

Cyclophilin A Controls *Salmonella* Internalization Levels and is Present at *E. coli* Actin-Rich Pedestals

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S. Typhimurium*), enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) commandeer the actin cytoskeleton of their host cells as a crucial step in their infectious processes. These pathogens depend on the injection of their own effectors directly into target host cells in order to usurp cellular signaling pathways that lead to morphological actin rearrangements in those cells. Here we show that the PPIase Cyclophilin A (CypA) is a novel component of *S. Typhimurium*-induced membrane ruffles and functions to restrict bacterial invasion levels, as in cells depleted of CypA, bacterial loads increase. We also demonstrate that CypA requires the EPEC effector Tir as well as pedestal formation for its recruitment to bacterial attachment sites and that its presence at pedestals also holds during EHEC infections. Finally, we demonstrate that CypA is found at lamellipodia; actin-rich structures at the leading edge of motile cells. Our findings further establish CypA as a component of dynamic actin-rich structures formed during bacterial infections and within cells in general. *Anat Rec*, 301:2086–2094, 2018. © 2018 Wiley Periodicals, Inc.

Key words: *S. Typhimurium*; membrane ruffles; EPEC; EHEC; pedestals; cyclophilin A

Cyclophilin A (CypA) is an abundant cytosolic and secreted eukaryotic protein that belongs to a family of evolutionary conserved prolyl *cis-trans* isomerases (PPIases) that catalyze a variety cellular processes that require protein folding and trafficking (Ryffel et al., 1991;

Sherry et al., 1992; Xu et al., 1992). These PPIases catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds (Lang et al., 1987). Previous studies have linked CypA expression to several cellular processes that require actin polymerization such as cell migration and general

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cytoskeletal remodeling (Xu et al., 1992; Calhoun et al., 2008; Saleh et al., 2015).

S. Typhimurium and pathogenic *E. coli* (EPEC and EHEC), manipulate the host actin cytoskeleton for their invasion of host cells and attachment to the cell surface respectively. These bacteria accomplish this by injecting specialized effectors directly into the host cytoplasm through a type three secretion systems (T3SS). *S. Typhimurium* bacteria inject an array of T3SS effectors directly into the host cell cytoplasm that either directly influence actin polymerization (SipA) or co-opt host signaling pathways (SopB, SopE, and SopE2) leading to the activation of host actin nucleators such as the Arp2/3 complex at the regions of *S. Typhimurium* interaction with the host cell membrane (Galyov et al., 1997; Hardt et al., 1998; Zhou et al., 1999a; Stender et al., 2000; Bakshi et al., 2000). Ultimately, this causes localized cytoskeletal remodeling in the form of short-lived actin-rich membrane protrusions (ruffles) that extend outward and engulf the bacteria (Francis et al., 1993).

EPEC and EHEC remain extracellular where they use their T3SS to inject an assortment of bacterial effectors into host cells to cause disease phenotypes (Jarvis et al., 1995). One of these effectors, the translocated intimin receptor (Tir), becomes embedded in the host plasma membrane where it promotes firm bacterial attachment by docking extracellularly with the bacterial surface protein intimin (Kenny et al., 1997). Intracellular phosphorylation of Tir at tyrosine 474 allows the attached bacteria to co-opt the actin polymerization machinery to generate an extensive meshwork of filamentous actin (F-actin) directly beneath the site of bacterial adherence (Rosenshine et al., 1992; Kenny, 1999; Kalman et al., 1999). These F-actin networks press up against the host plasma membrane and raise the bacteria off the natural surface of the host cell (forming motile structures called pedestals) enabling them to “surf” atop the epithelia (Sanger et al., 1996).

Here we demonstrate that both invasive and adherent bacterial pathogens hijack CypA during their cytoskeletal remodeling of host cells. We show that CypA is enriched at membrane ruffles generated by *S. Typhimurium* and functions to restrict bacterial internalization. We also demonstrate that CypA recruitment to EPEC requires the Tir effector and that EHEC pedestals also contain CypA. Finally we show that CypA is present at actin-rich lamellipodia generated at the leading edge of motile cells.

MATERIALS AND METHODS

Cell Culture

Human cervical (HeLa) and *Potorous tridactylus* kidney (Ptk2) epithelial cells were obtained from American Type Culture Collection (ATCC) (numbers CCL-2 and CCL-56, respectively). CypA WT (*PPIA*^{+/+}) and CypA KO (*PPIA*^{-/-}) mouse embryonic fibroblast cells (MEFs) were generated previously (Sun et al., 2014). Ptk2 cells were cultured in DMEM/F-12 (1:1) (Hyclone, GE Healthcare) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific). All other cell lines were cultured using Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (Hyclone, GE Healthcare) supplemented with 10% FBS. All cells were maintained in a cell culture incubator at 37°C and 5% CO₂. For experiments, cells were trypsinized and seeded into clear polystyrene

6-well or 24-well format plates (Corning) containing glass coverslips.

Bacterial Strains and Growth Conditions

The bacterial strains used included wild-type *S. Typhimurium* (strain SL1344), *S. Typhimurium* SL1344 mutants $\Delta sipA$, and $\Delta sopB/sopE/sopE2$, wild-type EPEC (strain E2348/69), E2348/69 mutant $\Delta escN$, wild-type EPEC (strain JPN15), JPN15 mutants Δtir , Δtir complemented with *tir*, and Δtir complemented with *tirY474F* and wild-type EHEC (serotype O157:H7). All bacterial strains were grown at 37°C using either LB agar or LB broth (BD Biosciences).

Bacterial Infections

For *S. Typhimurium* infections, overnight shaking cultures of the bacteria were subcultured at 37°C (with shaking) in LB broth for 3–4 hr to activate the bacteria. Cultured cells in well plates were infected with a 1:100 dilution of the bacteria for 15 min to observe membrane ruffle formation. For EPEC and EHEC infections, overnight broth cultures were used to infect cultured cells in well plates at a multiplicity of infection (MOI) of 10 for 6–8 hr.

Gentamicin Protection Assay

Cultured cells in well plates were incubated with wild-type *S. Typhimurium* for 20 min to allow for bacterial invasion. Following this, well plates were washed 3× with Dulbecco’s phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS [–/–]) (Gibco, Thermo Fisher Scientific) and then cells were left to incubate for 1 hr in media containing 50 µg/mL gentamicin (to kill extracellular bacteria). After the 1 hr, cells were rinsed 5× with Dulbecco’s phosphate buffered saline containing Ca²⁺ and Mg²⁺ (PBS [+/+]) (Gibco, Thermo Fisher Scientific) and intracellular bacteria released by treatment of cells with a 1% solution of Triton X-100 (made in PBS [+/+]) for 5 min. After the 5 min incubation, serial dilutions of the wells were performed in 96 well format assay blocks. Dilutions were selected (so as to give a count of between 30 and 300 bacterial colonies), spread onto LB plates and incubated for 24 hr at 37°C prior to their enumeration.

Antibodies and Reagents

Antibodies and reagents used in this study included: Alexa Fluor 594- and 488-conjugated phalloidin (Invitrogen); Alexa Fluor 594- and 488-conjugated goat anti-rabbit antibodies (2 µg/mL, Invitrogen); rabbit anti-cyclophilin A (10 µg/mL for immunofluorescence and 1 µg/mL for Western blot, Abcam, ab41684); mouse anti- α -tubulin (1:1,000 for Western blot, Developmental Studies Hybridoma Bank, 12G10); normal rabbit IgG control antibody (10 µg/mL for immunofluorescence, R&D Systems) and HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (1 µg/mL, Invitrogen). The mouse monoclonal anti- α -tubulin antibody (12G10) was deposited to the DSHB by Frankel, J./Nelsen, E.M.

Immunolocalization

For immunofluorescence studies, cells were fixed at room temperature for 15 min using pre-warmed (37°C) 3% paraformaldehyde (made in 150 mM NaCl, 4 mM Na/KPO₄, 5.0 mM KCl, pH 7.3) and then washed 3× with PBS [–/–]. Cells were treated with –20°C acetone for 10 min to permeabilize cells and then allowed to dry at room temperature for 30 min. Samples were then blocked with 5% normal goat serum (prepared in PBS [–/–]) for 20 min after which samples were incubated overnight at 4°C with primary antibodies prepared in TPBS/BSA (PBS [–/–], 0.5% Tween-20, 0.1% bovine serum albumin [BSA]). The following day, samples were washed 3X with TPBS/BSA and then treated with secondary antibodies (Alexa Fluor 594- or 488-conjugated goat anti-rabbit antibodies) at room temperature in the dark for 2 hr. To visualize F-actin, samples were incubated with Alexa Fluor 594- or 488-conjugated phalloidin (prepared in PBS [–/–]) for 20 min. Samples were rinsed 3× with PBS [–/–] and then mounted onto glass slides using Prolong Diamond antifade mountant containing DAPI (Invitrogen).

Lysate Preparation and Western Blotting

Lysates were prepared by washing cultured cells 3× with pre-warmed PBS [+/+] then treating the cells with ice-chilled RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 10% SDS) with cOmplete™ Mini EDTA-free protease inhibitor cocktail (Roche) on ice for 5 min. Cells were scraped and lysates collected into microcentrifuge tubes. The lysates were spun at 4°C and 10,000g for 10 min to pellet cellular debris. Top supernatants were collected into fresh microcentrifuge tubes and immediately stored at –80°C following flash freezing in liquid nitrogen. Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Pierce). For Western blotting, lysates samples were first prepared in 6× Laemmli buffer then boiled for 10 min. Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels and subsequently resolved by electrophoresis. Following protein separation, gels were rinsed in water for 5 min and then transferred onto nitrocellulose membranes using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad). Post transfer, membranes were washed for 5 min in TBST (Tris-buffered saline, 0.05% Tween-20), blocked with 4% Blotto (Santa Cruz Biotechnology (made in TBST) and then incubated with primary antibodies (diluted in TBST, 1% BSA) overnight at 4°C. The next day, membranes were rinsed 3× with TBST for 10 min each prior to incubating with secondary antibodies (HRP-conjugated goat anti-rabbit or goat anti-mouse) for 1 hr at room temperature. To visualize protein bands, membranes were treated with Western Lightning Plus-ECL (PerkinElmer) following the manufacturer's instructions and imaged using a Fuji-film LAS-4000 imager (Fujifilm). To confirm equal loading, membranes were stripped of bound antibodies using mild stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH 2.2) and re-probed as described above using the mouse anti- α -tubulin antibody.

DNA Constructs

The DNA plasmid encoding GFP-CypA was generated previously (Calhoun et al., 2008) and mKate-LifeAct was a gift from Dr. Michael Davidson (Addgene plasmid # 54697).

Microscopy and Live Cell Imaging

Images were acquired using a Leica DMI4000B (Leica Microsystems) inverted fluorescent microscope and Hamamatsu Orca R2 CCD camera (Hamamatsu Photonics). All components were controlled by MetaMorph Imaging System software (Universal Imaging). Images evaluated using MetaMorph Imaging System software or ImageJ. For live cell imaging, a Chamlide IC top stage incubator system was utilized to maintain a constant temperature and environment of 37°C and 5% humidified CO₂ gas in air.

Cell Culture Transfections

All DNA transfections of cultured cells were performed using jetPEI or jetPRIME transfection reagents (Polyplus Transfection) and carried out according to the manufacturer's instructions. Briefly, DNA:reagent complexes were added to cultured cells in 35 mm fluorodishes (World Precision Instruments) and allowed to incubate at 37°C for 4 hr. Following this, the culture media was replaced and cells were incubated for an additional 24 hr at 37°C to allow for sufficient expression of the plasmid gene product. Transfected cells were imaged live by time-lapse microscopy as described above.

Statistical Analysis

Statistical analysis was performed with Welch's t test using GraphPad Prism version 6.01. For Figure 1C, data were acquired from experiments performed three times (n = 3); graph shows nine bacterial CFU counts (3 averaged triplicate CFU values per genotype for each independent experiment) as well as average value (black bar; \pm standard deviation); 99% confidence intervals indicated by asterisks. All data involving immunofluorescence microscopy were obtained from experiments performed at least three times (n = 3). Additionally, all microscopy images shown are representative of experiments performed.

RESULTS

Cyclophilin A is Enriched at *S. Typhimurium* Membrane Ruffles

A crucial initial phase in *S. Typhimurium* pathogenesis is the ability of the bacteria to gain entry into their host cells. To do this, the bacteria exploit signaling pathways used by their hosts for actin polymerization through the use of injected bacterial effectors. The resulting effect exhibits as aggressive membrane ruffling that ultimately encapsulates any nearby *S. Typhimurium* and pulls them into the targeted cells (Francis et al., 1993). To determine if CypA is recruited to actin-rich membrane ruffles, we infected cultured cells with *S. Typhimurium* and immunolocalized fixed cells with antibodies against CypA. CypA was enriched at the *S. Typhimurium*-generated membrane ruffles (Fig. 1A) but did not colocalize completely with F-actin at those sites as bright CypA puncta were evident at areas with diminished actin signal (Fig. 1A).

As membrane ruffles are highly dynamic motile structures, we used live cell imaging to better understand the recruitment characteristics of CypA in relation to F-actin at the structures. To do this we co-transfected HeLa cells with mKate-LifeAct and GFP-CypA and found that CypA

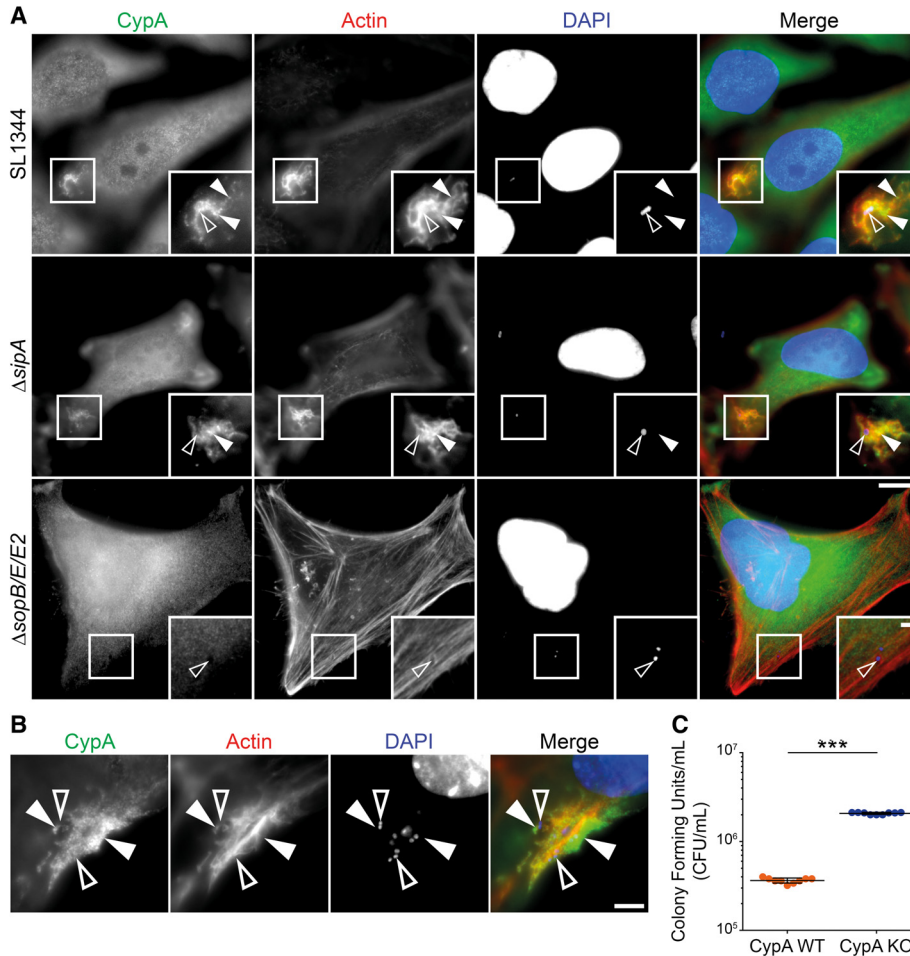


Fig. 1. Cyclophilin A is recruited to *S. Typhimurium* membrane ruffles and controls bacteria internalization levels. **(A)** HeLa cells infected with wild-type *S. Typhimurium* or the *S. Typhimurium* mutants ($\Delta sipA$ and $\Delta sopB/sopE/sopE2$) were fixed then stained with a rabbit polyclonal CypA targeting antibody (green), DAPI (blue) to visualize DNA, and Alexa594-phalloidin (red) to visualize F-actin. CypA recruitment depends on membrane ruffle generation. Solid arrowheads within insets (enlargement of boxed regions) indicate CypA at ruffles. *S. Typhimurium* bacteria are indicated by open arrowheads. Scale bars, 10 and 2 μm (insets). **(B)** CypA immunolabeling of CypA WT mouse embryonic fibroblasts infected with wild-type *S. Typhimurium*. Solid arrowheads indicate some of the CypA at ruffles. Some *S. Typhimurium* bacteria are indicated by open arrowheads. Scale bar is 5 μm . **(C)** Quantification of bacterial internalization levels during infections of CypA WT and CypA KO cells. Three independent experiments ($n = 3$) were run and triplicate counts of bacteria levels (as CFU/mL) from each experiment were obtained using gentamicin protection assays. The average CFU/mL values are as follows: $3.644e5$ in CypA WT cells and $2.073e6$; $***P < 0.0001$.

was recruited to ruffles at essentially the same time as F-actin (Video 1: https://players.brightcove.net/656326989001/default_default/index.html?videoId=5972139892001). The majority of CypA colocalized with actin as the ruffles formed, suggesting CypA redistribution within the structures could be linked to the polymerization of F-actin.

Localized cytoskeletal remodeling and subsequent membrane ruffling required for *S. Typhimurium* entry into cells is triggered by the injection of an array of T3SS effectors (Galyov et al., 1997; Hardt et al., 1998; Zhou et al., 1999a; Stender et al., 2000; Bakshi et al., 2000). To investigate the requirement of these effectors for CypA recruitment, we utilized *S. Typhimurium* T3SS effector mutants. The T3SS effector SipA is not essential for ruffle formation but enhances bacterial invasion rates through its ability to bundle actin as well as to inhibit actin depolymerization (Zhou et al., 1999a, 1999b; Lilic et al., 2003; McGhie et al.,

2004). To determine if SipA is necessary for CypA recruitment to membrane ruffles, we immunolocalized CypA in cells infected with a *S. Typhimurium* $\Delta sipA$ mutant. During these infections we found that there was no observable change in the localization characteristics as compared with the wild-type infections (Fig. 1A).

The *S. Typhimurium* effectors (SopB, SopE, and SopE2) are involved in the activation of cellular Rho GTPases that cause the dynamic cytoskeletal remodeling at the membrane during bacterial invasion events (Galyov et al., 1997; Hardt et al., 1998; Stender et al., 2000; Bakshi et al., 2000). To assess if CypA recruitment at bacterial attachment sites depends on membrane ruffle formation or simply bacterial attachment, we utilized a *S. Typhimurium* mutant lacking the abovementioned effectors (Boyle et al., 2006) and found that CypA was absent from bacterial contact sites (Fig. 1A).

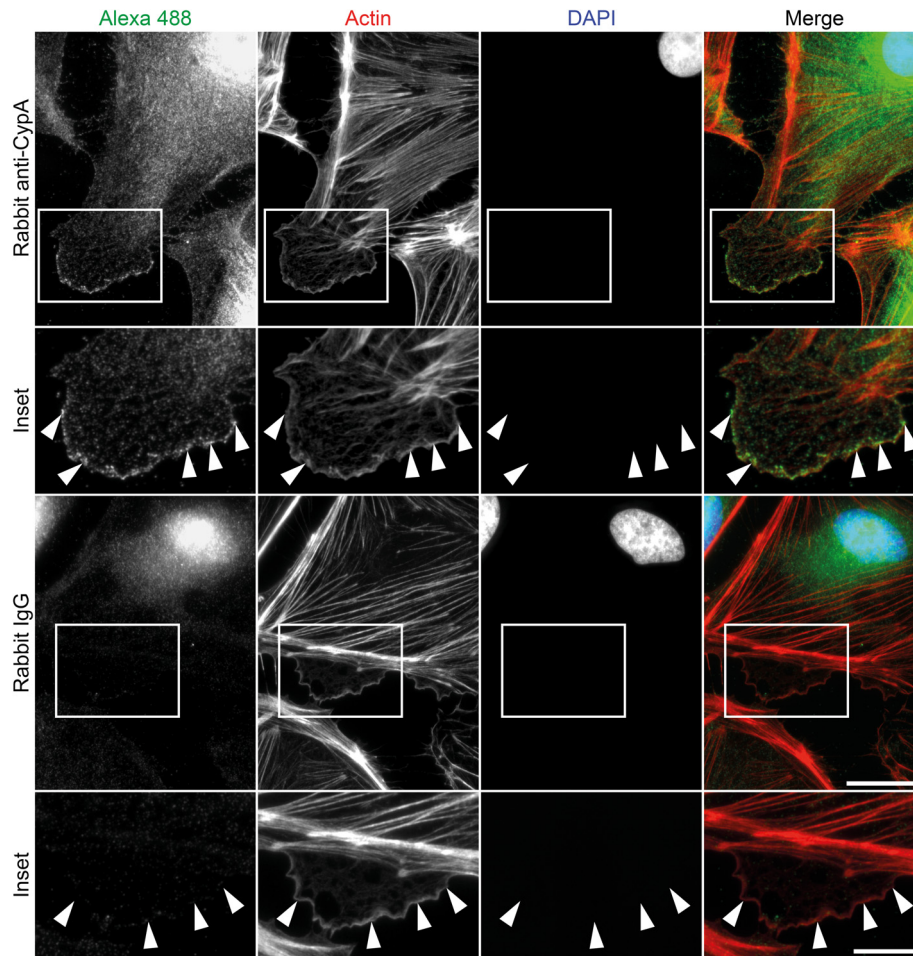


Fig. 2. Cyclophilin A is enriched at the lamellipodia leading edge of eukaryotic cells. Actively spreading Ptk2 cells were fixed and stained with a rabbit polyclonal CypA targeting antibody or rabbit IgG control antibody (green), DAPI (blue) to visualize DNA, and Alexa594-phalloidin (red) to visualize F-actin. Insets (enlargement of boxed regions) show the lamellipodium of motile cells; solid arrowheads point to the leading edge of the lamellipodia. Scale bars, 20 and 10 μm (insets).

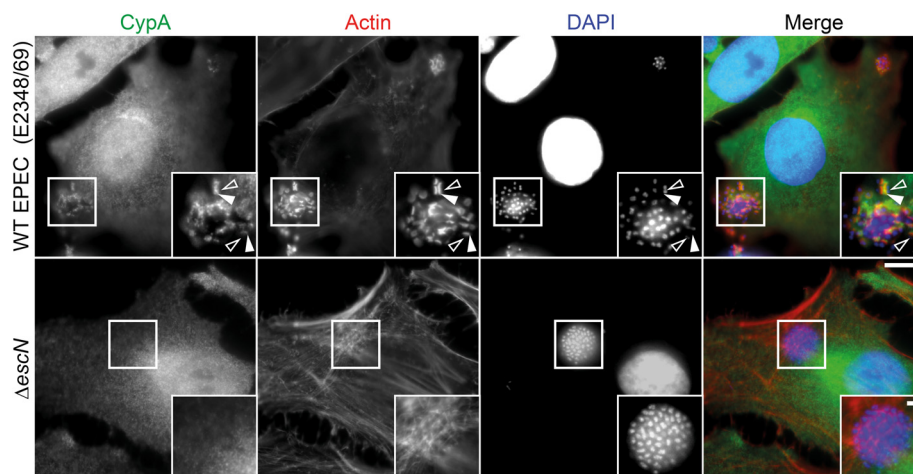


Fig. 3. Cyclophilin A recruitment to WT EPEC pedestals requires a functional T3SS. CypA (green), F-actin (red), and DNA (blue) were immunolocalized in HeLa cells infected with wild-type EPEC (WT EPEC E2348/69) and the T3SS mutant (ΔescN). CypA is enriched at wild-type EPEC pedestals (top) but not at bacterial attachment sites that do not form pedestals (bottom). Solid arrowheads indicate CypA at actin-rich pedestals while open arrowheads point to the corresponding attached bacteria. Scale bars, 10 and 2 μm (insets).

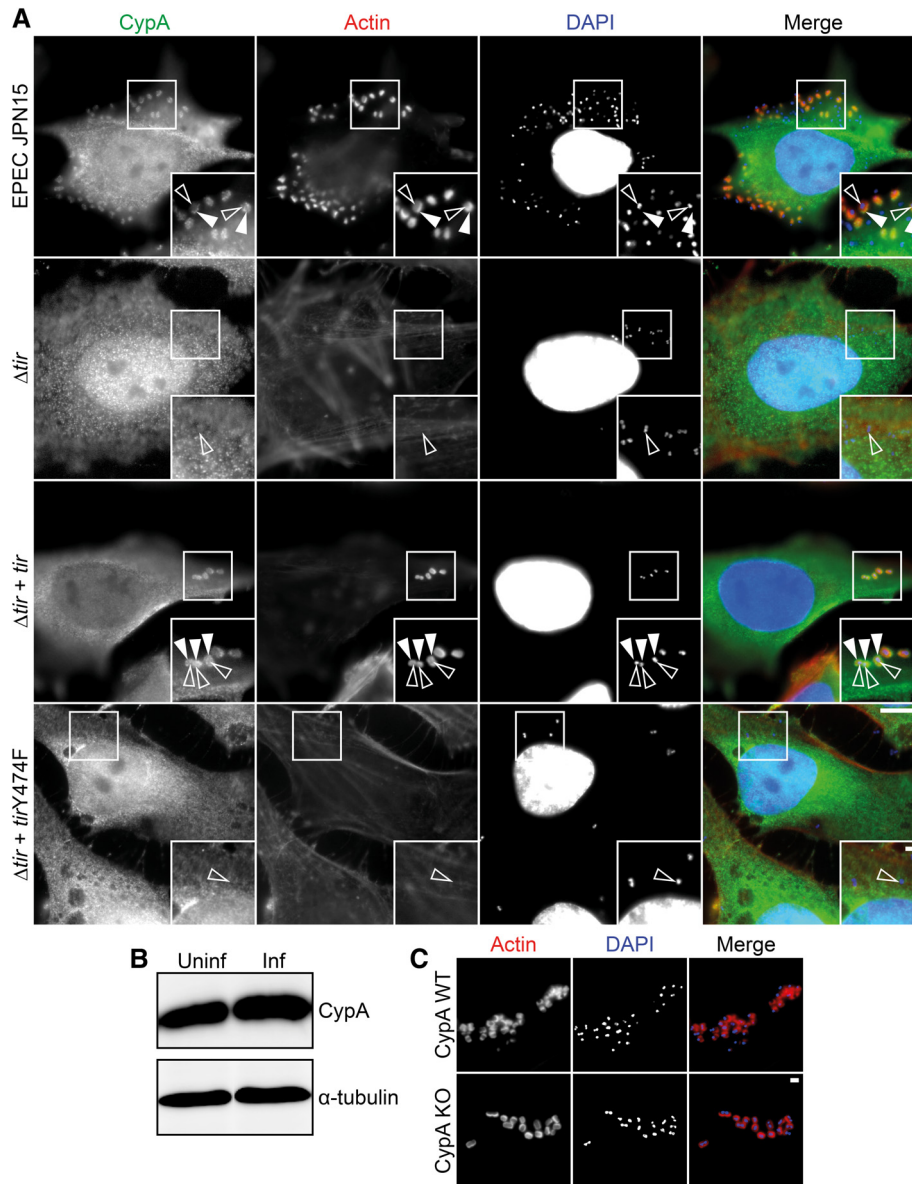


Fig. 4. Cyclophilin A recruitment at EPEC pedestals depends on Tir phosphorylation but its expression is not required for their formation. **(A)** Immunolocalization of CypA at sites of bacterial attachment during infections of HeLa cells with wild-type EPEC (JPN15) and JPN15 effector mutants: Δtir , $\Delta tir + tir$, and $\Delta tir + tirY474F$. Cells were stained with a rabbit polyclonal CypA targeting antibody (green), DAPI (blue) to visualize DNA, and Alexa594-phalloidin (red) to visualize F-actin. Insets depict regions of interest (boxes). Solid arrowheads indicate CypA at actin-rich pedestals while open arrowheads point to adhered bacteria. Scale bars, 10 and 2 μm (insets). **(B)** CypA protein levels are not altered during EPEC infections. Whole HeLa cell lysate from uninfected (Uninf) and 8 hr EPEC infections (Inf) were probed for endogenous CypA using a rabbit polyclonal anti-CypA antibody. The α -tubulin is shown as a loading control. **(C)** CypA expression does not affect pedestal formation. CypA WT and CypA KO cells were infected with wild type EPEC JPN15. Actin-rich pedestals were visualized with Alexa594-phalloidin (red) and bacteria with DAPI. Scale bar is 2 μm .

Cyclophilin a Regulates Host Cell Invasion by *S. Typhimurium*

To investigate the functional importance of CypA during *S. Typhimurium* invasion we utilized a mouse embryonic fibroblast (MEF) cell line deficient in CypA (Sun et al., 2014). We initially immunolocalized CypA in the parental wild-type (CypA^{+/+}) MEFs and found a similar staining pattern as observed previously (Fig. 1B).

Gentamicin protection assays were then run on the wild-type and CypA null MEFs to enumerate the number of intracellular bacteria during the infections. Surprisingly, we found a 5.7-fold increase in the number of bacteria internalized in CypA depleted cells as compared with those in wild-type cells (Fig. 1C). Collectively, these data suggest a surprising outcome whereby CypA expression restricts bacterial entry into host cells.

Cyclophilin a is a Novel Component of Lamellipodia

We have shown that CypA, a stimulator of eukaryotic cell motility (Saleh et al., 2015), is also a novel component of actin-rich membrane ruffles generated during *S. Typhimurium* infections. As these highly motile structures are enriched with actin filaments, we wanted to examine whether CypA was also present at the branched F-actin-containing leading edge of motile eukaryotic cells. To do this, we fixed and immunolocalized CypA in actively spreading Ptk2 cells; a highly motile epithelial cell line with easily observable lamellipodia. Immunofluorescence microscopy analysis of these cells showed an enrichment of CypA puncta at those sites whereas primary antibody controls lacked that localization (Fig. 2).

Cyclophilin A is Dispensable for Attaching and Effacing Bacteria

EPEC and EHEC are bacterial pathogens that hijack the actin cytoskeleton of their host's cells, causing actin-rich structures called pedestals at sites of bacterial adherence (Moon et al., 1983). In a previous study we identified CypA as a novel component of isolated EPEC pedestals and demonstrated CypA at pedestals during infections with an attenuated strain of EPEC referred to as JPN15 (Law et al., 2015). As this strain does not possess bundle-forming pili (BFP) which normally induces microcolonies on the surface of host epithelia, we sought to determine whether that localization was strain dependent. Using a strain of BFP positive WT EPEC (2,348/69) we found CypA enriched within the actin cores of pedestals (Fig. 3A). To ascertain whether CypA recruitment occurs simply as result of bacterial contact onto the host cell surface or because of the translocation of injected effectors, we infected cultured cells with the EPEC (2,348/69) Δ *escN* mutant which is unable to translocate T3SS effectors or form pedestals. When we examined regions of bacterial adherence to the host during these infections, we found no enrichment of CypA, indicating that recruitment of CypA requires a functional T3SS (Fig. 3A).

Firm attachment by EPEC depends on the translocation of the bacterial effector Tir directly into the host cell and its embedding in the host plasma membrane (Kenny et al., 1997). As EPEC mutants lacking *tir* alone cannot generate pedestals (Kenny et al., 1997), we determined if CypA could still be recruited to sites of bacterial adherence. We opted to do this using the JPN15 strain of EPEC as pedestals are larger and more easily resolved using

that strain. Using a JPN15 Δ *tir* strain, we found that CypA was absent from bacterial attachment sites indicating that translocated Tir plays a role in CypA recruitment (Fig. 4A). When we used a JPN Δ *tir* mutant complemented with *tir* (Δ *tir* + *tir*), CypA was present at EPEC pedestals and was indistinguishable from the wild-type JPN15 infections (Fig. 4A). EPEC pedestal formation also depends on the phosphorylation of tyrosine residue 474 of Tir, which ultimately results in the recruitment and activation of the Arp2/3 complex (Rosenshine et al., 1992; Kenny, 1999; Kalman et al., 1999). To test whether the phosphorylation of this residue is crucial for CypA recruitment, we utilized a JPN15 Δ *tir* strain complemented with a Tir variant that is non-phosphorylatable at residue 474 (Δ *tir* + *tir*-Y474F). Immunolabeling of CypA revealed that Y474 is necessary for CypA recruitment at bacterial attachment sites (Fig. 4A). Despite the altered localization of CypA during the infections, CypA expression remained unchanged as compared with uninfected controls (Fig. 4B).

Finally, we tested whether CypA expression itself is needed for EPEC pedestal formation. We infected wild-type and CypA null cells with EPEC (JPN15) and found that EPEC pedestals were still generated (Fig. 4C).

While EPEC and EHEC both generate actin-rich pedestals during their infections, each pathogen utilizes different molecular pathways to form pedestals (DeViny et al., 2001). To test if CypA is also a component of EHEC pedestals, we infected cultured cells with wild-type EHEC and stained fixed cells with CypA targeting antibodies. CypA was present at those pedestals; however, the staining intensity was noticeably decreased as compared with EPEC pedestals (Fig. 5).

DISCUSSION

In this study, we demonstrate that motile actin-rich structures generated by bacterial pathogens as well as those that occur normally during cell motility are highly enriched with CypA. Our most striking discovery occurred during our examination of CypA during *S. Typhimurium* infections which revealed a potentially novel CypA-dependent regulatory mechanism of restricting bacterial entry.

Among the array of T3SS effectors injected into host cells during *S. Typhimurium* infections a subset are responsible for the cytoskeletal rearrangements that cause membrane ruffling and thus bacterial internalization. We found that the abolishment of CypA increased,

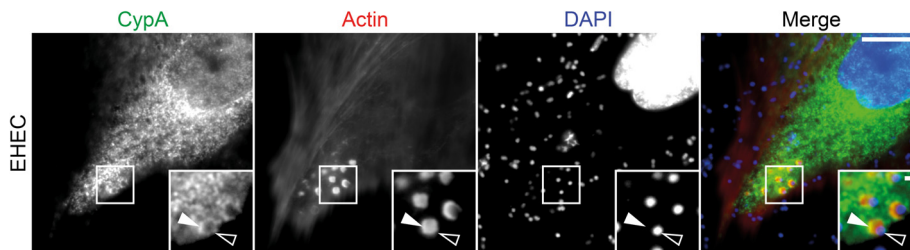


Fig. 5. Cyclophilin A is a component of EHEC-induced pedestals. HeLa cells infected with wild-type EHEC were fixed then stained with a rabbit monoclonal CypA targeting antibody (green), DAPI (blue) to visualize DNA, and Alexa594-phalloidin (red) to visualize F-actin. Solid arrowheads within insets (enlargement of boxed regions) indicate CypA at pedestals. EHEC bacteria are indicated by open arrowheads. Scale bars, 10 and 1 μ m (insets).

rather than decreased, bacterial invasion. One potential explanation for this may lie in *S. Typhimurium* effector dysfunction. We have previously demonstrated that CypA directly interacts with and stabilizes N-WASp, a eukaryotic activator of Arp2/3-mediated actin polymerization (Calhoun et al., 2008). This interaction depends on the GPXLP motif within N-WASp (Calhoun et al., 2008). Remarkably, protein sequence analysis of the *S. Typhimurium* effector SipA uncovered the GPXLP motif within the protein. Curiously, we did not find observable defects in CypA recruitment to $\Delta sipA$ ruffles. SipA normally augments bacterial infection via two mechanisms, first by promoting efficient membrane ruffling and thus bacterial entry (Zhou et al., 1999a), and second, by stimulating intracellular bacterial replication long after they enter cells via its presence at *Salmonella*-containing vacuoles (SCVs) (Brawn et al., 2007). Moreover, unlike SopB, SopE and SopE2, SipA promotes ruffling through its ability to directly bind actin, induce F-actin polymerization and elongation as well as shield filaments from ADF/cofilin-mediated depolymerization (Zhou et al., 1999a, 1999b; Lilic et al., 2003; McGhie et al., 2004). Based on this information, we can envision two scenarios during these infections whereby dynamic CypA interaction with the GPXLP motif of SipA attenuates intracellular bacterial levels: (1) by impeding SipA-mediated actin polymerization or (2) by interfering with SipA at SCVs. As we observed increased bacterial loads during early infections we postulate that the former scenario was at play during our study.

Several studies have provided strong evidence of a role for CypA in eukaryotic cell motility (Calhoun et al., 2008; Saleh et al., 2015). Here we show that endogenous CypA is present at the leading edge of motile cells, within the lamellipodia. This finding is line with recent work by Saleh et al. who showed that CypA can bind to CrkII to enhance cell motility (Saleh et al., 2015). Our finding of endogenous CypA at the leading edge of cells strengthens this points as cell motility induced by CrkII signaling is mediated by plasma membrane integrin-paxillin docks (Saleh et al., 2015).

In conclusion, we have shown that CypA is a component of motile actin-rich structures associated with the plasma membrane. Although CypA is not required for pedestal formation, our work demonstrates that CypA regulates bacterial loads during *S. Typhimurium* infections. The exact role this enzyme plays during these infections could have widespread applications for other pathogens that also hijack the actin cytoskeleton as part of their infectious processes.

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AUTHOR CONTRIBUTIONS

A.S.D. and J.A.G. conceived the study. A.S.D and K.E.W performed the experiments. R.H.C provided reagents and CypA expertise. All authors analyzed the data and wrote the manuscript.

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