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Investigating the Function of Rho Family GTPases during *Salmonella*/Host Cell Interactions

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Abstract

Salmonella enterica comprise a family of pathogenic gram-negative bacteria that have evolved sophisticated virulence mechanisms to enter non-phagocytic cells. The entry event is the result of a carefully orchestrated modulation of Rho family GTPase activity within the host cell, which in turn triggers localized remodeling of the actin cytoskeleton. These cytoskeletal rearrangements drive profuse membrane ruffling and lamellipodial extensions that envelop bacteria and trigger their internalization. This chapter describes a number of methods used to investigate the role of Rho family GTPases during *Salmonella*/host cell interactions. In particular, we detail a variety of complementary techniques, including affinity pull-down assays and bacterial-induced membrane ruffling and internalization assays to show that *Salmonella*-induced actin remodeling and entry require the Rho family members Rac and RhoG.

1. Introduction

Manipulation of the actin cytoskeleton is a prevailing theme in host/pathogen interactions (Cossart and Sansonetti, 2004; Munter *et al.*, 2006; Rottner *et al.*, 2005). A prime example is *Salmonella enterica*, gram-negative bacteria commonly associated with food-borne illness in humans. A hallmark of its close association with vertebrate hosts, *Salmonella* has evolved very sophisticated strategies to enter cells that are not normally phagocytic (Patel and Galan, 2005). Following host cell contact, *Salmonella* employs a specialized organelle termed the type III secretion system to inject multiple bacterial effector proteins directly into the host cell cytosol (Galan, 2001). A specific subset of these effector proteins taps into the eukaryotic signaling networks responsible for regulating actin dynamics and together they orchestrate profuse remodeling of the cytoskeleton at the site of entry (Patel and Galan, 2005). This reprogramming of the cytoskeletal machinery drives localized membrane ruffling and lamellapodial extensions that envelop bacteria and promote their internalization into membrane-bound vacuoles.

As key modulators of cytoskeletal remodeling in eukaryotes, the Rho family of small GTPases (Bustelo *et al.*, 2007; Etienne-Manneville and Hall, 2002) presents ideal targets for bacterial effector proteins to promote internalization into non-phagocytic cells. Like all small GTPases, the Rho family cycles between an active (GTP bound) state, competent for downstream signaling, and an inactive (GDP bound) state. This binary exchange of guanine nucleotides is largely controlled by two classes of regulatory proteins: guanine nucleotide exchange factors (GEFs), which activate Rho proteins by catalyzing the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), which accelerate the intrinsic GTPase activity inherent to Rho family members, leading to their inactivation (Moon and Zheng, 2003; Rossman *et al.*, 2005). *Salmonella* delivers three distinct effector proteins that functionally converge at the level of Rho family GTPases to elicit host cell entry. SopE and its homolog SopE2 act as bona fide GEFs for the Rho family members Cdc42, Rac and RhoG (Bakshi *et al.*, 2000; Hardt *et*

al., 1998; Stender *et al.*, 2000). A second effector protein, SopB, encodes a phosphoinositide phosphatase that feeds into the GTPase cycle by indirectly activating a host-encoded exchange factor, SGEF, specific for RhoG (Patel and Galan, 2006). A third effector protein, SptP, antagonizes the actions of SopE/E2 and SopB by functioning as a GAP for several Rho family members (Fu and Galan, 1999). This limited repertoire of effectors proteins provides *Salmonella* the capacity to reversibly modulate the Rho GTPase switch and thereby initiate the transient cytoskeletal remodeling events necessary for internalization. Moreover, Rho family GTPases play an essential role beyond bacterial entry, driving the accompanying *Salmonella*-induced macropinocytosis events and reprogramming of host gene expression (Chen *et al.*, 1996; Patel and Galan, 2006). The manipulation of GTPase function is thus essential to *Salmonella* pathogenesis, and the ability to fine-tune their activity ensures against overt cellular damage to the host.

This chapter describes a number of protocols used to investigate the differential activation and function of Rho family GTPases during *Salmonella*/host cell interactions. The different methods include affinity pull-down assays and *Salmonella*-induced membrane ruffling and internalization assays. Collectively, they demonstrate that *Salmonella* infection triggers activation of three distinct Rho family GTPases, Cdc42, Rac, and RhoG, and that *Salmonella*-induced actin remodeling and entry requires Rac and RhoG.

2. Determining *Salmonella*-Induced Rho GTPase Activation

The use of standard affinity pull-down assays to measure Rho GTPase activation in response to different cellular stimuli has been described extensively (Benard and Bokoch, 2002; Prieto-Sanchez *et al.*, 2006; Ren and Schwartz, 2000). These assays exploit downstream binding partners that specifically bind to the activated, GTP-bound form of their cognate GTPase. The binary complex can be isolated by coupling the GTPase-binding protein to glutathione *S*-transferase (GST) and using affinity precipitation. We utilize pull-down assays based on the Rac and Cdc42-binding partner PAK CRIB and the RhoG-binding target ELMO to evaluate GTPase activation in response to *Salmonella* infection.

2.1. Reagents

COS 1 cells: a monkey kidney fibroblast cell line (available at ATCC, Rockville, MD) used as host cells for *Salmonella* infection. Cells are cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Gemini) and 100 μ g/ml penicillin/streptomycin (Invitrogen) at 37°, 5% CO₂. **Bacterial strains:** the wild-type *S. enterica* serovar *typhimurium* (*S. typhimurium*) strain SL1344 is cultured to an invasion-competent state by inoculating 3 ml Luria broth (LB) with a freshly streaked colony and growing overnight ($\approx$ 16 h) at 37° on a rotating wheel ($\approx$ 20–30 rpm). Cultures are diluted in 2.5 ml LB containing 0.3 M NaCl, to an OD_{600 nm} of $\approx$ 0.125 and are grown on a rotating wheel at 37° until an OD_{600 nm} of 0.9, prior to immediate use for infection.

GST-PAK CRIB: the Cdc42/Rac-binding domain of PAK (amino acids 67–150) fused to GST is expressed and purified by binding to glutathione agarose beads as described previously (Benard and Bokoch, 2002).

GST-ELMO: a RhoG-binding fragment of ELMO-2 (amino acids 1–362) fused to GST is expressed and purified by binding to glutathione agarose beads as described previously (Prieto-Sanchez *et al.*, 2006).

PAK CRIB pull-down lysis buffer (PAK LB): 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1% (v/v) Triton X-100, 5% (v/v) glycerol, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\times$ complete protease inhibitor cocktail (Roche).

ELMO pull-down lysis buffer (ELMO LB): 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% (v/v) Triton X-100, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 5 mM β -glycerophosphate, 1× complete protease inhibitor cocktail.

Wash buffer: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 1× complete protease inhibitor cocktail.

Antibodies: endogenous levels of Cdc42 and Rac1 protein are probed using rabbit anti-Cdc42 (Santa Cruz Biotechnology) and mouse anti-Rac1 (clone 23A8, Upstate Biotechnology) antibodies. Because of a lack of suitable antibodies for endogenous RhoG, activation of this GTPase is determined by assaying levels of ectopically expressed flag-tagged RhoG using mouse anti-Flag M2 (Sigma).

2.2. Procedure

To assay activation of Cdc42 and Rac upon *Salmonella* infection, COS cells are seeded in 10-cm dishes overnight to ~80% confluency. In general, one 10-cm dish is sufficient to analyze activation of both GTPases at a selected time point postinfection. Cells are washed twice with 5 ml prewarmed Hanks buffered saline solution (HBSS; Invitrogen) and allowed to equilibrate for 10 min in 5 ml warm HBSS at 37°, 5% CO₂ prior to infection with invasion-competent *S. typhimurium*. Bacteria, resuspended in warm HBSS, are added to cells at a multiplicity of infection (MOI) of ~50 bacteria/cell, and infection is allowed to proceed by incubation at 37°, 5% CO₂. At desired time points, cell extracts are prepared by washing cells twice with ice-cold HBSS and lysing cells on ice by scraping into 1 ml PAK LB. Crude lysates are clarified by centrifugation at 16,000 g for 10 min at 4°. An aliquot of the cleared lysate (~50 μ l) is saved to assess total GTPase levels while the remainder is divided equally into two aliquots to measure levels of active Cdc42 and Rac. Cleared cell lysates should be used immediately for affinity precipitation by adding ~20 μ g GST-PAK CRIB glutathione agarose beads and rotating for 1 h at 4°. If multiple dishes are assayed for different time points, then it is important to maintain an equal volume for each pull down, diluting samples with PAK LB if necessary. Beads are collected by centrifugation at 1500 g, 4° for 2 min and washed gently four times with 1 ml cold wash buffer. Following the final wash, the wash buffer is removed carefully and the agarose pellet is resuspended in ~20 μ l Laemmli sample buffer containing 100 mM DTT. Samples are heated at 95° for 10 min and resolved by SDS-PAGE on a 13% polyacrylamide gel, together with aliquots of total lysate to determine both active and total GTPase levels, respectively. The separated proteins are transferred to polyvinylidene difluoride (PVDF) membranes for immunodetection with anti-Cdc42 or anti-Rac1 antibodies. Immunoblots are visualized using chemiluminescence reagents (Pierce) and can be quantified by scanning X-ray blots, provided that the detection system is in the linear range (this can be verified by ensuring linearity of twofold sample dilutions).

Salmonella-induced RhoG activation is determined using a similar affinity pull-down approach, with minor modifications. First, given the lack of a suitable antibody to detect endogenous RhoG, COS cells are transfected using Lipofectamine 2000 (Invitrogen) 1 day prior to infection with an expression plasmid encoding flag-tagged RhoG. Furthermore, activated RhoG is isolated by lysing infected cells in ELMO LB and affinity precipitation using GST-ELMO glutathione agarose as the affinity probe.

Salmonella-induced activation of Cdc42, Rac, and RhoG is evident as early as 10 min, reaching its peak 20–30 min postinfection (Fig. 11.1). In the case of RhoG, activation persists for up to 45 min after infection. The precise kinetics of activation depends greatly on the “invasion competency” of the bacteria, which is a function of the expression of the invasion-associated type III secretion system. To monitor infection and *S. typhimurium* “invasion competency,”

cells can be observed *briefly* during the assay by phase-contrast microscopy to ensure that the bacteria induce membrane ruffling.

2.3. Troubleshooting

A number of variables may affect the magnitude of GTPase activation induced by *S. typhimurium*. In addition to the invasion competence of bacteria, a major obstacle to this assay is excessive GAP activity in cell extracts. It is recommended that samples are processed rapidly and cell lysates are maintained at 4° throughout the pull down to minimize GTP hydrolysis of the target GTPase. If necessary, for example, during a time course experiment, a shorter centrifugation time (2 min) may be used to clarify crude lysates and make processing of multiple samples easier. Another important consideration is the detergent composition of the lysis buffer. Different cell types may require different detergents to achieve sufficient protein extraction and solubilization. Moreover, caution should be observed to ensure that detergents do not disrupt specific binding of GTPases during affinity precipitation (Benard and Bokoch, 2002).

3. Determining the Role of Rho GTPases in *S. typhimurium*-Induced Actin Remodeling

The role of Rho family GTPases in diverse cellular processes traditionally entails blocking their function by overexpression of dominant interfering mutants within the cell (Feig, 1999). Although this approach is very powerful and yields significant functional data regarding GTPase activity *in vivo*, concerns regarding its specificity have arisen (Czuchra *et al.*, 2005; Prieto-Sanchez and Bustelo, 2003; Wennerberg *et al.*, 2002). Indeed, as shown previously (Patel and Galan, 2006), dominant interfering mutants of Cdc42 (^{N17}Cdc42) drastically reduce *Salmonella*-induced Rac activation by affinity pull-down assays. The advent of RNA interference (RNAi) technology has offered a viable alternative to selectively deplete and hence block endogenous GTPase function, given that appropriate controls to assess potential “off-target” effects are observed. The principles of RNAi, together with rationale for experimental design and controls, have been reviewed extensively elsewhere (Endo *et al.*, 2006; Pei and Tuschl, 2006). The following protocol describes a microscopy-based assay using RNAi to examine the roles of Cdc42, Rac1, and RhoG in *Salmonella*-induced actin remodeling.

3.1. Reagents

Henle-407 cells: a human intestinal epithelial cell line (available at ATCC) used as host cells for *S. typhimurium* infection. Cells are cultured in DMEM supplemented with 10% heat-inactivated bovine calf serum (BSA; Hyclone) and 100 µg/ml penicillin/streptomycin at 37°, 5% CO₂.

Bacterial strains: *S. typhimurium* cultured as described earlier.

RNAi constructs: small interfering RNA (siRNA) or short hairpin RNA (shRNA) constructs engineered to silence the expression of endogenous Rho family GTPases are readily available commercially or have been described extensively. We employ the shRNA approach to deplete endogenous Cdc42 using a hairpin sequence targeting nucleotides 296–318 of human Cdc42. Oligonucleotide pairs A (ACAGTGGTGAGTTATCTCAGGAAGCTTGCTGAGATAACTCACCCTGTCCA TTTTTT) and B (GATCAAAAAATGGACAGTGGTGAGTTATCTCAGCA AGCTTCCTGAGATAACTCACCCTGTCTG) are annealed and ligated into the vector pSHAG (a gift from G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Hannon and Conklin, 2004). Attempts to knockdown endogenous Rac1 and RhoG using a similar vector-based approach proved unsuccessful. Therefore, to silence Rac1 and RhoG gene expression, we use synthetic SMARTpools, each comprising four

proprietary siRNA sequences (Dharmacon). A control nontargeting siRNA pool (siCONTROL; Dharmacon) is used as a negative control.

Lipofectamine 2000: transfection reagent (Invitrogen).

Microscope: we utilize an inverted Nikon Eclipse TE2000-U microscope equipped with oil immersion plan Apo 60× NA 1.4, 100× NA 1.4 or dry plan Fluor 40× NA 0.6 objectives and a charge-coupled device (CCD) camera (MicroMAX RTE/CCD-1300Y, Princeton Instruments). Acquisition and analysis are performed using MetaMorph imaging software (version 6.1, Universal Imaging Corp.).

3.2. Procedure

One day prior to transfection, Henle cells are seeded onto glass coverslips (13 mm) in a 24-well plate (Falcon) at a density of 5×10^4 /well in culture media without antibiotics. The absence of antibiotics is critical to increase transfection efficiency. Cells at ≈ 60 to 70% confluency are cotransfected with a GTPase-specific RNAi construct and an expression plasmid encoding GFP to identify transfected cells by microscopy. When working on a 24-well format, we routinely use 200 ng shRNA plasmid DNA or 1 pmol siRNA + 40 ng pEGFP diluted in 50 μ l serum-free media (SFM) per single well. Lipofectamine 2000 is diluted (1 μ l in 50 μ l SFM) prior to mixing gently with the RNAi/GFP solution. Once mixed, samples are incubated at room temperature for 30 min to generate transfection complexes, before adding drop wise to cells.

Cells are maintained at 37°, 5% CO₂ for a further 36 to 48 h to promote silencing and turnover of endogenous target GTPases. The RNAi efficacy for knockdown is verified at gene and protein levels by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting (Fig. 11.2C). To perform a *S. typhimurium*-induced cytoskeletal remodeling assay, cells are equilibrated into warm HBSS for 10 min at 37°, 5% CO₂ prior to infection with invasion-competent bacteria. *S. typhimurium* is added in warm HBSS at a MOI of 30, and infection is allowed to proceed at 37° for 30 min. This is sufficient time for bacteria to initiate cytoskeletal remodeling, clearly evident by phase-contrast microscopy as membrane ruffling. The assay is terminated by washing cells twice with 1 ml cold phosphate-buffered saline (PBS) and fixing with freshly prepared 4% paraformaldehyde (PFA)/PBS for 20 min. To visualize actin remodeling by fluorescence microscopy, cells are permeabilized with 0.1% Triton X-100 for 5 min at room temperature followed by incubation with ammonium chloride (2 mg/ml PBS) for 10 min to minimize autofluorescence. Cells are washed three times with 1 ml PBS and processed for immunofluorescence staining.

We routinely employ tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) to visualize polymerized F-actin and 4',6-diamidino-2-phenylindole (DAPI; Sigma) to detect bacteria. The latter also stains cellular nuclei, which, if necessary, may be avoided by specifically labeling bacteria with rabbit anti-*S. typhimurium* lipopolysaccharide (Difco Laboratories) followed by Alexa350-conjugated anti-rabbit IgG. Indeed, the only restriction when staining is to avoid the green (≈ 488 -nm emission) channel, which is used to detect the GFP expressing RNAi-transfected cells. All incubations are carried out at room temperature, in the dark, for 45 min, by inverting coverslips onto 75- μ l antibody solution (diluted in 1% BSA/PBS) drops. Coverslips are washed between each staining step by sequentially dipping three times into PBS, each time draining excess PBS. Following the final PBS wash, coverslips are further washed by dipping twice into ddH₂O and are mounted by inverting onto ProLong Gold antifade mounting media (Molecular Probes) on microscopy slides.

The actin remodeling elicited by *S. typhimurium* is manifested as a dense accumulation of phalloidin staining (F-actin), specifically at the sites of bacteria and host cell contact (see Fig. 11.2A). Under closer inspection, for instance by confocal microscopy, bacteria are evident at

the base of an actin rich “ruffle,” which protrudes from the apical cell surface. In order to quantify the cytoskeletal remodeling phenotype, the percentage of transfected cells, identified by GFP expression, displaying actin rearrangements as a consequence of bacterial infection is determined and standardized using the values obtained in control cells transfected with nontargeting siRNA (see Fig. 11.2B). By this measure, cells depleted of Cdc42 following RNAi are indistinguishable from control cells. In contrast, depletion of Rac1 or RhoG significantly reduces bacterial-induced actin remodeling, with a more pronounced effect seen upon simultaneous depletion of both these GTPases.

3.3. Troubleshooting

As in other infection experiments, it is essential to ensure the invasion competence of the bacteria. Moreover, the confluency of host cells at the time of infection should ideally be ≈ 80 to 90%. This may require some trial and error, as transfection and RNAi requirements dictate that cells are seeded ≈ 3 days prior to infection. It is important to proceed rapidly once *Salmonella* are ready for infection and maintain cells at 37° throughout the infection period. In general, *Salmonella*-induced actin ruffling should be clearly seen on ≈ 70 to 80% of control transfected cells. If necessary, an increased MOI can be used to promote increased actin remodeling.

When discerning Rho GTPase function, it is critical to verify the efficacy of their RNAi-mediated depletion (see Fig. 11.2C). This is a function of both the transfection efficiency, which may vary greatly between cell types, confluency and passage number of cells, and the efficacy of individual RNAi constructs. Whenever possible, cells should be seeded in duplicate to directly correlate RNAi knockdown of target GTPases by qRT-PCR and Western blotting with *S. typhimurium*-induced ruffling.

4. Determining the Role of Rho GTPases in *S. typhimurium* Internalization

The actin cytoskeletal rearrangements triggered by *Salmonella* are an essential prerequisite to bacterial internalization. Because the two events are tightly coupled, bacterial entry can serve as an alternative assay to complement and validate results gained from a ruffling assay. A variety of techniques have been developed to determine bacterial entry. The majority of these rely on differential staining protocols to directly discriminate internalized bacteria from those that are cell associated. The following protocol describes a variation on this theme, in which *Salmonella* carrying an inducible fluorescent marker are used to infect host cells. The assay involves stimulating fluorescence selectively within internalized bacteria following treatment of cells with gentamicin, a membrane-impermeable antibiotic that kills external bacteria by inhibiting protein synthesis. This strategy strongly correlates with the traditional approach of inside/outside staining of bacteria pre- and postcell permeabilization with different fluorophores (Van Putten *et al.*, 1994) and benefits from requiring only a single fluorescent channel. Together with RNAi, it can be used as a microscopy-based assay to determine the requirements for Rho family GTPases in *Salmonella* entry.

4.1. Reagents

Cell lines, *S. typhimurium*, and RNAi constructs are used as described earlier. To label *S. typhimurium* fluorescently, a bacterial strain is used that carries the plasmid pBAD DsRED T3.S4T (a generous gift from D. Bumann, Max-Planck Institute, Berlin, Germany) (Sorensen *et al.*, 2003), an expression vector that encodes the bacterially optimized fluorescent protein DsRED under the control of an arabinose-inducible promoter. Overnight but *not* subcultures of *Salmonella* are grown in the presence of ampicillin (100 $\mu\text{g/ml}$) to select for the plasmid-encoded marker.

Gentamicin: purchased from Sigma. Prepare fresh as a 100-mg/ml stock solution in ddH₂O.

L-(+)-Arabinose: purchased from Sigma. Prepare fresh as a 10% stock solution in ddH₂O.

4.2. Procedure

Henle cells are seeded onto coverslips and transfected with RNAi constructs as described previously. Following sufficient incubation (36–48 h) to deplete target GTPases, cells are equilibrated into warm HBSS and infected at an MOI of 20 with invasion-competent *S. typhimurium*/pBAD DsRED for 45 min at 37°, 5% CO₂. A longer incubation time than that used for cytoskeletal remodeling is used to ensure efficient bacterial entry. Cells are washed twice with 1 ml warm HBSS, and noninternalized bacteria are killed by incubating cells for 45 min in culture media containing 100 µg/ml gentamicin. To selectively visualize internalized bacteria, the culture medium is replaced with media containing 100 µg/ml gentamicin + 0.1% L-arabinose, and infection is allowed to proceed for a further 2 h. The arabinose chase may be shortened if necessary, as DsRED expression and detection of internalized bacteria are evident >1 h postinduction. However, extended chase times (>3–4 h) should be avoided to ensure against excessive bacterial replication. Finally, cells are washed twice with 1 ml cold PBS, fixed with freshly prepared 4% PFA/PBS for 20 min, and mounted on slides for analysis by immunofluorescence microscopy.

As with the actin-remodeling assay, RNAi-transfected cells are identified by coexpression of GFP. Therefore, the entry phenotype is scored based on the proportion of GFP expressing cells displaying internalized, that is, DsRED positive, *Salmonella* (Fig. 11.3A). As before, values are standardized considering the percentage of internalized bacteria in control siRNA-transfected cells to be 100% (see Fig. 11.3B). Depletion of Cdc42 has little effect on bacterial internalization, whereas depletion of Rac1 and RhoG by RNAi impairs *Salmonella* entry significantly.

4.3. Troubleshooting

This assay is based on the ability to score internalized bacteria unambiguously. A failure to kill external bacteria completely prior to inducing DsRED expression with arabinose can compromise its validity severely. Hence, it is important to ensure that gentamicin and arabinose solutions are freshly prepared. Another variable that may affect DsRED induction within internalized bacteria is excessive cell confluency. Thus, it is good practice to initially ensure that results parallel those seen by an alternative labeling method, such as inside/out staining. If necessary, variables, such as the duration of the gentamicin and arabinose chase, may need to be optimized for different conditions. This assay may also be coupled to fluorescence-activated cell sorting to simplify quantitation.

5. Summary

Rho family GTPases play a critical role during *Salmonella* infection. This chapter focused on the Rho family GTPase-dependent events that lead to bacterial internalization and described several complementary methods to showcase the remarkable adaptations that *Salmonella* has evolved to carefully modulate the Rho GTPase switch.

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Figure 11.1.

Activation of Cdc42, Rac, and RhoG upon infection with *S. typhimurium*. COS-1 cells were infected with wild-type (WT) *S. typhimurium* or its isogenic *invA* mutant (defective in type III secretion and thus unable to induce actin cytoskeletal rearrangements or bacterial entry) for 20 min (Cdc42 and Rac) or 30 min (RhoG). (Top) Relative levels of activated endogenous Cdc42 and Rac and ectopically expressed flag tagged RhoG as determined by affinity pull-down assays using GST-PAK CRIB (Cdc42 and Rac) or GST-ELMO (RhoG), respectively. (Bottom) Total GTPase levels in cell lysates.

Figure 11.2.

Salmonella-induced actin remodeling requires Rac and RhoG. (A) Henle-407 cells were cotransfected with GFP (to detect transfected cells) and RNAi constructs targeting Cdc42, Rac, or RhoG as indicated. Two days post-transfection, cells were infected with wild-type *S. typhimurium* for 30 min and stained with TRITC-conjugated phalloidin to visualize remodeling of the actin cytoskeleton. (B) The percentage of transfected (GFP positive) cells displaying actin rearrangements as a consequence of bacterial infection was enumerated and standardized considering the number of transfected cells exhibiting actin-rich ruffles in RNAi control transfections to be 100%. Results represent the mean $\pm$ SD of three independent experiments. (C) Western blot showing RNAi efficacy. Cell lysates were prepared 2 days post-transfection with RNAi constructs or a mock control, and protein levels were analyzed using antibodies directed against endogenous Cdc42, Rac, and actin.

Figure 11.3.

Salmonella internalization into non-phagocytic cells requires Rac and RhoG. (A) Intestinal Henle-407 cells were cotransfected with GFP (to detect transfected cells) and RNAi constructs targeting Cdc42, Rac, or RhoG as indicated. Two days post-transfection, cells were infected with wild-type DsRED *S. typhimurium* as described in the text. DsRED-positive *Salmonella* represent internalized bacteria only, as the expression of DsRED is induced once external bacteria are killed by gentamicin treatment. (B) The percentage of transfected (GFP positive) cells displaying internalized DsRED bacteria was enumerated and standardized considering the number of transfected cells with internalized bacteria in RNAi control transfections to be 100%. Results represent the mean $\pm$ SD of three independent experiments.