Group A Streptococcus Adheres to Pharyngeal Epithelial Cells with Salivary Proline-rich Proteins via GrpE Chaperone Protein*

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Background: Streptococcus pyogenes is often isolated from the oral cavity.

Results: Proline-rich protein in saliva interacted with streptococcal GrpE and the interaction promoted bacterial adhesion.

Conclusion: GrpE on S. pyogenes is involved in bacterial adherence to epithelial cells in the presence of saliva.

Significance: This is the first report that streptococcal GrpE works as multifunctional protein such as an adhesion and a component of septum formation.

Group A Streptococcus pyogenes (GAS) is an important human pathogen that frequently causes pharyngitis. GAS organisms can adhere to and invade pharyngeal epithelial cells, which are overlaid by salivary components. However, the role of salivary components in GAS adhesion to pharyngeal cells has not been reported precisely. We collected human saliva and purified various salivary components, including proline-rich protein (PRP), statherin, and amylase, and performed invasion assays. The GAS-HEp-2 association ratio (invasion/adhesion ratio) and invasion ratio of GAS were increased significantly with whole human saliva and PRP, while the anti-PRP antibody inhibited the latter. GAS strain NY-5, which lacks M and F proteins on the cell surface, was promoted to cohere with HEP-2 cells by whole human saliva and PRP. The 28-kDa protein of GAS bound to PRP and was identified as GrpE, a chaperone protein, whereas the N-terminal of GrpE was found to bind to PRP. A GrpE-deficient mutant of GAS strain B514Sm, TR-45, exhibited a reduced ability to adhere to and invade HEP-2 cells. Microscopic observations showed the GrpE was mainly expressed on the surface of the cell division site of GAS. Furthermore, GrpE-deficient mutants of GAS and Streptococcus pneumoniae showed an elongated morphology as compared with the wild type. Taken together, this is the first study to show an interaction between salivary PRP and GAS GrpE, which plays an important role in GAS infection on the pharynx, whereas the expression of GrpE on the surface of GAS helps to maintain morphology.

Group A Streptococcus pyogenes (GAS) is an important human pathogen that causes a number of serious suppurative infections of the throat, including pharyngitis and tonsillitis, and of the skin, such as impetigo. GAS infections occur widely throughout many countries and areas, are occasionally severe, and are referred to as streptococcal toxic shock syndrome. Streptococcal toxic shock syndrome mostly initiates from pharyngitis and sometimes causes necrotizing fasciitis, sepsis, or disseminated intravascular coagulation, which may result in death. Thus, the elucidation of the infection mechanism of GAS in host cells is critically important.

GAS adheres to and invades pharyngeal epithelial cells (1) and is also known to interact with different kinds of host cells such as breath epithelium A549 (1), HEP-2 (2), HeLa (2), navel band vein endothelium human umbilical vein endothelial cells (2), keratinocyte (3), Detroit 562 (4), and nasopharynx FaDu (4) cells. The initial interaction between GAS and pharyngeal cells is mediated by GAS surface molecules and specific receptors on the host cells (5). Reported examples of these interactions include the C-terminal portion of M protein of GAS and CD46 molecule of keratinocytes (6), hyaluronic capsule of GAS and CD44 molecule of keratinocytes (7), and the fibronectin-binding protein of GAS and integrin α5β1 molecule of host cells via the fibronectin molecule (8, 9).

In pharyngeal mucosa, various mucus molecules are secreted from mucosal epithelial and salivary glands, which modify the adhesion of GAS to host cells. More than 10 types of major proteins are known to exist in saliva, many of which interact with bacteria. Some proteins such as sIgA, mucin, proline-rich protein (PRP), amylase, statherin, lysozyme, and lactoferrin are thought to participate in oral bacterial adhesion to human host cells (10–12). In addition, PRP is known to be the most important part for adherence of many oral bacteria to host tissues (13).

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2 The abbreviations used are: GAS, group A Streptococcus pyogenes; PRP, proline-rich protein; WHS, whole human saliva; EGFP, enhanced GFP; THY, Todd-Hewitt broth with yeast extract.
PRP is a low molecular weight glycoprotein and classified into acidic and basic forms. The major function of PRPs is formation of the pellicle on the tooth surface, recalcification of enamel, dental calculus formation, and adsorption of the oral bacteria to enamel followed by dental plaque formation. PRPs show significant polymorphisms and are responsible for mediation of adherence of oral bacteria to oral cavity surfaces (14–16).

We investigated the effects of salivary components on GAS infection of pharyngeal epithelial cells. In addition, we analyzed the molecular mechanism of GAS adhesion via PRP and identified the molecule on the GAS surface that binds to PRP.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Eukaryotic Cells, and Growth Conditions**

GAS strain SSI-9, isolated from a patient with streptococcal toxic shock syndrome, was provided kindly by Dr. T. Murai (Toho University, Tokyo, Japan), whereas NY-5 (type M12) was kindly provided by Dr. H. Igarashi (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan), and JRS4 (M6) and B5145m (M50) was supplied by Dr. Hanski (The Hebrew University-Hadassah Medical School, Jerusalem, Israel). All strains were grown in Todd-Hewitt broth (BD Biosciences) supplemented with 0.2% yeast extract (THY). For antibiotic selection, erythromycin (10 μg/ml) or streptomycin (1,000 μg/ml) was added to the THY medium. *Escherichia coli* strain XL-10 Gold (Stratagene, La Jolla, CA) was grown in Luria-Bertani (LB) broth (Sigma-Aldrich) or on LB agar plates. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml and erythromycin, 250 μg/ml. A human laryngeal epithelial cell line, HEp-2 (ATCC CCL23), was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in an atmosphere containing 5% CO2 and 95% air.

**Purification of Salivary Component**—Whole human saliva (WHS) was collected from a healthy male donor. After treatment for protease inactivation by incubation at 50 °C for 30 min, the sample was centrifuged at 10,000 × g for 30 min at 4 °C, and then the supernatant was dialyzed extensively against cold distilled water using Spectra/Por 6 tubing (Spectrum Medical Industries, Los Angeles, CA) and lyophilized. The sample was then resuspended in 6 μM guanidine-HCl (Wako, Tokyo, Japan) and centrifuged at 10,000 × g for 30 min at 4 °C. The centrifuged supernatant was applied to a Sephacryl S-200 column (φ3 cm, 180 cm, GE Healthcare) equilibrated with 6 μM guanidine-HCl, and then gel filtration was done at a flow rate of 1 ml/min with 6 μM guanidine-HCl, and 3-ml fractions were collected. Each fraction was examined for proteins by SDS-PAGE.

**Purification of Acidic and Basic PRP**—PRP fractions were separated by gel filtration and dialyzed by distilled water and then lyophilized. Next, they were resuspended in potassium phosphate buffer (pH 6.0) and applied to a polyC column

| Name          | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| grpE-F1       | 5'−CGGGATCCCTGCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-F2       | 5'−CGGGATCCCTAGGGACGAGCCTCTTTCTCTG−3'                                    |
| grpE-F3       | 5'−CGGGATCCCTAGGACGAGCCTCTTTCTCTG−3'                                    |
| grpE-F4       | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-R1       | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-R2       | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-R3       | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-SpnKO-F  | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |
| grpE-SpnKO-R  | 5'−CGGGATCCCTGAGAAAATTAAAGGACG−3'                                     |

**FIGURE 1.** Separation of major salivary components and effects of those components on invasion by GAS. A, salivary amylase (lane 1), PRP (lane 2), and statherin (lane 3) in WHS separated by gel Sephacryl S-200 filtration. B, adhesion/invasion and invasion ratios of GAS strains SSI-9, JRS4, and NY-5 with HEp-2 cells. Black bars indicate adhesion/invasion ratio, and gray bars indicate invasion ratio. Ratios are shown as a percentage as compared with no addition of salivary components. *, p < 0.05; **, p < 0.01.
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Invasion Assay—Invasion of GAS into HEP-2 cells was determined as described previously (17). In brief, mid-log phase bacteria (1 \( \times \) \( 10^7 \) colony forming unit; CFU) were added onto semi-confluent HEP-2 cell monolayers (1 \( \times \) \( 10^5 \) cells) grown in 24-well culture plates with DMEM supplemented with WHS (0, 1, 5, 10 mg/ml) or a purified salivary component; i.e., PRP (0, 0.5, 1, 5 mg/ml), amylase (0, 0.5, 1, 5 mg/ml), or statherin (0, 0.5, 1, 5 mg/ml). After incubation for 3 h at 37 °C in 5% CO\(_2\), the wells were washed with PBS (pH 7.4), and then HEP-2 cells were disrupted with sterile distilled water, serially diluted in water, and plated on THY agar plates to determine the total number of cell-associated bacteria (i.e., both surface and invaded GAS). After non-adhered bacteria were washed out with PBS (pH 7.4), surface bacteria were killed by a 1-h incubation with gentamicin (50 \( \mu \)g/ml). After the medium was removed by aspiration, HEP-2 cells were detached from the THY agar plates to determine the total number of intercellular bacteria (i.e., both surface and invaded GAS). Alexa Fluor 568-conjugated anti-rabbit IgG antibody (Molecular Probes; 1:1,000 dilution). For microscopy, the excitation wavelength of EGFP was quenched by emission of Alexa Fluor 568, which caused red fluorescence to emanate from the adhered bacteria. Coverslips were placed on the slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained with a confocal microscope system.

Purification of PRP-binding Protein—Strain SSI-9 was cultured in 10 liters of TTY broth, which consisted of 7.2% tryptone, 0.72% yeast extract, 0.2% \( \text{K}_2\text{HPO}_4\), 0.2% \( \text{Na}_2\text{CO}_3\), 0.2% NaCl, 0.5% \( \text{KH}_2\text{PO}_4\), and 1.0% glucose, for 18 h. Bacterial cells were collected by centrifugation, washed twice with ice-chilled PBS, and centrifuged. The pellet was resuspended in 8 M urea for 2 h and dialyzed against 50 mM potassium phosphate buffer (pH 8.0) and then subjected to column chromatography using a DEAE 20 column (Bio-Rad) followed by a CHT-10 column (Bio-Rad). The PRP binding protein was examined by SDS-PAGE and Western blotting with biotinylated PRP prepared with an ECL biotinylation kit (GE Healthcare).

Amino Acid Sequencing—Proteins were separated by SDS-7.5% PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was stained with 0.1% Coomasie Brilliant Blue R-250 and then destained with 40% methanol. After washing with distilled water, N-terminal amino acid sequencing was performed by Edman degradation with an HP G1005A sequencer (Hewlett Packard, Palo Alto, CA) using the sample or its tryptic digest. A homology search was performed with the database of the Oklahoma GAS genome-sequencing project. Potential open reading frames were initially identified using GeneWorks software (version 2.4, IntelliGenetics, Campbell, CA).

Preparation of Recombinant GrpE and Antiserum—The primers used for PCR of the grpE gene are listed in Table 1. Primers grpE-F1 and grpE-R1 were used for whole grpE (rGrpE-1). Recombinant C-terminal fragments were produced by use of the primers grpE-F2 (rGrpE-2), grpE-F3 (rGrpE-3), grpE-F4 (rGrpE-4), and grpE-R1, and the N terminus using primers grpE-F1 and grpE-R2 (rGrpE-5), and grpE-R3 (rGrpE-6).
BamHI and EcoRI sites were added to the forward and reverse primers. The PCR products were digested with BamHI and EcoRI, which were ligated into a pGEX-6P-1 vector (GE Healthcare). Following transformation, the rGrpE series was purified according to the manufacturer’s instructions. rGrpE-specific antiserum samples were obtained from New Zealand White rabbits immunized subcutaneously four times with rGrpE-1.

**Binding Assays**—Streptococcal cell-associated proteins were extracted in 8 M urea with occasional stirring for 1 h at 25 °C followed by centrifugation (19). The supernatant was concentrated using 60% saturated ammonium sulfate precipitate and dialyzed against 50 mM sodium phosphate buffer. Proteins were transferred to a PVDF membrane (Millipore) either by an electroblotting using SDS-12% PAGE or a dot-blot apparatus (Bio-Rad), followed by treatment with the blocking agent (GE Healthcare). Hybridization was performed with biotinylated PRP (100 μg/ml; Calbiochem, La Jolla, CA) at 25 °C for 1 h, and the membrane was incubated with horseradish peroxidase (HRP)-conjugated streptavidin at 25 °C for 1 h. Binding was detected with an ECL Western blotting detection kit (GE Healthcare) and visualized by autoradiography with x-ray film at 25 °C.

**Immunofluorescence Observation of Cell Surface GrpE**—A broth culture of SSI-9 cells (1 ml) was centrifuged at 3,000 × g for 5 min, washed twice with PBS, and then blocked with blocking agent containing 50 μg/ml of human IgG (Calbiochem). Cell surface GrpE was detected using a rabbit anti-rGrpE polyclonal antibody and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Stained GAS organisms were analyzed using a confocal laser scanning microscope (model LSM 510).

**Reverse Transcription PCR (RT-PCR)**—Total RNA of GAS was prepared and RT-PCR performed as described previously (20). The forward and reverse primers were *grpE*-F and *grpE*-R, respectively.

**Production of GAS and Streptococcus pneumoniae GrpE Mutant**—PCR products obtained with primers *grpE*-F and *grpE*-R for GAS and GrpESpnKO-F and GrpESpnKO-R for *S. pyogenes*.

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**Figures**

**Figure 3.** Anti-PRP antibody abrogates PRP-promoted adhesion/invasion of HEp-2 cells by GAS. A, purification of acidic and basic PRP. Acidic and basic PRP were purified by HPLC from a PRP fraction obtained with gel filtration. B, inhibition of GAS adhesion and invasion by anti-PRP antibody. GAS strain SSI-9 with 0.5 mg/ml of acidic or basic PRP and 1:100 or 1:1000 diluted anti-PRP was inoculated into HEp-2 cells. The adhesion/invasion ratio was calculated based on the percentage of adherence/invasion bacteria as compared with inoculated bacteria. *, significant difference (p < 0.05) between the mean values, as determined with a Sheffe’s test. Three experiments were performed, with data presented as the mean of three wells from a representative experiment. S.E. values are represented by vertical lines.

**Figure 4.** Identification of PRP binding protein associated with GAS surface. A, reaction between 8 M urea extract of GAS and biotinylated PRP. Lane 1, silver staining of 8 M urea extract of GAS strain SSI-9. Lane 2, Western blotting with biotinylated acidic PRP. Lane 3, Western blotting with biotinylated basic PRP. B, the purified PRP binding protein was purified with DEAE and applied to a hydroxyapatite column. Lane 1, silver staining of purified PRP binding protein. Lane 2, Western blotting with biotinylated acidic PRP. C, open reading frame of PRP binding protein ( = GrpE). The amino acid sequence was predicted based on the GAS SF370 genomic DNA database. Gray highlighting indicates the sequence determined with amino acid sequencing.
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FIGURE 5. Structural similarities of GrpE proteins from different bacterial species based on deduced amino acid sequences. A, alignment of GrpE amino acid sequence from three species. Identical amino acid residues were consistently scattered among the sequences of GrpE in three species marked with gray boxes. #, DNA binding site in E. coli. B, a modeling program, SWISS-MODEL*, was used to simulate the structure of GAS GrpE and Homo sapiens mitochondrial chaperone protein (HMGE) (GrpE) (26) on E. coli GrpE. The three-dimensional structure was drawn using the Swiss-Pdb Viewer.

FIGURE 6. Binding activities of recombinant GrpE fragments. A, schematic maps of product recombinant proteins. B, SDS-PAGE of recombinant fragment protein. C, Western blotting with biotinylated PRP.
pneumoniae, the internal portion of grpE, were ligated into a pSF152 vector (21). The resultant plasmid pJM100, which possesses the add9 gene (spectinomycin-resistant), was transformed into GAS strain B514Sm and S. pneumoniae strain ATCC33400 by electroporation, and inactivated mutant strains were selected on spectinomycin-containing agar plates. In this manner, the GAS and S. pneumoniae GrpE-deficient mutant strains TR-45 and TR-68 were obtained by single crossover recombination. Integration and inactivation of grpE were demonstrated by PCR with the grpE-specific forward primer and add9-specific reverse primer. Morphologic microscopic observation of the GrpE-deficient mutant strain was performed with a scanning electron microscope.

Statistical Evaluations—Sheffe’s test was performed using StatView software (SAS Institute, Inc., Cary, NC) to evaluate differences between groups in the invasion assays. p values of < 0.05 were considered to indicate significance.

RESULTS

Effects of Salivary Components on Association between GAS and Host Cells—Gel filtration of WHS resulted in separation of PRP, amylase, and statherin (Fig. 1A). The ratios of adhesion and invasion of GAS strains SSI-9, JRS4, and NY-5 increased with increasing concentrations of WHS (p < 0.05, Fig. 1B). Addition of WHS (5.0 mg/ml) markedly promoted adhesion/invasion of strain NY-5, with no major cell surface proteins and low adhesion/invasion ability of HEp-2 cells, which served as host cells. Addition of PRP (0.5–1.0 mg/ml) also promoted the adhesion/invasion ratio in all three strains (p < 0.05, Fig. 1B). Amylase and statherin yielded no significant changes in the adhesion/invasion ability of GAS. In contrast, a confocal microscopic observation revealed increased adherence and invasion of GAS following addition of WHS or PRP (Fig. 2).

Acidic and basic PRP samples were obtained from PRP by HPLC (Fig. 3A), and both (0.5 mg/ml) significantly increased the adhesion/invasion and invasion ratios of strain SSI-9 with HEp-2 cells (p < 0.05). Furthermore, the anti-PRP antibody inhibited the promotion of adhesion and invasion by acidic PRP but not by basic PRP (Fig. 3B).

PRP Binding Protein of GAS—The 8 m urea extract of strain SSI-9 was found to cross-react with both acidic and basic PRPs (Fig. 4A). A PRP-binding 28-kDa protein was purified by ion exchange, followed by hydroxylapatite column chromatography (Fig. 4B). The N-terminal sequences of the 28-kDa band and its digest were found to be SEEIKKDDL, ADE, AILP, and ALAVEG, which were similar to the sequences of the 15–23, 56–58, 95–98, and 106–111 amino acids in GrpE, a chaperone protein. Southern blotting indicated that all 15 strains of GAS with different M types possessed the grpE gene (data not shown).

Modeling GAS GrpE on E. coli GrpE Structure—Despite the low level of amino acid identity among GAS GrpE, E. coli GrpE, and human mitochondrial chaperone protein (HMGE) (Fig. 5A), use of the modeling program SWISS-MODEL revealed that the model of the GAS GrpE and HMGE structures matched the crystal structure extremely well, with some deviations in the N terminus (Fig. 5B). The alignment program aligned GAS GrpE with E. coli GrpE, whereas HMGE showed poor consistency in the N-terminal that was proposed to interact with the substrate-binding domain of DnaK (22), suggesting that the structure of the substrate-binding interacting domain may not be conserved. A binding assay with the six lengths of recombinant GrpE fragments (Fig. 6, A and B) showed that whole GrpE and the 18- and 7-kDa N-terminal fragments of GrpE (rGrpE1, -5, and -6) bound to biotinylated PRP, whereas the C-terminal fragments (rGrpE2, -3, and -4) did not bind to PRP (Fig. 6C).

FIGURE 7. Localized expression of GrpE on surface of GAS SSI-9. A, lane 1, SDS-PAGE of 8 m urea extract of strain SSI-9. Lane 2, Western blotting with anti-GrpE antibody. Lane 3, Western blotting with biotinylated PRP. Lane 4, SDS-PAGE of culture supernatant (Sup.) of GAS. Lane 5, Western blotting with anti-GrpE antibody. Lane 6, Western blotting with biotinylated PRP. B, GrpE expression was observed using confocal microscopy. GAS was stained with SYBR Green II (green). a, staining with Alexa Fluor 568-conjugated antibody. b, staining with non-immune rabbit serum and Alexa Fluor 568-conjugated antibody. c, blocking with human IgG1 (50 μg/ml), followed by staining with anti-GrpE antibody and Alexa Fluor 568-conjugated antibody (red). d, fluorescent ring expression of GAS GrpE at cell-dividing position. C, scanning electron microscopic observations of GAS staining with anti-GrpE antibody and gold-conjugated antibody (a and b). Ring expression of GAS GrpE at cell-dividing position (b).
Expression of GrpE on Bacterial Surface and Role of GrpE—Western blotting revealed GrpE in the 8 M urea extract of GAS but not in the culture supernatant (Fig. 7A). The anti-GrpE and gold-conjugated antibodies cross-reacted with the bacterial surface protein located at the cell division site (Fig. 7, B and C). Non-immune rabbit serum or the Alexa Fluor 568-conjugated antibody alone did not react with the surface protein of GAS (Fig. 7B). Furthermore, GrpE mRNA expression levels did not show significant differences as compared with the growth phase (Fig. 8).

We then examined the differences in regard to invasion of HEp-2 cells between wild-type strain B514Sm and the GrpE-deficient mutant TR-45 using an invasion assay. Both adhesion/invasion and the invasion ratio of mutant TR-45 were found to be reduced markedly as compared with B514Sm despite the presence of PRP (Fig. 9).

Morphological Changes of GAS and S. pneumoniae Due to Loss of GrpE—Using scanning electron microscopy, both the GAS (TR-45) and S. pneumoniae (TR-67) mutants lacking GrpE showed morphological changes as compared with the
host cells. Both mutants had rod shapes, although they maintained their chain or diplococcal structure (Fig. 10).

**DISCUSSION**

In the present study, we found that WHS and PRP components promote the adherence of GAS to HEp-2 cells. The concentration of WHS is reported to range from 0.15 to 7.45 mg/ml, with an average of \( \frac{2.3}{2.3} \) mg/ml (23). In our study, the concentration required for GAS to adhere or maximally invade was determined to be 5 mg/ml, indicating that saliva clearly influences early infection by GAS in vivo. Although salivary amylase and statherin are known to have specific binding activities with some oral bacteria, no influence was observed in terms of adhesion of GAS to HEp-2 cells. In addition, the concentration of acidic PRP in whole saliva was reported to range from 288 to 521 \( \frac{9262}{9262} \) g/ml (24). We found that \( \frac{9262}{9262} \) g/ml of PRP, a similar level to that in fresh whole saliva, significantly promoted both GAS adhesion and invasion.

Following gentamicin treatment, a microscopic observation revealed that GAS adhesion/invasion remained unchanged with the addition of PRP for 1 h, suggesting that GAS attached to HEp-2 cells via PRP may survive even after gentamicin treatment. Protection of GAS by PRP from gentamicin may occur because GAS can aggregate with PRP (data not shown) and aggregated GAS organisms are protected from phagocytosis (25).

GrpE of GAS, identified as a PRP-binding protein in the present study, is a part of the heat shock protein 70 chaperone complex (GrpE, DnaJ, and DnaK), which is an important part of the cell machinery for protein folding and helps to protect cells from stress. Choglay et al. (26) reported that GrpE in *E. coli* organisms and humans have a functionally conserved C-terminal structure, which is a DnaK- and ATPase-binding site, despite a low level (28%) of amino acid identity. On the other hand, GAS and human GrpE have 23% of the same amino acid sequence, whereas the N-terminal structures of GrpE in GAS, *E. coli*, and humans vary. We found that the N terminus of GrpE of GAS binds to PRP and a drug that targets the GAS GrpE N terminus may specifically remove GAS. Nevertheless, the strength of the modeling and position of conserved residues in the regions that contact with Hsp70 proteins strongly suggest that GAS GrpE may also interact with Hsp70 proteins, possibly facilitating nucleotide exchange.

Some protein expression might be promoted by stimulation of salivary proteins, for example SspA and SspB, which are glycoprotein-binding proteins of *Streptococcus gordonii* DL1, and ClpE, a chaperone homologue protein of the *Lactobacillus lactis* ClpE-ATPase family that is promoted by their expression in saliva (27). The ClpE-ATPase family is highly conserved in many Gram-positive strains (28). In *E. coli*, ClpE family chaperone proteins cooperate with Dnak/Dnal/GrpE chaperone proteins (29). In GAS, some molecules that contain GrpE might be expressed in WHS or PRP, which in turn mediate the adhesion and invasion of GAS to host cells.

Our results showed that GrpE is expressed on the surface of GAS and localized in the center of the septum apparatus. GrpE and Dnak form a complex structure. Dnak is associated with cellular division and located in cellular membranes (30); thus, GrpE and Dnak may have the same distribution in GAS. GrpE forms a ring structure at the site of cell division, as FtsZ forms a ring that simultaneously constricts as the septum grows in *E. coli* (31). The gene order and organization of this genetic region associated with cell division and containing FtsZ has been found to be conserved in GAS, *Staphylococcus aureus*, and *Bacillus subtilis*, raising the possibility that previously unidentified loci may also be involved in division (32). In *E. coli*, the ring structure of FtsZ is associated with Dnak (33) and HscA (32), which are classified in the Hsp70 family. Dnak and GrpE might form a ring structure associated with FtsZ and cell divi-

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**FIGURE 10.** Elongated morphology of GrpE-deficient mutants of GAS and *S. pneumoniae*. A, GAS wild-type strain B514Sm. B, GAS GrpE-deficient mutant TR-45. C, *S. pneumoniae* wild-type strain ATCC33400. D, *S. pneumoniae* GrpE-deficient mutant TR-68.
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esion. In humans, GrpE is only expressed in mitochondria; thus, drugs that target GrpE on the bacterial surface are considered to be feasible.

The GrpE mutants showed changed morphology and were longer as compared with the wild type. Some bacterial species show morphology changes under various conditions. Streptococcus mutans glycoprotein IDG-60 (34) and Streptococcus agalactiae PcsN (35) are proteins required for cell wall separation, and these mutants revealed growth in clumps, cell separation in several planes, and multiple division septa within single cells. Sugai et al. (36) showed that cibacron blue 3G-A elongated Bacillus and Lactobacillus cells were filaments. Furthermore, an hscA conditional knock-out of the wild-type strain under non-permissive conditions exhibited longer rod cells with an abnormal localization of FtsZ (33, 37).

Cytosolic chaperones act to prevent the tight folding or aggregation of the precursor protein (38). A GrpE mutant might make use of the unfolding protein and change the shape of cells. The chaperone protein also takes part in protein transport in the cell; thus, a GrpE mutant might change the protein associated with the cell structure or surface proteins. The low adherence/invasion rate of the present GrpE mutants may have been caused by alterations of the cell binding-associated protein or difficulty with phagocytosis of elongated bacteria.

In summary, we found that the salivary component PRP binds to GAS GrpE and promotes GAS adherence to and invasion of HEp-2 cells. Our findings suggest that pathogenic GAS utilizes salivary PRP to infect the tonsils and pharynx.

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