million virulent new cases reported per year worldwide (3–5). Although GAS remains uniformly susceptible to penicillin, vaccines that can prevent GAS-associated diseases are inadequate (3, 4). Therefore, a better understanding of the cellular and molecular mechanisms of GAS infection is needed in designing and developing broad-spectrum drugs and diagnostic tools.

To establish infection, GAS must develop evolutionary strategies to evade initial host innate immune systems, especially complement-mediated elimination of the microbe. The complement system is primarily activated by three distinct pathways that are based on different recognition mechanisms: antigen-antibody complexes (classical pathway); host lectin binding to pathogen carbohydrate patterns (lectin pathway); and small amounts of spontaneously generated complement factor C3b, an opsonin, on microbial cell surfaces (alternative pathway) (6). Finally, after a series of cleavage reactions, all three pathways culminate in the formation of an enzyme, C3 convertase, which cleaves the central component, C3, into C3b and C3a. C3b can then either participate in further formation of the C3 convertase, leading to the amplification of complement activation, or deposit on a variety of target surfaces, including pathogenic cells, via covalent thioester binding. This triggers complement-mediated opsonization by phagocytic cells, thus clearing the pathogen from the host (6, 7). Hyperactivation of the complement system can be harmful to the host cells (e.g. anaphylactic response). Therefore, the host maintains several regulatory mechanisms to accelerate the dissociation of C3b convertase on host surfaces and/or promotes the degradation of surface-deposited C3b. More than 10 proteins are known to be involved in the regulation of the complement system (e.g. complement inhibitor factor H (FH) (8) and the serine protease, factor I (FI) (9)). As an example, when FH recruits FI degradation of C3b to inactivated C3b (iC3b) results in the inactivation of the complement pathway (9–11). Recent studies showed that the protease, hPm, can also act as complement inhibitor...
because it cleaves C3b and iC3b at multiple sites in vitro (12, 13). However, the functional consequences of this activity in vivo and on bacterial surfaces have not been established.

The ability to avoid the complement system is a key determinant in pathogen virulence. GAS can escape recognition by the complement system by employing various strategies: 1) recruiting host complement inhibitors, such as FH, to the bacterial surface via FH receptors (e.g., M protein, Fba) (14–16); 2) secretion of proteases, such as SpeB, that can cleave and inactivate C3b (17); and 3) production of the hyaluronic acid capsule around the bacterial surface that inhibits complement recognition (18). Bacterial receptors that recruit host complement inhibitors are considered critical to the complement inhibition mechanism, and there is a remarkable variability in the structure and function of these receptors among different GAS strains. For example, in many GAS strains (e.g., M5, M6, M18, M19, M24, and M28), M-protein transcribed from the emm gene is a major binding locus for sequestering host FH (14, 19).

In other strains (e.g., AP53) that express a direct hPg/hPm-binding M-protein (PAM), FH does not bind to the M-protein but instead uses a fibronectin-binding protein, Fba, to recruit FH (16). This indicates that complement inhibitors can bind to different receptors in GAS strains. Similarly, whereas hPg binds to PAM directly (20), it interacts with other M proteins indirectly, perhaps via fibrinogen (21). The manner in which differential binding of the FH-hPg/hPm to various receptors affects the inactivation of C3b has not been established.

In the present study, we examined the inactivation of C3b by the FH-FI complex and by hPm, along with their effects on phagocytosis, and the consequent lethality in mice using GAS strains AP53 and M23ND. These GAS strains express M-proteins that interact with the complement system in different manners. The results obtained serve to demonstrate the versatility of GAS in avoiding host immune surveillance through different mechanisms and further link GAS infectivity to conservation of components of the hemostasis system.

**Experimental Procedures**

**GAS Strains and Culture Conditions**—The isogenic GAS strains (viz. AP53/CovS⁺, AP53/CovS⁻, M23ND/CovS⁺, and M23ND/CovS⁻) used in this study have been described previously (22, 23). Isogenic mutants of strain AP53/CovS⁺, with targeted deletions of streptokinase (SK) (AP53/CovS⁻ΔSK), PAM (AP53/CovS⁻ΔPAM), and fibronectin-binding protein (Fbp) (AP53/CovS⁻ΔFbp), were constructed as described earlier (16, 22, 24). GAS strains were grown in Todd-Hewitt-1% yeast (THY) medium at 37 °C to an A₆₀₀nm of ~0.6 (mid-log phase). The cells were centrifuged, washed with PBS, and then used for further experiments. In all GAS experiments, equal numbers of cells were used, as determined by both A₆₀₀nm and bacterial counting assays on agar plates (16).

**Neutrophil Phagocytosis and Mouse Infection Assays**—GAS survival in the presence of neutrophils, isolated from fresh human blood drawn from nonimmune donors, was examined as described previously (16). Blood samples were taken by licensed phlebotomists (United States Code of Federal Regulations and International Conference on Harmonisation Guidelines on Good Clinical Practices). For this work, approval was obtained from the institutional review board, and all patients signed consent forms in accordance with the Declaration of Helsinki. For the mouse infection studies, transgenic C57BL/6 (hPg(Tg)) mice (provided by Dr. David Ginsburg, Ann Arbor, MI) expressing hPg were utilized (25). The mice were injected subcutaneously in the right flank with 4–5 × 10⁸ cells and observed twice daily for survival for up to 10 days.

**C3b/iC3b Degradation in Solution**—For in vitro C3b/iC3b degradation, the purified proteins, C3b, iC3b, FH, FI (Comptech, Tyler, TX), and hPm (ERL, South Bend, IN), were mixed in equimolar concentrations and incubated at 37 °C for 2 h. The proteins were then separated on SDS-PAGE under reducing conditions, and C3b/iC3b bands were detected by Western blotting. Mouse anti-human iC3b antibody (Thermo Scientific, Rockford, IL) that recognizes C3b and iC3b was used as the primary antibody followed by HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA) as the secondary antibody. The Clarity Western ECL kit (Bio-Rad) was employed to develop the protein bands in a ChemiDoc MP system (Bio-Rad).

**C3b/iC3b Degradation on GAS Cell Surfaces**—This assay was performed as described (16) with some modifications. All GAS strains were grown to A₆₀₀nm of ~0.6, washed twice with PBS, and resuspended in gelatin-veronal buffer (Sigma-Aldrich). Bacterial cells (~1 × 10⁹) were incubated with human plasma or hPg-deficient plasma for 60 min at 37 °C. In the case of the serum experiments, cells were incubated with 50% normal human serum (NHS; Atlanta Biologicals, Flowery Branch, GA) or FI-depleted serum (Comptech) for 60 min at 37 °C. After incubation, bacterial cells were washed twice with 10 mM EDTA in PBS. The bound proteins were eluted from cell surfaces in 60 μl of 4 mM Na₂CO₃, 46 mM NaHCO₃, pH 9.2, for 2 h at 37 °C. The eluted proteins were then separated on SDS-PAGE under reducing conditions, and the bound C3b/iC3b was detected by Western blotting as described above.

**Binding of FH to GAS Cells**—The binding of FH to GAS cells was performed in 50% NHS or 50% FH-depleted serum (Comptech) using Western blotting under non-reducing conditions, as described in our previous work (16). Mouse monoclonal anti-human FH (Thermo Scientific) and HRP-conjugated anti-mouse IgG were used as the primary and secondary antibodies, respectively. Image Lab Software (Bio-Rad) was used to quantify the band intensities. Purified FH was included as a positive control.

**Effects of Human Fibrinogen (hFg) on the Activation of hPg on GAS Cells**—Both AP53/CovS⁺ and M23ND/CovS⁻ cells were cultured in 30 ml of THY medium until an A₆₀₀nm of ~0.6 was obtained. The cells were collected by centrifugation and washed 3 times with sterile PBS. Cells were then resuspended to a final A₆₀₀nm of ~1.0. Aliquots of 400 μl of the suspension were incubated with 200 nm hPg in the absence and presence of 200 nm hFg (ERL). Tubes were then centrifuged, and the pellet was washed with PBS. Cells were resuspended in hPg activation buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). Aliquots of 100 μl of the cell suspension were distributed to individual wells of a protein-nonbinding 96-well plate along with 100 μl of activation buffer that also contained the chromogenic substrate for hPm, S2251 (0.5 mM), and 10 nm SK (24). Substrate cleavage was
**Results**

**FH Binding to GAS Strains**—To determine how C3b is cleaved on the surface of different GAS strains, we chose two different strains, AP53 and M23ND, because of important differences in the mode of generation of hPm. AP53 is a pattern D strain that expresses the *emm53* subtype M53 protein (plasminogen binding group A streptococcal M-like protein; PAM) as its primary M-protein (22). PAM interacts with hPg strongly and directly, resulting in rapid activation of hPg to hPm by SK (Fig. 1A), but shows no direct binding to hFg (26, 27). A second hypervirulent GAS strain was used that expresses the *emm23* subtype that activates hPg to hPm rapidly only in the presence of the direct hFg-binding M-protein, M23 (28) (Fig. 1B). Thus, the two GAS strains display important differences in the manner in which hPm is formed and assembled.

In our recently published work (16), we have shown that GAS strain AP53 can bind FH through the Fbp receptor, which is transcriptionally controlled by the two-component cluster of virulence sensor-responder (CovRS) system. Therefore, the overall binding of FH in AP53 can be regulated by the CovRS system. However, the mode of binding and regulation of FH to M23ND is not known. Like PAM, M23, cloned and expressed from M23ND, does not interact with FH, as shown by surface plasmon resonance experiments.3 Thus, in both cases, M-proteins PAM and M23 are not FH receptors.

In order to assess the ability of FH to bind to M23ND cells, the M23ND/CovS− strain, which contains a natural CovS-inactivating mutation, along with its isogenic M23ND/CovS+ derivative, were compared with AP53/CovS− and AP53/CovS+. We have previously shown that an inactivating mutation in CovS of AP53 leads to the derepression of Fbp receptor expression, thus facilitating enhanced binding of FH on the AP53 surface (16). To expand these observations, GAS cells were incubated in NHS, the FH bound to the cells was stripped, and its levels were measured using Western blotting (Fig. 2). As expected, AP53/CovS− cells show more FH binding, as compared with AP53/CovS+ cells. However, unlike AP53/CovS− cells, both M23ND/CovS− and M23ND/CovS+ cells displayed a very low level of binding of FH (Fig. 2, A and B). This indicated that M23ND does not utilize cell-bound FH in C3b inactivation to the same extent as AP53 and might employ other cofactors. As shown in Fig. 2, ~25% FH binding was observed in AP53/CovS−/ΔFbp cells, indicating that FH can still retain a small level of binding to the GAS cells in the absence of its primary receptor Fbp. The data also indicate the presence of other (secondary) receptors for FH in GAS cells.

**FI and hPm Work in Concert to Cleave C3b In Vitro**—To understand the cleavage pattern of C3b on the GAS surface by FI-FH and hPm, we first analyzed the FI-FH+ and hPm-catalyzed cleavage products of C3b (Fig. 3A), alone and in com-

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3 G. Agrahari, unpublished data.
nation, using purified proteins. The cleavage products were identified by Western blots using a C3b/iC3b-specific antibody (Fig. 3B). As expected, FI, in the presence of its cofactor FH cleaves C3b at Arg-1303 and Arg-1320 (blue arrow), giving rise to iC3b. hPm degrades iC3b through cleavage at Arg-945 (red arrow) and thus shows multiple smaller peptides on reduced SDS-polyacrylamide gels. Mouse anti-human iC3b antibody used in this study is known to recognize the C3dg region that contains the trieste domain (TED). B, cleavage of C3b and iC3b in the presence of FI-FH and/or hPm. The purified proteins were incubated in equimolar concentrations and incubated for 2 h at 37 °C, and the peptide bands generated after cleavage were visualized by Western blotting using anti-C3b/iC3b-specific antibody. C–E, time course of C3b/iC3b cleavage by FI-FH and hPm. Samples were removed at different time points, as indicated, and mixed with 2× SDS-PAGE sample buffer containing 2-mercaptoethanol. FI-FH cleaves C3b (lane 4 in C–E) much more rapidly than does hPm (lane 5 in C–E). Molecular masses are indicated on the gel (left) as determined from a standard molecular mass marker set.

**FIGURE 3. Cleavage of C3b and iC3b by FI-FH or hPm in solution.** A, schematic representation showing proteolytic cleavage of C3b by the FI-FH complex or by hPm. FI in the presence of its cofactor FH cleaves C3b at Arg-1303 and Arg-1320 (blue arrow), giving rise to iC3b. hPm degrades iC3b through cleavage at Arg-945 (red arrow) and thus shows multiple smaller peptides on reduced SDS-polyacrylamide gels. Mouse anti-human iC3b antibody used in this study is known to recognize the C3dg region that contains the trieste domain (TED). B, cleavage of C3b and iC3b in the presence of FI-FH and/or hPm. The purified proteins were incubated in equimolar concentrations and incubated for 2 h at 37 °C, and the peptide bands generated after cleavage were visualized by Western blotting using anti-C3b/iC3b-specific antibody. C–E, time course of C3b/iC3b cleavage by FI-FH and hPm. Samples were removed at different time points, as indicated, and mixed with 2× SDS-PAGE sample buffer containing 2-mercaptoethanol. FI-FH cleaves C3b (lane 4 in C–E) much more rapidly than does hPm (lane 5 in C–E). Molecular masses are indicated on the gel (left) as determined from a standard molecular mass marker set.

we hypothesize that AP53, which recruits FI via FH binding to Fbp, and recruits hPm through PAM binding, leads to the cleavage of C3b into a Pm-specific α’ 40 kDa band, similar to a cleavage pattern observed in lane 7 of Fig. 3. In addition, we hypothesize that an inactivating mutation in CovS that leads to the derepression of Fbp, without altering PAM expression, will result in the generation of a similar C3b cleavage pattern. To test these hypotheses, cells were incubated with NHS, and the cleavages of C3b by FI-FH and Pm present in serum were analyzed by Western blotting. As expected, incubation of AP53/CovS− with NHS gives rise to a Pm-specific α’ 40 kDa band (Fig. 4). The binding of FI-FH on the GAS surface determines the rate of amplification of C3b deposition. Therefore, the presence of increased FI-FH on AP53/CovS− inhibited C3b amplification by degrading the C3b to iC3b, which was cleaved by Pm into the α’ 40 kDa band. This results in an overall lower C3b deposition on AP53/CovS− cells. However, as compared with AP53/CovS−, AP53/CovS+ cells show a higher amount of α’ 40 production.
Despite having low FH binding (~25%; Fig. 2B). In this case, the C3b amplification is only partially repressed, thus leading to more C3b deposition when compared with AP53/CovS- cells. Additionally, only part of the total C3b is converted into iC3b due to lower FI-FH binding in GAS AP53/CovS+. However, given the excessively higher C3b deposition, the total amount of α’ 40 kDa band produced in AP53/CovS+ was higher as compared with GAS AP53/CovS-. The absence of FI in serum (NHS/ΔFI) severely attenuates the cleavage of C3b, indicating that FI is the primary factor in C3b degradation, and Pm only effectively functions on the FI-mediated cleavage product of C3b (viz. iC3b). The loss of C3b cleavage in the absence of FI and hPm further confirms the direct connection between FI and Pm in C3b degradation (Fig. 5).

With regard to GAS strain M23ND, the data in Fig. 4 clearly show that productive binding and activation of hPg on MD23 cells requires hFg. Furthermore, the hPm formed by this route does not efficiently cleave the iC3b generated by FI because a significant amount of the iC3b band was still present (Fig. 6). The most reasonable conclusion is that hPm bound directly to PAM via its kringle 2 (K2) domain will cleave iC3b (as in AP53 cells) but that hPm bound to M23 through hFg via its K1 and K4 domains (as in M23ND cells) lacks this particular activity. Further, FI was able to catalyze cleavage of C3b into iC3b, even in the absence of FH binding. These data suggest that other cofactors for FI are involved in protection against complement-mediated lysis of GAS M23ND.

**Acquisition of hPg by PAM and Activation of hPg to hPm by SK on AP53 Cells Are Necessary for Degradation of iC3b**—With the aim of demonstrating that GAS proteins participate in the cleavage FI-generated iC3b into smaller fragments by selectively recruiting and activating hPg to hPm on the bacterial surface, we first incubated GAS cells with complete plasma or hPg-deficient plasma and analyzed the pattern of C3b cleavage. As shown in Fig. 6A, in Pg-deficient human plasma, the Pm-specific α’ 40 kDa band was completely abolished concurrently with the appearance of iC3b, indicating that hPm is the main factor involved in the cleavage of iC3b into smaller fragments.

To determine whether PAM is the primary receptor involved in the sequestration of hPg/hPm on the bacterial surface, GAS AP53 lacking PAM (AP53/CovS-/ΔPAM) was incubated with NHS. The absence of PAM receptor on AP53 significantly inhibited the generation of the α’ 40 kDa band, indicating that PAM is the main receptor for hPg/hPm acquisition (Fig. 6B). Consistent with these data, incubation of AP53/CovS-/ΔPAM with Pg-depleted human plasma restored with exogenous hPg also significantly inhibited the generation of the α’ 40 kDa band (Fig. 6B). Interestingly, a weak 40 kDa Pm-specific band is still present in this sample (Fig. 6B, lane 8), suggesting that another hPm receptor may be present on GAS cells. This is not surprising because in addition to PAM, GAS is known to have other receptors (e.g., enolase and GAPDH) that are shown to bind hPg/hPm (29). It is possible that unlike PAM, these proteins might act as secondary receptors and in fact display weaker binding to hPg/hPm. Therefore, these receptors can show some activity in the absence of PAM.

PAM has been shown to bind hPg and hPm directly and with high affinity (30). hPg is the main component present in circulation, as compared with Pm, which is normally present at low levels. Therefore, hPg is the main form recruited by PAM on the bacterial surface during GAS infection and can be activated to Pm by GAS-secreted SK (31). To demonstrate that Pm present at the surface of GAS primarily originates from hPg by SK activation, an isogenic AP53 strain lacking SK (AP53/CovS-/ΔSK) was incubated in NHS in the absence and presence of exogenously added purified SK. As expected, deletion of SK in the AP53 strain completely abolished the generation of the α’ 40 kDa band of C3b originating from Pm cleavage (Fig. 6C), indicating that SK is required for activation of hPg to hPm that cleaves iC3b into smaller fragments.

**Phagocytic Resistance and Mouse Lethality of AP53 and M23ND Strains**—To better understand the impact of C3b cleavage on complement-mediated phagocytosis in both GAS AP53 and M23ND, these cells were incubated with purified human neutrophils in the presence of 20% nonimmune plasma. In normal human plasma, both AP53/CovS- and M23ND/
CovS− showed strong resistance to phagocytosis by neutrophils despite their differences in C3b cleavage pattern in blood ex vivo (Fig. 7A). As expected, AP53/CovS− and M23ND/CovS− displayed greater susceptibility to neutrophil-based killing, as compared with their respective CovS+ strains, confirming a role for the CovRS system in the regulation of phagocytosis in both GAS strains. This higher level of killing of AP53/CovS+ and M23ND/CovS+ by neutrophils, as compared with AP53/CovS− and M23ND/CovS−, was reflected in mouse survival studies of GAS infection, where CovS− strains were generally more lethal in mice (Fig. 7B). Interestingly, the M23ND/CovS+ strain shows higher mouse lethality and resistance against neutrophil killing, indicating that the CovRS system in the M23ND strain has a more limited role in the regulation of phagocytosis as compared with the AP53 strain.

Discussion

Bacterial pathogens, such as GAS, have evolved mechanisms to evade the host innate immune response system in order to ensure the survival of the invading bacteria. In more familiar cases, prevention of opsonin (host-derived C3b) formation and/or accumulation on bacterial cells inhibits phagocytic recognition of the bacterium by host phagocytic cells. Depending on the GAS strain and the types of receptors present on the cells, the resistance mechanisms displayed by bacteria may differ, but their ultimate aim is to inhibit the activation of C3 to C3b by C3 convertase on pathogen cells.

One method that bacteria employs to attenuate C3b accumulation on bacterial cells is to utilize complement inhibitory proteins, such as FH, on their surfaces to recruit the serine protease, FI. Once FI is bound on the surface of pathogens, this enzyme will degrade C3b into iC3b and lead to loss of C3b that is available to opsonize invading organisms. This strategy is employed by GAS to increase its survival in the host. Several streptococcal receptors for FH have been proposed, including several M-proteins (32) and, additionally, a fibronectin-binding protein, Fba/Fbp (16, 33). Another FH-based mechanism for attenuating the function of the complement pathway is centered on cells that contain the membrane protein DAF (decay-accelerating factor; CD55 in humans). FH is a positive regulator for DAF and in this way accelerates the inactivation of the C3 convertase complex generated from either the classical or alternate complement pathways (34).
In our present work, we elucidate two different mechanisms utilized by GAS to regulate the amount of C3b on their cell surfaces, and we further demonstrate how GAS has evolved distinct strain-specific strategies to avoid complement-mediated elimination by host cells (a summary of the events is provided in Fig. 8A). In one case, GAS AP53 interacts with FH through a distinct receptor, Fba/Fbp, leading to recruitment of FI and subsequent cleavage of C3b to iC3b on the bacterial surface (Fig. 8B).

Surprisingly, unlike AP53, GAS M23ND weakly binds FH but still cleaves C3b into iC3b, indicating that FH cannot act as a critical cofactor for FI in the M23ND strain (Fig. 8C). The other known cofactor for FI involved in C3 convertase inactivation in GAS strains is C4BP (16). The data presented in this study showed that M23ND also does not bind to C4BP (data not shown), indicating that M23ND either employs a cofactor distinct from FH/C4BP or may function without a co-factor. In the case of Staphylococcus aureus, cleavage of C3b by FI was inde-
independent of the presence of plasma co-factors. Instead, a bacterial receptor, clumping factor A, served as the ligand for FI binding, and a fragment of clumping factor A served as a cofactor for FI-mediated cleavage of C3b (35, 36). The identification and characterization of the co-factor/ligand as well as its receptor on M23ND remain to be determined.

Sequestration of the host serine protease, hPm, at the GAS cell surface is a key virulence determinant of GAS that facilitates bacterial dissemination to deeper tissue sites, resulting in severe invasive infection. To activate the zymogen of hPm (viz. hPg), GAS expresses a number of hPg receptors and a critical activator, SK. In GAS, the binding of hPg with M-protein receptors, combined with the generation of SK, plays an important role in the activation of hPg to hPm. Recent studies have shown that hPm plays a regulatory role in the complement cascade, where it acts as a complement inhibitor. hPm has the ability to degrade C5, C3b, and iC3b complement proteins at multiple sites in vitro (12, 13). However, the relevance of this function of Pm is not well understood. hPg and FH bind C3b at distinct sites and do not compete for binding (12). These observations suggest that C3b can be cleaved by both FI-FH and Pm. The manner in which two different proteases, FL and hPm, work on a single substrate, C3b, is not known. Our results demonstrated that FI specifically cleaves C3b, whereas hPm primarily cleaves iC3b both in vitro and on GAS cells. The sequential action of FI and hPm on C3b has significant impact on GAS virulence. Although inactivation of C3b to iC3b suppressed the formation of an amplification loop, GAS surface-bound iC3b can still modulate phagocytosis and adaptive immune responses. A recent study demonstrated that when an iC3b was bound to zymosan (iC3b-Zym) and exposed to THP-1 macrophages, most THP-1 cells phagocytosed iC3b-Zym (13). However, treatment of iC3b-Zym with hPm, which converted iC3b-Zym into Pm-iC3b-Zym, significantly reversed the phagocytosis by THP-1 cells. Therefore, we conclude that GAS employs multiple means to regulate complement pathways, such as utilization of hPm, which is at least as efficient as FI-FH but does not require a cellular cofactor to cleave iC3b, thus maximizing its ability to survive in the host.

In conclusion, our data demonstrate the manner in which GAS exploits two distinct host complement regulatory pathways to efficiently inhibit bacterial opsonization by sequentially degrading C3b and iC3b, thereby contributing to GAS evasion of the host complement system. Therefore, strategies aimed at selective inhibition of the FI-FH and Pm interactions with GAS receptors by small molecule inhibitors or strategies to suppress the expression of GAS receptors involved in these interactions will provide possible new treatments or diagnostic tools for GAS infection.

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