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Alexander J. Laarman, Bart W. Bardoel, Maartje Ruyken, Job Fernie, Fin J. Milder, Jos A. G. van Strijp and Suzan H. M. Rooijakkers

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Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways

Alexander J. Laarman,1 Bart W. Bardol,1 Maartje Ruyken, Job Fernie, Fin J. Milder, Jos A. G. van Strijp, and Suzan H. M. Rooijakkers

The complement system rapidly detects and kills Gram-negative bacteria and supports bacterial killing by phagocytes. However, bacterial pathogens exploit several strategies to evade detection by the complement system. The alkaline protease (AprA) of Pseudomonas aeruginosa has been associated with bacterial virulence and is known to interfere with complement-mediated lysis of erythrocytes, but its exact role in bacterial complement escape is unknown. In this study, we analyzed how AprA interferes with complement activation and whether it could block complement-dependent neutrophil functions. We found that AprA potently blocked phagocytosis and killing of Pseudomonas by human neutrophils. Furthermore, AprA inhibited opsonization of bacteria with C3b and the formation of the chemotactic agent C5a. AprA specifically blocked C3b deposition via the classical and lectin pathways, whereas the alternative pathway was not affected. Serum degradation assays revealed that AprA degrades both human C1s and C2. However, depletion assays demonstrated that the mechanism of action for complement inhibition is cleavage of C2. In summary, we showed that P. aeruginosa AprA interferes with classical and lectin pathway-mediated complement activation via cleavage of C2.

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The innate immune system rapidly detects and kills invading bacteria via different mechanisms, such as TLRs and the complement system. TLRs are expressed on immune cells and detect an enormous variety of microorganisms via recognition of highly conserved microbial molecular patterns, such as flagellin via TLR5 and LPS via TLR4 (1). Upon ligand binding, TLRs trigger an intracellular signaling cascade that leads to production of proinflammatory cytokines and activation of phagocytes. The complement system is a proteolytic cascade of plasma proteins, which results in direct killing of certain microorganisms and efficient bacterial recognition by phagocytes. The complement system consists of three distinct pathways: the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). All three recognition pathways culminate in the formation of C3 convertase enzymes that mediate deposition of C3b on foreign surfaces (2). The CP is initiated by binding of C1q to bacterium-bound Abs or repeating molecular patterns on bacteria. This binding initiates activation of the C1q-attached protease C1s because it cleaves C4 and C2 to induce formation of the C3 convertase complex. In the AP, microbial surfaces are recognized by properdin (5), which initiates assembly of the AP C3 convertase (C3bBb) on the surface. The AP also serves as an amplification loop to enhance the concentration of C3b on the surface. Cleavage of C3 by convertases is critical to opsonization of the bacterial surface with C3b, which is recognized by complement receptors on phagocytes and supports phagocytosis and killing. Further downstream in the cascade, formation of C5 convertases and cleavage of C5 result in generation of the potent anaphylatoxin C5a and the formation of C5b-9, the membrane attack complex (MAC) that can directly kill certain Gram-negative bacteria (6).

The Gram-negative bacteria Pseudomonas aeruginosa lives in water and soil and acts as an opportunistic pathogen in humans and plants. In healthy individuals, P. aeruginosa is efficiently killed via the innate immune system; however it causes chronic infections in immunocompromised patients. For instance, cystic fibrosis (CF) patients suffer from chronic infections caused by P. aeruginosa that cannot be eradicated after colonization. Strains isolated from CF patients rapidly adapt to the environment in the lung, resulting in dramatic genetic and morphological changes (7). Infection by P. aeruginosa is complicated by its inherent resistance to several classes of antibiotics and acquisition of resistance genes via mobile genetic elements (8). In addition, biofilm formation enhances the resistance to antibiotics in the CF lung (9). Remarkably, the strains isolated from CF patients are generally sensitive to direct complement killing, which is different from...
strains isolated from non-CF patients. These serum-resistant strains consume more C5b-9 complement components and have less C3b deposited on their surface (10).

Bacterial survival in the host is accompanied by the acquisition of different immune-evasive strategies. These strategies involve alteration of the bacterial surface, such as production of a capsule or immune-modulating membrane proteins, or secretion of soluble immune-evasion proteins, such as proteolytic factors. *P. aeruginosa* secretes a number of toxins and virulence factors, like elastase and alkaline protease (AprA). Elastase cleaves a wide variety of host proteins, such as collagen, IgG, and complement proteins (11). AprA is a 50-kDa zinc metalloprotease that degrades several components of the host immune system, such as complement C1q and C3 (12), or cytokines, like IFN-γ and TNF-α (13). In addition, AprA prevents TLR5 activation via the cleavage of monomeric flagellin (14). AprA is secreted via its own type I secretion system, which is encoded by three genes upstream of the aprA gene. *P. aeruginosa* also encodes a highly specific inhibitor of AprA named AprI, which is translocated to the periplasmic space according to its signal sequence. The role of AprA in pseudomonal virulence has been illustrated in a *Drosophila* infection model (15). AprA contributes to the persistence of *Pseudomonas entomophila* infection and protects against antimicrobial peptides produced by the innate immune system of *Drosophila*. Furthermore, AprA was found to be expressed in human CF patients infected with *P. aeruginosa* (16, 17).

In a previous study, Hong and Ghebrehiwet (12) showed that AprA cleaves complement components C1q and C3 and inhibits lysis of sheep erythrocytes via MAC. However, because *P. aeruginosa* is generally resistant to direct lysis via MAC, we wondered whether cleavage of complement components by AprA contributes to pseudomonal immune evasion. We found that AprA blocks various important complement functions hampering the bacterial clearance by human neutrophils. Furthermore, by using biological assays with more physiological relevant protease concentrations, we uncovered that AprA specifically blocks the CP and LP and predominantly cleaves complement protein C2.

**Materials and Methods**

**Proteins, sera, and bacterial strains**

C3 was purified from human plasma, as described (18). The purified components C1, C2, and C4 were purchased from Quidel. C1s was obtained from R&D, and C2-depleted serum was purchased from Sigma. Normal human serum was obtained from healthy volunteers, who gave informed consent. AprA and AprI of *P. aeruginosa* PAO1 were expressed and isolated from *Escherichia coli*, as described (14). Briefly, both proteins were expressed with a hexa-histidine tag in *E. coli* BL21 (Invitrogen). Recombinant proteins were isolated under denaturing conditions using a His trap column (GE Healthcare). AprA was refolded by dilution in 50 mM Tris-HCl (pH 9) containing 0.8 M l-arginine and 1 mM CaCl2. Refolding of AprI was performed by dilution in PBS. The His-tag of AprI was removed using enterokinase (Invitrogen). Both proteins were >95% pure, as assessed by SDS-PAGE and Instant Blue staining. Wild-type *P. aeruginosa* PAO1 was obtained from the Genome Sciences Manoil laboratory (University of Washington), and GFP-labeled PAO1 (19) was kindly provided by J.M. Beekman (University Medical Center Utrecht).

**Hemolytic assays**

The CP hemolytic assay was performed, as previously described (20), with minor modifications. Serum was preincubated with 22, 66, and 200 nM AprA or 200 nM AprA plus 1000 nM AprI for 30 min at 37°C. Subsequently, opsonized (anti-sheep IgM) sheep erythrocytes (Alsever) were incubated with AprA-treated serum in Veronal-buffered saline containing 0.5 mM CaCl2 and 0.25 mM MgCl2. After 30 min at 37°C, samples were centrifuged, and the absorbance of the supernatants at 405 nm was measured. The AP hemolytic assay was performed, as described above, using rabbit erythrocytes (Alsever) with Veronal-buffered saline containing 5 mM MgCl2 and 10 mM EGTA.

**FIGURE 1.** Secreted AprA concentration sufficient to inhibit complement activity. *A.* Overnight culture of *P. aeruginosa* (PAO1) was diluted 10 times in IMDM. After 2, 4, and 6 h, supernatant was collected and analyzed for AprA production by Western blot using a polyclonal Ab against AprA. *B.* Serum was preincubated with different concentrations of AprA (nM) or together with 1000 nM AprI for 30 min at 37°C. Subsequently, *E. coli* K12 (1 × 10⁵ CFU/ml) in RPMI-HSA was added to treated serum for 1 h at 37°C. Number of surviving bacteria/ml (CFU/ml) was determined by serial dilutions. Data represent mean ± SEM of three independent experiments. *C.* Serum was preincubated with AprA, with or without AprI (1000 nM), for 30 min at 37°C. Treated serum was mixed with opsonized sheep erythrocytes and incubated for 1 h at 37°C. Erythrocytes were pelleted, and OD of supernatant at 405 nm was measured. Data are expressed as relative lysis compared with lysed erythrocytes and represent mean ± SEM of three independent experiments.
E. coli killing

E. coli K12 was cultured in Luria–Bertani (LB) medium overnight at 37°C. The next day, the overnight culture was diluted 20 times in LB medium and grown to an OD600nm of 0.5. Bacteria were washed with RPMI 1640 supplemented with 0.05% human serum albumin (HSA). Human pooled serum was incubated with different concentrations of AprA, with or without 1000 nM AprI, for 30 min at 37°C in RPMI-HSA. Subsequently, bacteria were added (1 x 10^7/ml) and incubated for 1 h at 37°C. CFU were determined by plating serial dilutions on tryptic soy agar + 5% sheep blood.

Phagocytosis and killing

Phagocytosis assays were performed, as described (21). In short, serum was preincubated with buffer, 200 nM AprA, or 200 nM AprA plus 1000 nM AprI in RPMI 1640 containing 0.1% HSA for 30 min at 37°C. Then, 2.5 x 10^9 freshly isolated human neutrophils and 2.5 x 10^6 GFP-labeled, heat-killed P. aeruginosa were added and incubated for 15 min at 37°C while shaking at 600 rpm. The reaction was stopped by adding 1% ice-cold paraformaldehyde in RPMI 1640 containing 0.1% HSA. Phagocytosis was analyzed using the FACSCalibur (Becton Dickinson). Neutrophils were gated on the basis of forward- and side-scatter properties. Fluorescence intensity of 10,000 gated neutrophils was determined, and phagocytosis was expressed as the percentage of these cells containing fluorescently labeled bacteria (calculated using BD CellQuest Pro software). In another experiment, different concentrations of AprA were incubated with 5% serum in RPMI 1640 containing 0.1% HSA. In another experiment, different concentrations of AprA were incubated with 5% serum in RPMI 1640 containing 0.1% HSA. Phagocytosis was analyzed using the FACSCalibur (Becton Dickinson). Neutrophils were gated on the basis of forward- and side-scatter properties. Fluorescence intensity of 10,000 gated neutrophils was determined, and phagocytosis was expressed as the percentage of these cells containing fluorescently labeled bacteria (calculated using BD CellQuest Pro software). In another experiment, different concentrations of AprA were incubated with 5% serum in RPMI 1640 containing 0.1% HSA. Phagocytosis was expressed as the percentage of these cells containing fluorescently labeled bacteria (calculated using BD CellQuest Pro software).

Dose-dependent inhibition of phagocytosis by AprA

An overnight culture of P. aeruginosa strain PAO1-GFP in LB medium was washed in Veronal-buffered saline containing 0.5 mM CaCl_2, 0.25 mM MgCl_2, and 0.1% BSA. Serum was preincubated with different concentrations of AprA, with or without AprI, for 30 min at 37°C. Then, 2.5 x 10^9 bacteria were incubated with the preincubated serum for 30 min while shaking at 900 rpm. Bacteria were washed with PBS with 0.1% BSA. C3b deposition was detected using mouse anti-human C3b Abs (WM-1; American Type Culture Collection) and allophycocyanin-conjugated goat anti-mouse IgG (Protos). Median fluorescence of 10,000 bacteria was measured by flow cytometry.

C5a analysis

An overnight culture of P. aeruginosa strain PAO1 in LB medium was washed in RPMI 1640 with 0.1% HSA. Ten-percent serum and 200 nM AprA or 200 nM AprA plus 1000 nM AprI were preincubated for 30 min at 37°C and subsequently incubated with 5 x 10^7 bacteria at 37°C for 30 min while

**FIGURE 2.**

A. AprA inhibits complement-dependent effector functions important for P. aeruginosa. A and B. Phagocytosis of P. aeruginosa PAO1-GFP by human neutrophils in the presence of human serum and AprA. A. Serum was preincubated with buffer, AprA (200 nM), or AprA (200 nM) and AprI (1000 nM). B. Dose-dependent inhibition of phagocytosis by AprA in 5% serum with an IC_{50} value of 99.6 nM AprA (calculated with GraphPad Prism software using the sigmoidal dose-response function; R^2 = 0.8979). C. Inhibition of C3b deposition on P. aeruginosa by AprA. Serum was preincubated with buffer or different concentrations of AprA (nM) and mixed with bacteria. Deposition of C3b on the bacterial surface was quantified by flow cytometry, measuring the median fluorescence of 10,000 bacteria. The relative C3b deposition is the fluorescence relative to buffer. D. Inhibition of C5a release by AprA. Serum (10%) was preincubated with AprA, with or without AprI, and subsequently incubated with bacteria. Release of C5a in bacterial supernatants was measured by a calcium-mobilization assay using U937-C5aR cells. E. AprA blocks killing of P. aeruginosa by human neutrophils in 5% serum (AprA at 200 nM and AprI at 1000 nM). All figures represent mean ± SEM of three independent experiments. For D, the relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence. For E, the relative survival was calculated by dividing the number of CFU by the number of CFU at time 0.

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shaking at 600 rpm. Bacteria were centrifuged, and C5a was detected in collected supernatants by calcium mobilization: 10-fold–diluted supernatants were added to $5 \times 10^5$ Fluo-4-AM–labeled U937-C5aR cells (a generous gift from Prof. Eric Prossnitz, University of New Mexico, Albuquerque, NM), and the increase in intracellular calcium was measured by flow cytometry.

**Complement assays**

Complement ELISAs were performed, as described (22), with modifications. ELISA plates (MaxiSorp; Nunc) were coated overnight with 20 mg/ml LPS (*Salmonella enteriditis*; Sigma), 3 mg/ml IgM (Quidel), or 10 mg/ml mannan (*Saccharomyces cerevisiae*; Sigma) in 0.1 M sodium carbonate buffer (pH 9.6). Plates were blocked with 4% BSA in PBS with 0.05% Tween for 1 h at 37˚C. For the CP and LP, samples were diluted in Veronal-buffered saline containing 0.5 mM CaCl$_2$, 0.25 mM MgCl$_2$, 0.1% gelatin, and 0.05% Tween. For the AP, samples were diluted in Veronal-buffered saline containing 5 mM MgCl$_2$, 10 mM EGTA, 0.1% gelatin, and 0.05% Tween. Serum or C2-depleted serum was mixed with different concentrations of AprA or AprA together with AprI and subsequently added to the plates for 1 h at 37˚C. Deposited C3b and C4b were detected using Abs against C3d (WM-1–digoxigenin-labeled) and C4d (Quidel), respectively, followed by peroxidase-conjugated sheep anti-digoxigenin or a goat anti-mouse IgG (Southern Biotechnology), respectively. For the repletion experiment, IgM-coated plates were used, and the assay was performed, as described above. In short, 1% serum was preincubated with 200 nM AprA for 30 min at 37˚C. Then, 1000 nM AprI was added together with C1 (2 nM), C1s (3 nM), C2 (2 nM), C3 (67 nM), or C4 (20 nM), and the mixtures were added to the plate for 1 h at 37˚C. C3b deposition was detected, as described above.

**C2 cleavage**

C2 was incubated with different concentrations of AprA for 30 min at 37˚C in Veronal-buffered saline containing 0.5 mM CaCl$_2$ and 0.25 mM MgCl$_2$. Proteins were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Cleavage products were visualized with 0.1% Coomassie blue in 50% methanol, excised, and analyzed by N-terminal sequencing (Alphalyse).

**Western blotting**

The *P. aeruginosa* strain PAO1 was cultured overnight in LB medium and subsequently diluted 10 times in IMDM (Invitrogen). Bacterial supernatant was collected after 2, 4, and 6 h. The presence of AprA in supernatant of *P. aeruginosa* was analyzed by Western blotting using a protein G-purified polyclonal rabbit anti-AprA Ab (Genscript), followed by a goat anti-rabbit IgG Ab (Southern Biotech). Bands were quantified using ImageJ software.

Analysis of complement factor cleavage in serum was performed by Western blotting. Three-percent serum was incubated with different concentrations of AprA at 37˚C in PBS. The reaction was stopped by adding Laemmli sample buffer containing DTT. All samples were subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with 4% skimmed milk in PBS containing 0.1% Tween, C1s was detected by a mouse anti-human C1s Ab (Quidel), followed by HRP-labeled goat anti-mouse; C2, C3, and factor B (fB) were detected by goat anti-human C2 (Quidel), goat anti-human C3 (Protos), and goat anti-human fB (Quidel), respectively, followed by HRP-labeled donkey anti-goat (Jackson); and C4 was detected by biotin-labeled chicken anti-human C4 (Abcam), followed by HRP-labeled streptavidin (Southern Biotech). All Ab incubations were diluted in PBS with 0.1% Tween and 1% skimmed milk. ECL (GE Healthcare) was used for final signal detection.

**Results**

**Physiological AprA levels inhibit complement-mediated lysis**

To study the functional relevance of AprA secretion by *P. aeruginosa* on the complement system, we first estimated AprA concentrations in culture supernatants. Supernatants were collected at
different time points and analyzed for AprA expression by Western blotting using an Ab against AprA (Fig. 1A). The levels of AprA in the supernatant were semiquantified by comparison with fixed concentrations of recombinant AprA (Supplemental Fig. 1). The AprA concentrations in culture supernatants were between 8 nM (2-h culture) and 208 nM (10 μg/ml; 6-h supernatant). Because AprA was described to inhibit complement-dependent hemolysis via MAC, we repeated the assay to confirm AprA activity. AprA inhibited the lysis of Ab-sensitized sheep erythrocytes, a CP-mediated hemolysis assay, which was restored in the presence of AprI (Fig. 1B). Furthermore, we tested different concentrations of AprA in a serum-killing assay using *E. coli* as a model. In contrast to *P. aeruginosa* strain PAO1, *E. coli* K-12 is not serum resistant and is lysed at low serum concentrations as a result of incorporation of the lytic MAC. Indeed, AprA inhibited the complement-dependent lysis with an estimated IC₅₀ of 30 nM, which is within the concentration range detected in the supernatant (Fig. 1C). The natural inhibitor AprI blocks proteolytic activity of AprA (23), thereby allowing complement-dependent lysis of *E. coli*. Thus, AprA blocks complement-dependent lysis in a concentration range similar to AprA levels in *P. aeruginosa* supernatant.

AprA inhibits complement-dependent neutrophil functions important for *P. aeruginosa* killing

Because many *P. aeruginosa* strains are resistant to direct complement lysis (24), we investigated whether AprA facilitates resistance to other complement processes that are critical to neutrophil functioning. To study whether AprA blocks complement-dependent phagocytosis, we incubated GFP-labeled *P. aeruginosa* with human neutrophils in the presence of human serum and AprA. AprA blocked the phagocytosis compared with buffer or AprA supplemented with AprI (Fig. 2A), and its activity was dose dependent (Fig. 2B). The IC₅₀ of AprA is 100 nM, which is in the relevant range produced by *P. aeruginosa*. Incubation of *P. aeruginosa* with serum results in the deposition of C3b molecules on the bacterial surface, which are recognized by complement receptors on phagocytes. To study whether AprA inhibits the C3b deposition, we incubated bacteria with serum and detected surface-bound C3b using specific Abs and flow cytometry. AprA partially inhibited C3b deposition on *Pseudomonas* at comparable concentrations as observed for phagocytosis of *P. aeruginosa* (Fig. 2C). Further along the complement cascade, C5 is cleaved into the potent anaphylatoxin C5a that is recognized by the C5a receptor on neutrophils. Activation of the C5a receptor induces intracellular calcium mobilization and chemotaxis of the neutrophil to the site of inflammation. We incubated *P. aeruginosa* with serum and studied the release of C5a into the supernatant by measuring the supernatant-induced calcium mobilization on Fluo-3-AM–labeled U937 cells transfected with the C5a receptor (specificity of this assay is presented in Supplemental Fig. 2). When bacteria were incubated with AprA, supernatants could not induce calcium mobilization, meaning that there was no active C5a in the presence of AprA (Fig. 2D). Because AprA also blocks formation of C5b-9 (Fig. 1B, 1C), we believe that AprA does not specifically cleave C5a but prevents activation of C5 into C5a and C5b. Because AprA also blocks C3b deposition, it seems likely that it inhibits complement activation upstream of C3. To verify that AprA also inhibits the killing of *P. aeruginosa* by neutrophils, we incubated neutrophils with bacteria and serum and determined the percentage of survival after 15 min. After 15 min, 80% of the bacteria were killed. AprA inhibited the killing of *P. aeruginosa*, but not completely, showing a killing of 40% after 15 min. This effect was restored in the presence of AprI (Fig. 2E). In summary, AprA inhibited all complement-dependent effector functions, which allowed *P. aeruginosa* to escape neutrophil killing.

**FIGURE 5.** AprA depletes the limiting factor C2. A, AprA degrades C2 and C1s. Different concentrations of AprA were incubated with 3% serum for 30 min at 37°C. C1s, C2, C3, C4, and IB were detected by Western blotting. B, C2 is the limiting factor. Serum (1%) was preincubated with 200 nM AprA for 30 min at 37°C. Then, 0.2 μM AprI was added to stop the AprA cleavage, together with C1, C1s, C2, C3, or C4. Complement activation via the CP was determined by ELISA by detecting C3b deposition. For **B**, the data represent mean ± SEM of three independent experiments. Blots are representatives of three separate experiments.
MASP complexes. To study at what level AprA affects these pathways, we also analyzed the C4b deposition. In contrast to C3b deposition, AprA did not block C4b deposition in the CP and LP (Fig. 3D,3E). This indicated that AprA specifically blocks the CP upstream of C3 and downstream of C4 activation in the complement cascade.

AprA depletes the limiting factor C2

Many complement factors that are involved in the CP and LP induce C3b deposition; however, only two factors (C2 and C3) are involved after C4b deposition and before C3b deposition. To identify the critical factor that is cleaved by AprA, we incubated human serum with AprA for 30 min at 37°C. The complement factors C2, C3, and C4 were analyzed by Western blotting. As controls, C1s and fB were detected, which are crucial components of the CP and AP, respectively. AprA efficiently cleaved C2, whereas C3, C4, and fB were not affected (Fig. 5A). In addition, cleavage of C1s by AprA was observed; however, this was less efficient than C2 cleavage. We used the CP complement ELISA to discriminate the limiting factor that was cleaved by AprA. First, we incubated serum with AprA and inactivated the protease by adding the irreversible inhibitor AprI. Then, treated serum was used in the CP ELISA supplemented with the different purified complement factors. Only the serum that was supplemented with purified C2 restored the complement activation, whereas AprA-treated serum supplemented with C1, C1s, or C3 showed no activity (Fig. 5B). Thus, although AprA also cleaves C1s, the cleavage of C2 determines inhibition of complement activation.

AprA cleaves C2

C2 is a 100-kDa protein that consists of three complement control protein (CCP) regions, a von Willebrand factor A (vWFA) domain, and a peptidase domain. The cleavage of C2 by its natural proteases C1s and MASP results in formation of C2b (CCP1–3) and C2a (vWFA and peptidase domain) (Fig. 6B,6C). For the formation of a functional C3 convertase (C4b2a), full-length C2 first binds to C4b and is subsequently cleaved. C2b is then released, while C2a stays attached to C4b. The C4b2a convertase has a very short half-life due to the irreversible dissociation of C2a. C2 cleavage by AprA was further analyzed by SDS-PAGE and Coomassie staining. After incubating purified C2 with AprA, we observed three cleavage products of ∼75, ∼40, and ∼30 kDa, which were sent for N-terminal sequencing (Fig. 6A). Cleavage product 1 starts with NH2-IQIQRS, which is only 1 aa different from C2a (starts with NH2-KIQIQR). At higher protease concentrations, an increase in cleavage product 2 is observed, meaning that AprA secondarily cleaves between the vWFA and peptidase domain. Next to C2a, AprA cleaves C2b between CCP1 and CCP2. These data indicated that AprA efficiently interferes with CP and LP activation via the degradation of C2 (Fig. 7).

Discussion

Bacterial pathogens adopt many strategies to counteract the redundant attack of the human host defense. For instance, bacteria
secrete many proteins that specifically interact with the complement system, which can either be proteolytic or steric interactions (25, 26). In the defense against Gram-negative bacteria, the complement system directly destroys cells by inserting the pore-forming MAC into the bacterial membrane (27). Although all Gram-positive bacteria are protected from MAC lysis by their thick peptidoglycan layer, some Gram-negative bacteria have also become resistant to direct complement attack by altering their surface properties (28). The Gram-negative bacteria \textit{P. aeruginosa} does this by lengthening the \textit{O-Ag} side chains of LPS in the outer membrane (24). However, resisting the MAC is likely not sufficient for Gram-negative pathogens to fully overcome complement-mediated immune clearance. Complement also labels these bacteria with C3b to support phagocytic uptake and generates C5a to attract phagocytes to the site of infection. In vivo studies showed that complement receptor 3 (29, 30) and the C5a receptor (31–33) on neutrophils are important for protection against \textit{P. aeruginosa} lung infection. In this study, we showed that \textit{P. aeruginosa} also inhibited other steps in the complement cascade by secretion of the metalloprotease AprA. AprA blocks CP and LP activation and, thereby, prevents C3b-dependent uptake of \textit{Pseudomonas} by neutrophils and C5a-dependent neutrophil activation. Previously, it was shown that AprA inhibits MAC-dependent lysis of erythrocytes (12). In this study, we determined how AprA contributes to pseudomonal resistance against complement effector functions relevant to host clearance of this bacterium. Furthermore, we identified the molecular mechanism to be cleavage of C2.

AprA is the first bacterial protease demonstrated to cleave C2. Because C2 concentrations in serum (0.25 \mu M) are low compared with other complement molecules (C3 at 6.8 \mu M, C4 at 2.1 \mu M), this molecule is a vulnerable target for proteolytic degradation. Interestingly, we found that AprA cleaves C2 very close to the cleavage site of its natural proteases C1s and MASP. Strikingly, this is similar to the \textit{Staphylococcus aureus} metalloprotease aureolysin, which cleaves the C3 molecule 2 aa apart from the natural protease-cleavage site (34). This suggests that the natural cleavage site in these proteins is an accessible part of the protein that makes it vulnerable for cleavage by bacterial proteases. Fig. 7 provides a schematic overview of how we think AprA blocks complement activation. The formation of the C4b2a convertase is a multistep process (35). First, C2 binds surface-bound C4b and is subsequently cleaved into C2a, forming the active convertase. Because of its short half-life, C2a is released from the complex and cannot reassociate with C4b. The fluid-phase cleavage of C2 by AprA prevents formation of the proconvertase C4b2C and an active C4b2a complex. At present, it is not clear whether the additional cleavage sites of AprA in C2 (between CCP1 and CCP2 and between the vWFA and peptidase domain) are functionally relevant. Although some residues of the AprA-cleavage sites in C2 can also be found in factor B (the protein with the greatest homology to C2), we did not observe any cleavage of purified FB (data not shown) or FB in serum (Fig. 5). We expect that structural differences between FB and C2 could explain the difference in accessibility of the cleavage site. However, the lack of structural data on C2 makes it difficult to speculate about this point.

Production of AprA by \textit{P. aeruginosa} differs among strains and is highly dependent on culture conditions (36). This is illustrated by the enhanced production of AprA by culturing in the presence of sputum from CF patients (37). \textit{P. aeruginosa} produces detectable levels of AprA in vivo, as measured in samples obtained from patients with acute infections (38) and in sputum isolated from CF patients (16, 17). The observed in vivo concentrations tend to be in a different range than observed in \textit{P. aeruginosa} overnight cultures and the concentrations used in our study. However, determination of AprA concentration in vivo is complicated by the fact that AprA is produced locally in the bacterial environment and is subsequently diluted or degraded by host proteases in clinical samples. In a rat pouch model, only small amounts of injected recombinant AprA could be recovered after 6 h (39), suggesting that production of AprA in vivo is much greater than measured. In this article, we showed that AprA cleaves C2 in concentrations similar to levels secreted by \textit{P. aeruginosa} in functionally relevant time incubations. However, increasing AprA concentrations or incubation times allowed cleavage of more complement factors, such as C3 and C4 (data not shown). A previous article also showed that AprA cleaved C3 and C1q, but high concentrations of AprA (400 nM) and long incubation times (20 h) were used (12). Western blotting experiments showed that AprA (at 200 nM) effectively cleaved C1s in serum within 30 min (Fig. 5A). However, the functional-repletion assay (Fig. 5B) clearly showed that only C2 restored C3b deposition through the CP, suggesting that C1s is still active. Possibly, C1s is cleaved in a manner that does not block its function. This could be similar to natural activation of C1s, which is mediated by the protease C1r that cleaves thezymogen C1s into its active form, which can then cleave C4. Thus, by cleaving C1s, AprA may have paradoxically enhanced CP activity up to the stage of C4b deposition. Indeed, we observed that C4b deposition in the CP was increased in the presence of AprA. However, because AprA cleaves C2, we found that it blocked the CP at the level of C3b. The cleavage of C2 is in line with our findings that AprA does not affect the AP, because activation of this route is not dependent on C2. The AprA concentrations used for cleavage of C2 were in a similar range as previous studies examining AprA cleavage of flagellin and other human immune proteins (11, 14).

Thus, we showed that AprA blocks activation of the CP and LP by degrading C2. Thereby \textit{P. aeruginosa} evades complement-mediated phagocytosis and killing by neutrophils. Other known complement-modulating proteins of \textit{P. aeruginosa} are: the intracellular elongation factor tu that binds factor H (40), elastase that degrades C3 (41), and protease IV that degrades C1q and C3 (42). As observed for other bacterial pathogens (26), complement resistance factors in \textit{P. aeruginosa} are probably as redundant as the complement system itself. Especially because \textit{P. aeruginosa} strains from CF patients are sensitive to complement C5b-9 lysis in vitro (43, 44), we expect this bacterium to have multiple molecules that counteract complement activation and enable bacterial survival in the human host.

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Disclosures
The authors have no financial conflicts of interest.

References
COMPLEMENT INHIBITION BY P. AERUGINOSA


Supplemental Figure 1

AprA concentrations in bacterial supernatant. Quantification of the western blot presented in Figure 1 was calculated using ImageJ software. Left, standard curve of fixed AprA concentrations. Right, AprA concentration in PAO1 supernatant at different time points calculated using the standard curve.

Supplemental Figure 2

AprA inhibits calcium flux generated by C5a. (a) PAO1 or heat-killed (hk) PAO1 was incubated with buffer, 10% serum, 10% C5-deficient serum or 10% C5-deficient serum supplemented with C5. Release of C5a in bacterial supernatants was measured by a calcium mobilization assay using U937-C5aR cells. No calcium mobilization was observed for non-transfected U937 cells (b) Non-transfected U937 cells and U937 cells transfected with C5aR were stimulated with different concentrations C5a.
Supplemental figure 1

\[ y = 0.312 + 0.097x - 0.00021x^2 \]

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Supplemental figure 2

(a) Calcium Mobilization

- Pa + buffer
- Pa hK + buffer
- Pa + 10% serum
- Pa hK + 10% serum
- Pa + 10% C5 def serum
- Pa hK + 10% C5 def serum + C5

(b) Calcium Mobilization vs C5a (M)

- C5aR
- Control

Graph showing the effect of C5a on calcium mobilization in different conditions.