SopE and SopE2 from *S. typhimurium* activate different sets of RhoGTPases of the host cell.

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running title: Differential RhoGTPase specificity of SopE and SopE2

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Abbreviations: aa: amino acid; GDP: guanosine di-phosphate; mant: -O-(N-methylanthraniloyl); mGDP: mant-GDP; GEF: guanine nucleotide exchange factor; GST: glutathione S-transferase; *S. typhimurium*: *Salmonella enterica* subspecies *I* serovar Typhimurium; HUVEC: human umbilical vein endothelial cell.
ABSTRACT
The bacterial enteropathogen *Salmonella typhimurium* employs a specialized type III secretion system to inject toxins into host cells, which trigger signalling cascades leading to cell death in macrophages, secretion of pro-inflammatory cytokines or rearrangements of the host cell cytoskeleton and thus to bacterial invasion. Two of the injected toxins, SopE and the 69% identical protein SopE2, are highly efficient guanine nucleotide exchange factors (GEF) for the RhoGTPase Cdc42 of the host cell. However, it has been a puzzle why *S. typhimurium* might employ two toxins with redundant function. We hypothesized that SopE and SopE2 might have different specificities for certain host cellular RhoGTPases. *In vitro* guanine nucleotide exchange assays and surface plasmon resonance measurements revealed that SopE is an efficient GEF for Cdc42 and Rac1, while SopE2 was interacting efficiently only with Cdc42, but not with Rac1. Affinity precipitation of Cdc42-GTP and Rac1-GTP from lysates and characteristic cytoskeletal rearrangements of infected tissue culture cells confirmed that SopE is highly efficient at activating Cdc42 and Rac1 *in vivo*, while SopE2 was efficiently activating Cdc42, but not Rac1. We conclude that the translocated effector proteins SopE and SopE2 allow *S. typhimurium* to specifically activate different sets of RhoGTPase signalling cascades.
INTRODUCTION

The RhoGTPase subfamily of the Ras superfamily of small GTP-binding proteins comprises more than 10 different proteins (1). They act as molecular switches and cycle between GDP-bound (inactive) and GTP-bound (active) conformations (2). Activation and inactivation of RhoGTPases is controlled by guanine nucleotide exchange factors (GEFs), GTPase-activating enzymes and guanine dissociation inhibitors (3, 4). The eukaryotic GEFs for RhoGTPases share two common sequence motives, the DH (dbl-homology) and the PH (pleckstrin homology) domains, which are responsible for targeting, binding the RhoGTPases and catalysis of RhoGTPase\textsubscript{GDP} \rightarrow RhoGTPase\textsubscript{GTP} exchange (5). Only the active GTP-bound RhoGTPases can interact with downstream elements of signal transduction cascades mediating the cellular responses. GTPases of the Rho subfamily are central switches in the signalling cascades regulating motility, cellular adhesion, cell shape, cytokinesis, cell contraction and gene expression (6, 7). Each of the RhoGTPases can regulate a specific set of downstream signalling cascades leading to activation of specific cellular functions (4). For example, in Swiss 3T3 cells, activation of Cdc42 leads to the formation of filopodia, activation of Rac1 induces formation of lamellipodia and activation of RhoA leads to the formation of stress fibers and focal adhesions (8, 9, 10, 11). Thus, selective activation of specific RhoGTPases leads to specific cellular responses.

Invasion of the gram negative bacterial enteropathogen *S. typhimurium* (*Salmonella enterica* subspecies I serovar Typhimurium) into non-phagocytic mammalian cells is studied as a model system for the "trigger mechanism" of bacterial invasion (12). For triggering invasion, *S. typhimurium* employs the specialized type III secretion system encoded in "Salmonella pathogenicity island I" (SPI1) to inject/translocate a set of at least nine different bacterial toxins (called "effector proteins") into host cells (13, 14, 15, 16, 17, 18, 19, 20, 21, 22). Inside the host cell, the effector proteins activate signalling cascades leading to a variety of responses including cytoskeletal rearrangements and bacterial internalization/invasion (12).

It has been shown that Cdc42 is a key element in the *Salmonella* induced signalling cascades leading to transcriptional activation and bacterial invasion: disruption of Cdc42 signalling by
transfection with dominant negative Cdc42\textsubscript{N17} alleles interferes with bacterial invasion and activation of c-jun-kinase and PAK signalling (23, 24, 25). Rac1 plays a less prominent role and disruption of Rac1 signalling merely leads to reduced \textit{S. typhimurium} invasion rates, while inhibition of RhoA signalling does not affect invasion at all (23). This suggests that the translocated effector proteins of \textit{S. typhimurium} may preferentially address certain RhoGTPase signalling pathways.

Recent work has demonstrated that \textit{S. typhimurium} (strain SL1344) relies mainly on a set of three translocated effector proteins to trigger invasion: a triple mutant \textit{S. typhimurium} strain lacking SopB, SopE and SopE2 is non-invasive, even though the SPI1 type III secretion system is still fully functional (22, 24, 26, 27, 28, 29, 30). SopB has PI-phosphatase activity and a \textit{sopB}'-mutant is less invasive than wildtype \textit{S. typhimurium} (28, 31). Until now, however, the molecular mechanism explaining the role of SopB in triggering invasion is unclear.

SopE and SopE2 of \textit{S. typhimurium} are 69\% identical (21, 22, 27). Besides the lack of any recognizable sequence similarity to proteins with DH- or PH-domains, both proteins are highly efficient GEFs for Cdc42 \textit{in vitro} (22, 24, 32). In fact, the catalytic parameters of SopE mediated guanine nucleotide exchange of Cdc42 are similar to those reported for the active domains of eukaryotic GEFs for members of the Ras-superfamily (32). The specificity of SopE and SopE2 for other GTPases of the Rho subfamily has not been studied in detail. If both bacterial virulence factors had different preferences for different RhoGTPases, SopE and SopE2 might provide \textit{S. typhimurium} with a means to differentially activate specific signalling pathways inside the host cell.

In the present study we have analyzed the specificity of SopE and SopE2 for the RhoGTPases Cdc42 and Rac1. Biochemical analyses of purified recombinant proteins and analysis of RhoGTPase activity in infected tissue culture cells revealed that SopE is an efficient activator for both Cdc42 and Rac1 \textit{in vitro} and \textit{in vivo}. In contrast, SopE2 efficiently activates Cdc42, but not Rac1. This demonstrates for the first time that expression of two homologous translocated effectors with GEF activity for RhoGTPases allows \textit{S. typhimurium} to differentially activate specific signalling pathways within host cells.
MATERIALS AND METHODS

Bacterial Strains

All *S. typhimurium* strains used in this study have been described (26) and are isogenic derivatives of the virulent wild-type strain SL1344 (33). M516 (SL1344, ΔsopB, sopE::aphT, sopE2::tet) lacks the three major effector proteins necessary for tissue culture cell invasion (26). Transformation of M516 with pM136 (pBAD24, expresses SopE1-240-M45 under the control of the native *sopE*-promotor; (22)) or with pM226 (pBAD24, expresses SopE21-240-M45 under the control of the native *sopE2*-promotor; (22)) complements the invasion defect. For tissue culture cell infection experiments the bacteria were grown in high salt media as described (22).

Preparation of recombinant proteins

Preparation of recombinant proteins was performed essentially as described (32, 34). Briefly, all proteins used in this study were overexpressed as GST fusion proteins, recovered from bacterial extracts by binding to Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and either eluted with 20mM glutathione (GST, GST-SopE78-240, GST-SopE269-240, GST-Cdc42Hs1-192 and GST-Rac11-191) or cleaved off the column by digestion with thrombin protease (SopE78-240 and SopE269-240, Cdc42Hs1-192 and H-Ras) or with factor Xa (Rac11-191). Proteins were concentrated by ultrafiltration (MWCO 8000), snap frozen in liquid nitrogen and stored at -80°C.

Due to design of the expression vectors, the proteins carry the following additional amino acids: GST-SopE78-240 carries PGISGGGGGILEFEM between the thrombin cleavage site and L78 of SopE; GST-SopE269-240 carries PGISGGGGGIL between the thrombin cleavage site and G69 of SopE2; SopE78-240 carries the additional N-terminal amino acids: GSPGISGGGGGILEFEM; SopE269-240 carries the additional N-terminal amino acids:
GSPGISGGGGGIL; GST-Rac1_{1-191} carries GIDPGAT between the factor Xa recognition site and M1 of Rac1; Rac1_{1-191} carries the N-terminal amino acids GIDPGAT; H-Ras carries the additional N-terminal amino acids GS; GST-Cdc42Hs_{1-192} carries RRASVGSKIISA between the thrombin recognition site and M1 of Cdc42. Cdc42Hs_{1-192} carries the N-terminal amino acids GSRRASVGSKIISA.

The expression vector for the GST-fusion protein with the Rac1 and Cdc42 binding region (aa 56-272) of human PAK1B was generously provided by E. Sander and J.G. Collard (35) and purification of the protein bound to Glutathione-Sepharose beads was performed as described (35).

**Preparation of mGDP-Cdc42Hs_{1-192} complex**

To remove the associated GDP, GST-Cdc42Hs_{1-192} bound to a Glutathione-Sepharose 4B column was washed with 10 ml buffer D (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 2 mM EDTA, 2 mM DTT). Afterwards, beads were incubated as a batch in the presence of a 2.5-fold molar excess of fluorescent mGDP (Molecular Probes, Netherlands) for 10 min at 22°C in buffer D. After addition of excess MgCl2, mGDP-Cdc42Hs_{1-192} was cleaved off the column material using thrombin (Amersham Pharmacia Biotech) in buffer A (4°C; over night) and unbound mGDP was removed by gel filtration chromatography. Fractions containing the mGDP-Cdc42Hs_{1-192} complex were identified by fluorescence spectroscopy, pooled, concentrated by ultrafiltration (Millipore Ultrafree-15, MWCO 8000), snap frozen and stored at -80°C.

**Preparation of mGDP-Rac1_{1-191} complex**

For preparation of mGDP-Rac1_{1-191} we devised a new method based on the high stability of the SopE-Rac1 complex (32). 2 mg GST-SopE_{78-240} was bound to a Glutathione-Sepharose 4B column. Rac1_{1-191} (in buffer A: 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM MgCl2, 2 mM
DDTT was applied to the column and unbound Rac1-191 was removed by washing with 10ml of buffer A. The bound Rac1-191 was eluted as mGDP·Rac1-191 complex using buffer A (20°C) supplemented with 200µM mGDP and purified and concentrated as described above for mGDP·Cdc42Hs1-192.

Filter binding assays

Filter binding assays were performed in buffer B (50mM Tris-HCl, pH 7.6, 50mM NaCl, 5mM MgCl₂, 5mM DTT) as described (22, 32).

Surface Plasmon Resonance

Association and dissociation reactions involving GST-Cdc42Hs1-192, GST-Rac1-191, SopE78-240 and SopE269-240 were analyzed in buffer E (10mM HEPES/NaOH, pH 7.3, 150mM NaCl, 5mM MgCl₂, 0.005% Igepal CA-630 (Sigma)) using surface plasmon resonance (BIACore 2000 system) as described recently (32).

Fluorescence spectrometry

Fluorescence measurements were performed at 20°C in buffer F (40mM HEPES/NaOH pH 7.3, 100mM NaCl, 5mM MgCl₂) on an Aminco Bowman Series 2 Fluorescence Spectrometer (excitation: 366nm; emission: 440nm). Increasing concentrations of either mGDP·Rac1-191 or mGDP·Cdc42Hs1-192 were premixed with SopE78-240 or SopE269-240 (final concentrations: 25nM). Reactions were started by addition of unlabelled GDP (1mM final concentration) and dissociation of mGDP was recorded as decreased fluorescence at 440nm.

GST-PAK-CRIB affinity purification assay

To measure the amounts of Cdc42-GTP and Rac1-GTP in infected tissue culture cells we performed affinity purification assays as described (35). Briefly, confluent COS7 cells grown
in DMEM (5% FBS) were infected with *S. typhimurium* at a multiplicity of infection of 50 bacteria per cell. Cells were washed with cold PBS and lysed in 1ml GST-fish buffer (10% glycerol, 50mM Tris-HCl pH7.6, 100mM NaCl, 1% Igepal CA-630, 2mM MgCl₂ supplemented with „complete“ protease inhibitor cocktail (Roche)), lysates were cleared by centrifugation (4000x g; 4°C) and the activated Rac1/Cdc42 was recovered by binding to immobilized GST-PAK-CRIB-fusion protein (see above; 30', 4°C). The beads were washed and the amount of activated Cdc42-GTP and Rac1-GTP was determined by Western blot analysis using mouse-α-Rac1 (1:2500 in PBS, 5% non-fat milk; Upstate Biotechnology) or mouse-α-Cdc42 (1:500, Transduction Laboratories) antibodies. SopE1-240-M45 and SopE2-240-M45 proteins were detected using mouse-αM45 antibody (1:100 in PBS, 5% non-fat milk; (36)), a secondary horseradish peroxidase conjugated α-mouse antibody (1:12000; Dianova) and the ECL Plus detection kit, as recommended by the manufacturer (Amersham Pharmacia Biotech).

*Actin cytoskeletal rearrangements in HUVEC tissue culture cells*

Human umbilical vein endothelial cells (HUVEC) were grown to confluency in endothelial cell growth medium (EGM, Promo Cell, supplements: ECGS/H2, 10% FCS) on gelatin coated plastic coverslips (Thermanox, Nalge Nunc International) as described previously (37). The culture medium was replaced with serum free EGM and cells were infected with *S. typhimurium* (MOI = 50) for 40 minutes, washed, fixed with PBS, 4% paraformaldehyde, permeabilized with PBS, 0.1% Triton X-100 and stained with rhodamine-phalloidin (Molecular Probes, 1:20 in PBS, 3% BSA). Bacteria were stained with α-*Salmonella* O-1,4,5,12(8) antiserum (Difco, 1:400 in PBS, 3% BSA) and a secondary α-rabbit-FITC conjugate (Sigma, 1:250 in PBS, 3% BSA). Coverslips were mounted and analyzed by fluorescence microscopy. Cells with obvious rearrangments in the actin cytoskeleton (ca. 35% of all cells ) were evaluated and classified based on their cytoskeletal structure.
RESULTS

SopE and SopE2 display differential specificities for Cdc42Hs and Rac1.

Previous work had shown that SopE and SopE2 are efficient guanine nucleotide exchange factors for Cdc42 (22, 32). Guanine nucleotide exchange factor activity for other RhoGTPases has not been studied in detail. Here, we have compared the GEF-activity of SopE78-240 and SopE269-240 on Rac11-191 and Cdc42Hs1-192. Cdc42Hs1-192, Rac11-191 or H-Ras were loaded with [3H]GDP and we determined the rates of SopE78-240 or SopE269-240 mediated [3H]GDP release using filter binding assays (Fig. 1; MATERIALS AND METHODS). In line with earlier results (22), 1µM SopE78-240 and SopE269-240 catalyzed fast [3H]GDP release from Cdc42Hs1-192 (Fig. 1a (○); 1d (x)). In contrast, [3H]GDP release from Rac11-191 was much faster in the presence of SopE78-240 (Fig. 1b (●)) than in the presence of 1µM SopE269-240 (Fig. 1e (x)). Therefore, SopE78-240 is a highly efficient GEF for Cdc42Hs1-192 and Rac11-191, whereas SopE269-240 acts equally efficient on Cdc42Hs1-192, but is much less active on Rac11-191 in vitro.

Association/dissociation kinetics of the complexes between SopE, SopE2, Rac1 and Cdc42Hs.

To analyze the binding specificity of SopE and SopE2 we have measured the kinetics of formation and dissociation of the complexes between Cdc42 (or Rac1) and SopE2 (or SopE) using surface plasmon resonance. This technique allows one to study binding/dissociation kinetics by measuring the change in mass on the surface of a sensor chip. GST-Cdc42Hs1-192 or GST-Rac11-191 fusion protein (or GST as a control) was bound to a sensor chip and we measured the kinetics of binding of SopE78-240 (or SopE269-240; 100nM; Fig. 2a). The observed rates of complex formation between GST-Cdc42Hs1-192, GST-Rac11-191, SopE78-240 and SopE269-240 were dependent on the concentration of the RhoGTPase applied (Fig. 2b; data not shown). From the binding curves we calculated the kinetic constants for complex formation (kass) assuming simple one step bimolecular association reactions: SopE78-240 binds with similar kinetics to GST-Cdc42Hs1-192 and to GST-Rac11-191 (Tab. 1). In
contrast, the association rate constant for formation of the GST-Cdc42Hs1-192·SopE269-240 complex is 7-fold higher than for the GST-Rac11-191·SopE269-240 complex. We have also analyzed the dissociation of the complexes (Tab. 1). However, in the absence of GDP dissociation was slow and the dissociation rate constants are prone to experimental error and should be regarded as rough estimates. The GST-Cdc42Hs1-192·SopE78-240 complex and the GST-Rac11-191·SopE78-240 complex are roughly equally stable. In contrast, dissociation of the GST-Rac11-191·SopE269-240 complex is 6-fold faster than dissociation of the GST-Cdc42Hs1-192·SopE269-240 complex (Tab. 1). Overall, SopE78-240 binds with very similar equilibrium binding constants \( (K_D = k_{off}/k_{ass}) \) to GST-Cdc42Hs1-192 and to GST-Rac11-191 \( (K_D = 3.1 \times 10^{-10} \text{ M}) \), while equilibrium binding of SopE269-240 to GST-Cdc42Hs1-192 is 40-fold stronger than equilibrium binding to GST-Rac11-191 (Tab. 1).

In line with previous results for the GST-SopE78-240·Cdc42\(_{\Delta C}\) complex (32), dissociation of all complexes between GST-RhoGTPases and SopE78-240 or SopE269-240 was accelerated more than 1000-fold in the presence of 20µM GDP and the dissociation reactions were completed in less than 5 seconds (data not shown). Identical dissociation curves were obtained when we employed 20µM GTP (data not shown). However, the dissociation kinetics in the presence of guanine nucleotides were too fast to allow an accurate analysis in order to detect differences between the dissociation rates of the complexes with GST-Cdc42Hs1-192 and GST-Rac11-191.

**Multiple Turnover Kinetics of SopE and SopE2 mediated Nucleotide Exchange.**

We have also analyzed the SopE78-240 and SopE269-240 mediated nucleotide exchange in multiple turnover kinetic experiments using O-(N-methylanthraniloyl-GDP (mGDP), a fluorescent GDP derivative. The fluorescence of mGDP bound to Cdc42 is 4-fold higher than the fluorescence of unbound mGDP (32, 38, 39). The kinetics and concentration dependence of mGDP dissociation from Cdc42Hs1-192·mGDP or Rac11-191·mGDP was followed by fluorescence spectrometry (Fig. 3). In the SopE78-240 mediated nucleotide exchange reactions, Cdc42Hs1-192·mGDP nucleotide dissociation rate constants \( (v) \) reached a plateau at 20-40µM and the Michaelis-Menten parameters \( (k_{cat} = 5 \pm 1 \text{ s}^{-1} \text{ and } K_M = 6 \pm 2 \mu\text{M}; \text{Tab. } 2) \) were in the same order of magnitude as those reported for SopE78-240 mediated nucleotide exchange on
Cdc42V12-mGDP \( (k_{\text{cat}} = 0.95 \pm 0.06 \, \text{s}^{-1} \) and \( K_M = 4.5 \pm 0.9 \, \mu\text{M}; \) (32)). It is unclear whether the slight differences might be attributable to effects of the G12V mutation of Cdc42 used in the earlier study (32). SopE2_{69-240} is an even more efficient GEF for Cdc42Hs1-192 than SopE_{78-240} (Tab. 2).

Neither with SopE_{78-240} nor with SopE2_{69-240}, the observed nucleotide dissociation rate constants \( (v) \) of Rac1_{1-191}-mGDP did reach a plateau at concentrations up to 50µM Rac1_{1-191}-mGDP; Fig. 3a and b). Therefore, we could only estimate the catalytic efficiency of SopE_{78-240} and SopE2_{69-240} from the slopes of the liner plots shown in Fig. 3 (Tab. 2). This indicates that the catalytic efficiency of SopE2_{69-240} \( (k_{\text{obs}}/[\text{Rac1}_{1-191}-\text{mGDP}]) \) is about 6-fold lower than the catalytic efficiency of SopE_{78-240}.

**Affinity precipitation assays to determine substrate specificities of SopE and SopE2 in vivo.**

The biochemical analyses presented above show that SopE is an efficient GEF for Rac1 and Cdc42 while SopE2 is an efficient GEF for Cdc42 but not for Rac1. It was of interest to also analyze this specificity in vivo. The levels of GTP-bound Rac1 and Cdc42 in tissue culture cells can be analyzed directly in an affinity precipitation assay (35, 40). This assay is based on the ability of the Cdc42/Rac1-binding domain (CD; aa 56-272) of PAK-1 (p21-activated kinase 1) to specifically bind to activated Cdc42·GTP and Rac1·GTP, but not to inactive Cdc42-GDP or Rac1-GDP (35).

For the analysis of RhoGTPase activation by SopE and SopE2 during the course of an infection we have employed the *S. typhimurium* SL1344 mutant M516 (sopE::aphT; sopE2::pM218; ΔsopB; (26)), which lacks the three major effector proteins necessary for host cell invasion (26). This strain has a fully functional SPI1 typeIII secretion system (26) but it is unable to activate Cdc42- or Rac1-signalling (see Fig. 4). To analyze the in vivo specificity of SopE and SopE2 we have infected COS7 tissue culture cells for 40 min with the plasmidless control *S. typhimurium* strain M516 or with M516 complemented with expression vectors for epitope-tagged SopE_{1-240-M45} (pM136) or SopE2_{1-240-M45} (pM226; (22)). COS7 cell lysates
were subsequently analyzed using the GST-PAK-CD affinity precipitation assay (MATERIALS AND METHODS). M516 complemented with pM136 (SopE1-240-M45) was able to efficiently activate Cdc42 and Rac1 (Fig. 4, lanes 2a and 2b). In contrast, M516 complemented with pM226 (SopE21-240-M45) only activated Cdc42 but not Rac1 (Fig. 4, lanes 3a and 3b). Control experiments verified that the observed differences were not attributable to different amounts of Rac1 or Sop-proteins present in the lysates (Fig. 4, lanes 2c, 3c and lanes 1d, 2d, 3d). In conclusion, these are in line with the results from the biochemical analyses and show that SopE2 has the capacity to specifically activate Cdc42 signalling in vivo, while SopE activates both Rac1 and Cdc42.

**SopE and SopE2 have different effects on the actin cytoskeleton of HUVEC cells.**

In mammalian cells specific activation of Rho, Rac and Cdc42 leads to characteristic rearrangements of the actin cytoskeleton. Usually, activation of Cdc42 is associated with the formation of filopodia and activation of Rac1 with formation of lamellipodia ("ruffles"; (9, 10, 11)). Therefore, the differential signalling capacity of SopE and SopE2 might lead to different cytoskeletal rearrangements in infected tissue culture cells. In human umbilical vein endothelial cells (HUVEC) activation of Cdc42 and Rac1 induces formation of filopodia and lamellipodia, respectively (37, 41). Therefore, we have infected HUVEC for 40 min with S. typhimurium strain M516 or with M516 complemented with pM136 (SopE1-240-M45) or with pM226 (SopE21-240-M45). M516 did not induce actin cytoskeletal rearrangements (Fig. 5a). In contrast, M516 complemented with pM136 (SopE1-240-M45) induced the formation of lamellipodia. Infection with M516 complemented with pM226 (SopE21-240-M45) induced formation of filopodia whereas only a minority of infected cells formed lamellipodia (Fig. 5a and b). The small number of cells forming lamellipodia (Fig. 5b; M516 + pM226) might be attributable to indirect activation of Rac1 by activated Cdc42 (11). In conclusion, these data are in line with our observation that SopE can efficiently activate Rac1 (and Cdc42) signalling, while SopE2 activates Cdc42 but not Rac1 in vivo.
DISCUSSION

It is well established that the translocated *S. typhimurium* protein SopE acts as an efficient GEF for host cellular RhoGTPases both *in vitro* and *in vivo* (24, 32). This was of special interest, since SopE does not share any recognizable sequence similarity to eukaryotic GEFs or any other known proteins. Recently, it was discovered that *S. typhimurium* translocates an additional, 69% identical effector protein named SopE2 into host cells (21, 22). SopE2 is also capable of activating RhoGTPase signalling cascades leading to cytoskeletal rearrangements and bacterial entry (22). Why does *S. typhimurium* translocate two structurally and functionally similar effector proteins into host cells? We hypothesized that differences in the specificity of SopE and SopE2 for certain RhoGTPases might be one possible explanation. As activation of different RhoGTPases leads to specific changes in key cellular functions (i.e. actin cytoskeletal rearrangements, activation of transcription factors; (4)) this might enable *S. typhimurium* to precisely manipulate host cell physiology. Therefore, we have analyzed in the present study the specificity of SopE and SopE2 for Cdc42 and Rac1. Indeed, we found SopE is a potent GEF for Cdc42 and for Rac1 both *in vitro* and *in vivo*, while SopE2 is much more active on Cdc42 than on Rac1: 1) In the absence of free guanine nucleotide, the equilibrium binding of SopE2 to Cdc42 is about 40-fold stronger than binding of SopE2 to Rac1. 2) SopE2 mediated *in vitro* guanine nucleotide exchange is about 10-fold more efficient for Cdc42 than for Rac1. 3) Affinity precipitation assays revealed that upon translocation into COS7 tissue culture cells SopE2 activates Cdc42, but essentially no Rac1 4) Translocation of SopE2 into HUVEC tissue culture cells induces actin cytoskeletal rearrangements characteristic for specific activation of Cdc42. In contrast, SopE interacted efficiently with both Cdc42 and Rac1 *in vitro* and *in vivo*. Analysis of a SopE-SopE2 chimeric protein (promoter region and aa 1-95 of SopE (= translocation signal) fused to aa 96-240 of SopE2 (= catalytic domain)) verified that the observed specificities *in vivo* are really attributable to the
catalytic C-terminal domains (data not shown). Altogether, SopE and SopE2 provide \textit{S. typhimurium} with a means to specifically activate either Cdc42 and Rac1 or Cdc42 but not Rac1.

Eukaryotic GEFs share a common functional unit (DH- plus PH-domain) which facilitates GTPase-binding and catalysis. Yet, the eukaryotic GEFs for RhoGTPases display different specificities for different subsets of RhoGTPases (5, 42). The 3D-structure of Rac1 complexed with the DH- and PH-domains of the eukaryotic GEF Tiam1 has identified the amino acid residues determining the binding specificity (43). Unfortunately, as SopE and SopE2 do not share any recognizable sequence similarity with eukaryotic GEFs, it is impossible to predict the amino acid residues responsible for the different substrate preferences of SopE and SopE2 from the data of Worthylake \textit{et al.} (43).

Do all \textit{Salmonella} strains address Cdc42 and Rac1? Phylogenetic analyses have shown that sopE2 is present in all contemporary \textit{Salmonella} lineages (21, 22, 26). Therefore, the capacity to directly activate Cdc42 but not Rac1 (via SopE2) inside cells of the animal host is common to all \textit{Salmonellae}. In contrast, sopE is encoded in the genome of a bacteriophage which is only present in very few \textit{Salmonella} strains, including the \textit{S. typhimurium} strain SL1344 used in this study (26, 27, 44, 45). A second \textit{S. typhimurium} strain (ATCC 14028) that is commonly used to study virulence mechanisms does not carry SopEΦ and does not express SopE (Mirold and Hardt, unpublished). Therefore, the capacity to directly activate Rac1-signalling (via SopE) is not strictly required for \textit{Salmonella} virulence per se. However, it is well possible that SopE2 can activate Rac1 via an indirect mechanism as it is known that specific activation of Cdc42 will finally result in activation Rac1 in Swiss 3T3 fibroblasts (11). Nonetheless, expression of SopE improves virulence. Interestingly, SopE-expressing \textit{S. typhimurium} strains are associated with severe epidemics (44). It has been speculated that the improved epidemic virulence of these strains might simply be attributable to a higher "sopE"-gene dosage and higher total amounts of SopE-like proteins delivered into host cells (44).
However, the data presented here suggest that the improved virulence of sopE-positive *S. typhimurium* strains is much rather linked to the capacity of these strains to directly activate Rac1 and Cdc42 (via SopE) inside host cells.

Indeed, there are several lines of evidence from tissue culture experiments suggesting that direct activation of Cdc42 and Rac1 are needed to optimize host cell invasion: 1. Disruption of the *sopE* gene in *S. typhimurium* strain SL1344 leads to a 2-fold decreased invasiveness into COS7 tissue culture cells (27), while a *sopE2* mutant is equally invasive as the wildtype SL1344 strain (22). This argues that the presence of SopE can fully compensate for the loss of SopE2, while SopE2 (possibly due to its inability to directly activate Rac1) cannot completely compensate for the loss of SopE. 2. Complementation of a non-invasive *S. typhimurium* strain (M516 = SL1344, *sopE*, *sopE2*, *sopB*) which lacks all three translocated effector proteins triggering bacterial entry with a SopE expression vector is 2-fold more efficient than complementation with a SopE2 expression vector (26). 3. Disruption of Cdc42-signalling inside COS7 cells by transfection with Cdc42[$N_{17}$] expression vectors is 2-fold more efficient at blocking *S. typhimurium* SL1344 invasion than disruption of Rac1 signalling via Rac1[$N_{17}$] (23). In conclusion these observations suggest that the capacity to directly activate Rac1 (in addition to Cdc42) via SopE improves *S. typhimurium* virulence.

Taken together, our results show for the first time that *S. typhimurium* can specifically activate different RhoGTPases of the host cell via the translocated effector proteins SopE and SopE2. This allows the bacteria to fine tune host cellular responses very precisely. Future work will have to address how SopE/SopE2 triggered signalling may be further modulated by the other translocated effector proteins like the actin binding protein SipA (19), the PI-phosphatase SopB (Norris *et al.*, 1998) or the GTPase activating protein SptP (46). This will further advance our current knowledge of the intricate network of responses triggered by the translocated effector proteins of *S. typhimurium* in order to alter host cell signalling in a very precise manner.
REFERENCES


FIGURES AND LEGENDS

Fig. 1. Rates of guanine nucleotide exchange catalyzed by SopE and SopE2. [3H]GDP release from Cdc42Hs1-192-[3H]GDP (panels a and d), Rac1-191-[3H]GDP (panels b and e) and H-Ras-[3H]GDP (panels c and f) in the presence of 1 mM GDP and 1µM SopE78-240 (●), 1µM SopE269-240 (x), 10mM EDTA (Δ) or 1µM GST (◆) was analyzed using a filter binding assay. The data were acquired in three independent experiments.

Fig. 2. Surface plasmon resonance measurement of the SopE/SopE2 interaction with Cdc42Hs or Rac1. a. Binding kinetics of SopE78-240 (100 nM) and SopE269-240 (100 nM) to GST-Cdc42Hs1-192 and GST-Rac1-191. b. Concentration dependence of the rates of binding of SopE269-240 to GST-Cdc42Hs1-192 or to GST-Rac1-191. After washing with buffer E (0 - 90 sec), we applied SopE269-240 in buffer E at the indicated concentrations. The sensorgrams were corrected to show the specific signal changes. w: start of washing with buffer E.

Fig. 3. Multiple turnover kinetics of guanine nucleotide exchange by SopE or SopE2. Release of mGDP from Rac1-191·mGDP (1 - 45µM) or Cdc42Hs1-192·mGDP (1 - 60µM) was analyzed in the presence of 1 mM GDP and 25 nM SopE78-240 or SopE269-240 using fluorescence spectrometry (excitation wavelength = 366nm; emission wavelength = 440nm). The curves were fitted assuming a simple dissociation mechanism and the resulting rates (k_{obs.} = [pmol of mGDP released] / [pmol of Sop-protein] x s^{-1}) were plotted as a function of the concentrations of Rac1-191·mGDP (●) or Cdc42Hs1-192·mGDP (◇).

Fig. 4. Affinity precipitation assay to measure activation of host cellular Cdc42 and Rac1 by translocated SopE and SopE2. COS7 cells were infected for 40 min with M516 (lanes 1a-1d), M516 complemented with pM136 (SopE_{M45}; lanes 2a-2d) or M516 complemented with pM226 (SopE2_{M45}; lanes 3a-3d). Rac1-GTP and Cdc42-GTP present in the COS7 lysates was affinity precipitated using GST-PAK-CD beads (MATERIALS AND METHODS) and quantification of Rac1-GTP and Cdc42-GTP was performed by Western blot analyses using...
specific mouse-α-Cdc42 (lane a) or mouse-α-Rac1 (lane b) antibodies. Lane c: relative amounts of Sop-proteins (or Rac1; lane d) present in the lysates detected by Western blot using a specific mouse-α-M45 (lane c) or a mouse-α-Rac1 (lane d) antibody. Lane 4a-4d: Positive control. Rac1 and Cdc42 in lysate was activated by loading with GTPγS. Lane 5a-5d: Negative control. Rac1 and Cdc42 in lysate was inactivated by loading with GDP. The assay shown is representative for the five independent experiments performed.

Fig. 5. SopE/SopE2 induced rearrangements in the HUVEC actin cytoskeleton. a. HUVECs were infected for 40 min with the indicated S. typhimurium strains. Cells were fixed, f-actin was stained with rhodamine-phalloidin (red) and bacteria were stained with a polyclonal α-Salmonella antiserum and a secondary α-rabbit FITC antibody (green; MATERIALS AND METHODS). b. Quantitative analysis of the SopE/SopE2 induced cytoskeletal rearrangements. Cells with altered actin cytoskeletal morphology (ca. 35% of all cells) were classified based on their morphological features: profound membrane ruffling (i.e. Fig. 5a, panel 2), weak filopodia formation (<20 filopodia per cell), pronounced filopodia formation (>20 filopodia per cell; i.e. Fig. 5a, panel 3). For each S. typhimurium strain at least 100 cells with altered actin cytoskeletal morphology were evaluated in three independent experiments (experimental error: ±10%). *no cytoskeletal rearrangements observed.

ACKNOWLEDGEMENTS

We would like to thank Dr. Irmgard Assfal-Machleidt for her helpful instructions to perform and evaluate surface plasmon resonance measurements on the BIAcore 2000 instrument.
Friebel et al., Fig. 1
**Friebel et al., Fig. 2**
Table 1 Surface plasmon resonance measurement of the association/dissociation of SopE/SopE2 complexes with GST-Cdc42/GST-Rac1

<table>
<thead>
<tr>
<th></th>
<th>SopE</th>
<th>SopE2</th>
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<tbody>
<tr>
<td>GST-Cdc42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k_{\text{ass}} ) [M(^{-1}) s(^{-1})]</td>
<td>1.6 ± 0.1 * 10(^5)</td>
<td>4.0 ± 1.0 * 10(^5)</td>
</tr>
<tr>
<td>( k_{\text{off}} ) [s(^{-1})]</td>
<td>4.5 ± 1.5 * 10(^{-5})</td>
<td>2.1 ± 0.5 * 10(^{-5})</td>
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<tr>
<td>( K_D \left( k_{\text{off}} / k_{\text{ass}} \right) ) [M]</td>
<td>2.8 * 10(^{-10})</td>
<td>5.2 * 10(^{-11})</td>
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<tr>
<td>GST-Rac1</td>
<td></td>
<td></td>
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<tr>
<td>( k_{\text{ass}} ) [M(^{-1}) s(^{-1})]</td>
<td>3.2 ± 0.5 * 10(^5)</td>
<td>5.7 ± 1.4 * 10(^4)</td>
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<td>( k_{\text{off}} ) [s(^{-1})]</td>
<td>1.0 ± 0.2 * 10(^{-4})</td>
<td>1.3 ± 0.4 * 10(^{-4})</td>
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<tr>
<td>( K_D \left( k_{\text{off}} / k_{\text{ass}} \right) ) [M]</td>
<td>3.1 * 10(^{-10})</td>
<td>2.3 * 10(^{-9})</td>
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</table>

Friebel et al., Tab. 1
Friebel et al., Fig. 3
Table 2. Multiple turnover measurements of SopE/SopE2-mediated nucleotide exchange

<table>
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<tr>
<td></td>
<td>Cdc 42</td>
<td>Rac1</td>
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<tr>
<td>$K_M$ [µM]</td>
<td>6 ± 2</td>
<td>see figure 3</td>
</tr>
<tr>
<td>$k_{cat}$ [s$^{-1}$]</td>
<td>5 ± 1</td>
<td>see figure 3</td>
</tr>
<tr>
<td>$k_{obs} / [GTPase] [M$^{-1}$ s$^{-1}$]$^6$</td>
<td>29 ± 5 x 10$^4$</td>
<td>50 ± 15 x 10$^4$</td>
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</tbody>
</table>

$^6$ determined from measurements at low RhoGTPase concentrations corresponding to linear ranges of the curves shown in Fig. 3.
Friebel et al., Fig. 4

<table>
<thead>
<tr>
<th></th>
<th>control</th>
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<th>pSopE2</th>
<th>GTPγS</th>
<th>GDP</th>
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<tbody>
<tr>
<td>1</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
<td></td>
</tr>
</tbody>
</table>

- **a**  
  α-Cdc42 (activated Cdc42)

- **b**  
  α-Rac1 (activated Rac1)

- **c**  
  α-M45 (SopE/SopE2)

- **d**  
  1/12 of lysate
### a

![Immunofluorescence images](image)

<table>
<thead>
<tr>
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<th>M516</th>
<th>M516 + pM136</th>
<th>M516 + pM226</th>
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<tbody>
<tr>
<td>control</td>
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<tr>
<td>pM136 (SopE)</td>
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<tr>
<td>pM226 (SopE2)</td>
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### b

<table>
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<tr>
<th>Actin Cytoskeletal Morphology</th>
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<th>M516 + pM226</th>
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<tr>
<td>Profound Membrane Ruffling</td>
<td>0% *</td>
<td>100%</td>
<td>8%</td>
</tr>
<tr>
<td>Weak Filopodia Formation (&lt; 20 / cell)</td>
<td>0% *</td>
<td>0%</td>
<td>31%</td>
</tr>
<tr>
<td>Pronounced Filopodia Formation (&gt; 20 / cell)</td>
<td>0% *</td>
<td>0%</td>
<td>61%</td>
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</tbody>
</table>

_Friebel _et al._, Fig. 5_
SopE and SopE2 from S. typhimurium activate different sets of RhoGTPases of the host cell
Andrea Friebel, Heiko Ilchmann, Martin Aepfelbacher, Kristin Ehrbar, Werner Machleidt and Wolf-Dietrich Hardt

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