Heterologous expression of bacterial virulence factors in *Saccharomyces cerevisiae* is a feasible approach to study their molecular function. The authors have previously reported that the *Salmonella typhimurium* SigD protein, a phosphatidylinositol phosphatase involved in invasion of the host cell, inhibits yeast growth, presumably by depleting an essential pool of phosphatidylinositol 4,5-bisphosphate, and also that a catalytically inactive version, SigDR468A, was able to arrest growth by a different mechanism that involved disruption of the actin cytoskeleton. This paper describes marked differences between the phenotypes elicited by expression of SigD and SigDR468A in yeast. First, expression of SigDR468A caused accumulation of large unbudded cells and loss of septin organization, while SigD expression caused none of these effects. Second, growth inhibition by SigDR468A was mediated by a cell cycle arrest in G2 dependent on the Swe1 morphogenetic checkpoint, but SigD-induced growth inhibition was cell cycle independent. And third, SigD caused strong activation of the yeast MAP kinase Slt2, whereas SigDR468A rather inactivated another MAP kinase, Kss1. In a screen for suppressors of SigDR468A-induced growth arrest by overexpression of a yeast cDNA library, the Cdc42 GTPase was isolated. Furthermore, SigDR468A was co-purified with Cdc42 from yeast lysates. It is concluded that the *Salmonella* SigD protein deprived of its phosphatase activity is able to disrupt yeast morphogenesis by interfering with Cdc42 function, opening the possibility that the SigD N-terminal region might directly modulate small GTPases from the host during infection.
Salmonella & Miller, 1998; Norris synaptojanin (inositol 5-phosphatase)-like domain (Hong mammalian inositol 4-phosphatases, as well as a putative synaptojanin (inositol 5-phosphatase)-like domain (Hong mammalian inositol 4-phosphatases, as well as a putative phosphatase, bearing characteristic motifs 1 and 2 of phosphate substrates, but we have recently shown that the N-

entry in a Cdc42-dependent manner (Murli et al., 2001; Zhou et al., 2001). Recently, we have also reported that SigD produces a strong inhibitory effect in yeast (Alemán et al., 2005). SigD, a Salmonella homologue of IpgD from Shigella (Niebuhr et al., 2000), is an inositol polyphosphate phophatase, bearing characteristic motifs 1 and 2 of mammalian inositol 4-phosphatases, as well as a putative synaptojanin (inositol 5-phosphatase)-like domain (Hong & Miller, 1998; Norris et al., 1998; Marcus et al., 2001).

Although Salmonella cells lacking sigD are still able to invade host cells (Zhou et al., 2001), elimination of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂) from invaginating regions during phagocytosis induced by the bacteria depends on SigD (Terebiznik et al., 2002). SigD also seems to be important for the generation of the characteristic pools of phosphatidylinositol 3-phosphate in the membrane of the Salmonella-containing vacuole (Hernández et al., 2004). SigD might generate this phosphoinositide by dephosphorylation of phosphatidylinositol 3,5-bisphosphate, a lipid involved in endosomal maturation (Dukes et al., 2006).

There are clues that point towards the idea that SigD is involved in other functions, although the precise molecular mechanisms remain unknown: first, as stated above, it has been proposed to exert its function via modulation of small G proteins, like Cdc42 (Zhou et al., 2000); and, third, SigD seems to countermand the negative effect on yeast Cdc42-dependent signalling exerted by a phosphatase-dead version of SigD. This suggests that SigD might be able to modulate the activity of small GTPases in eukaryotic host cells and supports the value of the yeast model coupled to directed mutagenesis for performing basic functional studies on heterologous proteins.

**METHODS**

**Bacterial and yeast strains, media and growth conditions.** The *S. cerevisiae* strains used are described in Table 1. *S. typhimurium* C53 is a plasmidless derivative of the C5 wild-type strain (Pardon et al., 1986) and was kindly provided by F. Norel, Institut Pasteur, Paris, France. Escherichia coli DH5a F’ [K12 Δ(lacZYA-argF)U169 deor supE44 thi-1 recA1 endA1 hsdR17 gyrA96 relA1 (800lacZAM15) F’] was used for molecular biology techniques and protein expression.

YPD (1% yeast extract, 2% peptone and 2% glucose) broth or agar was the general non-selective medium used for growing the yeast strains. Synthetic minimal medium (SD) contained 0-17% yeast nitrogen base without amino acids and ammonium sulfate, 0-5% ammonium sulfate and 2% glucose, and was supplemented with appropriate amino acids and nucleic acid bases. SG and SR were SD with 2% galactose or raffinose, respectively, instead of glucose. Galactose induction experiments in liquid media were performed by growing cells in SR medium to exponential phase and then adding galactose to 2% for 6–8 h. SigD effects on yeast were tested by spotting growing cells onto plates of SD or of SG lacking the corresponding auxotrophic

**Table 1. *S. cerevisiae* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH499</td>
<td>MATa ade2-101 trp1-1 leu2-1 ura3-52 his3-Δ200 lys2-801</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>I783</td>
<td>MATa trp1-1 leu2-3,112 ura3-52 his4 can4</td>
<td>Lee et al. (1993)</td>
</tr>
<tr>
<td>TD28</td>
<td>MATa ura3-52 inol-131 canB</td>
<td>F. del Rey</td>
</tr>
<tr>
<td>MJY100</td>
<td>MATc can1-100 his3-11,15 leu2-3,112 lys2 trp1-1 ura3-1</td>
<td>Shulewitz et al. (1999)</td>
</tr>
<tr>
<td>MJY30</td>
<td>MATc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cdc28Δ (:: LEU2)</td>
<td>Shulewitz et al. (1999)</td>
</tr>
<tr>
<td>VBY16</td>
<td>MATc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 swe1Δ (:: LEU2)</td>
<td>Cid et al. (2001b)</td>
</tr>
<tr>
<td>DHY52</td>
<td>MATd ade2-loc his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 SPC29-YFP (:: HIS3)</td>
<td>T. Davis</td>
</tr>
<tr>
<td>BY4742</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>BY413a</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 knaMX4</td>
<td>Ruiz et al. (2003)</td>
</tr>
<tr>
<td>SY2002</td>
<td>MATa his3:: FUS1-HIS3 mfa2Δ Δ:: FUS1-lacZ ura3 leu2 trp1 his3 ade1</td>
<td>G. Sprague</td>
</tr>
<tr>
<td>YGS57</td>
<td>MATa his3:: FUS1-HIS3 mfa2Δ Δ:: FUS1-lacZ ura3 leu2 trp1 his3 ade1 rga2:: URA3 rga2:: TRP1</td>
<td>G. Sprague</td>
</tr>
<tr>
<td>HM53</td>
<td>MATa trp1Δ901 leu2-3,112 ade2-101 ura3-52 his3-Δ200 suc2Δ9 kss1Δ Δ:: URA3 slt2Δ Δ:: LEU2</td>
<td>H. Martin</td>
</tr>
<tr>
<td>BYAfus</td>
<td>MATa; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FUS3:: KanMX4</td>
<td>EUROSCARF</td>
</tr>
</tbody>
</table>

*P. Hieter, University of British Columbia, Vancouver, Canada; F. del Rey, Universidad de Salamanca, Spain; T. Davis, University of Washington, Seattle, USA; EUROSCARF, European Saccharomyces cerevisiae Archive for Functional Analysis (http://www.uni-frankfurt.de/fb15/mikro/euroscarf); G. Sprague, University of Oregon, Eugene, USA; H. Martin, Universidad Complutense de Madrid, Spain.
requirements to maintain plasmids as follows. Transformants were grown overnight in SD lacking uracil or leucine (SD–Ura or SD–Leu) and adjusted to an OD{sub}600 of 0.5. Five-microlitre aliquots of each sample plus three serial 1/10 dilutions were deposited on the surface of solid SG–Ura medium. Growth was monitored after 2 to 3 days at 28 °C.

**Molecular biology techniques and plasmid construction.** *E. coli* transformation and basic molecular biology techniques were performed by standard methods. Yeast transformation was achieved by the standard lithium acetate protocol. Two series of plasmids containing the *sigD* mutations F359A and K527A were constructed in this study, one series based on the pEG(KG) vector to express glutathione S-transferase (GST) fusion proteins in yeast (Mitchell et al., 1993) and a second series based on pGEX-KG (Guan & Dixon, 1991) for expression of GST-fused proteins in bacteria, by following the same strategy previously used to obtain pKG-SigD, pKG-SigD{sup}R468A-, pGEX-KG-SigD and pGEX-KG-SigD{sup}R468A- (Aleman et al., 1991–1993) and a second series based on pGEX-KG (Guan & Dixon, 1993) and a second series based on the pEG(KG) vector to express glutathione S-transferase (GST) fusion proteins in yeast (Mitchell et al., 1993) and a second series based on pGEX-KG (Guan & Dixon, 1991) for expression of GST-fused proteins in bacteria, by following the same strategy previously used to obtain pKG-SigD, pKG-SigD{sup}R468A-, pGEX-KG-SigD and pGEX-KG-SigD{sup}R468A- (Aleman et al., 2005). Plasmids from these series were named as follows: pKG-SigDF359A and pGEX-KG-SigD K527A for the pEG(KG) series; and pGEX-KG-SigDF359A and pGEX-KG-SigD K527A for the pGEX-KG series. These plasmids were constructed by PCR amplification from the SigD open reading frame from *S. typhimurium* genomic DNA. Site-directed mutagenesis was performed by sequential PCR. Oligonucleotides for these strategies are listed in Table 2. The primers used for amplification of the different SigD alleles had *Bam*HI- or *Hind*III-containing tails in their sequence. The PCR product was cloned into the pGEM-T vector (Promega) and cleaved with *Bam*HI for insertion into the same sites of either pEG(KG) or pGEX-KG. Wild-type *sigD* and the *sigD*{sup}R468A- mutant allele were also cloned in YCP15, a LEU2 centromeric vector with the GAL1 promoter, kindly provided by J. Thorner, University of California, Berkeley, USA, following the same *Bam*HI/ *Hind*III-based strategy. YCP15-SigD{sup}R468A-GFP was constructed by PCR amplification of *sigD*{sup}R468A- from pKG-SigD{sup}R468A- using the SigD{sup}1- and SigD-GFP2 primers (Table 2), which have *Bam*HI-containing tails in their sequence. The PCR product was cloned into the pGEM-T vector (Promega) and cleaved with *Bam*HI for insertion into the same site of the YCP15-GFP vector, a version of YCP15 produced in this work by inserting the GFP coding region into the *Bam*HI sites of the polylinker. For the pKG-SigD{sup}R468A-, A{sup}118–142 construct, site-directed mutagenesis using as template pKG-SigD{sup}A{sup}118–142- (Aleman et al., 2005) was performed to generate the R468A mutation by sequential PCR using the primers SigD{sup}1-1, SigD{sup}2-1, Arg-1 and Arg-2 (Table 2). The resulting PCR product was cloned into the pGEM-T vector (Promega), cleaved with *Bam*HI and *Hind*III and inserted into the same sites of the pEG-KG plasmid. Plasmid pEG(KG)-CDC24{sup}450–854- was constructed by PCR amplification from the CDC24 open reading frame from *S. cerevisiae* genomic DNA with oligonucleotides bearing *Bam*HI and *Hind*III restriction sites (underlined in Table 2), and cloned in the same sites of pEG(KG), pKG-CDC42{sup}G12V- (underlined) and L-CDC24 primers (Table 2), which contain XbaI flanking sites, and cloning the amplified product into the same restriction site of pEG(KG). In all cases, fidelity of the amplified DNA was verified by DNA sequencing. Other plasmids used in this work were pLA10H (Cid et al., 2001a), used to express GFP-tagged septin, and GAL-GST-CDC24, kindly provided by Daniel Lew (Duke University, *C. elegans*).

### Table 2. Oligonucleotides and PCR strategies for the yeast and bacterial expression constructs

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′–3′)*</th>
</tr>
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<tbody>
<tr>
<td>For <em>sigD</em>{sup}F359A</td>
<td>CACATTGGGCTGCGGGCGACGACGTC</td>
</tr>
<tr>
<td>Phe-1</td>
<td>GCCGCACGCCAATGGGTTAATGAGC</td>
</tr>
<tr>
<td>Phe-2</td>
<td>GGGATCCATGCAAAATACAGACTTC</td>
</tr>
<tr>
<td>SigD-1</td>
<td>CAAGCTTCAAGATGTTAATGAGG</td>
</tr>
<tr>
<td>SigD-2</td>
<td>CATTACGGCGTTTCCGCCGCCGCC</td>
</tr>
<tr>
<td>Lys-1</td>
<td>GGAAGGCGTGTAATGAAAAATTATGC</td>
</tr>
<tr>
<td>Lys-2</td>
<td>As above</td>
</tr>
<tr>
<td>SigD-1</td>
<td>As above</td>
</tr>
<tr>
<td>SigD-2</td>
<td>As above</td>
</tr>
<tr>
<td>For <em>pKG-CDC24</em>{sup}450–854</td>
<td>CGGGATCATGAGTATCAAGAAAAATCAA</td>
</tr>
<tr>
<td>Cdc24-1</td>
<td>CCCAGCTTTCAATACAGCAATGTT</td>
</tr>
<tr>
<td>Cdc24-2</td>
<td>As above</td>
</tr>
<tr>
<td>For SigD{sup}B468A-GFP</td>
<td>CGGGATCCAGATGTTAATGAGG</td>
</tr>
<tr>
<td>SigD-1</td>
<td>CCGCGACGCCAATGGGTTAATGAGC</td>
</tr>
<tr>
<td>SigD-GFP2</td>
<td>GGGATCCATGCAAAATACAGACTTC</td>
</tr>
<tr>
<td>For SigD{sup}B468A, A{sup}118–142</td>
<td>CGGGATCCAGATGTTAATGAGG</td>
</tr>
<tr>
<td>Arg-1</td>
<td>CCCCTGTGGCATTTGGCGCCTTTAC</td>
</tr>
<tr>
<td>Arg-2</td>
<td>AAAAGATGCCACAGGGATGATTGTC</td>
</tr>
<tr>
<td>SigD-1</td>
<td>As above</td>
</tr>
<tr>
<td>SigD-2</td>
<td>As above</td>
</tr>
<tr>
<td>For pKG-Cdc42{sup}G12V</td>
<td>CACTAGAGAAGAATTCTAGAAAAACGGCTAAGTG</td>
</tr>
<tr>
<td>U-CDC42</td>
<td>CTTCTAGAATTCTAGAAAAAGATG</td>
</tr>
<tr>
<td>L-CDC42</td>
<td>CTTCTAGAATTCTAGAAAAAGATGGGC</td>
</tr>
</tbody>
</table>

*Bam*HI and *Hind*III sites are underlined.
Durham, NC, USA). The cDNA library used in the screening for suppressors of SigD<sub>R66A</sub> by overexpression was constructed by Liu et al. (1992) on a PRS316 centromeric yeast expression vector.

**GST pull-down assays.** Cultures were grown overnight in raffinose-based medium to exponential phase, and expression of the GST or GFP fusions was induced with galactose for 8 h. Lysates were obtained from these cultures in ice-cold lysis buffer (10 %, v/v, glycerol, 50 mM Tris/HCl pH 7.5, 0.1 % NP40, 150 mM NaCl, 5 mM EDTA pH 8, 50 mM NaF, 5 mM sodium pyrophosphate, 50 mM β-glycerol phosphate, 1 mM sodium vanadate), clarified by centrifugation at 13 000 r.p.m. for 10 min at 4 °C, and 50 μl glutathione-Sepharose beads (Amersham Pharmacia) was added to 150 μl of each sample. After 1 h incubation at 4 °C, the beads were washed three times (for the Cdc42-SigD pull-down experiments) or five times (for the Cdc24-SigD pull-down experiment) with the same ice-cold buffer, and finally resuspended in 25 μl 2 × SDS-PAGE loading buffer (125 mM Tris/HCl pH 6.8, 5 % SDS, 25 % v/v, glycerol, 0.2 M DTT and 0.1% bromophenol blue). Samples were then loaded in polyacrylamide gels for immunodetection by Western blotting. In the gels, 10 μl aliquots of the original lysates were loaded as ‘inputs’, and 5 μl of the samples after affinity purification as ‘pull-downs’.

**Immunodetection by Western blotting.** Standard procedures were used for yeast cell growth, collection, breakage, protein separation by SDS-PAGE, and transfer to nitrocellulose membranes, as previously described (Rodríguez-Pachón et al., 2002). Anti-phospho-p44/p42 MAPK (Thr-202/Tyr-204) antibody (New England Biolabs) was used to detect dually phosphorylated Slt2, Kss1 and Fus3 MAPKs. Slt2 protein was detected using a polyclonal anti-Slt2 antibody (Martin et al., 1993). Kss1, Fus3 and Cdc42 were detected with specific polyclonal antibodies (sc-6775, sc-6773 and sc-87, respectively, from Santa Cruz Biotechnology). A monoclonal anti-actin antibody (Martín et al., 1993) was used to detect dually phosphorylated Slt2, Kss1 and Fus3 p44/p42 MAPK (Thr-202/Tyr-204) antibody (New England Biolabs) was used to detect dually phosphorylated Slt2, Kss1 and Fus3 MAPKs. Slt2 protein was detected using a polyclonal anti-Slt2 antibody (Martin et al., 1993). Kss1, Fus3 and Cdc42 were detected with specific polyclonal antibodies (sc-6775, sc-6773 and sc-87, respectively, from Santa Cruz Biotechnology). A monoclonal anti-actin antibody (MP Biomedicals) was used as a loading control. GST fusion proteins were detected using polyclonal or monoclonal anti-GST antibodies (Santa Cruz Biotechnology). The primary antibody was detected using horseradish-peroxidase-conjugated anti-rabbit or anti-mouse antibodies with the ECL detection system (Amersham Biosciences).

**Expression and purification of GST fusions from *E. coli*.** Three-mlilitre volumes of LB containing 100 μg ampicillin ml<sup>−1</sup> were inoculated with glycerol stocks and grown at 37 °C overnight. These cultures were used to inoculate experimental cultures to OD<sub>600</sub> 0.1 in a final volume of 10 ml, and IPTG was added at a final concentration of 0.1 mM. After 1 h incubation, the cultures were centrifuged for 5 min at 3000 r.p.m. Supernatants were poured off and pellets resuspended in 300 μl Tris/HCl pH 7.5 containing 1 mM EDTA, 1 mM DTT, 10 μg ml<sup>−1</sup> each of leupeptin and aprotinin; 15 μl of 25 mg lysozyme ml<sup>−1</sup> was added, followed by incubation on ice for 20 min. The suspensions were then sonicated three times for 30 s and centrifuged for 15 min at 13 000 r.p.m. To solubilize inclusion bodies, 300 μl Tris/HCl pH 7.5 containing 1 % Sarkosyl was added, followed by resuspension with a pipette, rotation on a spinning wheel for 20 min at 4 °C, then centrifuged for another 15 min. Three hundred microlitres of Tris/HCl pH 7.5 containing 4 % Triton X-100 was added to each Eppendorf tube, followed by 50 μl of a 50 % glutathione-adsorbed slurry (Amersham Pharmacia) and the mixture was incubated overnight at 4 °C. Next day, the slurry was washed three times with Tris/HCl pH 7.5 and resuspended in 100 μl of the same buffer. Efficiency of purification was monitored by SDS-PAGE analysis of 20 μl aliquots from these suspensions and staining gels with Coomassie blue. Standard techniques were performed for protein analysis.

**In vitro assay for phosphatase activity.** Phosphoinositide phosphatase activity was measured using a chromogenic assay based on the malachite green method, which was previously used for the analysis of protein phosphatases (Harder et al., 1994; Marcus et al., 2001). Briefly, recombinant purified protein was incubated with a reaction mixture consisting of 100 mM Tris/HCl pH 8, 10 mM diithiothreitol, 0.5 mM diC<sub>16</sub>-phosphatidylserine (Sigma P-1185) and 25 μM phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns (3,4,5)-P<sub>3</sub>) (diC<sub>16</sub> Biomol PH-107) (temperature 37 °C, reaction volume 50 μl). The reactions were stopped by addition of 50 μl malachite green reagent and absorbance was measured at 620 nm.

**Flow cytometric techniques.** For the analysis of DNA contents, samples were fixed and permeated by 5 min treatment with 70 % ethanol. They were then resuspended in 400 μl of 10 g l<sup>−1</sup> RNase A and incubated for 30 min at 37 °C. Then DNA was stained by addition of 0-005 % propidium iodide in PBS. Three thousand cells were analysed per second on a FACScan (Becton Dickinson) on the FL2 log scale. Data of forward scatter were simultaneously recorded. WinMDI 2.7 software was used to handle the graphics obtained.

**Microscopy techniques and immunofluorescence.** For fluorescence microscopy on live cells (for the observation of GFP and YFP), cells from exponentially growing cultures were centrifuged at 13 000 r.p.m washed once with sterile water and observed. For statistics on cell populations, an average of 200 cells was counted for each experiment.

Staining of actin in yeast cells with FITC-conjugated phalloidin (Sigma) was performed as previously described (Aleman et al., 2005). Indirect immunofluorescence on yeast cells was performed as previously described (Cid et al., 2001b). Anti-GST antibodies (Santa Cruz Biotechnology) were used at a 1:500 dilution. As secondary antibody, indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG was used at a 1:500 dilution. For both fluorescence microscopy and indirect immunofluorescence, cells were examined with an Eclipse TE2000U microscope (Nikon). Digital images were acquired with an Orca C4742-95-12ER charge-coupled device camera (Hamamatsu) and Aquacosmos Imaging Systems software.

**RESULTS**

**Mutational analysis of conserved residues within the phosphate domain of *Salmonella* SigD**

SigD is thought to exert its function mainly through dephosphorylation of cellular phosphoinositides (Norris et al., 1998; Steele-Mortimer et al., 2000; Feng et al., 2001; Marcus et al., 2001). We have reported that a conserved arginine residue (R468) within motif 2 of inositol polyphosphate 4-phosphatases is essential for SigD activity and function (Aleman et al., 2005). After alignment of the SigD catalytic domain and its homologue in *E. coli*, IpgD, with human inositol phosphate sequences (Fig. 1a), we decided to mutate two other residues: F359 within motif 1, conserved among phosphatases, and K527, within a putative synaptojanin-homology domain, which had been reported by Marcus et al. (2001) to partially impair lipid phosphatase activity in vitro. We made point mutations to change these residues to alanine and cloned the corresponding alleles as GST fusions, as well as wild-type SigD, to be expressed in *E. coli*. To learn whether the mutant proteins constructed had lost phosphatase activity, we purified the recombinant proteins and tested them by the malachite-green in vitro
phosphatase assay using PtdIns 3,4,5-P\(_3\) as substrate. The
GST-SigD\(^{F359A}\) and GST-SigD\(^{K527A}\) proteins showed an
in vitro activity on PtdIns 3,4,5-P\(_3\) about half of that of wild-
type GST-SigD (Fig. 1b), whereas activity for GST-
SigD\(^{R468A}\) was reduced to the levels of the negative GST
control, as reported previously (Aleman et al., 2005). We can
conclude that, unlike R468, the conserved phenylalanine
residue in motif 1 is not critical for SigD activity.

SigD accumulates in cytoplasmic vesicles in
yeast, whereas SigD\(^{R468A}\) displays a peripheral
localization

Next, we developed constructs that allowed expression of
the above-mentioned sigD mutant alleles in yeast. For this
purpose, the sigD alleles were cloned in N-terminal fusion
with glutathione S-transferase (GST) under the control of

![Diagram of SigD and IpgD alignments](image)

**Fig. 1.** (a). Alignment of the C-terminal region of *Salmonella* SigD with the equivalent region of its homologue in *E. coli* IpgD and human type II phosphatidyl inositol-4-phosphatase (Hs4PpaseII). Grey shading marks two conserved residues; black
shading depicts conservation in the three sequences (similarity, not identity, has been considered). Alignment was performed with Genedoc (http://www.psc.edu/biomed/genedoc). Motifs 1 and 2 of inositol-4-phosphatases are underlined. The point
mutations described in this work are substitutions for alanine in the residues marked with an asterisk. (b) *In vitro* phosphatase
activity of different alleles of SigD. Recombinant GST fusion proteins were tested for phosphatase activity in a malachite green
assay using PtdIns 3,4,5-P\(_3\) as substrate. Data are the means of three experiments; error bars represent SD.
the S. cerevisiae galactose-regulatable promoter of the GAL1 gene. When incubated in galactose medium, transformants expressing all versions of SigD tested were unable to grow (Fig. 2a), indicating that the GST-SigD$^{F359A}$ and GST-SigD$^{K527A}$ mutant versions were toxic for yeast, as reported previously for wild-type GST-SigD and GST-SigD$^{R468A}$ (Aleman et al., 2005). This effect is not a result of the fusion to GST, since sigD alone expressed from the GAL1 promoter behaved like the fusion (data not shown). We checked the expression of the GST fusions by hybridizing with anti-GST antibodies yeast lysates obtained after induction of the GAL1 promoter by growing the transformants in galactose-based medium for 6 h (Fig. 2b). In spite of their ability to arrest cell growth, the GST-SigD mutant proteins that retained some catalytic activity were undetectable, like the wild-type SigD, whereas the inactive GST-SigD$^{R468A}$ was clearly detectable in the lysates. This may suggest that catalytically active SigD is toxic at very low levels or that it is not present in lysates because it associates with particulate cellular fractions. To address the latter point, we studied the localization of the expressed proteins within fixed yeast cells by immunofluorescence with anti-GST antibodies. All the fusion proteins were detected by this technique, but distinct patterns of localization were observed for the different SigD mutants. As shown in Fig. 2(c), control GST-expressing cells showed a cytoplasmic localization, but GST-SigD, SigD$^{F359A}$ and GST-SigD$^{K527A}$ were observed as a dotted pattern. These patches were not associated with the cell surface and did not coincide with actin patches or nuclei (data not shown), suggesting that they correspond to intracytoplasmic compartments. Due to the low expression of these proteins, this signal was only detectable in a small percentage of cells (less than 5%), while most cells in the population showed no increase in fluorescence over the

![Fig. 2. Expression of wild-type and mutant Salmonella sigD alleles in yeast, and their effect on growth and subcellular localization. (a) Serial dilutions of S. cerevisiae YPH499 transformed with pEG-KG-based plasmids were dropped on solid SD (glucose) or SG (galactose) medium and incubated for 3 days at 28 °C. (b) Western blotting analysis with anti-GST antibodies of cell lysates from the same yeast transformants as in (a) expressing GST alone and GST fusions under the GAL1 inducible promoter as noted. (c) Immunofluorescence with anti-GST antibodies on the same transformants as above expressing GST or the different GST-SigD fusions as indicated. Representative cells from each experiment are shown. Bars, 5 μm.](image-url)
control. By contrast, GST-SigD{R468A} showed a clear peripheral localization, consistent with the plasma membrane, in more than 90% of the cells (Fig. 2c).

Expression of SigD{R468A} in yeast induces loss of growth polarity and septin ring assembly

The microscopic observations presented above revealed that cells expressing GST-SigD{R468A} were larger than control cells expressing GST alone or cells expressing wild-type GST-SigD, SigD{F359A} or GST-SigD{K527A} (Fig. 2c). We confirmed this by forward scatter analyses using flow cytometry. The average size of the population expressing GST-SigD{R468A} was significantly larger than that of control cells or cells expressing the other SigD versions (Fig. 3a). We have previously reported that phosphatase-dead alleles of SigD cause loss of actin polarity in yeast and that this effect is dependent on a region in the N-terminal non-catalytic part of the protein (Aleman et al., 2005). This phenotype of large un budded cells with depolarized actin is reminiscent of that of yeast mutants impaired in polarity establishment mechanisms, like those in the CDC24 and CDC42 genes (Johnson & Pringle, 1990). Therefore, we studied whether expression of SigD or the mutant versions would affect the establishment of primary cell polarity markers such as the septin ring. Septins are structural proteins that assemble at the yeast bud neck as a ring of microfilaments before the onset of bud emergence (see Gladfelter et al., 2001, for a review). By using a fusion protein of the yeast septin Cdc10 to GFP, we determined that expression of wild-type SigD (Fig. 3b), SigD{F359A} or SigD{K527A} (data not shown) did not affect septin ring assembly or shape. However, expression of SigD{R468A} led to disappearance of septin rings and the occurrence of abnormal septin patches randomly distributed along the cell surface (Fig. 3b). These patches were heterogeneous in size but did not assemble into ring-like structures. We can conclude that expression of wild-type SigD or the mutant versions that displayed some phosphatase activity in vitro causes growth inhibition without significantly distorting morphogenesis in yeast, whereas a catalytically impaired version of SigD dramatically alters general cell polarity mechanisms.

Expression of SigD{R468A} in yeast induces cell cycle arrest in G2

In order to learn whether SigD-induced growth arrest took place in a particular stage of the cell cycle, flow cytometry assays to assess the DNA content of cells expressing the above-described alleles were performed. As shown in Fig. 4(a), cells expressing GST alone, GST-SigD, GST-SigD{F359A} or GST-SigD{K527A} showed a balanced proportion of G1 and G2/M cells, as expected for an asynchronous culture in exponential phase. Therefore the dramatic growth arrest induced by expression of wild-type SigD and the aforementioned mutant derivatives is cell cycle independent. On the other hand, expression of GST-SigD{R468A} induced an accumulation of G2/M cells, indicating that the growth defect reflects a specific cell cycle arrest. To further study the cell cycle arrest induced by these sigD alleles, we carried out microscopic analyses and nuclear staining with DAPI. Typically, cells expressing GST-SigD{R468A} were mostly un budded. In spite of their 2n DNA content, as revealed by flow cytometry, these cells contained only one round nucleus (Fig. 4b). We expressed GST-SigD{R468A} in a strain which has the spindle pole body component Spc29 tagged with YFP, to allow the visualization of the SPBs. As shown in Fig. 4(b), the spindle poles were located on opposite sides of the periphery of the nucleus, indicating that cell cycle arrest takes place at the premitotic short spindle stage. In conclusion, expression of catalytically defective SigD in yeast cells yields un budded cells that are unable to commit to anaphase, arresting their nuclei in G2 with their SPBs separated to form a premitotic spindle.

Cell cycle arrest in SigD{R468A}-expressing cells is mediated by the morphogenesis checkpoint

A morphogenetic cell cycle checkpoint has been described in yeast that subordinates the G2–M transition to the correct progression of bud emergence (McMillan et al. 1999; Shulewitz et al., 1999; Barral et al., 1999). In brief, failures in bud site assembly, e.g. defects in septin ring assembly or actin polarization, are sensed by a molecular pathway which functions by negatively regulating the protein kinase Swe1.
This protein exerts an inhibitory role on the cyclin-dependent kinase (CDK) Cdc28/Cdk1 by phosphorylation on its Tyr-19 residue (Sia et al., 1996, 1998). Upon bud assembly failures, Swe1 remains active, thus phosphorylating the mitotic CDK so that cell cycle arrests in G2 until the morphogenetic apparatus is properly reassembled. So far we have shown that SigD R468A is able to cause a failure in morphogenesis and cytoskeletal assembly in yeast, as well as to induce nuclear mitotic arrest. This scenario might coincide with an activation of the morphogenetic checkpoint pathway. In that case, both swe1 and cdc28Y19F mutations, which eliminate such regulation, should overcome such arrest, allowing nuclear division. Confirming this hypothesis, about 60% of the cells were multinucleated when GST-SigD R468A was expressed in both mutants (Fig. 5a). Accordingly, we detected by flow cytometry a population of cells showing a DNA content corresponding to 4n or higher when GST-SigD R468A was produced in cdc28Y19F (Fig. 5b) or swe1Δ cells (data not shown). Thus all the evidence points to a very specific effect of this heterologous mutant protein on the loss of proper bud assembly, the cell cycle arrest being a consequence of this failure.

**Expression of different sigD alleles causes distinct effects on yeast MAPK signalling**

SigD, like other TTSS effectors, is thought to interfere with cell signalling in host cells. In this context, previous work has suggested that SigD mediates actin cytoskeleton rearrangements in a manner dependent on the small GTPase Cdc42 (Zhou et al., 2001). Moreover, signalling pathways, like those involving the PKB/Akt protein kinase, have been described to be activated by SigD (Steele-Mortimer et al., 2000). In yeast, small GTPases, namely Rho1 and Cdc42, act upstream of MAPK cascades. Activation of these pathways can be monitored by using specific antibodies against the dually phosphorylated activation domain of MAPKs (Martin et al., 2000). This methodology has been used to evaluate interaction of *S. typhimurium* SopE2 and SptP effector proteins with yeast signalling pathways (Rodríguez-Pachón et al., 2002). We made use of this approach to evaluate whether the different SigD mutants were able to induce the activation of yeast MAPK pathways. SigD expression did not affect the phosphorylation levels of the MAPKs in the wild-type strain, but it caused a phosphorylation increase in the mutated strains (Fig. 5b).

**Fig. 4.** Cells expressing catalytically defective SigD arrest nuclei in G2 with a premitotic spindle. (a) Flow cytometric analysis of RNase-treated propidium-iodide-stained YPH499 yeast cells expressing the different GST-SigD fusions as noted. The peak with lower fluorescence corresponds to cells with a DNA content corresponding to n and the peak of higher intensity corresponds to 2n. (b) DHYS2 yeast cells, which endogenously express a YFP-tagged version of the spindle pole body component Spc29, were transformed with pKG-SigD R468A. A transmitted-light image (left), DAPI staining (middle) and YFP signal (right) from a representative cell are shown.

**Fig. 5.** G2 arrest induced by catalytically defective SigD in yeast is mediated by the morphogenetic checkpoint. (a) DAPI staining on cells of MJY100 (wild-type), MJY30 (cdc28Y19F) and VBY16 (swe1) strains expressing GST-SigD R468A after 6 h in galactose medium. Bars, 5 μm. (b) Flow cytometric analysis of MJY100 (wild-type) and MJY30 (cdc28Y19F) cells, both transformed with pKG-SigD R468A.
p38-MAPK Hog1, as determined by using anti-phospho-p38 antibodies (data not shown). However, as shown in Fig. 6(a), the expression of GST-SigD, GST-SigD\(^{F359A}\) and GST-SigD\(^{K527A}\) led to higher levels of phosphorylation of the Slt2 MAPK compared to the control expressing GST, as detected with anti-phospho-p42/p44 antibodies. Slt2, also known as Mpk1, a key component of the Rho1-dependent cell integrity pathway, is activated by multiple stimuli related to cell wall morphogenesis and actin function (de Nobel et al., 2000; Martín et al., 2000; Harrison et al., 2001). Growth inhibition by GST-SigD was not rescued by individual deletion of SLT2 (data not shown), indicating that this effect is not exclusively caused by hyperactivation of the signalling pathway mediated by this MAPK. Expression of GST-SigDR468A also caused an increase in the phosphorylation of Slt2 over the basal levels, but significantly lower than that induced by the catalytically active versions of SigD (Fig. 6a). More interestingly, cells expressing GST-SigD\(^{R468A}\) displayed diminished levels of phosphorylation of another MAPK, namely Kss1, as compared to control conditions (Fig. 6b). The signal transduction pathway in which Kss1 participates is mediated by Cdc42 and is related to pseudofilamentation (Zhou et al., 1993; Cook et al., 1997; Lee & Elion, 1999). The expression of a mutant version of SigD bearing an internal deletion in the non-catalytic region (GST-SigD\(^{A118–142}\)), which retains some phosphatase activity but is unable to disrupt actin function either in yeast or in mammalian cells (Aleman et al., 2005), did not lower Kss1

**Fig. 6.** Expression of SigD\(^{R468A}\) in yeast downregulates Cdc42-dependent MAPK pathways. Anti-phospho-p42/p44 MAPK antibody was used to detect dually phosphorylated Slt2, Kss1 and Fus3. (a) Immunoblots with anti-phospho-p42/p44 or anti-Slt2, of cell extracts from *S. cerevisiae* YPH499 transformed with pEG-KG-based plasmids expressing GST alone (vector) or GST-SigD fusions as indicated. (b) Immunoblots with anti-phospho-p42/p44, anti-Kss1 and anti-actin of cell extracts from *S. cerevisiae* YPH499 transformed with pEG-KG-based plasmids expressing GST alone (vector) or GST-SigD fusions as indicated. The asterisk (*) shows a non-specific band detected with the anti-Kss1 antibodies that serves as an additional loading control. In both (a) and (b), control lysates from the double *slt2 kss1* mutant strain HM53 were processed in parallel to reveal that the detected bands corresponded to these MAPKs, as indicated. (c) Immunoblot with anti-phospho-p42/p44, anti-Kss1 and anti-Fus3 antibodies of cell extracts from *S. cerevisiae* BY4742 and an *itc1* deletant in the same background, as indicated, transformed with pEG-KG or derivatives expressing either GST-SigD or GST-SigD\(^{R468A}\). Strains bearing deletions in either the *KSS1* (HM53) or the *FUS3* (BYΔfus3) genes were processed in parallel to determine the bands corresponding to these proteins. (d) Immunoblot with anti-phospho-p42/p44, anti-Kss1 and anti-Fus3 of cell extracts from *S. cerevisiae* SY2002 and a triple *rga1 rga2 bem3* deletant in the same genetic background, bearing the YCpLG plasmid alone (Vector) or expressing wild-type or mutant SigD from the same vector as indicated.
phosphorylation levels (Fig. 6b). These results suggest that SigD<sup>R468A</sup>, which is defective in lipid phosphatase activity, triggers a response in yeast different to that of wild-type SigD or the mutant derivatives that retained a significant degree of enzymic activity in vitro. This response is dependent on the integrity of a fragment (residues 118–142) included within a region previously described to be important for association with host cell membranes (Marcus et al., 2002).

**SigD<sup>R468A</sup> eliminates Kss1 and Fus3 phosphorylation induced by stimulation of the mating pathway or upregulation of Cdc42**

We have shown above that basal phosphorylation levels of Kss1 are lowered by expression of GST-SigD<sup>R468A</sup>. Next we investigated whether SigD<sup>R468A</sup> was able to counteract Kss1 phosphorylation in activation conditions. It has been reported that disruption of the *ITC1* gene in MATα cells leads to autocrine stimulation due to a failure in the repression of *MATa*-specific genes (Ruiz et al., 2003). This leads to an abnormal situation in which the Cdc42-mediated mating pheromone pathway is activated during vegetative growth. As a consequence, the Kss1 and Fus3 MAPKs are activated (Ruiz et al., 2003). We expressed GST-SigD and GST-SigD<sup>R468A</sup> in a MATα *itc1Δ* strain and the isogenic wild-type. As shown in Fig. 6(c), activation of both Kss1 and Fus3 in a MATα *itc1Δ* strain was dramatically reduced by expression of GST-SigD<sup>R468A</sup>. These results suggest that expression of SigD<sup>R468</sup> in yeast downregulates signalling through MAPK pathways downstream of Cdc42. To further prove this point, we expressed GST, GST-SigD and GST-SigD<sup>R468A</sup> in a strain deleted for the genes encoding the three known GTPase-activating proteins (GAPs) for Cdc42, namely Rga1, Rga2 and Bem3. In the absence of its negative regulators, Cdc42 is constitutively activated in this triple mutant (Smith et al., 2002) and, as a consequence, phosphorylation of both Kss1 and Fus3 is enhanced (Rodriguez-Pachón et al., 2002). As shown in Fig. 6(d), SigD<sup>R468A</sup> was able to counteract the effect of the triple deletion of the GAPs on the activation of the Kss1 and Fus3 MAPKs downstream. Wild-type GST-SigD had no significant effect on basal phosphorylation levels of Kss1 (Fig. 6b–d), but it was able to partially decrease enhanced levels of phospho-Kss1 and phospho-Fus3 due to Cdc42 upregulation either by autocrine stimulation (Fig. 6c) or by elimination of its GAPs (Fig. 6d), further underscoring the idea that SigD can potentially downregulate this pathway, but that its phosphatase activity masks this effect.

**Overexpression of the Cdc42 PH domain mimics the phenotypes of SigD<sup>R468A</sup>**

The negative effects of SigD<sup>R468A</sup> on Cdc42 signalling could be due to a decrease in the levels of the protein. However, as shown in Fig. 7(b), the amount of Cdc42 in SigD<sup>R468A</sup>-expressing cells is not reduced as compared to control cells, as judged by immunoblotting with anti-Cdc42 antibodies. Given that the effect of SigD<sup>R468A</sup> on Cdc42 signalling is seen in the absence of the three GAPs of Cdc42 (Rga1, Rga2 and Bem3; Fig. 6d), we discarded the idea that SigD<sup>R468A</sup> is operating through any of these Cdc42 negative regulators. Alternatively, it could be impairing the function of the Cdc42 GDP–GTP exchange factor (GEF), Cdc24. However, we observed that overexpression of Cdc24, unlike Cdc42, was not able to rescue SigD<sup>R468A</sup>-induced phenotypes (data not shown). This suggests that the effect of SigD<sup>R468</sup> on Cdc42 signalling is not mediated by directly inactivating Cdc24. Nevertheless, we cannot discard the possibility that SigD<sup>R468A</sup> might interfere with Cdc24 function by other means, for instance by strongly competing with Cdc24 interactors. In this context, it has been reported that SigD is associated with cellular membrane fractions in the host cell (Marcus et al., 2002) and that Cdc24 possesses a putative lipid-binding PH domain (Yu et al., 2004). We have shown that SigD<sup>R468A</sup> strongly accumulates in the plasma membrane when expressed in yeast cells (see Fig. 2c). Therefore, it can be speculated that overexpression of SigD<sup>R468A</sup> impedes interaction of Cdc24 with its putative lipid targets at the plasma membrane. If that were the case, overproduction of the Cdc24 PH domain from the same plasmid as used for
SigD expression should be able to displace endogenous Cdc24, leading to a phenotype similar to that observed for SigD$_{R468A}$. As shown in Fig. 8(a), overproduction of an N-terminal-truncated version of Cdc24 (Cdc24$_{450-854}$) devoid of its GEF domain but preserving its PH domain, also inhibited growth of yeast cells. Actin staining of these cells revealed an effect of loss of actin cytoskeleton polarity (Fig. 8b) reminiscent of that described for SigDR468A overexpression (Aleman et al., 2005). Therefore, the effect of SigDR468A on Cdc42 signalling could be a consequence of an impaired accessibility of its positive regulator, Cdc24. However, this does not exclude the possibility that SigD might be able to directly downregulate Cdc42.

**SigD$_{R468A}$ co-purifies with Cdc42 and Cdc24 from yeast lysates**

SigD$_{R468A}$ might interfere with Cdc42 function by directly interacting with this GTPase. Although we were unable to detect a direct interaction between Cdc42 and SigD$_{R468A}$ by the yeast two-hybrid system (data not shown), we detected with specific anti-Cdc42 antibodies that endogenous Cdc42 co-purified with GST-SigD$_{R468A}$ from yeast lysates (Fig. 9a). In parallel, we used GST-SigD and GST-SigD$_{R468A}$, lacking a region that is essential for the effects of SigD$_{R468A}$ on actin (Aleman et al., 2005) and on Cdc42 signalling (see Fig. 6b). Whereas GST-SigD$_{R468A}$ was able to pull down endogenous Cdc42 very efficiently in these experiments, SigD$_{R468A}$, A$_{118-142}$ failed to do so (Fig. 9a), which suggests that the region of SigD from amino acids 118 to 142 is essential for the interaction. Since wild-type SigD is not detectable in the lysates (see also Fig. 2b), its ability to pull down Cdc42 could not be evaluated. We also tested whether Cdc42$_{G12V}$, a constitutively active mutant, was able to interact with SigD$_{R468A}$ by using a GST and a GFP fusion respectively, both expressed from the GAL1 promoter. Affinity purification of GST-Cdc42$_{G12V}$ led to co-purification of SigD$_{R468A}$-GFP (Fig. 9b), suggesting that SigD can bind the GTP-loaded active form of Cdc42. Finally, to determine whether Cdc24 would be able to pull down Cdc42$_{R468A}$-GFP, we co-expressed GST-Cdc24 and SigD$_{R468A}$-GFP, both again from the GAL1 promoter, and performed a similar co-purification experiment. As shown in Fig. 9(c), GST-Cdc24 – but not GST alone – was able to pull down SigD$_{R468A}$-GFP. This implies that phosphatase-dead SigD expressed in yeast is able to complex with both Cdc24 and Cdc42.

**DISCUSSION**

Many basic aspects of signal transduction and cytoskeletal dynamics have been thoroughly studied in the budding yeast *S. cerevisiae*. In recent years, researchers have taken advantage of this model to study the function of bacterial virulence factors, especially those translocated by TTSSs. Translocated bacterial effectors interfere with key host cell regulators, reprogramming cellular signalling to favour bacterial invasion (see Zaharik et al., 2002, for a review). For
Salmonella recently reported that the provide interesting tools for yeast researchers. We have effectors that target particular biological processes might or conserved signalling mechanisms, including those that through phylogeny, such as actin recruitment mechanisms of bacterial virulence factors when their intracellular targets mechanisms that bacterial effectors may target. Numerous between higher eukaryotes and yeast in the molecular genomic tools, but a major caveat lies in possible divergence manipulation and the availability of a growing variety of Valdivia (2004). Yeast has the advantage of its easy studying virulence factors have been recently discussed by et al. (2000; Lesser & Miller, 2001; Yoon et al., 2003; Skrzypek et al., 2003), SopE2 and SptP from Salmonella (Rodríguez-Pachón et al., 2002), effectors encoded by the LEE pathogenicity island from enteropathogenic E. coli (EPEC; Rodríguez-Escudero et al., 2005a) as well as proteins from Vibrio and Pseudomonas (Rabin & Hauser, 2003; Sato et al., 2003; Trosky et al., 2004) have been expressed in yeast. The pros and cons of the yeast system for studying virulence factors have been recently discussed by Valdivia (2004). Yeast has the advantage of its easy manipulation and the availability of a growing variety of genomic tools, but a major caveat lies in possible divergence between higher eukaryotes and yeast in the molecular mechanisms that bacterial effectors may target. Numerous reports prove, however, that yeast can shed light on the effect of bacterial virulence factors when their intracellular targets constitute basic molecular modules that are conserved through phylogeny, such as actin recruitment mechanisms or conserved signalling mechanisms, including those that involve small GTPases or MAPKs. Additionally, bacterial effectors that target particular biological processes might provide interesting tools for yeast researchers. We have recently reported that the Salmonella SigD/SopB TTSS effector may be a bifunctional protein, with a lipid phosphatase C-terminal domain and an N-terminal region that independently disrupts actin structures (Aleman et al., 2005). By using the yeast model, here we present evidence that the latter function can be attributed to downregulation of small GTPases, like Cdc42.

The hydrolytic activity of SigD on phosphatidylinositides is thought to play a role in Salmonella virulence, being important for proper formation of endocytic vesicles during the invasive process by hydrolysis of PtdIns 4,5-P_2 (Terebiznik et al., 2002) and contributing to the maintenance of high levels of PtdIns 3-P in Salmonella-containing vacuoles by hydrolysis of PtdIns 3,5-P_2 (Hernández et al., 2004; Dukes et al., 2006). The toxicity of SigD when expressed in yeast is probably due to depletion of PtdIns 4,5-P_2, which is essential for yeast viability (Audhya et al., 2004; Rodríguez-Escudero et al., 2005b). Supporting this view, we have shown that expression of SigD removes PtdIns 4,5-P_2 from the yeast plasma membrane in vivo (Aleman et al., 2005). In this work, we expressed in yeast three sigD alleles bearing point mutations in the conserved phosphatase domains: the novel sigD_{F359A} mutant, sigD_{R468A} (Aleman et al., 2005) and sigD_{K527A} (Marcus et al., 2001). F359 and R468 are conserved in all polyphosphate inositol 4-phosphatases found in databases, whereas K527 maps within a putative inositol 5-phosphatase domain proposed by Marcus et al. (2001). The mutation of F359, like that of K527, caused only a partial loss of activity in vitro and gave rise to effects close to those of wild-type SigD when expressed in yeast. These results imply that, in spite of being conserved throughout phylogeny, F359 in motif 1 is not essential for substrate recognition and catalytic activity. In contrast, mutation of R468 greatly diminished in vitro activity of the protein, but it was still toxic when expressed in yeast, causing a distinct phenotype that involves loss of actin polarization (Aleman et al., 2005). Here we report that SigD_{R468A} also causes septin disassembly, increase of cell size, cell cycle arrest in G2 and alterations in MAPK signalling. All these effects are reminiscent of the phenotype of cdc42 mutants: large round unbudded cells with depolarized actin cortical patches (Johnson & Pringle, 1990) and wrongly assembled septins (Cid et al., 2001a; Gladfelter et al., 2002), with nuclei arrested in G2 due to the function of the Swe1-dependent morphogenetic checkpoint (Sia et al., 1996; Shulewitz et al., 1999; McMillan et al., 1999). Our identification of Cdc42 as a suppressor of the toxicity induced by overexpression of SigD_{R468A} and our observation that SigD_{R468A} interacts with yeast Cdc42 in vivo confirm that all these effects are a consequence of the downregulation of Cdc42-mediated pathways.

Since Salmonella secreted proteins are thought to interfere with host cell signalling when translocated into the target cell, it is remarkable that wild-type SigD is able to induce the activation of the yeast cell integrity pathway mediated by the Slt2 MAPK. Activation of the same pathway is caused by expression of SopE2, another Salmonella effector that may
act in conjunction with SigD during bacterial infection (Rodríguez-Pachón et al., 2002; Zhou et al., 2001). SopE2 activates a second MAPK in yeast, Kss1, in accordance with its role as a GEF for small Rho-GTPases like Cdc42 (Hardt et al., 1998), since this GTPase is known to act upstream of Kss1 in yeast (Mösch et al., 1996). However, unlike SopE2, SigD does not activate Kss1, but rather reduces the phosphorylation levels of this kinase, an effect that becomes

Fig. 9. SigD<sub>R468A</sub> co-purifies with Cdc42 and Cdc24 from yeast lysates. (a) Yeast cell lysates from cultures expressing GST fusions to the indicated versions of SigD were collected and incubated with glutathione-Sepharose beads. Original lysates were loaded as 'inputs' (left) that represent about 5% of the total volume subjected to affinity purification. The 'pull-down' lanes (right) were loaded with one-fifth of the total volume of beads. Anti-Cdc42 antibodies were used to detect endogenous Cdc42, anti-GST antibodies as a control for expression and pull-down efficiency, and anti-actin antibodies as a control for specificity and loading. (b) Lysates from cells co-expressing SigD<sub>R468A-GFP</sub> with either GST or GST-Cdc24<sub>G12V</sub> were collected and subjected to affinity purification with glutathione-adsorbed beads as in (a). Membranes were immuno-blotted with anti-GFP antibodies to detect the SigD<sub>R468A-GFP</sub> fusion, with anti-GST antibodies as a control for expression and pull-down efficiency, and anti-actin antibodies as a control for specificity and loading. (c) Lysates from cells bearing combinations of the empty vectors pEG(KG) or YCpLG-GFP, or the same vectors expressing respectively SigD<sub>R468A-GFP</sub> and GST-Cdc24, as indicated, were collected, subjected to affinity purification, separated by SDS-PAGE and immunoblotted as in (b).
much more evident in the phosphatase-dead mutant SigD<sup>R468A</sup>. This effect is consistent with downregulation of Cdc42-dependent signalling in yeast by SigD, implying that this *Salmonella* effector does not act as a GEF. Activation of the Rho1-dependent Slt2 MAPK pathway is probably a reflection of the enzymatic activity of SigD, since phospho-Slt2 levels are not significantly enhanced when the phosphatase-dead mutant is expressed. In agreement with this view, we have recently reported that severe PtdIns 4,5-P<sub>2</sub> depletion in yeast leads to activation of the Slt2 pathway (Rodríguez-Escudero *et al.*, 2005b).

The fact that we do not see effects related to Cdc42 downregulation when expressing the wild-type SigD protein, in which the lipid phosphatase activity is intact, is probably due to these effects being masked by the more dramatic damage caused by removal of essential PtdIns 4,5-P<sub>2</sub> pools from the cell. Actually, wild-type SigD is able to counteract Cdc42 upregulation induced by mutation of its GAPs or by autocrine stimulation of the pathway. As judged by immunodetection of the expressed proteins from yeast lysates, SigD<sup>R468A</sup> is tolerated in higher levels than any of the versions that retain catalytic activity, suggesting that it does not exert such a strong toxic effect. In this context, a remarkable difference between SigD<sup>R468A</sup> and the catalytically competent forms of the protein is their localization: wild-type SigD or mutant forms that keep some degree of activity are retained in small cytoplasmic compartments, whereas the SigD<sup>R468A</sup> mutant localizes to the cell periphery. It is likely that the effect on phosphoinositides of catalytically active SigD will affect vesicular traffic. Therefore, elimination of the lipid phosphatase activity would not cause a blockage in the secretory pathway, allowing the mutant SigD<sup>R468A</sup> to proceed to the plasma membrane, where it might interfere with the function of membrane-bound Cdc42. Alternatively, SigD might primarily bind to the plasma membrane and promote endocytosis by a mechanism dependent on its catalytic activity, as it does during *Salmonella* invasion. This would allow accumulation of the catalytically active protein in such endosomes, while the phosphatase-dead version would eventually accumulate at the plasma membrane in amounts large enough to downregulate Cdc42 signalling. Since phosphatidylinositol phosphatases, like the conserved PIKfyve/Fab1 (Cabezas *et al.*, 2006; Gary *et al.*, 1998), bear phosphoinositide-binding domains outside the catalytic region, a feasible hypothesis is that the N-terminal region of SigD might strongly bind a certain species of plasma membrane lipid, competing with essential Cdc42 activators. A candidate would be the Cdc24 GEF, which has a putative phosphoinositide-binding PH domain. Our observation that overexpression in yeast of a fragment of Cdc42 containing its PH domain results in an alteration of actin similar to that caused by SigD<sup>R468A</sup>, together with the lack of Cdc42-related phenotypes induced by SigD<sup>A118–142</sup>, devoid of a reported membrane-association region, favours this hypothesis. Since the presence of a PH domain is a conserved common feature of GEFs for Rho GTPases (Blomberg *et al.*, 1999), SigD might be interfering with Cdc42 signalling through this mechanism in the scenario of infection. Nevertheless, it should be considered that such possible interference is observed in conditions of overexpression of the bacterial protein, a situation that does not occur in the host cell, in which relatively high levels of secreted effectors may be present only locally at the site of infection.

Regardless of a putative competition with Rho-GEFs, the interaction that we found between SigD<sup>R468A</sup> and Cdc42 hints at a direct regulation of the small GTPase itself. It is remarkable that the region of SigD (residues 118–142) that we previously found to be essential for disruption of actin structures both in yeast and in mammalian cell lines (Alemán *et al.*, 2005) is also required for interaction with Cdc42. The fact that Cdc24 is also able to co-purify with SigD<sup>R468A</sup> could indicate the presence of the bacterial effector in a complex integrated by both the GTPase and its activator. Interestingly, the *Yersinia* TTSS effector protein YopE also causes a budding defect and cell cycle arrest when expressed in *S. cerevisiae* (Lesser & Miller, 2001), reminiscent of that caused by SigD<sup>R468A</sup> described here. YopE is known to stimulate GTP hydrolysis *in vitro* of several members of the Rho family of G-proteins, including Cdc42 (Aili *et al.*, 2003; Von Pawel-Rammingen *et al.*, 2000). Sequence analysis does not reveal homology of SigD with YopE or with any known Rho GTPase-activating proteins (GAPs). However, our results open the appealing possibility that the non-catalytic domains of SigD might exert a negative regulation of host cell small GTPases. In concordance with this idea, we found that SigD<sup>R468A</sup> is able to relieve the increased Kss1 phosphorylation displayed by a mutant lacking all three Cdc42 GAPs. It has been hypothesized that SigD contributes to the initial stages of internalization of *Salmonella* by cooperating with SopE2 in actin rearrangements through local activation of Cdc42. The contribution of SigD to this activation is unknown, but it was reported to depend on its lipid phosphatase activity (Zhou *et al.*, 2001). It is also known that in later stages of invasion a downmodulation of the function of Rho-GTPases is needed for the host cell to regain its normal architecture. Although this function has been reported to be exerted by the bacterial effector SptP (Fu & Galan, 1999), it is tempting to speculate that SigD may cooperate in cell recovery.

In summary, our results in the yeast model point towards a novel involvement of SigD in a negative regulation of small GTPases that, in view of conservation of signalling mechanisms through evolution, might also operate upon infection of the host cell.

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