Molecular Characterization of a Novel Fibronectin-binding Protein of \textit{Streptococcus pyogenes} Strains Isolated from Toxic Shock-like Syndrome Patients*

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Group A \textit{Streptococcus pyogenes} has surface-located fibronectin (Fn)-binding proteins known to be a major virulence factor, which adheres to and invades host cells. We present a novel Fn-binding protein of group A streptococcus serotype M3 and M18 strains isolated from patients with toxic shock-like syndrome (TSLS). By searching the whole genome sequence of an M3 strain from a TSLS patient, an open reading frame was found among the putative surface proteins. It possessed an LPXTG motif and Fn-binding repeat domains in the C-terminal region and was designated as FbaB (Fn-binding protein of group A streptococci type B). The \textit{fbaB} gene was found in all M3 and M18 strains examined, although not in other M serotypes. Furthermore, FbaB protein was expressed on the cell surface of TSLS strains but not on non-TSLS ones. Enzyme-linked immunosorbent assay and ligand blotting revealed that recombinant FbaB exhibits a strong Fn-binding ability. An FbaB-deficient mutant strain showed 6-fold lower adhesion and invasion efficiencies to HEp-2 cells than the wild type. Moreover, mortality was decreased in mice infected with the mutant strain in comparison to the wild type. These data suggest that FbaB is etiologically involved in the development of invasive streptococcal diseases.

Group A streptococcus (GAS)§ is known as the pathogen of streptococcal pharyngitis, although it also causes severe invasive infections, including toxic shock-like syndrome (TSLS) and necrotizing fasciitis. All of these diseases are initiated by the adhesion of GAS to epithelial cells. GAS expresses a wide variety of structural and enzyme proteins that are associated with the bacterial cell wall. It has been demonstrated that several surface proteins have binding abilities to human host proteins, such as fibronectin (Fn) (1), laminin (2), plasmin (3), collagen (4), immunoglobulins (5), and C4b (6). Fn-binding proteins of GAS have also been reported to be adhesins and invasins, including protein F1/SfbI (1, 7), protein F2 (8), SfbII/SOF (9, 10), PFBP (11), and Fba (renamed FbaA) (12). It is interesting to note that each Fn-binding protein is distributed in a particular group of M serotype (7, 12, 13), and highly virulent GAS strains possess one or more Fn-binding proteins. M1 and M49 organisms express FbaA, whereas M6 and M12 organisms retain protein F1/SfbI, and M4 and M28 strains produce both proteins. On the other hand, M3 and M18 organisms do not possess FbaA as well as protein F1/SfbI and SfbII/SOF (7, 12, 13). M3 is a major serotype that has been isolated throughout the world from patients with TSLS and other severe invasive diseases (14–16), and genetic and epidemiological studies have shown that M18 strains are implicated in acute rheumatic fever (17). These findings led us to a hypothesis that there is another Fn-binding protein in M3 or M18 GAS organisms that is involved with their stronger pathogenic capability.

The availability of complete genome sequences of several serotype strains (17–19) has shifted focus to the identification and characterization of all gene products that are expressed in GAS, and as a result, several motifs that may be suitable for identification of functional proteins have been found. First, an LPXTG motif known to be the cell-anchoring sequence in Gram-positive cocci (21) has emerged as a target for the identification of new potential surface proteins using the genome sequencing data bases, with the result that GRAB (22), Sc1A (23), Sc1B (24), and FbaA (12) have been reported. Furthermore, various functional motifs among the eukaryotic and prokaryotic species have been added to the protein data bases. Here we report investigation of a novel Fn-binding protein specific for serotype M3 and M18 strains isolated from patients with TSLS in the complete genome data base of the M3 strain using biochemical and bioinformatics approaches.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Eukaryotic Cells, and Growth Conditions—**GAS strains were isolated from patients with pharyngitis and TSLS (25) and were grown in Todd-Hewitt broth (Difco) supplemented with 0.2% yeast extract (THY). For antibiotic selection, erythromycin (10 \( \mu \)g/ml) or

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EMBL Data Bank with accession number(s) AB084272.

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‡The abbreviations used are: GAS, group A streptococcus; TSLS, toxic shock-like syndrome; Fn, fibronectin; FbaB, fibronectin-binding protein of group A streptococci type B; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FBS, fetal bovine serum; BS, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; PVDF, polyvinylidene difluoride; CFU, colony-forming units; ORF, open reading frame.

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kanamycin (300 μg/ml) was added to THY medium. Escherichia coli strains XL-1 Gold (Stratagene, La Jolla, CA) and BL21 (Novagen, Madison, WI) were grown in Luria-Bertani (LB) broth (Sigma) or on LB agar plates. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), erythromycin (250 μg/ml), and kanamycin (30 μg/ml). Relevant characteristics of bacterial strains used in this study are listed in Table I. A human laryngeal epithelial cell line, HEp-2 (Applied Biosystems), was stained with 0.1% Coomassie Bril-

ing Blue R-250 and then destained with 40% methanol. After washing with distilled water, N-terminal amino acid sequencing was performed by Edman degradation using an ABI protein sequencer model 491HT (Applied Biosystems). The protein was separated by 10% SDS-PAGE (Invitrogen) at 37 °C in an atmosphere containing 5% CO₂ and 95% air. The complete genome sequence of GAS strain SSI-1 (M3, isolated from a patient with TSLS in Japan) was obtained from the genome data base at Osaka University (genome-info.osaka-u.ac.jp/bacteria/spyv/). Potential ORFs were initially identified using GeneWorks version 2.4 (IntelliGenetics, Campbell, CA), and ORF data were analyzed by PSI- and PHI-BLAST (www.gen-info.osaka-u.ac.jp/bacteria/spyv/BLAST/), whereas the prediction of signal sequence was done using the WWW Signal Scan Service (bimas.dccc.nih.gov/BLAST/), whereas the prediction of signal sequence was done using the WWW Signal Scan Service (bimas.dccc.nih.gov/BLAST/) and the predicted signal sequence was subsequently confirmed using ECL Western blotting detection reagents (Amer-

| Strain and plasmid | Relevant characteristics | Ref. |
|-------------------|--------------------------|-----|
| S. pyogenes strains | | |
| SSI-1 | M type 3; isolated from patient with TSLS<sup>a</sup> | T. Murai (Japan) |
| TR-47 | Isogenic mutant of SSI-1; derivative of pYT1185, fbaB-aphA3, Km<sup>+</sup> | This study |
| TR-47G | Isogenic mutant of TR-47; EGFP expression strain | This study |
| E. coli | | |
| BL-21 | F<sup>−</sup> ompT hsdS<sub>B</sub> (r<sub>hr</sub> m<sub>B</sub>) gal dcm | Novagen |
| Plasmids | | |
| pQE30 | Expression vector; Amp<sup>+</sup> | Qiagen |
| pSF151 | Suicide vector; for inserional mutagenesis; Km<sup>+</sup> | Tao et al. (30) |
| pYT1185 | pSF151 with internal region of the fbaB gene from SSI-1; Km<sup>+</sup> | This study |
| pQE-F2LB | pGEX-6P-1 carrying the fbaB gene; FbaB expression plasmid; Amp<sup>+</sup> | This study |

<sup>a</sup> Streptococcal toxic shock-like syndrome.

**TABLE II**

### Primers used in this study

| Designation | Sequence (5′ to 3′) | Ref. |
|-------------|---------------------|-----|
| Expression of recombinant protein | GAGGATCCGCTAGGACATGCGGAACACAGA | This study |
| N34-BamHI | GACAGTCGCATTACATGATGATGCGG | This study |
| N734-SalI | GTGCCACAAGATACAAACTTACAGGCAGG | This study |
| Construction of fbaB-deficient mutant strains | CATCAATAGGCTTACTATCTTCCCTC | This study |
| fbaBKO1 | GTTAAGCTTCCTGTTATCCC | This study |
| fbaBKO2 | HI GAGGATCCGTAGGACATGCGGAAACAAGA | This study |
| Distribution of the fbaB gene | N34- | This study |
| fbaBD1 | fbaBD2 | This study |

**FIG. 1.** Western blot analysis of 8 M urea extracts from M3 strains isolated from TSLS patients. A, Coomassie Brilliant Blue staining. B, Western blotting using rabbit anti-M3 serum. C, ligand blotting with biotinylated human Fn.
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Sham Biosciences) and visualized by autoradiography with an x-ray film (Fuji photo film, Kanagawa, Japan) at room temperature for 5 s.

Enzyme-linked Immunosorbent Assay (ELISA)—For measuring the binding ability of FbaB, ELISA was performed as described previously (32, 33). Fn at 1 or 10 μg/ml was coated onto 96-well flat-bottom microtiter plates (Nalge-Nunc, Naperville, IL) and left overnight at 4 °C. For blocking, 5% BSA was applied at 37 °C for 3 h. rFbaB was added and bound at 37 °C for 1 h. GAS was washed with phosphate-buffered saline (PBS) 5 times and added into wells for binding. After washing the plate with PBS containing 0.05% Tween 20, rabbit anti-FbaB or anti-M3 serum was added and incubated at 37 °C for 1 h. The plate was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Cell Signaling, Beverly, MA) at 37 °C for 1 h and reacted with 0.1% p-nitrophenyl phosphate disodium salt solution (Wako, Osaka, Japan) for 15 min. Color development was measured at 405 nm using an ELISA plate reader (model Titertek MK1; Flow Laboratories, Osaka, Japan). All results are presented as the means of triplicate determinations, and each assay was repeated at least 3 times. Statistical analysis was performed by a nonparametric Mann-Whitney U test.

Bacterial Cell Adhesion and Invasion Assays—Adhesion to and invasion of cells were quantified by standard procedures as described previously (12, 34). HEp-2 cells were cultured on 8-well chamber slides (Nalge-Nunc) and infected with 1 × 10^8 CFU bacteria per well (multiplicity of infection, 1:100) for 3 h. To determine bacterial adhesion, cells were washed 3 times with DMEM and lysed with 1 ml of sterile distilled water. Serial dilutions of the lysate were plated on THY agar plates to determine the number of viable GAS. For bacterial invasion, cells were washed 3 times and incubated for 1 h with DMEM containing gentamicin (100 μg/ml) and penicillin (100 units/ml). Cells were washed, lysed, and plated to count those invaded by GAS. Data are expressed as the means of the percentage of GAS recovered per well from 6 independent determinations ± S.E. The assays were repeated 3 times, and representative data are shown. Statistical analysis was performed by a nonparametric Mann-Whitney U test. All conclusions were based on p < 0.005 as significant.

Immunofluorescence Analysis—HEp-2 cells were cultured on 8-well chamber slides (Nalge-Nunc). After infection with GAS (multiplicity of infection, 1:100), the wells were fixed with 4% paraformaldehyde and blocked with 10% FBS containing 50 μg/ml human IgG (Calbiochem). Immunofluorescence staining was performed as described previously (31). Adhered GAS was detected using the rabbit anti-M3 polyclonal antibody and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Stained GAS was analyzed using a confocal laser scanning microscope (model LSM 510, Carl Zeiss, Oberkochen, Germany).

Virulence in Mice—CD-1 outbred mice (7 weeks old, female, 20 g in weight, 10 mice per group) were purchased from Charles River Laboratories, Japan. GAS strains were grown at 37 °C to an A_{oo} of 0.4 and washed twice with sterile PBS. They were then resuspended in sterile PBS, and concentrations were adjusted to ~4 × 10^8 CFU/ml. Each mouse was anesthetized with pentobarbital and injected with 0.1 ml of GAS (equivalent to 4 × 10^8 CFU) intraperitoneally. The mortality of infected mice was monitored every 24 h for 7 days. Statistical analysis was performed by a χ^2 test. All conclusions were based on p < 0.05 as significant.

RESULTS

Screening for Fn-binding Protein in Serotype M3 Strains—Surface proteins were extracted with 8 M urea from M3 GAS isolates from patients with TSLS or pharyngitis (29). Twenty seven strains of M3 were initially screened for expression of Fn-binding proteins by ligand blotting. On the basis of the reactions to biotinylated Fn by autoradiography, several isolates from TSLS patients were found to express Fn-binding protein (Fig. 1C). Approximately 80- and 88-kDa proteins were found to bind Fn at a binding capability much stronger than M3 protein, which is also an Fn-binding protein (35). However, the recovery of the 80- and 88-kDa proteins was much less than M protein at 55 kDa (Fig. 1, A and B).

Analysis of the Genomic Region of Neighboring the fbaB Gene—The complete genome sequencing of serotype M3 GAS strain SSI-1 from a TSLS patient was performed and annotated (DDBJ/GenBank™/EBI Data Bank accession number BA000034). When we searched for ORFs with a calculated molecular mass of ~88 kDa and harboring an LPXTG motif...
from all putative ORFs, we obtained one ORF. Because it possessed putative signal sequence in the N-terminal region and cell-anchoring LPATGE sequence and Fn-binding repeat motif (www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam02986&version=v1.54) in the C terminus, we presumed the ORF was an Fn-binding protein and designated it FbaB. Furthermore, the deduced sequence of FbaB had identical amino acid residues in the N terminus to the N-terminal amino acid sequence of the 88-kDa band of Fn-binding protein. Fig. 2 shows the regions of the fbaB gene that were analyzed by a BLAST search. The fbaB gene was located downstream of a global negative regulator gene, nra (4), and a putative regulator gene that has been annotated as the msmR gene (36). Furthermore, there were no phage or phage-like elements in this region. The fbaB gene had a streptococcal consensus Shine-Dalgarno sequence (GGAGAG), −10 (TAGGCT), and −35 (TGTGCC) sequences (1, 11, 37) preceding the start codon ATG. The ORF consisted of 2,202 nucleotides and encoded a protein of 733 amino acid residues with a deduced molecular mass of 81 kDa, and the mature protein was calculated to be 79 kDa.

Comparisons with the deduced amino acid sequence of other GAS proteins are shown in Fig. 2B. Similarities between FbaB and another Fn-binding protein, F2, of GAS (8) were confined to the C-terminal portions, which included the Fn-binding repeats. The homology between the region from amino acids 434 to 733 of FbaB and from 741 to 1039 residues of protein F2 was 97%. FbaB also showed a 49% homology to Cpa (collagen-binding protein of GAS; see Ref. 4) in the N-terminal region. Protein F1 (1) was found to have two sections that exhibited a high similarity to FbaB.

Inactivation of the fbaB Gene and Characterization of an FbaB-deficient Mutant—To inactivate the fbaB gene, we first constructed pYT1185 carrying the fbaB and aphA3 genes (Fig. 3A). pYT1185 was found to possess an internal fragment of the fbaB gene and the aphA3 gene (kanamycin-resistant). By using electroporation, the FbaB-deficient mutant strain TR-47 was obtained by a single crossover recombination. B, Western blot analysis with anti-FbaB and Fn binding of 8M urea extracts of GAS strains. Lane a, Coomassie Brilliant Blue staining. Lane b, Western blotting using anti-FbaB. Lane c, binding to biotinylated human Fn (10 μg/ml). C and D, binding of GAS strains to immobilized Fn (C, 1 μg/ml; D, 10 μg/ml). Fn-coated microtiter plates were incubated with various densities of GAS strains. Bound GAS organisms were detected using the anti-M3 antibody. Binding activity is presented as A405 values. These experiments were performed in triplicate, and data represent the mean of triplicate samples from one representative experiment. S.E. results are represented by vertical lines. *, p < 0.05.
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**TABLE III**

Distribution of the fbaB gene and FbaB protein among various M serotype strains of group A streptococci

| M serotype | Clinical status | fbaB gene | FbaB protein |
|------------|----------------|-----------|-------------|
| 1          | TSLS           | 0/9       | 0/9         |
| 2          | Pharyngitis    | 0/3       | 0/2         |
| 3          | TSLS           | 13/13     | 8/13        |
| 3          | Pharyngitis    | 14/14     | 0/14        |
| 4          | TSLS           | 0/2       | 0/2         |
| 4          | Pharyngitis    | 0/4       | 0/4         |
| 6          | Pharyngitis    | 0/5       | 0/5         |
| 9          | Pharyngitis    | 0/1       | 0/1         |
| 11         | Pharyngitis    | 0/4       | 0/4         |
| 12         | TSLS           | 0/4       | 0/4         |
| 12         | Pharyngitis    | 0/4       | 0/4         |
| 13         | Pharyngitis    | 0/4       | 0/4         |
| 18         | TSLS           | 2/2       | 1/2         |
| 18         | Pharyngitis    | 3/3       | 0/3         |
| 19         | Pharyngitis    | 0/1       | 0/1         |
| 22         | TSLS           | 0/2       | 0/2         |
| 22         | Pharyngitis    | 0/3       | 0/3         |
| 28         | TSLS           | 0/1       | 0/1         |
| 28         | Pharyngitis    | 0/6       | 0/6         |
| 49         | Pharyngitis    | 0/4       | 0/4         |
| 58         | Pharyngitis    | 0/3       | 0/3         |
| 75         | Pharyngitis    | 0/3       | 0/3         |
| 77         | Pharyngitis    | 0/3       | 0/3         |
| 80         | Pharyngitis    | 0/4       | 0/4         |
| 87         | Pharyngitis    | 0/3       | 0/3         |
| 89         | Pharyngitis    | 0/1       | 0/1         |

* a Chromosomal DNA of GAS strains was purified with a Puregene DNA isolation kit (Gentra Systems), and distribution was detected using the fbaB gene-specific PCR assay. Number of positive strains/number of total strains.

* b Cell surface proteins were extracted with 8 M urea. FbaB protein on the surface of GAS and suggest that the remaining Fn-binding ability of TR-47 may be ascribed to M3 protein (35). Distribution of the fbaB Gene and Fibronectin Binding to FbaB Protein from Various GAS Strains—The distribution of the fbaB gene was examined by PCR, and the expression of FbaB protein was investigated by Western blotting with anti-FbaB serum and ligand blotting with biotinylated Fn and ECL detection reagents (Amersham Biosciences). Number of positive strains/number of total strains.

* p < 0.0005 (χ² test).

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**Fig. 4.** Fn binding activity of recombinant FbaB. A, binding of Fn to rFbaB. Lane a, Coomassie Brilliant Blue staining. Lane b, binding to biotinylated human Fn (10 μg/ml). B, analysis of Fn binding activity by ELISA. Fn-coated microtiter plates (1 μg/ml) were blocked with BSA and bound with various concentrations of rFbaB for 1 h at 37 °C. Fn binding of rFbaB was detected with anti-FbaB serum. Three experiments were performed in triplicate, and data represent the mean of triplicate wells from one representative experiment. S.E. were represented by vertical lines.

**Fig. 5.** Polar localization of FbaB. A, distribution of FbaB on GAS surface by immunofluorescence analysis. Panel a, FbaB was visualized using rabbit anti-FbaB serum and Alexa Fluor 594-conjugated goat anti-rabbit IgG (red image). Panel b, EGFP expression of GAS strain SSI-1G can be observed as the green image. Panel c, merge image. Bars indicate 1 μm.

**Fig. 6.** Adhesion to and invasion of HEp-2 cells by GAS strains SSI-1 (wild type) and TR-47 (FbaB⁻). GAS strains were suspended in DMEM containing 10% FBS and added to HEp-2 cells (multiplicity of infection, 100:1; 100 bacteria per HEp-2 cells) as described under “Experimental Procedures.” A, the percentage of adhesion was calculated as (CFU of adhesion and invasion/CFU of inoculum) × 100. B, the percentage of invasion was calculated as (CFU of invasion/CFU of inoculum) × 100. Three experiments were performed, and the data represent the mean from six wells from one representative experiment. S.E. results are represented by vertical lines. *, p < 0.005.
coated microtiter plates, and bound rFbaB was quantified using rabbit anti-FbaB serum and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. The results showed that soluble rFbaB bound to immobilized Fn in a dose-dependent manner (Fig. 4B). BSA (Sigma) served as a negative control and did not bind to Fn.

Localization of FbaB from GAS—It was important to determine whether FbaB is surface-associated or localized intracellularly. Immunofluorescent microscopy using anti-FbaB and secondary Alexa Fluor 594-conjugated antibody revealed the polar distribution of FbaB on the bacterial cell surface (Fig. 5, red image). The EGFP-expressed strain SSI-1G was observed as a green image. Based on these results and those from ELISA with live GAS (Fig. 3, C and D), along with the presence of the LPXTG motif in the C terminus, we concluded that FbaB was localized on the surface of GAS, and we hypothesized that FbaB functions as an adhesin or an invasin.

Role of FbaB in Bacterial Invasion and Mouse Lethality—To investigate the role of FbaB, adhesion and invasion efficiencies of strain SSI-1 and its mutant TR-47 were compared. Adhesion to and invasion of the HEp-2 cells by these strains were measured, respectively. Adhesion to and invasion of FbaB-deficient mutant, TR-47, were reduced ~6-fold as compared with those of SSI-1, respectively (p < 0.005; Fig. 6).

The contribution of FbaB in adhesion to and invasion of epithelial cells was further studied (Fig. 7). Invasion of HEp-2 cells was assessed using EGFP expression in strain SSI-1G (EGFP<sup>-</sup>, FbaB<sup>+</sup>) and its isogenic mutant TR-47G (EGFP<sup>-</sup>, FbaB<sup>-</sup>). The adhesion of GAS to the surface of HEp-2 cells was visualized by staining with anti-M3 and Alexa Fluor 594-conjugated secondary antibodies (red image). The adhesion and invasion abilities of TR-47G were clearly abrogated when compared with wild type strain SSI-1G. The above results indicate that FbaB serves as an adhesin and an invasin with epithelial cells.

Lack of FbaB results in the reduction of mouse mortality after challenge with GAS. The wild type strain SSI-1 yielded 80% mortality on day 2 and 90% mortality on day 4, whereas that of its isogenic mutant strain TR-47 was only 40% on day 2 (Fig. 8). These results strongly suggest that FbaB contributed to the virulence of the GAS strain that resulted in mouse mortality.

**DISCUSSION**

Bacterial adhesion to host epithelial cells through extracellular matrix proteins is considered to promote both colonization and infection. Various bacterial pathogens possess Fn-binding proteins including *Staphylococcus aureus* (38), *Listeria monocytogenes* (39), and *Salmonella enterica* (40). A number of anchored proteins on the surface of GAS have shown binding ability to Fn. Furthermore, a correlation exists between the distribution of a particular Fn-binding protein and specific M serotypes, and a population of the M3 and M18 strains is known to be highly virulent (14–17, 41). In the present study, we elucidated a novel Fn-binding protein selectively isolated from M3 and M18 strains, which we termed FbaB.

Jaffe et al. (8) reported that a highly homologous repeat domain in protein F2 is the main Fn-binding region when compared with that of protein F1. The present results showed that the putative Fn-binding domain of FbaB was highly conserved at the C-terminal region (Fig. 2), and the domain contains three Fn-binding repeats consisting of 36-, 37-, and 38-amino acid residues. McGavin et al. (42) suggested that a contiguous sequence of 8 amino acids is essential for Fn binding activity in FnBB of *Streptococcus dysgalactiae*. We observed the conserved sequence, HFDNNXXP, at amino acids 671–684 in FbaB protein. Furthermore, the *fbaB* gene was shown to possess a potential ribosome-binding site and promoter-like sequences (~10 and ~35) upstream of the start codon and a putative signal sequence in the N terminus and a cell-anchoring LPXTG motif in the C terminus. Thus, it is reasonable to conclude that FbaB is expressed on the surface and has Fn binding ability.

To examine whether FbaB works as an adhesin and an invasin to host cells, it was important to determine the localization of the protein. The 8 μm urea extracts of several M3 GAS strains strongly reacted with biotinylated Fn by ligand blotting.
regulatory gene, RofA-binding box from GAS have been reported by McIver et al. (44). For example, the Mga-binding box and proteins in transcription utilize DNA binding activity for the gene that would function as an Nra-binding box. Several regulatory streams of the (GCTTCTAACTT) or similar sequences in the region up- 

stream of the nra gene of M3 strain SSI-1. We speculate that the fbaB gene is normally controlled under the negative regulator Nra. Podbielski et al. (45) also predicted that the negative regulatory activity of Nra could be attributed to the essential sequence that would function as an Nra-binding box. Several regulatory proteins in transcription utilize DNA binding activity for the regulator-binding box, which exists in the upstream region of the structural gene (44). For example, the Mga-binding box and RofA-binding box from GAS have been reported by McIver et al. (45) and Fogg and Caparon (46), respectively. However, in the present study we could not find the putative Nra-binding box (GCTTCTAAACTT) or similar sequences in the region upstream of the fbaB gene of M3 strain SSI-1.

Fna is a large and multifunctional molecule found in serum and basement membranes, and it is known to bind to a wide variety of proteins and host cells by a surface integrin (47). Various pathogenic bacteria bind to Fna and use it for mediation by forming a bridge across the host cells and then facilitating the escape from human immune systems by pretending to be autogenous components. Several reports (34, 48) have shown that adherence to and invasion of epithelial cells require both soluble Fna and integrin expression on the cell surface, which may be explained by the possibility that the Fn-binding protein cannot bind to integrins directly. Stockbauer et al. (49) identified three major SpeBs, a cysteine protease of GAS variants, and showed that one of these variants, SpeB2, contains a RGD motif that was found to be a minimum integrin-binding sequence (50). Furthermore, they demonstrated that only SpeB2, which is usually produced by highly virulent M serotype strains, binds to integrins. Because FnaB contained an RGD motif at amino acid positions 135–137 (Fig. 2), we speculated that it might be an integrin-recognizing ligand with the ability to bind to an integrin. Taken together, these results demonstrate that FnaB may promote bacterial adhesion to host cell surfaces and also contribute to the colonization and inva-

sion of organisms.

We also showed that FnaB indicated two molecular sizes by Western blot and ligand blot analyses (Figs. 1 and 3B). A band of 80-kDa FnaB from S. aureus extract reacted with Fn stronger than an 88-kDa protein, which was similar to the pattern shown between M1 protein and immunoglobulins by Raeder et al. (20). Their report demonstrated that SpeB, a cytotoxic protein, cleaved M1 protein to generate a protein with a higher level of immunoglobulin binding activity. To test the hypothesis that FnaB is also modified by SpeB protein, we incubated rFnaB with various concentrations of recombinant SpeB, which revealed two major bands (molecular mass of 80 and 88 kDa, data not shown). These results suggest that the proteolytic reaction by SpeB affects not only host cells but also the surface proteins of GAS to increase their virulence activities. This modification may be associated with the invasive capability of GAS and a key factor for analyzing the change to invasive strains. Furthermore, the cleavage of FnaB by SpeB in the N terminus may contribute to its escape from the host immune system by raising a distinct antigenicity. Although it is unclear how bacterial adhesion to and invasion of host cells affect streptococcal diseases in humans, findings from many studies have shown that intracellular invasion is associated with an important role of streptococcal virulence. Further study of FnaB may elucidate the mechanisms involved with invasive streptococcal infections, including bacterial colonization and the spread into deeper organs.

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