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Inhibition of *Salmonella typhimurium* Invasion by Host Cell Expression of Secreted Bacterial Invasion Proteins

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Pathogenic *Salmonella* species initiate infection of a host by inducing their own uptake into intestinal epithelial cells. An invasive phenotype is conferred to this pathogen by a number of proteins that are components of a type III secretion system. During the invasion process, the bacteria utilize this secretion system to release proteins that enter the host cell and apparently interact with unknown host cell components that induce alterations in the actin cytoskeleton. To investigate the role of secreted proteins as direct modulators of invasion, we have evaluated the ability of *Salmonella typhimurium* to enter mammalian cells that express portions of the *Salmonella* invasion proteins SipB and SipC. Plasma membrane localization of SipB and SipC was achieved by fusing carboxyl- and amino-terminal portions of each invasion protein to the intracellular carboxyl-terminal tail of a membrane-bound eukaryotic receptor. Expression of receptor chimeras possessing the carboxyl terminus of SipB or the amino terminus of SipC blocked *Salmonella* invasion, whereas expression of their chimeric counterparts had no effect on invasion. The effect on invasion was specific for *Salmonella* since the perturbation of uptake was not extended to other invasive bacterial species. These results suggest that *Salmonella* invasion can be competitively inhibited by preventing the intracellular effects of SipB or SipC. In addition, these experiments provide a model for examining interactions between bacterial invasion proteins and their host cell targets.

Salmonella infections continue to be an important health concern in both developed and undeveloped countries (14). These pathogenic bacteria possess many virulence determinants which they use to establish infection of a host. For example, lipopolysaccharide provides protection against host killing mechanisms (52, 54, 56). Additionally, the bacteria possess a set of genes that encode a type III secretion system which enables the bacteria to penetrate the membrane of host cells (1, 24, 27, 29, 35, 38, 42). Other determinants, including a virulence plasmid, permit these bacteria to survive and grow within the host lymphatic system (16, 17, 28, 30–33, 53, 58).

The ability to invade mammalian cells is critical to the ability of *Salmonella* to initiate infection of a host (39). Studies using microscopy and immunofluorescence to examine the internalization event have found that the cytoskeleton of the host is dramatically rearranged during entry (20, 27, 40) and depends on the polymerization of actin (15, 19, 26). Recent work from several laboratories has identified the genes responsible for *Salmonella* invasion (3, 12, 23, 42, 47). These genes reside on 35 kb of contiguous DNA that comprise pathogenicity island 1 (46) and maps to centisome 63 of the chromosome. The proposed functions of these gene products include transcriptional regulation of the invasion genes, protein secretion, and activation of host cell uptake pathways.

Numerous studies have identified proteins that are secreted into the extracellular media during the growth of invasive *Salmonella typhimurium* (34, 35, 42, 50). Two of these secreted invasion proteins, SipB and SipC, are likely the candidate proteins that interact directly with eukaryotic cell targets because they are essential for invasion (41), are the major secreted invasion proteins (50), and are translocated into the host cell in association with bacterial invasion (11). Furthermore, purified recombinant IpaC, the *Shigella* homologue of SipC (29), can activate cellular kinase activity and promote cellular uptake of noninvasive *Shigella flexneri* (44).

While it is clear that SipB and SipC have important roles in *Salmonella* internalization, the molecular details of their functions are unclear. We have explored the possibility of expressing SipB and SipC in cells sensitive to *Salmonella* invasion as a potential method for characterizing an interaction between these two invasion proteins and eukaryotic cells. Portions of SipB or SipC were fused to a plasma membrane-bound receptor to facilitate expression in mammalian cells. Surprisingly, expression of distinct portions of either SipB or SipC specifically blocked the ability of invasive *Salmonella* to penetrate cells.

MATERIALS AND METHODS

Construction of invasion protein chimera cDNA plasmids. Chromosomal DNA fragments from *S. typhimurium* SL1344 (60) invasion genes encoding SipB and SipC were amplified by PCR such that a *Bsp*E1 site was engineered into the 5′ end while an *Xho*I or *Not*I site was engineered into the 3′ end. PCR products were cloned and amplified in pGEM-T (Promega). Cloned invasion gene sequences were then ligated into pCR3 (Invitrogen) containing platelet-activating factor (PAF) receptor (PAFR) sequences (kindly provided by Rory Fisher, The University of Iowa), using a *Bsp*E1 site in the 3′ end of the PAFR cDNA and an *Xho*I or *Not*I site in the downstream region of the pCR3 multiple cloning site. Oligonucleotide primers used for sequencing and PCR were synthesized by the University of Iowa DNA Core Facility. Forward and reverse primer sequences are, respectively, 5′-TCCGGATGGTAAATGACGCAAGTAGC-3′ and 5′-GTTCGTTTCCTCGAGTTAGCGCGTCTG-3′ for the amino terminus of SipB, 5′-TCCGGAAGAAATCGGCTGAGTTCCAGG-3′ and 5′-ATGTCGACTTATGCGCGACTCTGGCGCA-3′ for the carboxyl terminus of SipB, 5′-TCCGGATGTTAATTAGTAATGTGGGAA-3′ and 5′-CATTCTCGAGCCCCTTTTATTTCCAGTT-3′ for the amino terminus of SipC, and 5′-TCCGGATGAATGCGTTGTCCGGTAGTA-3′ and 5′-ATGTCGACTTAAGCGCGAATATTGCCTG-3′ for the carboxyl terminus of SipC. Sequences were verified by automated fluorescent dideoxynucleotide sequencing by the University of Iowa DNA Core Facility.

Cell culture and transfections. Baby hamster kidney (BHK) cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's me-

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dium containing 10% fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere. Cells were split every 3 to 4 days via trypsinization. Cells were transfected by using lipofection or electroporation, with similar results. Electroporation was performed as described previously (48) in 0.4-cm cuvettes, using 3.8×10^6 cells, 25 μg of DNA, 220 V, 950 μF, and 90 ms. Lipofection was performed in 24-well tissue culture dishes as described previously (8), using 1 μg of DNA per well and 5 μl of Lipofectamine (GIBCO-BRL) per μg of DNA. Human embryonic kidney (HEK) and COS-7 cells were maintained and transfected in a similar fashion except that 0.5 μg of DNA per well was used in the transfection cocktail. For BHK cell dose-response experiments, total plasmid DNA was maintained at 1 μg per well by the addition of insertless pCR3 vector.

Invasion assays. Bacterial invasion, as determined by a gentamicin resistance assay (40), was assessed 48 to 54 h after transfection by inoculating 10^6 to 10^7 bacteria per well. Bacteria were incubated with transfected cells for 60 min. Cells were then incubated for 90 min with culture media containing gentamicin (50 μg/ml) to eliminate extracellular bacteria. Percent invasion was normalized based on the percent invasion observed in cells transfected with the PAFR cDNA. *S. typhimurium* SL1344 (60), *Listeria monocytogenes* 10403S (4), enteropathogenic *Escherichia coli* (EPEC) JPN15/pMAR7 (2), and *E. coli* HB101 expressing invasin (plasmid kindly provided by Ralph Isberg, Tufts University) were the invasive strains used in these studies.

Evaluation of ligand binding and receptor signaling in transfected cells. BHK cell transfectants were examined for the ability to bind [^{3}H]WEB 2086 and to accumulate inositol phosphates (IP) in response to PAF. Intact cell binding of [^{3}H]WEB 2086 was assessed at 25°C by a previously described method (49) in which saturation binding data were transformed to a Scatchard plot for the determination of receptor expression and affinity. PAF-induced IP accumulation was determined by chloroform-methanol extraction and ion-exchange chromatography as described previously (8).

RESULTS

Expression of *Salmonella* invasion proteins in BHK cells. Several groups have identified SipB and SipC as the major invasion proteins secreted by invasive *Salmonella* (34, 35, 42, 50), while another group documented the translocation of these two proteins into the host cell in association with invasion (11). Since it is unclear if invasion protein translocation precedes or follows bacterial invasion, we wished to evaluate the role of SipB and SipC as intracellular effectors of invasion. Therefore, we explored the feasibility of expressing SipB and SipC in cells as a means of evaluating their potential intracellular invasion-determining activities.

Since SipB and SipC are prokaryotic proteins, it is difficult to ensure their plasma membrane expression in eukaryotic cells. To ensure that they reach this putative site of action, we individually fused portions of either SipB or SipC to the seven-transmembrane-spanning guanine nucleotide-binding protein-coupled PAFR. This fusion-based approach was chosen because previous studies in our laboratory revealed that transfection of plasmids expressing SipB or SipC from the cytomegalovirus promoter had no effect on *Salmonella* invasion (data not shown). Fusions of invasion proteins to a receptor were used, as ligand-binding and agonist-induced second-messenger studies could verify the expression of the acceptor portion of the fusion protein. The PAFR was chosen since 40 of 46 amino acids from the intracellular carboxyl terminus of this protein can be removed without diminishing receptor expression. Additionally, a constitutively active PAFR is generated when the receptor is truncated at this site in the intracellular carboxyl-terminal tail (55). Based on this signaling property of the PAFR, failure of invasion protein fusion at this site should result in a constitutively active PAFR. Since none of the PAFR-invasion protein fusions were constitutively active (data not shown), the intracellular carboxyl-terminal tail of the PAFR was exploited as a site for expressing heterologous peptide sequences from SipB and SipC. Therefore, using this membrane anchor approach to express SipB and SipC in eukaryotic cells, we could evaluate their intracellular effects on *Salmonella* invasion.

Since both SipB and SipC are larger than the PAFR, invasion protein domains were fused to avoid potential steric hindrances imposed by fusion of the full-length invasion proteins. Additionally, previous studies have revealed that protein domains can be useful experimental tools when fused to other proteins or when individually expressed (discussed in reference 8). Therefore, we prepared cDNAs encoding PAFR fusions to peptide sequences derived from the amino terminus of SipB (PAFR/BN), the carboxyl terminus of SipB (PAFR/BC), the amino terminus of SipC (PAFR/CN), and the carboxyl terminus of SipC (PAFR/CC) (Fig. 1). Specifically, DNAs encoding secreted invasion protein domains were cloned onto the 3′ end of the coding region of a PAFR cDNA plasmid. Plasmid DNA was transfected into BHK cells. BHK cells were chosen because they are sensitive to *Salmonella* invasion, possess no endogenous PAFRs, and, unlike PAFR-expressing cells, do not accumulate IP second messengers in response to PAF (6–9). To assess receptor expression, studies were undertaken to evaluate ligand binding and PAF-induced IP accumulation in BHK cell transfectants. Ligand binding of intact cells was determined by using the PAFR antagonist WEB 2086 (13). The evaluation of receptor signaling and expression revealed that the PAFR and all four PAFR-invasion protein chimeras were expressed at similar levels (data not shown).

Attenuation of *Salmonella* invasion by transfection of cDNAs encoding PAFR-invasion protein chimeras. The ability of *S. typhimurium* to invade was evaluated in BHK cells transfected with cDNAs encoding one of the four PAFR-invasion protein chimeras. As presented in Fig. 2A, BHK cells transfected with cDNA encoding PAFR/BC or PAFR/CN were significantly less susceptible to *Salmonella* invasion than were PAFR transfectants. The invasion of *Salmonella* was decreased by 88% ± 5% and 84% ± 6% in PAFR/BC and PAFR/CN transfectants, respectively. However, no significant difference in invasion was detected in BHK cells transfected with PAFR/BN or PAFR/CC cDNA. Additionally, no synergistic or enhancing effects on invasion were detected in BHK cells simultaneously transfected with two, three, or four of the chimeric cDNAs (data not shown). To assess whether the effect was specific to BHK cells, we performed similar transfection-based experiments with HEK293 and COS-7 cells. An analogous pattern of invasion inhibition was observed when HEK293 or COS-7 cells were transfected with PAFR/BC or PAFR/CN cDNA (data not shown). As depicted in Fig. 2B, the levels of inhibition mediated by PAFR/BC and PAFR/CN were dependent on the amount of cDNA introduced into cells, as transfection of 0.5, 0.75, and 1 μg of DNA/well significantly inhibited invasion (68% ± 8% to 83% ± 4% inhibition versus PAFR controls), whereas no inhibition was detected following transfection of 0.125 or 0.25 μg of DNA/well. The latter response is consistent with cotransfection results (not shown) in which no inhibition was detected in cells cotransfected with 0.25 μg of DNA of all four constructs per well.

Relationship between MOI and *Salmonella* invasion in BHK cells expressing PAFR/BC or PAFR/CN. We also assessed the relationship between the inhibition of invasion and the number of bacteria in the invasion assays, i.e., the multiplicity of infection (MOI). Despite exponential increases in MOI, BHK cells transfected with cDNAs encoding PAFR/BC or PAFR/CN were uniformly resistant to *Salmonella* invasion compared with BHK cells transfected with the PAFR cDNA (Fig. 3). The extent of inhibition did not vary with the MOI and ranged from 86% ± 6% (PAFR/CN, MOI of 10) to 91% ± 2% (PAFR/BC, MOI of 1).

Specificity of the PAFR/BC- or PAFR/CN-mediated inhibition of *Salmonella* invasion of BHK cell transfectants. To evaluate the specificity of the inhibition of *Salmonella* invasion in cells expressing PAFR/BC or PAFR/CN, we examined the

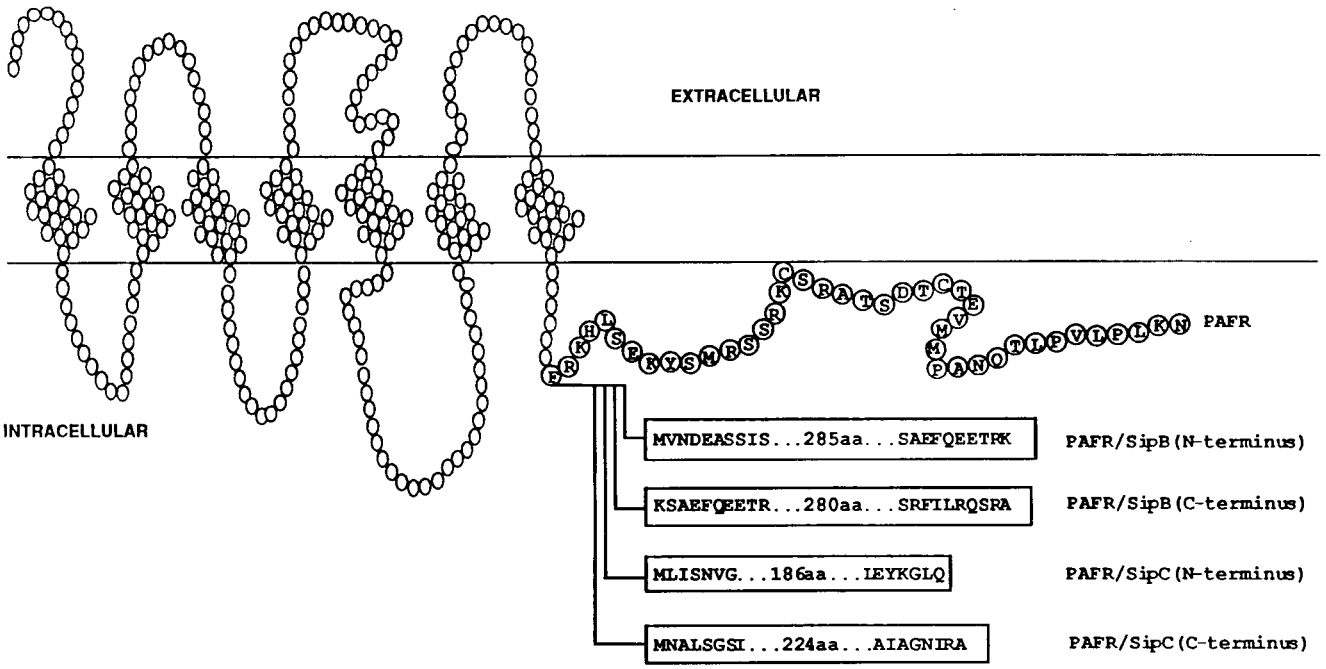

FIG. 1. Structures of PAFR-invasion protein chimeras. Open circles correspond to the PAFR backbone; blocks in the carboxyl-terminal tail contain letters corresponding to the amino- and carboxyl-terminal residues of the four invasion protein segments that were fused to the PAFR to generate the chimeric receptors. The introduction of invasion protein sequences following F301 resulted in a deletion of 40 amino acids (aa), represented by encircled letters, from the carboxyl-terminal tail of the PAFR.

effect of expressing these two hybrid receptors on the invasion of *Listeria* and EPEC and bacterial internalization mediated by the *Yersinia* invasin gene. Previous work has demonstrated that *Listeria* (22, 45), EPEC (5, 51), and bacteria expressing invasin (36, 37) invade by mechanisms different from that ascribed to *Salmonella*. The transient transfection system used for *Salmonella* invasion assays was employed to measure the effect of PAFR/BC or PAFR/CN on the invasion of these three other invasive organisms. Transfection of cDNAs encoding PAFR/BC and PAFR/CN, which inhibit *Salmonella* invasion 86% ± 7% and 87% ± 6%, respectively, of the level for the PAFR control, had no effect on bacterial entry mediated by *Listeria*, EPEC, or invasin (Fig. 4).

DISCUSSION

The data presented above indicate that cellular expression of portions of *Salmonella*-secreted invasion proteins results in specific inhibition of *Salmonella* invasion. This effect on invasion was dose dependent and specific for *Salmonella*. The significance of these results is underscored by other studies documenting an inhibition of *Salmonella* invasion. Many groups have inhibited *Salmonella* invasion by exposing tissue culture cells to the actin polymerization inhibitor cytochalasin D (15, 19, 25). Recent work has shown that *Salmonella* entry can be reduced by expressing a dominant negative CDC42 protein in COS-1 cells, thus implicating this molecule as a mediator of invasion (10).

The precise mechanisms underlying the inhibition of invasion presented in our study are unknown, although there are several possible explanations. First, the introduction of *Salmonella* invasion protein domains into tissue culture cells may activate host cell processes that generally prevent the uptake of particles. This does not appear likely since PAFR/BN and PAFR/CC did not affect invasion, although their inability to do so may be hindered by biophysical constraints imposed on these two proteins but not imposed on PAFR/BC and PAFR/CN. Our finding that uptake of three unrelated invasive bacteria was unaffected by the expression of PAFR/BC and PAFR/CN also argues against this explanation. Additionally, since transfected and untransfected cells appeared normal based on cytoskeletal staining experiments (not shown), PAFR/BC and PAFR/CN expression did not perturb normal actin rearrangement distribution.

A second explanation is that a region of the invasion protein portion of the hybrid receptor may be located extracellularly and sterically interfere with the ability of *Salmonella* to enter cells. Likewise, invasion protein sequences could traverse the membrane and function as a tethered antagonist for extracellular invasion protein receptors. This phenomenon is plausible since both SipB and SipC may possess putative membrane-spanning domains (42). However, increasing the MOI 100-fold had no effect on the inhibition of *Salmonella* entry whereas invasion was restored when transfection-mediated receptor expression was diminished by decreasing the dose of transfected cDNA. As the relationship between chimeric receptors and invasive bacteria does not directly correlate with the inhibition of invasion, it appears unlikely that the invasion protein sequences prevent invasion through a direct interaction with the bacteria or by binding to an extracellular invasion protein receptor.

A third explanation for the invasion-inhibiting activities of PAFR/BC and PAFR/CN is that the two invasion protein sequences interact with proteins capable of regulating the integrity of the cytoskeleton. That is, the invasion protein segments interact with the cellular target of the invasion proteins or with native invasion proteins translocated by the bacteria. This leads to a model in which the full-length invasion proteins activate a signal that induces cytoskeletal changes whereas the

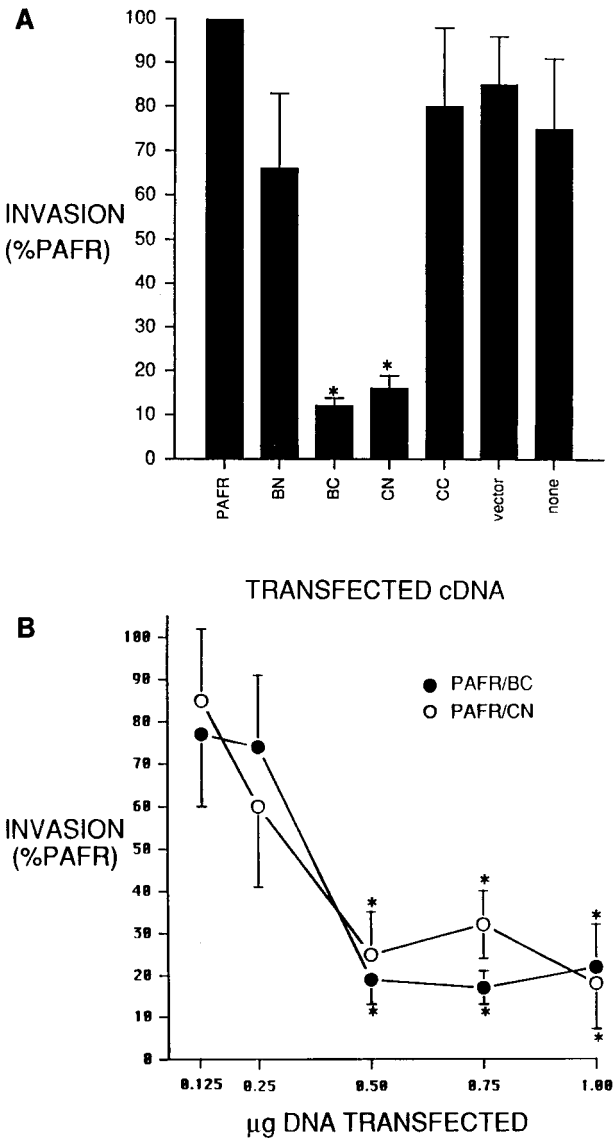

FIG. 2. Attenuation of *Salmonella* invasion by transfection of cDNAs encoding PAFR-invasion protein chimeras. (A) Effect of transfecting PAFR-invasion protein fusions on the invasion of *S. typhimurium* SL1344. BHK cells were transfected with insertless pCR3 vector (vector) or pCR3 containing a cDNA encoding the PAFR, PAFR/BN (BN), PAFR/BC (BC), PAFR/CN (CN), or PAFR/CC (CC). Invasion was also assessed in untransfected BHK cells (none). Cells were transfected, invasion assays were performed, and results were analyzed as described in Materials and Methods. Data represent the mean ± standard error of the mean from three separate transfections assayed simultaneously and repeated at least twice with similar results. Statistical significance was determined by analysis of variance with Scheffe's *F* test for multiple comparisons. *, $P < 0.05$ versus PAFR transfectants. (B) Effects of transfecting increasing amounts of cDNAs encoding PAFR/BC or PAFR/CN. Total plasmid DNA was maintained at 1 μg/well by the addition of insertless pCR3 vector. Cells were transfected, invasion assays were performed, and results were analyzed as described in Materials and Methods. Data presented are standardized based on invasion data obtained from cells transfected with an equal dose of the PAFR cDNA. Invasion data derived from PAFR transfectants was statistically indistinct at all transfection doses. Dose-response data represents the mean ± standard error of the mean from three separate transfections assayed simultaneously and repeated at least twice with similar results. Statistical significance was determined by Student's paired *t* test. *, $P < 0.05$ versus result from an equal dose of PAFR cDNA.

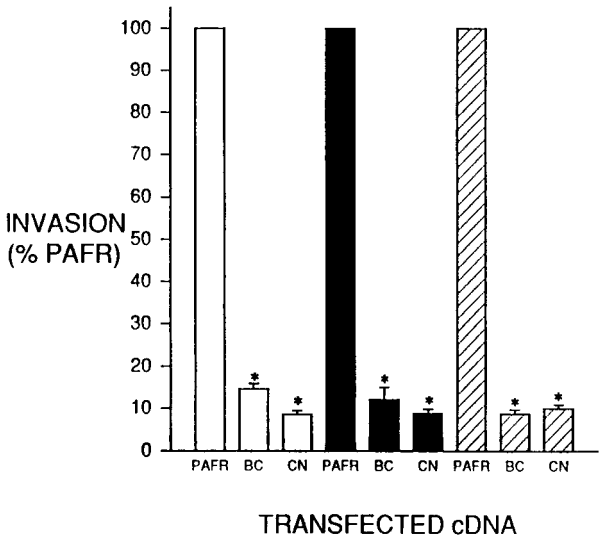

FIG. 3. Relationship between MOI and *Salmonella* invasion in BHK cells expressing PAFR/BC or PAFR/CN. Cells were transfected with 1 μg of plasmid DNA per well as described for Fig. 2A; invasion was assessed and analyzed as described for Fig. 2B. Bacteria were added at 5×10^5, 5×10^6, or 5×10^7/well to assess invasion at an MOI of 1 (open bars), 10 (black bars), or 100 (hatched bars), respectively. Data presented are standardized based on the percent invasion obtained from cells transfected with the PAFR cDNA and exposed to the same number of bacteria. Unstandardized invasion data derived from PAFR transfectants ranged from 7.4% ± 0.4% (MOI of 1) to 11.5% ± 0.6% (MOI of 100). Data represents the mean ± standard error of the mean from three separate transfections assayed simultaneously and repeated at least twice with similar results. Statistical significance among all responses was determined by analysis of variance with Scheffe's *F* test for multiple comparisons. *, $P < 0.05$ versus PAFR transfectants.

incomplete invasion proteins behave as weak partial agonists/competitive antagonists. This type of partial protein-based antagonism has been previously described with coexpression of a catecholamine receptor and peptides corresponding to its intracellular domains (43). Similarly, a peptide corresponding to the ligand-binding domain of the thyroid hormone receptor was able to compete with the thyroid hormone receptor for a transcriptional corepressor molecule (57). Extrapolation of these two competitive sequestration models to our results suggests that SipB and SipC interact with intracellular regulators of the cytoskeleton, consistent with studies demonstrating the translocation of these two proteins into the cell during bacterial invasion (11). Therefore, it is possible that the carboxyl terminus of SipB and the amino terminus of SipC retain binding sites for the proteins to which the full-length invasion proteins bind during the entry process. Alternatively, it is possible that the carboxyl terminus of SipB serves as a chaperone for SipC or that the amino terminus of SipC serves as a chaperone for SipB; that is, PAFR/BC sequesters wild-type secreted SipC or PAFR/CN sequesters wild-type secreted SipB. This latter possibility is supported by the notion that chaperone proteins involved in type III secretion can facilitate effector translocation (21) or prevent effector interactions with secretion apparatus proteins (59) by binding to secreted proteins. Additionally, the inhibitory chaperone phenomenon is supported by our finding that the fused invasion protein sequences do not appear to disturb cellular functions. In summary, while the exact mechanisms underlying the inhibition of invasion observed in PAFR/BC and PAFR/CN transfectants are unclear, our studies indicate that SipB and SipC serve as intracellular effectors of invasion.

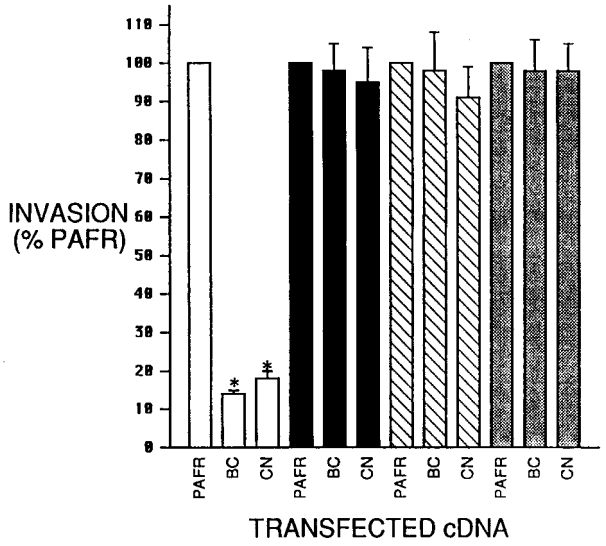

FIG. 4. Specificity of the PAFR/BC- or PAFR/CN-mediated inhibition of *Salmonella* invasion of BHK cell transfectants. Shown are effects of transfecting the PAFR/BC (BC) or PAFR/CN (CN) cDNA on the invasion of *Salmonella* (open bars), *Listeria* (filled bars), *E. coli* expressing invasin (hatched bars), and EPEC (gray bars). Cells were transfected by electroporation, and invasion assays were performed as described for Fig. 2B at an MOI of 10. Data presented are based on the percentage of bacterium-specific invasion observed in cells transfected with an equal dose of the PAFR cDNA. Data represents the mean ± standard error of the mean from three separate transfections assayed simultaneously and repeated at least twice with similar results. Statistical significance was determined by Student's paired *t* test. *, $P < 0.05$ versus PAFR transfectants.

The entrance of *Salmonella*, or any invasive organism, into eukaryotic cells represents a focus for studying and preventing the pathogenic effects of the bacteria (18). For *Salmonella*, SipB and SipC represent a direct line of communication between the bacteria and its target cell. In support of this premise is our finding that expression of portions of SipB and SipC at the cellular membrane significantly perturbs *Salmonella* invasion. This method of invasion inhibition may have implications for the pharmacotherapeutic prevention of *Salmonella* pathogenesis at the initial site of bacterium-host contact. Additionally, the fusion of *Salmonella* invasion proteins to a membrane-bound receptor may serve as a useful model for studying the molecular actions of other bacterial invasion proteins.

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