Actin Pedestal Formation by Enteropathogenic *Escherichia coli* Is Regulated by IQGAP1, Calcium, and Calmodulin*

Matthew D. Brown, Lynn Bry, Zhigang Li, and David B. Sacks

From the Department of Pathology Brigham and Women’s Hospital, Harvard Medical School, Thorn 530, Boston, Massachusetts 02115

During infection, enteropathogenic *Escherichia coli* (EPEC) injects effector proteins into the host cell to manipulate the actin cytoskeleton and promote formation of actin pedestals. IQGAP1 is a multidomain protein that participates in numerous cellular functions, including Rac1/Cdc42 and Ca\(^{2+}\)/calmodulin signaling and actin polymerization. Here we report that IQGAP1, Ca\(^{2+}\), and calmodulin modulate actin pedestal formation by EPEC. Infection with EPEC promotes both the interaction of IQGAP1 with calmodulin and the localization of IQGAP1 and calmodulin to actin pedestals while reducing the interaction of IQGAP1 with Rac1 and Cdc42. IQGAP1-null fibroblasts display a reduced polymerization of actin in response to EPEC. In addition, antagonism of calmodulin or chelation of intracellular Ca\(^{2+}\) reduces EPEC-dependent actin polymerization. Furthermore, IQGAP1 specifically interacts with Tir in *vitro* and in cells. Together these data identify IQGAP1, Ca\(^{2+}\), and calmodulin as a novel signaling complex regulating actin pedestal formation by EPEC.

Enteropathogenic *Escherichia coli* (EPEC)\(^2\) continues to be a major cause of morbidity and mortality worldwide (1). During infection, EPEC induces the formation of actin pedestals, which support the bacteria on the surface of epithelial cells (1). After initial adhesion to target epithelial cells mediated by the bundle forming pilus, EPEC uses a type III secretion system (TTSS) to inject into the host cell effector proteins, which manipulate the actin cytoskeleton. Key to this process is the translocated intimin receptor (Tir). After injection into the host cell, Tir is translocated into the cell membrane where it acts as a receptor for intimin, a bacterial adhesin facilitating close adhesion of the pathogen to the target host cell (2). Binding of intimin clusters Tir and induces phosphorylation of a critical tyrosine residue at position 474 by the tyrosine kinase c-Fyn (3). The phosphoryl-}

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*This work was supported, in whole or in part, by a National Institutes of Health grants (to D. B. S. and L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Pathology Brigham and Women’s Hospital, Harvard Medical School, Thorn 530, 75 Francis St. Boston, MA 02115. Tel: 617-732-6627; Fax: 617-278-6921; E-mail: dsacks@rics.bwh.harvard.edu.

2 The abbreviations used are: EPEC, enteropathogenic *E. coli*; BA*P*TA, 1,2-bis(o-aminophenoxy) ethane-N,N,N’,N”-tetraacetic acid tetra(acetoxymethyl)ester; CRIB, Cdc42/Rac1 interactive domain; GAP, GTPase activating protein; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; N-WASP, neuronal Wiskott-Aldrich syndrome protein; PBS, phosphate-buffered saline; Tir, translocated intimin receptor; TTSS, type III secretion system; GST, glutathione S-transferase; YFP, yellow fluorescent protein; PAK, p21-activated kinase; RFP, red fluorescent protein.

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EXPERIMENTAL PROCEDURES

Reagents—All tissue culture reagents were obtained from Invitrogen. The IQGAP1 polyclonal (10) and monoclonal antibodies (13) have been previously described. Other antibodies used were anti-Rac1 (Millipore), anti-Cdc42 (Santa Cruz), anti-tubulin (Sigma), anti-calmodulin (23), and anti-green fluores-
cent protein (GFP) (Santa Cruz) monoclonal antibodies. All fluorescent secondary antibodies were from Molecular Probes.

Cell Culture and Bacterial Strains—The following EPEC strains were kindly provided by Michael Donnenberg (University of Maryland): E2348/69 (wild type), CVD206 (intimin negative), UMD888 (bfpA, espA, and eaeA negative), UMD872 (espA negative), and UMD901 (bfpA negative). All have been previously documented (24–27). GFP- or dsRed-expressing EPEC strains were created by electroporating eGFP- (Invitrogen) or dsRed-containing plasmids into the respective EPEC strains. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). IQGAP1-null mouse embryonic fibroblasts (MEFs) have been previously described (14, 22). Cells were transiently transfected with 5 μg of DNA using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.

Plasmid Construction—Tir was cloned using an EPEC aliquot as a template. Using the primers 5’-CGGGATCCCTTAATGGAATACCCTTGTAAT and 3’-CGGAAATCTCTAGATATTAAACGAAACGTACTGTGCTCC. Tir DNA was amplified by PCR and cloned into RFP (provided by Roger Tsien, University of California, San Diego), Myc pcDNA3, and pGEX4T vectors using standard techniques. GST fusion proteins were expressed in E. coli and isolated with glutathione-Sepharose essentially as described (10). The GST tag was cleaved from GST-IQGAP1 using tobacco etch virus protease as described (28). To construct pEYFP-calmodulin, the human calmodulin sequence was amplified by PCR using the following primers: 5’-GGGATCCCTAATGGAATACCCTTGTAAT and 3’-GGGATCCCTAATGGAATACCCTTGTAAT. The PCR product was cloned into pEYFP (Clontech) using the BspE1 and BamH1 sites. All constructs were verified by sequencing and Western blotting.

Bacterial Culture Quantification—Bacteria were grown for 16 h at 37 °C with shaking, 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, plated in triplicate onto MacConkey Agar (Remel Inc.), and incubated at 37 °C overnight for quantification. After incubation for 16 h at 37 °C, the number of EPEC cells was determined, allowing the appropriate multiplicity of infection to be added to cultured cells.

EPEC Infection—Infection of HeLa cells or MEFs was performed essentially as described (20, 22). Briefly, E. coli cells 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 and equal amounts of protein lysate 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 5 times with buffer A, and resolved by SDS-PAGE. Western blots were probed with the antibodies indicated in the figures 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).

Immunoprecipitations—Immunoprecipitations were performed essentially as described (20, 22). Briefly, E. coli cells were lysed in buffer A before and after EPEC infection. Equal amounts of protein lysate were resolved by Western blotting or incubated with GST-PK-CRIB for 3 h at 4 °C. Glutathione beads were used to pull down complexes of PK-CRIB with GFP-bound Rac1 and Cdc42 and washed in buffer A. Samples were resolved by SDS-PAGE and probed for Cdc42 or Rac1 as indicated.

Immunocytochemistry—Immunocytochemistry was performed essentially as previously (22). Cells were fixed in 4% paraformaldehyde, PBS for 20 min at 22 °C and blocked/permeabilized in 0.2% Triton X-100, 3% bovine serum albumin, PBS for 1 h at 22 °C. Relevant antibodies were diluted 1:100 into 0.2% Triton, 1% bovine serum albumin/PBS and incubated at 4 °C overnight. Cells were washed with PBS, then incubated with the appropriate secondary antibody, diluted 1:1000 in PBS, for 1 h at 22 °C. For analysis of F-actin, 546- or 633-conjugated phalloidin (Molecular Probes) was included with secondary antibodies. To enhance the signal of GFP-IQGAP1 and YFP-calmodulin, cells were incubated with anti-GFP antibody followed by anti-mouse 488-conjugated secondary antibody. Coverslips were washed with PBS, mounted, and analyzed using a Zeiss LSM 510 confocal microscope, 100× oil objective and LSM software. Subsequent image processing was performed using Adobe Photoshop.

TNT Product Production—[35S]Methionine-labeled TNT products were produced with the TNT Quick Coupled Transcription/Translation system (Promega, Madison, WI) as described (28, 33). Briefly, 2 μg of IQGAP1 plasmid was incubated with 40 μl of TNT Quick Master Mix (Promega) and 2 μCi (1 Ci = 37 GBq) of [35S]methionine (PerkinElmer Life Sciences) at 30 °C for 90 min. 10 μl of relevant TNT product was incubated with 40 μl of GST or GST-Tir (>90%) pure) for 3 h at 4 °C. Glutathione-Sepharose beads were used to isolate GST proteins. Products were identified by SDS/PAGE and autoradiography.

In Vitro Binding Assays—Equal amounts of purified IQGAP1 (90% pure) were incubated for 3 h at 4 °C with 5 μg of GST-Tir in 1 ml of buffer A and protease inhibitor mixture. Complexes were isolated with glutathione-Sepharose, resolved by SDS/PAGE, and processed by Western blotting.

Quantification of Actin Pedestals—MEFs were treated as indicated and infected with EPEC as described above. Three hours later, cells were fixed and stained for actin. Using standard fluorescence microscopy, slides were analyzed, and the
number of EPEC that promoted polymerization of actin were quantified and expressed as a percentage of total bacteria adhered to the cells (34). Polymerized actin was detected using fluorescent phalloidin. Any accumulation of phalloidin staining at the site of bacterial adhesion was considered to indicate the presence of polymerized actin. Data are from at least three independent experiments.

### Statistical Analysis
Statistical analysis was performed using GraphPad Prism. One-way analysis of variance tests were used.

### RESULTS

**IQGAP1 Localizes to EPEC-induced Actin Pedestals**—A large body of work has shown that several actin regulatory proteins of the host cell are required for EPEC to form actin pedestals. These include Arp2/3, N-WASP, Nck, and α-actinin (2, 21, 35). To determine whether IQGAP1, a well characterized regulator of the actin cytoskeleton (15–17), participates in EPEC pathogenesis, we first examined the localization of IQGAP1 during the formation of actin pedestals by EPEC. HeLa cells were infected with EPEC, which stably express GFP (Fig. 1A). Cells were fixed 3 h post-infection and stained for endogenous IQGAP1 (red). EPEC were identified by expression of GFP (green). Analysis by confocal microscopy reveals strong staining of IQGAP1 at the sites of EPEC adhesion (Fig. 1A). To examine the time course of IQGAP1 localization to actin pedestals, HeLa cells were infected with GFP EPEC for 1, 2, or 3 h before fixing and staining cells for actin (red) and endogenous IQGAP1 (blue) (Fig. 1B). In agreement with previous work (for review, see Ref. 1), EPEC induces focused polymerization of actin at sites of bacterial adhesion (Fig. 1B). The polymerization of actin in the host cell is clearly seen from 1 h and persists to 3 h. Examination of endogenous IQGAP1 shows that it co-localizes with actin and EPEC at these pedestals at all the time points examined (Fig. 1B).

The interaction of IQGAP1 with Rac1 and Cdc42 has been well characterized (15). We previously reported that IQGAP1 is required for efficient host cell invasion by *Salmonella* through modulation of Rac1, Cdc42, and actin polymerization (22). Therefore, we examined the possible relationship of Rac1 and Cdc42 to EPEC-induced actin pedestals. HeLa cells were fixed before and after 3 h of infection with GFP EPEC (green). Cells were stained for IQGAP1 (blue) and either Cdc42 or Rac1 (red). The scale bar equals 10 μm. All data are representative of at least four independent experiments. The arrowhead indicates localization of IQGAP1 to sites of EPEC attachment.

EPEC Infection Inhibits the Interaction of IQGAP1 with Rac1 and Cdc42—Our data demonstrating that IQGAP1, but not Rac1 or Cdc42, localizes to EPEC-induced actin pedestals led to...
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FIGURE 3. EPEC infection reduces the interaction of IQGAP1 with Rac1 and Cdc42 and increases active Cdc42 but not Rac1. HeLa cells, plated in 10-cm culture dishes, were infected with EPEC. At the times indicated, cells were lysed, and endogenous IQGAP1 was immunoprecipitated (IP) from equal amounts of protein lysate as described under "Experimental Procedures." Non-immune rabbit serum (NIRS) was used as a negative control. A, Western blots of lysate and immune complexes were probed for IQGAP1, Rac1, and Cdc42. B, the amount of Rac1 and Cdc42 that co-immunoprecipitated with IQGAP1 was quantified by densitometry and corrected for the amount of IQGAP1 in the same sample. Data are from three separate experiments and show the mean ± S.E. *p < 0.05. C, MEFs were incubated with or without EPEC. At the times indicated, cells were lysed, and active (GTP-bound) Rac1 or Cdc42 was pulled down using GST-PAK CRIB as described under "Experimental Procedures." D, the level of active Rac1 and Cdc42 was quantified by densitometry and corrected for Rac1/Cdc42 in the lysate. Data are from 13 (Cdc42) or 12 (Rac1) independent experiments and show mean ± S.E. *p < 0.05.

the hypothesis that EPEC infection could disrupt the interaction between IQGAP1 and these GTPases. We tested this hypothesis by immunoprecipitation. HeLa cells were infected with EPEC, and IQGAP1 was immunoprecipitated. Compared with non-infected cells, the amount of Rac1 and Cdc42 that co-immunoprecipitated with IQGAP1 is significantly decreased by 48 and 55%, respectively, 2 h after the addition of EPEC (Fig. 3, A and B). Although there is a small and reproducible decrease in co-immunoprecipitation of both GTPases 1 h after the addition of EPEC, this change is not significant. The specificity of the co-immunoprecipitation is verified by the absence of Rac1 and Cdc42 from samples precipitated with non-immune rabbit serum (Fig. 3A). The total amount of IQGAP1, Rac1, and Cdc42 in the cell lysates is not altered by EPEC infection (Fig. 3A). These data suggest that EPEC alters the function of IQGAP1 and/or Cdc42 and Rac1 in the host cell during infection.

EPEC Infection Increases Activity of Cdc42 but Not Rac1—Many pathogenic microbes, such as Salmonella and Shigella, regulate small GTPases during infection (21). However, it is currently unknown whether EPEC is also able to manipulate the activity of the Rho GTPases. Therefore, to test whether EPEC is able to alter the GTP-loading of Rac1 and Cdc42, MEFs were infected with EPEC for 0, 1, and 2 h, and lysates were prepared. GTP-bound Rac1 and Cdc42 were isolated using GST-PAK CRIB (which recognizes only the active, GTP-bound forms of Rac1 and Cdc42) as previously (20, 22). The amount of GTP-bound Rac1 remains unchanged in response to incubation with EPEC (Fig. 3, C and D). Interestingly, although the activity of Cdc42 does not change after 1 h EPEC infection, after 2 h there is an 88% increase in the level of active Cdc42 (Fig. 3, C and D). These data suggest that during infection EPEC is able to specifically activate Cdc42 but not Rac1.

Infection by EPEC Promotes the Interaction between IQGAP1 and Calmodulin—The interaction of calmodulin with IQGAP1 inhibits the ability of IQGAP1 to interact with many of its other binding partners, including Rac1 and Cdc42 (7, 10, 16, 17). Therefore, we examined whether infection by EPEC alters the association of calmodulin with IQGAP1. After HeLa cells were infected with wild type EPEC for 1, 2, and 3 h, endogenous IQGAP1 was immunoprecipitated. Probing the Western blots for calmodulin reveals that EPEC promotes the interaction of calmodulin with IQGAP1 in a time-dependent manner (Fig. 4, A and B). Compared with non-infected cells (time = 0), the amount of calmodulin that co-immunoprecipitates with IQGAP1 after 2 and 3 h of infection with EPEC is increased by 72 and 170%, respectively. Note that the association of calmodulin and IQGAP1 is not altered after 1 h of infection. The total amounts of IQGAP1 and calmodulin in the cell lysates are unaltered by EPEC infection (Fig. 4). These data suggest that EPEC might manipulate calmodulin function. To test this hypothesis, the subcellular location of calmodulin in cells infected with EPEC was examined. MEFs were transiently transfected with YFP-calmodulin and infected with dsRed EPEC. Cells were fixed and stained for actin and YFP. YFP-calmodulin displays strong colocalization with actin along the entire length of the actin pedestal (Fig. 4C, arrowhead). Similarly, endogenous calmodulin also localizes to EPEC-induced actin pedestals (data not shown). We then aimed to examine the colocalization of calmodulin with IQGAP1 during EPEC infection. Our anti-IQGAP1 antibody has a lower affinity for mouse IQGAP1 compared with the human homologue, so we utilized HeLa cells for this experiment. HeLa cells expressing YFP-calmodulin were infected with dsRed EPEC and stained for YFP and endogenous IQGAP1. In the absence of EPEC, calmodulin (green) co-localizes with IQGAP1 (blue) around the cell periphery and in membrane ruffles (Fig. 4D). As shown earlier (see Fig. 1), IQGAP1 localizes to sites of bacterial attachment 1 h after the addition of EPEC. At this time point, only weak localization of calmodulin at the pedestal could be detected (Fig. 4D). Consistent with the co-immunoprecipitation data, 3 h after the addition of EPEC
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Calmodulin displays a strong localization to pedestals where it co-localizes with IQGAP1. Together these data suggest a role for calmodulin in actin pedestal formation by EPEC and suggest that the interaction of IQGAP1 with calmodulin is temporally regulated during the infection process.

IQGAP1 Is Required for Efficient Actin Pedestal Formation by EPEC—To examine whether IQGAP1 is required for actin pedestal formation, we compared normal and IQGAP1-null MEFs. Cells were infected with GFP EPEC for 3 h, then fixed and stained for actin. Using standard fluorescent microscopy, we quantified how many EPEC, which were adherent to the MEFs, had induced the polymerization of actin. These data are expressed as a percentage of the total number of EPEC counted. In normal MEFs, 76% of bacteria induce actin polymerization at 3 h under our assay conditions (Fig. 5). In the absence of IQGAP1, the percentage of EPEC that induce actin polymerization is significantly (p < 0.001) reduced to 43% (Fig. 5A, black bars).

To verify the requirement for IQGAP1 in actin pedestal formation, we examined its ability to “rescue” EPEC infection in IQGAP1-null MEFs. Cells were transfected with GFP-tagged IQGAP1 (or GFP alone as control), infected with EPEC, fixed, and subsequently stained for GFP and actin. In cells expressing GFP alone, 46% of EPEC induce actin pedestals (Fig. 5B). However, in MEFs expressing GFP-IQGAP1, this value increases to 75% (Fig. 5B), indicating exogenously expressed IQGAP1 completely rescues the ability of EPEC to induce actin pedestals in IQGAP1-null MEFs. Representative images of cells are shown (Fig. 5C). These data strongly support the concept that IQGAP1 is an important factor for efficient pedestal formation by EPEC.

Calmodulin Is Required for Efficient Actin Pedestal Formation by EPEC—The localization to actin pedestals of calmodulin and its increased association with IQGAP1 after EPEC infection suggest that calmodulin may be required for efficient pedestal formation. To test this hypothesis, we used the specific cell-permeable calmodulin antagonist, CGS9343B (32, 36–38). Normal MEFs and IQGAP1-null MEFs were preincubated with vehicle or CGS9343B for 6 h followed by infection with EPEC and fixation. Quantification of actin pedestals in normal MEFs reveals that antagonism of calmodulin significantly (p < 0.01) reduces the ability of EPEC to induce actin polymerization (Fig. 5A, compare black and white bars for MEF+/−). In contrast, CGS9343B does not significantly alter EPEC-dependent actin polymerization in IQGAP1-null MEFs (Fig. 5). Incubation of MEFs with CGS9343B for 9 h does not significantly alter cell viability (data not shown). These data argue that calmodulin participates in the process by which EPEC induces actin pedestals.

Ca²⁺ Is Required for Actin Pedestal Formation—The participation of Ca²⁺ during EPEC-induced actin pedestal formation is contentious, with publications both supporting (39, 40) and refuting (41) a role for Ca²⁺. The involvement of calmodulin in EPEC-induced pedestal formation implies that Ca²⁺ may participate in EPEC pathogenesis. In an attempt to clarify this issue, we used the cell-permeable Ca²⁺ chelator, BAPTA. Normal and IQGAP1-null MEFs were preincubated with BAPTA or vehicle for 16 h before infection with EPEC. EPEC-induced actin polymerization was quantified. As shown earlier (see Fig. 5), IQGAP1-null MEFs display significantly reduced ability to polymerize actin when infected by EPEC compared with normal MEFs (Fig. 6A, black bars). Chelation of [Ca²⁺], signifi-
cantly impairs the ability of EPEC to induce actin polymerization in normal MEFs (Fig. 6A, compare black and white bars for MEF^+/+). Analogous to the data obtained with CGS9343B, BAPTA does not further reduce actin polymerization in IQGAP1-null MEFs (Fig. 6A). Representative confocal images are shown (Fig. 6B). We verified that incubation of MEFs with 20 μM BAPTA for 19 h does not significantly alter cell viability (data not shown). These data suggest that Ca^{2+} is required for efficient formation of actin pedestals by EPEC. Furthermore, knock-out of IQGAP1 and chelation of Ca^{2+} do not have synergistic effects, implying that IQGAP1 and Ca^{2+} function in the same signaling pathway.

Localization of Actin, but Not IQGAP1, Requires Clustered Tir—To gain insight into the EPEC effector proteins that subvert IQGAP1 function, we utilized EPEC mutant strains that are deficient in specific virulence factors. HeLa cells were infected with the indicated EPEC mutants for 3 h, fixed, and stained for actin (red) and IQGAP1 (blue). EPEC were visualized by expression of GFP (green). As shown earlier (Fig. 1), wild type EPEC promotes the formation of actin pedestals, to which IQGAP1 localizes (Fig. 7A). Binding of intimin, a bacterial surface protein, induces clustering and tyrosine phosphorylation of Tir, promoting actin polymerization (2, 3, 42). Clustering of Tir and pedestal formation is, thus, dependent on binding to intimin (3, 42), as EPEC lacking intimin are unable to promote actin polymerization (3, 42, 43). Therefore, we examined whether IQGAP1 can localize to EPEC adhesion sites in the absence of intimin (Fig. 7). In agreement with work by others (3, 42), we observe that intimin-deficient EPEC are unable to induce polymerization of actin (Fig. 7B). Interestingly, IQGAP1 still localizes to sites of attachment of intimin-null EPEC but displays a more diffuse staining pattern than that seen with wild type EPEC (compare Figs. 7, A and B). EPEC lacking the bundle forming pilus (bfpA^-) promote actin polymerization and induce localization of IQGAP1 to pedestals (Fig. 7C). EPEC deficient in the TTSS (espA^-) or deficient in the TTSS, intimin, and bundle forming pilus (espA^- bfpA^- intimin^-) are unable to induce actin polymerization or localization of IQGAP1 (Fig. 7, D and E). These data suggest that injection of effector proteins via the TTSS is essential for EPEC to induce the localization of IQGAP1 to sites of EPEC attachment. In contrast, neither actin polymerization nor clustering of the Tir is necessary.

IQGAP1 Interacts Directly with Tir—The localization of IQGAP1 to intimin-deficient EPEC pointed to a potential interaction between IQGAP1 and Tir. To test this hypothesis, we

![Graph](image_url)

**FIGURE 5. Knock-out of IQGAP1 and antagonism of calmodulin reduce actin polymerization by EPEC.** Normal (MEF^+/+) or IQGAP1-null (MEF^-/-) MEFs were treated with ethanol (EtOH, black bars) or 40 μM CGS9343B (CGS, white bars) for 6 h before infection with GFP EPEC. 3 h post-infection, cells were fixed and stained for actin and analyzed by fluorescence microscopy. A, the number of EPEC inducing polymerization of actin at sites of adhesion was quantified. Data are expressed as a percentage of total bacteria adherent to the cells. Means ± S.E. from three independent experiments are shown. Total number of bacteria counted: MEF^+/+ EtOH, 3748; CGS, 4340; MEF^-/- EtOH, 1535; CGS, 1207. *, p < 0.01; **, p < 0.001, as determined by one-way analysis of variance. B, IQGAP1-null MEFs (MEF^-/-) were transfected with GFP (black column) or GFP-IQGAP1 (white column) before infection with GFP EPEC for 3 h. Cells were fixed, stained for actin and GFP, and analyzed by fluorescence microscopy. Means ± S.D. from two independent experiments are shown. Total number of bacteria counted: GFP, 821; GFP-IQGAP1, 714, *, p < 0.05, as determined by t test. C, representative confocal microscope images showing MEFs infected with GFP EPEC (green) and stained for actin (red). Merge represents composite of both channels. The scale bar equals 10 μm.
examined the ability of GST-Tir to bind pure IQGAP1. In vitro analysis shows that IQGAP1 binds directly to GST-Tir (Fig. 8A). The association is specific to Tir, because no IQGAP1 is present in the pull-down with GST alone. The interaction was confirmed using the transcription and translation (TNT) system; full-length IQGAP1 labeled with [35S]methionine binds specifically to Tir (Fig. 8B). In addition, Tir interacts only with the N-terminal half of IQGAP1. No binding to the C-terminal half is detected. To test whether IQGAP1 and Tir interact in cells, HeLa cells were transfected with Myc-Tir, and endogenous IQGAP1 was immunoprecipitated. Tir co-immunoprecipitates with IQGAP1 (Fig. 8C), showing that IQGAP1 and Tir interact in intact cells. Furthermore, IQGAP1 and RFP-Tir colocalize in HeLa cells (Fig. 8D). In the absence of EPEC, both proteins are distributed diffusely through the cell, with some enrichment in membrane ruffles. In cells infected with EPEC, RFP-Tir shows a strong co-localization with endogenous IQGAP1 at sites of bacterial adhesion (Fig. 8D). Together these data show that IQGAP1 interacts with Tir in vitro and in cells.

**DISCUSSION**

EPEC is a major cause of food-borne disease worldwide, causing severe diarrhea. Recent work has uncovered some of the molecular mechanisms contributing to EPEC infection (2, 21, 44). Despite these advances in our understanding of EPEC pathogenesis, questions concerning the signaling mechanism controlling actin pedestal formation remain (2, 21). Here we report that IQGAP1 is a novel host cell component required for efficient actin pedestal formation by EPEC. Consistent with this observation, IQGAP1 localizes to actin pedestals in normal host cells, whereas cells lacking IQGAP1 have significantly attenuated actin polymerization in response to EPEC infection. The magnitude of reduction in actin pedestal formation by IQGAP1 knock-out is similar to that reported for knockdown of dynamin, which reduced EPEC-dependent actin polymerization from 80 to 50% (34). Knockdown of other host cell proteins, such as Nck (45) and N-WASP (46), also reduce pedestal formation, but no quantification was performed in these studies, and we cannot directly compare our data to these reports.
Knock out of IQGAP1 does not abrogate pedestal formation by EPEC. Therefore, EPEC may utilize multiple signaling pathways during pedestal formation, with IQGAP1 functioning in one (or more) of these. In the absence of IQGAP1 the other pathways presumably compensate, enabling actin pedestal formation to still occur, albeit with significantly reduced efficiency. Alternatively, EPEC may activate a single pathway, within which IQGAP1 serves to promote efficient actin polymerization. This signaling pathway can still function without IQGAP1 but less efficiently. Further studies are required to identify which of these hypotheses is correct.

Interestingly, EPEC infection alters the interaction of IQGAP1 with Rac1, Cdc42, and calmodulin, suggesting IQGAP1 function is specifically regulated by EPEC. These data argue that EPEC usurps IQGAP1 function to facilitate actin pedestal formation. IQGAP1 participates in the normal function of the small GTPases, Rac1, and Cdc42 (15–17, 20). Rac1 and Cdc42 have a fundamental role in invasion by several microbial pathogens, including Salmonella, Yersinia, and Shigella (21). The participation of Rac1 and Cdc42 in EPEC-dependent pedestal formation is less well established. It has been reported that dominant negative Rac1 and Cdc42 constructs and Rho GTPase inhibitors did not impair the ability of EPEC to induce actin pedestal formation (47). We detected no co-localization of either Rac1 or Cdc42 with actin at EPEC-induced pedestals. To our knowledge, no prior publication has directly analyzed the effect of EPEC on the activation of the Rho GTPases in the host cell. We observed that whereas the amount of GTP-bound Rac1 did not change, levels of active Cdc42 increased by 88% 2 h after the addition of EPEC. Interestingly, EPEC infection reduces the interaction of Rac1 and Cdc42 with IQGAP1, with a concomitant increase in the association of calmodulin with IQGAP1. This would suggest that the increase in active Cdc42 during EPEC infection is not due to an interaction of Cdc42 with IQGAP1. Further studies are required to identify the mechanism by which EPEC activates Cdc42 and whether this is an important process for actin pedestal formation.

Prior studies document that calmodulin abrogates the binding of Cdc42 to IQGAP1 (7). Furthermore, calmodulin reduces the interaction of IQGAP1 with all of its binding partners examined to date, namely Rap1 (11), E-cadherin (32), β-catenin (48), S100B (49), B-raf (50), and actin (9, 10). It is, therefore, likely that enhanced association of calmodulin with IQGAP1 induced by EPEC impairs the ability of Cdc42 and Rac1 to bind to IQGAP1. It is not known whether the reduced association between Cdc42/Rac1 and IQGAP1 contributes to EPEC pathogenesis. An interesting, and perhaps pertinent, proteomic analysis documented that the expression of several small GTPases, including Rac1 and TC10 and their regulators, is reduced by TTSS-delivered EPEC effector proteins (51). Although we did not observe any change in Rac1 and Cdc42 expression levels during EPEC infection, there are some important experimental differences between our data and this report. Hardwidge et al. (51) used Caco-2 cells infected for 4 h, whereas we used HeLa cells infected for no more than 2 h. It is also possible the techniques we used are not sensitive enough to detect the change reported. The potential role of small GTPases in EPEC infection remains to be resolved.

The use of specific EPEC mutants has greatly facilitated the understanding of the mechanisms utilized during infection (24–27). We employed this strategy to gain insight into how EPEC exploits IQGAP1. Localization of IQGAP1 to EPEC requires the TTSS, as an espA-deficient mutant is unable to induce translocation of IQGAP1. These data suggest IQGAP1 recruitment to sites of bacterial adhesion is dependent on the injection of one or more bacterial proteins into the host cell. Indeed, IQGAP1 binds directly to the injected protein, Tir, and colocalizes with Tir at sites of EPEC adhesion. IQGAP1 localizes to EPEC attachment sites at a relatively short time interval post-infection, and so it would appear that the interaction with Tir is responsible for the recruitment of IQGAP1 to sites of EPEC adhesion. In support of this hypothesis, EPEC deficient in intimin are still able to cluster IQGAP1 at bacterial adhesion sites despite being unable to promote clustering and phospho-
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...rulation of Tir or actin polymerization. The localization of IQGAP1 to intimin^-EPEC is not identical to that induced by wild type EPEC. Although IQGAP1 is recruited to pedestals directly below wild type EPEC, it is much more diffusely associated with intimin^-EPEC. This finding is very similar to observations made by Goosney et al. (35) who documented that α-actinin interacts with Tir and localizes to wild type EPEC but is much more diffusely associated with intimin-deficient EPEC. Although no published reports have demonstrated a direct interaction between IQGAP1 and α-actinin, both of these proteins interact directly with Tir (this study and Ref. 35) and are components of receptor complexes, including α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, nphrin, and chloride channels (52–54). Furthermore, IQGAP1 was recently shown to bind directly to N-WASP, a crucial component of EPEC-dependent actin pedestal formation (2), and regulate N-WASP- and Arp2/3-dependent actin polymerization (55, 56). Although it has not yet been established what function IQGAP1 performs at the Tir, as a scaffold IQGAP1 can assemble many signaling proteins and complexes at the receptor. It is, therefore, tempting to speculate that IQGAP1, α-actinin, and N-WASP form a molecular link between Tir and the actin cytoskeleton, mediating signaling from Tir to promote polymerization of actin.

Although unable to promote actin polymerization, intimin-deficient EPEC can still activate other signaling pathways, including phosphorylation of host cell proteins (57, 58) and inositol trisphosphate fluxes (59). These inositol trisphosphate fluxes have been hypothesized to increase [Ca^{2+}], in response to EPEC (39, 59). The participation of Ca^{2+} in EPEC infection is contentious (1). There is evidence supporting the premise that Ca^{2+} is required for pedestal formation (39, 60), substantiated by reports that demonstrate an increase in [Ca^{2+}] during EPEC adhesion (39, 40). However, another publication disputes this finding, and the authors were unable to detect a change in [Ca^{2+}] after EPEC infection (41). The reason for the contradictory data is not known, but as identified in a review (1), this is clearly a controversial issue that requires clarification. Discrepancies in the published literature may be due to experimental differences or procedures used to measure [Ca^{2+}], (1). Indeed, these studies examine different time intervals of EPEC infection and report substantial differences in the basal [Ca^{2+}], from the same cell line (39, 41). Nevertheless, given the well documented role of Ca^{2+} and calmodulin in the regulation of the actin cytoskeleton (61), their participation in EPEC-induced actin pedestals is perhaps not surprising. Ca^{2+} has been associated with cell motility for 125 years (62), and [Ca^{2+}], are highest in the trailing edge and lowest at the leading edge of migrating cells (63). Ca^{2+}/calmodulin regulates cell shape by controlling the interaction of myosin with actin (62) and modulating the activity of several actin polymerization regulators, including the bundling protein, myristoylated alanine-rich protein kinase C substrate (MARCKS), and the actin severing protein, gelsolin (64).

Our data implicate Ca^{2+} in EPEC infection. By chelating [Ca^{2+}], with the cell-permeable agent BAPTA, we observed a significant reduction in EPEC-dependent actin polymerization. Consistent with these findings, antagonism of calmodulin impairs the ability of EPEC to induce pedestals. Collectively, these data suggest that normal Ca^{2+}/calmodulin signaling is required for optimum formation of actin pedestals. In support of this concept, we observed that infection by EPEC significantly increases the interaction between IQGAP1 and calmodulin. Ca^{2+} is known to modulate the association between calmodulin and IQGAP1; 1/2-fold more IQGAP1 binds to calmodulin in the presence of Ca^{2+} than in the absence of Ca^{2+} (7, 9, 10). Inhibition of EPEC-induced actin polymerization by either BAPTA or CGS9343B treatment is not further enhanced by knock-out of IQGAP1, suggesting IQGAP1 functions in the same pathways as Ca^{2+} and calmodulin. Therefore, we hypothesize that Ca^{2+}, calmodulin, and IQGAP1 form a novel regulatory signaling complex, controlling actin polymerization at the site of Tir injection. In this model (Fig. 9), EPEC binds to the surface of cells and injects effector proteins via the TTSS (Fig. 9A). This induces the binding of IQGAP1 to one of the injected protein...
proteins, the transmembrane receptor, Tir. Tir is retained at the site of injection through the association with intimin on the surface of the EPEC. Via its interaction with Tir, IQGAP1 accumulates at the site of bacterial adhesion (Fig. 9B). At the same time, EPEC also induce a localized increase in $[\text{Ca}^{2+}]$, which enhances the association of IQGAP1 with calmodulin and reduces the association of Rac1 and Cdc42 with IQGAP1. Consequently, calmodulin also accumulates at the site of bacterial adhesion. Through the recruitment of Nck, N-WASP and Arp2/3 also accumulate at the Tir, thereby inducing polymerization of actin. The presence of IQGAP1 and calmodulin and the interaction between IQGAP1 and Tir augments this localized actin polymerization, contributing to pedestal formation (Fig. 9C). Furthermore, IQGAP1 bundles actin filaments to ensure that an ordered structure of parallel actin filaments is formed (Fig. 9D).

Two reports require consideration in assessing this model. First, $\text{Ca}^{2+}$/calmodulin reduces the interaction of IQGAP1 with actin and attenuates actin-cross linking (9). However, IQGAP1 can still bind detectable amounts of actin even with a 10-fold molar excess of $\text{Ca}^{2+}$/calmodulin (9). Moreover, these studies were performed exclusively in vitro. Second, GTP-bound Cdc42 was reported to increase IQGAP1-dependent actin cross-linking (65). Similar to the previous report, this analysis was performed in vitro. In addition, because GST-Cdc42 was used, it is possible that the observed effect may be mediated by dimerization of GST. Importantly, EPEC-induced actin polymerization does not require active Rac1 and Cdc42 (47). Through the direct recruitment of the Cdc42 effector N-WASP and the Arp2/3 complex, EPEC appear to be capable of bypassing these GTPases during pedestal formation. This may explain why IQGAP1 does not interact with either Rac1 or Cdc42, well established binding partners of IQGAP1 (15, 17), during EPEC-induced actin pedestal formation.

IQGAP1 can form oligomers (28), and under physiological conditions it is likely that $\text{Ca}^{2+}$/calmodulin and Cdc42 do not provide such a rigid “all or none” and “clear-cut” regulation of IQGAP1 function but, rather, provide fine tuning of IQGAP1 interactions with binding partners and a more discrete modulation of IQGAP1 function. The observations of the effect of $\text{Ca}^{2+}$/calmodulin on the functional interactions between IQGAP1 and $\beta$-catenin support this notion. Although $\text{Ca}^{2+}$/calmodulin abrogates the binding of $\beta$-catenin to IQGAP1 in vitro, IQGAP1 stimulation of $\beta$-catenin-mediated transcriptional co-activation in cells is dependent on the interaction of IQGAP1 with calmodulin (48). During EPEC infection, $\text{Ca}^{2+}$/calmodulin regulation of IQGAP1 may ensure that the correct actin structures are formed (for example dense parallel actin filaments instead of the branched filaments formed during lamellipodia formation) or that actin polymerization occurs only at the correct site.

One concern that has been raised in regard to the concept of a change in $[\text{Ca}^{2+}]$, regulating actin polymerization during pedestal formation is how such a signal could promote a highly focused and localized response (41). Our data may ameliorate this concern. It is tempting to speculate that through the ability to integrate $\text{Ca}^{2+}$/calmodulin signaling with the regulation of actin dynamics, IQGAP1 could mediate the required localized polymerization of actin in response to $\text{Ca}^{2+}$. Collectively, our findings identify IQGAP1 as a previously unidentified host cell protein target of Tir and a component of a signaling pathway containing $\text{Ca}^{2+}$ and calmodulin, which regulates formation of EPEC-dependent actin pedestals.

Acknowledgments—Confocal analysis was performed at the Harvard NeuroDiscovery Center imaging facility. We thank Michael Donnenberg (University of Maryland) for EPEC mutant strains and Roger Tsien (University of California, San Diego) for plasmids.

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