IQGAP1 Regulates Salmonella Invasion through Interactions with Actin, Rac1, and Cdc42

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To infect host cells, Salmonella utilizes an intricate system to manipulate the actin cytoskeleton and promote bacterial uptake. Proteins injected into the host cell by Salmonella activate the Rho GTPases, Rac1 and Cdc42, to induce actin polymerization. Following uptake, a different set of proteins inactivates Rac1 and Cdc42, returning the cytoskeleton to normal. Although the signaling pathways allowing Salmonella to invade host cells are beginning to be understood, many of the contributing factors remain to be elucidated. IQGAP1 is a multidomain protein that influences numerous cellular functions, including modulation of Rac1/Cdc42 signaling and actin polymerization. Here, we report that IQGAP1 regulates Salmonella invasion. Through its interaction with actin, IQGAP1 co-localizes with Rac1, Cdc42, and actin at sites of bacterial uptake, whereas infection promotes the interaction of IQGAP1 with both Rac1 and Cdc42. Knockdown of IQGAP1 significantly reduces Salmonella invasion and abrogates activation of Cdc42 and Rac1 by Salmonella. Overexpression of IQGAP1 significantly increases the ability of Salmonella to enter host cells and required interaction with both actin and Cdc42/Rac1. Together, these data identify IQGAP1 as a novel regulator of Salmonella invasion.

Salmonella continues to be a major cause of morbidity and mortality worldwide, causing food poisoning and typhoid fever. Using a highly intricate system, Salmonella is able to hijack the actin regulatory machinery of the host cell to promote bacterial entry. To do this, Salmonella uses a type III secretion system to inject a battery of effector proteins into the host cell (reviewed in Ref. 1). These proteins are able to mimic host cell activators of actin polymerization, resulting in membrane ruffling and macropinocytosis (2). Key to these processes are three Salmonella effector proteins, SopE, SopE2, and SopB. SopE and SopE2 bind to and activate Rac1 and Cdc42 (3, 4). Despite sharing very little sequence similarity, SopE/SopE2 and eukaryotic cell guanosine nucleotide exchange factors (GEFs)2 use nearly identical catalytic mechanisms to activate Rac1 and Cdc42. SopB, a phosphoinositide phosphatase (5, 6), indirectly regulates actin polymerization through the activation of SH3-containing GEF and RhoG (7).

IQGAP1 is a multidomain protein that binds many targets, including Cdc42 (8–10), Rac1 (8, 11), actin (12, 13), calmodulin (10, 12), Rap1 (14), and components of the mitogen-activated protein (MAP) kinase cascade (15–17) (reviewed in Refs. 18–21). A primary function of IQGAP1 is to modulate cytoskeletal architecture. IQGAP1 enhances actin polymerization in vitro (22, 23) and co-localizes with actin in lamellipodia (8). In addition to a direct interaction with actin, IQGAP1 modulates the cytoskeleton indirectly through the Rho GTPases (18). IQGAP1 preferentially binds to active (GTP-bound) Cdc42 (8, 10) and Rac1 (8). IQGAP1 is not a GTPase-activating protein (GAP) and actually inhibits the intrinsic GTPase activity of Cdc42 in vitro, stabilizing Cdc42 in its active GTP-bound form (8, 12, 24). In cells, IQGAP1 substantially increases the pool of GTP-bound Cdc42, stimulating filopodia formation (24). As a consequence of its integral participation in cytoskeletal function, we examined the role of IQGAP1 in host cell invasion by Salmonella.

EXPERIMENTAL PROCEDURES

Reagents—All tissue culture reagents were obtained from Invitrogen. The IQGAP1 polyclonal (12) and monoclonal (16) antibodies have been previously described. Other antibodies used were anti-Rac1 monoclonal antibody (Transduction Laboratories), anti-Cdc42 monoclonal antibody (BD Transduction Laboratories), anti-Salmonella polyclonal antibody (BD Diagnostic Systems), anti-tubulin (Sigma), and anti-GFP monoclonal antibody (Santa Cruz Biotechnology). Monoclonal antibodies against SopE and SopE2 (4) were generously donated by Dr. Ed Galyov (Institute of Animal Health, Newbury, Berkshire, UK). For immunocytocchemical analysis of F-actin, 546- or 633-conjugated phalloidin (Molecular Probes) was used. All fluorescent secondary antibodies were from Molecular Probes. Mutant IQGAP1 plasmids (24–27) were cloned into pE green fluorescent protein (GFP) vector (Clontech) using standard restriction enzyme digests essentially as described previously for wild type IQGAP1 (28). GST–PAK–CRIB and GST–WASP–GBD were purified from bacterial lysates, as described previously (24).

Salmonella Infection—Infection of HeLa cells was performed essentially as described (29). HeLa cells, grown in Dulbecco’s fibroblast; PAK, p21-activated kinase; CRIB, Cdc42-Rac1-interactive binding domain; PBS, phosphate-buffered saline; GST, glutathione S-transferase; GBD, GTPase binding domain; ANOVA, analysis of variance.
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modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in 5% CO₂, were seeded into 24-well plates at ~5 × 10^4 cells/well (glass coverslips were added for immunofluorescence experiments). Salmonella were added at a multiplicity of infection (m.o.i.) of 20. Following a 10-min incubation at 37 °C in 5% CO₂, free bacteria were removed by washing cells four times with phosphate-buffered saline (PBS), and cells were processed for immunocytochemistry or immunoprecipitation as described below.

Immunoprecipitation—Immunoprecipitations were performed essentially as described previously (30). Cells were lysed in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, and 1% Triton X-100), containing protease inhibitors, and equal amounts of protein lysates were incubated with anti-IQGAP1 polyclonal antibodies for 3 h at 4 °C. Immune complexes were collected for 2 h with 40 µl of protein A-Sepharose (Amersham Biosciences), washed five times with buffer A, and resolved by SDS-PAGE. Western blots were probed with the antibodies indicated in Figs. 2–4 and developed by enhanced chemiluminescence (ECL). Densitometry of ECL signals was analyzed using UN-SCAN-IT software (Silk Scientific Corp.). Protein concentrations were determined using the DC protein assay (Bio-Rad).

Immunocytochemistry—Cells were fixed in 4% paraformaldehyde/PBS for 20 min at 22 °C and blocked/permeabilized in 0.2% Triton, 3% bovine serum albumin/PBS for 1 h at 22 °C. Relevant antibodies were diluted into 0.2% Triton, 1% bovine serum albumin/PBS and incubated at 4 °C overnight. Cells were washed with PBS and then incubated with the appropriate secondary antibody (546- or 488-conjugated anti-mouse or anti-rabbit antibodies, Molecular Probes), diluted 1:1000 in PBS, for 1 h at 22 °C. Coverslips were washed with PBS, mounted, and analyzed using a Zeiss LSM 510 confocal microscope and LSM software.

Cell Culture and Bacterial Strains—Wild type Salmonella typhimurium strain SL1344 (31) or the invasion-deficient strain, Salmonella HilA− (generously provided by Dr. Beth McCormick, Massachusetts General Hospital), were used. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). To generate stable IQGAP1 knockdown cells, siIQ5 or siIQ8 sequences cloned into the retroviral vector pSuper (25) were used. HeLa cells were infected with the viral vectors for 48 h and allowed to express for a further 48 h. Cells were then cultured in medium containing 2 µg/ml puromycin to select for incorporation of the construct. After 2 weeks, HeLa cells with stable incorporation of the siRNA were obtained. Cells were transiently transfected with 5 µg of DNA using Lipofectamine2000 (Invitrogen), according to the manufacturer’s instructions. Mouse embryonic fibroblasts (MEFs) were derived from control or IQGAP1 knock-out mice and immortalized as described (17). MEFs were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Bacterial Quantification—Bacteria were quantified by serial dilution (29). Bacteria were grown for 16 h at 37 °C with shaking and then subcultured at a dilution of 1:10 (1 ml in 10 ml) in fresh Luria-Bertani broth (Sigma) and incubated at 37 °C with shaking until an optical density (at 600 nm) of 0.5 was reached. The culture was serially diluted and plated in triplicate onto MacConkey agar (Remel Inc.) and incubated at 37 °C overnight. By quantifying the colony-forming units, the number of Salmonella present per ml of culture was determined, allowing the appropriate m.o.i. to be added to cultured cells.

Gentamicin Protection Assay—Salmonella invasion was analyzed using a gentamicin protection assay (29). Following infection for 40 min with Salmonella (m.o.i. of 20 for HeLa and 30 for MEFs), cells were extensively washed with PBS. Cells were then cultured in medium containing 50 µg/ml gentamicin for 2 h to kill extracellular bacteria. After additional washes, cells were lysed in 100 µl of 1% Triton X-100 for 5 min at 22 °C. The number of bacteria was quantified by serial dilution, as described above.

Immunocytochemical Analysis of Salmonella Invasion—Immunocytochemical quantification of invasion was performed essentially as described (7, 29, 32). Briefly, cells were infected for 40 min (HeLa) or 1 h (MEFs) and then cultured in gentamicin for 2 h. After extensive washing, cells were fixed. To detect extracellular bacteria, cells were incubated with anti-Salmonella antibodies without permeabilization followed by Alexa Fluor 488-labeled anti-rabbit secondary antibody, both for 1 h at 22 °C. Cells were washed and permeabilized in 0.2% Triton X-100, 3% bovine serum albumin/PBS for 1 h at 22 °C. Following permeabilization, cells were incubated for a second time with anti-Salmonella antibody (1 h at 22 °C) followed by Alexa Fluor 546-labeled anti-rabbit secondary antibody (1 h at 22 °C) to detect total Salmonella. For cells transfected with GFP-tagged IQGAP1 constructs, anti-GFP monoclonal antibodies were added during the second anti-Salmonella antibody incubation. The number of GFP-positive cells containing one or more intracellular bacteria was counted by fluorescent microscopy. Results were obtained from three separate experiments, each performed in quadruplicate. Over 700 cells were counted for GFP transfection, whereas between 100 and 200 cells were counted for transfections of GFP-tagged IQGAP1 constructs.

Rac1 and Cdc42 GTPase Assays—Assays were performed essentially as described (24, 25, 33). Briefly, HeLa cells were lysed in buffer A prior to and following Salmonella infection. Equal amounts of protein lysate were incubated with GST-PAK-CRIB (for Rac1 and Cdc42) or GST-WASP-GBD (for Cdc42) for 3 h at 4 °C. Glutathione beads were used to pull down complexes of WASP-GBD with GTP-bound Cdc42 or PAK-CRIB with GTP-bound Rac1 and Cdc42 and washed in buffer A. Samples were resolved by SDS-PAGE and probed for Cdc42 or Rac1, as indicated.

Statistical Analysis—Statistical analysis was performed using GraphPad Prism. One-way ANOVA tests or Student’s t tests were used where appropriate, as indicated.

RESULTS

IQGAP1 Localizes to Sites of Salmonella Invasion—To determine whether IQGAP1 is involved in Salmonella invasion, we first examined the localization of IQGAP1 during infection. Endogenous IQGAP1 is diffusely distributed throughout the cytoplasm of HeLa cells, with accumulation at the cell periphery (Fig. 1A, green). This distribution is similar to that documented with other cells (25, 30). IQGAP1 co-localizes with
endogenous actin, Rac1, and Cdc42 around the cell periphery in uninfected HeLa cells (Fig. 1A). A large body of work has shown that actin, Rac1, and Cdc42 all localize at sites of Salmonella invasion (reviewed in Ref. 1). To examine whether IQGAP1 localizes to the same sites, HeLa cells were infected with wild type S. typhimurium at an m.o.i. of 20 (Fig. 1). Twenty minutes after infection, cells were fixed and stained for endogenous IQGAP1 (green), and actin, Cdc42, or Rac1 (red) and examined by confocal microscopy. Salmonella were identified using 4′,6-diamidino-2-phenylindole (blue). In agreement with previous reports (32, 34), wild type Salmonella induce the formation of actin-rich phagocytic cups, which also contain Cdc42 and Rac1 (Fig. 1B, red). Analysis of these structures shows that IQGAP1 co-localizes with actin, Rac1, and Cdc42 at sites of Salmonella invasion (Fig. 1B). IQGAP1 also co-localizes with the GEFs SopE and SopE2 at these phagocytic cups (Fig. 1B). In contrast, infection with non-invasive Salmonella HilA/H11002 fails to induce formation of the phagocytic cup. Neither IQGAP1, actin, Cdc42, nor Rac1 are enriched in sites of Salmonella HilA− attachment (Fig. 1C). Thus, invasion by Salmonella, not merely attachment of the bacteria, is required to induce redistribution of IQGAP1, actin, Rac1, and Cdc42.

**IQGAP1 Is Required for Efficient Invasion by Salmonella**—To further examine the role of IQGAP1 in Salmonella invasion, we established HeLa cells with stable incorporation of a specific siRNA construct that reduces endogenous IQGAP1 (termed HeLa siIQ8 cells) or a control, non-silencing siRNA construct (termed HeLa siIQ5 cells) (Fig. 2A). (The siRNA oligonucleotides have been described previously (25).) Endogenous IQGAP1 levels are reduced ~75% in HeLa siIQ8 cells when compared with HeLa siIQ5 or untransfected cells (Fig. 2A). The ability of Salmonella to invade HeLa siIQ5 and HeLa siIQ8 cells was tested using a gentamicin protection invasion assay followed by quantification by serial dilution. The number of intracellular Salmonella is significantly decreased by 33% when endogenous IQGAP1 levels are reduced (Fig. 2B). The magnitude of the reduction is comparable with that observed with knockdown of Rac1 (7).

To validate the data derived from siRNA knockdown of IQGAP1, we tested the ability of Salmonella to invade cells lacking IQGAP1. We used a protocol described by Shi and Casanova (35) to determine Salmonella invasion into MEFs. IQGAP1-null MEFs and MEFs from littermate controls (17) were infected with wild type Salmonella at an m.o.i. of 30 for 1 h followed by the addition of gentamicin for 2 h. Salmonella invasion was assessed with an immunocytochemical assay (7, 29, 32, 35). Fixed cells were stained for extracellular and total Salmonella. Quantification revealed that the number of intracellular Salmonella in IQGAP1-null MEFs is 77% less than that in MEFs from littermate controls (Fig. 2, C and D). These data confirm that IQGAP1 is required for efficient invasion of host cells by Salmonella.

**FIGURE 1.** IQGAP1 localizes to sites of Salmonella invasion. HeLa cells were uninfected (A) or infected with wild type (WT) Salmonella (B) or invasion-defective Salmonella HilA− (C) for 20 min, at an m.o.i. of 20. Cells were fixed and stained for endogenous IQGAP1 (green) and actin, Cdc42, Rac1, SopE, or SopE2 (red). Salmonella were identified using 4′,6-diamidino-2-phenylindole (DAPI) (blue). Merge represents a composite of all three channels. In B, the arrowheads indicate IQGAP1 co-localization with actin, Rac1, and Cdc42 at sites of wild type Salmonella invasion. In C, the arrowheads identify the attachment of Salmonella HilA− without localization of IQGAP1, actin, Cdc42, and Rac1. Images are representative of at least four independent experiments. Scale bars = 10 μm.
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The effect of reducing intracellular IQGAP1 levels on the formation of the phagocytic cup was also examined. HeLa siIQ8 and MEF<sup>−/−</sup> cells, with the pertinent control cell lines, were infected with Salmonella for 20 min. Immunocytochemistry revealed that the intensity of actin in the phagocytic cup in HeLa siIQ8 cells is less than that in HeLa siQ5 cells (Fig. 2E). Essentially identical results were obtained with MEFs; actin staining in phagocytic cups of MEF<sup>−/−</sup> is reduced when compared with MEF<sup>+/+</sup> (Fig. 2E). These data suggest that IQGAP1 is required for efficient polymerization of actin at the site of Salmonella invasion. Together these results demonstrate that IQGAP1 is an important component of the signaling processes underlying Salmonella invasion.

Salmonella Invasion Promotes the Interaction of IQGAP1 with Rac1 and Cdc42—During host cell invasion by Salmonella, both Rac1 and Cdc42 are activated in response to bacterial effector proteins (1, 36). IQGAP1 binds to active Cdc42 and active Rac1 (8, 10), and we therefore examined whether Salmonella invasion altered the interaction of IQGAP1 with Rac1 and/or Cdc42. HeLa cells were infected with Salmonella for 10 or 20 min, and IQGAP1 was immunoprecipitated. When compared with non-infected cells, the amount of Rac1 and Cdc42 that co-immunoprecipitated with IQGAP1 is significantly increased by 77 and 42%, respectively, 20 min after the addition of Salmonella (Fig. 3). Although there is a small and reproducible increase in co-immunoprecipitation of both GTPases 10 min after the addition of Salmonella (7). Although these experiments are unable to establish whether IQGAP1 is upstream or downstream of Rac1 and Cdc42 (20), it is possible that IQGAP1 may act as a regulator of Rac1/Cdc42 during the actin dynamics induced by Salmonella infection (Fig. 3A). These observations are consistent with published data showing that GTP loading of Rac1 and Cdc42 peaks 20 min after the addition of Salmonella (7).

Knockdown of IQGAP1 Prevents Activation of Rac1 and Cdc42 by Salmonella—IQGAP1 has been previously shown to regulate the levels of active Cdc42 and Rac1 (24, 25). Therefore, to examine whether knockdown of IQGAP1 alters the ability of Salmonella to activate Rac1 and Cdc42, the levels of GTP-bound Rac1 and Cdc42 were analyzed at 1887 min after the addition of Salmonella (Fig. 3B). Although there is a small and reproducible increase in co-immunoprecipitation of both GTPases 10 min after the addition of Salmonella (7). Although these experiments are unable to establish whether IQGAP1 is upstream or downstream of Rac1 and Cdc42 (20), it is possible that IQGAP1 may act as a regulator of Rac1/Cdc42 during the actin dynamics induced by Salmonella infection (Fig. 3A). These observations are consistent with published data showing that GTP loading of Rac1 and Cdc42 peaks 20 min after the addition of Salmonella (7).

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abrogates the ability of Salmonella to activate Rac1 and Cdc42 in HeLa siIQ8 cells (Fig. 4). Therefore, IQGAP1 is required for efficient activation of Rac1 and Cdc42 by Salmonella effector proteins.

IQGAP1 Associates with Actin to Localize to Sites of Salmonella Invasion—To further elucidate the role played by IQGAP1 during Salmonella invasion, we examined mutant IQGAP1 constructs that selectively alter its interactions with Cdc42/Rac1 or actin. The mutant constructs used were IQGAP1ΔGRD, which is a dominant negative that decreases the amount of GTP-bound Cdc42 and Rac1 in cells (24, 25), IQGAP1ΔMK24, which neither binds Rac1 nor Cdc42 nor increases GTP-Cdc42 levels in cells (26), and IQGAP1G75Q, a point mutant that does not bind to actin but binds Cdc42 and Rac1 indistinguishably from wild type IQGAP1 (27). These mutant constructs, all tagged with GFP, were expressed in HeLa cells and compared with GFP-tagged wild type IQGAP1. Cells were allowed to express the plasmids for 48 h before infection. Twenty minutes after the addition of Salmonella, the HeLa cells were fixed and stained for actin (blue), GFP (green), and Salmonella (red). As expected, GFP does not localize with actin at the site of invasion (Fig. 5A, panels i–iv). Consistent with our observations with endogenous IQGAP1, wild type GFP-IQGAP1 demonstrates strong colocalization with both actin and Salmonella (Fig. 5B, panels i–iv). Similarly, the two IQGAP1 mutant constructs that bind actin, namely IQGAP1ΔGRD (Fig. 5C, panels i–iv) and IQGAP1ΔMK24 (Fig. 5D, panels i–iv), strongly localize to the actin-rich sites of invasion. In contrast, the IQGAP1 mutant that does not bind actin, IQGAP1G75Q, does not localize to these sites (Fig. 5E, panels i–iv). These data suggest that IQGAP1 requires an interaction with actin, but not Cdc42 or Rac1, to localize to sites of Salmonella infection.

IQGAP1 Promotes Salmonella Invasion through Interactions with Actin and Cdc42/Rac1—To gain insight into the molecular mechanism by which IQGAP1 contributes to Salmonella invasion, we examined the effect of mutant IQGAP1 constructs on the uptake of Salmonella. Due to a relatively low transfection efficiency, invasion was quantified by immunocytochemistry, using a technique that distinguishes intracellular from extracellular Salmonella.
lular *Salmonella* (7, 29, 32). Immunocytochemistry also enables identification of cells expressing the GFP-tagged IQGAP1 constructs, thereby permitting quantification of *Salmonella* uptake exclusively into cells expressing the relevant IQGAP1 constructs. Quantification of the average number of intracellular *Salmonella* per cell reveals that overexpression of wild type IQGAP1 significantly increases *Salmonella* invasion by 53% (Fig. 6, A, panels iv–vi, and B). This is likely due to the ability of IQGAP1 to stabilize the GTP-bound forms of Cdc42 and Rac1, thereby increasing the intracellular levels of the active GTPases (24). Expression of the IQGAP1 mutants that lack binding to either Cdc42/Rac1 or actin, namely IQGAP1/H9004MK24 (Fig. 6, A, panels x–xii, and B) and IQGAP1G75Q (Fig. 6, A, panels xiii–xv, and B), respectively, did not promote invasion of *Salmonella*. These data suggest that the interaction with Rac1/Cdc42 and actin is required for IQGAP1 to enhance *Salmonella* uptake into host cells. In contrast, expression of the dominant negative IQGAP1/GRD reduces *Salmonella* invasion by 48% (Fig. 6, A, panels vii–ix, and B). We have previously documented that IQGAP1/GRD reduces levels of both GTP-bound Rac1 and GTP-bound Cdc42 (24, 25). Therefore, this construct probably reduces invasion by preventing optimal activation of Rac1 and Cdc42 by *Salmonella* effectors.

![Figure 5](image.jpg)

**Figure 5.** IQGAP1 associates with actin to localize to sites of *Salmonella* invasion. HeLa cells were transfected with the GFP constructs indicated in panels A–E and infected with *Salmonella* at an m.o.i. of 20 for 20 min. Cells were stained with phalloidin to visualize actin (blue), anti-GFP antibodies to visualize GFP-tagged constructs (green), and anti-*Salmonella* antibodies (red). Merge represents a composite of all three channels. GFP–IQGAP1, GFP–IQGAP1/GRD, and GFP–IQGAP1/MK24 all localized to sites of *Salmonella* invasion, whereas GFP and GFP–IQGAP1/G75Q did not. The arrowheads indicate sites of invasion, determined by the presence of *Salmonella* and an accumulation of actin (blue). Representative images are shown. Scale bar = 10 μm.

![Figure 6](image.jpg)

**Figure 6.** IQGAP1 promotes *Salmonella* invasion through interactions with actin and Cdc42/Rac1. HeLa cells were transfected with GFP alone or the indicated GFP-tagged IQGAP1 constructs. Cells were infected with *Salmonella*, at an m.o.i. of 20, for 40 min, and then gentamicin was added for 2 h. Cells were stained for GFP (green), extracellular *Salmonella* (green), and total *Salmonella* (red). A, representative confocal images are shown. Scale bar = 10 μm. B, the number of intracellular *Salmonella* per cell was quantified. Cells expressing GFP alone were set as 100% invasion (7). Statistical analysis was performed using a one-way ANOVA and the Dunnett's post test from three independent experiments. Overexpression of GFP–IQGAP1 significantly increased invasion (*, p < 0.01), whereas GFP–IQGAP1/GRD (ΔGRD) significantly decreased invasion (*, p < 0.01) when compared with the GFP control. GFP–IQGAP1/MK24 (ΔMK24) and GFP–IQGAP1/G75Q (G75Q) did not alter invasion.

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**Table:**

| Actin | GFP Construct | *Salmonella* Merge |
|-------|---------------|-------------------|
| A     | i. GFP        | i. GFP            |
|       | ii. GFP–IQGAP1| ii. GFP–IQGAP1    |
|       | iii. GFP–IQGAP1/GRD | iii. GFP–IQGAP1/GRD |
|       | iv. GFP–IQGAP1/MK24 | iv. GFP–IQGAP1/MK24 |
| B     | i. GFP        | i. GFP            |
|       | ii. GFP–IQGAP1| ii. GFP–IQGAP1    |
|       | iii. GFP–IQGAP1/GRD | iii. GFP–IQGAP1/GRD |
|       | iv. GFP–IQGAP1/MK24 | iv. GFP–IQGAP1/MK24 |
| C     | i. GFP        | i. GFP            |
|       | ii. GFP–IQGAP1| ii. GFP–IQGAP1    |
|       | iii. GFP–IQGAP1/GRD | iii. GFP–IQGAP1/GRD |
|       | iv. GFP–IQGAP1/MK24 | iv. GFP–IQGAP1/MK24 |
| D     | i. GFP        | i. GFP            |
|       | ii. GFP–IQGAP1| ii. GFP–IQGAP1    |
|       | iii. GFP–IQGAP1/GRD | iii. GFP–IQGAP1/GRD |
|       | iv. GFP–IQGAP1/MK24 | iv. GFP–IQGAP1/MK24 |
| E     | i. GFP        | i. GFP            |
|       | ii. GFP–IQGAP1| ii. GFP–IQGAP1    |
|       | iii. GFP–IQGAP1/GRD | iii. GFP–IQGAP1/GRD |
|       | iv. GFP–IQGAP1/MK24 | iv. GFP–IQGAP1/MK24 |

**Notes:**

- *Salmonella* invasion is quantified as the number of intracellular *Salmonella* per cell.
- Statistical analysis is performed using a one-way ANOVA and the Dunnett’s post test from three independent experiments.
- GFP (green) is used for visualization.
- Extracellular *Salmonella* (green) is used for identification.
- Total *Salmonella* (red) is used for confirmation.
DISCUSSION

An accumulating body of work is elucidating many of the signaling pathways regulated by *Salmonella* during the invasion process. Here we identify IQGAP1 as a novel component of *Salmonella* infection. Through its interactions with actin and Cdc42/Rac1, IQGAP1 is important for successful invasion by *Salmonella*. Consistent with this observation, knockdown of IQGAP1 reduces invasion by 33%. Although comparable with the data obtained with knockdown of Rac1 (7), the magnitude of the reduction in invasion may be attenuated due to the presence of residual IQGAP1 in HeLa siQ8 cells. This concept is supported by data from IQGAP1-null MEFs, which demonstrate a 77% reduction in *Salmonella* invasion. Collectively, these data strongly suggest that IQGAP1 is necessary for optimal *Salmonella* infection into eukaryotic cells.

Previous work has highlighted the role of IQGAP1 in controlling the levels of active Rac1 and Cdc42. IQGAP1 binds active Cdc42 and Rac1 *in vitro* (8, 10, 11), and overexpression of IQGAP1 in cells increases the amount of active Cdc42 and Rac1, thereby modulating the cytoskeleton (24, 25). In contrast, expression of the dominant negative IQGAP1GRD reduces levels of active Cdc42 and Rac1 (24, 25). Here we observe that IQGAP1 co-localizes with Cdc42 and Rac1 at sites of *Salmonella* invasion. Moreover, *Salmonella* invasion augments the interaction of IQGAP1 with both Cdc42 and Cdc42. Importantly, knockdown of IQGAP1 prevents *Salmonella* from activating Rac1 and Cdc42. IQGAP1 also regulates the actin cytoskeleton directly and promotes actin polymerization (22, 23). We observed that IQGAP1 co-localizes with actin at sites of *Salmonella* entry. The attenuation of phagocytic cup formation by *Salmonella* in cells with reduced or absent IQGAP1 suggests that IQGAP1 may regulate actin polymerization in the phagocytic cup during invasion. Perhaps more interestingly, it is tempting to speculate that IQGAP1 may function to link Cdc42 and Rac1 to the actin cytoskeleton (19) to facilitate entry of *Salmonella* into host cells. Although binding to Cdc42/Rac1 is not necessary for IQGAP1 to localize to sites of invasion, we observed that an interaction with actin is required. However, analysis with mutant constructs suggests that IQGAP1 needs to interact with Cdc42/Rac1 to promote *Salmonella* entry. Based on these data, we propose a model of the molecular mechanism for the participation of IQGAP1 in the process by which *Salmonella* invades host cells (Fig. 7). *Salmonella* attaches to the host cell and injects effector proteins via the type III secretion system. Two of these effectors, SopE and SopE2, are GEFs that activate Rac1 and Cdc42, inducing actin polymerization and phagocytic cup formation. IQGAP1 binds to filamentous actin and accumulates in the phagocytic cup produced by the host cell in response to *Salmonella*. Once there, IQGAP1 binds to GTP-Rac1 and GTP-Cdc42 to maintain them in their GTP-bound form, enhancing actin polymerization. IQGAP1 thus couples Rac1 and Cdc42 to actin, augmenting actin polymerization, thereby further promoting formation of the phagocytic cup (Fig. 7). Knockdown of IQGAP1 (this study) or Rac1 (7) reduces, but does not completely abrogate, *Salmonella* invasion. These data suggest that in the absence of IQGAP1 or Rac1, *Salmonella* are able to enter host cells, albeit less efficiently, via an alternative signaling pathway(s).

GTP hydrolysis by Rac1 and Cdc42 is a slow process, but it can be accelerated by orders of magnitude through the actions of GAPs (37). *In vitro*, IQGAP1 inhibits both the intrinsic GT-Pase activity of Cdc42 (8, 24) and the GT-Pase activation of Cdc42 by p190rho GAP (8). Although the mechanism by which IQGAP1 inhibits active Cdc42 and Rac1 in eukaryotic cells has not been established, it is likely that the mechanism is similar to that documented *in vitro*. In addition to blocking the action of eukaryotic GAPs, in cells infected with *Salmonella*, IQGAP1 may prevent the action of SptP on the GT-Pases. SptP, a *Salmonella* effector protein injected into the host cell, is a GAP that inactivates Rac1 and Cdc42 by promoting hydrolysis of bound GTP (38). By this mechanism, SptP returns the actin cytoskeleton to normal following bacterial entry. In cells lacking IQGAP1, SptP may inactivate Rac1 and Cdc42 too early, preventing efficient formation of the phagocytic cup and consequently impairing optimal invasion. Consistent with this hypothesis, knockdown or knock-out of IQGAP1 reduces polymerization of actin in the phagocytic cup (Fig. 2). The mechanisms of action of IQGAP1 outlined above may not be mutually exclusive, and both may be operative.

Although it is generally accepted that the Rho GT-Pases are important for microbial pathogenesis (39), there is conflicting evidence regarding the specific roles of Cdc42 and Rac1 in *Salmonella* invasion. Several groups have documented that Cdc42 is essential for actin regulation during the invasion process (32, 38, 40, 41). In contrast, a recent study suggests that Rac1, but
not Cdc42, is required for the actin rearrangements initiated by "Salmonella" (7). In contradiction to the last observation, SopE2, which preferentially activates Cdc42 (with minimal effect on Rac1) in vitro and in intact cells (42), is sufficient to partially rescue invasion of a non-invasive strain of "Salmonella" (43). Moreover, a "Salmonella" strain that lacks SopE2 displays an ~80% reduction in the ability to invade HeLa cells (4). These observations reveal that Cdc42 contributes, at least in part, to invasion of host cells by "Salmonella". Discrepancies in documented roles for Rac1 and Cdc42 in "Salmonella" invasion may be due, at least in part, to the use of different cell types. Thus, the specific contribution of these GTPases to the mechanisms underlying the invasion process remains to be fully elucidated. Regardless of the mechanism, our data indicate that IQGAP1 is required for efficient "Salmonella" invasion, at least in part, through its interactions with Rac1 and/or Cdc42. Mutant IQGAP1 constructs that selectively lack binding to Cdc42 (whereas binding normally to Rac1, and vice versa) are necessary to discriminate whether one or both of these GTPases contribute to the mechanism by which IQGAP1 modulates "Salmonella" invasion.

IQGAP1 was recently shown to regulate neuronal WASP- and Arp2/3-dependent actin polymerization (23, 44). Therefore, IQGAP1 may control the formation of a complex that is required for efficient "Salmonella" entry. IQGAP1 appears to act as a molecular scaffold in numerous signaling pathways by virtue of its ability to interact with multiple proteins (20). By serving as a scaffold, IQGAP1 may assimilate many components of actin regulation at the phagocytic cup in response to "Salmonella" effector proteins. The interactions with actin, Rac1, and Cdc42 appear to be important for IQGAP1 function during host cell invasion by "Salmonella". Thus, our findings identify IQGAP1 as a novel regulator of "Salmonella" invasion, through its ability to regulate both Cdc42/Rac1 function and actin polymerization. Collectively, our data suggest that IQGAP1 may be a potential target for the development of novel therapeudic agents to ameliorate "Salmonella" infection.

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