IpgB1 Is a Novel *Shigella* Effector Protein Involved in Bacterial Invasion of Host Cells

**ITS ACTIVITY TO PROMOTE MEMBRANE RUFFLING VIA RAC1 AND CDC42 ACTIVATION**

Received for publication, March 7, 2005, and in revised form, April 22, 2005
Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.M502509200

Kenji Ohya‡§, Yutaka Handa‡, Michinaga Ogawa‡, Masato Suzuki‡, and Chihiro Sasakawa‡

From the ‡Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan and §CREST, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

*Shigella*, the causative agent of bacillary dysentery, is capable of inducing the large scale membrane ruffling required for the bacterial invasion of host cells. *Shigella* secrete a subset of effectors via the type III secretion system (TTSS) into the host cells to induce membrane ruffling. Here, we show that IpgB1 is secreted via the TTSS into epithelial cells and plays a major role in producing membrane ruffles via stimulation of Rac1 and Cdc42 activities, thus promoting bacterial invasion of epithelial cells. The invasiveness of the *ipgB1* mutant was decreased to less than 50% of the wild-type level (100%) in a gentamicin protection or plaque forming assay. HeLa cells infected with the wild-type or a *ipgB1*-hyperproducing strain developed membrane ruffles, with the invasiveness and the scale of membrane ruffles being comparable with the level of *ipgB1* production in bacteria. Upon expression of EGFP-IpgB1 in HeLa cells, large membrane ruffles are extended, where the EGFP-IpgB1 was predominantly associated with the cytoplasmic membrane. The IpgB1-mediated formation of ruffles was significantly diminished by expressing Rac1 small interfering RNA and Cdc42 small interfering RNA or by treatment with GGTI-298, an inhibitor of the geranylgeranylation of Rho GTPases. When IpgB1 was expressed in host cells or wild-type *Shigella*-infected host cells, Rac1 and Cdc42 were activated. The results thus indicate that IpgB1 is a novel *Shigella* effector involved in bacterial invasion of epithelial cells via the activation of Rho GTPases.

*Shigella* are highly adapted human pathogens and are the cause of bacillary dysentery. The prominent pathogenic feature of *Shigella* is the ability to invade a variety of cells during infection of the intestinal mucosa, including enterocytes, macrophages, dendritic cells, and neutrophils. When *Shigella* reach the colon, they translocate through the epithelial barrier via the M cells that overlie solitary lymphoid nodules. Once they reach the underlying M cells, *Shigella* invade the resident macrophages, and the infecting bacteria escape from the phagosome into the cytoplasm. *Shigella* multiply in the macrophage cytoplasm and finally induce cell death (1, 2). *Shigella* released from the dead macrophages enter the surrounding enterocytes through their basolateral surface by inducing large scale membrane ruffling. As soon as a bacterium is surrounded by a membrane vacuole, it immediately disrupts the vacuole membrane and escapes into the cytoplasm. *Shigella* multiply in the cytoplasm and move about by inducing actin polymerization at one pole of the bacterium, by which the pathogen moves within the cytoplasm as well as into the adjacent cells (3). Thus, the ability of bacteria to enter the colonic epithelial cells is essential for colonization within the intestinal epithelium.

For bacterial invasion of epithelial cells, *Shigella* (and *Salmonella*) use a special mechanism called the “trigger mechanism of entry,” which is characterized by macropinocytic and phagocytic events that allow cells to trap several bacterial particles simultaneously (4). When *Shigella* comes into contact with epithelial cells, the type III secretion system (TTSS) is stimulated and delivers the effectors into the host cells and surrounding bacterial space (5). The secreted effectors are capable of modulating various host functions engaged in remodeling the surface architecture of the host cell and escape from the host innate defense systems (4, 6). Studies have indicated that several *Shigella* effectors, including IpaA, IpaB, IpaC, IpgD, and VirA, secreted via the TTSS are involved in stimulating the reorganization of F-actin and microtubule cytoskeletons that trigger the bacterial uptake by host cells. IpaB, for example, is capable of binding to the hyaluronic acid receptor CD44 (7). The IpaB-CD44 binding occurring within rafts (8, 9) is capable of stimulating the cell signaling involved in promoting *Shigella* invasion via the accumulation of c-Src and stimulation of the phosphorylation of cortactin and Crk with the aid of the Abl tyrosine kinases (10–13). IpaC is capable of leading to the activation of Cdc42 and Rac1, since IpaC can be integrated into the host cytoplasmic membrane, which somehow leads to the recruitment of cortactin and activation of c-Src, thus promoting local actin polymerization (3, 11, 14). VirA delivered from *Shigella* into the vicinity of the bacterial entry site induces local degradation of the microtubules (15), thus resulting in the release of microtubule-associated host pro-
IpgB1 Acts as Shigella Invasin by Stimulating Rac1 and Cdc42

Experimental Procedures

Bacterial Strains, Plasmids, and Recombinant Protein Preparation—The bacterial strains and plasmids used in this study are listed in Table 1.

Previous study indicated that IpgB1 is secreted from Shigella via the TTSS into the medium (21). Since the ipgB1 gene is located upstream of the ipaBCDA operon on the large plasmid of Shigella, we speculated that IpgB1 would act as the effector protein. In this study, we have attempted to characterize the role of IpgB1 in Shigella infection and found that IpgB1 plays a major role in promoting the bacterial invasion of epithelial cells. The examination of HeLa cells infected with the wild-type, the ipgB1 mutant, or IpgB1-hyperproducing strain suggested that the invasive capacity and the scale of membrane ruffling were comparable with the level of IpgB1 production in bacteria. Since the size and the frequency of membrane ruffles in host cells induced by IpgB1 expression were diminished when Rac1 and Cdc42 activities were inhibited, such as by treatment with GGTI-298 (an inhibitor of the geranylgeranylation of Rho GTPases) or by the knocking down of rac1 or cdc42 by siRNAs, it was likely that IpgB1 is a novel Shigella effector acting as the invasin required for promoting bacterial entry into epithelial cells.

Table 1

| Strain or plasmid | Characteristic | Reference/Source |
|-------------------|----------------|------------------|
| Strains | **S. flexneri YSH6000** | S. flexneri serotype2a wild type | Ref. 22 |
| **S. flexneri S325** | YSH6000 mexA::Tn5 | This study |
| **S. flexneri IpgB1** | ipgB1 in-frame deletion mutant of YSH6000 | Ref. 23 |
| **Escherichia coli MC1061** | Employed for DNA technology | Ref. 51 |
| **E. coli BL21** | Employed for GST fusion protein expression | Amersham Biosciences |
| Vectors | **pMW119-Tp** | Tp', low copy cloning vector | Ref. 24 |
| **pTB101-Tp** | Expression vector with pTac promoter control, lacP and Tp' gene inserted into p חוק233-3 | Ref. 25 |
| **pGEX-4T-1** | GST fusion protein expression vector | Amersham Biosciences |
| **pGEX-6P-1** | GST fusion protein expression vector | Amersham Biosciences |
| **pKD4** | Helper plasmid for red recombinase-mediated recombination | Ref. 26 |
| **pCP20** | Template for PCR amplification of kanamycin cassette for red recombinase-mediated recombination | Ref. 26 |
| **pEGFP-C1** | EGFP-fusion protein expression vector | Clontech |
| **pMX-pur** | Retroviral expression vector | Ref. 30 |
| Plasmids | **pIpgB1-Spa15** | ipgB1-spa15 fragment cloned into pMW119-Tp | This study |
| **pTB-IpgB1-Spa15** | ipgB1-spa15 fragment cloned into pTB101-Tp under lacZ promoter control | This study |
| **pTB-IpgB1-Myc-Spa15** | ipgB1-myc-spa15 fragment cloned into pTB101-Tp under pTac promoter control | This study |
| **pGST-Spa15** | Spa15 cDNA cloned into pEGFP-C1 | This study |
| **pGST-CRIB** | CRIB-(67–150) gene cloned into pEGEX-4T-1 | This study |
| **pEGFP-IpgB1** | ipgB1 cDNA cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(29–208)** | ipgB1-(29–208) gene cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(1–153)** | ipgB1-(1–153) gene cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(153–508)** | ipgB1-(153–508) gene cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(1–105)** | ipgB1-(1–105) gene cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(105–208)** | ipgB1-(105–208) gene cloned into pEGFP-C1 | This study |
| **pEGFP-IpgB1-(1–153)** | ipgB1-(1–153) gene cloned into pEGFP-C1 | This study |
| **pMX-Myc-Rac1** | N-terminal Myc-tagged rac1 cDNA cloned into pMX-puro | This study |
| **pMX-Myc-Cdc42** | N-terminal Myc-tagged cdc42 cDNA cloned into pMX-puro | This study |

Bacterial Strains, Plasmids, and Recombinant Protein Preparation—The bacterial strains and plasmids used in this study are listed in Table 1.
IpgB1 Acts as Shigella Invasive by Stimulating Rac1 and Cdc42

of the regions before and after the coding sequences of ipgB1 (underlined). The resulting PCR product was introduced by electroporation into YSH6000 carrying pKD46, and the transformants were grown on L-agar supplemented with 50 μg/ml kanamycin. The transformants were cured of pKD46 by growth at 37 °C, and the kanamycin resistance genes were then eliminated by using the helper plasmid pCP20, which encodes the FLP recombinase. The helper plasmid was subsequently cured by growth at 37 °C.

Antibodies and Reagents—Polyclonal rabbit (New Zealand White) anti-IpgB1 antibody and anti-Spa15 antibody were raised against IpgB1 and Spa15, respectively, by using recombinant fragments of IpgB1 (IKDNSNSGNLQFWMWSQERTTY) and recombinant Spa15 removed of GST by PreScission protease (Amersham Biosciences). The antisera obtained were purified using epoxy-activated Sepharose beads (Amersham Biosciences) coupled to the rabbit antibody (PE and GST-PE) and the resulting IPG-B1 and GST-PE blotted on nitrocellulose membrane (Schleicher & Schuell). Rabbit polyclonal anti-IpaA (27), anti-IpaB, anti-IpaC, anti-IpaD, anti-Shigella lipopolysaccharide (LPS) (28), and anti-OspC2/3 2 were as described elsewhere. Anti-Rac1 monoclonal antibody (Transduction Laboratories), anti-Cdc4c2 monoclonal antibody (BD Biosciences), anti-Myc monoclonal antibody 9B11 (Cell Signaling), and anti-panaxatin monoclonal antibody (Chemicon) were commercial products. Rhodaminephalloidin for F-actin staining was purchased from Molecular Probes, Inc. (Eugene, OR). The anti-mouse IgG-alkaline phosphatase and anti-rabbit IgG-horseradish peroxidase used as secondary antibodies for immunoblotting were purchased from Sigma. The anti-rabbit IgG-fluorescein isothiocyanate, anti-rabbit IgG-TRITC, anti-rabbit IgG-Cy5, mouse IgG-fluorescein isothiocyanate, and anti-mouse IgG-TRITC used as secondary antibodies for immunohistochemistry were purchased from Sigma.

Cell Lines—HeLa cells were cultured in Eagle’s minimal essential medium (Sigma) supplemented with 10% fetal calf serum (FCS; Sigma). Madin-Darby canine kidney (MDCK) cells and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s minimal essential medium (Sigma) supplemented with 10% FCS in the presence of 5% CO2 at 37 °C. The retrovirus packaging Plat-E cells (29) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 6% fetal bovine serum, 200 μg/ml of puromycin (Sigma), and 10% FCS in the presence of 5% CO2 at 37 °C. For construction of stably Myc-tagged NIH3T3 cells, NIH3T3 cells were infected with retrovirus vectors introducing Myc-Rac1 and Cdc42 expressing NIH3T3 cells, pMX-Myc-Rac1 or pMX-Myc-Cdc42 were introduced into the Plate-E cells to produce retrovirus vectors expressing Myc-Rac1 or Myc-Cdc42, and NIH3T3 cells were infected as described elsewhere (29, 30). The resulting NIH3T3 cells expressing Myc-tagged Rac1 and Cdc42 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 μg/ml puromycin and 10% FCS in the presence of 5% CO2 at 37 °C and named 3T3/Myc-Rac1 and 3T3/Myc-Cdc42, respectively.

Analysis of Whole Cell Lysate and Congo Red-treated Supernatant from S. flexneri Derivatives—Whole cell lysates and the Congo red (CR)-treated supernatants of S. flexneri were prepared as described previously (31). Briefly, bacterial cultures incubated overnight at 30 °C were diluted 1:50 in brain-heart infusion broth (Difco) and subcultured for 4 h at 37 °C. To prepare whole cell lysates, cultures were washed with ice-cold phosphate-buffered saline (PBS) and then precipitated with PBS containing 6% trichloroacetic acid on ice for 15 min. The resulting precipitates were used as whole cell lysates. To prepare the CR supernatants, bacterial cultures incubated overnight at 30 °C were diluted 1:50 in brain-heart infusion broth (Difco) and subcultured for 4 h at 37 °C. To prepare whole cell lysates, cultures were washed with ice-cold phosphate-buffered saline (PBS) and then precipitated with PBS containing 6% trichloroacetic acid on ice for 15 min. The resulting precipitates were used as whole cell lysates. To prepare the CR supernatants, bacterial cultures incubated overnight at 30 °C were diluted 1:50 in brain-heart infusion broth (Difco) and subcultured for 4 h at 37 °C. After incubation for 15 min, samples were pelleted down at 4 °C, and then the supernatants were filtered through a 0.45-μm pore size filter (Sartorius) and precipitated with PBS containing 6% trichloroacetic acid; the resulting precipitates were used as CR supernatants.

Whole cell lysates and CR supernatants from the same number of bacteria (grown overnight at 30 °C with 50 μg/ml kanamycin and 100 μg/ml gentamicin) were collected by using the insulin-agar overlay method. The lysates were incubated with antibodies specific for IpaA, IpaB, IpaC, IpaD, IpgB1, OspC2/3, and Spa15, respectively.

Injection of Infected Cells and Immunofluorescence Microscopy—Bacteria grown overnight at 30 °C were diluted 1:50 in brain-heart infusion broth and grown for 2 h at 37 °C. For the induction of IpgB1-Spa15 in WT/IpTB-IpgB1 Spa15 (Table 1), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at a final concentration of 0.2 mm. The bacteria were then incubated with a multiplicity of infection (MOI) of 10 to 300, and infection was initiated by centrifuging the plates at 700 × g for 10 min. After incubation for 15 min at 37 °C in the presence of 5% CO2, the cells were extensively washed with prewarmed PBS, fixed with 4% paraformaldehyde in PBS for 15 min, and stained with the antibodies or reagents indicated. The coverslips were mounted in Vectashield (Vector Laboratories). Confocal fluorescence microscopy (LSM510; Carl Zeiss). To inhibit protein geranylation, cells were pretreated at 37 °C for 40 h with 5 or 10 μM geranylgeranylation transferase inhibitor (GGT-298) (Calbiochem). To inhibit protein farnesylation, cells were pretreated at 37 °C for 40 h with 5 or 10 μM farnesyltransferase inhibitor (FTI-277) (Calbiochem). Quantification of Shigella-induced F-actin foci was performed as described by Bougnères et al. (13) with slight modification. The surface area of the F-actin foci around the sites of entry of bacteria was measured on the acquired Z-series images with confocal laser-scanning microscopy (LSM510 version 3.2 software (Carl Zeiss)). The data shown are the interquartile range and median values for more than 60 entry structures.

Virulence Assay—The mouse pulmonary infection of shigelloides was performed as described previously (32). Six-week-old female C57BL/6 mice (CLEA Japan) were housed for a week in the animal facility of the Institute of Medical Science, University of Tokyo, in accordance with guidelines drafted by the university. Mice (more than 10 mice for each Shigella strain) were inoculated intranasally with 5 × 106 colony-forming units of bacteria under anesthesia and monitored for survival until 10 days postinfection. The Shigella plaque-forming assay was performed as described previously (31). MDCK cells were seeded on 24-well plates and cultured for 5 days to a confluent monolayer. Before infection, cells were treated with Hanks’ balanced salt solution supplemented with 100 μM EGTA for 1 h and infected with the Shigella strains indicated for 2 h. After washing with PBS twice, Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 100 μg/ml gentamicin, and 60 μg/ml kanamycin were added, and the cells were cultured at 37 °C for 3 days in the presence of 5% CO2. The diameter of plaques was measured with a phase-contrast microscope equipped with a CCD camera using the IPLab spectrum software (Scanalytics). The epithelial cell invasiveness of Shigella was measured by the gentamicin protection assay (33). HeLa cells were inoculated with bacteria at an MOI of 300, and infection was initiated by centrifuging the plates at 700 × g for 10 min. After incubating the cells at 37 °C for 20 min in the presence of 5% CO2, the culture medium was replaced with the medium supplemented with 200 μg/ml of gentamicin, and incubated for an additional 20 min. After washing the cells with ice-cold PBS twice and lysing them in PBS supplemented with 0.5% Triton X-100, the intracellular bacteria were counted by plating serial dilutions of the lysates on L-agar plates and incubating at 37 °C overnight.

Intracellular Expression of EGFPIpgB1 Fusion Proteins and Small Interfering RNA—Cells were transfected with each peGFP-IpgB1 construct by using FuGENE6 transfection reagent (Roche Applied Science) for immunofluorescence analysis, or by using Lipofectamine 2000 (Invitrogen) to prepare cell lysates by following the manufacturer’s instructions. Duplex small interfering RNAs (siRNAs) for Rac1 (RAC1 Validated Stealth RNAi) and Cdc42 (CDC42 Validated Stealth RNAi) were purchased from Invitrogen. Duplex siRNAs were transfected into HeLa cells by using Lipofectamine 2000 according to the manufacturer’s instructions 24 h before peGFP-IpgB1 transfection. Stealth RNAi negative control duplexes (Invitrogen) whose GC content is similar to that of each duplex siRNA were used as negative controls. The transfection efficiency of each duplex siRNA (~90%) was confirmed by using Block-iT Fluorescent Oligo (Invitrogen) according to the manufacturer’s instructions.

Cdc42 and Rac1 Activation Assay—The GST-CRIB binding assay to assess Cdc42 and Rac1 activation was essentially performed as described elsewhere (10). Serum-starved cells transfected with the plasmids indicated for 20 h or infected with the Shigella strains indicated for an MOI of 300 for 15 min were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1% Nonidet P-40, and 5% glycerol supplemented with the Complete, EDTA-free protease inhibitor mixture (Roche Applied Science)). Equal amounts of the lysates were incubated with 15 μg of GST-CRIB bound to the glutathione-Sepharose 4B beads for 60 min at 4 °C, and the beads were washed four times with the lysis buffer. To provide a positive control, cells were treated with 100 ng/ml platelet-derived growth factor (Peprotech) for 5 min at 37 °C. The samples were separated by SDS-PAGE and immunoblotted with anti-Myc antibody.

2 R. Akakura, unpublished material.
**RESULTS**

*IpgB1 Is Secreted via the TTSS—* WT (a wild-type YSH6000), Δ*ipgB1* (nonpolar *ipgB1* mutant), and S325 (a TTSS-deficient mutant) were tested for their ability to secrete IpgB1 into PBS containing 0.003% Congo red (CR supernatant), a conditioned medium for stimulating the TTSS activity (34). As shown in Fig. 1B, IpgB1 was secreted from WT, but not from Δ*ipgB1* or S325 into the CR supernatant by 15 min after the addition of CR into PBS. Under these conditions, IpaB, IpaC, and IpaD were secreted from WT and Δ*ipgB1*, but not from S325 into the CR supernatant. Since Spa15 acts as the chaperone for IpgB1 (35) and *ipgB1* alone could not restore the IpgB1 secretion completely, p*IpgB1*-Spa15 was constructed. IpgB1 secretion was restored with Δ*ipgB1*IpaB1-Spa15, confirming that IpgB1 was secreted from *Shigella* via the TTSS. The level of IpgB1 secretion from WT/pTB-IpgB1-Spa15 (pTB-IpgB1-Spa15 is an IPTG-inducible IpgB1-Spa15 plasmid) was greatly increased when IpgB1 production was induced by adding IPTG at 0.2 mM. Under the conditions, IpgB1 secretion from WT, but not from S325 or S325/pTB-IpgB1-Spa15, was detected in a similar manner to Ipa secretion. Note that addition of IPTG had no effect on the level of IpaA, OspC2, or OspC3, which are also known to be chaperoned by Spa15 (35) (Fig. S1). These results clearly indicated that IpgB1 is a protein secreted via the TTSS.

*Shigella Delivers IpgB1 into Host Cells—* The visualization of IpgB1 secreted from YSH6000 into HeLa cells was difficult using the confocal immunofluorescence microscopy with anti-IpgB1 antibody. Therefore, pTB-IpgB1-Myc-Spa15 was constructed and introduced into WT (WT/pTB-IpgB1-Myc-Spa15) to allow us to visualize IpgB1-Myc secreted from invading bacteria. The immunoblotting with anti-Myc antibody revealed that IpgB1-Myc was produced in WT/pTB-IpgB1-Myc-Spa15 or S325/pTB-IpgB1-Myc-Spa15, although the secretion of the protein into the CR supernatants was only detected from WT/pTB-IpgB1-Myc-Spa15 (Fig. 2A), confirming the fusion protein can be secreted from the bacteria via the TTSS. HeLa cells were subsequently infected for 15 min with bacteria grown in medium containing 0.2 mM IPTG and then examined by immunofluorescence microscopy by staining with anti-LPS antibody (blue in Fig. 2B), anti-Myc antibody (green in Fig. 2B), and rhodamine-phalloidin (red in Fig. 2B). After infecting the HeLa cells with WT/pTB-IpgB1-Myc-Spa15 for 15 min, IpgB1-Myc signals were detected around the invading bacteria, in which the IpgB1-Myc signals were also co-localized with *Shigella*-induced membrane ruffles (see the green signals). Under the conditions, Myc signal alone (used as the negative control for anti-Myc antibody) was not detected in HeLa cells infected with WT, indicating that the observed IpgB1-Myc signal was not a nonspecific reaction of anti-Myc antibody. Thus, the results indicated that IpgB1 is secreted from *Shigella* during entry into host cells and that the secreted IpgB1 is associated with the *Shigella*-induced membrane ruffles protruding from the cell periphery.

*IpgB1 Activity Is Involved in Promoting Shigella Invasiveness—* To establish the status of IpgB1 as a *Shigella* virulence-
associated protein, C57BL/6 mice were intranasally infected with $5 \times 10^6$ colony-forming units of $\Delta_{ipgB1}$, $\Delta_{ipgB1}/p_{ipgB1}$-Spa15, WT (positive control), or S325 (negative control) (Fig. 3A). At that dose, 100% of the mice were killed by WT by day 4, but none was killed by S325 by day 10. Under these conditions, 40% of the mice infected with the $\Delta_{ipgB1}$ mutant survived by day 10, whereas 100% of the mice were killed by $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 by day 8 (Fig. 3A), clearly demonstrating that IpgB1 contributes to the pathogenesis. To determine which of the steps in Shigella infection requires the IpgB1 activity, polarized MDCK cell monolayers were basolaterally infected with WT, $\Delta_{ipgB1}$, or $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 (see “Experimental Procedures”), and the number (representing the bacterial invasiveness) or diameter (representing the bacterial ability to multiply and intra- and intercellular spreading) of the plaques that had developed by day 3 were compared (Fig. 3B). The number of plaques produced by $\Delta_{ipgB1}$ was 11.2 ± 5.0% (WT - 100%), whereas the number produced by $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 was 84 ± 8.8%, which was greater than that produced by $\Delta_{ipgB1}$ (Fig. 3C), suggesting that the absence of IpgB1 function resulted in a decrease in bacterial invasiveness. The plaques that had developed 3 days after infection with WT or $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 were over 0.6 mm in diameter and were clear. The diameter of the plaques produced by $\Delta_{ipgB1}$ was less than 0.2 mm, and the plaques were turbid (Fig. 3, B and D) (see “Discussion”). To ensure the above notion, we compared the invasive capacity of WT with that of $\Delta_{ipgB1}$ or $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 using the gentamicin protection assay. As shown in Fig. 3E, the invasive capacity of $\Delta_{ipgB1}$ decreased by ~50% relative to that of WT (100%), whereas the wild-type level of invasiveness was restored in $\Delta_{ipgB1}/p_{ipgB1}$-Spa15. To further investigate the impact of IpgB1 activity on bacterial entry, HeLa cells were infected with WT/pTB-IpgB1-Spa15 or WT grown in medium with 0.2 mM IPTG, and the invasive capacity of WT/pTB-IpgB1-Spa15 was compared with that of WT. As shown in Fig. 3E, the invasive capacity of WT/pTB-IpgB1-Spa15 in the gentamicin protection assay was dramatically increased up to 30-fold the WT level. Although the bacterial invasiveness varied among assay conditions, the results of the series of experiments further corroborate that the IpgB1 activity contributes to enhancement of Shigella invasive efficiency.

IpgB1 Activity in Host Cells Contributes to the Formation of Membrane Ruffles—The invasive capacity of bacteria in the gentamicin protection assay was dramatically increased when HeLa cells were infected with the IpgB1-hyperproducing strain (WT/pTB-IpgB1-Spa15). Hence, WT, $\Delta_{ipgB1}$, $\Delta_{ipgB1}/p_{ipgB1}$-Spa15 (the $ipgB1$-spa15 was cloned in a low copy vector, pMW19-Tp), or WT/pTB-IpgB1-Spa15 (the $ipgB1$-spa15 was IPTG-inducible by cloning in a high copy vector, pTB101-Tp) was investigated for the ability to induce membrane ruffling in HeLa cells infected for 15 min with each of the bacteria grown in the medium in the presence of 0.2 mM IPTG, and the invasive capacity of WT/pTB-IpgB1-Spa15 was dramatically increased up to 30-fold the WT level. Although the bacterial invasiveness varied among assay conditions, the results of the series of experiments further corroborate that the IpgB1 activity contributes to enhancement of Shigella invasive efficiency.

To directly demonstrate the IpgB1 activity in epithelial cells to induce the formation of membrane ruffles, HeLa cells transfected with pEGFP-IpgB1 enhanced green fluorescent protein (EGFP) fused with the N terminus of IpgB1; Fig. 5A) for 20 h were examined with an immunofluorescence microscope. Al-
though the expression of EGFP alone in HeLa cells did not result in the formation of ruffles, cells expressing EGFP-IpgB1 protruded membrane ruffles along their periphery, where the EGFP signals were confined to the extending membrane periphery (Fig. 5B and Supplemental Movie 1). To identify the IpgB1 domain responsible for inducing membrane ruffling, we created EGFP fusions with various truncated forms of IpgB1 (EGFP-IpgB1-(29–208), EGFP-IpgB1-(1–153), EGFP-IpgB1-(53–208), EGFP-IpgB1-(1–105), EGFP-IpgB1-(105–208), and EGFP-IpgB1-(53–153)) and investigated the percentage of each EGFP-positive cells that formed membrane ruffles. The results showed that the percentage of transfectants expressing EGFP-IpgB1-(29–208) (Fig. 5B and Supplemental Movie 1) was 94%, and the percentage of EGFP-IpgB1-(53–208) that formed ruffles was 75%. Since the other truncated EGFP-IpgB1 versions barely induced membrane ruffling, we concluded that almost the entire IpgB1 molecule, except residues 1–29, is involved in the formation of membrane ruffles (Fig. 5A and B).

IpgB1-induced Membrane Ruffling Is Dependent on Rac1 and Cdc42 Activities—Since Shigella-induced ruffle formation depends on Rac1 and Cdc42 activities (36), HeLa cells were transfected with Rac1 siRNA, Cdc42 siRNA, or a mixture of the two, and the effect of the knocking down of rac1, cdc42, or rac1/cdc42 expression on the level of Rac1 or Cdc42 production was checked (Fig. 6A). Total cell lysates from the transfectants with nonsilencing control siRNA (RNAi cont), Rac1, and/or Cdc42 siRNA (Rac1 RNAi, Cdc42 RNAi, and Rac1/Cdc42 RNAi) were analyzed by the immunoblotting with anti-Rac1, anti-Cdc42, and anti-panactin antibodies. As seen in Fig. 6A, the levels of Rac1 and Cdc42 were significantly decreased in HeLa cells transfected with Rac1 siRNA and/or Cdc42 siRNA, respectively. Therefore, we investigated HeLa cells expressing EGFP-IpgB1 for the effect of any of the RNAis (RNAi cont, Rac1 RNAi, Cdc42 RNAi, and Rac1/Cdc42 RNAi) on the EGFP-IpgB1-mediated membrane ruffling. After the transfection of HeLa cells containing control siRNA with pEGFP-IpgB1, the percentage of ruffle-positive transfectants was similar to the percentage of HeLa cells expressing EGFP-IpgB1 alone (100%), whereas the percentage of ruffle-positive transfectants containing Rac1 siRNA and Cdc42 siRNA was only 20 and 55%, respectively (Fig. 6B). Note that the transfectants containing Cdc42 siRNA showed a moderate reduction in the percentage of ruffle-positive transfectants as compared with the transfectants containing Rac1 siRNA; however, the size of the membrane ruffles was significantly smaller than that of the transfectants containing control siRNA (Fig. 6B). Furthermore, the percentage of ruffle-positive transfectants containing Rac1/Cdc42 siRNAs was dramatically reduced, and membrane ruffles were barely detectable (Fig. 6B).
Membrane Ruffling Induced by IpgB1 Is Inhibited by a Geranylgeranyltransferase Inhibitor—The above results indicated that induction of membrane ruffling by IpgB1 in HeLa cells is strongly dependent on Rac1 and Cdc42 activities. To confirm this, HeLa cells were treated with GGTI-298, an inhibitor of the geranylgeranylation of Rho GTPases (37, 38), for 40 h, and after being infected with WT or WT/pTB-IpgB1-Spa15 for 15 min, they were investigated for the effect of GGTI-298 on the formation of membrane ruffles by immunofluorescence microscopy with anti-Shigella LPS antibody or rhodamine-phalloidin. As a control, HeLa cells treated with FTI-277, an inhibitor of farnesylation of the Ras family (37, 38), for 40 h were infected with WT or WT/pTB-IpgB1-Spa15 for 15 min. As shown in Fig. 7, although FTI-277 had no effect on HeLa cell ruffle formation by Shigella infection at all, GGTI-298 inhibited the formation of membrane ruffles. The size of the membrane ruffles induced by WT or WT/pTB-IpgB1-Spa15 was greatly diminished by GGTI-298 in a dose-dependent manner, but not by FTI-277 (Fig. 7B), indicating that the formation of IpgB1-induced membrane ruffles is dependent on the geranylgeranylation of Rho family GTPases, such as Rac1 and Cdc42, required for the association with the cytoplasmic membrane.

IpgB1 Leads to Activation of Rac1 and Cdc42—To further confirm the ability of IpgB1 to stimulate Rac1 and Cdc42 activities, NIH3T3 cells expressing Myc-Rac1 (3T3/Myc-Rac1) or Myc-Cdc42 (3T3/Myc-Cdc42) were investigated by immunoblotting with anti-Rac1, anti-Cdc42, and anti-Myc antibodies (Fig. 8A), thus facilitating detection of the activated Myc-Rac1 and Myc-Cdc42 in the GST-CRIB pull-down assay (see “Experimental Procedures”) of platelet-derived growth factor-treated cells (Fig. 8, B and C). As shown in Fig. 8, B and C, expression of EGFP-IpgB1 was associated with higher levels of activated Rac1 and Cdc42 than the expression of EGFP alone. To confirm this in vivo, the 3T3/Myc-Rac1 cells were infected with WT, ΔipgB1, or WT/pTB-IpgB1-Spa15 for 15 min, and activated Rac1 was measured by immunoblotting with anti-Myc antibody. The results showed that the level of activated Rac1 in the WT-infected cells was higher than that of mock control or ΔipgB1-infected cells and that it was even higher after infection with WT/pTB-IpgB1-Spa15 (Fig. 8D). The same was true for Cdc42, although the levels of activated Cdc42 after WT/pTB-IpgB1-Spa15 infection and WT infection were similar to each other (Fig. 8E, see “Discussion”), suggesting that IpgB1 has the ability to stimulate Rac1 and Cdc42 activities in host cells.

DISCUSSION

In this study, we have identified IpgB1 protein as an effector secreted from Shigella via the TTSS, which acts to promote bacterial entry into host cells. We also found that IpgB1 expression in mammalian cells is able to induce membrane ruffling via the stimulation of Rac1 and Cdc42 activities. Our conclusions were based on the following results: (i) the ipgB1 mutant partially but significantly attenuated pulmonary infec-
IpgB1 Acts as Shigella Invasin by Stimulating Rac1 and Cdc42

FIG. 5. EGFP-fused IpgB1 proteins induce membrane ruffling in HeLa cells. A, construction of a series of truncated IpgB1 molecules fused to the C terminus of EGFP in pEGFP-C1. The right column shows the ability to induce membrane ruffling by each construct observed in B. B, representative images of pEGFP-IpgB1-transfected HeLa cells. HeLa cells were transfected with each of the pEGFP-IpgB1 constructs for 20 h. The left columns are F-actin images (red), the middle columns are EGFP images (green), and the right columns are merged double fluorescence images. Each value represents the percentage of transfected HeLa cells showing marked changes in cell shape with membrane ruffles relative to that of pEGFP-IpgB1-transfected cells (100%) (right side, numbers). Scale bar, 10 µm.

tion in the murine model; (ii) when the MDCK cell monolayer was infected with the ipgB1 mutant, the number of plaques was significantly lower than that with the wild type; (iii) the invasive efficiency or the scale of membrane ruffles in HeLa cells infected with Shigella was comparable with the level of IpgB1 production in bacteria; (iv) ectopic expression of IpgB1 in HeLa cells induced the formation of large membrane ruffles; (v) the IpgB1-mediated formation of membrane ruffles in host cells
Fig. 6. *IpgB1*-induced membrane ruffling is dependent on Rac1 and Cdc42 activity. A, Rac1 and Cdc42 siRNAs reduced expression of each protein. Total cell lysates from HeLa cells transfected with nonsilencing control siRNA (RNAi cont), Rac1, and/or Cdc42 siRNAs (Rac1 RNAi, Cdc42 RNAi, and Rac1/Cdc42 RNAi) were analyzed by immunoblotting with anti-Rac1, anti-Cdc42, and anti-panactin antibodies. B, the
IpgB1 Acts as Shigella Invasin by Stimulating Rac1 and Cdc42

This bacterial activity is gained through the interaction of several effectors such as IpaA, IpaB, IpaC, and VirA with their host target molecules (see Introduction). IpgB1 is shown to be rapidly secreted from Shigella via the TTSS. Indeed, under in vitro conditions for stimulating the TTSS such as by adding Congo red to the medium, IpgB1 secretion from Shigella can be detected only 15 min after the addition of Congo red into the medium (this study). When we visualized the IpgB1-Myc protein secreted from Shigella into HeLa cells using immunofluorescence microscopy, we observed that the IpgB1 signal within the cells was localized around the cytoplasmic membrane as well as within the membrane ruffles. Furthermore, when HeLa cells were infected with the IpgB1-hyperproducing Shigella (WT/pTB-IpgB1-Spa15), the invasive efficiency was dramatically increased by ~30-fold compared with the wild-type level (see Fig. 3E). Consistently, the size of membrane ruffles extending from HeLa cells infected with the IpgB1-hyperproducing Shigella was also more than 2 times larger than that with wild type. Thus, the results suggest that a functional IpgB1 is required at an early stage of Shigella infection, where the IpgB1 activity engages in promoting entry into host cells.

The diameter of the plaques that had developed 3 days after infection with the ipgB1 mutant was significantly smaller (one-third the diameter of that with wild type) than that developed after infection with wild-type or ipgB1 complement strain (see Fig. 3B), suggesting that IpgB1 function may also be involved in a later step in Shigella infection such as the cell-cell dissemination step. Dissemination of Shigella through a cell monolayer involves at least four distinctive steps: the actin-based movement of intracellular bacteria, formation of membrane protrusions, engulfment of the protrusions by adjacent cells, and lysis of the two cellular membranes that surround the bacterial protrusion (39). Since the ipgB1 mutant showed no defects in bacterial motility, the formation of membrane protrusions, or the ability to disrupt the host plasma membrane,3 we speculate that IpgB1 also takes part in the uptake of bacteria by adjacent cells or in some undefined function required for bacterial cell-cell spreading.

Since HeLa cells ectopically expressing EGFP-IpgB1 formed large membrane ruffles and the EGFP-IpgB1 signal was predominantly associated with the membrane ruffles including the cell periphery, we investigated various truncated versions of IpgB1 fused with EGFP for their membrane association. The results showed that although EGFP-IpgB1-(53–208) was still able to induce membrane ruffling, the degree of association was significantly less than that with EGFP-IpgB1 (full-length IpgB1) or EGFP-IpgB1-(29–208), implying that the N-terminal 52 amino acids may be involved in the association (see Fig. 5B). The other truncated IpgB1 versions (i.e., those lacking the N-terminal 104 amino acids (EGFP-IpgB1-(1–153)), or both terminal portions (EGFP-IpgB1-(53–153)) were yet unable to induce membrane ruffling in HeLa cells. Therefore, the entire IpgB1 sequence, except amino acid residues 1–29 perhaps required as the TTSS translocating signal (40), appears to be required to induce membrane ruffling. Since the amino acid sequence of IpgB1 contains neither a predictable transmembrane domain nor significant similarity to other known proteins in the database, the membrane association of IpgB1 may be achieved through the interaction with some host protein(s).

EGFP-IpgB1-induced membrane ruffling was inhibited by reduced expression of Rac1 and Cdc42. HeLa cells transfected with control, Rac1, Cdc42, and Rac1/Cdc42 siRNAs were subsequently transfected with pEGFP-IpgB1 for 20 h. Representative EGFP images (green), F-actin images (red), and merged images of the transfected cells are shown. The percentage of EGFP-IpgB1-positive cells transfected with the siRNAs indicated that showed marked changes in cell shape with membrane ruffles is shown (graph). Data are means ± S.D. of the results of three independent experiments. Scale bar, 10 μm.
The IpgB1-mediated membrane ruffling was strongly inhibited in HeLa cells treated with GGTI-298, an inhibitor of geranylgeranylation of Rho GTPases (see Fig. 7B), suggesting that the IpgB1-mediated formation of membrane ruffles is dependent on the activity of Rho GTPases such as Rac1 and Cdc42. A similar inhibitory effect of GGTI-298 on *Salmonella* entry into host cells was reported (41, 42). Note that there is another mechanism to lead to formation of membrane ruffles in a Rho GTPase-independent manner (43, 44). For example, p21-activated kinase 1 directly interacts with filamin FLNa, and the p21-activated kinase 1-FLNa interaction is involved in inducing ruffle formation independently of Rho GTPases (44). Indeed, some low level of IpgB1-mediated ruffle formation was still detected upon treatment of HeLa cells with GGTI-298 at 5 μM (Fig. 7B), suggesting that a Rho GTPase-independent ruffle formation may also be partly involved in *Shigella* invasion. Since the formation of large membrane ruffles induced by expression of EGFP-IpgB1 in HeLa cells was greatly diminished by introducing the Rac1 or Cdc42 siRNAs into the cells, the IpgB1-mediated ruffle formation appears to mostly be dependent on the Rac1 and Cdc42 activities. Indeed, when IpgB1 was expressed in NIH3T3 cells or the cells were infected with *Shigella*...
IpgB1 acts as Shigella invasin by stimulating Rac1 and Cdc42

...the levels of activated Rac1 and Cdc42 were significantly increased according to the results of the CRIB-binding assay. The levels of activated Rac1 and Cdc42 in NIH3T3 cells were significantly increased after infection with wild-type Shigella for 15 min in comparison with that with ipgB1 mutant or the uninfected control, which also agreed with the previous study (10). It is noteworthy that the enhancement of the IpgB1-dependent Rac1 activation was greater than that of Cdc42 (see Fig. 8). Furthermore, the inhibitory effect of Rac1 siRNA on IpgB1-mediated ruffle formation was more striking than that of the Cdc42 siRNA (see Fig. 6). Although the mechanism underlying the activation of the Rho GTPases by IpgB1 remains to be elucidated, our results suggest that IpgB1 is capable of mediating stimulation of both Rac1 and Cdc42 activities, in which IpgB1 appears to act on Rac1 more efficiently than Cdc42. 

SopE2) effectors (possessing GEF activity) secreted via the bacterial entry, because the sizes of the membrane ruffles and bacterial invasion were greatly enhanced as the level of IpgB1 expression increased in epithelial cells and in bacteria, respectively. Of importance, the IpgB1-mediated membrane ruffles in HeLa cells were apparently larger than those induced by IpaC and VirA expression (14, 15). Since none of the above host proteins has so far been found to directly interact with IpgB1 as examined by a yeast two-hybrid or GST pull-down assay, we speculate that IpgB1 may stimulate Rac1 and Cdc42 by interacting with some host protein(s) associated with the host membrane. In any event, identification of the host protein(s) that interacts with IpgB1 will be necessary to understand the precise role of IpgB1 in Shigella invasion of host cells.

Acknowledgments—We thank S. Yoshida, H. Mimuro, T. Toyotome, H. Iwai, Y. Yoshikawa, and all other members of the Sasakawa laboratory for critical reading of the manuscript, technical advice, and help. We are also grateful to T. Suzuki for valuable discussion throughout the study, T. Kitamura for providing pMX vector and Plat-E cells, G. Tran Van Nhieu for providing IpaA antibody, and S. Imao-Ohmi for helpful advice.

REFERENCES

1. Zychlinsky, A., Prevost, M. C., and Sansonetti, P. J. (1992) Nature 358, 167–169
2. Suzuki, T., Nakanishi, K., Tsutsui, H., Iwai, H., Akira, S., Inohara, N., Chamaillard, M., Nunez, G., and Sasakawa, C. (2005) J. Biol. Chem. 280, 14042–14050
3. Cossart, P., and Sansonetti, P. J. (2004) Science 304, 242–248
4. Sasakawa, P. (2004) in Bacterial Invasion of Host Cells (Lamont, R., ed) Vol. 5, pp. 25–57, Cambridge University Press, Cambridge, UK
5. Blocker, A., Komoriya, K., and Aizawa, S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3027–3030
6. Ogawa, M., Yoshimori, T., Suzuki, T., Sagara, H., Mizushima, N., and Sasakawa, C. (2005) Science 307, 727–731
7. Skowron, A., Mounier, J., Ohayon, H., Gounon, P., Sansonetti, P., and Tran Van Nhieu, G. (2000) Cell Microbiol. 2, 19–33
8. van der Goot, F. G., Tran Van Nhieu, G., Aliooua, A., Sansonetti, P., and Payrastre, B. (2004) J. Biol. Chem. 279, 4779–4789
9. Lafont, F., Tran Van Nhieu, G., Hanada, K., Sansonetti, P., and van der Goot, F. G. (2002) EMBO J. 21, 4449–4457
10. Burton, E. A., Plattner, R., and Pendergast, A. M. (2003) EMBO J. 22, 5471–5479
11. Tran Van Nhieu, G., Enninga, J., Sansonetti, P., and Grompone, G. (2005) Curr. Opin. Microbiol. 8, 16–20
12. Dumont, G., Sansonetti, P., and Tran Van Nhieu, G. (2000) J. Cell Sci. 113, 71–80
13. Bougères, L., Girardin, S. E., Weed, S. A., Kargavin, A. V., Olivo-Marin, J. C., Parsons, J. T., Sansonetti, P. J., and Tran Van Nhieu, G. (2004) J. Cell. Biol. 166, 225–235
14. Tran Van Nhieu, G., Caron, E., Hall, A., and Sansonetti, P. J. (1999) EMBO J. 18, 3249–3262
15. Yoshida, S., Katayama, E., Kuwae, A., Mimuro, H., Suzuki, T., and Sasakawa, C. (2002) EMBO J. 21, 2923–2935
16. Yoshida, S., and Sasakawa, C. (2003) Trends Microbiol. 11, 139–143
17. Matsuzawa, T., Kuwae, A., Yoshida, S., Sasakawa, C., and Abe, A. (2004) EMBO J. 23, 3570–3582
18. Kendel, M., Zenke, F. T., and Bokoch, G. M. (2002) Nat. Cell Biol. 4, 294–301
19. Niebuhr, K., Giuriato, S., Pedron, T., Philpott, D. J., Gates, F. S., Sable, J., Shecht, M. P., Parsot, C., Sansonetti, P. J., and Payrastre, B. (2005) EMBO J. 24, 5069–5078
20. Bourdôt-Sicard, R., Rüdiger, M., Jockusch, B. M., Gounon, P., Sansonetti, P. J., and Tran Van Nhieu, G. (1999) Mol. Microbiol. 36, 760–771
21. Sasakawa, C., Kamata, K., Sakai, T., Murayama, S. Y., Makino, S., and Yoshikawa, M. (1986) Infect. Immun. 51, 473–475
22. Sasakawa, C., Makino, S., Kamata, K., and Yoshikawa, M. (1986) Infect. Immun. 54, 32–36
23. Durand, J. M., Okada, N., Tobe, T., Watarai, M., Fukuda, I., Suzuki, T., Nakata, N., Komatsu, K., Yoshikawa, M., and Sasakawa, C. (1994) J. Bacteriol. 176, 4627–4634
24. Tobe, T., Nagai, S., Okada, N., Adler, B., Yoshikawa, M., and Sasakawa, C. (1991) Mol. Microbiol. 5, 887–893
25. Datsenko, K. A., and Wanner, B. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645
26. Tran Van Nhieu, G., Ben-Zeev, A., and Sansonetti, P. J. (1997) EMBO J. 16, 2717–2729
27. Watarai, M., Tobe, T., Yoshikawa, M., and Sasakawa, C. (1995) EMBO J. 14, 2461–2470
28. Morita, S., Kojima, T., and Kitamura, T. (2000) Gene Ther. 7, 1063–1066
29. Kitamura, T. (1998) Int. J. Hematol. 67, 351–359
30. Ogawa, M., Suzuki, T., Tatsuoka, I., Abe, H., and Sasakawa, C. (2003) Mol. Microbiol. 48, 913–931
31. Phalipon, A., Kaufmann, M., Michetti, P., Cavaillon, J. M., Huerre, M., Sansonetti, P., and Krahenbuhl, P. J. (1995) J. Exp. Med. 182, 769–778
32. Silver, P. L., Isberg, R. R., and Falkow, S. (1987) Infect. Immun. 55, 1674–1679
33. Parsot, C., Ménard, R., Gounon, P., and Sansonetti, P. J. (1995) Mol. Microbiol. 16, 291–300
34. Page, A. L., Sansonetti, P., and Parsot, C. (2002) Mol. Microbiol. 43, 1533–1542
35. Mounier, J., Laurent, V. H., Hall, A., Fort, P., Carlier, M. F., Sansonetti, P. J., and Egile, C. (1999) J. Cell. Sci. 112, 2069–2080
36. Casey, P. J., and Seabra, M. C. (1996) J. Biol. Chem. 271, 5289–5292
37. Vogt, A., Qin, Y., McGuire, T. P., Hamilton, A. D., and Sehbi, S. M. (1996) Oncogene 15, 1991–1999

4 K. Ohya and Y. Handa, unpublished data.
39. Page, A. L., Ohayon, H., Sansonetti, P. J., and Parsot, C. (1999) *Cell. Microbiol.* 1, 183–193
40. Ghosh, P. (2004) *Microbiol. Mol. Biol. Rev.* 68, 771–795
41. Forsberg, M., Blomgran, R., Lerm, M., Sarndahl, E., Sebit, S. M., Hamilton, A., Stendahl, O., and Zheng, L. (2003) *J. Leukocyte Biol.* 74, 620–629
42. Tafazzoli, F., Magnusson, K. E., and Zheng, L. (2003) *Infect. Immun.* 71, 872–881
43. Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S., and Knaus, U. G. (1998) *J. Biol. Chem.* 273, 8137–8144
44. Vadlamudi, R. K., Li, F., Adam, L., Nguyen, D., Ohtia, Y., Stossel, T. P., and Kumar, R. (2002) *Nat. Cell Biol.* 4, 681–690
45. Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galán, J. E. (1998) *Cell* 93, 815–826
46. Fu, Y., and Galán, J. E. (1999) *Nature* 401, 293–297
47. Stender, S., Friebel, A., Linder, S., Rohde, M., Mirolid, S., and Hardt, W. D. (2000) *Mol. Microbiol.* 36, 1206–1221
48. Schmidt, A., and Hall, A. (2002) *Genes Dev.* 16, 1587–1609
49. Katoh, H., and Negishi, M. (2002) *Nature* 424, 461–464
50. Brugnara, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) *Nat. Cell Biol.* 4, 574–582
51. Casadaban, M. J., and Cohen, S. N. (1980) *J. Mol. Biol.* 138, 179–207