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Cooperative Interactions between Flagellin and SopE2 in the Epithelial Interleukin-8 Response to *Salmonella enterica* Serovar Typhimurium Infection

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Flagellin is an important stimulus for epithelial interleukin-8 (IL-8) secretion because of its ability to activate Toll-like receptor 5 (TLR5). SopE2, a *Salmonella* guanine nucleotide exchange factor (GEF), is also involved in intestinal inflammation. To clarify the proinflammatory mechanisms of these proteins, we examined their effects on IL-8 secretion and intracellular signaling in T84 epithelial cells. A *Salmonella* strain lacking SopE2 (and its homolog SopE) induced lower levels of IL-8 than the wild type and exhibited reduced activation of mitogen-activated protein kinases (MAPKs). Overexpression of wild-type SopE2 in this strain restored MAPK activation and augmented IL-8 production, whereas a mutant lacking GEF activity failed to increase IL-8 expression. Additional effects on signaling were demonstrated in transient transfection experiments, in which SopE2 enhanced the ability of TRAF6, a signal transducer downstream of TLR5, to activate the NF-κB transcription factor in 293 cells. Flagellin was also found to be required for IL-8 induction in T84 cells. In its absence, the ability of SopE2 overexpression to increase IL-8 secretion was impaired. Part of this impairment was related to the decreased motility of the flagellin-deficient strain, but lack of flagellin also affected translocation of SopE2 into the infected cells. Our results indicate that flagellin and SopE2 interact functionally at multiple levels to increase IL-8 secretion by epithelial cells—flagellin facilitating the translocation of SopE2, and SopE2 enhancing signaling pathways activated by flagellin. These observations offer a mechanistic explanation for the involvement of these proteins in the pathogenesis of *Salmonella*-induced gastroenteritis.

Salmonella enterica serovar Typhimurium is a major cause of acute gastroenteritis in humans, accounting for about 40,000 cases reported annually in the United States (37). The molecular basis for *Salmonella*-induced enteritis has been elucidated by studies carried out with cell culture and with animal models of intestinal inflammation (43). A number of factors have been found to contribute to pathogenesis, including increased enterocyte fluid secretion, recruitment of inflammatory cells to the site of infection, decreased epithelial barrier function, and enterocyte death. These cellular abnormalities have been associated with several alterations at the molecular level, such as increased inositol phosphate flux, increased prostaglandin synthesis, and global changes in gene expression that include the induction of inflammatory cytokines and chemokines, such as interleukin-8 (IL-8) (16–18, 38). Some of these alterations have been linked to the effects of bacterial proteins that interact with specific host target molecules. For instance, the SopB protein, introduced into the host cell via the *Salmonella* pathogenicity island 1 (SPI1) type III secretion system (TTSS), functions as an inositol phosphate phosphatase that increases levels of inositol tetrakisphosphate, an antagonist of chloride channel closure (36). Similarly, the *Salmonella* SipA protein is involved in inducing the transepithelial migration of neutrophils by eliciting the apical secretion of a chemoattractant molecule (31, 34).

IL-8 released from the basolateral aspect of infected epithelial cells plays an important role in the initial movement of neutrophils from the circulation into the subepithelial region (15, 29, 33). Recently, *Salmonella* flagellin has been shown to play a major role in the induction of IL-8 via interactions with its cellular receptor Toll-like receptor 5 (TLR5), which is expressed on the basolateral aspect of polarized enterocytes (22, 23, 25, 46). However, other studies have implicated the SPI1 TTSS and bacterial guanine nucleotide exchange factors (GEFs) that are translocated into the host cell via this system in the activation of epithelial proinflammatory signals, although not specifically in the induction of IL-8 (10, 24, 26). It is not clear how these earlier observations are related to the more recent findings on flagellin, specifically, whether the signals activated by *Salmonella* GEFs, such as SopE and SopE2, contribute to the production of IL-8 and whether they act redundantly or cooperatively with signals activated by flagellin-TLR5 interactions.

SopE2, a protein expressed by all strains of *Salmonella*, is introduced into host cells via the SPI1 TTSS (4, 42). Like its homolog, SopE, which is expressed by only some *Salmonella* strains, it is a GEF that activates mammalian Rho GTPases

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(21, 42). This activation contributes to the induced cytoskeletal rearrangements that are involved in bacterial invasion of epithelial cells (4, 48). SopE2 has also been implicated in the pathogenesis of the diarrhea and enteritis associated with *Salmonella* infection in calves (47). It is not clear whether this effect of SopE2 is related to its role in bacterial invasion or to some other function. However, our earlier work indicating that SopE2 is involved in the up-regulation of macrophage inducible nitric oxide synthase (iNOS) independently of effects on invasion would be consistent with a specific role in inflammation (11). To further characterize the proinflammatory functions of SopE2, we examined its role in IL-8 secretion by the T84 human enterocyte cell line, particularly with respect to interactions with flagellin. In the results described here, we present evidence to indicate multiple functional interactions between flagellin and SopE2 that lead ultimately to increased epithelial IL-8 production. Our findings shed light on the contribution of these molecules to the pathogenesis of *Salmonella*-induced enteritis.

MATERIALS AND METHODS

Bacterial strains. The wild-type serovar Typhimurium strain F98 (which lacks SopE), the corresponding SopE2-deficient mutant, SE2.1, and the SE2.1/pSE2 derivative of SE2.1 that is transcomplemented with a SopE2-encoding plasmid have been described previously (4). The wild-type strain SL3201 and its flagellin-deficient derivative, SL3201 Δ*fljB* Δ*fliC*, which lacks expression of flagellin B and flagellin C (23, 46), were obtained from Andrew Gewirtz (Emory University, Atlanta, Ga.). They were transformed with the SopE2 expression plasmid, using electroporation as described in detail earlier (11), to derive the corresponding SopE2-overexpressing strains SL3201/pSE2 and SL3201 Δ*fljB* Δ*fliC*/pSE2. All bacteria were grown in Luria-Bertani medium, with ampicillin included at a concentration of 100 μg/ml when selection for the SopE2 plasmid was required. For T84 infections, the bacteria were grown overnight in static cultures with minimal aeration. The bacteria were collected by centrifugation at 14,000 × *g* for 5 min, washed with sterile phosphate-buffered saline (PBS), and resuspended in tissue culture medium without antibiotics at a density of 4×10^9/ml. Twenty-five-microliter aliquots of this suspension (10^8 bacteria) were used to infect the cells.

Analysis of SopE2 expression. Overnight static cultures were centrifuged to collect the bacteria. After washing the bacterial pellet, it was resuspended in sample buffer. The resuspended bacterial pellets were boiled in the sample buffer for 5 min and centrifuged at 14,000 × *g* for 5 min to pellet insoluble material, and equal volumes of the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In some cases, as indicated in specific experiments, the amount of bacterial extract loaded was adjusted, based on the results of initial experiments, so that the SopE2 signal obtained on the Western blot was roughly comparable in all the lanes. After SDS-PAGE, the proteins were transferred to nitrocellulose by semidry blotting as previously described (11). SopE2 was detected on the blots using SopE2-specific murine monoclonal antibodies (4), followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin G and enhanced chemiluminescence (Super Signal West Pico kit; Pierce Chemical Co., Rockford, Ill.). The blots were exposed to X-ray film for appropriate times, the autoradiographs were then scanned, and band densities were quantitated with NIH Image software. For detection of secreted SopE2, supernatants of overnight bacterial static cultures were precipitated with trichloroacetic acid as detailed earlier (11). The precipitated proteins were separated by SDS-PAGE, and SopE2 was detected by immunoblotting as described above.

Cell culture and infection. T84, a transformed human colonic epithelial cell line (35), was obtained from Beth McCormick (Massachusetts General Hospital, Boston, Mass.) and grown in F12-Dulbecco modified Eagle medium with 10% heat-inactivated fetal calf serum. Passages 50 to 62 were used for all experiments. For IL-8 assays, the cells were seeded on collagen-coated 6.5-mm, 5-μm-pore-size Costar Transwell filter inserts (Corning Corporation, Corning, N.Y.) so that the upper chamber would allow access to either the apical or basolateral surfaces of the polarized cells as required. The cells were grown until a transepithelial resistance of at least 3,000 Ω/cm^2 was achieved. Before addition of bacteria, the cells were washed and placed in antibiotic-free medium. Infection was started by addition of bacteria to the upper chamber of the Transwell in a volume of 150 μl and was allowed to proceed for 1 h at 37°C. In some experiments, a brief centrifugation (500 × *g* for 10 min) was carried out to facilitate deposition of the bacteria on the cells, as has been described by others (19). The medium was aspirated from both the upper and lower chambers at the end of the infection period. The cell monolayer was washed twice with sterile PBS, and medium containing gentamicin at a concentration of 100 μg/ml was added to both chambers. After incubation for 5 h at 37°C, the basolateral medium was collected and used for IL-8 assays. The cells were washed with PBS and lysed with 0.2 ml of 1% Triton X-100. An aliquot of the lysate was used to determine the protein concentration by using the DC protein assay (Bio-Rad, Hercules, Calif.), following instructions provided by the manufacturer. For experiments with nonpolarized cells, the cells were grown in 24-well tissue culture plates until each well was 50 to 75% confluent. They were then infected with bacteria for 1 h as described above, washed, and placed in gentamicin-containing medium, and the supernatants were collected for IL-8 enzyme-linked immunosorbent assay (ELISA) after 5 h. All infections were carried out in triplicate.

IL-8 assay. IL-8 in the basolateral medium was measured by ELISA, using anti-human IL-8 antibodies from Pharmingen (San Diego, Calif.). Recombinant human IL-8 (R & D Systems, Minneapolis, Minn.) of known concentration was used to generate a standard curve. To allow comparison between multiple experiments, the amount of IL-8 produced was normalized to the protein content of the cell monolayer. Because of variations in baseline IL-8 production, the results are expressed as *n*-fold increase, representing the normalized IL-8 produced by infected monolayers divided by the normalized IL-8 produced by control, uninfected monolayers.

Invasion assay. T84 cells were infected as described above. After the 1 h of infection, the cells were washed and placed in medium with 100 μg of gentamicin/ml to kill extracellular bacteria. Two hours later, the cells were washed and lysed in sterile 1% Triton X-100. The number of bacteria inside the cells was determined by plating 100-μl aliquots of serial dilutions of the lysates on Luria-Bertani plates and counting the colonies after overnight incubation at 37°C. This number was expressed as a percentage of the infecting dose for each strain (obtained by plating serial dilutions of the inoculum).

Mutagenesis of SopE2. A SalI-EcoRI fragment containing the carboxy-terminal region of the SopE2 open reading frame was cloned into the plasmid pBluescript (Stratagene, La Jolla, Calif.), such that the insert was flanked by T3 and T7 primer sites. Glycine 168 of SopE2 was converted to alanine by site-directed mutagenesis using a PCR-based method (12). Briefly, in a first round of PCR, two overlapping DNA fragments were generated using the pBluescript/SopE2 construct as the template and the following primer pairs: T3 (5′ AATTAACC CTCACTAAAGGG 3′) paired with mutagenic primer 1 (5′ AGGTGGGGCA GCGGCGAATCCTT 3′) and T7 (5′ GTAATACGACTCACTATAGGGC 3′) paired with mutagenic primer 2 (5′ AAGGATTCGCCGCTGCCCCACCT 3′). The two fragments were gel purified and annealed. Mutual priming by the overlapping fragments allowed extension, followed by a second round of PCR carried out using only the T3 and T7 primers. The fragment thus generated was digested with SalI and EcoRI and cloned into pBluescript. It was sequenced using T3 and T7 primers, as well as custom primers complementary to regions adjoining the mutation (sequencing primer 1, 5′ CCATATTATCAGCCGTG 3′; sequencing primer 2, 5′ CTGAAATTGTTGTGGCG 3′). After the results of the sequencing confirmed the mutation, the mutated fragment was cloned back into the original SopE2 expression construct to restore the complete open reading frame.

Analysis of MAPK activation in T84 cells. T84 cells were grown in 24-well tissue culture plates until they reached 50 to 75% confluence. They were infected for different times with the various *Salmonella* strains as described above. At each time point, the cells were placed on ice, washed quickly with cold PBS, and lysed in 0.2 ml of 1% Triton X-100 in 10 mM Tris-HCl (pH 8.0)–150 mM NaCl, containing a cocktail of protease inhibitors (10 μg of aprotinin and leupeptin/ml, 2 mM phenylmethylsulfonylfluoride) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate). The lysates were cleared by centrifugation at 14,000 × *g* for 10 min, and the protein concentrations were determined using the DC protein assay kit (Bio-Rad). Equal amounts of total protein were separated by SDS-PAGE and then transferred to nitrocellulose membranes by semidry blotting as previously described (11). After the membranes were blocked with 5% nonfat dry milk, they were probed with antibodies to either phosphorylated extracellular growth factor-regulated kinase (ERK) (Santa Cruz Biotechnology, Santa Cruz, Calif.) or phosphorylated p38 kinase (Cell Signaling, Beverly, Mass.) and then developed with horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories, San Francisco, Calif.) and enhanced chemiluminescence. Appropriate exposures to X-ray film were made, and the filters were then stripped and reprobed with antibodies to total ERK or total p38 kinase, as relevant (both from Santa Cruz Biotechnology). Autoradiograms were

scanned and analyzed with NIH Image software to quantitate band densities. The mean density of the phosphorylated mitogen-activated protein kinase (MAPK) band was divided by the mean density of the corresponding total MAPK band to yield a normalized band density value.

Transfections and NF-κB reporter assays. Transfection of 293 human embryonic kidney epithelial cells and NF-κB reporter assays were carried out essentially as described in detail earlier (9). In brief, 293 cells were transfected by the calcium phosphate precipitation method with 1 μg of a NF-κB-dependent chloramphenicol acetyltransferase (CAT) expression construct and 0.5 μg of a constitutively expressed β-galactosidase expression construct (both plasmids were kindly provided by Ranjan Sen, Brandeis University), along with 1 μg of a Flag epitope-tagged TRAF6 expression construct (a gift of David Goeddel, Tularik Corporation) with or without 0.6 or 1.2 μg of a c-*myc* epitope-tagged SopE2 expression construct. Forty hours after the transfection, cell lysates were prepared and used to determine β-galactosidase activity by a colorimetric assay (39) and CAT concentration by an ELISA-based method (CAT-ELISA kit; Boehringer-Mannheim, Indianapolis, Ind.). CAT concentrations were normalized to β-galactosidase activity to control for transfection efficiency, and then expressed as *n*-fold increases over the values of control cells transfected only with the CAT and β-galactosidase constructs.

Construction of SopE2-GFP fusion expression plasmid. A PCR product was generated from the pBluescript-SopE2 plasmid described above, using the T7 primer and a downstream primer that replaced the last two amino acids of the SopE2 reading frame with a BglII site (5′ GGAGATCTCATTCTGAAGATA CTTATTCGC 3′). The PCR product was digested with SalI and BglII and gel purified. At the same time, a second PCR product was generated from the plasmid pUC19GFP (kindly provided by Donald Smith [MGH, Boston, Mass.]) that contained the entire open reading frame of the *Aequoria victoria* green fluorescent protein (GFP) gene, such that the reading frame was flanked by a BglII site at the 5′ end and an EcoRI site at the 3′ end. The GFP primers used were 5′ GAAGATCTCCATGAGTAAAGGAGAAGA 3′ and 5′ CGGAATTC TTATTTGTATAGTTCATCCAT 3′, respectively. The PCR product was digested with BglII and EcoRI and gel purified. The two restriction enzyme-digested and gel-purified PCR products were ligated into the pSE2 plasmid isolated from the SE2.1/pSE2 strain so as to replace the carboxy-terminal region of the SopE2 reading frame with the corresponding region of SopE2 fused in-frame to GFP. The new plasmid, pSE2-GFP, was transformed into the SopE2-deficient SE2.1 strain to confirm that the fusion protein could functionally complement SopE2 and also into the SL3201 and SL3201 Δ*fljB* Δ*fliC* strains. Expression of the fusion protein was confirmed in all the transformant strains by Western blotting of bacterial lysates with an anti-GFP antibody (Molecular Probes, Eugene, Ore.).

Analysis of SopE2 translocation into T84 cells. Translocation of SopE2 into the infected cells was analyzed using the protocol described recently by Kubori and Galan (28). In brief, T84 cells grown to 50 to 75% confluence in six-well plates were pretreated with 10 μM MG132 (Calbiochem, San Diego, Calif.) to inhibit proteasomal degradation of SopE2 (28) and then infected with the SopE2-GFP-expressing strains of *Salmonella*. At the end of the 1-h infection period, the cells were washed with cold PBS and then lysed with 0.1% Triton X-100 in PBS containing the cocktail of protease inhibitors described above. Insoluble material, including cell-associated bacteria, was removed by centrifugation at 16,000 × *g* for 15 min, and the supernatant, containing translocated *Salmonella* effector proteins, was analyzed for the presence of the SopE2-GFP fusion protein by Western blotting with the anti-GFP antibody. The blot was then stripped and reprobed with an antibody to the housekeeping protein glyceraldehyde phosphate dehydrogenase (Research Diagnostics Inc., Flanders, N.J.) to confirm equivalent loading of lanes.

Statistical analysis. Statistical significance was determined using the Student *t* test.

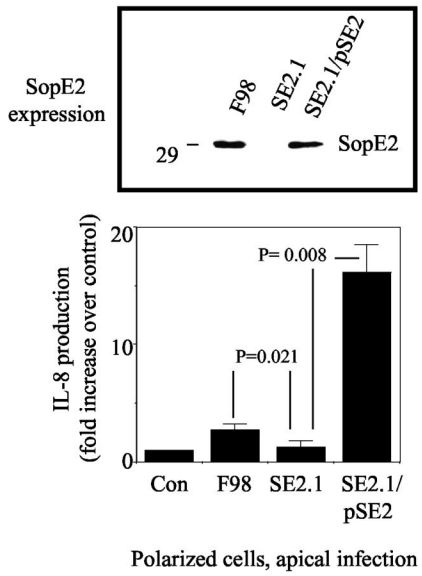
RESULTS

SopE2 regulates epithelial IL-8 production in a GEF activity-dependent manner and independently of effects on invasion. To examine the effect of SopE2 on IL-8 secretion, polarized T84 monolayers were infected apically with the wild-type serovar Typhimurium strain F98, the isogenic SopE2-deficient mutant SE2.1, or the SopE2-transcomplemented strain SE2.1/pSE2, which expresses about sixfold-higher levels of SopE2 (based on band quantitation) than F98 (upper part of Fig. 1A). IL-8 in the basolateral supernatant was measured 5 h later by ELISA. As shown in Fig. 1A, the amount of IL-8 induced by the SopE2-deficient strain was lower than that induced by the wild type. This difference was small, but it was reproducible over the course of eight separate experiments (representing 24 individual infections for each strain) and was statistically significant ($P = 0.021$). More strikingly, overexpression of SopE2 resulted in a marked increase in IL-8 induction compared to results with either the wild-type or SopE2-deficient strains ($P = 0.008$). Similar results were obtained when the infection was carried out using nonpolarized T84 cells (Fig. 1B) and also when polarized T84 cells were infected from the basolateral side of the monolayer (data not shown). These observations indicate that SopE2 is involved in the regulation of IL-8 production by *Salmonella*-infected T84 cells and that this regulation is independent of cell polarity.

To determine whether this effect of SopE2 was dependent on GEF activity, we introduced a point mutation that converted glycine 168 to alanine, a change in the catalytic core region that has been shown previously to inactivate SopE (8). The plasmid encoding the mutant protein was transformed into SE2.1 to yield the strain SE2.1/pSE2*. Western blot analysis indicated that the wild-type and mutant SopE2s were expressed at comparable levels (upper part of Fig. 1C). When polarized T84 cells were infected apically with SE2.1/pSE2*, the amount of IL-8 produced was close to baseline and was significantly lower than that induced by SE2.1/pSE2 ($P = 0.0006$). These results indicate that the effect of SopE2 on IL-8 induction is dependent on GEF activity.

It has been reported previously that SopE2 influences bacterial invasiveness in HeLa cells (4). We confirmed that observation for infection of polarized T84 cells under the conditions used in the IL-8 assays described here. Figure 2A demonstrates that the SopE2-deficient SE2.1 strain was less invasive than the wild-type F98 strain, whereas the SopE2-transcomplemented strain SE2.1/pSE2 displayed wild-type or greater levels of invasion. The invasiveness of SE2.1/pSE2* was comparable to that of SE2.1, indicating a requirement for GEF activity in this function. Based on these differences, a potential explanation for the effect of SopE2 on IL-8 production could be related to its role in invasion. To test this idea, we examined the effect of cytochalasin D, an agent that interferes with rearrangements of the actin cytoskeleton, on cell invasion and IL-8 production during the course of *Salmonella* infection of T84 cells. Cytochalasin D treatment resulted in an approximately 90% reduction in the number of intracellular bacteria (Fig. 2B, left histogram) but produced only an approximately 30% decrease in IL-8 induction (Fig. 2B, right histogram). These results indicate that the influence of SopE2 on IL-8 secretion cannot be explained entirely by its role in bacterial entry into cells.

SopE2 influences the activation of several intracellular signaling pathways. Based on the previously reported importance of flagellin and TLR5 in inducing epithelial IL-8 expression (22, 23, 25, 46) and following on our observations that flagellin and TLR5 expression were not affected by SopE2 (data not shown), we considered the possibility that SopE2 exerted its effects on IL-8 production by influencing the signaling pathways downstream of TLR5. *Salmonella* infection of intestinal epithelial cells has been shown previously to lead to activation



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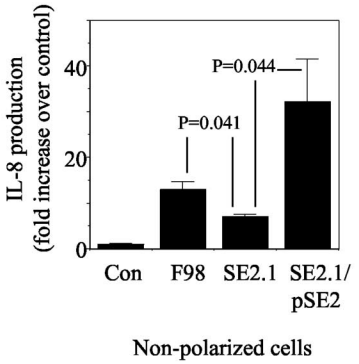

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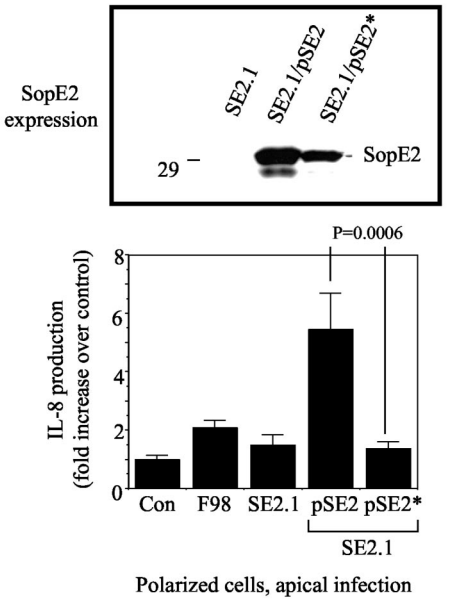

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FIG. 1. (A) *Salmonella*-induced IL-8 secretion by T84 cells is regulated by SopE2. Polarized T84 monolayers were left uninfected (control) or infected apically for 1 h with wild-type (F98), SopE2-deficient (SE2.1), or SopE2-transcomplemented (SE2.1/pSE2) strains of *Salmonella*. IL-8 in the basolateral culture medium was assayed by ELISA 5 h later. The results represent the means and standard deviations from eight separate experiments, with each experiment consisting of triplicate infections. *P* values are indicated above the corresponding bars. The Western blot in the upper part of the figure indicates the expression of SopE2 in lysates of the various bacterial strains. The amount of SE2.1/pSE2 lysate loaded was approximately 1/10 that of the F98 lysate. The number to the left of the blot indicates the position of the 29-kDa molecular mass standard. (B) IL-8 production by T84 cells in response to *Salmonella* infection in nonpolarized T84 cells. Nonpolarized T84 monolayers were left uninfected (control) or infected for 1 h with wild-type (F98), SopE2-deficient (SE2.1), or SopE2-transcomplemented (SE2.1/pSE2) strains of *Salmonella*. IL-8 in the supernatant was assayed by ELISA 5 h later. The results indicate the means and standard deviations of triplicate wells and are representative of two separate experiments. *P* values are indicated above the corresponding bars. (C) The effect of SopE2 on IL-8 secretion requires GEF activity. Polarized T84 monolayers were left uninfected (control) or infected apically for 1 h with *Salmonella* strains F98 (wild-type), SE2.1 (SopE2-deficient), SE2.1/pSE2 (SE2.1 expressing wild-type SopE2), or SE2.1/pSE2∗ (SE2.1 expressing mutant SopE2). IL-8 in the basolateral culture medium was assayed by ELISA 5 h later. The results represent the means and standard deviations of triplicate wells and are representative of two separate experiments. The *P* value for the difference between SE2.1/pSE2- and SE2.1/pSE2∗-infected cells is indicated. The Western blot in the upper part of the figure indicates the expression of SopE2 in bacterial supernatants of SE2.1, SE2.1/pSE2, and SE2.1/pSE2∗. The number to the left of the blot indicates the position of the 29-kDa molecular mass standard.

of the MAPKs ERK, p38, and the c-jun N-terminal kinase (26), pathways that are also activated by TLRs (5). Furthermore, inhibition of p38 activation has been shown to attenuate *Salmonella*-induced IL-8 production (26), and recently published work indicates that p38 has effects on the translation of IL-8 mRNA (45). Therefore, we examined the effect of SopE2 on the activation of ERK and p38 kinases. T84 cells were infected with either the wild-type (F98), SopE2-deficient (SE2.1), or SopE2-overexpressing (SE2.1/pSE2) strains, and cell lysates were analyzed by Western blotting at different times, using antibodies specific for the phosphorylated (activated) forms of the kinases. As shown in Fig. 3A, MAPK activation induced by the SopE2-deficient strain was appreciably altered when compared to activation by the wild-type strain. In the case of the p38 kinase, the major effect of SopE2 deficiency was on the duration of activation. At the 1-h time point, the magnitude of p38 activation was similar in both the wild-type and SopE2-deficient strains. However, at later time points, activated p38

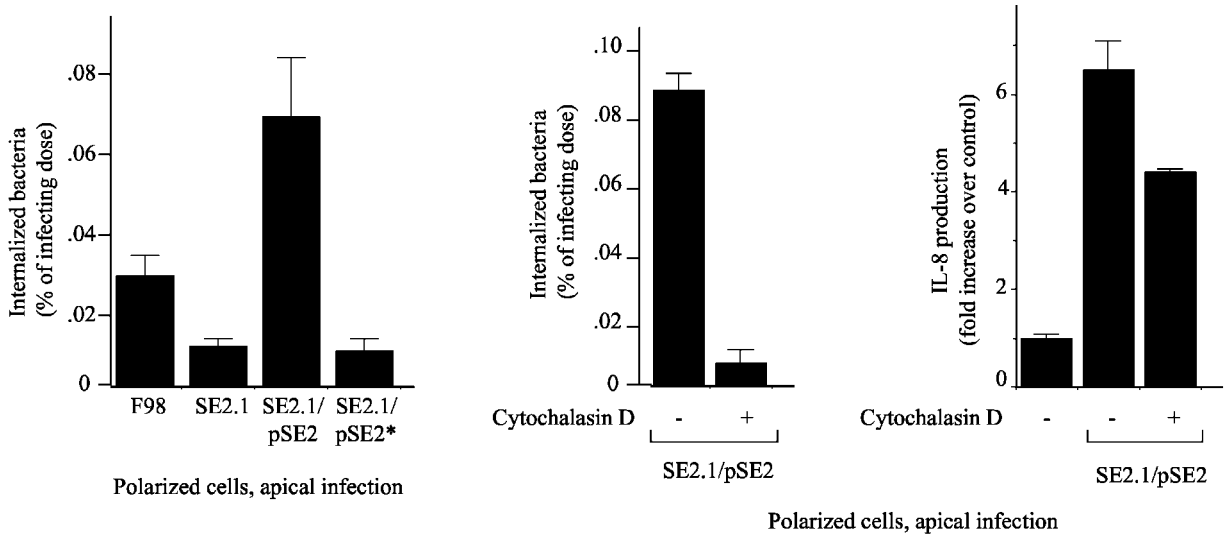

FIG. 2. (A) Invasiveness of the *Salmonella* strains for T84 cells is regulated by SopE2. Polarized T84 monolayers were infected apically for 1 h with the wild-type (F98) or SopE2-deficient (SE2.1), SopE2-transcomplemented (SE2.1/pSE2), or SopE2 (SE2.1/pSE2*) mutant strains. The number of internalized bacteria was determined by gentamicin protection assay and expressed as a percentage of the number of infecting bacteria. The results indicate the means and standard deviations from triplicate wells and are representative of two separate experiments. (B) Effect of cytochalasin D on cell invasion and IL-8 induction by *Salmonella*. Left histogram, polarized T84 monolayers were infected apically for 1 h with the SopE2-transcomplemented strain SE2.1/pSE2 in the presence (+) or absence (−) of 15 μg of cytochalasin D/ml. The number of internalized bacteria was determined by gentamicin protection assay and expressed as a percentage of the number of infecting bacteria. The results indicate the means and standard deviations for triplicate wells and are representative of two separate experiments. Right histogram, polarized T84 monolayers were left uninfected or were infected apically for 1 h with the SopE2-transcomplemented strain SE2.1/pSE2 in the presence (+) or absence (−) of 15 μg of cytochalasin D/ml. IL-8 in the basolateral culture medium was assayed by ELISA 5 h later. The results indicate the means and standard deviations for triplicate wells and are representative of two separate experiments.

could be detected only in the wild-type infection. In the case of the ERK kinase, the major effect of SopE2 deficiency was on the magnitude of activation, with the kinetics being similar to wild-type kinetics. Overexpression of SopE2 in the transcomplemented SE2.1/pSE2 strain corrected the abnormalities of both p38 and ERK activation, whereas overexpression of the GEF-inactive mutant SopE2* did not (data not shown). These results indicate that SopE2 regulates ERK and p38 kinase activation during *Salmonella* infection, and they are consistent with the idea that SopE2 increases IL-8 production by altering the activation of these kinases.

We also examined the effect of SopE2 on activation of the NF-κB pathway. In initial experiments, we used the degradation of IκBα as an indicator of activation of this pathway and found that SopE2 had little or no effect on this function (data not shown). Recently, TLR2 was shown to activate a Rac1-dependent signaling pathway that increased NF-κB transcriptional activity without altering IκBα degradation (3). To determine whether SopE2 might have a similar effect, we took a transfection-based approach to investigate the influence of SopE2 on NF-κB transcriptional activity stimulated by tumor necrosis factor receptor-associated factor 6 (TRAF6), a signaling molecule that functions downstream of TLR5 and other TLRs (5). The human embryonic kidney epithelial cell line 293 was transfected with an NF-κB-dependent CAT expression construct, as well as a constitutively expressed β-galactosidase construct. Cotransfection of SopE2 resulted in an approximately twofold increase of NF-κB transcriptional activity, as measured by elevated normalized CAT expression, compared with that of control cells transfected with only the CAT and β-galactosidase constructs (Fig. 3B). TRAF6 by itself increased CAT expression by about eightfold over that for control cells. When SopE2 was expressed together with TRAF6, a dose-dependent enhancement of NF-κB activity was observed, amounting to a 15- to 20-fold increase over that for control cells ($P = 0.018$ for the comparison of TRAF6 alone with TRAF6 plus 1.2 μg of SopE2). This effect was not related to a SopE2-dependent increase in TRAF6 expression (upper part of Fig. 3B), suggesting that the effect on NF-κB-dependent transcription was caused by cooperative interactions between SopE2- and TRAF6-activated signals.

The effect of SopE2 on IL-8 secretion is influenced by flagellin. To further explore potential functional interactions between SopE2 and flagellin in inducing IL-8, we made use of the observation that overexpression of SopE2 significantly enhanced the amount of IL-8 produced (Fig. 1A). The SopE2 expression plasmid was transformed into either the wild-type serovar Typhimurium strain SL3201 or its isogenic, flagellin-deficient derivative, SL3201 Δ*fljB* Δ*fliC*. SopE2 expression in both sets of transformants was similar and comparable to that seen with SE2.1/pSE2 (upper part of Fig. 4A). The parental and transformed strains were first tested for invasiveness. As shown in Fig. 4A, overexpression of SopE2 in the wild-type SL3201 strain resulted in a significant increase in invasiveness

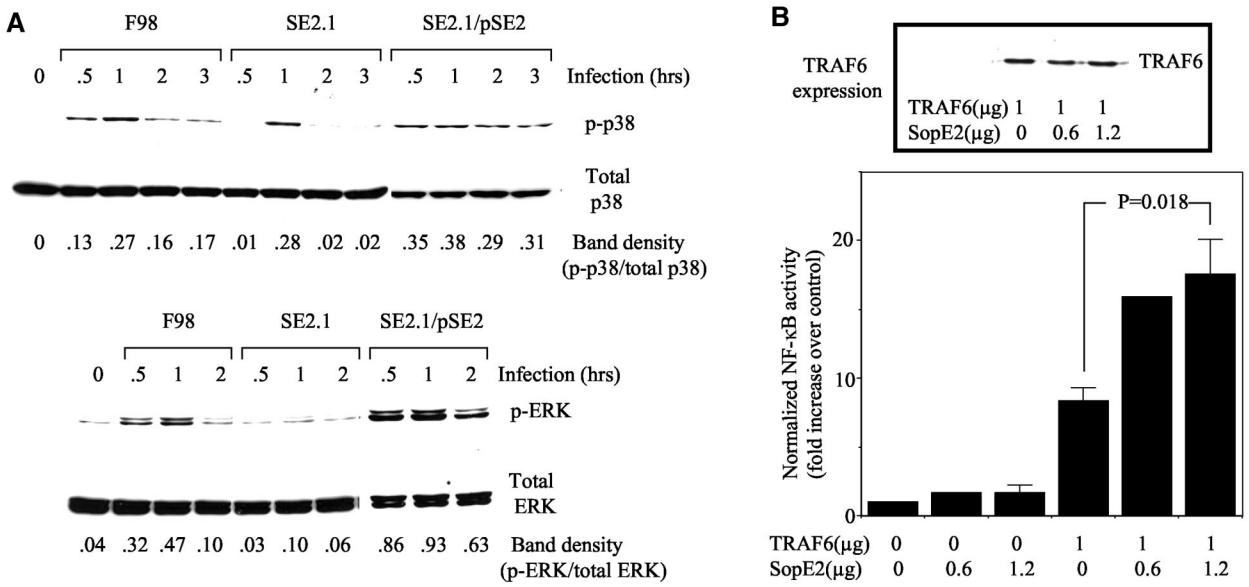

FIG. 3. (A) Activation of the p38 and ERK kinases by *Salmonella* is regulated by SopE2. T84 cells were infected with wild-type (F98), SopE2-deficient (SE2.1), or SopE2-transcomplemented (SE2.1/pSE2) strains of *Salmonella* for the indicated times. Cell lysates were prepared at each time point and analyzed by Western blotting for the presence of phosphorylated p38 (p-p38) or phosphorylated ERK (p-ERK). The blots were then stripped and reprobed with antibodies to total p38 and total ERK, respectively. The images were analyzed with NIH Image software, and the mean density of each band measured. The density of the phosphorylated MAPK band was divided by the density of the corresponding total MAPK band to obtain the normalized band density, which is indicated below each lane. The results shown are representative of three separate experiments (the indicated quantitation of band densities refers specifically to the Western blots shown). (B) SopE2 enhances TRAF6-induced NF-κB activation. Human embryonic kidney 293 cells were transfected with the indicated amounts of the TRAF6 and SopE2 expression constructs, along with 1 μg of an NF-κB-dependent CAT reporter plasmid and 0.5 μg of a constitutively expressed β-galactosidase plasmid. Forty hours after the transfection, cell lysates were prepared and the quantities of β-galactosidase and CAT were determined. CAT levels were normalized to β-galactosidase activity and expressed as the *n*-fold increase over control cells transfected with just the CAT and β-galactosidase reporters. The Western blot in the upper part of the figure indicates the expression of TRAF6 in the various transfections. The results shown indicate the means and standard deviations for three separate experiments. The *P* value for the comparison of NF-κB activation by TRAF6 alone versus TRAF6 plus 1.2 μg of SopE2 is indicated.

($P = 0.001$), whereas equivalent overexpression in the flagellin-deficient mutant did not have a significant effect on invasion. Since the flagellin-deficient strains are nonmotile, we examined whether actively depositing the bacteria onto the cells by centrifugation ($500 \times g$, 10 min) might alter the results. As has been noted by others (19, 41), the centrifugation appreciably enhanced the invasiveness of the flagellin-deficient SL3201 Δ*fljB* Δ*fliC* strain (albeit not up to wild-type levels), increasing the number of internalized bacteria from 0.002% of the infecting dose to 0.013%. The centrifugation also revealed that SopE2 overexpression had a striking effect on the invasiveness of the flagellin-deficient strain (increasing it from 0.013% for the SL3201 Δ*fljB* Δ*fliC* strain to 0.239% for the SL3201 Δ*fljB* Δ*fliC*/pSE2 strain; $P = 0.018$). The invasiveness of the flagellin-expressing strains was not significantly altered by the centrifugation.

We then proceeded to examine the ability of these strains to induce IL-8 following infection of nonpolarized T84 cells (Fig. 4B). As expected from previous reports indicating an important role for flagellin in this process (22, 23, 46), the amount of IL-8 induced by the flagellin-deficient SL3201 Δ*fljB* Δ*fliC* strain was significantly less than that for the wild-type SL3201 strain ($P = 0.003$), and this outcome was not altered by centrifugation. In the absence of centrifugation, SopE2 overexpression significantly increased IL-8 induction by the wild-type SL3201 strain ($P = 0.004$ for the comparison of SL3201/pSE2 with SL3201) but did not appreciably increase the amount of the cytokine induced by the flagellin-deficient strain (SL3201 Δ*fljB* Δ*fliC*/pSE2 versus SL3201 Δ*fljB* Δ*fliC* in Fig. 4B). This finding suggested that the effect of SopE2 on IL-8 production was flagellin dependent. With centrifugation, however, SopE2 significantly increased the amount of IL-8 produced, even in the flagellin-deficient background ($P = 0.003$ for the comparison of SL3201 Δ*flj* Δ*fliC*/pSE2 with SL3201 Δ*fljB* Δ*fliC*), indicating that a good deal of the apparent flagellin dependence was related to the decreased motility of the mutant strain. Decreased motility was not the complete explanation, however, since even after centrifugation, the amount of IL-8 induced by the SopE2-overexpressing, flagellin-deficient strain was significantly less than that induced by the SopE2-overexpressing, wild-type strain ($P = 0.011$ for the comparison of SL3201 Δ*fljB* Δ*fliC*/pSE2 with SL3201/pSE2), an effect similar to the findings of the invasion assay (Fig. 4A).

Flagellin is required for translocation of SopE2 into infected cells. We considered a further explanation for the influence of flagellin on SopE2 function, namely, that flagellin and/or an intact flagellar apparatus might be required for the translocation of SopE2 into the infected cell. We addressed this possibility initially by attempting to detect translocated SopE2 by using the anti-SopE2 monoclonal antibodies available to us.

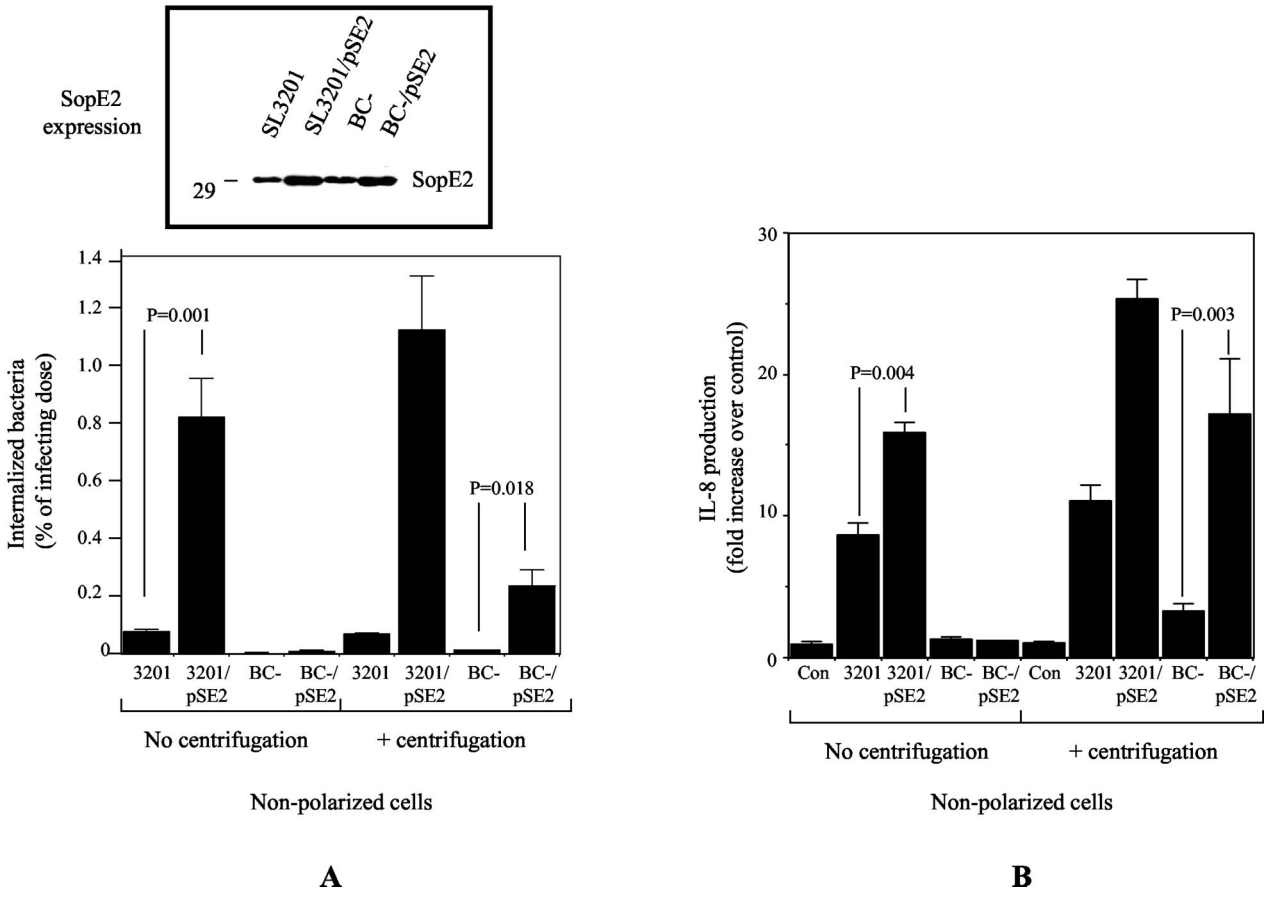

FIG. 4. (A) The effect of SopE2 on cell invasion in flagellin-deficient and wild-type strains. T84 cells were infected with SopE2-overexpressing or parental strains of SL3201 and SL3201 Δ*fljB* Δ*fliC* (indicated as BC−), with brief centrifugation (500 × *g* for 10 min) applied where noted. The number of internalized bacteria was determined by gentamicin protection assay and expressed as a percentage of the number of infecting bacteria. The results indicate the means and standard deviations for triplicate wells and are representative of two separate experiments. *P* values are indicated above the corresponding bars. The Western blot in the upper part of the figure indicates the expression of SopE2 in bacterial lysates of the various strains. The number to the left of the blot indicates the position of the 29-kDa molecular mass standard. (B) The role of SopE2 and flagellin in IL-8 secretion by nonpolarized T84 cells. Cells were left uninfected (control) or infected with SopE2-overexpressing or parental strains of SL3201 and SL3201 Δ*fljB* Δ*fliC* (indicated as BC−), with brief centrifugation (500 × *g* for 10 min) applied where noted. IL-8 in the supernatant medium was assayed by ELISA 5 h later. The results shown indicate the means and standard deviations from two separate experiments, each consisting of triplicate infections. *P* values are indicated above the corresponding bars.

However, the sensitivity of detection with these reagents proved to be inadequate for the purpose. Accordingly, we proceeded to generate a SopE2-GFP fusion protein expression plasmid as described in the Materials and Methods section. The plasmid was transformed into *Salmonella* strains SE2.1, SL3201, and SL3201 Δ*fljB* Δ*fliC* to generate the corresponding derivatives SE2.1/pSE2-GFP, SL3201/pSE2-GFP, and SL3201 Δ*fljB* Δ*fliC*/pSE2-GFP. Western blotting of bacterial lysates revealed the presence of an anti-GFP-reactive band of the expected size (approximately 55 kDa based on the combined sizes of SopE2 and GFP) in the transformants but not the parental strains (Fig. 5A). The invasiveness of the SE2.1/pSE2-GFP transformant strain was significantly enhanced relative to that of the parental SE2.1 strain and was comparable to the invasiveness of the SE2.1/pSE2 strain overexpressing wild-type SopE2 (Fig. 5B), indicating that the SopE2-GFP fusion was functionally active. To examine translocation of the fusion protein, nonpolarized T84 cells were infected with the transformants for 1 h, and the soluble fraction of detergent lysates of the infected cells was examined by Western blotting with the anti-GFP antibody. Since it has been reported recently that the SopE2 homolog SopE is rapidly degraded by the proteasome after translocation (28), we took the precaution of treating the cells with the proteasome inhibitor MG132. As shown in Fig. 6A, translocation of the SopE2-GFP fusion protein was readily detected after infection with SE2.1/pSE2-GFP or SL3201/pSE2-GFP. However, very little SopE2-GFP could be detected in the cells after infection with the flagellin-deficient SL3201 Δ*fljB* Δ*fliC*/pSE2-GFP strain, even though this strain expresses considerably higher levels of the fusion protein than the wild-type SL3201/pSE2-GFP strain (Fig. 5A) and even though centrifugation of the bacteria onto the cells was used to compensate for the decreased motility of the strain. In the deliberately overexposed blot of Fig. 6B, it can be seen that centrifugation does increase the amount of SopE2-GFP translocated from the flagellin-deficient strain, consistent with the effects on invasive-

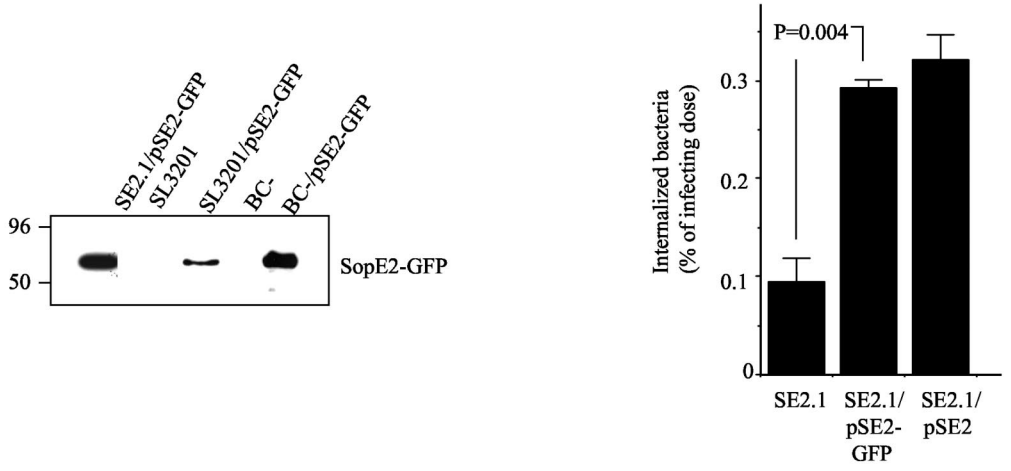

FIG. 5. Expression and function of the SopE2-GFP fusion protein. A SopE2-GFP fusion expression plasmid was constructed as described in the text and transformed into the SE2.1, SL33201, or SL3201 Δ*fljB* Δ*fliC* (indicated as BC−) strains. Expression of the fusion protein in bacterial lysates of the various strains is shown in panel A. For panel B, a gentamicin protection assay was used to compare the invasiveness of transformants of SE2.1 expressing either the SopE2-GFP fusion or SopE2 with the invasiveness of the parental SE2.1 strain. The results shown indicate the means and standard deviations for triplicate infections and are representative of two separate experiments. The *P* value for the difference between SE2.1 and SE2.1/pSE2-GFP is indicated.

ness and IL-8 production seen in Fig. 4. Similar results were obtained in the absence of MG132 treatment (data not shown). We conclude from these observations that optimal translocation of SopE2 into the infected cell requires flagellin and/or an intact flagellar apparatus.

DISCUSSION

The results described in this paper indicate that the SopE2 effector protein of *Salmonella* regulates epithelial IL-8 production. Flagellin is currently considered a major *Salmonella* com-

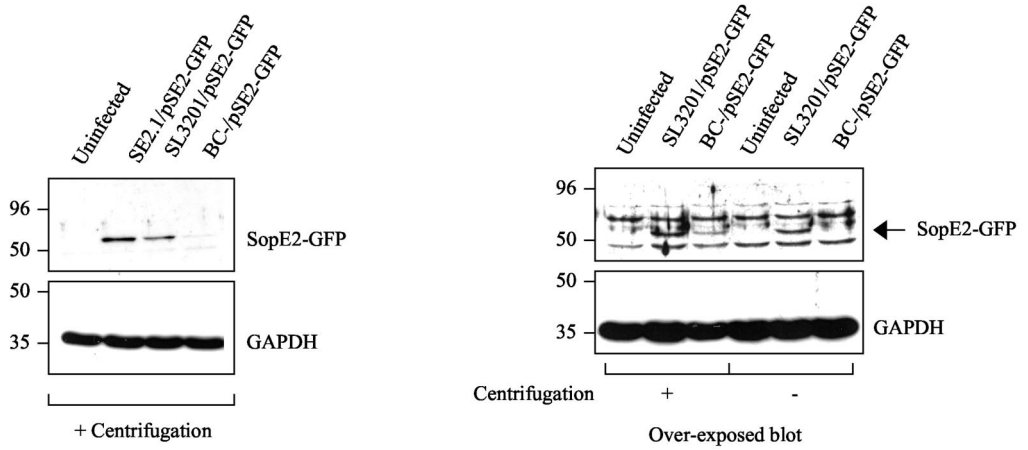

FIG. 6. Translocation of the SopE2-GFP fusion protein into T84 cells. T84 cells pretreated with the proteasome inhibitor MG132 (to inhibit degradation of SopE2) were left uninfected or infected for 1 h with SopE2-GFP-expressing transformants of SE2.1, SL3201, or SL3201 Δ*fljB* Δ*fliC* (indicated as BC−). Cytosolic extracts were prepared as described in the text and analyzed by Western blotting with an anti-GFP antibody for the presence of translocated SopE2-GFP. The blot was then stripped and reprobed with an anti-glyceraldehyde phosphate dehydrogenase antibody to confirm equivalent loading of lanes. The results shown in panel A indicate the appreciable impairment of translocation of SopE2-GFP in the flagellin-deficient strain, even though the bacteria were centrifuged onto the cells. However, in the deliberately overexposed blot shown in panel B, it can be seen that centrifugation does increase the amount of translocated SopE2-GFP. The results are representative of at least two separate experiments.

ponent involved in eliciting epithelial IL-8 secretion because of its ability to activate TLR5. In its absence, *Salmonella*-induced IL-8 production is markedly diminished (22, 23, 46). Nevertheless, a role for the SPI1 TTSS has been shown previously, suggesting the involvement of an effector protein dependent on this secretory apparatus (26). The identity of this putative IL-8-inducing effector protein(s) has not been elucidated so far. We believe that the data presented here argue strongly that SopE2 is one of the SPI1 TTSS-dependent effectors involved in IL-8 induction, and furthermore, that its function in this process is based on cooperative functional interactions with flagellin. Our experiments clearly indicate that a SopE2-deficient mutant of *Salmonella* induces significantly less epithelial IL-8 than the isogenic wild-type strain, while a SopE2-overexpressing strain induces significantly higher-than-wild-type levels of IL-8 (Fig. 1). The influence of SopE2 on IL-8 secretion is associated with effects on the activation of the p38 and ERK kinases (Fig. 3A) and on TRAF6-dependent activation of NF-κB (Fig. 3B), all of which are signaling pathways that function downstream of TLRs, such as TLR5 (5), and that are involved in the intestinal epithelial IL-8 response to bacterial infection (1, 6, 13, 20, 26, 40, 45).

Our findings are consistent with observations indicating a requirement for the *Salmonella* TTSS and p38 kinase activation in inducing IL-8 production in epithelial cells (26) and with the ability of the SopE2 homolog, SopE, to activate signaling pathways that target transcription factors (24). Together with our earlier observations on the role of SopE2 in iNOS induction in macrophages (11), the involvement in IL-8 production supported by the data here indicates that this effector protein has multiple proinflammatory effects. SopE2 and its downstream signaling pathways may thus be attractive targets for the control of *Salmonella*-induced enteritis.

SopE2 has been shown previously to play an important role in cell invasion (4), an observation that we were able to reproduce here (Fig. 2A). However, our results indicate that cell entry is not absolutely required for the effect of SopE2 on IL-8 secretion (Fig. 2B), consistent with our earlier observation that SopE2-dependent up-regulation of iNOS in macrophages is independent of the number of intracellular bacteria (11). Effector proteins such as SopE2 are delivered into the host cytosol via the *Salmonella* SPI1 TTSS without a requirement for bacterial entry into the cell (7, 44), so it is not unexpected that their functional effects can be dissociated from cell invasion. Furthermore, distinct signaling pathways connect the *Salmonella* GEFs either to cytoskeletal changes or to MAPKs and changes in gene expression, so that blocking the former would not affect the latter (10, 21, 30, 42). It should be mentioned, however, that there are examples of cytoskeletal changes influencing MAPK signaling. For instance, cortical actin structures have been shown to regulate epidermal growth factor-induced MAPK activation (2). Thus, the results shown in Fig. 2B should not be interpreted to completely exclude the possibility that the cytoskeletal changes associated with cell invasion might contribute to signals regulating IL-8 production.

The mechanism by which SopE2 influences intracellular signaling and IL-8 production requires further elucidation. Our results indicate that it involves the GEF activity of SopE2 (Fig. 1C), but the identity of the Rho GTPases that are activated and the nature of the molecular events downstream of such activation are currently unknown. The p21-activated kinase, PAK, has been shown previously to connect SopE and Cdc42 to the activation of MAPK pathways (10), making it a likely candidate for mediating at least some of the effects of SopE2 on IL-8 secretion. To our knowledge, SopE and SopE2 have not been previously implicated in the regulation of NF-κB activity, as we have shown here (Fig. 3B), and clearly, it will be important to determine how such regulation occurs. Rac1-dependent activation of NF-κB has been shown to occur downstream of TLR2 (3). This process occurs without altered IκBα degradation, and appears to increase the transcriptional activity of the p65 subunit of NF-κB via activation of phosphatidylinositol-3-kinase and the Akt kinase. It is an intriguing possibility that similar mechanisms may be involved in the effect of SopE2 on NF-κB. Interestingly, TLR5, unlike TLR2, does not appear to be connected to the Rac1-dependent pathway of NF-κB activation, since it does not have the cytoplasmic motif required to recruit phosphatidylinositol-3-kinase (3). Therefore, the ability of SopE2 to activate NF-κB by this unusual mechanism would complement TLR5 signals that lead to increased IκBα degradation and nuclear translocation of NF-κB (14).

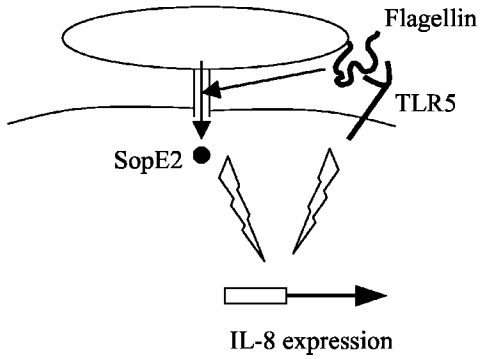

FIG. 7. A model of cooperative interactions between SopE2 and flagellin in the induction of IL-8. Flagellin is required for the optimal translocation of SopE2. Signals activated by SopE2 and flagellin act together to increase production of IL-8.

Our experiments indicate that the ability of SopE2 to regulate IL-8 production is influenced by the presence of flagellin (Fig. 4). This functional interaction between flagellin and SopE2 appears to be based on multiple factors. As indicated by the consequences of centrifugation, the decreased motility of the flagellin-deficient strain has a clear impact on the effect of SopE2 on both invasion and IL-8 production (Fig. 4A and 4B). A close association between *Salmonella* and the target cell is required to facilitate the translocation of effector proteins such as SopE2, and decreased motility is likely to affect the development of the appropriate cell-bacterium interactions. Indeed, we found that centrifugation enhanced the translocation of SopE2 from the flagellin-deficient strain (Fig. 6B).

Even after using centrifugation to correct the motility defect, it is apparent that the SopE2-dependent enhancement of IL-8 production is less in the flagellin-deficient strain than in the wild-type strain (Fig. 4B). These findings suggest a requirement for flagellin in the effects of SopE2 that is unrelated to motility. Part of this flagellin dependence can be ascribed to the impaired SopE2 translocation that persists in the flagellin-deficient strain even after centrifugation (Fig. 6A). Several

earlier studies have documented that mutations in *Salmonella* transcriptional regulatory genes (*flhC*, *flhD*, *fliA*, or *fliZ*) that control the expression of flagellar components can also affect the expression of SPI1-encoded genes, including those involved in the translocation of effector proteins (19, 27, 32). However, to our knowledge, there are no previous reports of mutations in flagellar structural components, such as flagellin, altering effector translocation. In fact, there are at least two studies showing that mutations in flagellar structural genes have little or no effect on expression of SPI1 TTSS components (19, 27). Thus, the mechanism linking defective flagellin expression and/or flagellar structure with decreased SopE2 translocation is not immediately obvious. Based on observations that the invasiveness of flagellin-deficient strains can be partially corrected by centrifugation (19) (Fig. 4A), it seems unlikely that such strains completely lack a functional SPI TTSS, suggesting a more subtle defect in the introduction of effectors into the host cytosol. Clearly, this is an issue that deserves further investigation, especially since it raises the possibility that some of the diminished inflammatory effects of flagellin-deficient bacterial strains (22, 23, 46, 49) may be secondary to abnormal effector translocation.

Another component of the SopE2-flagellin interaction is at the level of signaling. As discussed above, we have shown that SopE2 contributes to the activation of at least three signaling pathways (ERK, p38, and NF-κB) that are also activated by the binding of flagellin to TLR5 and that are known to be involved in the induction of IL-8 expression. The net amount of IL-8 produced would thus depend on the combined effects of signals activated by SopE2 and flagellin.

In conclusion, our observations have demonstrated multiple functional interactions between SopE2 and flagellin in the induction of epithelial IL-8 (Fig. 7). Flagellin is required for the efficient translocation of SopE2 into the infected cell, and the translocated SopE2 in turn activates signals that function together with those activated by flagellin/TLR5 to increase IL-8 expression. These cooperative interactions between SopE2 and flagellin highlight the importance of both proteins in the pathogenic effects of *Salmonella* in the intestine. Furthermore, the involvement of SopE2 in the regulation of epithelial IL-8 production offers a molecular explanation for the reported role of this effector in the *Salmonella*-induced diarrhea and intestinal inflammation seen in the bovine model of infection (47). Thus, our observations provide new mechanistic insights into the pathogenesis of *Salmonella* gastroenteritis and the role of SopE2 in this disease.

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