Opacity Factor Activity and Epithelial Cell Binding by the Serum Opacity Factor Protein of *Streptococcus pyogenes* Are Functionally Discrete*[S]

Christine M. Gillen[‡1]**, Harry S. Courtney**[§2]**, Kai Schulze**[¶]**, Manfred Rohde**[||]**, Mark R. Wilson**[‡]**, Anjuli M. Timmer**[**]**,
Carlos A. Guzman**[¶]**, Victor Nizet**[**]**, G. S. Chhatwal**[||]**, and Mark J. Walker**[‡3]

From the ‡*School of Biological Sciences, University of Wollongong, New South Wales 2522, Australia, the* §*Veterans Affairs Medical Center and Department of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38104, the* ¶*Department of Vaccinology, Helmholtz Centre for Infection Research, Braunschweig, Germany, the* ||*Department of Microbial Pathogenicity, Helmholtz Centre for Infection Research, Braunschweig, Germany D-38124, and the* **Department of Pediatrics, Division of Pharmacology and Drug Discovery, University of California, San Diego, La Jolla, California 92093-0687*

Serum opacity factor (SOF) is a unique multifunctional virulence determinant expressed at the surface of *Streptococcus pyogenes* and has been shown to elicit protective immunity against GAS infection in a murine challenge model. SOF consists of two distinct domains with different binding capacities: an N-terminal domain that binds apolipoprotein AI and a C-terminal repeat domain that binds fibronectin and fibrinogen. The capacity of SOF to opacify serum by disrupting the structure of high density lipoproteins may preclude its use as a vaccine antigen in humans. This study generated mutant forms of recombinant SOF with reduced (100-fold) or abrogated opacity factor (OF) activity, for use as vaccine antigens. However, alterations introduced into the N-terminal SOF peptide (SOFΔFn) by mutagenesis to abrogate OF activity, abolish the capacity of SOF to protect against lethal systemic *S. pyogenes* challenge in a murine model. Mutant forms of purified SOFΔFn peptide were also used to assess the contribution of OF activity to the pathogenic processes of cell adhesion and cell invasion. Using latex beads coated with full-length SOF, SOFΔFn peptide, or a peptide encompassing the C-terminal repeats (FnBD), we demonstrate that adhesion to HEp-2 cells is mediated by both SOFΔFn and FnBD. The HEp-2 cell binding displayed by the N-terminal SOFΔFn peptide is independent of OF activity. We demonstrate that while the N terminus of SOF does not directly mediate intracellular uptake by epithelial cells, this domain enhances epithelial cell uptake mediated by full-length SOF, in comparison to the FnBD alone.

Streptococcus pyogenes (group A streptococcus, GAS)[4] is an important human pathogen responsible for a wide variety of

skin and mucosal infections ranging from pharyngitis and impetigo to more severe invasive infections, such as necrotizing fasciitis and streptococcal toxic shock-like syndrome (1–3). The serum opacity factor (SOF) is a large protein of ~110 kDa, which is expressed at the cell surface by approximately half of all clinical isolates (4, 5). Similar to a number of other surface proteins expressed by *S. pyogenes*, SOF binds fibronectin via a C-terminal-repeated domain (FnBD) (6–8), a function that has been implicated in the adhesion of GAS to epithelial cells (9). In contrast to the conserved C terminus, the N terminus of SOF (SOFΔFn) is highly variable, exhibiting ~55% identity between different serotypes of *S. pyogenes*. The N-terminal domain of SOF was originally thought to cleave apolipoprotein A1 (apoAI) in human serum leading to the precipitation of high density lipoproteins (HDLs) (10, 11). However, it has recently been demonstrated that the OF activity of SOF is not enzymatic; rather, the direct binding of apoAI by SOF triggers the release of the HDL lipid cargo of apoAI, initiating the opacity reaction (12). This OF domain of SOF promotes GAS invasion of epithelial cells (13), but it is not known whether the OF activity itself or a discrete domain within the N terminus of SOF contributes to this phenotype. SOF is a virulence determinant of GAS, with insertional inactivation or allelic replacement of *sof* reducing mortality in an intraperitoneal and subcutaneous murine infection model (13, 14). SOF is also a vaccine candidate, parenteral immunization of mice with SOF protects against lethal intraperitoneal challenge (15).

It is not known what physiological effect that the precipitation of HDL would have upon the human host or how the interaction of SOF with apoAI contributes to the pathogenesis of GAS. ApoAI exerts a potent anti-inflammatory effect by preventing contact between infected T-cells and monocytes, thereby inhibiting cytokine production (namely tumor necrosis factor-α (TNF-α) and interleukin-1) in monocytes (16). *In vitro*, both HDL and apoAI exert anti-inflammatory effects against the potent bacterial endotoxins, Gram-negative LPS and Gram-positive lipoteichoic acid, binding strongly to both endotoxins and inhibiting the production of TNF-α (17–19). *In vivo*, trans-

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ride; SOFΔFn, N-terminal SOF domain; FnBD, C-terminal fibronectin binding domain; WT, wild type; HDL, high density lipoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum.

genic animal studies of the toxicity of LPS have shown that expression of human *apoAI* transgenes protected mice from a lethal dose of LPS (20). ApoAI also possesses specific anti-bacterial and anti-viral properties (21–23).

The administration of active SOF protein as a vaccine when the downstream effects of disrupting HDL and its activity *in vivo* are unknown would not be recommended, as the most effective protection against GAS infection is delivered when the SOF protein is administered parenterally (15), and the localized depletion of HDL may reduce the body's defense against other pathogens or may result in inflammation at the site of immunization. Thus, before SOF could be used as a potential vaccine antigen (alone or as part of a multivalent vaccine formulation) it would be prudent to eliminate the OF activity of the protein. To this end, this study generated mutant forms of recombinant SOF protein with attenuated or eliminated OF activity, for use as vaccine formulations. These mutant SOF proteins have also been used to further delineate OF activity and cell binding activity within the N terminus of SOF.

MATERIALS AND METHODS

Site-directed Mutagenesis—A pQE30-based vector encoding a fusion protein of residues 33–872 of SOF from a M75 GAS strain (lacking signal sequence and fibronectin binding repeat region, pSOF75ΔFn) (24), was used as the template for all mutagenesis reactions. Site-directed mutagenesis was performed as previously described (25). Primers for site-directed mutagenesis are given in supplemental Table S1. Two mutagenesis strategies were employed, amino acid residues were substituted with alanine (single residues up to 5 residues) and small deletions were made within rSOF75ΔFn. Deletions within rSOF75ΔFn were generated by using site-directed mutagenesis to introduce two AvrII restriction sites flanking the region to be deleted, followed by digestion with AvrII. The digested fragments were then separated by agarose gel electrophoresis, and the DNA fragment containing the portion of pSOF75ΔFn of interest was extracted from the gel and re-ligated.

Expression and Purification of Wild Type and Mutant Forms of rSOF75 and rSOF75ΔFn Protein—Large scale expression and purification of rSOF75 proteins was conducted essentially according to the manufacturer's instructions (Qiagen), and has been previously described (13, 26). To ensure correct refolding of proteins was achieved, wild type purified proteins were subsequently tested for opacity factor activity using an agarose overlay method (7).

Structural Characterization of Wild Type and Mutant Forms of rSOF75ΔFn Protein—To determine the structural integrity of mutant rSOF75ΔFn proteins in comparison to the wild type, a comparison of the secondary structure of wild type and mutant rSOF75ΔFn proteins was conducted using circular dichroism (CD) spectroscopy. CD spectra were acquired using a Jasco J-810 Spectropolarimeter (Jasco). Experiments were conducted at room temperature with proteins at a concentration of ~0.2 mg/ml in 10 mM sodium phosphate buffer, pH 7.5 containing 50% trifluoroethanol (27, 28). Far UV spectra were recorded from 190–250 nm in a 0.1-cm pathlength cell (Starna) containing 400 μl of protein solution. The data shown represents an average of ten scans, corrected for a buffer baseline. Mean residue ellipticity (MRE; $[\theta]$) was calculated using Equation 1 (29).

$$MRE[\theta] = \frac{\theta \times 100 \times \text{molecular weight}}{\text{conc (mg/ml)} \times \text{path length} \times 1000 \times \text{no. of residues}}$$

(Eq. 1)

The α-helical content of wild type and mutant forms of rSOF75ΔFn was calculated from the MRE value at 222 nm using Equation 2 as described by Ref. 30.

$$\% \alpha\text{-Helix} = \frac{MRE[\theta]_{222} - 2340}{30300} \times 100$$

(Eq. 2)

Opacity Activity Assays—Qualitative opacity factor assays were conducted using the serum agarose overlay method (7). The serum overlay method permits visual confirmation of OF activity, as binding of apoA1 by SOF causes precipitation of apoAI and HDL, which appears as an opaque white band on the solid serum/agarose medium. Data are presented as an inversion of the actual blot with opacity activity appearing as a dark band on a light background. Quantitative opacity factor assays were conducted using purified HDL or human serum using the method of Courtney *et al.* (12), with opacification measured as absorbance at 405 nm.

ApoAI Binding Capacity—Wells of a microtiter plate were coated with 20 μg/ml rSOF75 protein or gelatin in 0.01 M sodium bicarbonate for 1 h at 37 °C. Plates were washed with PBS and blocked with gelatin (1 mg/ml in PBS) for 1 h at 37 °C, 100 μl of biotinylated (Pierce) apoAI (Calbiochem) was added and incubated at 37 °C for 1 h. Following washing, 100 μl of avidin-peroxidase (0.5 μg/ml) was added to the wells and incubated at 37 °C for 1 h. The plates were then washed and 3,3′-5,5′-tetramethylbenzidine substrate added. Color development was stopped using 1 M phosphoric acid, and the absorbance at 450 nm was recorded. Assays were performed in triplicate.

Interaction of SOF-coated Latex Beads with HEp-2 Cells—Assays of the interaction of SOF-coated latex beads with HEp-2 cells were conducted per previously published methods (13, 31, 32). Preliminary assays were conducted in Dulbecco's modified Eagle's medium HEPES supplemented with either 10% FCS or 1% FCS. However, differential binding was observed between these two assay conditions, the rSOF75ΔFn only mediated binding to HEp-2 cells when incubated in DMEM HEPES 1% FCS, and thus these conditions were used for all further latex bead adherence assays. The efficiency of protein loading onto latex beads was measured by FLUOstar fluorescent plate reader (BMG Labtech) using anti-SOF75 rabbit serum and fluorescent labeling with goat anti-rabbit Alexa 488 (green) (Molecular Probes) (data not shown), protein loading efficiency was found to be comparable for all protein domains. To determine the effect of exogenous addition of the rFNBD domain on the adhesion and internalization of rSOFΔFn-coated latex beads, purified rFnBD at 1, 5, or 10 μg/ml was preincubated with the HEp-2 cells for 1 h prior to addition of the coated latex beads, and was maintained throughout the subsequent 4-h incubation with the latex beads.

Confocal Microscopy Studies—HEp-2 cells (after incubation with the coated latex beads) were fixed for 30 min on ice in 500 μl/well of prechilled 4% paraformaldehyde in PBS, and then washed twice in PBS. Cells were then blocked by the addition of 200 μl/well of PBS containing 10% fetal calf serum and incubated for 30 min at room temperature. The blocking solution was then removed, and the cells were incubated with either protein-G-purified rabbit polyclonal anti-SOF75 antibodies (26) (30 μg) or rabbit polyclonal anti-FnBD antibodies (1:100 dilution) for 45 min at room temperature. Following washing with PBS, cells were incubated with goat anti-rabbit Alexa 488 diluted 1:400 in PBS containing 10% BSA (Molecular Probes) for 1 h at room temperature and subsequently washed with PBS. Cells were permeabilized with 200 μl/well of 0.1% (v/v) Triton X-100 in PBS for 30 min on ice, washed in PBS, followed by storage at 4 °C overnight. The following day, cells were treated with goat anti-rabbit Alexa 633 diluted 1:400 in PBS containing 10% BSA (Molecular Probes) for 1 h and washed three times in PBS. Cells were then mounted onto a glass slide using Mowiol solution (Calbiochem). Images were recorded using a Leica TCS SP confocal microscope mounted on a Leica DM IRBE inverted microscope with Leica TCS NT software (Version 2.61; Leica Microsystems).

Mouse Immunization and Challenge—To determine the protective efficacy of rSOF75ΔFn proteins challenge studies were performed. BALB/c mice ($n = 10$) were immunized subcutaneously with 25 μg of wild type or mutant forms of rSOF75ΔFn protein in incomplete Freund's adjuvant. Control mice received a subcutaneous injection of PBS. After 2 weeks, the mice were boosted with an intramuscular injection of another 25 μg of each protein in PBS. Control mice received a PBS injection. Two weeks after the booster injections, all mice were challenged by an intraperitoneal injection of ~1×10^9 CFU of the SOF-positive M49 GAS strain 591 (33). The number of surviving mice was recorded daily. Moribund mice were sacrificed and recorded as dead.

Serum samples were collected on days 0 and 28, and stored at -20 °C prior to determination of rSOF75ΔFn-specific antibodies. In brief, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc) were coated with 2 μg/ml of wild type rSOF75ΔFn in coating buffer (bicarbonate, pH 9.4). After overnight incubation at 4 °C, plates were blocked with 1% BSA in PBS (pH 7.4) for 1 h at 37 °C. Serial 2-fold dilutions of serum in PBS with 1% BSA were added (100 μl/well), and plates were incubated for 1 h at 37 °C. After four washes, secondary biotinylated antibodies were added followed by 1 h of incubation at 37 °C. After six washes, 50 μl/well of peroxidase-conjugated streptavidin (Pharmingen), diluted 1:1000, was added, and plates were further incubated for 45 min at room temperature. After a final six washes, the substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) in 0.1 M citrate-phosphate buffer containing 0.1% H_2O_2 was added, and plates were incubated for 30–60 min at room temperature. The absorbance was measured at a wavelength of 405 nm.

Statistical Analyses—For apoAI binding experiments, an unpaired Student's t test was used to determine if there was any significant difference in the apoAI binding ability of wild type and mutant SOF proteins. For latex bead experiments, a one way analysis of variance using Bartlett's test for equal variance was used to determine whether there was any significant variation in the median number of beads attached to or taken up by
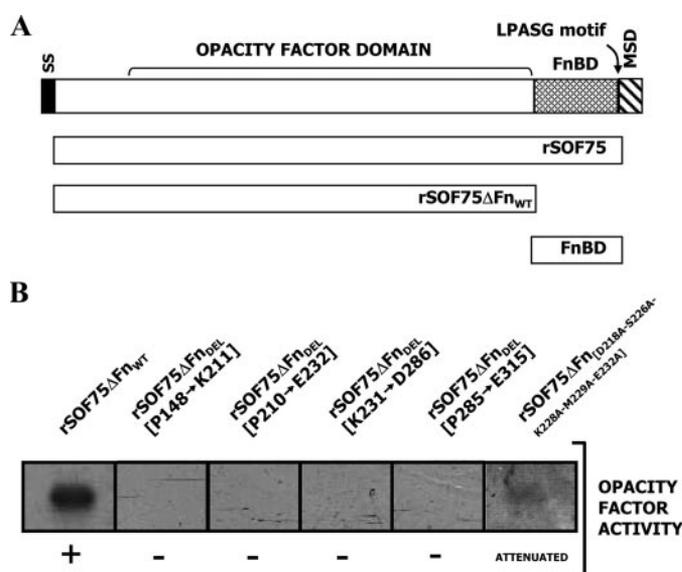
FIGURE 1. **The domain structure, opacity factor activity, and adherence phenotypes of SOF, rSOF75, rSOF75ΔFn$_{WT}$, and the rSOF75ΔFn mutants generated in this study.** *A*, schematic representation of the SOF75 protein. *SS*, signal sequence; *FnBD*, fibronectin binding domain; *MSD*, membrane spanning domain. The domain mediating OF activity is indicated by a *bracket*. *Boxes* represent the domains encompassed by the rSOF75 proteins used in this study. *B*, opacity factor phenotype of wild type and mutant forms of the rSOF75ΔFn peptide as indicated by the serum agarose overlay method.

HEp-2 cells, followed by a Tukey's Multiple Comparison Test for individual comparison of adherence and internalization mediated by two different proteins. For immunization and challenge experiments, a Kruskal-Wallis test was used to determine whether there was any significant variation in the median titers of the four groups of antisera. Dunn's Multiple Comparison test was used for individual comparison of two groups of antisera. Difference in survival curves was determined by log rank test. All statistics were performed using GraphPad Prism version 4.02 (Graph-Pad Software Inc., San Diego, CA).

RESULTS

SOF is a unique multifunctional protein, capable of binding fibronectin via a C-terminal domain designated FnBD (8, 24) and apoAI via an N-terminal domain (12) (Fig. 1*A*). The N-terminal domain of SOF (SOFΔFn) has been shown to mediate adhesion to HEp-2 cells and promote HEp-2 cell invasion by whole GAS cells. To assess the contribution of the OF activity of SOF in the processes of HEp-2 epithelial cell adhesion and invasion, deletion mutagenesis was used to eliminate the OF activity of recombinant rSOF75ΔFn. Deletion was undertaken to remove between 22 and 63 amino acid residues of the rSOF75ΔFn protein (between Pro[148] and Lys[211], Pro[210] and Glu[232], Lys[231] and Asp[286], Val[285] and Glu[315]). Each of the rSOF75ΔFn deletion mutants lacked OF activity (Fig. 1*B*). The rSOF75ΔFn$_{DEL}$[P210→E232] mutant was also found to lack OF activity when incubated in human serum or human HDL (Fig. 2). To delineate specific amino acid residues that contribute to OF activity, site-directed mutagenesis to alanine was undertaken on 52 amino acids of SOF75ΔFn that are 100% conserved in 16 different SOF sequences (supplemental Table S1). A mutant form of rSOF75ΔFn with attenuated OF activity (100-fold reduction in activity) was constructed by simultaneously sub-
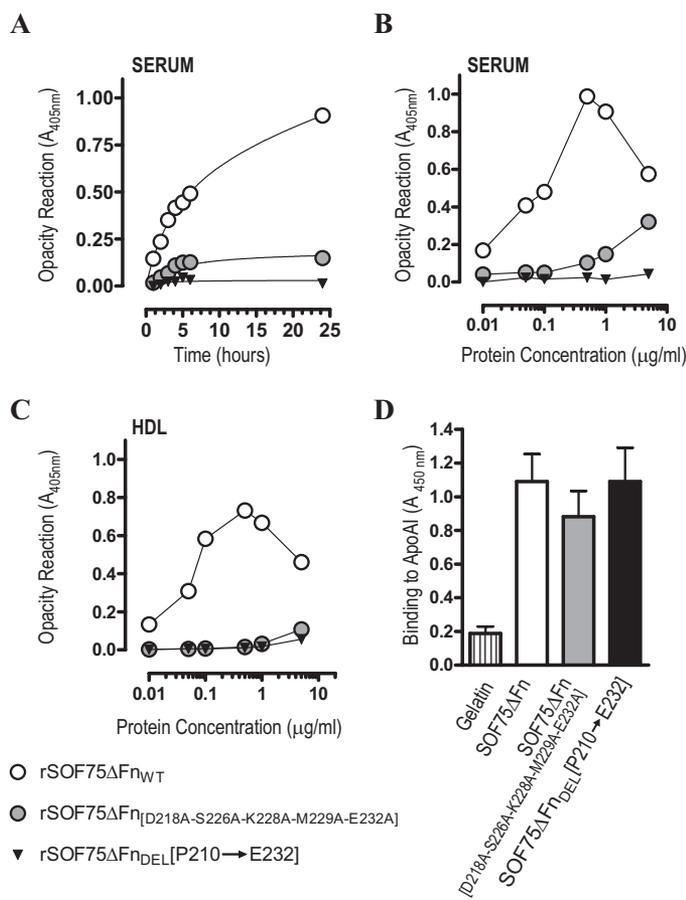
FIGURE 2. **The OF activity and apoAI binding capacity of wild type and mutant forms of rSOF75ΔFn.** *A*, OF activity over 24 h was determined by adding 1 μg/ml of either rSOF75ΔFnBD_{WT}, rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]}, or rSOF75ΔFn_{DEL}[P210→E232] to human serum and recording the $A_{405\,nm}$ at timed intervals. *B* and *C*, the opacification of serum (*B*) or HDL (*C*) as a function of protein concentration. Human serum or purified human HDL was treated with the indicated concentration of protein for 24 h and the absorbance determined at 405 nm. *D*, binding of biotinylated apoAI by either rSOF75ΔFnBD_{WT}, rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]}, rSOF75ΔFn_{DEL}[P210→E232], or gelatin.

FIGURE 3. **rSOF75ΔFn mutants have perturbed secondary structure.** The spectra of wild type rSOF75ΔFn (*solid line*) compared with mutant forms of recombinant SOF75ΔFn (*dotted line*). *A*, rSOF75ΔFn_{DEL}[P148→K211]; *B*, rSOF75ΔFn_{DEL}[P210→E232]; *C*, rSOF75ΔFn_{DEL}[V285→D315]; *D*, rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]}. All proteins exhibit CD emission spectra characteristic of proteins containing both α-helices and β-sheets, displaying a characteristic minimum at ~220 nm, a second minimum at ~207 nm, and a maximum at 190 nm. CD spectra in *A*, *B*, and *C* show a shift in the latter minimum to a shorter wavelength, indicating an increase in the proportion of the protein having disordered structure.

stituting 5 amino acids with alanine (Asp[218], Ser[226], Lys[228], Met[229], Glu[232]). The rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]} protein has attenuated OF activity when incubated with either human serum or human HDL (Fig. 2). To determine whether the loss or attenuation of OF activity was due to a decrease in the apoAI binding capacity of SOF, the ability of rSOF75ΔFn_{WT}, rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]}, and rSOF75-ΔFn_{DEL}[P210→E232] to bind biotinylated apoAI was assayed (Fig. 2D). There was no significant decrease in apoAI binding by the mutant proteins, suggesting that the loss of OF activity occurs via an alternative mechanism.

The impact of the mutations to rSOF75ΔFn on protein structure was analyzed using far-UV CD spectroscopy. rSOF75ΔFn_{WT} had a CD emission spectrum typical of proteins containing both α-helices and β-sheets, with a characteristic minimum at ~220 nm, a second larger minimum at ~207 nm and a maximum at 190 nm (34). Of the rSOF75ΔFn_{DEL} mutant proteins generated, rSOF75ΔFn_{DEL}[K231→D286] could not be purified for structural analysis. Far-UV CD spectra obtained for rSOF75ΔFn_{DEL}[P148→K211], rSOF75ΔFn_{DEL}[P210→E232], and rSOF75ΔFn_{DEL}[V285→D315] indicate perturbation to the

secondary structure of the proteins. The rSOF75ΔFn_{WT} is predicted to contain 27% α-helicity, with a predicted 24% α-helical content in rSOF75ΔFn_{DEL}[P148→K211], 21% in rSOF75-ΔFn_{DEL}[P210→E232], and 22% in rSOF75ΔFn_{DEL}[V285→D315]. A concomitant shift toward a more disordered structure was observed for each deletion mutant, as indicated by a shift in the 207 nm minima to a shorter wavelength of 204 nm for rSOF75ΔFn_{DEL}[P148→K211] and rSOF75-ΔFn_{DEL}[P210→E232] and 205.5 nm for rSOF75-ΔFn_{DEL}[V285→D315] (Fig. 3) (34, 35). In contrast to the loss of secondary structural elements in the rSOF75ΔFn_{DEL} mutants, the rSOF75ΔFn_{[D218A-S226A-K228A-M229A-E232A]} mutant had increased secondary structure when compared with rSOF75ΔFn_{WT}, with an increase in predicted α-helicity from 27 to 29% (Fig. 3).

To assess the direct contribution of the OF activity of SOF in the processes of HEp-2 epithelial cell adhesion and invasion, the ability of latex beads coated with wild type OF-positive, mutant OF-negative, and OF-attenuated forms of the rSOF75ΔFn protein to bind to the human pharyngeal epithelial cell line HEp-2 was assayed. These studies indicate that the SOFΔFn domain mediates attachment to HEp-2 cells, with the latex beads coated with rSOF75ΔFn_{WT} adhering to HEp-2 cells in numbers equivalent to latex beads coated with the full-length rSOF75 ($p > 0.05$), and significantly more latex beads coated
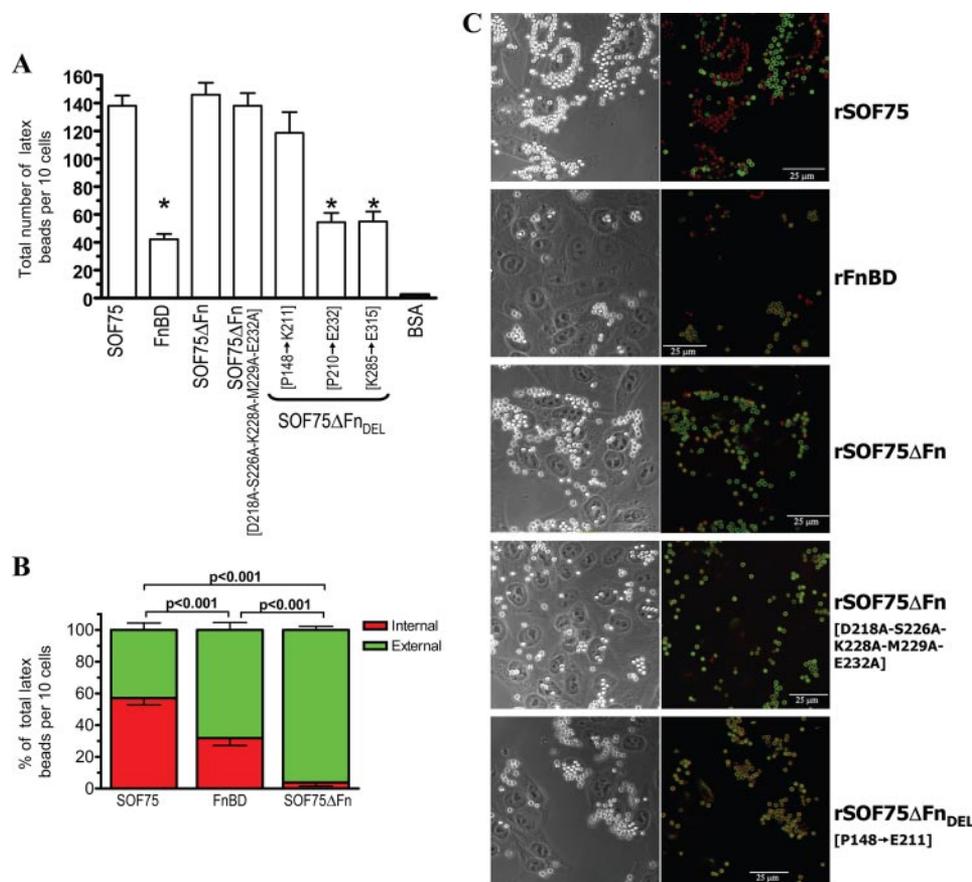
FIGURE 4. **SOFΔFn promotes association of latex beads to HEp-2 cells independent of OF activity.** *A*, total number of SOF-coated latex beads per 10 HEp-2 cells. SOF-coated latex beads were incubated with HEp-2 cells for 4 h in Dulbecco's modified Eagle's medium HEPES supplemented with 1% FCS. *Asterisks* indicate adherence significantly lower than that mediated by the recombinant full-length SOF, rSOF75 ($p < 0.001$). *B* and *C*, binding and uptake of SOF-coated latex beads by HEp-2 cells. Following incubation with latex beads, internal and external beads were differentiated using double immunofluorescence confocal microscopy. External beads were labeled by incubation with SOF-specific rabbit anti-serum, followed by goat anti-rabbit Alexa-488 conjugated antibody (*green*). Internal beads were labeled by subsequently permeabilizing the cells using 0.1% Triton X-100, and subsequently incubating the cells with SOF-specific rabbit antiserum, followed by goat anti-rabbit Alexa-633-conjugated antibody (*red*). *C*, sample of images obtained using double immunofluorescence microscopy. Binding and uptake mediated by rSOF75, rFnBD, and rSOF75ΔFn peptides is shown. Binding mediated by SOF75ΔFn[D218A-S226A-K228A-M229A-E232A] with attenuated OF activity and rSOF75ΔFn_DEL[P148→K211] with abrogated OF activity is also shown.

strains or non-invasive *Lactococcus lactis* (13). However, while the SOFΔFn promotes epithelial cell invasion in these backgrounds, this study has shown that the SOFΔFn protein domain is not sufficient *per se* to mediate intracellular invasion of HEp-2 cells (Fig. 4, *B* and *C*). However, while it is apparent that the N terminus of SOF does not directly mediate epithelial cell invasion, it may be concluded that the higher uptake by epithelial cells of latex beads coated with full-length SOF, in comparison to the FnBD domain alone, is attributable to the N-terminal domain of SOF. A significantly greater proportion of latex beads coated with rSOF75 were found to be intracellular (57.1%) than latex beads coated with FnBD alone (31.8% intracellular) ($p < 0.01$) (Fig. 4*B*).

While SOF is a protective antigen in murine vaccination studies, the capacity of SOF to opacify serum raises questions about its use in humans. The capacity to knock-out OF activity while retaining the structural and functional integrity of the molecule may be a requirement for further evaluation of SOF as a human vaccine candidate. To this end, the protective efficacy of rSOF75ΔFn_WT, rSOF75-ΔFn[D218A-S226A-K228A-M229A-E232A] and the corresponding deletion mutant rSOF75ΔFn_DEL[P210→E232] were assessed in a murine

with rSOF75ΔFn_WT adhering to HEp-2 cells than latex beads coated with a protein encompassing only the fibronectin binding domain of the SOF protein (rFNBD) ($p < 0.01$). Furthermore, this HEp-2 adherence mediated by the SOFΔFn domain is not dependent on OF activity, with latex beads coated with rSOF75ΔFn[D218A-S226A-K228A-M229A-E232A] (attenuated OF activity) and rSOF75ΔFn_DEL[P148→K211] (abolished OF activity) mediating adherence at the same level as the rSOF75ΔFn_WT protein ($p > 0.05$) (Fig. 4, *A* and *C*). Latex beads coated with rSOF75ΔFn_DEL[P210→E232] and rSOF75ΔFn_DEL[V285→D315] have a significantly reduced capacity to attach to HEp-2 cells when compared with rSOF75ΔFn_WT ($p < 0.01$), however, there were significantly more latex beads coated with rSOF75ΔFn_DEL mutants attached to HEp-2 cells than were observed with the BSA control ($p < 0.01$). It has been previously demonstrated that the SOFΔFn domain of SOF possesses pro-invasive properties when expressed on the surface of non-invasive GAS

model of lethal GAS challenge. rSOF75ΔFn_WT, rSOF75ΔFn[D218A-S226A-K228A-M229A-E232A], and rSOF75-ΔFn_DEL[P210→E232] all elicited significantly elevated titers of anti-rSOF75ΔFn_WT serum IgG ($p < 0.001$; $p < 0.01$, $p < 0.01$) (Fig. 5*A*). In concordance with the results of Courtney *et al.* (15), mice immunized with rSOF75ΔFn_WT showed significantly increased survival when challenged with the SOF-positive M49 GAS strain 591 compared with PBS control mice ($p = 0.0069$). While mice immunized with rSOF75-ΔFn[D218A-S226A-K228A-M229A-E232A], and the corresponding deletion mutant rSOF75ΔFn_DEL[P210→E232] showed increased survival when compared with the control mice, this increase was not statistically significant ($p > 0.05$) (Fig. 5*B*). While Courtney *et al.* (15) have previously shown that parenteral immunization with rSOF2ΔFn protects against lethal systemic challenge with an M2 GAS strain, this is the first study to show that immunization with SOF can protect against lethal challenge with a heterologous GAS strain.
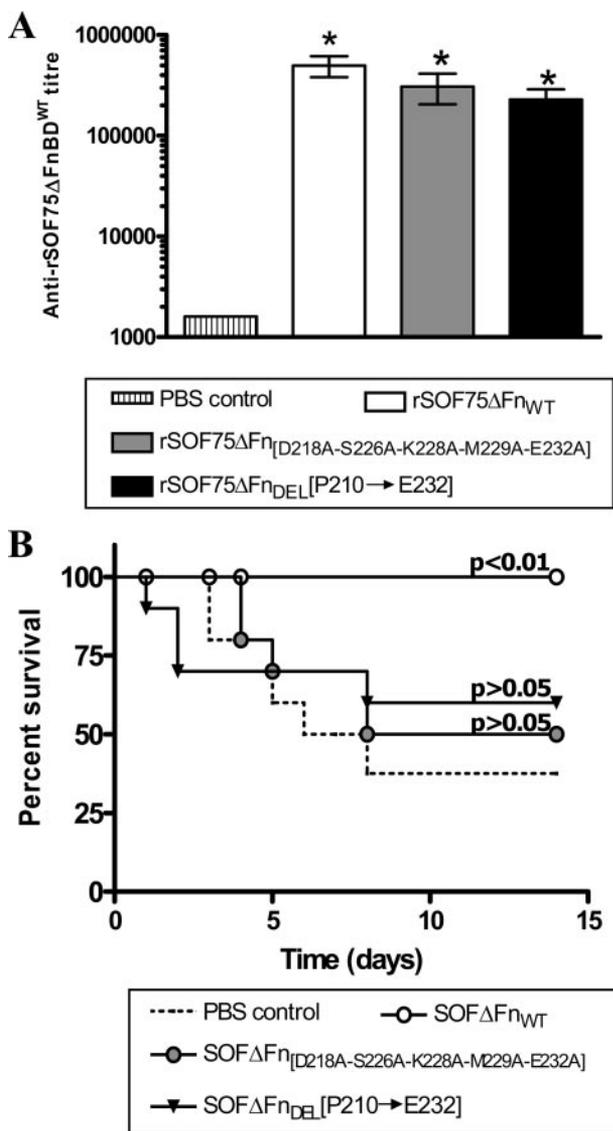
FIGURE 5. **The murine response to parenteral immunization with wild type and mutant forms of rSOF75ΔFn and subsequent lethal intraperitoneal challenge.** *A*, antibody titers directed against rSOF75ΔFn$_{WT}$ 28-days postintravenous immunization with either rSOF75DFn$_{WT}$, rSOF75ΔFn$_{[D218A-S226A-K228A-M229A-E232A]}$, or rSOF75ΔFn$_{DEL}$[P210→E232]. *Bars* represent specific IgG present in the serum of control and vaccinated mice, with results expressed as geometric means, error bars represent S.E. ($n = 10$). *Asterisks* indicate titers significantly greater than the control. *B*, survival of intravenously immunized mice after lethal challenge with a heterologous *S. pyogenes* strain. Mice ($n = 10$) vaccinated with either rSOF75ΔFn$_{WT}$, rSOF75ΔFn$_{[D218A-S226A-K228A-M229A-E232A]}$, or rSOF75-ΔFn$_{DEL}$[P210→E232] were challenged with ~1×10^9 CFU of virulent M49 *S. pyogenes* strain 591. Mortality was recorded daily. Significance was determined using the log rank test.

DISCUSSION

SOF is a unique multifunctional protein which may contribute to GAS pathogenesis by virtue of its abilities to interact with a variety of components of plasma and the extracellular matrix. A number of GAS surface proteins have a role in promoting host epithelial cell invasion including SfbI (36, 37), M protein (31, 38, 39), PrtF2 (40, 41), FbaA (42), and SOF (13). M protein and SfbI mediate intracellular invasion via two distinct pathways, both of which are dependant on fibronectin binding. M protein-mediated ingestion of GAS depends on co-engagement of the CD46 receptor and fibronectin via separate domains of the M protein; fibronectin in turn acts as a bridging molecule binding $\alpha5\beta1$ receptors at the host epithelial cell surface (43–46). The formation of the M protein-fibronectin-$\alpha 5\beta1$ complex and engagement of the CD46 receptor results in intracellular signaling cascades that lead to cytoskeletal rearrangement for ingestion of the bacteria (31, 47). As opposed to M protein, SfbI requires only an interaction with fibronectin to trigger intracellular invasion, because the two fibronectin binding domains on SfbI co-operatively bind fibronectin which in turn binds $\alpha5\beta1$ integrins on the host cell surface (44, 45). The resultant complex triggers the formation and recruitment of caveosomes which may allow GAS to persist within host cells without exposure to the acidic environment of phagosomes or lysosomes (48). While it has been clearly demonstrated through specific gene deletion that SOF, PrtF2 and FbaA are mediators of intracellular invasion of epithelial cells, the mechanism via which these surface protein mediate internalization is not known. The work of Timmer *et al.* (13), using SOFΔFn expressed at the surface of SOF-negative GAS and the heterologous species *L. lactis*, found that the N-terminal OF domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin binding domain. However, using latex beads coated with SOF protein, we demonstrate that the N-terminal OF domain is not intrinsically sufficient to mediate epithelial cell invasion, but will significantly enhance intracellular invasion in the presence of FnBD. Different GAS strains express a wide array of surface anchored proteins that interact with fibronectin, the M49 GAS strain used in the experiments of Timmer *et al.* (13) expresses both FBP54 and PrtF2 (40, 49). Thus, in this case the SOFΔFn expressed on the surface of M49 GAS may enhance cellular uptake, as the requirement for fibronectin binding may be achieved by FBP54 and PrtF2 expressed at the surface of the M49 GAS cells. However, the exogenous addition of rFnBD does not induce internalization of rSOFΔFn (results not shown), suggesting that fibronectin binding must be coupled at the surface of the latex bead or GAS cell for enhanced internalization to occur.

Incubation of cells with exogenous rSOFΔFn does not enhance uptake of GAS cells but inhibits internalization in a concentration-dependent manner (13), suggesting that the interaction of the N-terminal domain of SOF with the epithelial cell surface occurs via a specific receptor on the surface of HEp-2 cells.

We have previously demonstrated (13) that the N-terminal OF domain of SOF mediates tight adherence to epithelial cells. Thus, SOFΔFn-mediated adherence to the surface of epithelial cells most likely occurs via a mechanism independent of that required for the opacity reaction. The finding that these mutations clearly diminished the ability of SOF to opacify HDL but did not alter its binding to apoA1 indicated that other functions of SOF related to the opacity reaction were altered. Recent data suggest that SOF is a heterodivalent fusogenic protein that opacifies HDL by binding and cross-linking HDL particles resulting in the displacement of apoA1, fusion of the HDL particles, and the extrusion of a delipidated HDL particle (50). This mechanism produces a very large lipid particle that is enriched in cholesterol esters and essentially depleted of apolipopro-

teins. Thus, we propose that the described mutations of SOF alter the opacity reaction by interfering with one or more of these processes. The expression of multifunctional macromolecules at the surface of GAS is a common theme. For instance, M protein, SfbI, and the hyaluronic acid capsule each have dual functions in epithelial cell interactions and phagocytosis resistance (31, 36–39, 51–54), while glyceraldehyde-3-phosphate dehydrogenase functions as a glycolytic enzyme and binds multiple serum proteins including plasmin and fibronectin (55, 56). SOF joins an increasing list of multifunctional surface proteins of GAS.

As an immunogenic surface protein of GAS, SOF is a candidate vaccine antigen. While SOF was shown to lack protective efficacy against mucosal challenge when administered intranasally (57), SOF is a promising vaccine against systemic infection as parenteral immunization of mice with SOF2ΔFn protects against lethal intraperitoneal challenge (15). Unlike in Europe and the United States, where the throat is often the primary tissue reservoir, the skin is the major site of infection among a number of populations in which GAS infection is endemic including the Australian aboriginal population (58), populations of India and Trinidad, American Indians, and Polynesians living in New Zealand (59–61). Thus, there is a need for protective antigens for use in areas such as the tropical north of Australia, where the skin is the primary route of GAS entry. Two of the prime vaccine candidates from the surface proteome of GAS, SfbI and the conserved C-terminal epitopes of M protein, while effective at eliciting protection against mucosal colonization, have proven ineffectual at reducing the rate of mortality due to systemic GAS infection in murine models (62, 63). While SOF has proven to be protective against systemic infection, it is not known what physiological effect the interactions between HDL and SOF would have upon the human host following vaccination. It would be prudent to eliminate the OF activity of the SOF protein to ensure undesirable side effects do not occur. To this end the OF-attenuated (rSOF75ΔFn$_{[D218A-S226A-K228A-M229A-E232A]}$) and corresponding OF-negative (rSOF75ΔFn$_{DEL}$[P210→E232]) mutants generated in this study were examined to determine the protective efficacy of the mutants against lethal systemic GAS infection. Unfortunately, while the mutant proteins remained immunogenic, the structural alterations introduced upon mutagenesis may have prevented accessibility of protective epitopes or altered the protective epitopes such that mice immunized with rSOF75ΔFn$_{[D218A-S226A-K228A-M229A-E232A]}$ or rSOF75ΔFn$_{DEL}$ were not significantly protected. Other protective epitopes in streptococcal antigens such as the Group B streptococcus polysaccharide capsule and alpha C protein (64, 65) will only be protective if presented to the host immune system in their native conformation. In addition, maintenance of the conformational structure may enhance the immune response to epitopes of M proteins of GAS (66). The protective efficacy of the main protective epitope of SOF may have been altered by a loss of conformation associated with mutagenesis performed in this study. This study has shown that SOF can protect against intraperitoneal challenge by a heterologous GAS strain. These data, in conjunction with previous findings that antiserum against one serotype of GAS was bactericidal for multiple GAS serotypes (15), suggest that SOF contains common protective epitopes.

The SOF protein is a virulence determinant in GAS with affinity for multiple serum proteins including fibrinogen (67), fibronectin (8, 24), and apoAI (12). Additionally, SOF plays a role in epithelial cell adhesion and invasion (9, 13) and phagocytosis resistance (68). This study has shown that the N terminus of SOF mediates binding to HEp-2 cells and delineates this binding activity from the ability of the N terminus of SOF to opacify serum. This study is the first to indicate that vaccination with SOF can protect against infection by a heterologous GAS serotype in an animal model.

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