Streptococcus pyogenes is an important pathogen that causes pharyngitis, sepsis, and rheumatic fever. Cell-associated streptococcal C5a peptidase (ScpA) protects S. pyogenes from phagocytosis and has been suggested to interrupt host defenses by enzymatically cleaving complement C5a, a major factor in the accumulation of neutrophils at sites of infection. How S. pyogenes recognizes and binds to C5a, however, is unclear. We detected a C5a-binding protein in 8 M urea extracts of S. pyogenes by ligand blotting using biotinylated C5a. Searching of genome databases showed that the C5a-binding protein is identical to the streptococcal plasmin receptor (Plr), also known as streptococcal surface dehydrogenase (SDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the present study we identified a novel function of this multifunctional protein. Western blotting and immunofluorescence microscopy with anti-Plr/SDH/GAPDH showed that Plr/SDH/GAPDH is located on the bacterial surface and released into the culture supernatant. Next, we examined whether the streptococcal Plr/SDH/GAPDH inhibits the biological effects of C5a on human neutrophils. We found that soluble Plr/SDH/GAPDH inhibits C5a-activated chemotaxis and H₂O₂ production. Furthermore, our results suggested that soluble Plr/SDH/GAPDH captures C5a, inhibiting its chemotactic function. Also, cell-associated Plr/SDH/GAPDH and ScpA were both necessary for the cleavage of C5a on the bacterial surface. Together, these results indicate that the multifunctional protein Plr/SDH/GAPDH has additional functions that help S. pyogenes escape detection by the host immune system.

Group A Streptococcus pyogenes is a human pathogen that causes streptococcal pharyngitis as well as more severe invasive infections, including necrotizing fasciitis, sepsis, and streptococcal toxic shock syndrome. The initial step of S. pyogenes infection is bacterial adhesion to host epithelial cells through extracellular matrix proteins (e.g. fibronectin) (1–3). Fibrinogen-binding proteins of S. pyogenes have been identified as adhesins/invasins, and how S. pyogenes invade epithelial cells has been examined in several studies (1, 3, 4–8).

To cause systemic and septic infections, it is apparent that S. pyogenes must escape from detection by the host immune system and survive in plasma, after which they invade and spread into various organs via the blood stream. Recently, it was reported that infiltrating neutrophils were not observed at sites of S. pyogenes infection (9). The complement system plays an important role in the innate immune system, which acts as a protective shield in the early phases of infection as well as an effector in the acquired immune system. During activation of the complement system, complement fragments C3a, C4a, and C5a are generated, whereupon they act as anaphylatoxins. C5a is the most important anaphylatoxin because it acts as a chemoattractant for neutrophils and macrophages and because it integrates activation of the classical, alternative, and lectin binding pathways. Furthermore, C5a activates the expression of various inflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, -6, and -8 in macrophages and endothelial cells (10–12). Of these, interleukin-8 is particularly important because it recruits neutrophils to sites of infection. Recently, Edwards et al. (13) found a novel cell envelope protease as an interleukin-8-cleaving enzyme and suggested that this chemokine-cleaving activity contributes to the pathogenesis of necrotizing S. pyogenes infection.

Wexler and Cleary (14) reported that S. pyogenes expresses a C5a-specific protease on its surface that modulates the host chemotactic response. This protease, which they named streptococcal C5a peptidase of group A streptococci (ScpA), was isolated and sequenced by Chen and Cleary (15, 16) although how it recognizes and binds C5a has not been clear. In the present study, we tested the hypothesis that ScpA must be recruited to the cell surface for cleavage by cell-associated ScpA. Using genome sequencing databases and a BLAST homology search, we found that the gene encoding the C5a-binding protein is most closely related to those for the streptococcal plasmin receptor (Plr) (17), streptococcal surface dehydrogenase (SDH) (18), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19). Recently, SDH and GAPDH were shown to be the same as Plr, and our current findings suggest that the multifunctional protein Plr/SDH/GAPDH exists as cell-associated and soluble forms that have distinct functions in inhibiting the host immune system.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Reagents—S. pyogenes strain SSI-9, isolated from a patient with streptococcal toxic shock syndrome, was provided by Drs. T. Murai (Toho University, Japan) and K. Ooe and Y. Shimizu (Asahi General Hospital, Japan). The organism was grown in Todd-Hewitt broth (Difco) supplemented with 0.2% yeast extract. Kanamycin

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2 The abbreviations used are: ScpA, streptococcal C5a peptidase of group A streptococci; Plr, streptococcal plasmin receptor; rPlr, recombinant Plr; SDH, streptococcal surface dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGF, enhanced green fluorescent protein; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue R-250; GST, glutathione S-transferase.
S. pyogenes Evades Neutrophils by Its Multifunctional GAPDH

TABLE 1

Primers used in this study

| Designation          | Sequence (5’ to 3’)                                             | Reference         |
|----------------------|-----------------------------------------------------------------|-------------------|
| For expression of recombinant protein |                                                                 |                   |
| pfr/sdhi/gapdh-Fw1   | CCGGAATCCCCATGCTGATCGATTAAGGTGCTATTACGCG                      | This study        |
| pfr/sdhi/gapdh-Rv1   | GGAACTTCTTATACACTGAATGTTGTTGATTGAG                            | This study        |
| pfr/sdhi/gapdh-Rv2   | GGAACTTCTTATACACTGAATGTTGTTGATTGAG                            | This study        |
| pfr/sdhi/gapdh-Rv3   | GGAACTTCTTATACACTGAATGTTGTTGATTGAG                            | This study        |
| pfr/sdhi/gapdh-Rv4   | GGAACTTCTTATACACTGAATGTTGTTGATTGAG                            | This study        |
| scpA-Fw1             | CGGGGATCCAAATCTGCGAACAGAAGCAGATTACCG                           | Stafslien et al. (23) |
| scpA-Rv1             | CGGGGATCCAAATCTGCGAACAGAAGCAGATTACCG                           | Stafslien et al. (23) |
| For insertional mutagenesis |                                                              |                   |
| scpA-KO1             | AATCTGGGGATGAAAGAAGAT                                          | This study        |
| scpA-KO2             | GATGGCGTTCCTCGGTCTCTTGG                                         | Terao et al. (1)  |
| aplcA3-Fw2           | TCCGTTACTTTTATAGACTGAGGG                                        | Terao et al. (1)  |
| aplcA3-Rv2           | GGGATGGCTCCGTGATGCGG                                          | Terao et al. (1)  |

(300 µg/ml) was added to the medium to select the isogenic mutant strain. Escherichia coli strains XL-10 Gold (Stratagene) and BL21 (DE3) pLysE (Novagen) were grown in Luria-Bertani broth (Sigma) or on Luria-Bertani agar plates supplemented with 100 µg/ml ampicillin and 30 µg/ml kanamycin.

DNA Procedures—Extraction and purification of genomic DNA from S. pyogenes were performed using standard procedures (20). Streptococcal chromosomal genes were mutated by electroporation as described previously (1–3).

N-terminal Amino Acid Sequencing and Genome Sequence Analysis—C5a-binding protein was purified by diethylaminoethyl column chromatography (Bio-Rad) from 8 ml urea extracts of S. pyogenes strain SSI-9 (serotype M1). The isolated protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Protein on the membrane was stained with Coomassie Brilliant Blue R-250 (CBB; Fluka), after which N-terminal amino acid sequencing was performed by Edman degradation using an ABI protein sequencer (model 491HT; Applied Biosystems). To identify C5a-binding protein, the complete genome sequences of S. pyogenes strains SSI-1 (21) and SF370 (22) were obtained from the genome databases at Osaka University and Oklahoma University, respectively.

Construction and Purification of Recombinant Proteins—A 1008-bp DNA fragment from the pfr/sdhi/gapdh gene was amplified by PCR from chromosomal DNA of strain SSI-9. The scpA gene of strain 90-226 (M1) was amplified by PCR from plasmid pScpA 90-226 (gift from Dr. P. P. Cleary, University of Minnesota) (23). The primers used are listed in Table 1. PCR products were ligated into expression vector pGEX-6P-1 (Amersham Biosciences). Plasmids for expression glutathione-S-transferase (GST)-C5a-enhanced green fluorescent protein (EGFP) and GST-EGFP fusion proteins were constructed as described previously (23). The human C5a gene was amplified by reverse transcription-PCR from total RNA of Hep-G2 cells (Riken). Recombinant proteins were induced with isopropyl-1-thio-β-D-galactopyranoside in E. coli BL21 (DE3) pLysE containing the appropriate plasmids and purified using glutathione-Sepharose 4B and PreScission protease (Amersham Biosciences). The recombinant proteins were dialyzed against phosphate-buffered saline. The purified recombinant proteins were found to contain less than 0.25 ng/ml endotoxin using a Limulus ES-II single test (Wako).

Preparation of Rabbit Anti-Pfr/SDH/GAPDH Serum—All animal procedures were in compliance with the Osaka University Graduate School of Dentistry guidelines and were approved by the institutional animal care and ethics committee (accession number 00-018). On days 0, 14, 28, and 42, New Zealand White rabbits (Charles River Laboratories Japan) were anesthetized with pentobarbital as previously described (24) and injected with 500 µg of recombinant protein in TiterMax Gold (CytRx). Serum was collected on day 56 and stored at −80 °C until use. Anti-ScpA serum was provided by Dr. P. P. Cleary (University of Minnesota) (23). Western blotting was performed as described previously (25).

Insertional Mutagenesis Using a Targeting Plasmid—Inactivation of the scpA gene in S. pyogenes was carried out as described previously (1, 26). The PCR product of an internal portion of scpA was ligated into pSF151 vector (27). The primers used for inactivation are listed in Table 1. S. pyogenes SSI-9 was transformed with the resulting plasmid, pYT1161, by electroporation, and the mutant strains were selected on kanamycin-containing agar plates.

Ligand Blotting—Ligand blotting was performed as described previously (1). Briefly, human C5a (Sigma) and bovine serum albumin (BSA; Sigma) were biotinylated using an ECL protein biotinylation kit (Amer sham Biosciences), and the concentrations were adjusted to 50 µg/ml. Streptococcal surface components were extracted with 8 M urea at room temperature (28), separated by electrophoresis on 12% SDS-PAGE gels, and transferred to PVDF membranes. The membranes were blocked overnight at 4 °C with 10% membrane blocking agent (Amersham Biosciences) and then incubated with biotinylated C5a for 1 h. After incubation with horseradish peroxidase-labeled streptavidin at room temperature for 1 h, bands were detected using ECL. Western blotting detection reagents (Amersham Biosciences) and visualized by exposure to x-ray film (Fuji photo film) at room temperature for 15 s.

Biacore Analysis—Biacore analysis was performed as described previously (29). Human C5a, recombinant Pfr (rPfr)/SDH/GAPDH, or BSA was diluted to 100 µg/ml in 10 mM sodium acetate, pH 4.0 (Biacore International AB) and immobilized on the surface of a Sensor Chip CM5 (Biacore International AB) using an Amine-coupling kit (Biacore International AB). Lyophilized rPfr/SDH/GAPDH, rScpA, or heat-inactivated rScpA was suspended in HBSP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20) and adjusted to 0.125, 0.25, 0.5, and 1 µM. Anti-Pfr/SDH/GAPDH serum was also diluted with HBSP buffer (1:10). For binding analysis, rPfr/SDH/GAPDH was injected at a flow rate of 30 µl/min at 25 °C. Relative units for C5a binding were corrected by subtraction of those for BSA binding.

Isolation and Labeling of Neutrophils—Venous blood samples were obtained from healthy volunteers, immediately treated with heparin, and diluted with RPMI 1640 (Sigma). Mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences) (30). The pellets, which contained neutrophils and erythrocytes, were treated at 4 °C for 5 min with ACK lysis buffer (150 mM NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA, pH 7.2) to lyse the erythrocytes. Neutrophils were collected by centrifugation (400 × g for 15 min at 25 °C), washed twice with RPMI 1640, and resuspended in RPMI 1640.
at $5 \times 10^6$ cells/ml. Neutrophils were shown to be 99% viable by trypan blue exclusion and 98–99% pure by staining with Giemsa. The neutrophils were labeled with 1 mM biscarboxyethyl carboxyfluorescein pentacetoxymethylester (Dojindo) as described previously (31). After 30 min the neutrophils were washed twice with RPMI 1640 and immediately used for analysis of chemotactic activity.

Fluorescent-based Chemotaxis Assay—Chemotactic migration was evaluated as described previously (32) in 96-well chemotaxis chambers containing polycarbonate filters (pore size 5 μm; Kurabo). The wells of the bottom plate were filled with 10 nM C5a and 0–100 nM rPlr/SDH/GAPDH and then incubated for 2 h at 37 °C. The reaction was terminated by the addition of the equal volume of trichloroacetic acid, and then the samples were centrifuged for 10 min at 5000 × g. Ferrous ammonium sulfate (1.5 mM) and potassium thiocyanate (0.25 mM) were added to the supernatant, and the absorbance of the ferrithiocyanate complex was measured at 492 nm.

Results

Measurement of $H_2O_2$ Production by Neutrophils—The effect of Plr/SDH/GAPDH on the oxidative function of neutrophils was evaluated by established assay (33). Neutrophils were suspended in Hanks’ balanced salt solutions (Invitrogen) supplemented with 1 mM sodium azide to inhibit endogenous peroxidase. C5a (10 nM) was mixed with 0–100 nM rPlr/SDH/GAPDH or 500 nM rabbit anti-human C5a IgG. After 2 h the solution was incubated with neutrophils (5 × 10^6 cells/ml) for 1 h at 37 °C. The reaction was terminated by the addition of the equal volume of trichloroacetic acid, and then the samples were centrifuged for 10 min at 500 × g. Ferrous ammonium sulfate (1.5 mM) and potassium thiocyanate (0.25 mM) were added to the supernatant, and the absorbance of the ferrithiocyanate complex was measured at 492 nm.

Immunofluorescence and Confocal Microscopy—Immunofluorescent staining was performed as described previously (2, 29). Streptococcal organisms were washed with phosphate-buffered saline and blocked with 10% goat serum (Tissue Culture Biologicals) containing 50 μg/ml human IgG (Calbiochem). Bacterial cells were stained with SYBR green II, and surface Plr/SDH/GAPDH was detected using a rabbit anti-Plr/SDH/GAPDH serum followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes). Stained bacteria were analyzed using an LSM 510 confocal laser scanning microscope (Carl Zeiss).

Assay of ScpA Proteolytic Activity—To examine the enzymatic activity of cell-associated Plr/SDH/GAPDH and ScpA, S. pyogenes cells were washed with sterile phosphate-buffered saline and resuspended in reaction buffer (100 mM NaCl, 100 mM Tris, pH 8.5, 5 mM CaCl₂, 1 mM dithiothreitol, 0.1% NaN₃) at 1 × 10⁸ colony forming units/ml as described previously (34). Next, GST-ScpA-EGFP was added, and the mixture was incubated for 1 h at 37 °C (final concentration 1 μM). GST-EGFP served as a negative control. In some reactions, 20 nM E-D4 (Calbiochem) was added to block the truncation of ScpA and GST-ScpA-EGFP by streptococcal cysteine protease SpeB (34). Also, the effects of cell-associated Plr/SDH/GAPDH and ScpA on the proteolysis of C5a were evaluated using rabbit anti-Plr/SDH/GAPDH IgG and anti-ScpA IgG. To examine the effect of antibodies, the bacterial suspension was incubated for 1 h with 100 nM antibodies that had been purified with an MAbTrap Kit (Amersham Biosciences) and then mixed with the substrate. After the reaction, the mixture was separated without heating by nonreducing 10% SDS-PAGE. Fluorescent fusion proteins were visualized with a Typhoon 9400 system (Amersham Biosciences). Control reactions contained 100 nM rScpA.

Statistical Analysis—The significance of differences between the means of groups was determined using a nonparametric Mann-Whitney U test. All tests were carried out using StatView J-5.0 software (SAS Institute Inc.). Differences with $p < 0.05$ were considered significant.

Identification of the C5a-binding Protein from S. pyogenes—The targeted protein, which was bound to biotinylated C5a, was purified by diethylaminoethyl column chromatography. The N-terminal amino acid sequence of this protein was VXVGINGFGXGXL, which is identical to the N terminus of the open reading frame of SPy0274 (DDBJ/EMBL/GenBank™ accession number M95569) in the complete genome sequence of serotype M1 S. pyogenes strain SF370. We performed a BLAST search using the SPy0274 sequence and limited the results to streptococcal proteases. We found that this protein is the most similar (>98%) to Plr (17). SDH (18), and GAPDH (19). SDH and GAPDH were recently reported to be identical to Plr. The plr/sdh/gapdh gene has a streptococcal consensus Shine-Dalgarno sequence (GGAGGA) as well as −10 (AATAAT) and −35 (TTTTCA) sequences (35–37) preceding the start codon (ATG). The open reading frame consists of 1008 nucleotides and encodes a protein of 336 amino acid residues with a predicted molecular mass of 36 kDa.
Characterization of C5a Binding by Plr/SDH/GAPDH and Identification of the Region Necessary for C5a Binding Activity—We constructed rPlr/SDH/GAPDH by PCR amplification of the SPy0274 nucleotide sequence. The purified protein was separated on a 12% SDS-PAGE gel and analyzed by ligand blotting with biotinylated C5a. We confirmed that rPlr/SDH/GAPDH bound to soluble C5a (Fig. 2A). To further analyze the binding activity of soluble rPlr/SDH/GAPDH, we immobilized C5a on a Sensor Chip CM5 and analyzed its interaction with various concentrations of rPlr/SDH/GAPDH using a Biacore X system. The Biacore profiles demonstrated that soluble rPlr/SDH/GAPDH bound to immobilized C5a with a high affinity. A global fitting procedure (BIAevaluation software version 3.02) determined that the association ($k_{ass}$) and dissociation ($k_{diss}$) rate constants were $1.04 \times 10^4 \pm 100$ M$^{-1}$ s$^{-1}$ and $4.24 \times 10^{-3} \pm 3$ s$^{-1}$, respectively. Also, the $K_A$ and $K_D$ values were calculated to be $2.44 \times 10^6 \pm 6$ M$^{-1}$ and $4.09 \times 10^{-7} \pm 9$ M, respectively. BSA, which served as a negative control, did not bind to C5a. Our results suggest that both cell-associated and soluble Plr/SDH/GAPDH of S. pyogenes bind C5a.
We further determined the region of Plr/SDH/GAPDH necessary for binding to C5a using a series of truncated rPlr/SDH/GAPDHs (Fig. 2B). Fragments with C termini at residue 164 or higher retained C5a binding, but fragment 1–114 did not bind C5a. Therefore, it appears that the sequence between residues 114–163 is an important element for C5a binding. A schematic diagram of Plr/SDH/GAPDH is shown in Fig. 2C.

**Expression and Localization of Plr/SDH/GAPDH**—Originally, Plr/SDH/GAPDH was found in the 8 M urea extract, which contained the bacterial surface proteins. Because ligand blotting and Biacore analyses demonstrated that both immobilized and soluble Plr/SDH/GAPDH could function as complement-binding proteins, we postulated that Plr/SDH/GAPDH exists as cell-associated and soluble forms in vivo. We, therefore, investigated whether Plr/SDH/GAPDH is surface-associated, secreted into the culture supernatant as a soluble protein, or localized intracellularly. Western blotting with an anti-Plr/SDH/GAPDH antiserum showed that Plr/SDH/GAPDH is expressed on the bacterial surface and released from cells (Fig. 3A).

To confirm the localization on the surface, we analyzed *S. pyogenes* cells by immunofluorescent microscopy using the anti-Plr/SDH/GAPDH antiserum followed by an Alexa Fluor 594-conjugated secondary antibody. We found that Plr/SDH/GAPDH was localized at the poles of the bacterial cell surface (red in Fig. 3B). These results confirm

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**FIGURE 3. Localization of Plr/SDH/GAPDH on the bacterial surface.** A, the urea extracts of *S. pyogenes* strain SS1–9 (cell), cell culture supernatant (sup), and purified recombinant rPlr/SDH/GAPDH were analyzed by Western blotting with an antiserum against Plr/SDH/GAPDH. Rabbit preimmune serum was used as a negative control. B, to confirm the localization of Plr/SDH/GAPDH on the surface of *S. pyogenes*, cells were analyzed by immunofluorescence microscopy with anti-Plr/SDH/GAPDH serum. Cells were washed with phosphate-buffered saline, blocked with 10% goat serum containing 50 μg/ml human IgG, and incubated with SYBR green II (green) and rabbit anti-Plr/SDH/GAPDH serum. Immunoreactive proteins were detected with Alexa Fluor 594-conjugated goat anti-rabbit IgG (red). C, immunofluorescence was carried out as described in B except using rabbit preimmune serum as a negative control.
that some of the Plr/SDH/GAPDH is expressed on the surface of S. pyogenes.

Role of Cell-associated Plr/SDH/GAPDH in Bacterial Proteolysis of C5a—We next examined the ability of cell-associated Plr/SDH/GAPDH to enhance C5a cleavage on the surface of S. pyogenes using a fluorescent substrate for ScpA. This fluorescent substrate, rGST-C5a-EGFP, was a fusion of GST (for purification), the full-length of C5a, and EGFP, a fluorescent substrate for ScpA. This fluorescent substrate, rGST-C5a-EGFP, cleaved GST-C5a-EGFP fusion protein migrated as a 28-kDa fragment. Because EGFP is a 27-kDa protein, our results are consistent with cleavage of rGST-C5a-EGFP at the predicted site. Finally, we found that blocking cell-associated Plr/SDH/GAPDH or ScpA with specific antibodies inhibited C5a cleavage (Fig. 4A, green rectangle). Also, cleavage of rGST-C5a-EGFP by soluble ScpA was blocked by the anti-ScpA antibody but not by the anti-Plr/SDH/GAPDH antibody (Fig. 4A, red rectangle). Moreover, we examined whether anti-Plr/SDH/GAPDH serum directly bound to C5a. Biacore analysis revealed that anti-Plr/SDH/GAPDH serum specifically associated with immobilized rPlr/SDH/GAPDH but not with immobilized C5a (Fig. 4B). Preimmune serum (negative control) bound neither rPlr/SDH/GAPDH nor C5a (Fig. 4C). These results suggest that only Plr/SDH/GAPDH can bind C5a on the bacterial surface.

Cell-free Plr/SDH/GAPDH Captures C5a, Forming a Complex—We next examined whether C5a forms a complex with rPlr/SDH/GAPDH or native Plr/SDH/GAPDH in S. pyogenes culture supernatant. After incubation of the mixtures for 1 h at 37 °C, they were separated without boiling by nonreducing SDS-PAGE and then analyzed by Western blotting with anti-C5a and anti-Plr/SDH/GAPDH antibodies. We found that several high molecular weight proteins reacted with both antibodies (Fig. 5, A and B). We also found that anti-Plr/SDH/GAPDH serum did not cross-react with C5a and that anti-C5a serum did not cross-react with Plr/SDH/GAPDH (Fig. 5, A and B, lanes 6 and 8). Interestingly, the same high molecular weight bands were detected with both antibodies.

Based on these results considering the lack of antibody cross-reactivity, we concluded that C5a and Plr/SDH/GAPDH form a complex. This led us to hypothesize that soluble Plr/SDH/GAPDH captures C5a in the plasma and inhibits its ability to activate neutrophils.

rPlr/SDH/GAPDH Inhibits Neutrophil Activation—When we examined the chemotactic responses of neutrophils, we found that maximal chemotactic activity was induced by 10 nM C5a (data not shown). We further investigated the effect of various concentrations (0–100 nM) of rPlr/SDH/GAPDH on C5a-induced chemotaxis. As shown in Fig. 6A, rPlr/SDH/GAPDH significantly inhibited chemotactic activity toward C5a in a dose-dependent manner. In independent experiments, we found that both 100 nM Plr/SDH/GAPDH and 500 nM anti-C5a IgG inhibited chemotaxis to 10 nM C5a by ~60%.

C5a is known to promote bacterial killing by inducing neutrophil production of H₂O₂ (39). We first determined the amount of C5a required for eliciting a maximal H₂O₂ response in neutrophils. In preliminary assays, we found that 10 nM C5a induced maximal H₂O₂ production, and neutrophils were dose-dependently activated by C5a in a narrow concentration range (data not shown). We also found that reacting C5a with 100 nM rPlr/SDH/GAPDH reduced C5a-induced H₂O₂ production by 34% (Fig. 6B). Furthermore, Fig. 6, A and C, show that neutrophils were dose-dependently activated by C5a in a narrow concentration range. Together, these results suggest that entrapment of C5a by soluble Plr/SDH/GAPDH in host tissues and blood inhibits the recruitment of neutrophils to sites of infection as well as the production of H₂O₂ by neutrophils. Finally, a Limulus test indicated that the purified 1 µM rPlr/SDH/GAPDH solution contained less than 0.25 ng/ml lipopolysaccharide. A 10-fold concentration of lipopolysaccharide did not induce chemotactic activity or production of H₂O₂ (Fig. 6, A and B), indicating that the effects of rPlr/SDH/GAPDH were not due to contamination by lipopolysaccharide.

**DISCUSSION**

The innate immune system acts as an initial protective barrier against bacterial pathogens and defends the host until the acquired immune system is activated. Several reports have recently clarified the mechanism by which
pathogens are recognized via Toll-like receptors (40). The complement system also plays a major role in the innate immune system. Complement activation product C5a is known to act as a chemical mediator of neutrophils and to bind to a specific receptor on the cell surface (39). C5a is a 74-amino acid polypeptide generated after activation of the classical, alternative, and lectin binding pathways, and it plays a central role in the complement system (41). In addition, C5a production is associated with many clinical conditions including sepsis (42). For instance, activation of the complement system causes the excessive production of C5a during sepsis (43). Another study showed that the concentration of C5a is stable in the blood of healthy individuals but changes significantly in patients with sepsis (44). Furthermore, Czermak et al. (42) showed that symptoms of sepsis correspond with the excessive production of C5a in rats. These results suggest that a change in the concentration of C5a can lead to immune system dysfunction. Degradation of C5a by ScpA and Plr/SDH/GAPDH may suppress host immunity.

FIGURE 5. rPr/SDH/GAPDH binds to C5a and forms complexes. A, rPr/SDH/GAPDH from S. pyogenes, C5a, or both were incubated for 1 h at 37 °C, separated without heating by nonreducing SDS-PAGE, transferred to PVDF membranes, and analyzed by staining with CBB (lanes 1–6) or analyzed by Western blotting with anti-Pr/SDH/GAPDH (lanes 5–7) or anti-C5a serum (lanes 8–10). Rabbit preimmune serum was used as a negative control (lanes 11–13). B, the cell culture supernatant (sup) from strain SS-9 S. pyogenes, C5a, or both were incubated for 1 h at 37 °C and analyzed as described above. CBB-stained molecular weight standards are shown in the far left lanes of both panels.

S. pyogenes Evades Neutrophils by Its Multifunctional GAPDH

FIGURE 6. Effect of soluble rPr/SDH/GAPDH on neutrophil chemotaxis and H2O2 production. A, human neutrophils were isolated from whole blood samples, and their chemotaxis to combinations of C5a, rPr/SDH/GAPDH, anti-C5a IgG, and lipopolysaccharide (25 pg/ml; negative control) was examined. Chemotaxis was shown as the percentage of neutrophils that migrated to the lower wells compared with the control group (Ctrl). B, the production of H2O2 by human neutrophils was determined in the combinations of C5a, rPr/SDH/GAPDH, anti-C5a IgG, and lipopolysaccharide. The assay was performed in the presence of 1 mM sodium azide to prevent endogenous peroxidase from destroying H2O2. Results represent the means ± S.E. (n = 6) for the relative fluorescence (%) compared with 10 nM C5a (Ctrl), p < 0.01 (*) and p < 0.001 (**) versus control (10 nM C5a).
S. pyogenes Evades Neutrophils by Its Multifunctional GAPDH

FIGURE 7. Proteolytic and C5a binding activities of heat-treated rScpA. A, rGST-C5a-EGFP was incubated with or without heat-treated rScpA. The left lane contained rGST-C5a-EGFP alone. The numbers 0, 10, 20, 30, 60, and 120 represent the amount of time (min) of heating at 70 °C. Samples were separated by SDS-PAGE without reduction or heating. The gel was visualized with a Typhoon 9400 laser scanner. B, biomolecular interaction of C5a and heat-inactivated rScpA. C5a was immobilized on a sensor chip. rScpA (1 μl) was heated at 70 °C for 120 min and injected at a flow rate of 30 μl/min. The resonance units (RU) indicate the extent of antiserum binding by immobilized proteins. The interaction of unheated rScpA (1 μl) with rPlr/SDH/GAPDH (1 μl) was examined as a control.

Only a few bacteria possess molecules that specifically inhibit C5a. For example, S. pyogenes expresses ScpA on its surface, a molecule that inhibits phagocytosis (45, 46). Contrary to our expectations, ligand blotting showed that immobilized ScpA did not bind to soluble C5a (Fig. 1). In addition, Biacore analysis showed that soluble ScpA had a low affinity for immobilized C5a (K_D = 1.38 × 10^7 M^-1 and K_D = 7.25 × 10^7 M^-1). We suspected that S. pyogenes recruits C5a to the bacterial surface via a C5a binding molecule, whereupon it is degraded by cell-associated ScpA. Thus, we searched for a C5a binding molecule on the surface of S. pyogenes. We identified Plr/SDH/GAPDH as a C5a-binding protein; however, it was also possible that C5a was bound, rapidly cleaved, and released by ScpA during the ligand blotting experiments. To determine whether this occurred, we heated rScpA at 70 °C for 10–120 min as described by Anderson et al. (34) and then incubated it with rGST-C5a-EGFP. As shown in Fig. 7A, the C5a-degrading activity of rScpA was eliminated by the heat treatment. Continuous analysis of the interaction between heat-inactivated rScpA and C5a with the Biacore system showed that, in contrast to the heat-treated rScpA, which bound weakly to C5a, rPlr/SDH/GAPDH bound to C5a with a high affinity (Fig. 7B). This suggests that the C5a binding activity on the bacterial surface was mainly due to Plr/SDH/GAPDH.

Another report indicated that the streptococcal surface M protein inhibits phagocytosis by neutrophils (47); however, DeMaster et al. (31) demonstrated that S. pyogenes are phagocytosed and killed after association with neutrophils regardless of whether S. pyogenes express M protein. DeMaster et al. (31) also reported that the efficiency of phagocytosis and bactericidal activity of neutrophils are significantly increased by C5a. Therefore, we considered it important to determine whether C5a could be destroyed or captured by S. pyogenes in host tissues before its recognition by C5a receptors on neutrophils. Although the bactericidal activities of neutrophils are highly effective at preventing the spread of bacterial infections, S. pyogenes expresses several molecules on its surface that inhibit phagocytosis by neutrophils. Pandiripally et al. (49, 50) found that the surface protein FbaA, also known as ORF (open reading frame) X (48), binds to two complement regulatory proteins, specifically, factor H and factor H-like protein 1. Also, Horstmann et al. (51) showed that the M protein binds to factor H, and Kotarsky et al. (52) reported that the M protein binds to both factor H and factor H-like protein 1. Because factor H and factor H-like protein 1 regulate human complement activation by depositing opsonin C3b on bacteria, they proposed that the anti-phagocytic activity of S. pyogenes is due to inhibition of the complement system. Together, these findings indicate that S. pyogenes may have evolved multiple surface components that allow it to avoid and disarm the innate immune system and, therefore, survive in the host.

Here, we demonstrated that Plr/SDH/GAPDH is expressed on the bacterial surface and partially released into the culture supernatant (Fig. 3, A and B). We also found that for effective degradation of C5a, both ScpA and Plr/SDH/GAPDH, must be expressed on the bacterial surface (Fig. 4). We further hypothesized that soluble Plr/SDH/GAPDH plays a role that helps the bacteria evade detection by the host immune system. In addition, we showed that soluble rPlr/SDH/GAPDH captures additional human C5a and forms a complex in the culture supernatant (Fig. 5, A and B) and that neutrophil chemotaxis and H_2O_2 production are significantly inhibited by soluble rPlr/SDH/GAPDH (Fig. 6, B and D).

To further examine the function of Plr/SDH/GAPDH on live S. pyogenes, we attempted to construct a plr/sdh/gapdh-deficient S. pyogenes mutant. We integrated a linearized plr/sdh/gapdh gene replacement plasmid in the highly competent strain S. pyogenes SSI-9 (1, 2, 26) using a double-crossover allelic-replacement method. Also, we transformed S. pyogenes with a circular kanamycin-resistant suicide plasmid to cause single-crossover mutagenesis in the plr/sdh/gapdh gene. Construction of a plr/sdh/gapdh-deficient mutant strain of S. pyogenes should allow investigation of the role of Plr/SDH/GAPDH; however, we were unsuccessful (data not shown), suggesting that the targeted gene is essential for the viability of S. pyogenes. This is not surprising because, in S. pyogenes, glycolysis, which is the initial metabolic pathway of carbohydrate catabolism, is mediated exclusively by the Embden-Meyerhof pathway (22) in which GAPDH is an essential enzyme. Furthermore, Winram and Lottenberg (53) and Jin et al. (54) reported that the plr/sdh/gapdh gene is required for the survival of S. pyogenes.

Previous reports have demonstrated that Plr/SDH/GAPDH plays multiple roles in virulence. The plasmin binding activities of Plr/SDH/GAPDH are known to contribute to streptococcal pathogenesis, whereas conversion of human plasminogen in blood by plasmin initiates the proteolytic degradation of fibrin clots. S. pyogenes secretes the bacterial plasminogen activator streptokinase, which causes venous thrombosis (55) and, in mice, functions as a critical virulence factor by forming local fibrin clots (56). Thus, like Plr/SDH/GAPDH and streptokinase, Plr/SDH/GAPDH and ScpA may work synergistically. In addition, Pancholi and Fischetti (57, 58) reported that Plr/SDH/GAPDH functions as an ADP-ribosylation protein and regulates intracellular signaling pathways in host cells, and Jin et al. (54) showed that Plr/SDH/GAPDH promotes bacterial adherence to pharyngeal cells.

In summary, our results show that the Plr/SDH/GAPDH protein plays multiple roles in bacterial virulence because it allows the evasion of the complement system through cleavage and trapping of C5a. Plr/SDH/GAPDH creates energy via the glycolytic pathway, induces clot formation, disrupts intracellular signaling in the host, and interferes with adherence of the bacteria to host cells. In the initial step of infection, this multifunctional protein works as an adhesin. After internalization of S. pyogenes in the tissue, Plr/SDH/GAPDH functions together with ScpA to help the bacteria escape detection by neutrophils, whereas it functions together with streptokinase to form fibrin clots. How S. pyogenes...
evades the first line of host defense remains unclear, and additional studies of Pfr/SDH/GAPDH are needed to determine how it participates in invasive streptococcal infections, including its role in avoidance of detection by the innate immune system and the deep invasion of organs.

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