A synaptojanin-homologous region of *Salmonella typhimurium* SigD is essential for inositol phosphatase activity and Akt activation

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Abstract The Ser–Thr kinase Akt is activated in epithelial cells by *Salmonella enterica* serovar *typhimurium*. The bacterial effector SigD, which is translocated into host cells via the specialized type III secretion system, is essential for Akt activation. Here, we investigated the inositol phospholipid substrate preferences of SigD. Recombinant SigD preferentially dephosphorylated phosphatidylinositol 3,5-biphosphate and phosphatidylinositol 3,4,5-triphosphate over other phosphatidylinositol lipids. Phosphatidylinositol 3-phosphate was not a substrate, suggesting the 5′ phosphate moiety is one of the preferred substrates. Database searches revealed that SigD bears a small region of homology to the mammalian type II inositol 5-phosphatase synaptojanin. Mutation of two conserved residues in this region, Lys527 and Lys530, decreased or abrogated phosphatase activity, respectively. The *Shigella flexneri* SigD homologue, IpgD, displayed a similar activity in vitro and also activated Akt when used to complement a Δ*sigD Salmonella* strain. A mutation in IpgD at Lys507, analogous to Lys530 of SigD, also failed to activate Akt. Thus, we have characterized a region near the carboxyl-terminus of SigD which is important for phosphatase activity. We discuss how dephosphorylation of inositol phospholipids by SigD in vivo might contribute to the activation of Akt. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: SopB; IpgD; PKB; Synaptojanin; Phosphatidylinositol 3-kinase; *Shigella*

1. Introduction

The facultative intracellular pathogen *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) is an important causative agent of food-borne gastroenteritis in humans. The ability to breach the intestinal barrier and to survive within a variety of mammalian cell types is required for pathogenicity and is dependent on multiple virulence factors. Many of the genes encoding these factors are located on the bacterial chromosome within five distinct *Salmonella* pathogenicity islands (SPIs) (reviewed in [1]). SPI1 encodes a type III secretion system (TTSS), which translocates effectors directly into the cytoplasm of host cells and is essential for invasion of non-phagocytic cells [2]. These bacterial effectors interact with and alter components of eukaryotic signaling pathways and the actin cytoskeleton, resulting in membrane ruffling on the cell surface followed by bacterial internalization (Fig. 1A) [3–5].

Phosphoinositide 3-kinases (PI3Ks) are activated by a variety of extracellular signals to generate specific inositol lipids that are involved in multiple cellular activities, including the regulation of cell growth, proliferation, survival and cytoskeletal changes (reviewed in [6]). One of the best-characterized targets of PI3K lipid products is the Ser–Thr kinase Akt (also known as PKBα). Upon cellular stimulation, Akt is recruited from the cytosol in an inactive conformation to cellular membranes by interaction of its pleckstrin homology domain with the PI3K lipid products phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol 3,4-biphosphate (PtdIns(3,4)P_2) [7]. Akt activity is not stimulated by translocation itself but is dependent upon phosphorylation at two residues (Thr308 and Ser473). Thr308 is phosphorylated by the phosphoinositide-dependent kinase 1, which also contains a pleckstrin homology domain, and may also target Akt to the membrane [8–10]. The mechanism of phosphorylation of Ser473 remains elusive. Possible candidates are the integrin-linked kinase, phosphoinositide-dependent kinase 1 in a complex with a fragment derived from protein kinase C-related kinase 2, and Akt autophosphorylation [11–13]. It is likely a combination of these mechanisms is at play, depending on the local membrane environment and the cell type.

Our previous work showed that *S. typhimurium* activates Akt and that this activation is absolutely dependent on the SPI1-secreted effector SigD (also known as SopB) [14] (Fig. 1A). SigD dephosphorylates a variety of soluble inositol polyphosphates as well as inositol phospholipids in vitro, and has been shown to convert inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) into inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4) in vivo [15–17] (Fig. 1A). *Shigella flexneri* contains a TTSS homologous to the SPI1 TTSS of *Salmonella* and a *sigD* homologue, *ipgD*. SigD and IpgD both contain two motifs similar to those found in mammalian inositol 4-phosphatases. A conserved cysteine residue in the second motif is essential for the activity of mammalian inositol 4-phosphatases, as well as the in vitro phosphatase activity of SigD and activation of Akt by SigD [14,17]. Intriguingly, SigD is

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Abbreviations: PKBα, serine–threonine kinase Akt; PI3K, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-biphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-biphosphate; PtdIns(3,5)P_2, phosphatidylinositol 3,5-biphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,5,6)P_5, inositol 1,3,4,5,6-pentakisphosphate; TTSS, type III secretion system; SPI, *Salmonella* pathogenicity island; PBS, phosphate-buffered saline; TBS, Tris-buffered saline

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not required for *Salmonella*-induced membrane translocation of Akt, revealing a novel pathway in which the membrane translocation of Akt does not lead to its activation unless an exogenous bacterial protein is present. The mechanism through which SigD activates Akt is unknown, prompting us to investigate the inositol phospholipid substrate preferences of SigD.

A sequence database search against SigD revealed that it also contains a small region of homology to the type II inositol 5-phosphatase synaptojanin located within a region downstream of the inositol 4-phosphatase motifs (Fig. 1B). Mammalian type II 5-phosphatases hydrolyze water-soluble substrates such as inositol 1,4,5-triphosphate and Ins(1,3,4,5)P_4, as well as phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) and PtdIns(3,4,5)P_3. We show here that recombinant SigD and IpgD both dephosphorylate several inositol phospholipids, and mutation of conserved lysine residues in SigD leads to a gradual reduction in catalytic activity. Moreover, this reduced phosphatase activity correlates with a reduced ability of SigD to activate Akt in vivo. These studies reveal a previously uncharacterized region of SigD and synaptojanin, which, at least for SigD, is important for phosphatase activity.

2. Materials and methods

2.1. Cell culture and bacterial strains

HeLa cells, *S. typhimurium* SL1344 wild type and the Δ*sigD* mutant were as described previously [14,18].

2.2. Plasmid construction and protein purification

pACDE, which encodes both SigD and its chaperone SigE, was constructed by amplification of the *sigDE* promoter and operon starting at position −416 with *Pfu* DNA polymerase (Stratagene) using the primers D1 (5′-GCG AAT TCT ATC TGT TCA AGC ATG-3′) and D6 (5′-CGT GAA TTC TCA TTA AGA AAG TAT GTT G) and inserting into pCRTOPO2.1 (Invitrogen). The resulting plasmid was digested with *Xho*I and *Hin*dIII and inserted into the *Sal*I/*Hin*dIII sites of pACYC184 (New England Biolabs). SigD point mutants K527A and V528A were constructed by site-directed mutagenesis using SM9 (5′-GGC GGG GCG GGA AAC gca GTA ATG AAA AAT TTA TCG CCA G-3′) and SM10 (5′-CGG GGC GGG AAA CAA Agc aAT GAA AAA TTT A-3′), respectively, and the Sculptor Mutagenesis kit (Amersham). To construct the SigD point mutant L508A, two DNA fragments were amplified with *Pfu* DNA polymerase using the primer pairs D1 (see above) with SM12 (5′-ATT CAG tgc TAC TTT TTG GAA AAT TTT CTG TCC-3′) and D6 (see above) with SM13 (5′-CAA AAA GTA gca CTG AAT AGC GGT AAC CTG G-3′). The two fragments were isolated, mixed, and used as a template in a PCR reaction with D1 and D6. The resulting DNA was digested with *Kpn*I and *Stu*I, and the fragment containing the mutation was inserted into the corresponding sites of pACDE. The SigD point mutants, L539A and K530A, were constructed similarly. The primers for the first amplification used with D1 were SM14 (5′-GAT AGG AAG CAT TGA GCA CCT CTG GCG-3′) and SM019 (5′-GAT AAA TTt gcC ATT ACT TTG TTT CCC GCC C-3′), respectively. The primers for the second amplification used with D6 were SM15 (5′-TGC TCA ATg ctT CCT ATC AAA AAC GAG TTG-3′) and SM020 (5′-AAC AAA GTA ATG gca AAT TTA TCG CCA GAG GTG C-3′), respectively.

To construct the pACipgDE complementing plasmid, the *sigD* promoter, including the ribosome binding site, from position −416 to −9 relative to the initiating ATG was amplified with *Pfu* DNA polymerase using the primers SM4 (5′-CCG TAG ATA TCG CGA ATT CTA TCT GTT CAA GC-3′) and SM5 (5′-GTA CGG GAT CCT AAT ATT CCT GAA TAG GGG GA-3′) and inserted as an *Eco*RV–*Bam*HI fragment into the corresponding sites of pACYC184 to generate pSigD-400. The *S. flexneri ipgD* and *ipgE* open reading frames from position −2 relative to the initiating *ipgD* ATG codon to the *ipgE* stop codon were similarly amplified using the primers SM6 (5′-ACT GGA TCC AAA TGC ACA TAA CTA ATT TGG-3′) and SM7 (5′-ACT GTC GAC ATT AAT ACC CCT TCA TTC-3′) and inserted as a *Bam*HI/*Sal*I fragment into pSigD-400.

pACipgDE* containing a C439S mutation was constructed as follows: two DNA fragments were amplified with *Pfu* DNA polymerase using the primer pairs SM7 with ipgC439f (5′-CTG GAA TTC GAA GAG TGG GAA GGA CAG AAC-3′), and SM6 with ipgC439r (5′-TTC GAA TTC CAG CAA GGTACA GCA CC-3′). The two DNA fragments were isolated, mixed, and used as a template in a PCR reaction with primers SM6 and SM7. The mutated *ipgD* was inserted as a *Bam*HI/*Sal*I fragment into pSigD-400.

For protein purification, *sigD* missing the first 29 codons was amplified with *Pfu* DNA polymerase using SM018 (5′-AAC GCT AGC CAG ATT CTC TCA GGC CAG GGC-3′) and LK032 (5′-CGC GGA TCC TCA AGA TGT GAT TAA TGA AGA-3′). The resulting DNA was inserted as a *Nhe*I/*Bam*HI fragment into pET28a (Novagen), which generates polyhistidine tagged SigD. The *ipgDE* operon was similarly amplified and inserted in pET28a using SM032 (5′-CCA CGC GCT AGC ATG CAC ATA ACT AAT TTG GGA-3′) and SM033 (5′-CCA CGC GGA TCC TTA ATA CCC CTT CAT TCT TCG-3′). Fusion proteins were affinity-purified on nickel-NTA agarose (Qiagen) according to the manufacturer's instructions.

2.3. Phosphoinositide phosphatase assay

Phosphoinositide phosphatase activity was measured using a chromogenic assay based on the malachite green method previously used for the analysis of protein phosphatases [19,20]. Briefly, recombinant purified protein (50–100 ng) was incubated with synthetic short chain (octanoyl) phosphoinositides (Echelon Research Laboratories, Salt Lake City, UT, USA) (50–100 μM) as indicated (temperature $T = 37$°C, reaction volume $V = 25$ μl). The reactions were stopped by addition of 80 μl malachite green reagent [20] and absorbance measured at 620 nm. The assay provided linear detection in the range of up to 1000 pmol of Pi.

2.4. Bacterial infection of eukaryotic cells

Bacterial infections were carried out as described previously [14]. Briefly, bacteria were grown in Luria–Bertani broth at 37°C to late log phase. The culture was centrifuged at 10 000×*g* for 2 min at room temperature and resuspended in phosphate-buffered saline (PBS). Invasion was initiated by addition of 30 μl bacteria directly to cultured cells. Cells were then incubated at 37°C in 5% CO_2 for 25 min, and free bacteria were removed by washing with PBS.

2.5. Akt kinase assay

Materials for the Akt kinase assay were obtained as part of a kit (New England Biolabs), and used according to the manufacturer's instructions. Briefly, cells were lysed by the addition of 200 μl cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin), and transferred to microfuge tubes. Insoluble material was pelleted by centrifuging for 10 min at 16 000×*g*. Total Akt was immunoprecipitated from lysates with 20 μl of immobilized Akt 1G1 monoclonal antibody. The antibody–resin was pelleted, washed twice in 1 ml lysis buffer, and twice in 1 ml kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). For the kinase reaction, pellets were resuspended in kinase buffer supplemented with 200 μM ATP and 1 μg GSK-3 fusion protein, and incubated for 30 min at 30°C. Proteins were electrophoresed on a standard 12% SDS–PAGE gel, transferred to nitrocellulose membrane, and immunoblotted as described below.

2.6. Immunoblotting

Immunoblotting was performed as described [14]. Briefly, cell extracts were prepared by adding 80 μl of hot SDS sample buffer containing 50 mM DTT directly to the monolayer in 35 mm dishes. Samples were collected and boiled for 5 min. Proteins were separated by SDS–PAGE (10%) and transferred to nitrocellulose membranes. Blots were blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 and 5% skim milk powder (Carnation). Blots were then incubated with primary antibody overnight at 4°C, at 1:1:000 dilution in primary antibody dilution buffer (5% bovine serum albumin, TBS, 0.1% Tween-20). Rabbit anti-PKB-ser^{473} and rabbit phospho-GSK-3α/β (Ser21/9) antibodies were obtained from NEB.

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies were used at 1:2000 dilution. The PhotoTope-HRP detection system (NEB) was used according to the manufacturer's directions.

3. Results

3.1. Lys527 and Lys530 are essential for full phosphatidylinositol phosphatase activity of SigD

In addition to two motifs bearing homology to mammalian inositol 4-phosphatases, SigD contains a region of homology to the inositol 5-phosphatase synaptojanin (Fig. 1B). This region is near the carboxyl-terminus of SigD (residues 507–563) and within a domain of synaptojanin similar to other type II inositol 5-phosphatases (residues 495–550). This prompted us to investigate the substrate preferences of SigD. Recombinant SigD was incubated with a number of short chain phosphatidylinositol lipids. Activity of SigD towards the various substrates was assessed in a colorimetric assay that measures phosphate release (Fig. 2A). Of the monophosphorylated inositol phospholipids, the wild type protein showed significant activity towards PtdIns(4)P and PtdIns(5)P, but not PtdIns(3)P. Of the inositol phospholipids with multiple phosphate moieties, SigD showed the greatest activity towards PtdIns(3,4,5)P_3 and phosphatidylinositol 3,5-biphosphate (PtdIns(3,5)P_2), followed by PtdIns(3,4)P_2 and

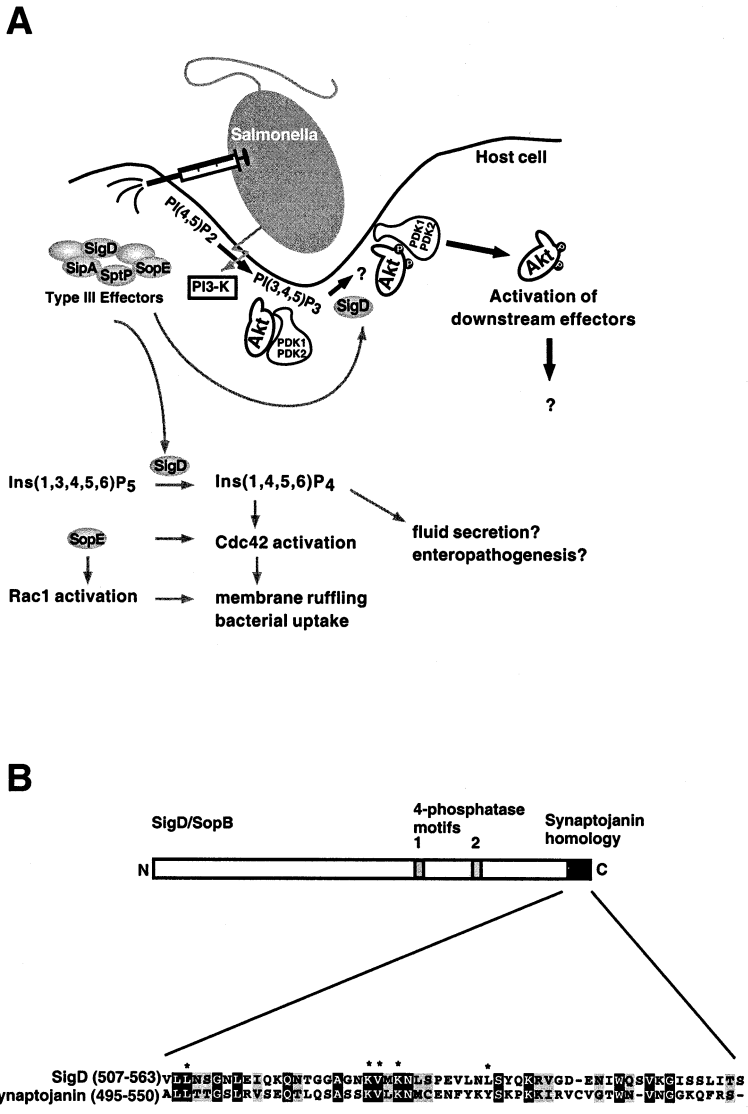

Fig. 1. Cellular functions of SigD and homology to synaptojanin. A: The SPI1 TTSS injects effectors into the host cell to activate multiple signaling pathways, some of which result in membrane ruffling and bacterial uptake. For example, SopE catalyzes exchange of GDP for GTP on Rac1 and Ccd42 [4], thus activating them and inducing membrane ruffling. SigD converts Ins(1,3,4,5,6)P_5 into Ins(1,4,5,6)P_4, which can indirectly activate Cdc42 [15]. Ins(1,4,5,6)P_4 may also be responsible for the increased fluid secretion and recruitment of subepithelial neutrophils in a ligated bovine intestinal loop model of diarrhea [17,24]. PI3K is activated by an unknown bacterial effector(s), thought to induce the recruitment of Akt to PtdIns(3,4,5)P_3 at membrane ruffles. The mechanism through which SigD induces the phosphorylation and activation of Akt is unknown, prompting us to investigate the inositol phospholipid substrate preferences of SigD. B: Partial sequence alignment of SigD and rat synaptojanin. Identical residues are shaded in black, and similar residues are shaded in gray. Asterisks indicate residues mutated in SigD in this study. Sequences were aligned with Clustal W and shaded with Boxshade through the Biology Workbench 3.2 server (http://workbench.sdsc.edu/CGI/BW.cgi).

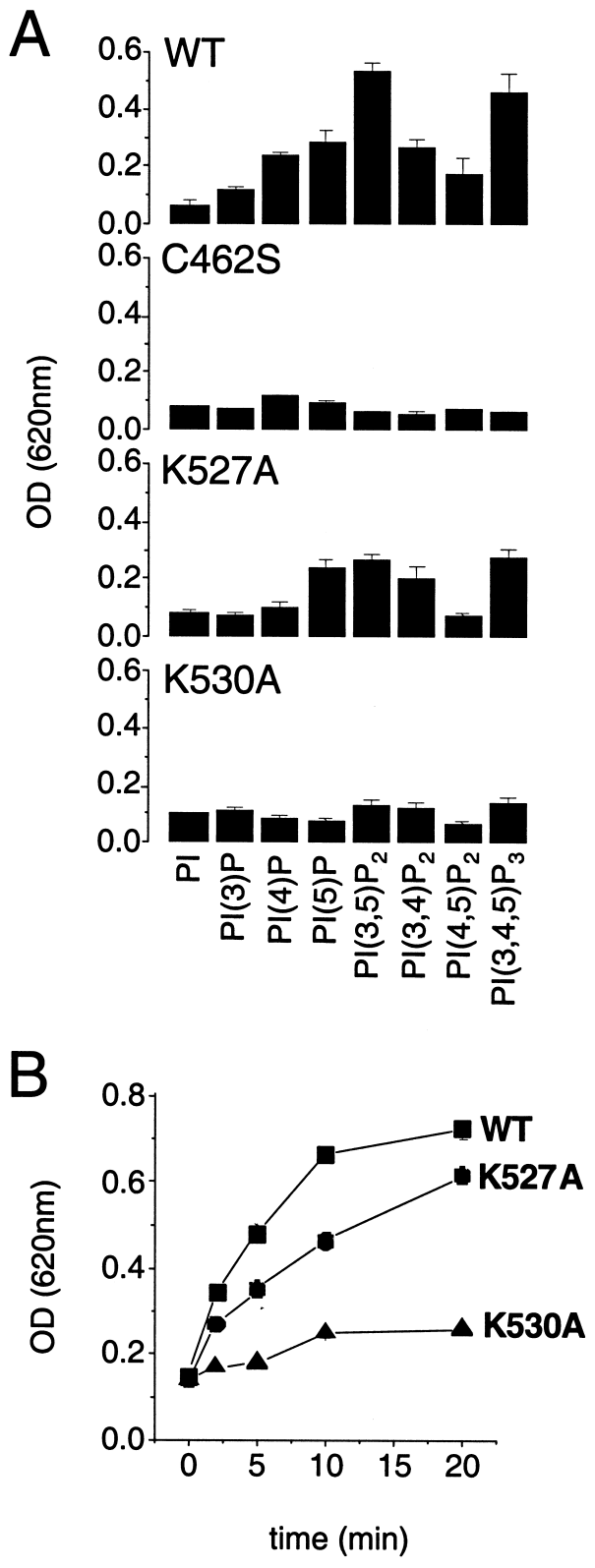

Fig. 2. Phosphoinositide phosphatase activity of SigD and SigD point mutants. Recombinant purified protein was incubated with phosphoinositides and release of phosphate was detected colorimetrically by absorbance at 620 nm. A: Substrate preference profile of wild type SigD (WT) and SigD point mutants. Proteins (80 ng) were incubated with substrate phosphoinositides (50 μM) for 20 min at 37°C. Phosphatidylinositol (PI) was used as a background control incubation and gave the same values as incubations of protein without any lipid (data not shown). Data are represented as mean ± S.D. of at least three separate experiments. B: Time course of phosphate release. PtdIns(3,4,5)P_3 (100 μg) was incubated with SigD wild type and mutant proteins and the reaction stopped at indicated times. Data are shown as mean values ($n = 3$), the standard deviations are within the symbol size.

PtdIns(4,5)P_2. As expected, changing Cys462 within the second 4-phosphatase motif to a serine residue completely abrogated activity, since this is considered to be a critical catalytic residue ([14,21] and Fig. 2A). To determine the functional relevance of the homology between SigD and synaptojanin, two conserved lysine residues were changed to alanine (Fig. 1B, marked with *). Altering Lys527 and Lys530 significantly reduced the phosphatase activity of SigD (Fig. 2A). Kinetic analysis of the reaction shows that phosphate release by wild type SigD increased sharply within the first 5 min and plateaued by 10–20 min (Fig. 2B). The Lys530 mutant had essentially no activity, while the Lys527 mutant had an intermediate level of activity (Fig. 2B).

3.2. Reduced phosphatase activity of Lys527 and Lys530 SigD mutants correlates with reduced activation of Akt in vivo

We wished to determine whether the reduced phosphatase activity of the SigD mutants correlated with a reduced ability to activate Akt in vivo. To further assess the importance of residues in the area of homology to synaptojanin, additional residues were individually mutated (Fig. 1B, marked with *). A conserved valine (Val528), a conserved leucine (Leu508) and a non-conserved leucine (Leu539) were each changed to alanine. Mutations were incorporated into wild type *sigD* in pACYC184 (designated pACDE), and used to complement *S. typhimurium* devoid of SigD (Δ*sigD*). HeLa cells were serum-starved for 3 h and then infected with either wild type *S. typhimurium* (strain SL1344) or the Δ*sigD* strain carrying pACDE or derivatives with the indicated mutations. After infection, the cells were lysed, and the phosphorylation state of Akt was assessed by probing Western blots with phospho-specific antibodies recognizing Ser473 or Thr308 phosphorylation (Fig. 3A,B). As we have previously reported [14], mutation of the critical catalytic cysteine residue (C462S) abrogated SigD activation of Akt. When Lys527 was mutated, the activation of Akt was significantly reduced, with phosphorylation at Thr308 barely detectable. Mutation of Lys530, however, completely abrogated the phosphorylation and activation of Akt by SigD. Altering the other residues, Leu508, Val528, and L539, had no effect on Akt activation compared to wild type SigD. The expression level and competence for secretion of the point mutants were also assessed. When *S. typhimurium* SL1344 is grown under conditions which induce secretion of SPI1 TTSS, SigD is visible on a Coomassie-stained gel among other proteins secreted into the growth medium [22]. The SigD point mutants were all secreted by the SPI1 TTSS in amounts comparable to the wild type protein (Fig. 3C), indicating these mutant proteins are still synthesized and exported by the TTSS.

3.3. S. flexneri IpgD is also an inositol phosphatase

IpgD is 59% similar and 41% identical to SigD/SopB, at the amino acid level. It has been shown to be secreted by the TTSS of *S. flexneri* and is involved in the modulation of the

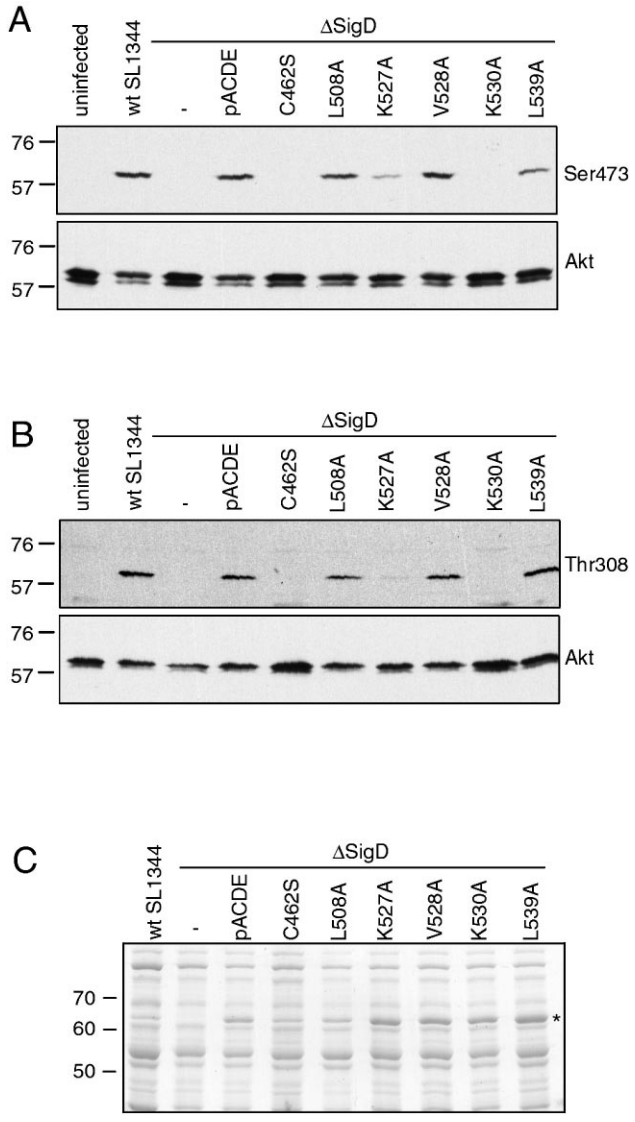

Fig. 3. Lys527 and Lys530 of SigD are essential for full Akt activation. A and B: Cells were incubated with either wild type *S. typhimurium* (SL1344) or the Δ*sigD* strain carrying control plasmid pACYC184 (−), plasmids encoding wild type SigD (pACDE) or the indicated point mutations for 25 min. Western blot analysis was with phosphospecific antibodies (upper panels) to Ser473 (A) or Thr308 (B), after which the blots were stripped and probed with anti-Akt to detect total Akt (lower panels). C: The SigD point mutants are synthesized and secreted by *Salmonella* in amounts comparable to the wild type protein. The *S. typhimurium* strains from B and C were grown under conditions that induce the secretion of SPI1 proteins into the culture media [22]. Proteins were precipitated with trichloroacetic acid and separated by SDS–PAGE. The gel was subsequently stained with Coomassie R-250.

host's cytoskeletal response upon bacterial entry [23]. IpgD, when expressed alone, is unstable in bacteria ([23] and our unpublished results). Therefore, IpgD was purified together with its chaperone IpgE and used in a substrate preference assay. SigD, when purified together with its chaperone SigE, had a similar substrate preference profile to SigD alone (data not shown). The substrate preference of IpgD was similar to that of SigD, however IpgD used PtdIns(3,4,5)P_3 significantly more than the other substrates (Fig. 4A). As expected, a mutation at Cys439, which is the critical catalytic cysteine residue, completely abrogated IpgD phosphatase activity.

3.4. Activation of Akt by IpgD requires Lys507

To determine whether IpgD is also able to activate Akt, *ipgD* and *ipgE* were cloned into pACYC184 under the transcriptional control of the *sigD* promoter and introduced into the Δ*sigD* strain. As before, HeLa cells were infected with bacteria and the phosphorylation state of Ser473 of Akt was determined (Fig. 4B, upper two panels). To assess Akt enzymatic activity, total Akt was immunoprecipitated from cell extracts using an immobilized antibody that recognizes both phosphorylated and non-phosphorylated forms. Immunoprecipitates were then incubated with a recombinant protein containing the consensus sequence surrounding Ser21 of GSK-3α or Ser9 of GSK-3β, which are Akt substrates, and ATP. The products were analyzed by Western blotting with an antibody specifically detecting phospho-GSK-3 (Fig. 4B, lower panel). Wild type IpgD (pACipgDE) induced both the phosphorylation of Akt at Ser473 and activation of Akt. As expected, mutating the conserved catalytic cysteine residue (C439S) abrogated this activity (pACipgDE*). Mutating IpgD Lys507, analogous to Lys530 of SigD, also abrogated phosphorylation and activation of Akt (Fig. 4C). Therefore, this conserved lysine residue is functionally conserved between these two bacterial species.

4. Discussion

Our previous study demonstrated that the *S. typhimurium* SPI1 TTSS translocated effector SigD induces Akt activation, the first bacterial effector identified to activate this signaling pathway [14]. Intriguingly, both wild type *S. typhimurium* and the Δ*sigD* strain stimulate both translocation of Akt to membrane ruffles and the association of PI3K with phosphotyrosine-containing proteins [14]. Therefore, another bacterial effector must be responsible for the initial activation of PI3K upstream of the action of SigD.

Recent reports have shown that in vivo, SigD/SopB functions specifically as a 3-phosphatase to convert Ins(1,3,4,5,6)P_5 into Ins(1,4,5,6)P_4 [15,16]. SopB, the nearly identical SigD homologue of *Salmonella dublin*, is required for both increased fluid secretion and recruitment of subepithelial neutrophils in a ligated bovine intestinal loop model of diarrhea [17,24]. This activity is attributed to an increase in intracellular Ins(1,4,5,6)P_4, a product of the inositol phosphatase activity of SopB in *S. dublin*-infected T84 colonic epithelial cells [25] (Fig. 1A). Ins(1,4,5,6)P_4 produced by SigD has also been proposed to act in concert with SopE to activate Rho GTPases, promoting host cell actin cytoskeleton rearrangements and bacterial internalization [15]. We have extended previous observations that SigD can hydrolyze inositol phospholipids [17] by testing a wider range of lipid substrates. Of the monophosphorylated inositol phospholipids, SigD had the greatest activity towards PtdIns(5)P, while PtdIns(3)P was not a substrate. Of the inositol phospholipids with multiple phosphate moieties, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 were the best substrates. IpgD also hydrolyzed phosphate groups from inositol phospholipids, and preferred substrates in the order PtdIns(3,4,5)P_3 > PtdIns(3,4)P_2 > PtdIns(3,5)P_2. Taken together, these results suggest that both SigD and IpgD are capable of hydrolyzing phosphate groups from the 5′ position of lipid substrates, including PtdIns(3,4,5)P_3. We have found that Lys527 and Lys530 in SigD are absolutely required for full inositol phosphatase activity. Moreover, the activity level

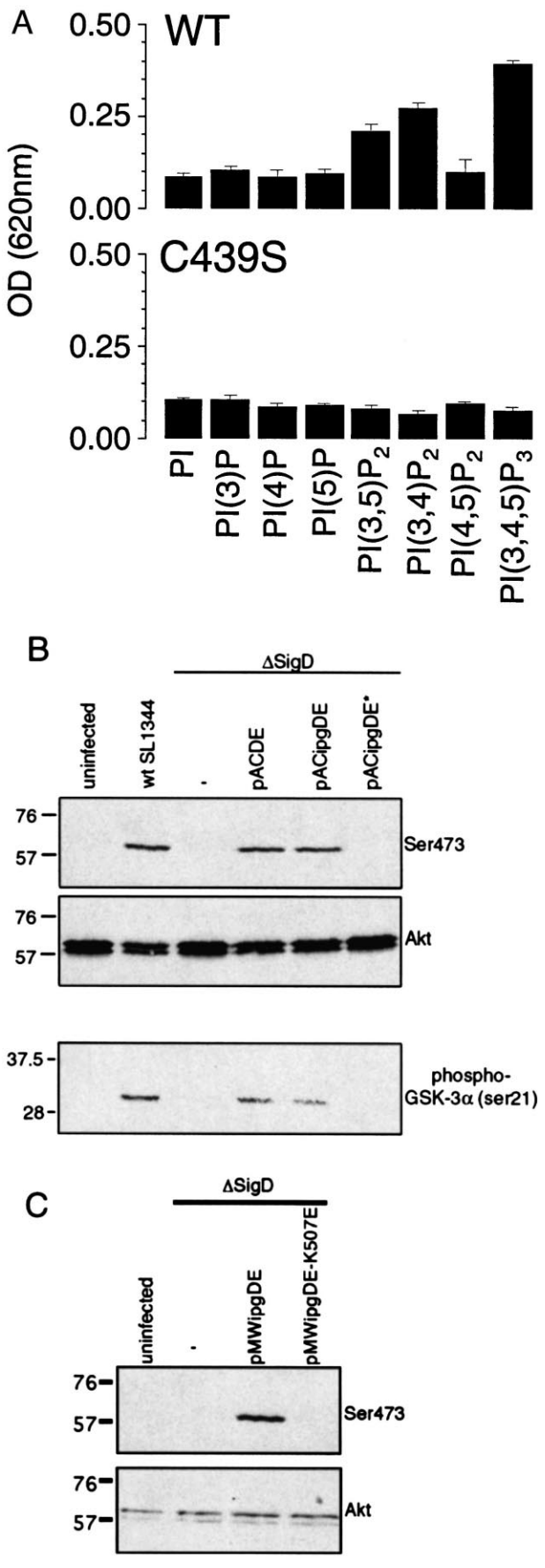

Fig. 4. *S. flexneri* IpgD hydrolyzes inositol phospholipids and activates Akt. A: *S. flexneri* wild type IpgD and mutant proteins (200 ng) were incubated with substrate phosphoinositides (150 μM) for 1 h at 37°C ($n = 3$). B: Activation of Akt by IpgD. Cells were infected with *S. typhimurium* as described in Fig. 3. Plasmids expressing *ipgD* and its chaperone *ipgE* (pACipgDE) or the C439S mutant (pACipgDE*) were introduced into the Δ*sigD* strain and tested in parallel with SL1344. Western blot analysis was performed as described in Fig. 3 (upper two panels). Bottom panel: cell extracts were incubated with immobilized antibody recognizing both phosphorylated and non-phosphorylated forms of Akt. Antibody beads were washed extensively and resuspended in kinase buffer supplemented with 200 μM ATP and 1 μg GSK-3 fusion protein. Western blotting was with phosphospecific antibodies to GSK-3α/β (Ser21/9). C: Mutation of Lys507 abrogates Akt activation. Δ*sigD* strains transformed with either control pMW119 (−) or pMW119 containing wild type *ipgD* and ipgE (pMWipgDE), or an *ipgD* point mutant (pMWipgDE-K507E) were incubated with HeLa cells as described. Western blotting was as described in Fig. 3.
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of these mutants in vitro correlates with Akt activation in vivo. These residues lie within a region of homology to the mammalian 5-phosphatase synaptojanin, in a region that is similar to other type II 5-phosphatases. These observations suggest that this domain of SigD and synaptojanin is important for substrate recognition and/or catalysis.

We demonstrated that *S. flexneri* IpgD activates Akt in epithelial cells when translocated by *Salmonella*, a previously uncharacterized activity of this protein. Moreover, a mutant in IpgD at Lys507, analogous to SigD Lys530, completely abrogates Akt activation, and presumably phosphatase activity. However, it is not known if *S. flexneri* activates Akt upon invasion. An increase in intracellular Ins(1,4,5,6)P_4 is not observed in *S. flexneri*-infected T84 colonic epithelial cells, as it is in *S. dublin*-infected cells [25]. Thus, whereas previous studies do not show a similar function of IpgD and SopB/SigD, we demonstrate that these two proteins do indeed have similar activities.

We propose a two-step mechanism for how Akt might be activated by *Salmonella*. The SPI1 TTSS of *Salmonella* injects effectors that promote membrane ruffling, bacterial uptake, and the activation of PI3K. The subsequent increased concentration of PtdIns(3,4,5)P_3 at membrane ruffles results in recruitment of Akt, and its activating kinase(s). We have previously shown Akt recruitment to be a SigD-independent process. We propose that the 5′ phosphatase activity of SigD converts PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2, inducing a conformational change in recruited Akt, favoring its phosphorylation at Thr308 and Ser473 and allowing full activation. The relative contribution of PtdIns(3,4,5)P_3 versus PtdIns(3,4)P_2 to Akt activation is controversial [8,26], and may be related to cell type and the local environment. Indeed, there is evidence that PtdIns(3,4)P_2 is required for Akt activation in mast cells (V. Duronio, personal communication). Membrane recruitment of Akt is very localized in this system, occurring only at membrane ruffles in the vicinity of bacteria. Upon stimulation by growth factors, for example, membrane recruitment of Akt occurs over the whole cell surface. This would explain why overexpression of SigD/SopB does not result in measurable differences in cellular inositol lipids [16]. We believe that the changes in PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 levels may be too small and localized to detect, relative to the abundant PtdIns(4,5)P_2 in membranes.

Our study reveals a previously unrecognized homologous

domain of SigD and synaptojanin. That this important region of both SigD and IpgD lies within the type II 5-phosphatase domain of synaptojanin supports our model for Akt activation by *Salmonella*. It also suggests that these residues play an important role in the recognition or hydrolysis of the 5′ phosphate moiety of inositol lipid substrates used by both phosphatases.

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