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Serum opacity factor promotes group A streptococcal epithelial cell invasion and virulence

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Summary

Serum opacity factor (SOF) is a bifunctional cell surface protein expressed by 40–50% of group A streptococcal (GAS) strains comprised of a C-terminal domain that binds fibronectin and an N-terminal domain that mediates opacification of mammalian sera. The *sof* gene was recently discovered to be cotranscribed in a two-gene operon with a gene encoding another fibronectin-binding protein, *sfb*X. We compared the ability of a SOF(+) wild-type serotype M49 GAS strain and isogenic mutants lacking SOF or SfbX to invade cultured HEp-2 human pharyngeal epithelial cells. Elimination of SOF led to a significant decrease in HEp-2 intracellular invasion while loss of SfbX had minimal effect. The hypoinvasive phenotype of the SOF(–) mutant could be restored upon complementation with the *sof* gene on a plasmid vector, and heterologous expression of *sof*49 in M1 GAS or *Lactococcus lactis* conferred marked increases in HEp-2 cell invasion. Studies using a mutant *sof*49 gene lacking the fibronectin-binding domain indicated that the N-terminal opacification domain of SOF contributes to HEp-2 invasion independent of the C-terminal fibronectin binding domain, findings corroborated by observations that a purified SOF N-terminal peptide could promote latex bead adherence to HEp-2 cells and inhibit GAS invasion of HEp-2 cells in a dose-dependent manner. Finally, the first *in vivo* studies to employ a single gene allelic replacement mutant of SOF demonstrate that this protein contributes to GAS virulence in a murine model of necrotizing skin infection.

Introduction

Group A *Streptococcus* (GAS) is responsible for a wide spectrum of human disease, from simple pharyngitis to serious invasive infections including necrotizing fasciitis and toxic shock syndrome. Approximately half of GAS strains are capable of opacifying mammalian serum, a unique phenotype attributable to the presence of serum opacity factor (SOF) encoded by the *sof* gene (Kreikemeyer *et al*., 1995; Beall *et al*., 2000). SOF is a 110 kDa protein with an LP(X)SG cell wall anchor motif expressed in both membrane bound and extracellular forms (Rakonjac *et al*., 1995; Courtney *et al*., 1999; Katerov *et al*., 2000). SOF is a bifunctional protein comprised of an N-terminal opacification domain, and C-terminal domain of highly conserved tandem repeat sequences capable of binding the host extracellular matrix components fibronectin and fibrinogen (Rakonjac *et al*., 1995; Kreikemeyer *et al*., 1999; Courtney *et al*., 2002). Once thought to be a lipoproteinase, SOF has recently been shown to bind apoA-I and apoA-II, causing release of high-density lipoprotein (HDL) lipid cargo, which in turn coalesces to form lipid droplets, resulting in serum opacification (Courtney *et al*., 2006a).

Studies with SOF-coated latex beads indicate the protein can promote adherence to human HEp-2 pharyngeal epithelial cells including fibronectin-specific interactions (Oehmcke *et al*., 2004). A role in GAS virulence has been attributed to SOF, because insertional inactivation of the *sof* gene in a serotype 2 GAS isolate reduced mortality in a mouse intraperitoneal (I.P.) infection model (Courtney *et al*., 1999). SOF also triggers host production of opsonic antibodies that protect against infection by SOF(+) strains of GAS, suggesting it may represent a useful vaccine antigen (Courtney *et al*., 2003).

The *sof* gene is recently recognized to be a member of a two gene operon, invariably cotranscribed with a gene encoding a second surface anchored protein, *sfb*X (Jeng *et al*., 2003). SfbX possesses a C-terminal domain of tandem repeat sequences very highly homologous to the fibronectin-binding domain of SOF, but a unique

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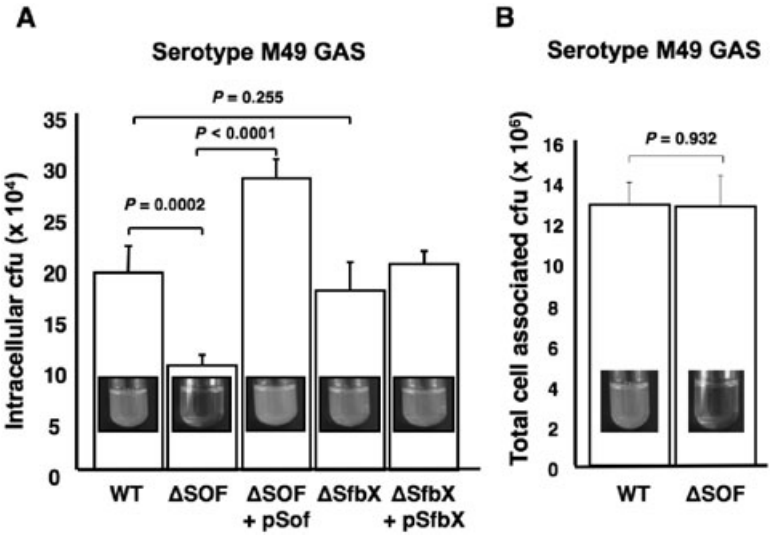

Fig. 1. A. Invasion of HEp-2 human pharyngeal epithelial cells by WT M49 GAS strain NZ131, isogenic allelic replacement mutants lacking either SOF or SfbX, and each mutant complemented by replacement of the corresponding gene on a plasmid vector. B. Adherence to HEp-2 cells of WT M49 GAS stain and the isogenic ΔSOF mutant. Figure insets show serum opacification phenotype of each tested strain.

N-terminal domain. SfbX is capable of binding fibronectin, but the function of its N-terminal sequences has yet to be elucidated (Jeng *et al*., 2003). Given previous insertional mutagenesis studies targeting *sof* were performed before the operon structure was recognized, polar effects on *sfb*X would be anticipated, and the precise contribution of each individual gene is therefore uncertain.

In this study, we utilize non-polar allelic replacement mutants of *sof* and *sfb*X, coupled with heterologous expression of each gene, to study their contribution(s) to GAS epithelial cell invasion and animal virulence. Unexpectedly, our results uncovered a key contribution of the N-terminal SOF opacification domain to GAS cellular invasion independent of the C-terminal fibronectin-binding motifs.

Results

Serum opacity factor and not SfbX contributes to epithelial cell invasion by M49 GAS

Allelic replacement mutants of M49 GAS lacking SOF or SfbX were compared with the wild-type (WT) parent strain for their ability to invade cultured monolayers of HEp-2 human pharyngeal epithelial cells *in vitro*. In the graphical representation of our results, photo insets depicting the serum opacity phenotype of the individual bacterial strains are provided within the corresponding bars. As measured by antibiotic protection assay, the isogenic ΔSOF mutant exhibited a 50% decrease in cellular invasion ($P = 0.0002$). The invasion phenotype of the ΔSOF mutant could be restored above WT levels by return of the single *sof* gene on a multicopy number complementation plasmid ($P < 0.0001$) (Fig. 1A). The isogenic ΔSfbX mutant did not differ significantly from WT GAS in HEp-2 cell invasiveness ($P = 0.255$) (Fig. 1A). No difference in overall adherence of WT and ΔSOF mutant GAS was observed, suggesting the contribution of SOF to HEp-2 cell invasion was not simply secondary to increased bacteria–epithelial cell interaction (Fig. 1B). Furthermore, as SOF and SfbX share highly homologous C-terminal fibronectin-binding repeat domains, these results provided a clue that N-terminal sequences in the 'opacification' domain of SOF could possess unique properties facilitating cellular invasion.

Gain of function analysis by heterologous expression of SOF and SfbX

To determine if *sof* and *sfb*X are sufficient to confer increased invasion of epithelial cells, expression vectors for each GAS M49 gene were transformed into bacteria lacking this operon. Under the standard assay condition, overexpression of *sof*49 in GAS M1 strain 5448 increased HEp-2 cell invasion 100-fold, while overexpression of *sfb*X produced a 10-fold gain in invasion frequency (Fig. 2A). When parallel studies of heterologous expression were performed in *Lactococcus lactis*, which lacks significant fibronectin binding, *sof* conferred a nearly 1000-fold increase in HEp-2 cell invasion compared with a 10-fold increase for *sfb*X (Fig. 2B). These analyses in heterologous bacterial strains are also consistent with the key role of SOF, but not SfbX, in epithelial cell invasion by GAS (Fig. 1A). Though SOF did not appear to contribute significantly to adherence in the GAS M49 parent strain (Fig. 1B), heterologous expression of *sof*49 in *L. lactis* led to a 10-fold increase in the organism's ability to adhere to HEp-2 cells (Fig. 2C). We speculate that the lack of baseline fibronectin binding by *L. lactis* (Jeng *et al*., 2003) allows the fibronectin binding domain of SOF to promote cellular adherence upon its introduction, whereas in GAS

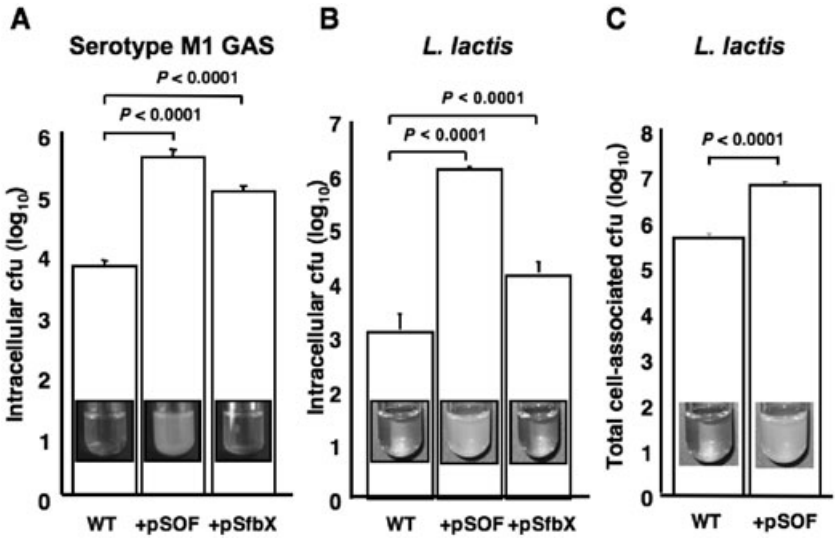

Fig. 2. Invasion of HEp-2 human pharyngeal epithelial cells upon heterologous expression of the *sof* and *sfb*X genes from M49 GAS in (A) M1 GAS that normally lacks the corresponding operon and (B) *L. lactis* strain NZ9000. (C) Adherence to HEp-2 cells of *L. lactis* NZ9000 with and without expression of GAS *sof* gene. Figure insets show serum opacification phenotype of each tested strain.

the loss of SOF does not reduce overall adherence because of the existence of several additional fibronectin binding surface proteins.

Visualization of SOF-dependent bacterial uptake in HEp-2 cells

To corroborate observed differences in HEp-2 cell invasion detected in the antibiotic protection assays, fluorescence techniques were used to differentiate surface bound and intracellular bacteria. Bacteria were stained with calcein-AM (green) prior to use in a standard HEp-2 cell invasion assay as above, but at the assay end-point the fluorescence of external bacteria was quenched with ethidium bromide (red). As seen in Fig. 3, noticeably fewer ΔSOF mutant GAS were observed inside HEp-2 cells compared with the WT parent strain. Likewise, the number of M1 GAS and *L. lactis* internalized by HEp-2

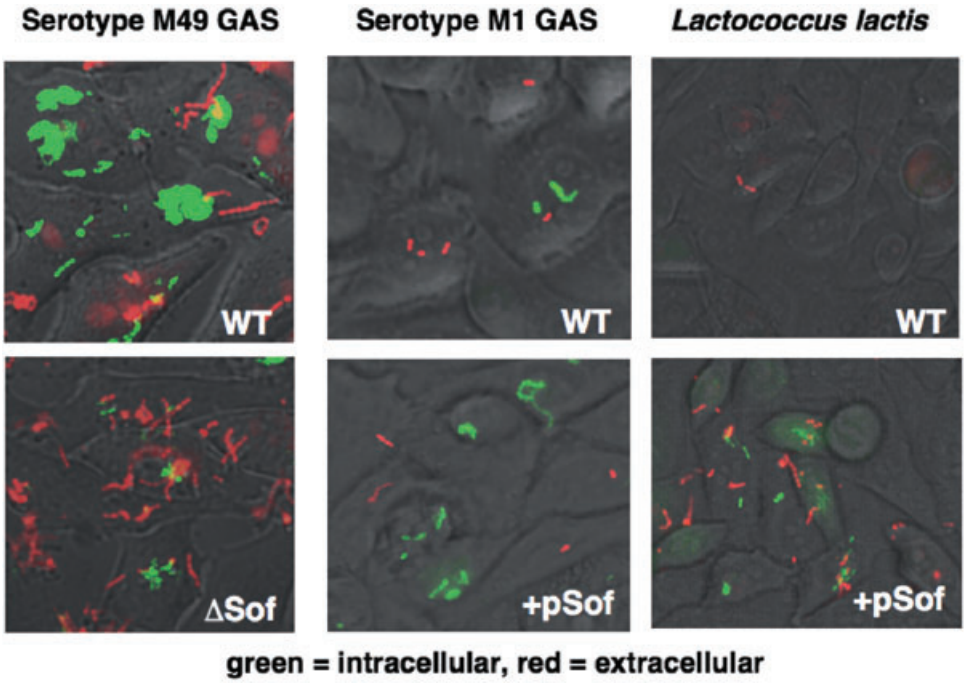

Fig. 3. Fluorescent imaging demonstrating the contribution of SOF to interactions with HEp-2 epithelial cell monolayers. Green fluorescence represents the intracellular bacteria, and red fluorescence the extracellular bacteria. Decreased intracellular invasion is seen upon deletion of SOF from WT SOF(+) GAS and increased intracellular invasion observed upon heterologous expression of SOF in WT SOF(–) GAS and *L. lactis*.

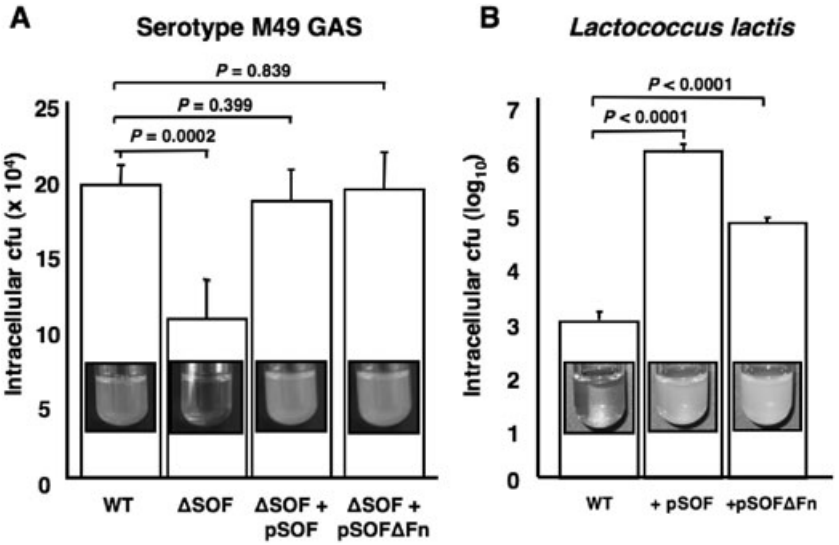

Fig. 4. Deletion of the fibronectin-binding domain of SOF protein does not eliminate the contribution of SOF to HEp-2 epithelial cell invasion as tested in (A) complementation studies using the M49 GAS ΔSOF mutant and (B) heterologous expression studies in *L. lactis*. Figure insets show serum opacification phenotype of each tested strain.

cells were increased when these bacteria expressed the cloned M49 *sof* gene (Fig. 3).

The N-terminal opacification domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin-binding domain

We observed in our mutagenesis and heterologous expression studies that SOF contributed much more significantly than SfbX to epithelial cell invasion (Figs 1 and 2), though both proteins possess homologous C-terminal fibronectin-binding domains. We hypothesized that the N-terminal enzymatic domain of SOF must therefore play a direct role in promoting epithelial cell invasion. To test this hypothesis, we generated an in-frame deletion of the entire fibronectin-binding repeat domain of SOF within our expression plasmid (pAT-SOFΔFn) to produce a truncated derivative of the protein that maintained opacification activity. Plasmid pAT-SOFΔFn expressing the truncated SOFΔFn protein was observed to complement the invasion defect of the M49 NZ131Δ*sof* mutant to WT levels (Fig. 4A), and to confer a 100-fold increase in epithelial cell invasion when expressed in *L. lactis* (Fig. 4B). To corroborate our above findings, the opacification domain of GAS SOF75, lacking the fibronectin-binding repeats, was expressed in *Escherichia coli* and purified (SOF75ΔFn). Addition of the exogenous SOF75ΔFn peptide produced a dose-dependent inhibition of HEp-2 cell invasion by a WT SOF(+) M75 GAS strain (Fig. 5B), consistent with competitive inhibition of a GAS–host cell interaction mediated by surface-bound SOF. As an irrelevant peptide control, exogenous actin at 1 or 10 μg ml^{-1}

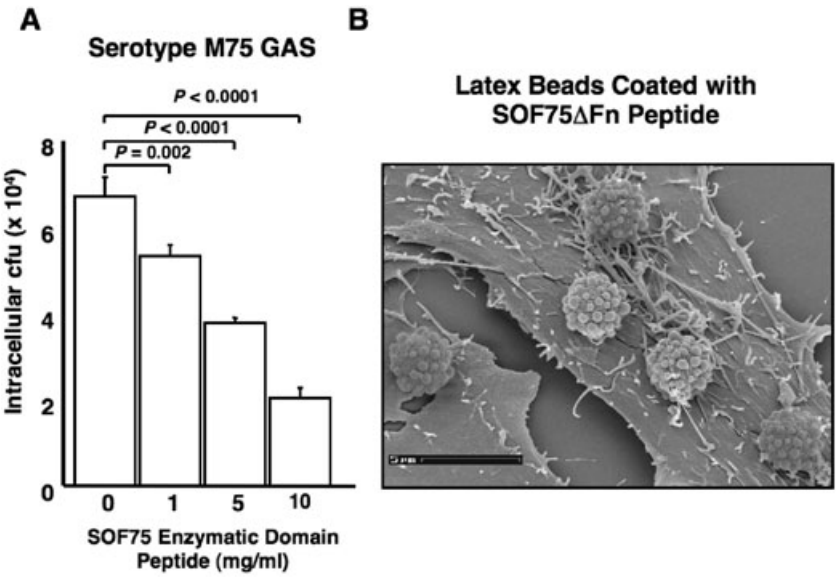

Fig. 5. A. A SOF peptide lacking the fibronectin-binding repeats produces a dose-dependent inhibition of HEp-2 epithelial cell invasion by WT SOF(+) GAS.
B. A SOF peptide lacking fibronectin-binding repeats promotes association of latex beads to HEp-2 cells.

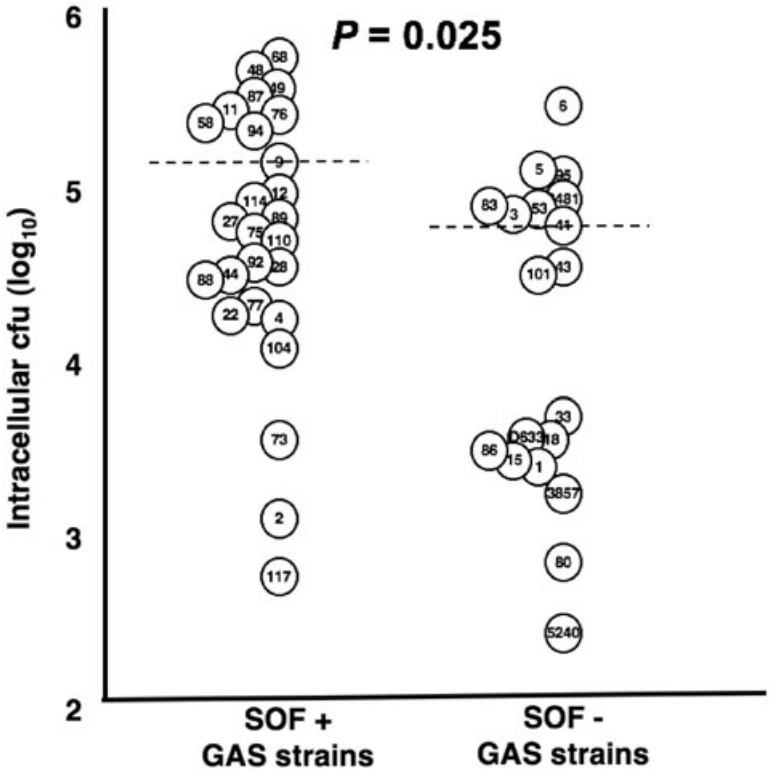

Fig. 6. Survey of panel of 45 GAS strains of varying M-protein (*emm*) genotypes reveals a correlation of SOF-positivity with increased capacity for HEp-2 cell invasion.

did not impair M75 GAS invasion (not shown). When complexed to latex beads, the above mentioned SOF75ΔFn peptide promoted tight adherence of the beads to HEp-2 cells as visualized by scanning electron microscopy (Fig. 5B). Taken together, these results indicate that the N-terminal opacification domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin-binding domain.

Correlation of SOF with HEp-2 cell invasiveness among GAS strains

To ascertain whether the observed contribution of SOF to GAS invasiveness in our molecular genetic studies could be reflected broadly across strains of varying SOF phenotype, we studied a panel of GAS strains of various *emm* (M protein) genotypes from the Centers for Disease Control including both SOF(+) ($n = 26$) and SOF(–) ($n = 19$) types. These strains were screened for invasion of HEp-2 cells in a 96 well format modification of the standard assay (Fig. 6). Mean intracellular colony-forming unit (cfu) recovered from the SOF(+) strains was 130 274 compared with 50 233 for the SOF(–) strains; median values were 55 150 for the SOF(+) strains and 29 700 for the SOF(–) strains. A significant positive correlation was thus apparent between GAS expression of SOF and invasiveness in HEp-2 epithelial cells ($P = 0.025$).

Serum opacity factor contributes to GAS virulence in murine infection

The specific contribution of SOF to GAS virulence was tested in murine models of infection. In Fig. 7A, Kaplan–Meyer survival curves were established for groups of 10 male CD1 mice challenged by I.P. injection with 2×10^7 cfu of either the WT M49 GAS strain or its isogenic ΔSOF and ΔSfbX mutants. No difference was seen between the WT strain and the ΔSfbX mutant, with 100% mortality by day 3–4; a slight delay in the kinetics of killing of the ΔSOF mutant was observed with 25% of mice surviving and asymptomatic at the end of the 10 day assessment period. SOF was next expressed heterologously in the WT M1 GAS strain, and groups of 12 mice compared with vector only control using an I.P. challenge dose of 2×10^6 cfu. An increase in mortality from 50% to 75% was observed by 10 days (Fig. 7B). Though neither tendency following loss or gain of SOF reached statistical significance, the effect of SOF expression in the M1 background could theoretically have been underestimated due to plasmid loss in the absence of *in vivo* antibiotic selection. We next conducted a further assessment of the contribution of SOF to GAS virulence in a murine subcutaneous infection model. Sixteen male CD1 mice were shaved and injected subcutaneously in one flank with 1×10^8 cfu WT

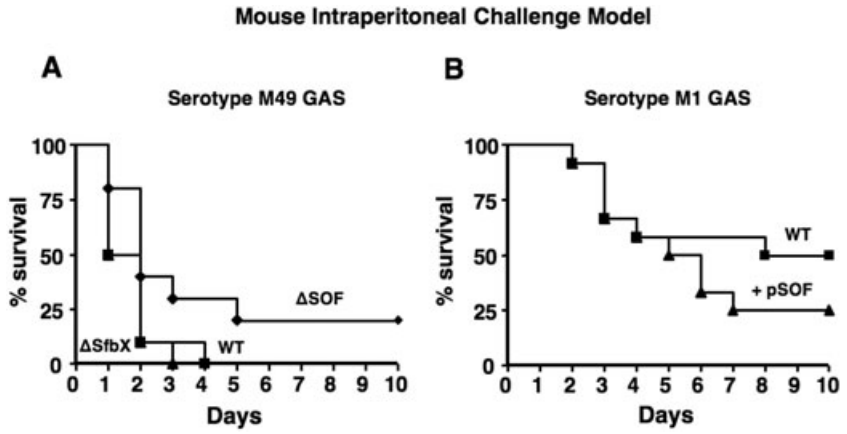

Fig. 7. A. Effect of deletion of SOF or SfbX on mouse survival in an I.P. challenge model using a SOF(+) M49 WT parent strain.
B. Effect of heterologous expression of SOF in the M1 GAS background on mouse survival in the same challenge model.

GAS M49 strain and in the contralateral flank with an equivalent dose of the isogenic ΔSOF mutant. In this way, each mouse served as its own control. The development of necrotic ulcers was monitored and lesion area calculated at day 7 (Fig. 8A). Lesions produced by the WT strain were larger in 12/16 mice, lesions equivalent in size in 3/16 mice, and in only 1/16 mice was the lesion produced by the ΔSOF mutant larger. The lesions were excised and the cfu/g skin tissue calculated by plating dilutions of the tissue homogenate. Bacterial counts were significantly ($P = 0.002$) higher for the WT strain compared with the ΔSOF mutant in 14/16 mice, with a median 7.7-fold increase of cfu g^{-1} tissue recovered for the WT strain (Fig. 8B). Photos of two representative animals from the skin challenge experiment are shown in Fig. 8C. Our results confirm a unique contribution of SOF to GAS virulence independent of SfbX and are the first to demonstrate a role of SOF in GAS pathogenicity using a localized infection model.

Discussion

Serum opacity factor is a unique bifunctional protein produced by many GAS strains and possesses roles in host cell interaction, immunogenicity and disease pathogenesis. Here we have used isogenic mutants and heterologous expression to show for the first time that SOF promotes GAS invasion of human pharyngeal epi-

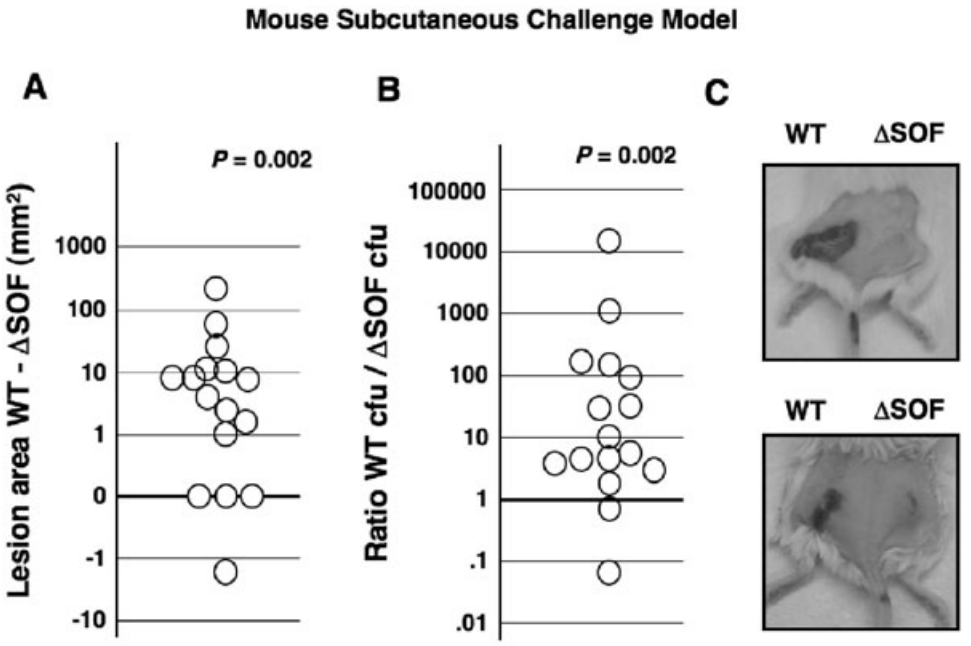

Fig. 8. Effect of deletion of SOF on GAS virulence in a murine skin infection model, including (A) decreased lesion size (represented by difference in lesion size), (B) decreased bacterial counts in the infected skin tissues (represented by ratio of WT bacterial counts divided by ΔSOF counts). (C) Representative images of necrotic lesions developing in animals infected using this model.

thelial cells and contributes to GAS virulence independent of the coexpressed SfbX protein.

The affinity of several GAS surface anchored proteins to fibronectin and other extracellular matrix components has been shown to play a major role in the organism's adherence to and invasion of host epithelial cell surfaces (Hanski and Caparon, 1992; Kreikemeyer *et al*., 1995; Molinari *et al*., 1997; Rocha and Fischetti, 1999). Our experiments with a domain deletion construct and purified protein reagent demonstrate that the N-terminal enzymatic domain of SOF, in addition to its activity in serum opacification, possesses distinct pro-invasive properties. The N-terminal domain of SOF thus may cooperate with the C-terminal fibronectin-binding repeats in promoting GAS–host cell interactions. We found SfbX, with homologous C-terminal repeats to SOF, can confer increased invasion in the *L. lactis* background where fibronectin-binding is otherwise absent. However, deletion of *sfb*X was insufficient to impair invasion of GAS, where the presence of additional fibronectin-binding surface molecules may provide compensation.

The *sof*/*sfb*X operon is located approximately 10 kb from the tandemly linked genes of the GAS *mga* regulon on a 5500 bp fragment that appears in *sof* positive strains as a precise insertion relative to the same chromosomal region of *sof*-negative M1, M3 and M18 strains (Jeng *et al*., 2003). There is a strict correlation of the presence of *sof* with specific *emm* sequence types. It is interesting that this strict correlation is observed, even though many *emm* types are shared by *sof*-positive strains that have unrelated *sof* genes and multilocus sequence types (Beall *et al*., 2000). Expression of *sof*/*sfb*X is influenced by the multiple gene regulator (*mga*) that lies closely upstream of *emm* and controls several GAS virulence genes involved in colonization, cellular invasion and immune resistance (McIver and Myles, 2002); *mga* has recently been shown to bind to a specific promoter element upstream of *sof*/*sfb*X in a fashion sufficient to activate transcription (Almengor *et al*., 2006). We found specific deletion of SOF in the M49 GAS background significantly reduced virulence in the mouse model of necrotizing skin infection. The importance of SOF in this model may reflect contributions not only to host cell adherence and intracellular invasion, but also to enhanced resistance to phagocytic clearance, as recently demonstrated in human whole blood killing assays (Courtney *et al*., 2006b).

Group A streptococcal strains have been defined as belonging to either class I or class II M types based on epitopes present within the conserved C repeat region of M proteins (Bessen *et al*., 1989; Bessen and Fischetti, 1990) and the architecture of the *mga* regulon (Haanes *et al*., 1992). The expression of SOF is found exclusively in Class II GAS (Bessen *et al*., 1989). Given that SOF is expressed by only a subset of GAS strains (40–50%) the opacity factor is not a requirement for virulence in all GAS strains. However, this study found that specific deletion of SOF in the Class II M49 GAS background significantly reduced virulence in the mouse model of necrotizing skin infection. Different GAS strains express a wide array of surface anchored proteins that interact with components of the extracellular matrix (Ramachandran *et al*., 2004), and a number of these have been shown to play a role in adherence of the organism to host epithelial cell surfaces and the invasion of the GAS bacterium into these cells (Caparon *et al*., 1991; Hanski and Caparon, 1992; Wang and Stinson, 1994; Jaffe *et al*., 1996; Courtney *et al*., 1997; Molinari *et al*., 1997; Kreikemeyer *et al*., 2004). Differential expression of multiple adhesins in response to varying environmental cues and extracellular matrix components at specific tissue sites may be important for colonization of a tissue specific site. Thus, SOF may represent a critical virulence factor of Class II SOF(+) GAS, the absence of which in Class I GAS may be compensated for by the presence of other fibronectin-binding surface adhesins.

In summary, our studies demonstrate that SOF can contribute to epithelial cell invasion and animal virulence in the approximately 50% of clinical isolates that express this protein. Previous assumptions that interactions with the host cell were mediated predominantly through the C-terminal repeat domains should now be reconsidered, as both opacification and promotion of host cell invasion appear to be mediated by the SOF N-terminal domain.

Experimental procedures

Bacterial strains and culture conditions

Serotype M49 GAS strain NZ131 is a well-characterized T14 SOF(+) strain originally isolated from the skin of a patient with glomerulonephritis (Simon and Ferretti, 1991). Isogenic mutants NZ131ΔSOF and NZ131ΔSfbX contain a precise, in-frame allelic replacement of the corresponding open reading frame with a chloramphenicol acetylase (*cat*) gene marker (Jeng *et al*., 2003). Serotype M1 GAS strain 5448 is a T1, SOF(–) isolate from a patient with necrotizing fasciitis and toxic shock syndrome (Kansal *et al*., 2000). *L. lactis* strain NZ9000 is derived from MG1363 and lacks the *nis* operon (Kuipers *et al*., 1998). SOF and SfbX amplified from NZ131 were cloned in vector pDCerm to yield plasmids pAJ-SOF and pAJ-SfbX (Jeng *et al*., 2003), and used to transform M1 GAS 5448 and *L. lactis* for heterologous expression studies in comparison with vector only controls. GAS were grown in Todd–Hewitt broth (THB), on Todd–Hewitt agar plates (THA), or on plates of Trypticase Soy Agar + 5% sheep red blood cells (SBA). Antibiotic selection for plasmid maintenance utilized 2 μg ml^{-1} of erythromycin (Em) for GAS and 5 μg ml^{-1} Em for *L. lactis*. To prepare log phase GAS for use in tissue culture assays or animal infections, bacteria were grown to $OD_{600} = 0.4 = 2 \times 10^8$ cfu ml^{-1} at 37°C, pelleted,

washed, resuspended and diluted in PBS or tissue culture media to the desired concentration. *L. lactis* was prepared as above ($OD_{600} = 0.4 = 10^8$ cfu ml^{-1}) but incubation performed at 30°C. No difference in bacterial chain length or logarithmic phase growth rate related to mutagenesis of SOF or SfbX in GAS or heterologous expression of the two proteins in *L. lactis* was observed.

Serum opacification assays

Bacteria were grown to stationary phase overnight, pelleted by centrifugation, and 100 μl of supernatant added to 1 ml of heat inactivated (55°C, 30 min) horse serum (Gibco) in a 24 well cell culture plate. The plate was rocked for 15 min, sealed in a plastic bag with moisture, and incubated overnight at 37°C. Opacification was observed and photographed on a dark background.

Epithelial cell invasion and adherence assays

Human pharyngeal epithelial cells (HEp-2) were obtained from ATCC and propagated as monolayers in RPMI 1640 (Mediatech) supplemented with 10% FBS + 2 mM L-glutamine + 100 μM nonessential amino acids at 37°C with 5% CO_2.) For assays, HEp-2 cells were plated at 2×10^5 cells well^{-1} in 1 ml of media in a tissue culture treated 24 well plate and incubated at 37°C × 24 h. Bacterial cultures were inoculated from a single colony and grown overnight in THB with corresponding antibiotic selection. The following day, cultures were diluted 1:10 in fresh THB without antibiotics to an OD_{600} of 0.4. Immediately prior to assay, the culture media on the HEp-2 cells was replaced with fresh RPMI 1640 + 2% FBS. Bacteria were diluted in PBS and 10^6 cfu added to each well for a multiplicity of infection of 5:1 (bacteria : cell). The plate was centrifuged at 800 *g* × 5 min to ensure contact of the bacteria with the cell monolayer, then incubated at 37°C in 5% CO_2. After 2 h, monolayers were washed ×3 with PBS to remove unattached bacteria. Media containing antibiotics (100 μg ml^{-1} gentamicin + 5 μg ml^{-1} penicillin) was added to each well, and the plates incubated an additional 2 h to kill extracellular bacteria. The wells were washed again, and 100 μl of trypsin added to each well for 3–5 min at 37°C to facilitate cell detachment. Triton-X 100 was added to each well at a final concentration of 0.02% and triturated with a micropipette for 20 s to liberate intracellular bacteria. Dilutions were plated on THB at 37°C overnight for enumeration of cfu. All assays were carried out in triplicate. For the M75 strain invasion assays, the HEp-2 cells were plated in 96 well plates at 4×10^4 cells well^{-1} in 200 μl media on the previous day. The purified SOFΔFn protein was pre-incubated with the cell for 1 h prior to addition of the bacteria at a moi of 5:1 and maintained throughout the 2 h incubation step. Adherence assays were performed as described above, but after 2 h of incubation, wells were washed ×6 with PBS to remove non-adherent bacteria. Cells were then lysed, diluted and plated as described. HEp-2 cells did not display differences in cell morphology or viability during the time frame of bacterial exposures; Trypan blue exclusion showed HEp-2 cell viability ≥ 98% at all assay endpoints.

Fluorescence microscopy

HEp-2 cells were prepared as described in the invasion assay protocol. Bacteria were grown to an OD_{600} of 0.3 (GAS) or 0.2 (*L. lactis*), then 1 ml pelleted by centrifugation at 10 000 *g*. Bacteria were resuspended in 100 μl PBS + 10% THB, 0.5 μl 10 mM calcein-AM (Molecular Probes) added, and staining carried out for 1 h at 37°C (GAS) or 2 h at 30°C (*L. lactis*). Stained bacteria were washed ×3 with PBS to remove residual calcein-AM, resuspended in 1 ml PBS, 100 μl added to each HEp-2 monolayer, the plate centrifuged, and incubated for 2 h at 37°C to allow for internalization. After incubation, wells were washed with PBS, counterstained for 1–3 min with 100 μg ml^{-1} ethidium bromide, and washed again. Fluorescence was visualized with a Nikon TE200 inverted microscope using a green filter, a red filter, and white light. Images were captured with a CCD camera and assembled using Adobe Photoshop Creative Suite 2.

Construction and expression of pAT-SOFΔFn

This plasmid was generated by modification of pAJ-*sof* (Jeng *et al*., 2003) through inverse PCR and blunt-end ligation. Primers were designed (forward: 5′-ttcttccactttgataacgag-3′, reverse: 5′-ggcagtaatggattcaatgga-3′) to create an inverse PCR product that consisted of the entire plasmid and gene, excluding the C-terminal fibronectin binding repeat region (residues 831Q to 960R). The resultant PCR product was purified (Qiagen Gel Extraction Kit), blunt-end ligated for 2 h at room temperature using Invitrogen T4 ligase and used to transform *E. coli* MC1061 with selection for Em resistance. Resultant plasmid pAT-SOFΔFn was purified and confirmed by restriction analysis and direct sequencing. The pAT-SOFΔFn was used to transform the GAS M49 ΔSOF mutant and *L. lactis* by electroporation at 1700 V, recovery in THB 0.25% sucrose, and Em selection. Transformants were confirmed by PCR analysis and gain of serum opacification phenotype.

Survey of GAS clinical strains for correlation between SOF phenotype and HEp-2 cell invasion

Single representatives of 45 different *emm* types were obtained from invasive isolates collected through the Centers for Disease Control Active Bacterial Core surveillance program in the United States during 2002–2004 (see http://www.cdc.gov/ncidod/dbmd/abcs/). These isolates were collected from patients of a wide age range (< 1 to > 90 years) and varied geographic locations, which represented a wide range of clinical manifestations. Strains were grown in 200 μl THB within individual wells of a 96 well plate with passage over two nights prior to use in the assay. HEp-2 cells were plated in a 96 well tissue culture treated plate at 4×10^4 cells well^{-1} in 200 μl of media and incubated ×24 h. On the day of the assay, 18 h stationary phase GAS cultures were serially diluted 1:100 in 96 well plates in RPMI 2% FBS, then 100 ml added to the HEp-2 cell monolayers in quadruplicate wells for each strain. The balance of the study was carried out as for the standard invasion assay, with serial dilutions plated on THB for enumeration of cfu.

Expression and purification of recombinant SOF75ΔFn protein

A His_6-tagged fusion protein of residues 33–872 of SOF from M75 GAS strain (lacking signal sequence and fibronectin-binding repeat region, SOF75ΔFn) was constructed using the pQE30 vector system (Kreikemeyer *et al*., 1999). Large-scale expression and purification of SOF75ΔFn was conducted essentially according to manufacturer's instructions (Qiagen, Chatsworth, CA). Briefly, recombinant protein was expressed in *E. coli* M15 (pREP4) (pSOF75ΔFn), 1 l culture in LB was grown to mid-log phase, and protein expression was induced by the addition of IPTG to a final concentration of 1 mM and incubation for a further 4 h at 37°C with shaking. Cells were harvested by centrifugation at 4000 *g* for 20 min at 4°C and cell pellets stored at −20°C overnight prior to protein extraction and purification. Large-scale purification of (His_6)-tagged SOF75ΔFn was performed using Ni-NTA chromatography under denaturing conditions according to manufacturer's instructions (Qiagen, Chatsworth, CA). To ensure slow buffer exchange for refolding of the denatured proteins, the proteins were initially dialysed against two changes of 0.5–1 l PBS followed by two changes of 3 l PBS. To ensure correct refolding of proteins was achieved, purified proteins were subsequently tested for opacity factor activity using an agarose overlay method (Kreikemeyer *et al*., 1999).

Interaction of SOF coated latex beads with HEp-2 cells

As per previously published methods (Molinari *et al*., 1997; Dombek *et al*., 1999), latex beads (3 μm; Sigma-Aldrich, St Louis, MO) were coated with purified recombinant SOF75ΔFn. Briefly, 10^8 bead particles in 50 μl of PBS were incubated with 5 μg of purified protein in PBS overnight at 4°C. After washing steps, free binding sites on the bead surface were blocked by incubation with 200 μl of 10 mg ml^{-1} BSA in PBS for 1 h at room temperature. Followed by washing and resuspension in a final volume of 1 ml Dulbecco modified Eagle medium (DMEM) (Invitrogen, Karlsruhe, Germany) with HEPES and 1% fetal calf serum (FCS). The efficiency of protein loading was verified by incubation of $\sim 2 \times 10^7$ coated beads with polyclonal anti-SOF75 rabbit serum (diluted 1:1000 PBS + 5 mg ml^{-1} BSA) (Gillen *et al*., 2002) followed by washing in PBS and incubation with goat anti-rabbit Alexa 488 (green) (50 μl of a 1:40 dilution in PBS + 5 mg ml^{-1} BSA) (Molecular Probes, Eugene, OR) for 1 h at room temperature and subsequently washed with PBS. Bead fluorescence was then visualized by fluorescent microscopy (Zeiss inverted microscope × 100 M), where 100% of observed beads were coated with protein (data not shown). HEp-2 cells were seeded on 12-mm-diameter glass coverslips (Nunc, Wiesbaden, Germany), placed on the bottom of 24 well tissue culture plates (Nunc, Wiesbaden, Germany) at 2×10^5 cells per well and allowed to grow in DMEM supplemented with 10% FCS (FCS; Gibco), 5 mM glutamine (FlowLaboratories, McLean, VA), penicillin (100 U ml^{-1}), and streptomycin (100 mg ml^{-1}) to semiconfluent monolayers at 37°C in a 5% CO_2 atmosphere. Cells were washed twice with DMEM HEPES, and 500 μl of DMEM HEPES + 1% FCS was added to the cells followed by the addition of 200 μl of the bead suspension and incubation for 2 h at 37°C in a 5% CO_2 atmosphere. Cells were washed vigorously with PBS to remove unbound beads and processed for scanning electron microscopy.

Electron microscopy studies

Cells were fixed in a solution containing 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer for 45 min on ice and then washed with cacodylate buffer. For scanning electron microscopy, samples were dehydrated in a graded series of acetone and subjected to critical-point drying with CO_2. Samples were then covered with a 10-nm-thick gold film and examined with a Zeiss 982 Gemini digital scanning microscope.

Mouse infection models

For systemic challenges, 10-week-old male CD-1 mice were infected I.P. with the specified dose of GAS strains (2×10^7 cfu for WT GAS M49 and isogenic mutants, 2×10^6 cfu for WT M1 GAS and transformant expressing SOF49) in 5% sterilized porcine gastric mucin (MP Biomedicals). Mortality was monitored and recorded for a 10 day period. An established model of GAS necrotizing subcutaneous infection was also used (Datta *et al*., 2005; Buchanan *et al*., 2006). Briefly, logarithmic phase GAS mutant were pelleted, resuspended in PBS, diluted 1:1 with sterile Cytodex beads (Sigma), and injected subcutaneously at the specified dose in 100 μl volume into shaved 10-week-old male CD-1 mice. WT SOF(+) GAS strain M49 was injected into one flank and its isogenic ΔSOF mutant simultaneously in the opposite flank for direct comparison in each mouse. The developing lesion sizes were measured daily. At the end of the experiment, mice were sacrificed, the lesion removed by excisional biopsy, homogenized, and serially diluted in PBS for plating on THA and enumeration of cfu $lesion^{-1}$.

Statistical analyses

Data sets were compared by Student's *t*-test using the Microsoft Excel statistical package; a P-value < 0.05 was considered significant.

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