Group A Streptococcal Cysteine Protease Cleaves Epithelial Junctions and Contributes to Bacterial Translocation*\(^{[3]}\)

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**Background:** Group A Streptococcus (GAS) translocates across the host epithelial barrier.

**Results:** Streptococcal pyrogenic exotoxin B (SpeB) directly cleaves junctional proteins.

**Conclusion:** The proteolytic efficacy of SpeB allows GAS to translocate across the epithelial barrier.

**Significance:** SpeB-mediated dysfunction of the epithelial barrier may have important implications for not only bacterial invasion but also dissemination of other virulence factors throughout intercellular spaces.

**Streptococcus pyogenes** (group A Streptococcus; GAS)\(^{3}\) is well known as a human-specific pathogen responsible for numerous diseases, ranging from pharyngitis and impetigo to life-threatening invasive diseases, including necrotizing fasciitis and streptococcal toxic shock syndrome (1, 2). Serious postinfectious immune sequelae such as rheumatic fever and acute glomerulonephritis occasionally develop following repeated GAS exposure. Despite the availability of sequence information for several GAS genomes and detailed characterization of their virulence factors, a safe and effective commercial GAS vaccine has yet to be developed (3).

**Group A Streptococcus** (GAS) is an important human pathogen that possesses an ability to translocate across the epithelial barrier. In this study, culture supernatants of tested GAS strains showed proteolytic activity against human occludin and E-cadherin. Utilizing various types of protease inhibitors and amino acid sequence analysis, we identified SpeB (streptococcal pyrogenic exotoxin B) as the proteolytic factor that cleaves E-cadherin in the region neighboring the calcium-binding sites within the extracellular domain. The cleaving activities of culture supernatants from several GAS isolates were correlated with the amount of active SpeB, whereas culture supernatants from an speB mutant showed no such activities. Of note, the wild type strain efficiently translocated across the epithelial monolayer along with cleavage of occludin and E-cadherin, whereas deletion of the speB gene compromised those activities. Moreover, destabilization of the junctional proteins was apparently relieved in cells infected with the speB mutant, as compared with those infected with the wild type. Taken together, our findings indicate that the proteolytic efficacy of SpeB in junctional degradation allows GAS to invade deeper into tissues.

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been extensively investigated and shown to be important for pathogenesis, its participation in epithelial barrier dysfunction is as yet unproven. Herein, we provide the first direct evidence that SpeB (streptococcal pyrogenic exotoxin B), a broad spectrum secreted cysteine protease, effectively cleaves transmembrane proteins associated with the epithelial barrier to permit bacterial penetration. Our results reveal a new mechanism to explain how GAS directly disrupts the epithelial barrier.

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Culture Conditions—**Invasive GAS clinical isolates, strains NIH35 (serotype M28), SSI-1 (serotype 3), SSI-9 (serotype M1), and #30 (serotype M12), were isolated from patients with streptococcal toxic shock syndrome. Other GAS clinical isolates, strains SE370 (serotype M1), TW3358 (serotype M3), TW3337 (serotype M12), TW3339 (serotype M28), NZ131 (serotype M49), and 591 (serotype M49), were used as noninvasive GAS strains. *Escherichia coli* XL10-Gold (Stratagene) served as a host for plasmids pAT18 and pSET4s (14, 15). GAS strains and *E. coli* strains were cultured at 37 °C in Todd–Hewitt broth (*Becton, Dickinson and Company*) supplemented with 0.2% yeast extract (*Becton Dickinson*) (THY medium). *E. coli* strains were cultured in LB medium (Sigma–Aldrich) at 37 °C with agitation. For selection and maintenance of the mutants, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; erythromycin, 150 μg/ml for *E. coli* and 1 μg/ml for GAS; and spectinomycin, 100 μg/ml for *E. coli* and GAS.

**Construction of Recombinant SpeB and GAS Mutant Strains—**Preparation of recombinant SpeB has been previously described (16). An in-frame speB deletion mutant, its complemented strain, and *sagA-speB* double mutant were constructed using pSET4s, as previously reported (11, 17). Primers speBkoF1 (5′-GGGATCCGTGTTATATCAGAATGTGTTT-TTGAAATG–3′), speBkoR1 (5′-ACTTTGTTAACCCTGTTAGAGCCCATTTTTTATACTCTTTCT–3′), speBkoF2 (5′-GAAAGGATTTATAAAAATATGGCTTACACGTTACCAAGT–3′), and speBkoR2 (5′-AAGCAGGTTTAAAAAATCTTTACACGTATCTTTCT–3′) were used for deletion of speB gene. For construction of EGFP-expressing GAS strains, a pAT18-EGFP vector was transformed into the GAS strains by electroporation (8).

**Cell Cultures—**Caco-2 cells (Riken Cell Bank) were maintained in minimum essential medium (Invitrogen) supplemented with 20% fetal bovine serum (SAFC Biosciences) and 20 μg/ml gentamicin, 17.75 mM NaHCO₃ (Wako), and 15 mM HEPES (Dojindo) at pH 7.4. HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (Wako) supplemented with 10% fetal bovine serum (SAFC Biosciences), 20 μg/ml gentamicin. Detroit 562 cells (ATCC CCL-138; American Type Culture Collection) were maintained in minimum essential medium-α (Wako) supplemented with 10% fetal bovine serum (SAFC Biosciences) and 20 μg/ml gentamicin.

For translocation assays, Caco-2 cells were seeded at 2 × 10⁵ cells/well onto polycarbonate Millicell culture plate inserts (12-mm diameter, 3-μm pore size; Millipore) and cultured for 5 days at 37 °C under a 5% CO₂ atmosphere, as described previously (11). Transepithelial electrical resistance (TER) of the fil-ter-grown monolayers was measured using a Millicell-ERS device (Millipore), and monolayers exhibiting TER values of 450–500 Ω cm² were used in the experiments.

**Translocation Assay—**GAS strains were grown to the exponential phase (A₆₀₀ = 0.4) and centrifuged at 7000 × g for 5 min. Pelleted cells were washed with PBS and then resuspended in cell growth medium. Polarized monolayers were infected with GAS at a multiplicity of infection (MOI) of 10. The ability of GAS strains to translocate into monolayers was assessed by quantitative cultures of media obtained from the lower chambers at various times after infection as described previously (11).

**Paracellular Flux of FITC-Dextran—**Caco-2 cells were grown on Millicell filters and infected with GAS at an MOI of 10 for 2 h. To remove nonadherent bacteria, the medium in the upper chamber was replaced with fresh medium at 2 h after infection. After removing nonadherent bacteria, FITC-dextran with a molecular mass of 4, 10, or 70 kDa (Sigma) was added to the apical surface of the cell monolayers. At 8 h after infection, the amount of FITC-dextran in the basolateral medium was measured using a Wallac 1420 ARVOx fluorometer (excitation, 485 nm; emission, 535 nm; PerkinElmer Life Sciences).

**Analysis of GAS-induced Cleavage of Junctional Proteins—**Overnight cultures of GAS clinical isolates were centrifuged at 7000 × g for 5 min, and then the supernatants were incubated with 0.5 μg of occludin (Abnova) or E-cadherin (R&D Systems or Advanced BioMatrix) for 6 h at 37 °C. To search for a bacterial protease that cleaves E-cadherin, GAS supernatants were individually pretreated for 30 min at room temperature with the following protease inhibitors; N-ethylmaleimide (1 mM), E-64 (10 μM), chymostatin (330 μM), leupeptin (100 μM), AEBSF (1 mM), aprotinin (800 μM), benzamidine HCl (1 mM), trypsin inhibitor (100 μM), 6-aminoheoxaenoic acid (38 mM), pepstatin (1.5 μM), phosphoramidom (10 μM), bestatin (1 mM), or EDTA (1 mM). All protease inhibitors were purchased from Sigma–Aldrich.

For infection assays, epithelial cells were seeded at 2 × 10⁵ cells/well (35-mm diameter; Corning), cultured for 3 days, and then infected with GAS strains at an MOI of 10. At the end of the infection period, the infected cells were lysed with Laemmli gel loading buffer containing 6% 2-mercaptoethanol. Cleavage of intercellular junctions was detected by Western blot analysis (11). Western blot signals were quantified using Scion Image 4.0.3.2 software (Scion).

For identification of the cleavage site in E-cadherin, E-cadherin was incubated with DTT-activated recombinant SpeB (200 nM) for 6 h at 37 °C. Proteins were separated by SDS-PAGE and stained with Coomasie Brilliant Blue. N-terminal amino acid sequencing was performed using the Edman degradation method with an ABI protein sequencer model 491HT (Applied Biosystems).

**Measurement of SpeB Activity—**For quantitative assays of SpeB activity, casein hydrolysis was analyzed using FITC-labeled casein (18). Filter-sterilized supernatants from overnight GAS cultures or recombinant SpeB were activated in assay buffer (0.1 M phosphate buffer, pH 7.6, 0.01 mM EDTA) supplemented with 10 mM DTT and incubated for 30 min at 37 °C. The activated supernatants were added to an equal volume of
FITC-labeled casein (Sigma-Aldrich) prepared with or without 10 μM E-64. At 6 h after incubation, the reactions were stopped by adding 5% trichloroacetic acid, and then the mixtures were stored overnight at 4 °C. Following centrifugation (15,000 × g for 5 min), the resultant supernatants were diluted with 0.5 M Tris buffer (pH 8.5), and fluorescence intensity was measured using a Wallac 1420 ARVOsx fluorometer (excitation wavelength, 485 nm; emission wavelength, 535 nm; PerkinElmer Life Sciences).

Statistical Analysis—Statistical analysis was performed using a Mann-Whitney U test. A confidence interval with a p value of < 0.05 was considered to be significant.

RESULTS

GAS Supernatant Induces Cleavage of E-cadherin—Culture supernatants from several serotypes of GAS strains recovered from invasive (strains SSI-9, SSI-1, #30, and NIH35) and noninvasive (strains SF370, TW3358, TW3337, TW3339, NZ131, and 591) diseases were incubated with the extracellular domain of human E-cadherin fused to the Fc region of human IgG1, after which cleavage was analyzed using Western blot analysis (Fig. 1). Utilizing an antibody against the extracellular domain of E-cadherin, the intact protein was detected as an ~120-kDa band under a reducing condition. Loss of the 120-kDa band and appearance of cleavage products with apparent molecular masses of 60–100 kDa were detected in culture supernatant samples obtained from SF370, SSI-1, TW3358, #30, TW3337, NIH35, TW3339, and 591. Interestingly, cleavage was completely abrogated by pretreatment with a protease inhibitor mixture (data not shown). On the other hand, neither the culture supernatant from strain SSI-9 nor that from strain NZ131 was capable of cleaving the E-cadherin fragment. These results indicate that the culture supernatants from strains SF370, SSI-1, TW3358, #30, TW3337, NIH35, TW3339 and 591 include proteases that cleave the extracellular domain of E-cadherin. We also noted that the ability of GAS culture supernatants to cleave E-cadherin was not related to disease severity, i.e., strains from invasive as compared with those from noninvasive diseases.

Identification of Bacterial Protease Responsible for Cleavage of E-cadherin—To search for bacterial proteases that contribute to the cleavage of E-cadherin, the culture supernatant of strain NIH35, a prominent degrader, was pretreated with several types of protease inhibitors before co-incubation with recombinant E-cadherin protein (Fig. 2). The cysteine protease inhibitors N-ethylmaleimide, E-64, and chymostatin completely inhibited cleavage of E-cadherin, whereas protease activity partially remained in the leupeptin-treated GAS supernatant. In contrast, protease inhibitors that target serine protease, aspartate protease, and metalloprotease had no detectable effect on E-cadherin cleavage, whereas EDTA did have an acceleratory effect. This result may reflect a chelating effect of EDTA on stabilization of the E-cadherin structure, because E-cadherin forms a homodimer in the extracellular domain in a Ca²⁺-dependent manner. In experiments with strain SSI-1, even though the degradation pattern of E-cadherin was slightly different from that observed with other strains (Fig. 1), the appearance of cleavage products was completely inhibited by cysteine protease inhibitors (data not shown). It has been reported that GAS secretes two major cysteine proteases, SpeB and immunoglobulin G-degrading enzyme of S. pyogenes (IdcS or Mac-1). E-64, a cysteine protease, specifically inhibits the proteolytic activity of SpeB, but not that of IdcS (19). In the present study, supernatant-induced cleavage of E-cadherin was completely inhibited by E-64 (Fig. 2). These results suggest that SpeB is a proteolytic factor for cleavage of E-cadherin. Moreover, restoration of the full-length band was observed in a leupeptin concentration-dependent manner, whereas the cleavage product did not disappear even in the presence of leupeptin at 50 μM (supplemental Fig. S1). Leupeptin is an arginine-dependent protease inhibitor. Because Cys at position 192, His at position 340, and Trp at position 357 play critical roles in the enzymatic activity of SpeB (20), leupeptin might be less effective for inhibition of SpeB activity as compared with those other cysteine proteases. Consequently, we investigated whether SpeB is involved in cleavage of E-cadherin.

SpeB Cleaves Intercellular Junctional Proteins—To examine whether SpeB is responsible for GAS supernatant-induced cleavage of intercellular junctions, an in-frame speB deletion mutant and its complemented strain were constructed. Equivalent growth rates of wild type and mutant strains were observed in conventional liquid medium (data not shown). GAS culture supernatant-induced cleavage of E-cadherin was nearly abolished by mutagenesis of the speB gene, whereas it was completely restored by the complementation (Fig. 3A). A similar phenomenon was observed with strains #30, TW3337, and TW3339 (supplemental Fig. S2), indicating that SpeB-dependent protease activity toward E-cadherin is conserved among clinical isolates with no relation to disease severity. These observations prompted us to compare the SpeB-specific protease activities in the culture supernatants of the previously examined GAS strains (Fig. 3B). High caseinolytic activities were detected in culture supernatants from strains #30, TW3337, NIH35, TW3339, and 591, which are findings consistent with its ability to cleave recombinant E-cadherin (Fig. 1). In contrast, lower levels of SpeB activity were observed in supernatants from strains SSI-9, SF370, SSI-1, TW3358, and NZ131. Together, these results indicate that SpeB-associated cysteine protease activity is correlated with the cleavage of intercellular junctions.
SpeB is secreted as a 42-kDa zymogen and autocatalyzed into an active 28-kDa cysteine protease (18, 20), and its substrate specificity is similar to that of the papain family of proteases (21). Active SpeB cleaves the host extracellular matrix (22), immunoglobulins (23, 24), and complement components (16). In addition to its ability to cleave host proteins, SpeB exerts proteolytic activity toward streptococcal surface proteins (25–27). Numerous investigations have reported that this proteolytic activity contributes to bacterial evasion from the host defense system and systemic dissemination (reviewed in Refs. 28 and 29). Therefore, we postulated that SpeB directly cleaves intercellular junctional proteins, and GAS translocates through the opening of paracellular junctions.

To examine whether SpeB directly cleaves transmembrane junctions, recombinant E-cadherin was treated with various concentrations of recombinant SpeB (Fig. 3C). An SpeB-dependent loss of the full-length band and appearance of several cleaved products, which reacted with an antibody against the extracellular domain of E-cadherin, were detected. Thus, five N-terminal residues in the cleavage products of E-cadherin in the presence of 200 nM SpeB were determined by peptide N-terminal residues in the cleavage products of E-cadherin in extracellular domain of E-cadherin, were detected. Thus, five N-terminal residues in the cleavage products of E-cadherin in the presence of 200 nM SpeB were determined by peptide sequence analysis, which identified the N-terminal sequence of fragments with molecular masses of ~80 and 65 kDa as DWVIP, which is identical to the N terminus of the EC1 domain, i.e., the N terminus of the recombinant protein. SpeB has been reported to cleave IgG in the hinge region (23). Because recombinant E-cadherin is the extracellular domain fused to the IgG Fc region at the C terminus, it is likely that an 80-kDa band represents a fragment with intact extracellular E-cadherin and a truncated Fc fragment. Deducing from this size, the 65-kDa band represents a fragment cleaved within the EC domains, possibly between EC4 and EC5. Meanwhile, the sequence of cleavage fragments with molecular masses of ~55 and 35 kDa revealed VTDTN, which corresponds to the neighboring sequence of the calcium-binding site between the EC2 and EC3 domains. Speculating from these sizes, the C terminus of the 55- and 35-kDa fragments would be within the Fc region and within the region between EC4 and EC5, respectively. Because each EC domain of E-cadherin contains DXD or DXNDN motifs responsible for mediating calcium-dependent adhesion, SpeB may cleave the regions neighboring these motifs between each EC domain. Furthermore, SpeB-mediated cleavage of occludin, the most prominent member of TJ, was examined using an antibody against extracellular domain of E-cadherin and horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected with a peroxidase substrate tetramethylbenzidine.
polarized Caco-2 cells, which express typical epithelial markers of differentiation and are widely used as an in vitro model of the epithelial barrier (30), were utilized for the translocation assay (Fig. 5A). The apical surfaces of Caco-2 monolayers were infected with strain NIH35 or the speB deletion mutant, and then the ability of GAS to translocate across the monolayers was assessed for up to 8 h at 37 °C. The wild type strain was detected in basolateral media, indicating migration of bacteria through the monolayers. Although deletion of the speB gene had no effect on the production of capsule and SLS (data not shown), translocation of the speB mutant was significantly decreased. The translocation phenotype of the complemented strain was restored to the wild type level. To further investigate the generality of the role of SpeB in the translocation process, we also constructed speB deletion mutants with a background of #30, TW3337, TW3339, and 591, which showed a relatively high level of SpeB activity (Fig. 3B). For all of the tested strains except strain 591, the translocation ability of their speB deletion mutants was decreased as compared with the wild type (Fig. 5B). An antibiotic protection assay confirmed that strain 591 tends to internalize in an intracellular niche (data not shown). These results suggest that SpeB-dependent translocation is widely conserved among a majority of clinical isolates.
values between the wild type and speB deletion mutant was observed (data not shown). Our previous study reported that SLS, which is encoded by the sagA gene, facilitates GAS translocation via degradation of epithelial intercellular junctions, accompanied by a decrease in TER. Therefore, decreased TER value in the Caco-2 cell monolayers infected with the speB mutant was attributed to SLS-induced loss of intercellular junction integrity. Furthermore, the bacterial translocation rate of the speB mutant was considerably lower than that of the wild type and speB deletion mutant (supplemental Fig. S3A). To clarify the relative contribution of SpeB in bacterial translocation, we constructed a double deletion mutant lacking the sagA and speB genes. Unexpectedly, no significant difference in the ability of translocation across Caco-2 monolayers between sagA mutant and the sagA-speB double mutant was observed. We next assessed the effects of SpeB on junctional integrity using a biochemical assay that measured transepithelial transport of FITC-labeled dextrans (supplemental Fig. S3B). Interestingly, only passive diffusion of dextran with a molecular mass of 4 kDa across the cell monolayers was decreased in cells infected with the sagA-speB double mutant, as compared with those infected with the sagA mutant. These results suggest that the proteolytic activity of SpeB contributes to dysfunction of the paracellular barrier in concert with SLS through a different mechanism. Additionally, we emphasize that single deletion of the speB gene compromised the ability of bacteria to translocate across the epithelial monolayer (Fig. 5). Therefore, we argue that SpeB is one of the determinants for the bacterial translocation across epithelial barrier.

DISCUSSION

Epithelial barrier integrity is maintained by physical interactions of cell-cell junctional complexes, including TJs and AJs (33). TJs seal intercellular space between adjacent epithelial cells to protect subepithelial tissue against microbial invaders, whereas AJs are required for integrity of the TJs. Several human pathogens have evolved strategies to target intercellular junctions and their components (34, 35). GAS also possesses some tools, such as a hyaluronic acid capsule (10) and SLS (11), to trigger signals that disrupt intercellular junctions and permit bacterial penetration via a paracellular route. In this study, we sought to define the role played by potential proteolytic activity of SpeB in destabilization of epithelial cell-cell junctional complexes. Here, we report for the first time that SpeB directly cleaves intercellular junctions and that its proteolytic activity contributes to GAS translocation across the epithelial barrier.

E-cadherin is a constituent of AJs and spans paracellular space through five extracellular domains repeated in tandem, EC1–EC5 (36). The extracellular portion of E-cadherin contains the conserved motifs DXD and DANDN, which bind Ca$^{2+}$ and serve as templates for homophilic binding with E-cadherin molecules on the surface of neighboring cells. It has been reported that single mutations introduced into calcium-binding motifs resulted in abrogated calcium binding activity and cell adhesion potential (37). Notably, we revealed in the present study that SpeB cleaves the ectodomain of E-cadherin at a site close to the region of calcium-binding sites. Together, these
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In the present study, we found a positive correlation between the amount of active SpeB and cleavage of E-cadherin, whereas its proteolysis activity was not associated with the severity of disease related to GAS isolates derived from invasive infections. In clinical epidemiology surveillance, the association of SpeB expression with disease severity in GAS infections remains controversial. Although the cysteine protease activities of strains SSI-9 and SSI-1, isolates from invasive diseases, were relatively low in our in vitro experiments, we previously demonstrated that SpeB is a key factor for survival of SSI-9 in a mouse model (16). Therefore, it is likely that the protease activities of these strains are increased in vivo. Because we also noted that several non-invasive isolates possess a potential ability to destabilize intercellular junctions, disruption of cell-cell adhesion is likely related to a variety of clinical symptoms involving the skin and soft tissue. Consequently, the proteolysis action of SpeB may be involved in the development of skin and purulent infections.

It was recently reported that naturally occurring single amino acid replacement in ropB (38), a critical positive regulator of the speB gene, may have a role in expression of active SpeB in in vivo conditions. Furthermore, it has been suggested that spontaneous mutations within genes encoding the CovRS two-component system affect transcriptional activity of several virulence factors including SpeB during dissemination in vivo (27, 39, 40). A dynamic shift of SpeB expression in vivo caused by these mutations or sensing the surrounding environment would affect the translocation phenotype of GAS, especially in the early stages of infection.

In summary, the present findings provide evidence that SpeB can cleave intercellular junctions, thereby allowing bacterial translocation across the epithelial barrier. We speculate that SpeB-induced opening of paracellular junctions also permits penetration of several virulence factors, including toxins and superantigens, and leads to exacerbation of clinical manifestations. Following bacterial translocation across the epithelial barrier, evasion of the host immune system is essential for development of invasive infections. Accordingly, a part of colonized bacteria in superficial epithelial cells of the pharynx or skin might preferentially invade through a paracellular route to avoid being killed in intracellular space. However, a universal issue with the in vitro model of the epithelial barrier used in this study is differences in epithelial stratification between the polarized cell line and in vivo conditions. Both the pharynx and skin, primary sites of GAS infection, are guarded by multilayered stratified epithelium, whereas the cells used in this study differentiate into a polarized monolayer. Therefore, further investigations such as in vivo animal studies are necessary for proper interpretation of our in vitro findings. Although the precise role of SpeB in the pathogenesis of invasive GAS disease remains controversial, the present results support the notion that SpeB acts as a multifunctional element in the initial stages of infection.

Acknowledgments—We thank H. Watanabe and T. Murai for providing the GAS strains. We gratefully acknowledge T. Sekizaki and D. Takamatsu for the pSET4s plasmid. We thank Y. Fujinaga for offering helpful advice.
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