Inhibition of Cdc42-dependent signalling in *Saccharomyces cerevisiae* by phosphatase-dead SigD/SopB from *Salmonella typhimurium*

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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, SigD^{R468A}, 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 SigD^{R468A} in yeast. First, expression of SigD^{R468A} caused accumulation of large unbudded cells and loss of septin organization, while SigD expression caused none of these effects. Second, growth inhibition by SigD^{R468A} 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 SigD^{R468A} rather inactivated another MAP kinase, Kss1. In a screen for suppressors of SigD^{R468A}-induced growth arrest by overexpression of a yeast cDNA library, the Cdc42 GTPase was isolated. Furthermore, SigD^{R468A} 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.

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INTRODUCTION

The biology of the budding yeast *Saccharomyces cerevisiae* is remarkably well known. In consequence, this micro-organism has been widely used in approaches aimed at shedding light on general biological questions. A feasible strategy is the use of *S. cerevisiae* as a model eukaryotic host cell for the study of bacterial proteins related to virulence (reviewed by Valdivia, 2004). Such bacterial effectors are often translocated by the pathogen into the host cytoplasm by a molecular syringe-like structure known as the type III secretion system (TTSS) to modulate signalling events and cytoskeletal rearrangements favouring infection (Zaharik *et al.*, 2002; Patel & Galán, 2005). The reliability of yeast as a simple eukaryotic host cell model depends on the assumption that basic molecular modules are conserved between lower and higher eukaryotes. For instance, actin nucleation, cell cycle regulation and basic signalling modules, such as

those involving GTPases or mitogen-activated protein kinase (MAPK) cascades, are universal for eukaryotic cells. Strengthening this view, it is known that some higher eukaryotic orthologues of key signalling components, such as the Rho GTPase Cdc42, are able to complement the corresponding yeast mutants (Shinjo *et al.*, 1990). Yeast morphogenesis is very likely to be affected by bacterial effectors which interfere with host cell signalling and the cytoskeleton, since budding is supported by cytoskeletal structures, such as actin filaments and septin collars (Pruyne & Bretscher, 2000). Thus, when studied in yeast, virulence factors that target conserved mechanisms may yield conclusions translatable to the real scenario of infection.

The function of Cdc42 from the host cell is required for invasion by *Salmonella* (Chen *et al.*, 1996). We have previously shown that the *Salmonella typhimurium* TTSS-translocated proteins SopE2 and SptP are able to interfere with Cdc42-dependent cell signalling when produced in yeast in a way which is consistent with their effects on higher eukaryotic cells (Rodríguez-Pachón *et al.*, 2002). SopB/SigD, another *Salmonella* virulence factor translocated by the TTSS (Patel & Galán, 2005), has also been proposed to mediate actin cytoskeleton rearrangements and bacterial

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GAP, GTPase-activating protein; GEF, GDP–GTP exchange factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; PtdIns 4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns 3,4,5-P₃, phosphatidylinositol-3,4,5-trisphosphate; TTSS, type III secretion system.

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 phosphatase, 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.*, 2001); second, it has been shown to activate cellular protein kinase B (PKB/Akt) (Knodler *et al.*, 2005; Marcus *et al.*, 2001; Steele-Mortimer *et al.*, 2000); and, third, SigD seems to countermand the epidermal growth factor (EGF)-mediated signalling that regulates chloride secretion (Bertelsen *et al.*, 2004). It cannot be discounted that these effects of SigD are a consequence of its activity on inositol and phosphatidylinositol polyphosphate substrates, but we have recently shown that the N-terminal region of SigD may exert a function on the actin

cytoskeleton independent of the catalytic domain (Alemán *et al.*, 2005).

In the present work, we expressed wild-type and mutant alleles of SigD in yeast and studied their effects. We show here that SigD causes different effects on yeast morphogenesis and cell cycle depending on whether catalytically active or inactive alleles are expressed. Our results point towards a 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* strain DH5α F' [K12 Δ(lacZYA-argF)U169 deoR supE44 thi-1 recA1 endA1 hsdR17 gyrA96 relA1 (φ80lacZΔM15) 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 cells onto plates of SD or of SG lacking the corresponding auxotrophic

Table 1. *S. cerevisiae* strains

Strain	Genotype	Reference or source*
YPH499	MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-Δ200 lys2-801	P. Hieter
1783	MATa trp1-1 leu2-3,112 ura3-52 his4 can ^R	Lee <i>et al.</i> (1993)
TD28	MATa ura3-52 ino1-131 can ^R	F. del Rey
MJY100	MATa can1-100 his3-11,15 leu2-3,112 LYS2 trp1-1 ura3-1	Shulewitz <i>et al.</i> (1999)
MJY30	MATx can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cdc28 ^{Y19F} (::LEU2)	Shulewitz <i>et al.</i> (1999)
VBY16	MATx can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 swe1Δ(::LEU2)	Cid <i>et al.</i> (2001b)
DHY52	MATx ade2-loc his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 SPC29-YFP(::HIS3)	T. Davis
BY4742	MATx his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF
BYitc1α	MATx his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 itc1::kanMX4	Ruiz <i>et al.</i> (2003)
SY2002	MATa his3::FUS1-HIS3 mfa2-Δ1::FUS1-lacZ ura3 leu2 trp1 his3 ade1	G. Sprague
YGS57	MATa his3::FUS1-HIS3 mfa2-Δ1::FUS1-lacZ ura3 leu2 trp1 his3 ade1 rga1::URA3 rga2::TRP1 bem3::TRP1	G. Sprague
HM53	MATa trp1Δ901 leu2-3,112 ade2-101 ura3-52 his3-Δ200 suc2Δ9 kss1Δ::URA3 slt2Δ::LEU2	H. Martín
BYΔfus3	MATa; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FUS3::KanMX4	EUROSCARF

*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. Martín, 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 OD₆₀₀ 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^{R468A}, pGEX-KG-SigD and pGEX-KG-SigD^{R468A} (Alemán *et al.*, 2005). Plasmids from these series were named as follows: pKG-SigD^{F359A} and pKG-SigD^{K527A} for the pEG(KG) series; and pGEX-KG-SigD^{F359A} 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* C53 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 the upper and lower oligonucleotides respectively (underlined in Table 2) to allow directed cloning. PCR products were cloned into the pGEM-T vector (Promega) and cleaved with *Bam*HI/*Hind*III for insertion into the same sites of either pEG(KG) or pGEX-KG. Wild-type *sigD* and the *sigD*^{R468A}

mutant allele were also cloned in YCpLG, 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. YCpLG-SigD^{R468A}-GFP was constructed by PCR amplification of *sigD*^{R468A} from pKG-SigD^{R468A} using the SigD-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 YCpLG-GFP vector, a version of YCpLG produced in this work by inserting the GFP coding region into the *Bam*HI/*Xba*I sites of the polylinker. For the pKG-SigD^{R468A, Δ118–142} construct, site-directed mutagenesis using as template pKG-SigD^{Δ118–142} (Alemán *et al.*, 2005) was performed to generate the R468A mutation by sequential PCR using the primers SigD-1, SigD-2, Arg-1 and Arg-2 (Table 2). The resulting PCR product was cloned into the pGEM-T vector (Promega), cleaved with *Bam*HI/*Hind*III and inserted into the same sites of the pEG-KG plasmid. Plasmid pEG(KG)-CDC24^{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^{G12V} was constructed by amplifying the *cdc42*^{G12V} mutant allele from pRS315-CDC42(G12V), a gift of Alan Bender, Indiana University, Bloomington, USA, with the U-CDC42 and L-CDC42 primers (Table 2), which contain *Xba*I 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,

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

Oligonucleotide	Sequence (5'–3')*
For SigD^{F359A}	
Phe-1	CACATTGGCTGCGGCGACGACGTC
Phe-2	GCCGCAGCCAATGTGGGTGTTAATGAGC
SigD-1	GGGATCCATGCAAATACAGAGCTTC
SigD-2	CAAGCTTTCAAGATGTGATTAATGAAG
For SigD^{K527A}	
Lys-1	CATTACGGCGTTTCCCGCCCCGCC
Lys-2	GGAAACGCCGTAATGAAAAATTTATCGCC
SigD-1	As above
SigD-2	As above
For pKG-CDC24^{450–854}	
Cdc24-1	CGGGATCCATGAGTATCAACGAAAATCAAA
Cdc24-2	CCCAAGCTTTCAATACAGACGAATGTTCAA'
For SigD^{R468A}-GFP	
SigD-1	As above
SigD-GFP2	CGGGATCCAGATGTGATTAATGAAG
For SigD^{R468A, Δ118–142}	
Arg-1	CCCTGTGGCATCTTTGCCGCTTTTAC
Arg-2	AAAGATGCCACAGGGATGATGGATTC
SigD-1	As above
SigD-2	As above
For pKG-Cdc42^{G12V}	
U-CDC42	CCTCTAGAAGAATTCATGAAAACGCTAAAGTGTG
L-CDC42	CCTCTAGAATTCGATTAAGAAAAGATGGGC

**Bam*HI and *Hind*III sites are underlined.

Durham, NC, USA). The cDNA library used in the screening for suppressors of SigD^{R468A} 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 μ l glutathione-Sepharose beads (Amersham Pharmacia) was added to 150 μ l 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 μ l 2 $\times$ 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 μ l aliquots of the original lysates were loaded as 'inputs', and 5 μ l 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 (Martín *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-millilitre volumes of LB containing 100 μ g ampicillin ml⁻¹ were inoculated with glycerol stocks and grown at 37 °C overnight. These cultures were used to inoculate experimental cultures to OD₆₀₀ 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 μ l Tris/HCl pH 7.5 containing 1 mM EDTA, 1 mM DTT, 10 μ g ml⁻¹ each of leupeptin and aprotinin; 15 μ l of 25 mg lysozyme ml⁻¹ 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 μ l Tris/HCl pH 7.5 containing 1.5% 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 μ l 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 μ l of the same buffer. Efficiency of purification was monitored by SDS-PAGE analysis of 20 μ l 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, 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 dithiothreitol, 0.5 mM diC₁₆-phosphatidylserine (Sigma P-1185) and 25 μ M phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns 3,4,5-P₃) (diC₁₆, Biomol PH-107) (temperature 37 °C, reaction volume 50 μ l). The reactions were stopped by addition of 50 μ l 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 μ l of 10 g l⁻¹ 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 (Alemán *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 phosphatase 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 (Alemán *et al.*, 2005). After alignment of the SigD catalytic domain and its homologue in *E. coli*, IpgD, with human inositol phosphatase 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 (Alemán *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

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 (Hs4PaseII). 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} (Alemán *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.

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 (Alemán *et al.*, 2005). This phenotype of large unbudded 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 unbudded. 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 unbudded cells that are unable to commit to anaphase, arresting their nuclei in G2 with their SPBs separated to form a premitotic spindle.

Fig. 3. Cell size and septin distribution in yeast cells expressing *sigD* and *sigD*^{R468A}. (a) Flow cytometric graphics showing the forward scatter parameter of cells expressing GST and GST-SigD fusions as marked. A shift of the peaks to the right along the *x* axis indicates a larger average size in the population. (b) Cdc10-GFP distribution in cells expressing GST, GST-SigD and GST-SigD^{R468A}. Yeast cells were co-transformed with expression vectors containing the septin-GFP fusion (pLA10H) and the corresponding GST-SigD fusions as marked. Bars, 5 μ m.

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.

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) DHY52 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.

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 *cdc28*^{Y19F} 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 *cdc28*^{Y19F} (Fig. 5b) or *swe1*Δ 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 (Martín *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

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 (*cdc28*^{Y19F}) and VBY16 (*swe1*) strains expressing GST-SigD^{R468A} after 6 h in galactose medium. Bars, 5 μ m. (b) Flow cytometric analysis of MJY100 (wild-type) and MJY30 (*cdc28*^{Y19F}) 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-SigD^{R468A} 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^{Δ118–142}), which retains some phosphatase activity but is unable to disrupt actin function either in yeast or in mammalian cells (Alemán *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* (BYΔ*fus3*) 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^{R468A}, 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^{R468A} 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^{R468A}. Next we investigated whether SigD^{R468A} was able to counteract Kss1 phosphorylation in activation conditions. It has been reported that disruption of the *ITC1* gene in *MAT α* cells leads to autocrine stimulation due to a failure in the repression of *MAT α* -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^{R468A} in a *MAT α itc1 Δ* strain and the isogenic wild-type. As shown in Fig. 6(c), activation of both Kss1 and Fus3 in a *MAT α itc1 Δ* strain was dramatically reduced by expression of GST-SigD^{R468A}. These results suggest that expression of SigD^{R468A} in yeast downregulates signalling through MAPK pathways downstream of Cdc42. To further prove this point, we expressed GST, GST-SigD and GST-SigD^{R468A} 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 (Rodríguez-Pachón *et al.*, 2002). As shown in Fig. 6(d), SigD^{R468A} 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 Cdc42 rescues toxicity of SigD^{R468A}, but not wild-type SigD

In order to better understand the effects of SigD^{R468A} in the yeast cell, we performed a genetic screen for the isolation of suppressors of SigD^{R468A} toxicity by overexpression. For this purpose, we co-transformed a yeast cDNA library in a *URA3*-based centromeric vector under the control of the *GAL1* promoter (Liu *et al.*, 1992) in cells bearing a

LEU2-based vector that expressed *sigD^{R468A}* under the same promoter. Viable colonies were recovered from galactose plates lacking leucine and uracil. In these conditions, only clones bearing cDNAs able to rescue yeast cells from SigD^{R468A}-induced toxicity would be able to grow. From parallel plating of 1/10 dilutions of the transformation mixture on glucose plates, we calculated that a total of 35 000 clones were screened, and only 12 of them were recovered on galactose medium. *URA3*-based plasmids extracted from these clones retained the ability to rescue viability of SigD^{R468A}-expressing cells after retransformation. The sequence of all 12 clones corresponded to the *CDC42* cDNA. Thus, overexpression of Cdc42 can overcome SigD^{R468A} toxicity, as shown in Fig. 7(a). However, Cdc42 overproduction did not rescue cells expressing wild-type SigD, suggesting that the main toxic effect for the yeast cells of catalytically active SigD operates through Cdc42-independent mechanisms, probably by its effects on essential cellular pools of phosphatidylinositol (Alemán *et al.*, 2005). In agreement with the idea that growth inhibition caused by SigD^{R468A} in yeast is specifically due to downregulation of Cdc42 signalling, co-overexpression of Cdc42 in SigD^{R468A}-expressing cells restored phosphorylation of Kss1 (Fig. 7b). However, overexpression of Cdc42 had no effects on MAPK phosphorylation levels in cells expressing wild-type SigD.

Overproduction of the Cdc24 PH domain mimics the phenotypes of SigD^{R468A}

The negative effects of SigD^{R468A} 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^{R468A}-expressing cells is not reduced as compared to control cells, as judged by immunoblotting with anti-Cdc42 antibodies. Given that the effect of SigD^{R468A} 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^{R468A} 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^{R468A}-induced phenotypes (data not shown). This suggests that the effect of SigD^{R468A} on Cdc42 signalling is not mediated by directly inactivating Cdc24. Nevertheless, we cannot discard the possibility that SigD^{R468A} 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 membranous 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^{R468A} strongly accumulates in the plasma membrane when expressed in yeast cells (see Fig. 2c). Therefore, it can be speculated that overexpression of SigD^{R468A} 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

Fig. 7. Overexpression of Cdc42 rescues SigD^{R468A}-induced toxicity in yeast. (a) Serial dilutions of transformants bearing empty vectors (YCpLG and pRS316), or combinations of the same plasmids expressing SigD^{R468A} (YCpLG-SigD^{R468A}), SigD (YCpLG-SigD) or Cdc42 (pRS316-CDC42), were dropped on solid SD (glucose) or SG (galactose) medium and incubated for 3 days at 28 °C. (b) Immunoblot with anti-phospho-p42/p44, anti-Kss1, anti-actin (as a loading reference) and anti-Cdc42 of cell extracts from *S. cerevisiae* YPH499 transformed with the same combinations of plasmids as in (a).

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 SigD^{R468A} overexpression (Alemán *et al.*, 2005). Therefore, the effect of SigD^{R468A} 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, Δ118–142}, lacking a region that is essential for the effects of SigD^{R468A} on actin (Alemán *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, Δ118–142} 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 SigD^{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

Fig. 8. Overexpression of a version of Cdc24 lacking the N-terminal GEF domains inhibits cell growth and disrupts actin polarity. (a) Serial dilutions of *S. cerevisiae* YPH499 transformed with pEG-KG and pEG(KG)-Cdc24⁴⁵⁰⁻⁸⁵⁴ plasmids, as indicated, were dropped on solid SD (glucose) or SG (galactose) medium and incubated for 3 days at 28 °C. (b) Actin staining with FITC-conjugated phalloidin of the same transformants, expressing either GST alone or a fusion to the C-terminal half of Cdc24, which includes its PH domain, as indicated, grown in galactose-based medium for 6 h. Bars, 5 µm.

example, YopE, YopJ and YopM from *Yersinia* (Von Pawel-Rammingen *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 (Alemán *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-P₂ (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-P₂ (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-P₂, 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-P₂ from the yeast plasma membrane *in vivo* (Alemán *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} (Alemán *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 (Alemán *et al.*, 2005). Here we report that SigD^{R468} 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 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

Fig. 9. SigD^{R468A} 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^{R468A}-GFP with either GST or GST-Cdc42^{G12V} were collected and subjected to affinity purification with glutathione-adsorbed beads as in (a). Membranes were immunoblotted with anti-GFP antibodies to detect the SigD^{R468A} 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^{R468A}-GFP and GST-Cdc24, as indicated, were collected, subjected to affinity purification, separated by SDS-PAGE and immunoblotted as in (b).

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

much more evident in the phosphatase-dead mutant SigD^{R468A}. 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 enzymic 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-P₂ 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-P₂ 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^{R468A} 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^{R468A} 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^{R468A} mutant localizes to the cell periphery. It is likely that the effect on phosphoinositides of catalytically active SigD would affect vesicular traffic. Therefore, elimination of the lipid phosphatase activity would not cause a blockage in the secretory pathway, allowing the mutant SigD^{R468A} 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 Cdc24 containing its PH domain results in an alteration of actin similar to that caused by SigD^{R468A}, together with the lack of Cdc42-related phenotypes induced by SigD^{Δ118–142}, 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^{R468A} 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^{R468A} 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^{R468A} 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^{R468A} 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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