might represent a universal link between small G proteins of the Rho-family deserves further investigation. □

Methods

Expression vectors and antibodies

The expression vectors used were pCR–myc–eps8, pCR3–ΔSH3Eps8 (ref. 13), pDCR–H–RasV12 (ref. 14), prCD2p110 (ref. 17), pSG5–HA–PKB[16], pMT2–Sos-1 (ref. 18), pCEFL–HA–E3b1 (ref. 4), pCDNA3RacQ61L (RacQL) and pCDNA3CDC42Q61L (Cdc42QL)[19]. Corresponding empty vectors were used as controls when appropriate (Ctr in Figures). Antibodies (Ab) used were: anti-eps8 and anti-e3b1 sera[3,4]; an anti-Eps8 (amino-acid positions 32–56 of the murine Eps8 protein) peptide serum; anti-ERK1 and anti-Sos-1 polyclonal sera (Santa Cruz Biotechnology); a rat monoclonal anti-v-H-Ras (AB-2, Oncogene Science); and a mouse monoclonal anti-Rac-1 (Transduction Laboratory).

Generation of *eps*8-null mice and fibroblasts

Mouse genomic *eps*8 clones were isolated from a 129SV library (Stratagene). A 7-kilobase (kb) *Xho*I–*Xho*I fragment was used for 5′ homology and a 2.5-kb *Eco*RV–*Not*I fragment for 3′ homology. A PGK–*neo* cassette replaced an exon-containing *eps*8 genomic 1.7-kb *Xho*I–*Eco*RV fragment. The *eps*8 SH3 domain is encoded by 2 exons (Fig. 1a) and the targeting construct excluded the first and part of the second of these exons. A genomic probe, flanking the targeting construct at the 5′ end, was used to detect the wild-type (2.6 kb) and the targeted (9.5 kb) alleles (Fig. 1a). Electroporation into mouse E14 ES cell clones, and subsequent manipulations leading to mice heterozygous and homozygous for the mutant *eps*8 allele, were as described[20]. Immortalized fibroblasts from +/+, +/− or −/− embryos were established as described[20] (see also Supplementary Information).

Microinjection, immunofluorescence and biochemical assays

Fibroblasts were seeded on gelatine-coated glass coverslips and serum-starved for 24 h. Expression vectors (0.1 mg ml^{-1}) were microinjected in the cell nuclei. Successful injections were assessed either by direct staining of the protein encoded by the injected complementary DNA or by detection of a co-injected GFP expression plasmid. At least 100 microinjected cells were analysed for each experiment. Three hours after injection (4 h in the case of Cdc42QL), cells were treated with 10 ng ml^{-1} of PDGF or 100 ng ml^{-1} of EGF (Upstate Biotechnology) or mock treated for 10 min, fixed and stained with TRICT-labelled phalloidin to detect actin filaments, using standard procedures[9].

GEF and CRIB assays were performed as described[21,22]. All presented data are the mean ± s.e. of at least three independent experiments. In GEF assays, results are expressed as a percentage of the [^{3}H]GDP released after 20 min relative to time 0, after subtracting the background counts released in control reaction (buffer alone).

In vitro bindings, immunoprecipitation and co-immunoprecipitation were performed as described[3].

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Supplementary Information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion

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An essential feature of the bacterial pathogen *Salmonella* spp. is its ability to enter cells that are normally non-phagocytic, such as those of the intestinal epithelium[1]. The bacterium achieves entry by delivering effector proteins into the host-cell cytosol by means of a specialized protein-secretion system (termed type III), which causes reorganization of the cell's actin cytoskeleton and ruffling of its membrane[2–4]. One of the bacterial effectors that stimulates these cellular responses is SopE, which acts as a guanyl-nucleotide-exchange factor on Rho GTPase proteins such as Cdc42 and Rac (ref. 5). As the actin-cytoskeleton reorganization induced by *Salmonella* is reversible and short-lived, infected cells regain their normal architecture after bacterial internalization[6,7]. We show here that the *S. Typhimurium* effector protein SptP, which is delivered to the host-cell cytosol by the type-III secretion system, is directly responsible for the reversal of the actin cytoskeletal changes induced by the bacterium. SptP exerts this function by acting as a GTPase-activating protein (GAP) for Rac-1 and Cdc42.

We identified a *S. Typhimurium* mutant that can induce actin-cytoskeleton rearrangements and membrane ruffling in host cells but is unable to reverse these effects after bacterial internalization (Fig. 1). This *S. Typhimurium* mutant strain carries a null mutation in *sptP*, a gene that encodes an effector protein for delivery to the host cell by the invasion-associated type-III secretion system[8,9]. Ref52 cells infected with wild-type *S. Typhimurium* or the complemented mutant strain began to regain a normal appearance of their

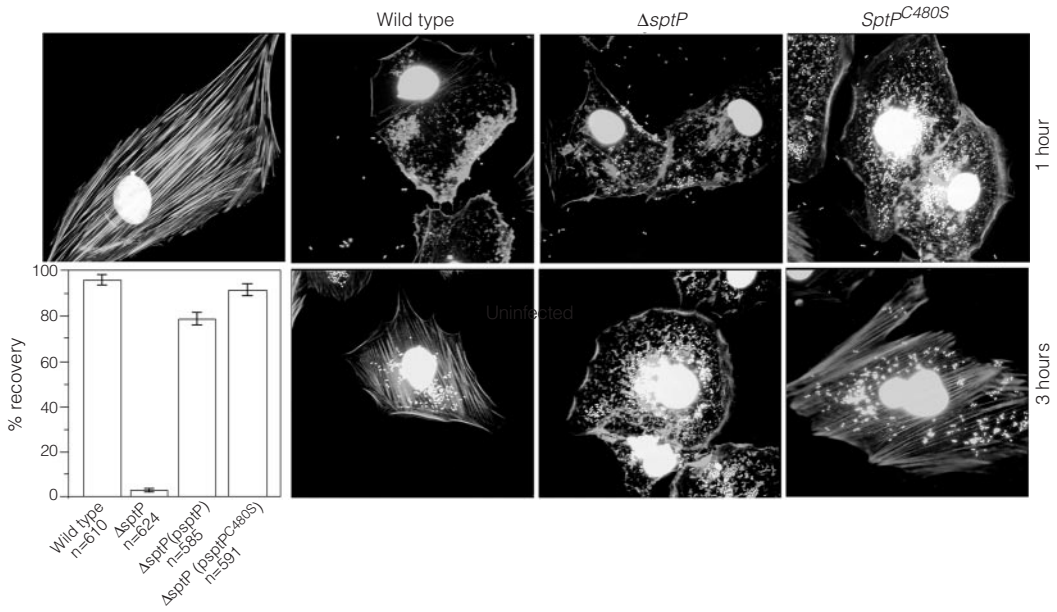

Figure 1 SptP mediates the recovery of the normal organization of the actin cytoskeleton after *S. Typhimurium* internalization. Ref52 cells were infected with different strains of *S. Typhimurium* and the actin cytoskeleton visualized by rhodamine–phalloidin staining at 1 h and 3 h after infection. Bacteria were visualized by DAPI staining. Phalloidin and DAPI images were individually captured with a Hammatsu 75i CCD camera and merged using Adobe Photoshop. The percentage of cells that recovered their actin-cytoskeleton architecture 3 h after infection with different bacterial strains is shown. Values are the mean and standard deviations from three independent experiments with wild-type, *ΔsptP*, or the mutant strain expressing either wild-type SptP (p*sptP*) or mutant SptP(C480S) (p*sptP*^{C480S}) from integrated plasmids.

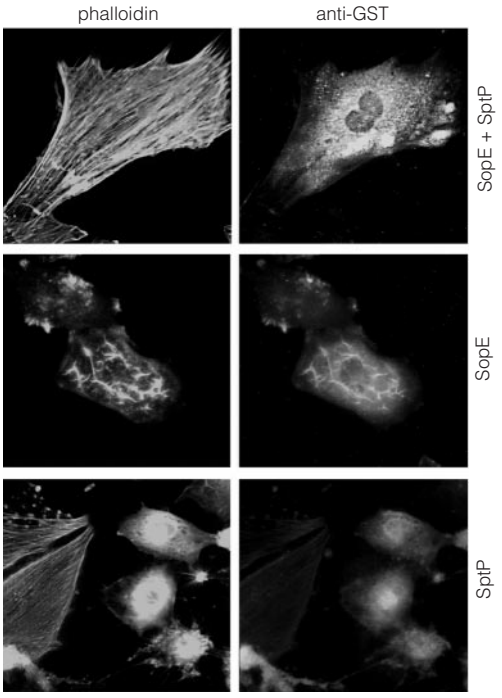

Figure 2 SptP and SopE antagonize each other's function. Ref52 cells were microinjected with GST–SopE or GST–SptP, or with a mixture of both. The concentration of bacterial protein was 400 μg ml^{-1}. When administered individually, equivalent amounts of purified GST were added to the microinjection solution to maintain a constant total protein concentration. The actin cytoskeleton was visualized by rhodamine–phalloidin staining 45 min after microinjection. Microinjected cells were identified by staining with an antibody against GST (Sigma). In each case, at least 100 microinjected cells were examined in 3 independent experiments and gave the same results as those shown here.

actin cytoskeleton as soon as 80 min after infection, and by 3 h most of the cells had fully recovered, despite the large number of internalized bacteria (Fig. 1, and data not shown). In contrast, cells infected with the *sptP* mutant strain continued to ruffle 3 h after infection and so did not regain a normal actin cytoskeletal architecture (Fig. 1, and data not shown). These results indicate that *S. typhimurium* is active in the process that leads to the rebuilding of the actin cytoskeleton after bacterial infection, presumably through the function of the type-III secreted protein SptP.

SptP is a modular protein consisting of two discrete domains[8]. The amino-terminal region shares sequence similarity with ExoS of *Pseudomonas aeruginosa*[10] and YopE of *Yersinia* spp.[11,12]. The carboxy-terminal domain of SptP shares sequence similarity with the *Yersinia* YopH protein and other tyrosine phosphatases. Purified SptP disrupts the actin cytoskeleton when microinjected into host cells, although it does so in a tyrosine-phosphatase-independent manner[8,9]. ExoS, YopE and YopH also disrupt the actin cytoskeleton and are delivered into host cells by related type-III secretion systems encoded by these bacteria[13–16].

We investigated whether the tyrosine phosphatase activity of SptP was necessary for rebuilding the actin cytoskeleton after *S. Typhimurium* infection by constructing a *S. Typhimurium* strain that expresses a mutant form of SptP (SptP(C480S)) with a critical cysteine residue at its catalytic site substituted by serine, which therefore has no tyrosine phosphatase activity[9]. Ref52 cells infected with this mutant strain rebuilt their actin cytoskeleton in a way that was indistinguishable from that observed after infection with wild-type *S. Typhimurium* (Fig. 1). Results were similar with strains expressing a truncated form of StpP lacking its last 60 amino acids which was therefore also devoid of tyrosine phosphatase activity (data not shown). These results indicate that the SptP-mediated rebuilding of the actin cytoskeleton is independent of its tyrosine phosphatase activity.

We have previously shown that microinjection of purified *S. Typhimurium* SopE protein stimulates extensive membrane ruffling and actin cytoskeleton reorganization, consistent with its role as

an exchange factor for Rho GTPases[5]. To investigate the effect of SptP on SopE-mediated actin cytoskeletal rearrangement, we microinjected purified SopE and SptP together and examined the architecture of the actin cytoskeleton by rhodamine–phalloidin staining. Simultaneous microinjection of SptP and SopE prevented the membrane ruffling and actin cytoskeleton rearrangement normally stimulated by microinjection of SopE alone[5] (Fig. 2) and blocked disruption of the actin cytoskeleton that is seen after microinjection of SptP alone[9] (Fig. 2). These results indicate that when simultaneously applied to cells, SptP and SopE can antagonize each other's effect on the actin cytoskeleton.

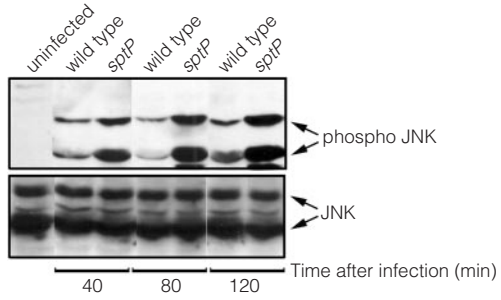

Figure 3 Effect of SptP on *S. Typhimurium*-mediated JNK activation. Ref52 cells were infected for various times with either wild-type *S. Typhimurium* or a *sptP*-deletion mutant at a multiplicity of infection of 40. The levels of phosphorylated (active) JNK were determined by western-blot analysis and densitometric scanning (see Methods; upper panel). The blot was stripped and reprobed with an antibody directed against JNK (Santa Cruz Biotechnology) (lower panel).

As well as causing actin cytoskeleton rearrangement, *S. Typhimurium* infection results in the activation of the MAP-kinase pathways involving p38 protein and JNK kinase, which trigger a nuclear response and cause proinflammatory cytokines to be produced[17]. These responses depend on Cdc42 and Rac-1 and are mediated by bacterial effectors delivered by the type-III secretion system, such as that of SopE (refs 2, 3, 5). We investigated whether SptP could also antagonize bacterial signals leading to the activation of SAPK/JNK kinases. Ref52 cells were infected for various times with either wild-type *S. Typhimurium* or the *sptP* isogenic mutant and the activation of SAPK/JNK kinase was tested. As shown in Fig. 3, SAPK/JNK activation was lower in cells infected with wild-type *S. Typhimurium* than in cells infected with the *sptP* mutant. Activation of SAPK/JNK peaked 30 to 40 min after infection with wild-type *Salmonella* and declined slightly over time (Fig. 3, and data not shown). In contrast, the amount of the phosphorylated (active) form of SAPK/JNK present was significantly higher (3–5-fold in three independent experiments) in cells infected with the *sptP* mutant, and remained high even 120 min after infection (Fig. 3). These results indicate that SptP downregulates the SAPK/JNK activation stimulated by *S. typhimurium* infection.

Rho GTPases exert a variety of effects through different downstream proteins (reviewed in refs 18, 19), and the activation of the SAPK/JNK pathway and the actin cytoskeleton rearrangements mediated by the Rho GTPases Cdc42 and Rac-1 are thought to be mediated by different effector proteins. The results indicating that SptP can antagonize signals leading to both cytoskeletal and nuclear responses stimulated by either *S. Typhimurium* infection or microinjection of SopE suggest that SptP may act directly on the Rho GTPases. We therefore investigated whether SptP could interact with Rac-1 by using a glutathione-*S*-transferase (GST)

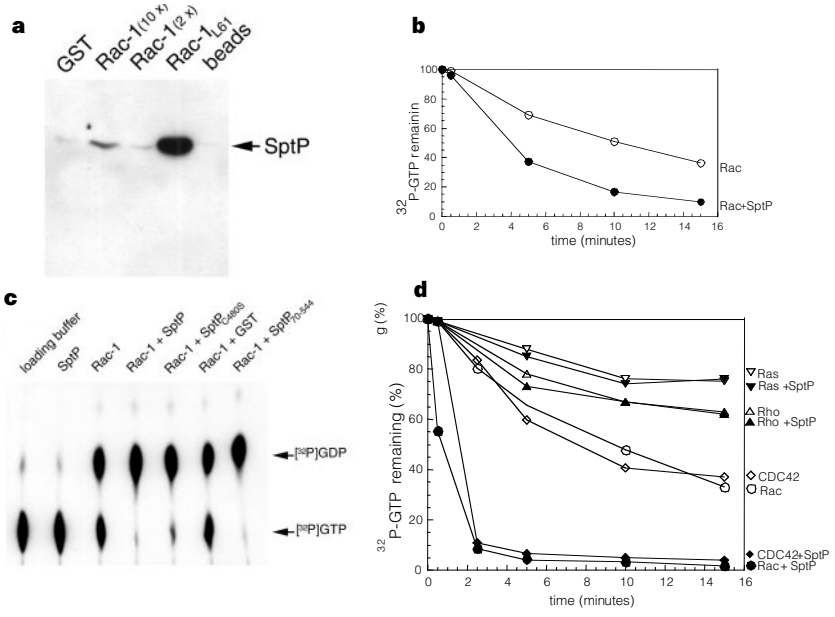

Figure 4 SptP is a GAP for CDC42 and Rac. **a**, SptP binds Rac(L61). Bacterial extracts containing SptP were incubated with glutathione–agarose beads loaded with GST, GST–Rac and GST–Rac(L61), and bound proteins were examined by western blot using a monoclonal antibody against SptP. The amount of GST-Rac protein used in the assay was 2 (2×) and 10 (10×) fold more than GST–Rac(L61). Equivalent results were obtained when purified SptP was used in the assay (see Methods). **b**, **c**, SptP stimulates the GTPase activity of Rac. The effect of addition of purified GST–SptP(70-544) (10 nM) to purified [γ-^{32}P]GTP-loaded GST–Rac-1 (400 nM) was examined in a filter-binding GAP assay (see Methods) (**b**) (values represent results from one of four experiments that gave equivalent results, with a standard deviation of less than 5%). Alternatively, the GAP activity of GST–SptP (100 nM) towards purified [α-^{32}P]GTP-loaded GST–Rac-1 (400 nM) was examined by TLC (**c**) (this experiment was repeated three times with identical results). **d**, GAP activity of SptP shows preference for Rac and Cdc42. The GAP activity of GST–SptP(70-544) towards Rac-1, Cdc42Hs, RhoA and H-Ras was examined by a filter-binding assay (see Methods). The concentration of GST–SptP(70-544) was 40 nM for reactions with Cdc42 and Rac, 160 nM for reactions with Rho, and 400 nM for reactions with Ras. The concentration of all small G proteins in the reactions was 400 nM (values represent the results of one of three experiments that gave equivalent results, with a standard deviation of less than 5%).

pull-down assay. Wild-type Rac-1 and its constitutively active mutant form Rac-1(L61) were purified as GST-fusion proteins and tested for their ability to interact with wild-type SptP expressed in *Escherichia coli*. SptP was able to interact with GST–Rac-1(L61) but not with either GST–Rac-1 or GST alone (Fig. 4a), indicating that SptP can bind Rac-1 in an interaction that is preferentially or exclusively directed to the GTP-bound form of this GTPase.

The function of small GTP-binding proteins is negatively regulated by a family of GTPase-activating proteins (GAPs) which preferentially bind the active (GTP-bound) form of the cognate G protein and stimulate its intrinsic GTPase activity[18–20]. The observations that SptP antagonizes bacterial responses resulting from the activation of the Rho GTPases Cdc42 and Rac-1 and that SptP preferentially or exclusively binds the GTP-bound form of Rac-1 suggested that SptP might function as a GAP to downregulate signalling through Rho GTPases. We therefore tested the ability of SptP to stimulate the intrinsic GTPase activity of Rac-1 and Cdc42 and found that SptP had potent GAP activity for Rac-1 (Fig. 4b, c) and Cdc42 (Fig. 4d). The SptP GAP activity was readily detected even at 40-fold molar excess of Rac-1 over SptP (Fig. 4b) and showed a slight preference for Rac-1 over Cdc42 (Fig. 4d, and data not shown). In contrast, SptP had no measurable GAP activity towards Ras and its GAP activity towards Rho was 100-fold less than towards Rac (Fig. 4d, and data not shown), indicating specificity for a subset of Rho GTPases. SptP(C480S), which has no tyrosine phosphatase activity, retained wild-type GAP activity (Fig. 4c), consistent with our observation that tyrosine phosphatase activity is not required for modulating effects of SptP on the actin cytoskeleton.

Proteins with GAP activity towards Rho GTPases share a sequence motif containing an invariant arginine that is essential for efficient catalysis[20]. Inspection of the amino-acid sequence of SptP, as well as of the related bacterial proteins ExoS and YopE, revealed the presence of a similar sequence motif (Fig. 5a). We therefore tested whether this motif was involved in the catalytic activity of SptP by constructing an SptP mutant in which the invariant arginine was replaced by alanine. The resulting protein (SptP(R209A)) could still bind Rac(L61) (data not shown), indicating that the mutation did not cause a gross conformational change. As shown in Fig. 5b, SptP(R209A) had no GAP activity towards Rac, indicating that SptP works by a mechanism similar to that used by other GAPs[20]. To determine whether this GAP activity of SptP mediated the rebuilding of the actin cytoskeleton following infection with wild-type *Salmonella*, we constructed a *S. Typhimurium* strain expressing SptP(R209A). This strain expressed and secreted the mutant protein at wild-type levels (data not shown). Cells infected with the *S. Typhimurium* strain expressing SptP(R209A) failed to regain the normal appearance of the actin cytoskeleton, confirming the importance of SptP GAP activity in this process (Fig. 5c).

We have shown that the *S. Typhimurium* type-III secreted protein SptP modulates the actin cytoskeleton by acting as a GAP for Rac-1 and Cdc42. A similar mechanism may account for the activity of the related toxins ExoS and YopE which also disrupt the actin cytoskeleton. The GAP activity of SptP results in downregulation of the actin-cytoskeleton rearrangements stimulated by other bacterial effectors delivered by the type-III secretion system, such as the Rho-GTPase exchange factor SopE. Our results provide a molecular explanation for the rapid reversibility of cellular responses associated with *S. Typhimurium* entry into cells. Also relevant may be the selectivity of SptP for Rac-1 and Cdc42, and its lack of GAP activity towards Rho. As SopE activates Rho, at least *in vitro*[5], such activity may help to rebuild the actin cytoskeleton after bacterial internalization, given that activation of Rho leads to the assembly of stress fibres and focal adhesions[18,19]. The downregulation of cellular responses may help to maintain the viability of cells that provide a permissive environment for the bacteria to replicate or evade host defences, because prolonged signalling through Cdc42 and Rac mediated by other bacterial effectors may harm the cell. Our results also indicate that *Salmonella* delivers SopE and SptP into the host cell either sequentially or in different amounts in order to stimulate the responses associated with the infectious process. The coordinated regulation of Rho GTPases by bacterial effector proteins that can sequentially step up and reduce their activities is a remarkable example of a strategy evolved by a pathogen to manipulate the cellular functions of its host. □

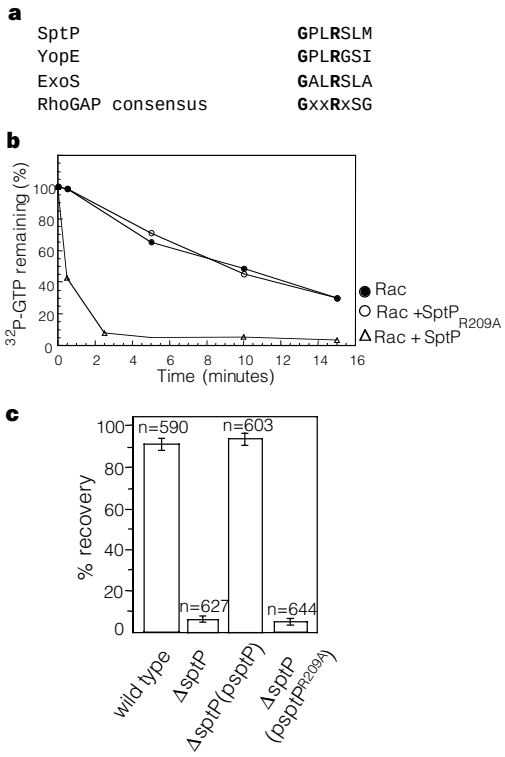

Figure 5 An 'arginine finger' characteristic of Rho-GTPase-activating proteins is required for SptP function. **a**, SptP displays a conserved motif (arginine finger) present in RhoGAPs and related bacterial toxins. **b**, SptP(R209A), which carries a mutation in the arginine finger motif, is devoid of GAP activity. The GAP activity of GST–SptP(70–544,R209A) (1 μM) towards [γ-^{32}P]-GTP-loaded GST–Rac-1 (400 nM) was examined by a filter-binding assay. The concentration of the positive control GST–SptP(70–544) in this reaction was 40 nM (values represent the results of one of three experiments that gave equivalent results, with a standard deviation of less than 5%). **c**, The GAP activity of SptP is required for the recovery of the actin cytoskeleton after bacterial infection. Ref52 cells were infected with wild-type *S. Typhimurium*, the isogenic Δ*sptP* strain, the isogenic Δ*sptP* carrying a plasmid encoding either wild-type SptP (p*sptP*) or the GAP-deficient mutant SptP(R209A) (p*sptP*^{R209A}), and 3 h after infection the integrity of the actin cytoskeleton in infected cells was examined by fluorescence microscopy (see legend to Fig. 1). The results are the mean and standard deviations of three independent experiments.

Methods

Bacterial strains and plasmids

Wild-type *S. Typhimurium* strain SB300 has been described[9]. The isogenic strains SB749, which carry a complete deletion of the *sptP* gene, SB1001 and SB1002 which express wild-type SptP or the catalytic mutant SptP^{C480S}, respectively, from integrated plasmids were constructed by site-directed mutagenesis and allelic exchange. Plasmids expressing fusion proteins between glutathione-*S*-transferase (GST) and wild-type or mutant forms of SptP have been described[9]. Plasmids expressing fusion proteins between GST and different Rho GTPases were from different sources and have also been described[2,5]. SptP^{R209A} was

constructed by site-directed mutagenesis using PCR. Plasmids pSB759 and pSB1473, which express wild-type SptP and the GAP-defective mutant $SptP^{R209A}$, respectively, from an arabinose-inducible promoter were constructed by standard recombinant-DNA techniques.

Cell lines, bacterial infections, microinjection of cultured cells and immunofluorescence microscopy

Ref52 cells were used as they possess a well developed actin cytoskeleton. Microinjection of these cells, bacterial infections, antibody staining and fluorescence microscopy were all carried out as described[9].

GST pull-down assay

GST–Rac1 or GST–$Rac\text{-}1^{L61}$ purified as described[5], were bound to glutathione-4B agarose beads in 100 μl PBS buffer at 4 °C for 30 min. Beads were then washed with binding buffer (20 mM phosphate buffer, pH 7.4, containing 1% Triton-X100, 1 mM DTT) before being added to a lysate of *E. coli* DH5α carrying plasmid pSB759 or pSB1473, which expresses *sptP* or $sptP^{R209A}$ under an arabinose-inducible promoter, or to a solution of purified SptP protein cleaved from the GST moiety by thrombin (data not shown). Bacterial lysates were prepared by sonication in binding buffer after growth under inducing conditions (growth for 5 h in LB containing 0.005% arabinose). Cleavage of SptP from the GST moiety was carried out as described[5]. The beads prebound to GST-fusion proteins were incubated with the bacterial lysates or purified SptP protein at 4 °C for 30 min and washed three times in binding buffer. The bound proteins were eluted in sample buffer and analysed by standard western blotting using a monoclonal antibody against SptP.

JNK activation assay

Detection of the phosphorylated (active) form of JNK was carried out by western blotting with a phosphospecific antibody according to the manufacturer's instructions (New England Biolabs). Quantification of phosphorylated JNK was carried out by densitometric analysis of films exposed in the linear intensity range. Polyclonal antibody against JNK was from Santa Cruz Biotechnology.

GAP assays

Purified small GTP-binding proteins fused to GST were loaded with 50 nM of GTP (1:30 ratio of [α-^{32}P] GTP to cold GTP) in 20 μl GTP-loading buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM DTT) at room temperature for 15 min. GTP hydrolysis was initiated upon addition of $MgCl_2$ and different forms of SptP or control protein, as indicated. After 15 min, GTP and GDP were eluted from the samples by 0.4% SDS, 5 mM EDTA, 5 mM GTP and 5 mM GDP at 65 °C for 5 min and separated by thin-layer chromatography on polyethyleneimine–cellulose sheets (Scientific) with 0.75 M KH_2PO_4, pH 3.5, as the developing solvent, followed by autoradiography and PhosphorImager quantification (Molecular Dynamics). Alternatively, GAP activity was measured by a filter-binding assay[21]. All filter-binding assays were carried out using GST–$SptP^{70\text{-}544}$, which lacks the secretion and translocation signals and is more soluble than GST–$SptP^{1\text{-}544}$.

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Deregulated cyclin E induces chromosome instability

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Cyclin E, a regulatory subunit of cyclin-dependent kinase 2 (Cdk2), is an important regulator of entry into S phase in the mammalian cell cycle. In normal dividing cells, cyclin E accumulates at the G_1/S-phase boundary and is degraded as cells progress through S phase[1,2]. However, in many human tumours cyclin E is overexpressed[3] and the levels of protein and kinase activity are often deregulated relative to the cell cycle[4–7]. It is not understood how alterations in expression of cyclin E contribute to tumorigenesis. Here we show that constitutive cyclin-E overexpression in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosome instability (CIN). In contrast, analogous expression of cyclin D1 or A does not increase the frequency of CIN. Cyclin-E-expressing cells that exhibit CIN have normal centrosome numbers. However, constitutive overexpression of cyclin E impairs S-phase progression, indicating that aberrant regulation of this process may be responsible for the CIN observed. These results indicate that downregulation of cyclin-E/Cdk2 kinase activity following the G_1/S-phase transition may be necessary for the maintenance of karyotypic stability.

There is considerable evidence that alteration of expression of cyclin E contributes to the development of many types of human tumours. Overexpression of cyclin E correlates with advanced grades and stages of tumour and has prognostic significance in many tumour types[5–11]. Expression of cyclin E is elevated in premalignant lesions of the breast and skin, indicating an early event in the development of these tumours[8,11]. Additionally, constitutive overexpression of cyclin E specifically targeted to the mammary epithelium of transgenic mice increases the incidence of breast hyperplasias and carcinomas[12].