Streptococcus pneumoniae Phosphoglycerate Kinase Is a Novel Complement Inhibitor Affecting the Membrane Attack Complex Formation*

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The Gram-positive bacterium Streptococcus pneumoniae is a major human pathogen that causes infections ranging from acute otitis media to life-threatening invasive disease. Pneumococci have evolved several strategies to circumvent the host immune response, in particular the complement attack. The pneumococcal glycolytic enzyme phosphoglycerate kinase (PGK) is both secreted and bound to the bacterial surface and simultaneously binds plasminogen and its tissue plasminogen activator tPA. In the present study we demonstrate that PGK has an additional role in modulating the complement attack. PGK interacted with the membrane attack complex (MAC) components C5, C7, and C9, thereby blocking the assembly and membrane insertion of MAC resulting in significant inhibition of the hemolytic activity of human serum. Recombinant PGK interacted in a dose-dependent manner with these terminal pathway proteins, and the interactions were ionic in nature. In addition, PGK inhibited C9 polymerization both in the fluid phase and on the surface of sheep erythrocytes. Interestingly, PGK bound several MAC proteins simultaneously. Although C5 and C7 had partially overlapping binding sites on PGK, C9 did not compete with either one for PGK binding. Moreover, PGK significantly inhibited MAC deposition via both the classical and alternative pathway at the pneumococcal surface. Additionally, upon activation plasmin(ogen) bound to PGK cleaved the central complement protein C3b thereby further modifying the complement attack. In conclusion, our data demonstrate for the first time to our knowledge a novel pneumococcal inhibitor of the terminal complement cascade aiding complement evasion by this important pathogen.

Background: Pneumococci employ multiple strategies to escape host complement attack.

Results: PGK directly inhibits MAC formation and aids degradation of C3b by plasminogen.

Conclusion: PGK is a novel complement inhibitor.

Significance: The findings expand our knowledge of complement evasion by pneumococci and encourage a search for the role of the MAC in defense against Gram-positive pathogens.

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The complement system consisting of both soluble and cell surface-bound proteins is an essential arm of the host’s innate immune system (1). Upon contact with bacterial or non-self surfaces, the complement components initiate a tightly regulated proteolytic cascade resulting in the opsonization of bacteria with C3b for phagocytosis, generation of anaphylatoxins C3a and C5a, and formation of the cytolytic membrane attack complex (MAC)3 or C5b-9 for direct bacterial killing (1–3). Depending on the mode of recognition the complement system can be activated by three different routes, the classical, the lectin, and the alternative pathway all of which results in the formation of C3 convertases. This enzyme complex cleaves the central complement protein C3 into C3a and C3b (opsonin). The generated C3b molecules then interact with the C3 convertases resulting in the formation of C5 convertases that cleave C5 into C5a and C5b. The generated C5b is first bound by C6 to form a C5b6 complex followed by C7 resulting in a hydrophobic C5b-7 complex that attaches and is inserted into the target membranes. Subsequently, C8 is incorporated followed by multiple (10–15) C9 molecules that polymerize to form the lytic transmembrane pore-forming structure (4, 5).

Considering the destructive potential of complement and to restrict its action on non-self surfaces, the cascade is under tight regulation of both soluble and surface-bound inhibitors such as soluble vitronectin and clusterin or the membrane-bound glycoprotein CD59 that all inhibit MAC formation (6–9). The lack of these inhibitors predisposes the cells for complement-dependent cytolysis.

The complement is a particular target of evasion strategies developed by pathogens that produce a plethora of virulence factors that modulate almost all stages of the complement cas-

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3 The abbreviations used are: MAC, membrane attack complex; Abs, antibodies; C4BP, C4b-binding protein; GMFI, geometric mean fluorescence intensity; GVB2, gelatin-Veronal buffer; DGV8, dextrose-containing GVB buffer; NHS, normal human serum; o/n, overnight; PGK, phosphoglycerate kinase; RT, room temperature; FH, Factor H; uPA, urokinase like plasminogen activator; tPa, tissue plasminogen activator; ANOVA, analysis of variance.
C3b was labeled with 125I using the chloramine-T method. C5b-6, C7, C8, and C9 were from Complement Technology. Serum were purchased from Quidel, whereas C3b, C5, C6, purified from human plasma (29). C1q-, C5-, and C9-depleted N-terminal His6 tag was expressed and purified as described (19, 20). The Alexa Fluor (488)-labeled rabbit anti-mouse Ig conjugate was from (Invitrogen), whereas the low M₉ heparin was from the National Institute for Biological Standards and Control.

Normal human serum (NHS) was prepared from freshly drawn blood obtained from healthy volunteers with informed consent and according to the recommendations of the local ethical committee in Lund (permit 418/2008). The pooled blood was allowed to clot for 30 min at room temperature (RT) and then incubated for 1 h on ice. After two centrifugations, the serum fraction was frozen in aliquots and stored at −80 °C.

Binding from Serum—Microtiter plates were coated with PGK or BSA in PBS overnight (o/n) at 4 °C. After incubation with blocking solution (50 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20, 3% fish gelatin (Norland)) for 2 h at RT, NHS diluted in binding buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl₂, and 50 µg/ml BSA) was added to the wells at concentrations indicated in the figures. After incubation for 1 h and 30 min at RT bound plasminogen was detected. The plates were developed with o-phenylenediamine (Dako) substrate and H₂O₂, and the absorbance at 490 nm (Varian Cary 50 MPR Microplate Reader) was measured.

125I-C3b Degradation—Microtiter plates were coated with 50 µl of PGK (5 µg/well) in PBS o/n at 4 °C. BSA was used as the negative control. Blocking was performed with 250 µl of blocking solution for 2 h at RT. After washing, tPA or urokinase like plasminogen activator (uPA; 10 units/well) diluted in binding buffer was incubated with the immobilized PGK for 1.5 h at RT. After washing, plasminogen (0.5 µg/well) together with 125I-labeled C3b (100 kcpm) were added and incubated for 1.5 h at 37 °C. The positive and negative control reactions for C3b degradation were prepared with Factor H and with or without Factor I, respectively. The reaction was stopped by the addition of reducing SDS-PAGE sample buffer and boiling at 95 °C for 5 min. Thereafter, the samples were separated by SDS-PAGE. Degradation products of C3b were visualized using phosphorimaging (Fuji).

Hemolytic Assay—The activity of the classical pathway of complement activation was assessed using the sheep erythrocytes (Håtunalab). The erythrocytes were washed 3 times with ice-cold DGVB²⁺ buffer (2.5 mM Veronal buffer (containing sodium-diethyl-barbiturate and 5,5’-diethyl-barbiturate acid) (pH 7.3), 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂), resuspended to a concentration of 10⁹ cells/ml, and incubated with gentle shaking for 20 min at 37 °C with an equal volume of amboceptor (Dade Behring) diluted 1/3000 in DGVB²⁺ buffer. Amboceptor is an anti-sheep erythrocyte Ab that activates the classical pathway of complement. After 2 washes, 8 × 10⁷ cells/ml were incubated for 1 h at 37 °C with 0.2% NHS and increasing concentrations of PGK, diluted in DGVB²⁺ buffer, in a total volume of 150 µl. BSA (Applichem) was used as a negative control. To assess the activ-

Pneumococcal PGK Inhibits MAC

cade. This includes inhibition of complement recognition and amplification, interference with complement receptor interaction on phagocytes, or cleavage of complement proteins (10–12). Although Gram-negative bacteria are highly susceptible to lysis by MAC, the Gram-positive organisms are not (13) even though the presence of some terminal pathway components on the bacterial surface has been reported (14–16). Protection against MAC-derived lysis is explained by the presence of a thick peptidoglycan layer that interferes with the insertion of C5b9 into the target membrane (17, 18). Therefore, the clearance of these Gram-positive organisms from the host is believed to depend predominantly on opsonization and phagocytosis. Interestingly, the deficiency of MAC components in patients was associated with increased susceptibility to bacterial infections not only with Neisseria but also some Gram-positive species (19). Moreover, despite the currently perceived non-essential role of MAC in Gram-positive bacterial killing, many Gram-positive bacteria such as Streptococcus pyogenes (Group A streptococci) and Staphylococcus aureus not only express proteins that inhibit MAC formation but also recruit the host inhibitors of MAC (20, 21).

Interestingly, the Gram-positive bacterium Streptococcus pneumoniae (pneumococcus) has also evolved several strategies to evade the host immune attack. Pneumococci express several surface proteins that recruit the host complement inhibitors such as Factor H, vitronectin, and C4b-binding protein (C4BP) to the bacterial surface to control the complement-mediated attack (22–26). Although S. pneumoniae are commensals that asymptomatically colonize the upper respiratory tract, any alteration in host-pathogen homeostasis allows pneumococci to gain access to the normally sterile parts of the airways and to cause local infections. These include mild infections such as otitis media and sinusitis or life-threatening pneumonias that asymptomatically colonize the upper respiratory tracts of children >5 years of age in developing countries (27).

The pneumococcal glycolytic enzyme phosphoglycerate kinase (PGK) has recently been identified as a surface-exposed and secreted protein that binds plasminogen and its activator tissue plasminogen activator (tPA) (28). In the present study we demonstrate that PGK has additional function in modulating the complement attack. PGK interacts with the MAC components thereby blocking their assembly and membrane insertion. To our knowledge, we provide the first data presenting S. pneumoniae PGK as a terminal pathway inhibitor.

EXPERIMENTAL PROCEDURES

Proteins and Abs—Pneumococcal PGK (41.9 kDa) with an N-terminal His6 tag was expressed and purified as described (28). Polyclonal antiserum against purified PGK was raised in rabbits (28). C4BP (570 kDa) and Factor H (FH, 150 kDa) were purified from human plasma (29). C1q-, C5-, and C9-depleted serum were purchased from Quidel, whereas C3b, C5, C6, C5b-6, C7, C8, and C9 were from Complement Technology. C3b was labeled with 125I using the chloramine-T method. Human glu-plasminogen and sheep anti-human plasminogen antibodies (Abs) were purchased from Hematologic Technologies, goat anti-human C5 and C7 Abs were from Quidel, and goat anti-human C6, C8, and C9 were from Complement Technology. The mAb e11 that recognizes a neo-epitope present in polymeric C9 of C5b9-MAC complex but absent in monomeric C9 was purchased from Hycult, and the peroxidase-conjugated swine anti-rabbit and rabbit anti-goat IgG were from Dako. The Alexa Fluor (488)-labeled rabbit anti-mouse Ig conjugate was from (Invitrogen), whereas the low M₉ heparin was from the National Institute for Biological Standards and Control.

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Pneumococcal PGK Inhibits MAC

The presence of the alternative pathway, rabbit erythrocytes (obtained from a local farm) were washed 3 times with Mg$^{2+}$-EGTA buffer (2.5 mM Veronal buffer (pH 7.3) containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl$_2$, and 10 mM EGTA). Erythrocytes at a concentration of 6 × 10$^7$ cells/ml were incubated for 1 h at 37 °C with 1.25% NHS together with increasing concentration of PGK, FH, or BSA diluted in Mg$^{2+}$-EGTA buffer in a total volume of 150 μl. After the incubation the samples were centrifuged at 800 × g to pellet intact cells, and the hemolytic activity (i.e. the number of lysed erythrocytes and released hemoglobin) was determined by spectrophotometric measurement of absorbance at 405 nm. The lysis obtained in the absence of inhibitor was defined as 100% hemolytic activity.

To analyze the effect of PGK on inhibition of MAC formation using purified MAC components, amboceptor-coated sheep erythrocytes were first preincubated with C5b-6 (1 μg/ml) for 10 min at 37 °C in DGVB$^{2+}$ buffer with shaking. The cells were then washed with DGVB$^{2+}$ and suspended in a solution of C7 (1 μg/ml), C8 (0.2 μg/ml), and C9 (1 μg/ml) that had been preincubated for 10 min on ice with increasing amounts (0–80 μg/ml) of PGK or BSA (negative control) for 30 min at 37 °C with shaking. Alternatively, C7 alone was preincubated for 10 min on ice with 50 μg/ml PGK or BSA before incubation with the C5b-6 coated erythrocytes for 30 min at 30 °C. This was followed by washing and the addition of C8 (0.2 μg/ml) and C9 (1 μg/ml). After 30 min at 37 °C, cells were centrifuged, and the extent of cell lysis was determined by measuring the release of hemoglobin at 405 nm.

To investigate the effect of PGK-C5 interaction on inhibition of MAC formation, amboceptor-coated sheep erythrocytes were mixed with 1% C5-depleted serum diluted in DGVB$^{2+}$ and incubated at 37 °C for 5 min with shaking to allow formation of C5 convertases on the erythrocyte surface (30). The cells were then washed with DGVB$^{2+}$ and suspended in solution of C5 (0.5 μg/ml) and C6 (0.75 μg/ml) that had been preincubated for 45 min on ice with increasing amounts of PGK or BSA (negative control) for 30 min at 37 °C with gentle shaking. The cells were then washed with DGVB$^{2+}$ and suspended in solution of C7 (1 μg/ml), C8 (0.2 μg/ml), and C9 (1 μg/ml). Cell lysis was allowed to proceed for 30 min at 37 °C after which the cells were sedimented, and the absorbance of the supernatant at 405 nm was measured.

Complement Activation Assays—To assess the ability of PGK to activate the complement pathway, microtiter plates (Maxisorp; Nunc) were coated with 50 μl of PGK (5 μg/ml), aggregated human IgG (2.5 μg/ml for the classical pathway), mannan (100 μg/ml (Sigma) for the lectin pathway), or zymosan (20 μg/ml (Sigma) for the alternative pathway) in PBS o/n at 4 °C. Wells coated with BSA were used as a control. The plates were washed with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 between each step. Plates were blocked with 250 μl of 1% BSA in PBS for 2 h at RT. After blocking, the plates were washed, and dilutions of NHS or serum deficient in C1q in GVB$^{2+}$ (5 mM Veronal buffer (pH 7.4), 144 mM NaCl, 1 mM MgCl$_2$, 0.15 mM CaCl$_2$, and 1% gelatin) for classical and lectin pathway, respectively, were added to the plates and incubated for 15 min (for detection of C3b) or 45 min (for C9 detection) at 37 °C. For the alternative pathway, the plates were incubated with NHS diluted in Mg$^{2+}$-EGTA buffer and incubated for 20 min (C3b) and 60 min (C9) at 37 °C. Following the incubation deposited complement proteins were detected using specific rabbit polyclonal Abs against C3d or C9 diluted 1:2000 in 1% BSA in PBS and horseradish peroxidase-labeled secondary Abs against rabbit (1:2000 in blocking solution) for 1 h each at RT. The plates were developed with o-phenylenediamine substrate and H$_2$O$_2$, and the absorbance at 490 nm was measured. For inhibition of deposition of terminal complement components (MAC components C5b-9) from all three complement pathways by PGK, microtiter plates were coated with respective positive activators as described above in PBS o/n at 4 °C. After washing, the plates were blocked using 1% BSA in PBS for 2 h at RT. Different concentrations of NHS (1.0% for classical, 0.75% for lectin, and 2.5% for alternative pathway) diluted in GVB$^{2+}$ or Mg$^{2+}$-EGTA buffer were incubated with increasing concentrations of PGK, C4BP, FH, or BSA at 37 °C for 15 min at 37 °C after which the mixtures were added to the activator-coated plates. The plates were incubated at 37 °C, and deposited MACs were detected using the mAb aE11.

Direct Binding Assays—Microtiter plates were coated with PGK (5 μg/ml), C7 (5 μg/ml), or C9 (5 μg/ml) in PBS o/n at 4 °C. Wells coated with BSA were used as the control. After incubation with blocking solution for 2 h at RT, the plate was incubated with increasing concentrations of plasma-purified terminal complement components C5, C6, C7, C8, and C9 for binding to PGK or PGK for binding to C7 or C9 in binding buffer and incubated for 1 h and 30 min at RT. For investigation of the effect of ionic strength and the presence of heparin on PGK-C7 or PGK-C9 interactions, the binding buffer was supplemented with NaCl to final concentrations ranging from 0 to 800 mM or with 0–800 μg/ml heparin. In competition binding assays, a mixture of C5, C7, and C9 or plasminogen and C9 was added to PGK-coated wells. Bound MAC components, plasminogen, or PGK were detected using specific polyclonal Abs followed by peroxidase-conjugated secondary Abs. The plates were developed with o-phenylenediamine substrate and H$_2$O$_2$, and the absorbance was measured at 490 nm. $K_D$ values for the binding of C7 or C9 with PGK were calculated from the binding curves using nonlinear regression analysis, applying the equation for one-site binding (GraphPad Prism 5.0).

Inhibition of C9 Polymerization—Purified human C9 (0.5 μM) was incubated with or without PGK (at concentrations of 0–5 μM) in 20 mM Tris buffer (pH 7.2) overnight at 37 °C. BSA (5 μg) was used as negative control. The samples were mixed with reducing sample buffer, incubated at 95 °C for 5 min, run on 2.5–10% gradient SDS-PAGE, and transferred onto a PVDF membrane. The membrane was blocked with 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 30% fish gelatin at pH 8.0. C9 was detected with goat anti-C9 Abs followed by horseradish peroxidase-conjugated rabbit anti-goat secondary Abs and visualized with a 3,3′-diaminobenzidine tetrahydrochloride colorimetric substrate system (Sigma).

Inhibition of C9 Incorporation into MAC—Amboceptor-coated sheep erythrocytes were prepared as described above and resuspended in DGVB$^{2+}$ to a concentration of 5 × 10$^8$ cells/ml. The cell suspension (30 μl) was mixed with 0.5% C9-depleted serum diluted in DGVB$^{2+}$ and incubated at 30 °C
for 30 min with shaking to allow formation of a C5b-8 complex on the erythrocyte cell surface. The cells were thereafter washed twice with DGVB²⁺ and resuspended in 1 µg/ml C9 that had been preincubated for 30 min on ice with increasing amounts of PGK or BSA (negative control). Cell lysis was allowed to proceed for 30 min at 37 °C after which the cells were sedimented, and the absorbance of the supernatant was measured at 405 nm.

**Bacterial Strains and Culture Conditions**—All pneumococcal strains used in this study, except S. pneumoniae NCTC10319 (serotype 35A) and D39 (serotype 2), were clinical isolates collected at the Clinical Microbiology Laboratory, Skåne University Hospital (Malmö, Sweden) (Table 1). Pneumococci were cultured on blood agar plates at 37 °C and 5% CO₂.

**Flow Cytometry Analysis of MAC Deposition on Pneumococci**—The deposition of MAC from NHS via the classical and alternative complement pathway on pneumococcal surface was measured using flow cytometry. Pneumococci were cultured o/n on blood agar plates, washed in PBS, and adjusted to 10⁹ cfu/ml. Bacteria (5 × 10⁷ cfu) were incubated with 1.0 and 2.0% NHS or at concentrations indicated in figure for 1 h at 37 °C in GVB²⁺ or Mg²⁺EGTA buffer for the classical and alternative pathway, respectively. In inhibition assays, pneumococci NCTC10319 (5 × 10⁷ cfu in 100 µl) were incubated with NHS (0.2 and 2.0% for classical and alternative pathway, respectively) in the absence or presence of PGK that was used as inhibitor. Deposited MAC was detected using mAb aE11 and rabbit anti-mouse Alexa488-labeled Ig. Finally, bacteria were fixed using 1% paraformaldehyde (Sigma), and the flow cytometry analysis was performed using CyFlow space (Partec) to detect the MAC deposition. Bacteria were analyzed using log-forward and log-side scatter dot-plot, and a gating region was set to exclude debris and larger aggregates of bacteria. 15,000 bacteria/events were analyzed for fluorescence using log-scale amplifications. The geometric mean fluorescence intensity (GMFI) was used as a measure for binding activity.

**RESULTS**

**Plasminogen Bound to PGK Is Activated to Plasmin and Cleaves C3b**—The pneumococcal glycolytic enzyme PGK binds plasminogen and its activator tPA (31). However, it is not clear whether PGK can also recruit plasminogen from human serum. To assess this, microtiter plates coated with PGK were incubated with several dilutions of human serum, and binding of plasminogen was detected with specific Abs. A dose-dependent increase in binding of plasminogen proportional to the serum concentration was obtained (Fig. 1A).

It is known that plasminogen interacts with complement protein C3 and upon activation to plasmin inactivates complement at the level of C3 (32). To determine, if PGK-bound plasminogen can also affect complement, tPA or uPa bound to immobilized PGK was incubated with plasminogen and ¹²⁵I-labeled C3b. After the incubation samples were separated by SDS-PAGE, and C3b cleavage products were analyzed. The resulting protein signal pattern revealed cleavage of C3b by activated plasminogen (plasmin) (Fig. 1B). Importantly, no C3b cleavage was observed upon incubation with the activators alone or in the presence of BSA (Fig. 1B), suggesting that the plasminogen bound to PGK was converted to proteolytically active plasmin, which in turn cleaved C3b.

**PGK Inhibits the Hemolytic Activity of Human Serum**—Because PGK is also a secreted protein, we asked whether it has any additional role in modulating the complement attack. To investigate whether the pneumococcal PGK inhibits the activity of the classical and the alternative complement pathways, hemolytic assays were performed. Human serum was mixed with various concentrations of recombinant PGK and was subsequently incubated with erythrocytes. The results demonstrate a significant dose-dependent inhibition of the lysis of erythrocytes through the classical pathway (Fig. 2A), whereas PGK had only a negligible effect on lysis mediated by the alternative pathway even at the highest concentrations (Fig. 2B).
FIGURE 2. PGK inhibits the hemolytic activity of human serum. To measure the inhibitory effect of PGK on the classical pathway, amboceptor-coated erythrocytes were subjected to complement attack from NHS in the presence of increasing amounts of pneumococcal PGK in DGVB2 \(-\) buffer (A). BSA was used as the negative control. The degree of lysis was estimated by measurement of the release of hemoglobin. To study inhibition of the alternative pathway, rabbit erythrocytes were incubated with NHS with increasing concentrations of pneumococcal PGK in Mg\(^{2+}\) \(-\) EGTA buffer (B). FH and BSA were used as positive and negative controls, respectively. Cell lysis was measured as in A. The absorbance obtained without an inhibitor was set to 100%. The graphs show the mean \pm S.D. of three independent experiments performed in duplicate. Statistical significance of differences was calculated using two-way analysis of variance and Bonferroni post-test. ***, \(p < 0.001\).

FIGURE 3. PGK inhibits but not activates the complement pathways. PGK was preincubated with NHS in GVB2 \(-\) or Mg\(^{2+}\) \(-\) EGTA buffer and added to microtiter plates coated with aggregated human-IgG (A), mannan (B), and zymosan (C). C4BP (A and B) and FH (C) were used as positive controls, whereas BSA was used as a negative control. Deposition of MAC (C5b-9) was measured using monoclonal Ab aE11. The amount of deposited MAC components in the absence of inhibitor was set to 100%. The data represent the mean \pm S.D. of three independent experiments performed in duplicate. Statistical significance of differences was calculated using two-way analysis of variance and the Bonferroni post-test. ***, \(p < 0.001\), D, for analysis of classical pathway activation, the plates were coated with PGK, with aggregated human-IgG as a positive control or BSA as a negative control. The plates were incubated with indicated concentrations of NHS in GVB2 \(-\) buffer. C3b deposition was detected with specific Abs. E, for analysis of alternative pathway activation, NHS diluted in Mg\(^{2+}\) \(-\) EGTA buffer was added to zymosan-coated plates, and deposition of C3b was measured. The means \pm S.D. of three independent experiments performed in duplicates are presented.
To corroborate the above results and to demonstrate that PGK indeed inhibits the complement cascade, microtiter plates were coated with complement-activating ligands, aggregated IgG (classical pathway), mannan (lectin pathway), and zymosan (alternative pathway). Afterward, NHS preincubated with PGK was added. Deposition of MAC (C5b9) was measured with monoclonal Abs specifically recognizing a neo-epitope present in polymeric C9 of C5b9-MAC. The pretreatment of serum with PGK significantly inhibited the deposition of MAC in a dose-dependent manner via all the three pathways (Fig. 3, A–C). Taken together, our data indicated that PGK specifically inhibits the complement cascade.

PGK Does Not Activate Complement—As PGK significantly inhibits complement, we asked whether the observed inhibition is due to the ability of PGK to activate and consequently consume complement as has recently been demonstrated for pneumococcal endopeptidase O (33). To this end microtiter plates coated with PGK, with complement activating agents for the three pathways as positive controls and BSA as a negative control, were incubated with NHS followed by detection of the deposited complement component C3b with specific Abs. PGK did not induce any deposition of C3b via the classical pathway and alternative pathway (Fig. 3, D and E). For investigation of the lectin pathway, human serum depleted of C1q was used.

FIGURE 4. PGK binds MAC components and inhibits its formation. A, PGK inhibits hemolysis of erythrocytes using purified terminal complement components. PGK (0–80 μg/ml) was preincubated with C7, C8, and C9 and added to C5b-6-coated sheep erythrocytes. After the incubation, cell lysis was measured, and the lysis in the absence of inhibitor was set to 100%. B, PGK binds terminal complement components C5, C7, and C9. Microtiter plates were coated with PGK (5 μg/ml), and increasing amounts of C5, C6, C7, C8, and C9 were added. Binding was detected using specific polyclonal Abs. For A and B, BSA was used as the negative control. The mean ± S.D. of three independent experiments performed in duplicate is presented. Statistical significance was calculated using two-way ANOVA. ns, not significant; ***, p < 0.001. C, PGK simultaneously binds C5, C7, and C9. PGK was immobilized on microtiter plates, and a mixture of C5, C7, and C9 (2.5 μg/ml each) was added. Bound C5, C7, and C9 were detected separately using specific Abs that did not cross-react with other proteins. Data presented are from three independent duplicate experiments ± S.D. One-way ANOVA was used to calculate statistical significance between the binding in the absence and the presence of proteins. D, PGK inhibits the MAC formation at the level of C5. PGK preincubated with C5 (0.5 μg/ml) and C6 (0.75 μg/ml) was added to sheep erythrocytes incubated with 1% C5 depleted serum. After incubation, the erythrocytes were washed and further incubated with C7, C8, and C9. The degree of lysis was estimated by measurement of the release of hemoglobin. The mean values ± S.D. from three separate experiments are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Similar to the results obtained for the other two pathways, negligible deposition of C3b was triggered by PGK (data not shown). Taken together, our data indicate that PGK does not activate any of the complement pathways.

**PGK Binds MAC Components and Inhibits Its Formation**

The above results showed that although PGK does not activate complement, its presence in serum significantly inhibited the deposition of MAC through all three pathways (Fig. 3). To substantiate the data, we tested the ability of PGK to block the MAC formation using purified MAC components. Monitoring of lysis of erythrocytes was performed as a read out. PGK was preincubated with purified C7, C8, and C9 and later added to C5b6-treated and amboceptor-sensitized sheep erythrocytes. The presence of PGK significantly inhibited the lysis of erythrocytes in a dose-dependent manner (Fig. 4A). PGK at 80 μg/ml inhibited the lysis of erythrocytes by almost 90%, whereas BSA included as a negative control did not (Fig. 4A). The data further confirm the inhibitory role of PGK in MAC formation.

To understand the mechanism of MAC inhibition, we studied the ability of PGK to interact with purified MAC components. Microtiter plates coated with PGK were incubated separately with purified C5, C6, C7, C8, and C9, and the binding was detected using specific polyclonal Abs. BSA was used as negative control. One-way ANOVA was used to calculate statistical significance: *ns*, not significant; **, *p* < 0.01; ***, *p* < 0.001.

**FIGURE 5. C7 binds PGK.** Microtiter plates were coated with either PGK (5 μg/ml) (A) or C7 (5 μg/ml) (B), and increasing amounts of C7 or PGK was added. Binding was detected using specific polyclonal Abs. BSA was used as negative control. Statistical significance was calculated using two-way ANOVA and the Bonferroni post-test. Microtiter plates were coated with PGK, and the effect of different concentrations of NaCl (C) or heparin (D) on binding of C7 (2.5 μg/ml) to PGK was analyzed. The amount of C7 bound in the absence of NaCl or heparin was set at 100%. Specific polyclonal Abs were used to detect bound C7. One-way ANOVA and Dunnett’s post-test were performed to calculate statistical differences compared with the binding at 150 mM NaCl or without heparin. E, PGK does not affect binding of C7 to C5b-6. Complement protein C7 preincubated without or with PGK or BSA was added to immobilized C5b-6, and the binding was detected using specific polyclonal Abs. F, PGK inhibits the MAC formation at the level of C7. PGK (50 μg/ml) preincubated with C7 was added to C5b-6-coated sheep erythrocytes. BSA was used as negative control. After washing, the erythrocytes were incubated with a mixture of C8 and C9, and the hemolysis was measured. Data presented are from three independent duplicate experiments ± S.D. One-way ANOVA was used to calculate statistical significance: *ns*, not significant; **, *p* < 0.01; ***, *p* < 0.001.
To verify the effect of PGK-C5 interaction on MAC formation, the hemolytic activity was studied. For this, PGK preincubated with C5 and C6 was added to Ab-sensitized sheep erythrocytes that have been treated with C5-depleted serum. After the incubation, purified C7, C8, and C9 proteins were added, and the lysis of erythrocytes was assessed. A dose-dependent inhibition of erythrocyte lysis was observed in the presence of PGK (Fig. 4D). Taken together the data clearly indicated that PGK affects the process of formation of the functional MAC by virtue of its interaction with C5.

**FIGURE 4.** To determine whether the interaction between C7 and PGK is hydrophobic or ionic in character, the aforementioned binding assay was conducted in the presence of varying NaCl concentrations. Binding of C7 to PGK substantially decreased with increasing NaCl concentrations (Fig. 4C). At 800 mM NaCl, the C7 binding to PGK was reduced by 60% as compared with binding at the physiological concentration of 150 mM NaCl (Fig. 4C). Because C7 binds heparin, the effect of heparin on the PGK-C7 interaction was determined (34). A dose-dependent modulation of binding of C7 to PGK in the presence of heparin was observed. The amount of 400 μg/ml heparin reduced the binding by 45% (Fig. 4D).

To dissect the effect of PGK-C7 interaction on MAC formation in more detail, we analyzed whether PGK inhibits binding of C7 to the C5b6 complex. PGK was preincubated with C7, and thereafter the mixture was added to immobilized C5b6. The resulting data indicated that C7 bound to C5b6, and C7 complexed with PGK bound with the same intensity (Fig. 4E). Thus, PGK-C7 interaction does not block the attachment of C7 to the

**FIGURE 5.** To determine whether the interaction between C7 and PGK is hydrophobic or ionic in character, the aforementioned binding assay was conducted in the presence of varying NaCl concentrations. Binding of C7 to PGK substantially decreased with increasing NaCl concentrations (Fig. 5C). At 800 mM NaCl, the C7 binding to PGK was reduced by 60% as compared with binding at the physiological concentration of 150 mM NaCl (Fig. 5C). Because C7 binds heparin, the effect of heparin on the PGK-C7 interaction was determined (34). A dose-dependent modulation of binding of C7 to PGK in the presence of heparin was observed. The amount of 400 μg/ml heparin reduced the binding by 45% (Fig. 5D).
C5b6 complex, but an effect on its membrane insertion cannot be excluded. To verify this hypothesis, hemolytic activity was analyzed. PGK was preincubated with C7 and then added to C5b6-treated sheep erythrocytes. This was followed by incubation with C8 and C9, and the extent of lysis was determined. PGK (50 μg/ml) significantly reduced erythrocyte lysis by ~80% compared with the lysis in the absence of PGK (Fig. 5F). Taken together, PGK-C7 interaction inhibits MAC formation by affecting the insertion of C7 into the target membrane.

PGK Binds C9—To assess PGK-C9 interaction, microtiter plates coated with PGK were incubated with increasing concentrations of purified human C9 followed by detection using specific Abs. A dose-dependent binding of C9 to immobilized PGK was observed (K_D = 37 ± 3 nM) (Fig. 6A). Additionally, the binding of pneumococcal PGK to immobilized C9 was also detected in reverse settings (Fig. 6B). In further assays the effect of NaCl and heparin on PGK-C9 interaction was investigated. Similar to PGK-C7, the binding of C9 to PGK was affected in the presence of increasing NaCl and heparin concentrations, respectively (Fig. 6, C and D). At 800 mM NaCl the binding of C9 to PGK was reduced by 45% as compared with 150 mM NaCl (Fig. 6C). Similarly, the presence of 800 μg/ml heparin reduced the binding up to 55% (Fig. 6D). Taken together, PGK is a C9-binding protein, and the interaction is influenced by the ionic strength.

Because PGK also binds plasminogen, we asked whether C9 and plasminogen bind simultaneously to PGK and whether binding sites for these two proteins on PGK are independent of each other. For this a constant concentration of C9 (2 μg/ml) was added together with increasing concentrations of plasminogen or a constant concentration of plasminogen (2 μg/ml) in the presence of an increasing amount of C9 to immobilized PGK. The bound C9 and plasminogen were detected using specific Abs. C9 binding to PGK was not affected by the presence of plasminogen (Fig. 6E). Similarly, the presence of increasing concentrations of C9 did not influence the binding of plasminogen to the immobilized PGK (Fig. 6F). Taken together the two proteins do not compete with each other for binding to PGK, indicating that C9 and plasminogen can bind simultaneously and to non-overlapping binding sites on PGK.

PGK Inhibits C9 Polymerization and MAC Formation—Due to the fact that PGK interacts with C9, we investigated the effects on either C9 self-polymerization or its interaction with C5b8. To examine whether PGK inhibits C9 polymerization, 0.5 μM C9 was incubated with increasing amounts of PGK (0–5 μM) overnight at 37 °C, and the samples were analyzed by SDS-PAGE under reducing conditions followed by Western blotting with anti-C9 Abs. BSA (5 μM) was used as the negative control. The resulting pattern indicates that PGK inhibited C9 polymerization in a concentration-dependent manner (Fig. 7A). The intensities of the bands for polymerized C9 were determined by densitometry (Fig. 7B). To further evaluate the significance of inhibition of C9 polymerization on the attenuation of functional MAC formation, Ab-sensitized sheep-erythrocytes were subjected to complement attack from C9-depleted serum to induce C5b8 formation on their surface. C9 had been preincubated with increasing amounts of PGK and was then added to the cells. Lysis was evaluated by measuring hemoglobin release. BSA was used as a negative control. PGK inhibited erythrocyte lysis in a dose-dependent manner (Fig. 7C), indicating that the PGK-C9 interaction affects the MAC formation.
PGK Limits MAC Deposition on Pneumococci, Which Is Serotype-independent—The capsular polysaccharide is an essential virulence factor of pneumococci and to date >91 serologically distinct capsular polysaccharides have been described (35, 36). It is known that the capsular polysaccharides render pneumococci resistant to complement-mediated opsonophagocytosis and interfere with pneumococcal interaction with host cells and protein (37). To demonstrate the functional relevance of pneumococcal PGK, we investigated the extent of MAC deposition on the pneumococcal surface after incubation with various serum concentrations. The flow cytometry analysis demonstrated dose-dependent deposition of MAC on the pneumococcal surface via the classical and alternative complement activation pathway, proportional to the serum concentration (Fig. 8, A and B). To study whether capsular polysaccharide would also affect the extent of MAC deposition, a collection of 12 clinical isolates of S. pneumoniae (Table 1) belonging to 12 different serotypes was analyzed for the level of MAC deposition after treatment with human serum. Flow cytometry analyses demonstrated MAC deposition with a variable extent on all the tested clinical isolates mediated via the classical and the alternative complement activation pathway (Table 1). Moreover, the highly encapsulated strains like the serotype 3 strains were relatively resistant to MAC deposition, whereas the NCTC10319 type 35A had a relatively strong signal for MAC deposition. Thus the data

FIGURE 8. Detection of MAC (C5b-9) deposition on S. pneumoniae using flow cytometry. Pneumococci NCTC10319 were incubated with increasing concentration of NHS in GVB Buffer for the classical pathway (A) or Mg²⁺ EGTA buffer for the alternative pathway (B) and incubated for 1 h at 37 °C. MAC (C5b-9) deposition was detected using the mAb aE11 Ab that recognizes a neo-epitope present in polymeric C9 but absent in monomeric C9. The GMFI data from at least three independent experiments are shown (mean ± S.D.) as a measure of MAC deposition from classical and alternative pathway. Inset, representative flow cytometry histogram for MAC deposition via the two pathways is shown. Statistical significance for MAC deposition compared with bacteria background (Bac.) was calculated using one-way ANOVA. PGK restricts MAC deposition on pneumococcal surface. The deposition of MAC (C5b-9) via the classical (C and D) or alternative pathway (E and F) onto the pneumococcal surface (NCTC10319) from 0.2 and 2% NHS, respectively, was measured in the absence or presence of exogenous added PGK protein. BSA was used as negative control. Deposited MAC was analyzed by flow cytometry using mAb aE11. A representative flow cytometry histogram from three independent experiments is shown (C and E), and GMFI values (D and F) from three independent experiments performed in duplicate are presented. Statistical significance was calculated using one-way ANOVA. ns, not significant; *, p < 0.05; ***, p < 0.001.
suggest that the MAC deposition is a general mechanism that is influenced by the extent of capsule.

To verify the role of PGK in inhibiting MAC deposition, blocking experiments were performed. The deposition of MAC via the classical complement activation pathway on pneumococci was measured by flow cytometry in the presence of PGK. Flow cytometry analyses indicated a dose-dependent competitive inhibition of MAC on pneumococci by PGK (Fig. 8, C and D). In contrast, BSA showed no inhibitory effect. Similar inhibition was observed on MAC deposition via the alternative complement activation pathway (Fig. 8, E and F). In summary, our data identify for the first time PGK as a novel complement inhibitor from the Gram-positive species S. pneumoniae that inhibits the formation of functional MAC. PGK specifically interacts with the terminal complement components C5, C7, and C9 thereby modulating the complement attack (Fig. 9).

DISCUSSION

During the course of evolution, pathogens have evolved intricate mechanisms for coexistence with the human host. These include mechanisms for adherence, colonization, invasion, dissemination to other tissues, and most importantly the survival by escaping the host immune attack. The complement system, as a major arm of innate immune system, functions as a first line of deterrence, and therefore, is a key target for evasion strategies developed by invading pathogens. The Gram-positive human pathogen S. pneumoniae has evolved a broad spectrum of weapons in its arsenals for perpetuation within its host. In this study we identified another complement inhibitory factor of S. pneumoniae; PGK as a novel inhibitor of the MAC formation.

The pneumococcal PGK (41.9 kDa) is a monomeric, two-domain-containing glycolytic enzyme that mediates conversion of 1,3-biphospho-D-glycerate to 3-phospho-D-glyerate yielding ATP within glycolytic metabolism. PGK has recently been classified as an additional “moonlighting protein” expressed on the surface of pneumococci (31). Moreover, PGK is also found in the culture supernatants, although the underlying mechanism of its surface presentation and secretion is not yet known. PGK was recently shown to interact simultaneously with

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TABLE 1

MAC deposition on various isolates of S. pneumoniae

| S. pneumoniae strains | Serotypes | MAC deposition |   |   |
|----------------------|-----------|----------------|---|---|
|                      |           | CP (GMFI)      | AP (GMFI) |
| KR405                | 3         | 0.82 ± 0.02    | 0.8 ± 0.02 |
| KR408                | 4         | 11.00 ± 2.87   | 3.89 ± 1.44 |
| KR409                | 6A        | 11.46 ± 6.64   | 5.31 ± 1.52 |
| KR430                | 6B        | 28.53 ± 4.89   | 12.19 ± 2.26 |
| KR412                | 7F        | 4.48 ± 0.65    | 2.05 ± 0.51 |
| KR414                | 8         | 1.59 ± 0.61    | 1.38 ± 0.63 |
| KR416                | 9V        | 12.9 ± 4.29    | 10.59 ± 2.7 |
| KR419                | 11        | 43.33 ± 5.26   | 41.48 ± 5.35 |
| KR420                | 14        | 23.02 ± 4.02   | 10.35 ± 3.69 |
| KR426                | 19F       | 8.9 ± 2.22     | 3.31 ± 0.68 |
| KR411                | 23A       | 10.84 ± 3.53   | 3.82 ± 1.34 |
| NCTC10319            | 35A       | 73.42 ± 5.45   | 70.52 ± 16.81 |
| D39                  | 2         | 1.21 ± 0.1     | 1.12 ± 0.11 |

FIGURE 9. Schematic model of pneumococcal PGK-mediated complement inhibition. PGK inhibits all three pathways by interacting with C5, C7, and C9 proteins and interfering with C9 polymerization.
Pneumococcal PGK Inhibits MAC

plasminogen and its activator, which upon activation to plasmin assists pneumococci in tissue migration and dissemination by cleaving fibrin thrombi and extracellular matrix proteins (31). In this study we tested whether PGK has any additional role in pneumococcal infections and in particular whether it has any complement modulatory activity. Indeed, here we attribute MAC inhibition as an additional function to PGK. In addition, plasminogen bound to PGK also cleaved C3b upon activation to plasmin, thereby mediating additional complement control.

Although the MAC formation directly kills the Gram-negative bacteria either by lysis or disturbance of metabolic processes, the role of MAC deposition-mediated lysis or attenuation of the Gram-positive organisms is perceived to be ineffective. Nevertheless, to date two MAC inhibitors have been identified in Gram-negative bacteria that include streptococcal inhibitor of complement (SIC) from Group A streptococci and staphylococcal supernantigen-like protein 7 (SSL7) from S. aureus (20, 21, 38). In addition, several other MAC inhibitors are known that include the recently identified terminal pathway inhibitor CspA and the CD59-like protein from Gram-negative bacteria Borrelia burgdorferi, schistosome C inhibitory protein type 1 (SCIP-1) from Schistosoma mansoni, paramyosin from S. mansoni and Trichinella spiralis, and the galactose-specific adhesin from Entamoeba histolytica (39–43). Recently Berends and co-workers (14) demonstrated that the distinct surface localization of complete MAC (C5b-9) on Gram-positives is determined by the subcellular localization of cell wall components. Therefore, it may be appropriate to speculate that depending on the specific site of MAC insertion and considering the dynamic nature of the constantly changing bacterial cell wall during growth, these bacteria might be sensitive to MAC. Indeed, a recent study demonstrated that peptidoglycan recognition proteins initiate killing of Gram-positive bacteria by entering the cell wall at the site of daughter cell separation where the cell wall is thin. This mechanism occurs independent of MAC insertion and leads to activation of internal bacterial stress responses (44). Similarly, other host immune components such as antimicrobial peptides may cooperate with the inserted MAC and support killing of Gram-positive bacteria.

We observed that PGK inhibits all pathways of complement cascade in both hemolytic and complement deposition assays. This was attributed to the interaction between PGK and MAC components. PGK binds the complement proteins C5, C7, and C9 and thereby inhibits the assembly and the membrane insertion of the MAC (C5b-9 complex). Importantly, the inhibition of complement cascade by soluble recombinant PGK was independent of its inherent enzymatic activity, as similar inhibition was observed while performing hemolytic assay using serum or purified MAC components. Interestingly, PGK does not activate any of the complement pathways. This is in sharp contrast to another moonlighting protein, the pneumococcal endopeptidase O, that upon its localized release strongly activates, consumes, and inhibits classical complement pathways as a result of its interaction with C1q (33, 45).

Recombinant PGK interacted in a dose-dependent manner with C7 and C9. As these proteins are abundant plasma proteins, it seemed highly possible that they may compete with one another for binding to PGK. However, our results indicate that PGK simultaneously binds these proteins. Although C5 and C7 had partially overlapping binding sites on PGK, C9 did not compete with either of these for PGK binding. We observed a dose-dependent interaction between PGK and C7 and C9 at physiological ionic strength, which was influenced by increasing the ionic strength. Although PGK–C7 interaction significantly reduced the lysis of erythrocytes, the effect was not a consequence of binding inhibition of PGK–C7 complex to C5b-6 but rather due to inhibition of the membrane insertion of C7. In other words the PGK binding site on C7 and its attachment site to C5b-6 are distinct. Moreover, PGK binds C7 and C9 with comparable affinity.

In this study we also demonstrate that the PGK–C9 interaction inhibits MAC by decreasing C9 polymerization both in the fluid phase and on the surface of sheep erythrocytes, thereby preventing lytic pore formation. Moreover, the presence of PGK significantly inhibited MAC deposition via both the classical and alternative pathway at the pneumococcal surface. Because PGK also binds plasminogen and both C9 and plasminogen are abundant plasma proteins, it appeared highly possible that they may compete with each other for binding to PGK. However, our results indicate that binding of C9 is not outcompeted by plasminogen and vice versa, suggesting that both proteins interact with different motifs on pneumococcal PGK.

The current study provides novel insights into the mechanism of pneumococcal manipulation of the terminal complement pathway and adds new facts to our knowledge about pneumococcal infections and its interaction with the human host. It is intriguing that although MAC formation is considered inconsequential against Gram-positive bacteria because of the thick peptidoglycan layers, many pathogens encode inhibitors for the terminal complement pathway. Therefore, the functional relevance of MAC deposition on Gram-positives and its inhibition needs to be further investigated in detail, and we would expect that in the future more MAC inhibitors would be identified. Moreover, identification and characterization of such inhibitors of the terminal complement pathway could add promising targets to the repertoire of potentially useful drugs for treatment of inflammatory diseases in future. Taken together we describe here PGK as a first novel pneumococcal surface-exposed and secreted immune evasion protein that directly affects the terminal complement pathway by blocking MAC formation.

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