Salmonella enterica serotype Typhimurium usurps the scaffold protein IQGAP1 to manipulate Rac1 and MAPK signalling

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Salmonella enterica serotype Typhimurium invades eukaryotic cells by re-arranging the host-cell cytoskeleton. However, the precise mechanisms by which Salmonella induces cytoskeletal changes remain undefined. IQGAP1 (IQ motif-containing GTPase-activating protein 1) is a scaffold protein that binds multiple proteins including actin, the Rho GTPases Rac1 and Cdc42 (cell division cycle 42), and components of the MAPK (mitogen-activated protein kinase) pathway. We have shown previously that optimal invasion of Salmonella into HeLa cells requires IQGAP1. In the present paper, we use IQGAP1-null MEFs (mouse embryonic fibroblasts) and selected well-characterized IQGAP1 mutant constructs to dissect the molecular determinants of Salmonella invasion. Knockout of IQGAP1 expression reduced Salmonella invasion into MEFs by 75%. Reconstituting IQGAP1-null MEFs with wild-type IQGAP1 completely rescued invasion. By contrast, reconstituting IQGAP1-null cells with mutant IQGAP1 constructs that specifically lack binding to either Cdc42 and Rac1 (termed IQGAP1ΔMK24), actin, MEK (MAPK/ERK (extracellular-signal-regulated kinase) kinase) or ERK only partially restored Salmonella entry. Cell-permeant inhibitors of Rac1 activation or MAPK signalling reduced Salmonella invasion into control cells by 50%, but had no effect on bacterial entry into IQGAP1-null MEFs. Importantly, the ability of IQGAP1ΔMK24 to promote Salmonella invasion into IQGAP1-null cells was abrogated by chemical inhibition of MAPK signalling. Collectively, these results imply that the scaffolding function of IQGAP1, which integrates Rac1 and MAPK signalling, is usurped by Salmonella to invade fibroblasts and suggest that IQGAP1 may be a potential therapeutic target for Salmonella pathogenesis.

Key words: IQ motif-containing GTPase-activating protein 1 (IQGAP1), microbial pathogenesis, protein scaffold, Salmonella.

INTRODUCTION

Salmonella enterica serotype Typhimurium is a highly virulent Gram-negative pathogen that causes severe systemic disease, including gastroenteritis and typhoid fever in humans [1,2]. During infection, Salmonella usurps host-cell signalling pathways, particularly those that regulate the actin cytoskeleton [3,4]. Salmonella is equipped with a T3SS (type III secretion system) that injects host cells with several bacterial proteins [5]. These include SopE and SopE2, which mimic the function of GEFs (guanine-nucleotide-exchange factors) and activate the Rho GTPases Rac1 and Cdc42 (cell division cycle 42) by stimulating the exchange of GDP for GTP [6,7]. Active Rac1 and Cdc42 induce the activation of the N-WASP (neuronal WASP (Wiskott–Aldrich syndrome protein)), WAVE2 (WASP family member 2) and the Arp (actin-related protein) 2/3 complex, which triggers actin polymerization and membrane ruffling [8–10]. The generation of membrane ruffles substantially facilitates bacterial invasion into host cells [3,4]. After entry, Salmonella inactivates Rac1 and Cdc42 using SpP, a GAP (GTPase-activating protein) that helps restore the host cell’s original cytoskeletal architecture [3]. Although it is generally accepted that Rho GTPases participate in Salmonella invasion, the exact roles of Rac1 and Cdc42 during Salmonella uptake are unclear. For example, Chen et al. [11] reported decreased Salmonella invasion into COS-1 cells expressing a dominant-negative Cdc42 construct, suggesting that Cdc42 is the pivotal GTPase manipulated during host-cell invasion. However, the same group showed that Salmonella invasion into COS-2 fibroblasts and intestinal Henle 407 cells was abrogated following siRNA (short interfering RNA)-mediated knockdown of Rac1, but not Cdc42, indicating that Rac1 is the more important small GTPase for Salmonella entry [12]. Another group observed that siRNA-mediated knockdown of Rac1 and Cdc42 had no significant effect on Salmonella invasion into human foreskin fibroblasts [13]. Although some of the discrepant data have been ascribed to differences among cell types, these studies indicate that the mechanisms underlying Rac1 and Cdc42 function in Salmonella pathogenesis are incompletely understood.

The MAPK (mitogen-activated protein kinase) pathway relays extracellular signals to various intracellular targets, including the actin cytoskeleton [14–16]. The most extensively studied module of the MAPK pathway is the MEK (MAPK/ERK (extracellular-signal-regulated kinase) kinase)/ERK cascade. In this cascade, extracellular stimuli induce activation of the small GTPase Ras, which activates B-Raf. B-Raf then phosphorylates and activates MEK, resulting in phosphorylation of ERK [16]. The MEK/ERK pathway regulates cell adhesion and motility, processes that are governed by changes in the actin cytoskeleton [14]. Importantly, Salmonella stimulates MAPK activation in host cells [17–19] and treatment of cells with the MEK inhibitor PD98059 reduces Salmonella uptake [13,19]. These findings suggest that Salmonella may also target the actin cytoskeleton via the MAPK cascade to achieve infection, although the precise mechanism by which this occurs is unknown.

Abbreviations used: Arp, actin-related protein; Cdc42, cell division cycle 42; CRIB, Cdc42/Rac-interacting binding region; ERK, extracellular-signal-regulated kinase; EGFP, enhanced green fluorescent protein; GAP, GTPase-activating protein; GBD, GTPase-binding domain; GST, glutathione transferase; GTP[S], guanosine 5′-O[(3′-thio)triphosphate]; IQGAP1, IQ motif-containing GTPase-activating protein 1; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; MOI, multiplicity of infection; PAK, p21-activated kinase; siRNA, short interfering RNA; T3SS, type III secretion system; WASP, Wiskott–Aldrich syndrome protein; N-WASP, neuronal WASP.

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Iqgap1 (IQ motif-containing GAP1) is a ubiquitously expressed 189-kDa protein that is a pivotal element of cytoskeletal architecture and function [20,21]. Iqgap1 cross-links actin filaments [22,23] and influences actin assembly both by virtue of its association with actin, N-WASP and the Arp 2/3 complex [24], and by modulating the active state of Rac1 and Cdc42 [25,26]. Despite its name, IQGAP1 is not a GAP, but preferentially binds to Rac1 and Cdc42, stabilizing the GTPases in their active forms [26,27]. In addition, IQGAP1 binds to numerous other proteins, including actin, calmodulin and growth factor receptors [28]. It has become apparent that IQGAP1 functions as a scaffold, integrating diverse signalling pathways [28]. For example, IQGAP1 binds to and regulates the activation of B-Raf [29], MEK [30] and ERK [31], thereby facilitating MAPK signalling. A recently uncovered role for IQGAP1 is in microbial pathogenesis [32]. Published evidence has demonstrated that Salmonella manipulates IQGAP1 to invade HeLa cells [33] and EPEC (enteropathogenic Escherichia coli) requires IQGAP1 to form actin pedestals in host cells [34]. Moreover, the Salmonella effector SseI binds directly to IQGAP1 and exploits IQGAP1 to reduce macrophage motility, thereby promoting chronic infection [35]. In order to further elucidate the molecular mechanisms by which Salmonella enters host cells, we reconstituted IQGAP1-null MEFs (mouse embryonic fibroblasts) with selected IQGAP1 mutant constructs that lack binding to specific target proteins. Using this approach, we report a novel IQGAP1-dependent mechanism for Salmonella invasion that involves both Rac1 and MAPK signalling.

**EXPERIMENTAL**

**Reagents**

All cell culture reagents were obtained from Invitrogen. The anti-IQGAP1 polyclonal antibody has been described previously [36]. The monoclonal antibodies used were anti-Rac1, anti-Cdc42 (BD Biosciences), anti-ERK, anti-(phospho-ERK) (Cell Signalling Technology) and anti-β-tubulin (Sigma). Anti-mouse and anti-rabbit secondary antibodies (conjugated to horseradish peroxidase) were obtained from GE Healthcare. Fluorescent polystyrene beads were purchased from Polysciences. Bovine collagen solution and BSA were obtained from Sigma and Invitrogen respectively. The EGFP (enhanced green fluorescent protein) vector (pEGFP) was obtained from Clontech. The mutant IQGAP1 constructs that lack binding to Rac1/Cdc42 (IQGAP1ΔMK24), actin (IQGAP1-G75Q), ERK (IQGAP1ΔWW) or MEK (IQGAP1ΔIQ) have been described previously [37–39]. The mutant IQGAP1 construct that binds neither Rac1/Cdc42 nor actin (IQGAP1-G75QΔMK24) was prepared in two steps. Initially, PCR was performed using IQGAP1 in pcDNA3 as a template, with the following primers: 

5′-GCTGTACAAGTCCGCCGCAGACGAGGTTGACG-3′ (forward) and 5′-TTCAGCAAGATCACTGTGGTAGAAG-3′ (reverse). The forward primer included the BsrGI restriction site. The PCR product was cut with BsrGI and Pac1 restriction enzymes. pEGFP-IQGAP1 was digested with BsrGI and Pac1 and the PCR product was ligated into the pEGFP-IQGAP1 plasmid, yielding pEGFP-IQGAP1-G75Q. IQGAP1-G75QΔMK24 was prepared by ligating the Pac1/Spe1 fragment of pcDNA3-IQGAP1ΔMK24 into the Pac1/Spe1-digested product of pcDNA3-IQGAP1ΔMK24. The sequence of all constructs was confirmed by dideoxy sequencing. The constitutively active (Q61L) Rac1 and Cdc42 constructs were a gift from Professor Anne Ridley (King’s College London, London, U.K.). Trans-IT transfection reagent was purchased from Mirus Bio. GST (glutathione transferase)-tagged fragments of the CRIB (Cdc42/Rac-interacting binding region) of PAK (p21-activated kinase) (GST-PAK-CRIB) and the GBD (GTPase-binding domain) of WASP (GST–WASP-GBD) were purified from bacterial lysates as described previously [25]. Chemical inhibitors of Rac1 (NSC23766) and MEK1 (PD98059) were purchased from Calbiochem. GTP[S] (guanosine 5′-[γ-thio]triphosphate) was obtained from Cytoskeleton.

**Cell culture and transfection**

MEFs were obtained from IQGAP1-knockout mice and control mice, and immortalized as described previously [29]. MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum and 5% (v/v) antibiotic solution (Invitrogen). Wild-type and mutant IQGAP1 cDNA constructs were transiently transfected into IQGAP1-null MEFs using Trans-IT transfection reagent according to the manufacturer’s instructions. To optimize protein expression, the transfection was performed twice on consecutive days.

**Bacterial quantification**

DsRed-tagged *Salmonella* (strain SL1344, a gift from Dr Lynn Bry, Brigham and Women’s Hospital, Boston, MA, U.S.A.) was used for all experiments. Bacteria were grown for 16 h at 37°C with shaking prior to subculture at a dilution of 1:10 in fresh Luria–Bertani medium and incubated at 37°C with shaking until an attenuation (at 600 nm) of 0.5 was reached. The culture was serially diluted and plated in triplicate on to MacConkey agar plates and incubated at 37°C overnight. The number of *Salmonella* present per ml of culture was determined based on calculations using the number of colony-forming units and the dilution factor.

**Gentamycin protection assay**

*Salmonella* invasion was quantified using a gentamycin protection assay [33]. MEFs were infected with *Salmonella* at an MOI (multiplicity of infection) value of 30 for 30 min. Cells were then washed with PBS and incubated with gentamycin (50 μg/ml; Gibco) to kill extracellular bacteria. After extensive washing, cells were lysed in 1% Triton X-100. The number of bacteria was quantified by serial dilution, as described above.

**Immunocytochemical analysis of *Salmonella* invasion**

MEFs grown on glass coverslips were infected with DsRed-tagged *Salmonella* at an MOI value of 30 for 30 min, then incubated with gentamycin (50 μg/ml) to kill extracellular bacteria [40]. After extensive washing, cells were fixed in 4% paraformaldehyde (Fisher Scientific) for 20 min, permeabilized in 0.1% Triton X-100, and stained with Alexa Fluor® 647 phalloidin (Molecular Probes) to visualize actin. Coverslips were washed with PBS, mounted and analysed using a Zeiss spinning-disk confocal microscope and SlideBook software. *Salmonella* invasion was assessed by (i) counting the number of cells containing one or more intracellular bacteria and (ii) by quantifying the average number of intracellular bacteria per cell. At least 50 cells from a minimum of three fields of view were analysed per experiment. Moreover, each experiment was performed at least three times.

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Bead internalization assay
FITC-conjugated polystyrene beads (2 μm diameter) were coated with fibrillar collagen or 3% (v/v) BSA essentially as described previously [41,42]. MEFs cultured in six-well plates were incubated overnight with coated beads at a ratio of six beads per cell, to allow binding and internalization of the beads. BSA-coated beads bind to the cell surface and are phagocytosed in a non-specific manner, whereas collagen-coated beads are taken up through cell surface β1 integrins. Extracellular fluorescence was quenched with trypan blue; the fluorescence from internalized beads was analysed by flow cytometry. Internalization was quantified based on the percentage of cells with one or more internalized beads. Approximately 10,000 cells were analysed in each experiment for each condition.

Rac1 and Cdc42 GTPase assay and ERK activation assay
Assays to determine the amounts of active Rac1 and Cdc42 were performed essentially as described previously [25,26,43]. Following Salmonella infection, control and IQGAP1-null MEFs were lysed in buffer A [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA and 1% Triton X-100] containing protease inhibitors. Equal amounts of protein lysate were incubated with GST–PAK-CRIB (for Rac1) or GST–WASP-GBD (for Cdc42) for 3 h at 4°C. Glutathione beads were used to pull-down active, GTP-bound Rac1 or Cdc42 and washed with buffer A. Samples were resolved by SDS/PAGE (15% gel) and immunobotted for Rac1 or Cdc42. In some experiments, protein lysates were preincubated with GTPγS as a positive control. To determine the amount of active ERK, control and IQGAP1-null MEFs were lysed and equal amounts of protein lysate were resolved with SDS/PAGE (20% gel gradient) and immunobotted for phospho-ERK.

Statistical analysis
Statistical analysis was performed using SPSS software. One-way ANOVA and Bonferroni post-hoc multiple comparison tests were used to test the effect of IQGAP1 expression on bacterial invasion. Statistical significance was set at P < 0.05.

RESULTS

Maximal invasion of Salmonella into MEFs requires IQGAP1
To further our understanding of the molecular basis of Salmonella infection, we used cells from IQGAP1-null mice. Knockout of IQGAP1 was verified by Western blotting (Figure 1A). To test the hypothesis that IQGAP1 is required for optimal Salmonella invasion, MEFs from control (+/+), IQGAP1 null (−/−) mice were infected with dsRed-tagged Salmonella. Microscopic analysis revealed that control MEFs harboured considerably more intracellular Salmonella than IQGAP1-null MEFs (Figure 1B). The extent of Salmonella invasion was quantified by three different methods. (In all assays, control MEFs were indistinguishable from those without IQGAP1). MEFs were incubated with FITC-conjugated beads coated with BSA and bead internalization was quantified by flow cytometric analysis of bead fluorescence. The number of beads phagocytosed into control MEFs was indistinguishable from that into IQGAP1-null MEFs (Figure 1G). In addition, we analysed beads coated with fibrillar collagen to engage the β1 integrin receptors [46]. Control and IQGAP1-null MEFs internalized similar numbers of beads (Figure 1G). These results indicate that IQGAP1 is a specific target for Salmonella invasion, but is not of central importance for the non-specific phagocytosis of beads.

Salmonella invasion into MEFs is partially dependent on binding of IQGAP1 to Rac1/Cdc42
The Rho GTPases Rac1 and Cdc42 are exploited by Salmonella to effect entry into host cells [3,4,6], but the mechanism by which this occurs is incompletely understood. To directly evaluate the role of IQGAP1 in the manipulation of Rac1 and Cdc42 by Salmonella, we reconstituted IQGAP1-null MEFs with an IQGAP1 construct, termed IQGAP1ΔMK24, that specifically lacks binding to Rac1 and Cdc42 [37]. Successful reconstitution with GFP-tagged IQGAP1ΔMK24 was visualized by both Western blotting and confocal microscopy (Figures 2A and 2C). IQGAP1ΔMK24 significantly augmented Salmonella entry into IQGAP1-null MEFs, but reached only $3.9 \pm 7.7\%$ (mean ± S.E.M., n = 3) of that with control cells (Figure 2D). To assess the role for IQGAP1 in the manipulation of actin by Salmonella, we reconstituted IQGAP1-null MEFs with an IQGAP1 point mutant construct, termed IQGAP1-G75Q (Figures 2A and 2B), that specifically lacks binding to actin [38]. As was observed with IQGAP1ΔMK24, IQGAP1-G75Q partially rescued Salmonella entry into IQGAP1-null MEFs (Figure 2D). A third mutant IQGAP1 construct termed IQGAP1-G75QΔMK24 binds neither identically results: Salmonella invasion into IQGAP1-knockout cells was 75% lower than in the control cells (Figures 1D–1F). The experimental approach (using dsRed-null Salmonella) was validated against a dual-colour immunofluorescence assay that differentially stains extracellular and intracellular bacteria [33,44]; essentially identical results were obtained using the two assays (results not shown).

If the reduced entry of Salmonella is due to the absence of IQGAP1, one would anticipate that restoring IQGAP1 in IQGAP1-null cells would rescue Salmonella entry. To test this hypothesis, IQGAP1-null MEFs were reconstituted with GFP-tagged full-length IQGAP1 (Figures 1A and 1C). (Previous studies from both our group and other investigators have shown that the GFP tag does not interfere with IQGAP1 function [33,45].) Reconstitution of IQGAP1-null MEFs with wild-type IQGAP1 restored Salmonella invasion to that seen in control MEFs when evaluated by confocal microscopy (Figures 1D and 1E). In the gentamycin protection assay, whereas wild-type IQGAP1 also promoted Salmonella infection, this was consistently (albeit not statistically significant) less than that into control cells (Figure 1F). The last observation can be explained by the incomplete (~50%) transfection efficiency of the IQGAP1-null MEFs (results not shown). Collectively, these data validate the importance of IQGAP1 for efficient Salmonella invasion into MEFs.

Our findings may result from specific targeting of IQGAP1 by Salmonella during invasion. Alternatively, IQGAP1 may be an essential component of the phagocytosis signalling machinery and the reduced Salmonella uptake may be due to a general reduction of phagocytosis. In order to discriminate between these possibilities, we compared phagocytosis of beads by cells with IQGAP1 with cells without IQGAP1. MEFs were incubated with FITC-conjugated beads coated with BSA and bead internalization was quantified by flow cytometric analysis of bead fluorescence. The number of beads phagocytosed into control MEFs was indistinguishable from that into IQGAP1-null MEFs (Figure 1G). In addition, we analysed beads coated with fibrillar collagen to engage the β1 integrin receptors [46]. Control and IQGAP1-null MEFs internalized similar numbers of beads (Figure 1G). These results indicate that IQGAP1 is a specific target for Salmonella invasion, but is not of central importance for the non-specific phagocytosis of beads.
Figure 1  Optimal Salmonella invasion requires IQGAP1

(A) Equal amounts of protein lysate obtained from control MEFs (+/+), IQGAP1-null MEFs (−/−) and IQGAP1-null MEFs reconstituted with full-length GFP–IQGAP1 (WT) were resolved by SDS/PAGE. Western blots were probed with anti-IQGAP1 and anti-tubulin (loading control) antibodies. Compared with endogenous IQGAP1, GFP–IQGAP1 (WT) exhibits reduced migration on SDS/PAGE due to the mass of the GFP tag. Results are representative of three independent experiments. *P < 0.05. (B) Control (+/+ ) and IQGAP1-null (−/−) MEFs were infected with DsRed-tagged Salmonella at an MOI value of 30 for 30 min, incubated with gentamycin for 2 h and washed extensively to remove extracellular bacteria. Cells were then fixed and stained for F-actin (filamentous actin) using Alexa Fluor® 647 phalloidin. Representative confocal micrographs are shown, indicating intracellular Salmonella (left-hand panels) and F-actin (centre panels). Merged image (far right panel) represents a composite of both channels (Salmonella in red and F-actin in blue). Scale bar = 10 μm. (C) IQGAP1-null MEFs were reconstituted with GFP–tagged full-length IQGAP1 prior to infection with DsRed-tagged Salmonella. Confocal micrograph illustrates GFP–IQGAP1 (far–left panel), intracellular Salmonella (centre–left panel) and F-actin (centre–right panel). Merged image (right–hand panels) represents a composite of all three channels (GFP–IQGAP1 in green, Salmonella in red and F-actin in blue). Yellow indicates co-localization. Representative images are shown. Scale bar = 10 μm. (D) Histograms depict the average number of intracellular Salmonella per cell in control (+/+), IQGAP1-null (−/−) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT). Data, expressed as means ± S.E.M. with control MEFs set at 1, were obtained from three independent experiments. *P < 0.05. (E) Histograms depict the percentage of cells showing at least one intracellular Salmonella in control (+/+), IQGAP1-null (−/−) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT). Data, expressed as means ± S.E.M. with control MEFs set at 100 %, were obtained from three independent experiments. *P < 0.05. (F) Control (+/+ ), IQGAP1-null (−/−) and IQGAP1-null MEFs reconstituted with wild-type IQGAP1 (WT) were infected with Salmonella at an MOI of 30 for 30 min, incubated with gentamycin for 2 h and lysed, and the number of intracellular Salmonella were quantified (based on the number of colony forming units (CFU)). Data, expressed as means ± S.E.M. with control MEFs set at 1, and were obtained from three independent experiments. *P < 0.05. (G) Control (+/+ ) and IQGAP1-null (−/−) MEFs were incubated with FITC-conjugated beads coated with either BSA or with collagen. The number of cells with internalized bead(s) was evaluated by flow cytometry. Histograms depict the percentage of cells showing at least one intracellular bead. Data, expressed as means ± S.E.M., were obtained from three independent experiments.

Salmonella invasion into MEFs requires the interaction of Rac1, but not Cdc42, with IQGAP1

IQGAP1ΔMK24 binds neither Rac1 nor Cdc42 [37], therefore it is not possible to determine whether Rac1 and/or Cdc42 are needed for optimal Salmonella uptake. In order to dissect the relative contributions of the two GTPases in Salmonella invasion, we employed two complementary strategies: a cell-permeant Rac1 inhibitor (NSC23766) and constitutively active Rac1 and Cdc42. Chemical inhibition of Rac1 activation reduced Salmonella entry into control MEFs by 52.5 ± 12.4 % (mean ± S.E.M., n = 3, P < 0.05) (Figure 3A). Pull-down assays of active Rac1 (using GST–PAK-CRIB, which binds only GTP-Rac1) confirmed that NSC23766 abrogated Salmonella-induced Rac1 activation without changing levels of total Rac1 (Figure 3B). Importantly, no active Rac1 was detected in IQGAP1-null cells infected with Salmonella (Figure 3B). Notably, the number of Salmonella that entered control MEFs incubated with the Rac1 inhibitor is reduced by 52.5 % compared to control cells, whereas Salmonella uptake in IQGAP1-null cells is reduced by 70.8 %, suggesting that IQGAP1 is required for Salmonella invasion into MEFs.
Cdc42. Collectively, these results, which validate the importance of Rac1 activation in *Salmonella* invasion into host cells, reveal that IQGAP1 is required for *Salmonella* to usurp Rac1 function to promote entry.

The second approach to dissect the roles of Rac1 and Cdc42 employed constitutively active (Q61L) forms of the Rho GTPases. Cells transfected with the Q61L constructs were identified by cell morphology: Q61L Rac1 induced membrane ruffling, while cells expressing Q61L Cdc42 had visibly enhanced formation of actin-rich cell extensions (Figure 3D). Neither constitutively active Rac1 nor constitutively active Cdc42 significantly altered *Salmonella* uptake into control MEFs (Figures 3D and 3E). *Salmonella* invasion into IQGAP1-null MEFs was significantly augmented by transfection of Q61L Rac1, reaching 69.5 ± 8.8% (mean ± S.E.M., n = 3, P < 0.05) of levels observed in control MEFs (Figures 3D and 3E). By contrast, constitutively active Cdc42 had no significant effect on *Salmonella* invasion into IQGAP1-null MEFs. These results reinforce the concept that optimal *Salmonella* infection requires the binding of Rac1, but not Cdc42, to IQGAP1.

**Salmonella invasion into MEFs is partially dependent on binding of MEK and ERK to IQGAP1**

The results shown above reveal that the interactions of Rac1 and actin with IQGAP1 comprise only part of the mechanism by which *Salmonella* usurps IQGAP1 function to enter host cells. We therefore set out to identify other IQGAP1-regulated pathways that account for the decreased uptake of *Salmonella* into IQGAP1-null cells. We chose to explore the MEK/ERK module of the MAPK pathway for several reasons. ERK is activated upon *Salmonella* infection [17] and inhibition of MEK/ERK signalling attenuates *Salmonella* uptake into eukaryotic cells [13,19]. Moreover, IQGAP1 binds and modulates the activity of both MEK and ERK [30,31]. We therefore hypothesized that *Salmonella* may usurp the IQGAP1-dependent activation of MEK/ERK. We again employed a multi-faceted approach, using both selected discrete IQGAP1 mutant constructs and a cell-permeant MAPK inhibitor. We used two well-characterized IQGAP1 mutant constructs; one termed IQGAP1ΔIQ that does not bind MEK [30], the other termed IQGAP1ΔWW that selectively lacks binding to ERK [31] (Figure 2B). *Salmonella* invasion into IQGAP1-null MEFs reconstituted with IQGAP1ΔIQ or IQGAP1ΔWW (Figure 4A) was evaluated by confocal microscopy. IQGAP1ΔIQ and IQGAP1ΔWW each partially rescued *Salmonella* invasion to 54.6 ± 7.6% and 57.5 ± 12.5% (mean ± S.E.M., n = 3, P < 0.05) respectively of that seen in control MEFs (Figures 4B and 4C). These results suggest that the efficiency of *Salmonella* entry is partly dependent on MEK and ERK binding to IQGAP1. The MEK inhibitor PD98059 reduced *Salmonella* invasion into control MEFs by 42.5 ± 9.0% (mean ± S.E.M., n = 3, P < 0.05) (Figure 4C). Importantly, PD98059 did not impair *Salmonella* invasion into IQGAP1-null MEFs (Figure 4C). These observations imply that IQGAP1 is necessary for *Salmonella* to usurp MAPK signalling. This postulate is supported by the finding that IQGAP1ΔIQ or IQGAP1ΔWW partially rescues *Salmonella* invasion into MEFs despite treatment with PD98059 (Figure 4C).

To confirm the integrated role of MAPK and IQGAP1 in *Salmonella* infection, we evaluated activation of the MAPK cascade by measuring levels of phospho-ERK by Western blotting. Uninfected MEFs, both +/+ and −/−, have very low levels of active ERK (Figure 4D). Infection of control cells with *Salmonella* markedly increased phospho-ERK, without altering total ERK. The ability of *Salmonella* to activate ERK was significantly attenuated in IQGAP1-null MEFs (Figures 4D
Figure 3  *Salmonella* utilizes IQGAP1 interactions with Rac1, but not Cdc42, to invade MEFs

(A) Control (+/+ ) and IQGAP1-null (−/− ) MEFs were pre-incubated with vehicle or the Rac1 inhibitor NSC23766 for 30 min and then infected with DsRed-tagged *Salmonella* at an MOI value of 30 for 30 min. Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+ , white bars) and IQGAP1-null (−/− , black bars) MEFs. Data, expressed as means ± S.E.M. with control (+/+ ) MEFs treated with vehicle set at 1, were obtained from three independent experiments. *Significantly different from control (+/+ ) MEFs treated with vehicle (P < 0.05). †Significantly different from IQGAP1-null (−/− ) MEFs treated with vehicle and from IQGAP1-null MEFs treated with NSC23766 (P < 0.05). (B) Equal amounts of protein lysate were prepared from control (+/+ ) and IQGAP1-null (−/− ) MEFs infected with *Salmonella* at an MOI of 30 for 30 min. Samples were resolved by Western blotting and probed with an anti-Rac1 antibody (total Rac1). Equal amounts of protein lysate were also incubated with GST–PAK-CRIB as described in the Experimental section. Complexes were collected with glutathione–Sepharose and resolved by SDS/PAGE. Western blots were probed with an anti-Rac1 antibody (active Rac1). (C) Equal amounts of protein lysate were prepared from control (+/+ ) and IQGAP1-null (−/− ) MEFs infected with *Salmonella* at an MOI value of 30 for 30 min. Western blots were probed with anti-Cdc42 antibody (total Cdc42). Equal amounts of protein were also incubated with GST–WASP–GBD as described in the Experimental section. Complexes were collected with glutathione–Sepharose and resolved by SDS/PAGE. Samples were resolved by Western blotting and probed with anti-Cdc42 antibody (active Cdc42). As a positive control, protein lysates were pre-incubated with GTP[S] prior to probing with anti-Cdc42 antibody (right-hand panels). (D) Control (+/+ ) and IQGAP1-null (−/− ) MEFs were transfected with constitutively active (Q61L) Rac1 or constitutively active (Q61L) Cdc42 prior to infection with DsRed-tagged *Salmonella* at an MOI value of 30 for 30 min. Representative confocal micrographs are shown, indicating intracellular *Salmonella* (left-hand panels) and F-actin (filamentous actin centre panels). Merged image (right-hand panels) represents a composite of both channels (*Salmonella* in red and F-actin in blue). Scale bar = 10 μm. (E) Histograms depict the average number of intracellular *Salmonella* per cell in control MEFs (+/+ , white bars) and IQGAP1-null MEFs (−/− , black bars), which were either untransfected, transfected with constitutively active (Q61L) Rac1 or transfected with constitutively active (Q61L) Cdc42. Data, expressed as means ± S.E.M. with untransfected control (+/+ ) MEFs set at 1, were obtained from three independent experiments. *Significantly different from control (+/+ ) MEFs (P < 0.05). †Significantly different from IQGAP1-null (−/− ) MEFs, either untransfected or transfected with Q61L Cdc42 (P < 0.05).

and 4E). Importantly, ERK activation by *Salmonella* was completely rescued by reconstituting IQGAP1-null MEFs with full-length IQGAP1. In contrast, reconstitution of IQGAP1-null cells with IQGAP1ΔIQ or IQGAP1ΔWW failed to significantly increase activation of the MAPK pathway (Figures 4D and 4E). Collectively, these results show that MAPK signalling is exploited by *Salmonella* during invasion into MEFs, and that this function is mediated through IQGAP1.

**IQGAP1 integrates Rac1 and MAPK signalling for *Salmonella* entry**

In order to dissect the functional roles of IQGAP1, Rac1, MEK and ERK during *Salmonella* invasion, we pre-treated control MEFs, IQGAP1-null MEFs and IQGAP1-null MEFs reconstituted with IQGAP1ΔMK24 with the MEK inhibitor PD98059. *Salmonella* invasion into IQGAP1-null MEFs reconstituted with the non-Rac1-binding IQGAP1ΔMK24 construct was essentially identical with that observed in control MEFs in which MEK signalling had been blocked with PD98059 (Figure 5A). Importantly, PD98059 significantly reduces *Salmonella* entry into IQGAP1-null MEFs expressing IQGAP1ΔMK24, suggesting that the binding of MAPK and Rac1 to IQGAP1 are additive. Moreover, concurrent disruption of Rac1 binding to IQGAP1 (with IQGAP1ΔMK24) and MAPK signalling (with PD98059) reduces *Salmonella* infection by the same magnitude as elimination of IQGAP1 (Figure 5A).
Salmonella usurps IQGAP1 during infection

Figure 4  Salmonella invasion is partially dependent on MEK and ERK binding to IQGAP1

(A) Equal amounts of protein lysate obtained from control MEFs (+/+), IQGAP1-null MEFs (−/−) as well as IQGAP1-null MEFs transfected with GFP–IQGAP1ΔIQ (ΔIQ) or GFP–IQGAP1ΔWW (ΔWW) were resolved by SDS/PAGE. Western blots were probed (immunoblot, IB) with anti-IQGAP1 and anti-tubulin (loading control) antibodies. Results are representative of three independent experiments. (B) IQGAP1-null MEFs transfected with GFP–IQGAP1ΔIQ (top panels) or GFP–IQGAP1ΔWW (bottom panels) were infected with DsRed-tagged Salmonella at an MOI value of 30 for 30 min, incubated with gentamycin for 2 h and washed extensively to remove extracellular bacteria. Cells were then fixed and stained for F-actin (filamentous actin). Confocal micrographs illustrate GFP–IQGAP1ΔIQ (top row, left-hand panel) and GFP–IQGAP1ΔWW (bottom row, left-hand panel), intracellular Salmonella (centre-left panels) and F-actin (centre-right panels). Merged image (right-hand panels) represents a composite of all three channels (GFP–IQGAP1ΔIQ and GFP–IQGAP1ΔWW in green, Salmonella in red and F-actin in blue). Representative images are shown. Scale bar = 10 μm. (C) Histograms depict the average number of intracellular Salmonella per cell in control (+/+), IQGAP1-null (−/−) and IQGAP1-null MEFs transfected with GFP–IQGAP1ΔIQ (ΔIQ) or GFP–IQGAP1ΔWW (ΔWW) constructs. Where indicated, cells were pre-incubated with vehicle or the MEK1 inhibitor PD98059 for 30 min prior to Salmonella infection. Data, expressed as means ± S.E.M. with (+/+ ) MEFs incubated with vehicle set at 1, were obtained from three independent experiments. *Significantly different from control (+/+ ) MEFs treated with vehicle (P < 0.05). †Significantly different from IQGAP1-null (−/−) MEFS treated with vehicle (P < 0.05). §Significantly different from IQGAP1-null (−/−) MEFS reconstituted with GFP–IQGAP1ΔIQ or with GFP–IQGAP1ΔWW (P < 0.05). (D) Control (+/+) and IQGAP1-null (−/−) MEFS were uninfected (−) or infected (+) with Salmonella at an MOI value of 30 for 30 min. In addition, IQGAP1-null MEFS transfected with full-length GFP–IQGAP1 (WT), GFP–IQGAP1ΔIQ (ΔIQ) or GFP–IQGAP1ΔWW (ΔWW) were infected with Salmonella. Equal amounts of protein lysate were resolved by SDS/PAGE. Western blots were probed (immunoblot, IB) with anti-(phospho-ERK) (pERK) antibodies, then stripped and reprobed with anti-ERK antibodies. Results are representative of three independent experiments. (E) The amounts of phospho-ERK in cells infected with Salmonella in the experiments depicted in (D) were quantified by densitometry. Data, expressed as means ± S.E.M., were obtained from three independent experiments. *Significantly different (P < 0.05) from both control (+/+) MEFS and IQGAP1-null MEFS reconstituted with full-length IQGAP1 (WT).

Collectively, these results indicate that Salmonella usurps both Rac1 and MAPK signalling to achieve entry into MEFs and that these activities require IQGAP1.

DISCUSSION

Invasion into eukaryotic cells is an essential component of microbial pathogenesis, but the molecular mechanisms underlying infection are incompletely understood. Taking advantage of the availability of IQGAP1-null cells that we generated [29], we observe that the absence of IQGAP1 impairs entry of Salmonella into host cells by 75%. Verification that Salmonella requires IQGAP1 for efficient entry is provided by our results that invasion is completely restored when IQGAP1-null cells are reconstituted with wild-type IQGAP1. Uptake of beads occurs normally in cells lacking IQGAP1, indicating that our observation is not merely a non-specific effect of IQGAP1 on phagocytosis.

IQGAP1 binds to and regulates a diverse variety of proteins, thereby co-ordinating several fundamental cellular activities [28]. A number of IQGAP1-binding partners link pathogenic microbes...
Figure 5  *Salmonella* targets IQGAP1 function for entry into MEFs in a Rac1- and MAPK-dependent manner

(A) Control (+/+), IQGAP1-null (−/−) MEFs and IQGAP1-null MEFs reconstituted with GFP–IQGAP1ΔMK24 were pre-incubated with vehicle or the MEK1 inhibitor PD98059. After 30 min, cells were infected with DsRed-tagged *Salmonella* at an MOI value of 30 for 30 min. Histograms depict the average number of intracellular *Salmonella* per cell in control (+/+), IQGAP1-null (−/−) and IQGAP1-null MEFs reconstituted with GFP–IQGAP1ΔMK24 (ΔMK24). Data, expressed as means ± S.E.M. with (+/+ ) MEFs incubated with vehicle set at 1, were obtained from three independent experiments. *Significantly different from control (+/+ ) MEFs treated with vehicle (\( P < 0.05 \)).†Significantly different from IQGAP1-null (−/−) MEFs reconstituted with GFP–IQGAP1ΔMK24 and control (+/+ ) MEFs treated with PD98059 (\( P < 0.05 \)). (B) Proposed model by which IQGAP1 integrates Rac1 and MAPK signalling during *Salmonella* invasion into MEFs. a, IQGAP1 binds to Rac1, actin, MEK and ERK, facilitating efficient entry of *Salmonella* into control (+/+ ) MEFs. Hatched areas indicate sites of *Salmonella* entry at nascent membrane ruffles. b, Disruption of Rac1 binding to IQGAP1 or inhibition of Rac1 activation (with NSC23766) reduces the ability of *Salmonella* to usurp Rac1 function, and consequently, to enter the host cell. Disruption of actin binding to IQGAP1 also precludes optimal *Salmonella* uptake. MAPK signalling is unaffected under these conditions, allowing partial *Salmonella* invasion. c, Disruption of MEK or ERK binding to IQGAP1 or inhibition of MAPK signalling with PD98059 partially reduces *Salmonella* invasion. Rac1 signalling is unaffected under these conditions, allowing partial *Salmonella* invasion. d, Loss of IQGAP1 expression abrogates both Rac1- and MAPK-mediated signalling required for efficient *Salmonella* invasion.

to invasion of host cells [32]. In order to dissect out the host-cell pathways that are regulated by *Salmonella* via IQGAP1, we used a series of well-characterized mutant IQGAP1 constructs. The first construct examined was IQGAP1ΔMK24, which is unable to bind Rac1 or Cdc42 and maintain them in the active state [37]. This was selected both because the *Salmonella* effectors SopE and SopE2 facilitate invasion by activating Rac1 and Cdc42 in the host cell [3,4,6] and because IQGAP1 maintains Rac1 and Cdc42 in their active GTP-bound forms [25–27]. Reconstitution of IQGAP1-null MEFs with IQGAP1ΔMK24 or transfection with constitutively active Rac1 increased *Salmonella* entry to 50% of that into control cells. Consistent with this observation, a selective Rac1 inhibitor reduced by 50% *Salmonella* invasion into control cells, but had no influence on entry into IQGAP1-null cells. Moreover, the presence of IQGAP1 in cells incubated with *Salmonella* was required to detect active Rac1. The uniformity of the results observed with these different strategies strongly suggests that IQGAP1 is required for *Salmonella* to fully usurp Rac1 function to infect host cells. Our analyses also identified some differences between Cdc42 and Rac1. Unlike Rac1, constitutively active Cdc42 failed to increase *Salmonella* invasion into IQGAP1-null cells, suggesting that *Salmonella* does not target interactions of IQGAP1 with Cdc42 during infection of MEFs.

Reconstitution of IQGAP1-null MEFs with IQGAP1-G75Q, which lacks actin binding, reveals that optimal *Salmonella* infection also requires association of actin with IQGAP1. The ability of IQGAP1 to cross-link actin filaments is enhanced by Rho GTPases [23] and one interpretation of our results is that *Salmonella* manipulates IQGAP1 to link Rac1 to actin. However, because the effects of IQGAP1ΔMK24 and IQGAP1-G75Q could be independent, we generated IQGAP1-G75QΔMK24 that binds neither Rac1 nor actin. Invasion of *Salmonella* into IQGAP1-null MEFs reconstituted with the double mutant IQGAP1-G75QΔMK24 was essentially the same as that into cells...
reconstituted with each of the single mutant constructs, validating that IQGAP1, Rac1 and actin share a common pathway targeted by Salmonella during infection of MEFs. Thus our results suggest that IQGAP1 serves as a scaffold that links Rac1 activation to actin assembly required for membrane ruffling and Salmonella internalization.

Interactions of Rac1 with IQGAP1 account for only part of the Salmonella invasion defect observed with IQGAP1-null cells. Therefore a separate IQGAP1-associated pathway is also likely to be targeted by Salmonella during entry into MEFs. In addition to Rho GTPases, Salmonella usurps other host-cell functions to effect entry. One of these is the MAPK pathway. Salmonella stimulates the activity of MEK and ERK in human intestinal cells [17,18] and macrophages [19]. Because IQGAP1 is a MAPK scaffold [30,31], we reconstituted IQGAP1-null cells with IQGAP1ΔIQ and IQGAP1ΔWW, which lack binding to MEK and ERK respectively. Disruption of IQGAP1 binding to MEK or to ERK yielded Salmonella invasion 45% lower than that measured with wild-type IQGAP1. We also observed that the MEK/ERK inhibitor PD98059 reduced Salmonella invasion into control MEFs by 45%, which is the same magnitude of attenuation as that recently reported with human foreskin fibroblasts [13]. In contrast, PD98059 had no effect on Salmonella entry into IQGAP1-null cells, regardless of whether they were reconstituted with IQGAP1ΔIQ, IQGAP1ΔWW or neither. Consistent with these findings, the ability of Salmonella to activate ERK is significantly attenuated in MEFs lacking IQGAP1. ERK activation by Salmonella is completely restored when IQGAP1-null cells were reconstituted with wild-type IQGAP1, but no rescue was seen with IQGAP1 constructs that lack MEK or ERK binding. Collectively these results strongly suggest that an interaction of IQGAP1 with MEK and ERK is necessary for Salmonella to usurp MAPK signalling, implying that IQGAP1 may link Salmonella infection to MAPK activation.

One of the most striking results from the present study is that Rac1 and MAPK signalling are additive in terms of their contributions to Salmonella invasion into MEFs. This is substantiated by the finding that concurrent disruption of Rac1 and MAPK signalling reduces Salmonella invasion by 75%, whereas inhibition of Rac1 alone or MAPK alone reduces invasion by 45%. Interestingly, simultaneous inhibition of Rac1 and MAPK signalling attenuates Salmonella infection to the same extent as complete absence of IQGAP1. This observation is congruent with accumulating evidence that IQGAP1 is a scaffold that integrates multiple intracellular signalling pathways [28,47]. Scaffold proteins control specificity and spatio-temporal aspects of cell function [48,49]. The participation of scaffolds in yeast and mammalian cell function has been the focus of considerable attention [14–16]. Although scaffolds are less recognized in bacterial infection, a recent study showed that the E. coli T3SS effector EspG assembles a signalling complex containing ADP-ribosylation factor and PAK [50]. To our knowledge, the present study is the first to identify a host cell protein scaffold that links three endogenous proteins in two distinct signalling pathways that are usurped by Salmonella to infect cells. Based on our results, we propose a model in which Salmonella exploits the Rac1-, actin-, MEK- and ERK-binding properties of IQGAP1, allowing efficient infection (Figure 5B). Disruption of the interaction between Rac1 and IQGAP1, actin and IQGAP1 or chemical inhibition of Rac1 activation, reduces Salmonella uptake into MEFs, but the pathogen continues to exploit interactions of IQGAP1 with MEK and ERK to achieve invasion (Figure 5B). Similarly, disruption of the interaction of MEK or ERK with IQGAP1 reduces Salmonella invasion, but partial uptake is observed by virtue of ongoing interactions between Rac1 and IQGAP1 (Figure 5B).

Finally, simultaneous suppression of Rac1 and MAPK signalling (and the greatest inhibition of Salmonella infection) is observed following complete loss of IQGAP1 expression (Figure 5B). In conclusion, the present study identifies IQGAP1 as an integrator of Rac1- and MAPK-dependent signalling usurped by Salmonella to achieve infection in the host. Further research into the molecular determinants of microbial pathogenesis is essential for the development of novel therapeutic agents for infectious diseases.

AUTHOR CONTRIBUTION

Hugh Kim designed and performed the research, analysed data and wrote the paper; Colin White contributed experimental methods and analysed data; Zhigang Li contributed biochemical reagents; and David Sacks designed the research, analysed data and wrote the paper.

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