Invasion of HeLa cells by group B streptococcus requires the phosphoinositide-3-kinase signalling pathway and modulates phosphorylation of host-cell Akt and glycogen synthase kinase-3

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The group B streptococcus (GBS) is an opportunistic bacterial pathogen with the ability to cause invasive disease. While the ability of GBS to invade a number of host-cell types has been clearly demonstrated, the invasion process is not well understood at the molecular level. What has been well established is that modulation of host-cell actin microfilaments is essential for GBS invasion to occur. Phosphoinositide-3 kinase (PI3K) is a key regulator of the cytoskeleton in eukaryotic cells. Our goal in this investigation was to explore the role of the PI3K/Akt signalling pathway in epithelial cell invasion by GBS. The epithelial cell invasion process was mimicked using the HeLa 229 cell-culture model. Treating HeLa cells with chemical inhibitors of PI3K, Akt or Ras prior to bacterial infection inhibited GBS invasion but not attachment; treatment with 30 μ M LY294002 (PI3K inhibitor) reduced GBS invasion by 75 %, 20 μ M L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (ICIO) (Akt inhibitor) reduced GBS invasion by 50 %, and 10 μ M manumycin A (Ras inhibitor) inhibited GBS invasion by 90 %. Genetic inactivation of the p85 α or p110 α PI3K subunits in HeLa cells also reduced GBS invasion by 55 and 30 %, respectively. Western blot analysis revealed that phosphorylation of host-cell Akt and glycogen synthase kinase-3 (GSK-3) occurs in response to GBS infection, and that this is mediated upstream by PI3K. Infection of HeLa cells with GBS triggers pro-survival signalling and protects the HeLa cells from camptothecin-induced caspase-3 cleavage. The results from this investigation show that GBS both requires and activates the PI3K/Akt host-cell signalling pathway during invasion of epithelial cells.

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INTRODUCTION

The group B streptococcus (GBS) is a Gram-positive, opportunistic bacterial pathogen with the ability to cause invasive disease in humans. GBS is best known for its

Abbreviations: CPT, camptothecin; GAS, group A streptococcus/streptococci; GBS, group B streptococcus/streptococci; GSK-3, glycogen synthase kinase-3; HK-GBS, heat-killed GBS; ICIO, L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; PDK1, phosphoinositide-dependent protein kinase 1; PH, plesktrin homology; PI3K, phosphoinositide-3 kinase; PIP₂, phosphatidylinositol 3,4-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-triphosphate; PKC, protein kinase C.

A supplementary figure showing the results of experiments to determine whether the inhibitors used affected bacterial membrane permeability to antibiotics is available with the online version of this paper.

ability to cause severe morbidity and mortality in neonates, and is the leading cause of meningitis in infants less than 1 month of age (Schuchat *et al.*, 1997; Schuchat, 1998; Tyrrell *et al.*, 2000). Other forms of invasive GBS neonatal disease include pneumonia and sepsis. GBS infections are not restricted to infancy, but can also occur in adulthood. Those at highest risk include the elderly and those with another underlying illness, such as diabetes mellitus (Farley *et al.*, 1993; Schuchat *et al.*, 1990; Schuchat, 1998; Tyrrell *et al.*, 2000). Disease manifestations in adulthood can be similar to those observed in neonates, in addition to bone and soft-tissue infections.

It has been well established previously that GBS are able to invade a variety of host-cell types, including epithelial cells, endothelial cells and macrophages (Gibson *et al.*, 1993;

Greco *et al.*, 1995; Lalonde *et al.*, 2000; Nizet *et al.*, 1997; Shin *et al.*, 2006; Tyrrell *et al.*, 2002; Valentin-Weigand *et al.*, 1996, 1997). The attachment and invasion of GBS into host cells is thought to involve numerous pathogen–host-cell interactions, but an understanding of these events at the molecular level is only in the early stages of characterization. What has been well established is that host-cell actin microfilaments are recruited to the site of GBS attachment and invasion, and that modulation of actin microfilaments by GBS is essential for invasion to occur. This has been demonstrated by the finding that the addition of cytochalasin D (a potent inhibitor of actin polymerization) to epithelial cells prior to infection inhibits GBS invasion (Gibson *et al.*, 1993; Greco *et al.*, 1995; Tyrrell *et al.*, 2002; Valentin-Weigand *et al.*, 1997), as well as by microscopic demonstration of actin recruitment to the site of GBS attachment (Tyrrell *et al.*, 2002).

Phosphoinositide-3 kinase (PI3K) is a lipid kinase that catalyses the addition of a phosphate to phosphoinositides; the resulting phospholipid molecules modulate the actin cytoskeleton with precise spatial and temporal control (Stokoe, 2005; Vanhaesebroeck & Alessi, 2000). The formation of phosphatidylinositol 3,4,5-triphosphate (PIP₃) from phosphatidylinositol 3,4-bisphosphate (PIP₂) by PI3K leads to recruitment of Akt to the host-cell membrane, and subsequent Akt phosphorylation and activation (for overview, see Fig. 1). The role of PI3K and phosphoinositide metabolism in bacterial pathogenesis is becoming increasingly appreciated. A requirement for PI3K in host-cell invasion has been identified for the pathogens group A streptococcus (GAS) (Purushothaman *et al.*, 2003), *Helicobacter pylori* (Kwok *et al.*, 2002), *Chlamydia pneumoniae* (Coombes & Mahony, 2002) and *Listeria monocytogenes* (Ireton *et al.*, 1999), to name just a

few. The result of manipulation of this pathway by a pathogen is coordination of actin rearrangement, leading to internalization of the organism.

The epithelium is a key component of innate immunity for humans. A breach of the epithelial barrier by a bacterial pathogen is often the precipitating event in invasive disease. In order to better understand the GBS disease process, our goal in this investigation was to explore the role of the PI3K/Akt signalling pathway in epithelial cell invasion by GBS.

METHODS

Bacterial strains, cell lines, and culture conditions. The GBS strain used in this investigation is NCS13, a serotype V organism isolated from a soft-tissue wound of an elderly patient, and has been previously described (Tyrrell *et al.*, 2002). The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain NCTC 10241 was obtained from the National Collection of Type Cultures (NCTC), London, and the *Yersinia enterocolitica* subsp. *enterocolitica* (*Y. enterocolitica*) strain 23715 was obtained from the American Type Culture Collection (ATCC). All of the organisms were cultured at 35 °C on sheep blood agar plates (BAPs; Dalynn Biologicals) or in Todd–Hewitt broth (THB; Difco Laboratories).

The human epithelial cell line HeLa 229 (ATCC CCL-2.1) was obtained from the ATCC and was grown and maintained in Opti-MEM I reduced serum medium (Invitrogen) supplemented with 4% fetal bovine serum (FBS; Gibco), unless otherwise noted.

Chemical inhibitors. Staurosporine, bisindolylmaleimide, manumycin A and camptothecin (CPT) were purchased from Sigma–Aldrich. The PI3K inhibitor LY294002 was obtained from Cell Signaling Technology. The Akt inhibitor 1-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (ICIO), Akt inhibitor V (Triciribine) and Akt inhibitor VII were obtained from Calbiochem. Table 1 provides a summary of the actions of these inhibitors.

Fig. 1. Schematic diagram illustrating events in Akt activation by the PI3K pathway. An external stimulus binds to a receptor on the host-cell surface, leading to activation of PI3K (1), catalysing the formation of membrane-bound PIP₃ from PIP₂ (2). Akt is then recruited to the cell membrane, where it is anchored via binding of PIP₃ to the PH domain of Akt (3). This is followed by phosphorylation of a threonine residue (Thr³⁰⁸) on the Akt kinase domain by PDK1 (4); this results in a conformational change in Akt and phosphorylation of a serine residue (Ser⁴⁷³) by PDK2 (5), leading to full activation of Akt kinase activity. Akt then interacts with various effector molecules in the host cell (6), resulting in activation or inactivation of these host-cell components.

Table 1. Pharmacological agents used in this investigation

Agent	Target or mode of action in host cell
Akt inhibitor ICIO	Inhibits Akt by preventing PIP ₃ formation
Akt inhibitor V	Inhibits Akt; mechanism yet to be characterized, but does not involve PI3K or PDK1
Akt inhibitor VII	Interacts with the PH domain of Akt to prevent phosphoinositide binding by Akt
Bisindolylmaleimide	Interacts with PKC catalytic subunit to competitively inhibit ATP binding
CPT	Topoisomerase inhibitor; induces caspase-3 cleavage
LY294002	PI3K inhibitor; competes with ATP for binding to and activation of PI3K
Manumycin A	Ras farnesyltransferase inhibitor; blocks membrane binding essential for Ras activation
Staurosporine	Broad-spectrum protein kinase inhibitor

For the Akt inhibitor assays, HeLa cells were serum-starved by replacing the Opti-MEM I + 4 % FBS with Opti-MEM I (no FBS) for 18–24 h preceding treatment with inhibitor for 3 h prior to the addition of bacterial inoculum. For all other inhibitors, HeLa cells were incubated for 1 h with inhibitor prior to adding bacterial inoculum. The inhibitors were present during the invasion assays until the first wash, when fresh media was added, and during the entire duration of the attachment assays.

Antibodies and plasmids. Rabbit anti-phospho-Akt (Ser 473) (9271) and rabbit anti-Akt (9272) were purchased from Cell Signaling Technology, as were mouse anti-phospho-GSK-3 α (Ser21) (46H12; 9337), rabbit anti-phospho-GSK-3 β (Ser9) (9336) and rabbit anti-GSK-3 β (9315). Rabbit caspase-3 antibody was obtained from Stressgen Bioreagents.

Plasmids expressing the dominant-negative forms of the PI3K subunits (p110 α ; p85 α) and the wild-type p110 α subunit (p110WT) were generously provided by Dr David Stokoe (University of California, San Francisco Cancer Research Institute) (Purushothaman *et al.*, 2003; Wang *et al.*, 2002).

Invasion (antibiotic protection) assay. Invasion of bacteria into an epithelial cell (HeLa) monolayer was quantified using a standard antibiotic protection invasion assay with minor modifications (Rubens *et al.*, 1992; Tyrrell *et al.*, 2002). Briefly, a monolayer of HeLa cells was grown to confluence in 24-well plates (Corning), and was then treated with either a pharmacological agent or the solvent used as a carrier for the agent (solvent control; this is represented as the 0 μ M concentration of inhibitor in the graphical representations of the data). Assays were performed in triplicate for each condition. The bacterial inocula (GBS, *S. typhimurium* or *Y. enterocolitica*) were grown overnight in THB at 35 °C with agitation. The next day, approximately 1.5×10^5 GBS, 4×10^4 *S. typhimurium* or 1.5×10^4 *Y. enterocolitica* were added to the monolayer. The differences in inocula reflect the variation in invasion efficiency of the different species.

Following inoculation, the cell-culture plates were centrifuged at 100 g for 5 min at room temperature. After a 2 h incubation at 35 °C to allow internalization, the monolayer was washed three times with PBS to remove unbound bacteria. Bacteria that had bound but not been internalized were killed by incubation for 2 h with fresh media containing 5 μ g penicillin ml⁻¹ and 100 μ g gentamicin ml⁻¹. After the 2 h incubation, an aliquot of medium from each well was applied to a BAP to ensure that the antibiotic treatment was sufficient to kill all extracellular bacteria. The monolayers were washed with PBS, trypsinized and then lysed with 0.1 % Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate was counted to determine the number of c.f.u. that had invaded the monolayer.

The HeLa cell monolayers were examined at the end of each incubation period during the assay to observe their integrity in the presence of the

chemical inhibitors. Portions of the HeLa cell-culture supernatants were plated onto BAPs following the initial 2 h infection to ensure that the inhibitor treatments did not reduce bacterial viability. A subset of control invasion assays was also performed where the chemical inhibitor was added during the 2 h incubation with antibiotic-containing media rather than prior to adding the bacterial inoculum.

Transient transfection of HeLa cells. For the invasion assays into HeLa cells with altered genetic backgrounds, a Lipofectamine transient transfection procedure with plasmid DNA expressing a dominant-negative form of the cell moiety in question was utilized. HeLa cells were grown to approximately 80–90 % confluence and the culture medium was replaced with serum-free Opti-MEM I. Then, 1 μ g plasmid DNA and 2 μ g Lipofectamine 2000 transfection reagent (Invitrogen) were added to the HeLa cells in accordance with the manufacturer's instructions. After 5 h incubation at 37 °C, the cell-culture medium was replaced with fresh Opti-MEM I with 4 % FBS and the cells were incubated overnight at 37 °C prior to performing the invasion assay.

Bacterial attachment assay. A quantitative attachment assay was performed to assess the ability of GBS to attach to a HeLa cell monolayer in the presence of the various pharmacological inhibitors. HeLa cells were grown to confluence in 24-well tissue-culture plates, then treated with inhibitor or the inhibitor's solvent, with each assay condition tested in triplicate. GBS were grown in THB at 35 °C with agitation overnight. The following day, approximately 3×10^4 GBS were added to the monolayer. For trypsinized GBS, a 0.5 McFarland standard of organisms was made in 0.25 % trypsin/PBS; this was incubated at 35 °C for 1 h and diluted in THB prior to inoculation of HeLa cells.

Following inoculation, the cell-culture plates were centrifuged at 100 g for 5 min at room temperature, and then incubated at 4 °C [a temperature that permits bacterial attachment but not internalization (Tamura *et al.*, 1994)] for 2 h. Following incubation, the HeLa cells were washed six times with PBS to remove unbound bacteria. The cells were then trypsinized and lysed with 0.1 % Triton X-100. The lysates were spread onto BAPs and incubated overnight at 35 °C. The number of colonies on each plate was counted to determine the number of GBS that had attached to the monolayer under each assay condition.

Statistical analysis. For the attachment and invasion assays, relative percentage attachment/invasion was calculated as follows: [Number of c.f.u. attached (invaded) to treated cells/number of c.f.u. attached (invaded) to control cells] $\times$ 100 %.

For each assay, every condition was performed in triplicate, and each assay was repeated a minimum of three times. GraphPad InStat3 software was used for statistical analysis of the data. Analysis of variance using Dunnet's multiple comparisons test was used to compare attachment to or invasion of inhibitor-treated or transfected

cells with that observed in the control cells. Where indicated, variance between inhibitor treatments was also analysed with the Bonferroni multiple comparisons test. A *P* value <0.05 compared to control cells was deemed to be statistically significant and is denoted by “*” in the graphical representations of data. The graphical representations are illustrative of the mean of one representative experiment; error bars represent SEM.

Immunoblotting. To assess modulation of Akt and glycogen synthase kinase-3 (GSK-3) phosphorylation in response to GBS infection, HeLa cells were grown in six-well culture plates to approximately 80 % confluence before being serum-starved for 20–24 h. The cells were then challenged with an inoculum of 3×10^8 GBS for 5, 15, 30 or 60 min, or left uninfected (0 min). The reactions were immediately transferred to ice at the appropriate time points, the cells washed with cold PBS, and then harvested in ice-cold buffer containing $1 \times$ Cell Lysis Buffer (Cell Signaling Technology), 0.2 % Protease Inhibitor Cocktail for Mammalian Cell Extracts (Sigma), and 1 mM PMSF (Sigma). The lysates were centrifuged at 13 000 g for 10 min and the protein concentration of the supernatant fraction was determined using the Bio-Rad protein assay, which is based on the method of Bradford (1976). Samples, 100 μ g for 10-well gels or 50 μ g for 15-well gels, of cell lysate were resolved via SDS-PAGE, and electroblotted to a 0.45 μ m pore-size nitrocellulose membrane (Bio-Rad) following standard protocols (Bollag & Edelstein, 1991). Each blot was then probed with antibody that detected a phosphorylated form of Akt, GSK-3 α or GSK-3 β , and detected on BioMax Light Film for Chemiluminescence (Kodak) using the Amersham Biosciences Enhanced Chemiluminescence (ECL) detection system. The blots were then stripped with Western ReProbe Solution (Calbiochem), washed, and reprobed with antibodies detecting either total Akt (for phospho-Akt) or total GSK-3 β (for phospho-GSK-3 α and β) as loading controls. Where indicated, a subset of the cells was treated with 50 μ M LY294002 or 20 μ M ICIO prior to bacterial infection. For studies with heat-killed GBS (HK-GBS), a 3 McFarland standard of GBS was made in THB and then placed in an 80 °C water bath for 30 min. A portion of the culture was inoculated to a BAP to ensure the treatment was adequate for complete killing.

For immunoblots to detect caspase-3 cleavage, HeLa cells were grown to approximately 90 % confluence in six-well culture dishes, serum-starved for 18–20 h and then infected with 2×10^8 c.f.u. of exponential-phase GBS. Following a 30 min incubation to allow bacterial internalization to occur, the cells were washed and the medium replaced with fresh medium containing 5 μ g penicillin ml⁻¹ and 100 μ g gentamicin ml⁻¹. At this time, CPT, at the concentrations indicated in the assays, was also added to the cells for the incubation times indicated. Cell lysates were then collected, resolved and probed as described for the phosphorylation studies, with the exception that the primary antibody used was to caspase-3.

RESULTS

GBS invasion requires activation of PI3K

To delineate host-cell signalling events involved in GBS invasion of HeLa cells, we sought to explore the role of the PI3K signalling pathway in GBS invasion. PI3K regulates numerous moieties related to cytoskeletal rearrangement, which is known to be a key event in GBS invasion of host cells. Wortmannin is a fungal metabolite that inhibits PI3K, mitogen-activated protein kinase and myosin light-chain kinase (Davies *et al.*, 2000). It has been shown previously that Wortmannin is a potent inhibitor of GBS

epithelial cell internalization, suggesting that PI3K could be required in the invasion process (Tyrrell *et al.*, 2002). However, as Wortmannin can inhibit other host-cell kinases, we sought to confirm the specificity of this previous finding using LY294002, a pharmacological inhibitor that is specific to PI3K (Vlahos *et al.*, 1994). LY294002 inhibits PI3K by competing with the binding of ATP to PI3K, preventing PI3K activation, a different mechanism than that of Wortmannin. Furthermore, LY294002 has been demonstrated to inhibit GBS invasion of a different class of host cell, human brain microvascular endothelial cells (Shin *et al.*, 2006).

LY294002 reduced GBS invasion of HeLa cells at a concentration of 10 μ M, and at 30 μ M invasion was inhibited by approximately 75 %. (Fig. 2a). In contrast, the internalization of *S. typhimurium*, a highly invasive bacterium that is internalized independently of PI3K (Finlay & Cossart, 1997; Tafazoli *et al.*, 2003), was not reduced in response to LY294002 treatment.

To determine if treatment of HeLa cells with LY294002 caused alterations in HeLa cell permeability that allowed antibiotics to enter the cells, thereby reducing intracellular viability of the bacteria, an assay was performed in which LY294002 (as well as the other chemical inhibitors used in subsequent portions of this investigation) was added when the cell-culture medium was replaced with antibiotic-containing medium following the 2 h bacterial infection period. No reduction in GBS or *S. typhimurium* invasion was observed when LY294002 was added during antibiotic treatment. These data are included in Supplementary Fig. S1.

PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. Transient transfection with dominant-negative forms of the PI3K subunits was used to genetically inactivate PI3K and corroborate the findings of the chemical inhibitor studies. Invasion of GBS into HeLa cells expressing dominant-negative forms of the p110 α and p85 α subunits of PI3K was reduced by approximately 30 and 55 %, respectively, compared to cells exposed to the Lipofectamine transfection reagent alone (Fig. 2c). While these figures are statistically significant, and the trend is in concurrence with the chemical inhibitor studies, the level of reduction in invasion was not as great as that observed with the chemical inhibitor treatment; this is likely due to the fact that this was a transient transfection assay and thus only a subset of the epithelial cell monolayer will have taken up and be expressing the dominant-negative form, and consequently possess inactive PI3K. HeLa cell invasion by *S. typhimurium* was not reduced by the genetic inactivation of either the p85 α or the p110 α subunit (Fig. 2c). As a further control, HeLa cells were transiently transfected with a wild-type p110 α construct, which did not reduce GBS invasion of HeLa cells (Fig. 2c). These results, together with the previously reported Wortmannin data (Tyrrell *et al.*, 2002), indicate that PI3K activation is required for the GBS invasion process.

In order to examine whether PI3K is required for the adhesion of GBS to the host cell, a quantitative attachment assay was performed. Treatment with LY294002 did not reduce GBS attachment to HeLa cells at any concentration assayed (Fig. 2b). Since it has been demonstrated elsewhere that although trypsinization does not reduce GBS viability, it cleaves surface proteins important in adherence to epithelial cells (Tamura et al., 1994), trypsinized GBS were employed as a control for detection of reduced attachment in each of the attachment assays presented in this study, thus ensuring that this assay system would detect an alteration in GBS attachment to host cells should one be present.

Fig. 2. HeLa cell invasion (but not attachment) by GBS requires PI3K. Filled bars, GBS; open bars, *S. typhimurium*. (a) The HeLa cell monolayer was treated with the PI3K inhibitor LY294002 for 1 h prior to bacterial infection; this treatment inhibits GBS invasion but not that of *S. typhimurium*. (b) Treatment of HeLa cells with LY294002 for 1 h prior to GBS infection does not reduce bacterial attachment to these cell lines; attachment of GBS to HeLa cells is impaired if the bacteria are treated with a 0.25 % trypsin solution for 1 h at 35 °C prior to infection. (c) Transfection of HeLa cells with dominant-negative (DN) PI3K catalytic (p110α) or regulatory (p85α) subunits inhibits GBS invasion but not *S. typhimurium* invasion. In contrast, transfection of the cells with wild-type (WT) p110α does not inhibit the ability of GBS to invade the cells. Relative percentage attachment/internalization was calculated as described in Methods. Bars show mean ± SEM (error bars) of one representative experiment in which each assay condition was tested in triplicate; each assay was performed at least three times. **P* < 0.05 compared to control cells, as evaluated using Dunnett's multiple comparisons test.

Entry of GBS into epithelial cells requires protein kinase activity

PI3K activation results in the production of phosphorylated lipid products in the cell membrane, which can lead to the recruitment of other host-cell proteins (Takenawa & Itoh, 2001), and activation of downstream signalling moieties. One class of effectors influenced by PI3K are protein kinases, and thus we investigated if these are also involved in GBS invasion. We began with staurosporine, a broad-spectrum protein kinase inhibitor. Previous studies have suggested that this inhibitor reduces but does not abolish GBS invasion of Hep-2 cells (Valentin-Weigand et al., 1997). We found that staurosporine completely abolished GBS invasion of HeLa cells, even at the very low concentration of 0.2 μM, and failed to inhibit *S. typhimurium* invasion, even at 1.0 μM concentration, suggesting that protein kinases are involved in the GBS internalization pathway (data not shown). Protein kinase C (PKC) forms a major proportion of the protein kinase family and is a downstream target of PI3K (Chou et al., 1998). Furthermore, PKC has been shown to interact with host-cell proteins involved in the anchoring of actin microfilaments in the host cell, and thus seemed to be a possible protein kinase candidate. We assayed bisindolylmaleimide, a PKC inhibitor, for an effect on GBS invasion of HeLa cells. Bisindolylmaleimide did not inhibit GBS invasion at the maximal concentration assayed, 1.0 μM (Fig. 3a), and *S. typhimurium* was also unaffected by the treatment. Another control organism, *Y. enterocolitica*, was also assayed to ensure that the bisindolylmaleimide was having the expected effects on the host cell at the concentrations used. The invasion of *Y. enterocolitica* was reduced by all the concentrations assayed, from 0.1 to 1.0 μM (Fig. 3a). These data suggested that PKC is not involved in GBS invasion, and that other protein kinases or protein kinase

Fig. 3. GBS invasion requires Akt but not PKC. Filled bars, GBS; open bars, *S. typhimurium*; grey bars, *Y. enterocolitica*. (a) Bisindolylmaleimide, a PKC inhibitor, was used to treat HeLa cells for 1 h prior to bacterial infection. The treatment did not inhibit GBS or *S. typhimurium* invasion but did reduce invasion by *Y. enterocolitica*, indicating that PKC is not required for GBS invasion. (b) HeLa cells were serum-starved for 18 h then treated with the Akt inhibitor ICIO for 3 h prior to infection. This treatment inhibits GBS invasion in a dose-dependent manner. (c) GBS attachment is not inhibited by ICIO treatment but is inhibited if the GBS are treated with 0.25% trypsin solution at 35 °C for 1 h prior to infection. (d) HeLa cells were serum-starved for 20 h and then treated with ICIO or the Akt inhibitors Akt V or Akt VII for 3 h prior to infection. Each of these treatments inhibited GBS invasion; however, the level of inhibition induced by the different agents was not significantly different ($P \geq 0.05$ with Bonferroni multiple comparisons analysis of variance comparing pairs of columns). Relative percentage attachment/internalization was calculated as described in Methods. Bars show mean $\pm$ SEM (error bars) of one representative experiment in which each assay condition was tested in triplicate; each assay was performed at least three times. * $P < 0.05$ compared to control cells, as evaluated using Dunnett's multiple comparisons test.

pathways must be utilized; thus, an alternative protein kinase, Akt (protein kinase B), was considered.

Akt is required for HeLa cell invasion by GBS

The Akt inhibitor ICIO is a phosphatidylinositol ether analogue that inhibits Akt by preventing PIP₃ from forming and binding to Akt (Fig. 1, step 2) (Hu *et al.*, 2000). This inhibitor is reported to be selective and specific for Akt up to a concentration of 83 μM, and was utilized to screen for the involvement of this protein kinase in GBS infection. ICIO treatment resulted in dose-dependent inhibition of GBS that became statistically significant at 20 μM, with an ~50% reduction in invasion, suggesting

that Akt is involved in GBS invasion (Fig. 3b). A quantitative attachment assay indicated that ICIO did not reduce GBS adhesion to HeLa cells at any concentration used (Fig. 3c).

As illustrated in Fig. 1, activation of Akt occurs in a series of sequential steps involving binding to PIP₃ followed by two sequential phosphorylation events. Akt inhibitors are available that function at different levels of Akt activation; we wanted to investigate whether different modes of Akt inhibition would affect GBS invasion differently. To this end, Akt inhibitor V (Akt V) and Akt inhibitor VII (Akt VII) were used in conjunction with the invasion assay. Akt V inactivates Akt by targeting a process that is yet to be clearly characterized, but is known not to involve PI3K or

phosphoinositide-dependent protein kinase 1 (PDK1) (i.e. it blocks a process other than 1 or 4 in Fig. 1) (Yang *et al.*, 2004). Akt VII is a peptide that has been engineered to permeate the host cell and inactivates Akt by binding to the plexstrin homology (PH) domain of Akt, thereby preventing phosphoinositide binding by Akt (Fig. 1, step 3) (Hiromura *et al.*, 2004). All of the Akt inhibitors significantly reduced GBS invasion, but the level of invasion inhibition did not differ significantly between these three inhibitors (Fig. 3d).

Ras is involved in GBS invasion of HeLa cells

It has been demonstrated elsewhere that Ras can activate PI3K by targeting the p85 domain, which leads to activation of the PI3K/Akt pathway (Chan *et al.*, 2002). In light of the finding that PI3K and Akt are required for GBS invasion, the pharmacological inhibitor manumycin A was used to investigate the role of Ras in GBS internalization. This compound is a Ras farnesyltransferase inhibitor which functions by blocking the post-translational isoprenylation of Ras required for proper membrane binding and targeting, which is essential for Ras activation (Sattler *et al.*, 1998).

Treatment of HeLa cells with manumycin A for 1 h prior to GBS infection had a potent, dose-dependent inhibitory effect on invasion. At a concentration of 2.5 μM , approximately 30 % inhibition of invasion occurred (Fig. 4a), and at 10 μM , invasion was reduced by 90 %. In contrast, the invasion of *S. typhimurium* was not affected by the treatment (Fig. 4a). Manumycin A did not significantly alter the ability of GBS to attach to HeLa cells (Fig. 4b). These data suggest that Ras is also involved in the signalling pathway that leads to the internalization of GBS by epithelial cells.

Phosphorylation of Akt in HeLa cells occurs in response to GBS infection

Akt is a serine/threonine kinase that is phosphorylated in its active form. Although a need for active Akt was demonstrated by the chemical inhibitor studies, it was not clear if GBS was able to modulate Akt phosphorylation directly. To assay for Akt phosphorylation during GBS invasion, HeLa cells were either infected with GBS or left uninfected, then lysates were collected and assayed via Western blotting using an antibody recognizing the activated form of Akt that is phosphorylated on Ser⁴⁷³. The blot was then stripped and reprobed for total Akt; this confirms that the effect seen was due to modulation of Akt phosphorylation status and not due to an increase in protein synthesis in the cell.

Akt phosphorylation in HeLa cells is induced by GBS infection at the early time point of 5 min and continues for at least 60 min post-infection (Fig. 5a). Akt phosphorylation was not observed in HeLa cells infected with HK-GBS (Fig. 5d).

Fig. 4. Ras is required in GBS invasion of HeLa cells. Manumycin A, an inhibitor of Ras farnesyltransferase, was used to treat HeLa cells for 1 h prior to bacterial infection (a); this treatment inhibited GBS invasion in a dose-dependent manner but did not inhibit *S. typhimurium* invasion; the treatment did not inhibit GBS attachment to HeLa cells (b). Relative percentage attachment/internalization was calculated as described in Methods. Bars show mean $\pm$ SEM (error bars) of one representative experiment in which each assay condition was examined in triplicate; each assay was performed at least three times. * $P < 0.05$ compared to control cells, as evaluated using Dunnet's multiple comparisons test.

The demonstration of PI3K and Akt involvement in GBS internalization suggested transduction through PI3K as an upstream modulator of Akt phosphorylation. To explore this, a subset of cells were treated with 50 μM LY294002 for 1 h prior to GBS infection. This treatment abolished Akt phosphorylation (Fig. 5a), indicating that GBS-induced Akt phosphorylation occurs downstream of PI3K activation.

GBS infection results in phosphorylation of GSK-3

Akt has been demonstrated to play a role in the balance between host-cell survival and cell death as a mediator of apoptosis. Akt phosphorylates many targets that modulate apoptotic function in the host cell, with one major target being GSK-3. GSK-3 exists in two isoforms, GSK-3 α

Fig. 5. GBS invasion of HeLa cells induces phosphorylation of Akt and GSK-3. (a) Immunoblot for Akt phosphorylation. Serum-starved HeLa cells were either left untreated (0 min) or treated with GBS for 5, 15, 30 or 60 min. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated Akt was detected using an anti-phospho-Akt (Ser⁴⁷³) antibody followed by detection of total cellular Akt with a polyclonal antibody. A subset of cells were also treated with 50 μ M LY294002 for 1 h prior to GBS infection. GBS induces Akt phosphorylation quickly after infection, but this is not observed if PI3K is inhibited. HK-GBS do not induce Akt phosphorylation (d). (b, c) Immunoblots for GSK-3 phosphorylation. Serum-starved HeLa cells were either left untreated (0 min) or infected with GBS for 5, 15, 30 or 60 min. Subsets of HeLa cells were treated with 50 μ M LY294002 for 1 h or 20 μ M ICIO (Akt inhibitor) for 3 h prior to GBS infection. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies recognizing GSK-3 α phosphorylated on Ser²¹ or GSK-3 β phosphorylated on Ser⁹. Blots were then reprobed with an antibody that detects total cellular GSK-3 β . GBS induces phosphorylation of both GSK-3 α and GSK-3 β in HeLa cells; this phosphorylation is abrogated if the cells are treated with LY294002 or ICIO prior to infection. HK-GBS do not induce phosphorylation of either GSK-3 subunit (d).

(51 kDa) and GSK-3 β (47 kDa), and is primarily regulated through inhibition. Phosphorylation of GSK-3 is inhibitory and is a pro-survival signal in the host cell. To explore modulation of host-cell GSK-3 phosphorylation by GBS, HeLa cells were either left uninfected, or infected with GBS for 5, 15, 30 or 60 min, then cell lysates were collected and analysed by Western blotting with antibodies recognizing the phosphorylated form of GSK-3 α (Ser²¹), or GSK-3 β (Ser⁹). The blots were then stripped and reprobed with an antibody recognizing total GSK-3 β as a control for protein loading.

GBS infection results in an increase in GSK-3 α phosphorylation at 5 min post-infection, and this continues for at least 60 min post-infection (Fig. 5b). GBS infection also stimulates GSK-3 β phosphorylation, but the effect is more subtle and does not occur until 15 min post-infection (Fig. 5b). HK-GBS do not induce GSK-3 phosphorylation (Fig. 5d). GBS-induced phosphorylation of GSK-3 α and β is abolished by treatment with LY294002 for 1 h prior to GBS infection (Fig. 5b); however, PI3K does have downstream effectors other than Akt, so a subset of cells were also treated with ICIO for 3 h prior to GBS infection.

Inhibition of Akt prevents GBS-induced GSK-3 phosphorylation until 30 min post-infection, although at 60 min, some phosphorylation was observed (Fig. 5c). However, at this point, some phosphorylation of Akt was also observed (Fig. 5c), suggesting that after that amount of time, GBS infection of the cells overwhelms the ability of the inhibitor to completely eliminate phosphorylated Akt in the HeLa cell. These data indicate that GSK-3 phosphorylation induced by GBS occurs downstream of PI3K and Akt.

Infection with GBS protects HeLa cells from CPT-induced caspase-3 cleavage

CPT is a topoisomerase inhibitor that induces apoptosis transduced by caspase-3 cleavage. As Akt phosphorylation and GSK-3 phosphorylation are well-recognized pro-survival (anti-apoptotic) signals, we considered the possibility that GBS infection protects HeLa cells from the activity of CPT. HeLa cells were either infected with GBS for 30 min or left uninfected. The medium was then replaced with fresh medium containing antibiotics and 0, 10 or 50 μ M CPT for 4 h, at which time HeLa cell lysates were collected and analysed by Western blotting. Caspase-3 was detected using an antibody that recognizes both the uncleaved, procaspase form ($\sim$ 35 kDa) and the 20 kDa processed (activated) form. In the uninfected cells, caspase-3 cleavage was observed with both 10 and 50 μ M CPT treatments. In contrast, caspase-3 cleavage was not observed in the infected cells at either CPT concentration (data not shown). The assay was then extended to examine the ability of GBS to protect HeLa cells from caspase-3 cleavage at later time points post-infection, and to observe whether the balance would shift to being pro-apoptotic at later time points. At 18 h post-infection, low levels of caspase-3 cleavage were observed in CPT-treated, GBS-infected cells, but the amount was much lower than that observed in uninfected, CPT-treated cells (Fig. 6). The assay could not be performed at later time points, because at approximately 20 h post-infection, some caspase-3 cleavage

began to appear in the untreated control cells, likely owing to the advanced age and prolonged period of serum starvation of the cells by this point in time (not shown). These data suggest that GBS promotes host-cell survival at early points post-infection, and can prevent activation of caspase-3, the executioner caspase of apoptosis.

DISCUSSION

The PI3K/Akt signalling pathway has been implicated in an array of cellular functions, including cytoskeletal regulation, vesicle trafficking, and the balance between cellular survival and regulated cell death (Stokoe, 2005). PI3K activation has been specifically implicated in phagocytosis, pseudopod formation and membrane ruffling; the formation of these cellular structures is dependent upon modification and manipulation of the actin cytoskeleton, a key event in GBS invasion of host cells (Cox *et al.*, 1999; Pizarro-Cerda & Cossart, 2004; Stokoe, 2005; Tyrrell *et al.*, 2002). In this study, we used HeLa cells as a model of the host epithelial cell barrier. LY294002 treatment of HeLa cells inhibited GBS internalization, confirming previous findings that suggest that PI3K is involved in the GBS epithelial cell invasion process.

PI3K activation triggers activation of a number of downstream signalling molecules, including protein kinases. We found that while protein kinases play a role in GBS invasion of HeLa cells, PKC appears not to be involved. Purushothaman *et al.* (2003) found this to be the case for invasion of GAS into HEP-2 cells as well. However, for *Streptococcus suis*, it has been found that PKC inhibitors reduce phagocytosis into J774 macrophage cells (Segura *et al.*, 2004). *S. suis* infection of these cells resulted in rapid phosphorylation of PKC post-infection, and this occurred downstream of PI3K activation (Segura *et al.*, 2004). Thus, while we have demonstrated that inhibition of PKC does not affect GBS internalization into HeLa cells, it is possible that the scenario is different in other cell types.

Fig. 6. GBS infection protects HeLa cells from CPT-induced caspase-3 cleavage. HeLa cells were either uninfected or infected with exponential-phase cultures of GBS for 30 min. Cell lysates were either collected at this point (time 0) or the cell medium was replaced with fresh, antibiotic-containing medium and 10 μ M CPT was added to a subset of the cells. The cells were further incubated for 3 or 18 h, and then the cell lysates were collected, separated by SDS-PAGE and transferred to nitrocellulose. Caspase-3 was detected using an antibody that detects both the uncleaved, procaspase form of caspase-3 (35 kDa) and the 20 kDa processed (activated) form.

Ras is a GTP-binding switch protein that cycles between the active, GTP-bound state and the inactive, GDP-bound state (Reuther & Der, 2000; Taylor & Shalloway, 1996). Ras signalling is initiated by ligand binding to different receptors at the host-cell surface, and functions downstream from receptor kinases (Reuther & Der, 2000). Ras itself also activates downstream effector molecules; PI3K can be recruited to the host-cell membrane for activation via activated Ras (Reuther & Der, 2000). In light of the identification of a requirement for PI3K in GBS invasion, it was not surprising that manumycin A, a Ras inhibitor, also inhibited GBS internalization.

One category of PI3K effector is the Rho family GTPases; these GTPases are also implicated in many aspects of actin regulation. For example, Rac, Rho and Cdc42 link extracellular signals to the formation of stress fibres, lamellipodia or filopodia (Hall, 1998; Tapon & Hall, 1997). Rho-family GTPases are important in GBS invasion of HeLa cells (Burnham *et al.*, 2007) as well as that of human brain microvascular endothelial cells (HBMEC; Shin & Kim, 2006), and thus it is possible that the interplay between PI3K and Rho GTPases mediates GBS invasion as well. However, the PI3K–Rho GTPase relationship can be complex; these molecules can act both upstream and downstream of one another, and thus further investigation would be required to confirm an interaction between these signalling pathways in GBS invasion.

Akt inhibitor assays indicated that the PI3K effector Akt is required for efficient GBS invasion of HeLa cells. The suggestion that Akt is involved in GBS invasion is strengthened by the observation that Akt is phosphorylated in response to GBS infection; Akt phosphorylation occurs rapidly after GBS is added to HeLa cells and persists for at least 60 min post-infection. The ability of Akt to regulate so many facets of the host cell makes it an attractive target for manipulation by a bacterial pathogen to augment the invasion process.

In addition to cytoskeletal regulation, Akt plays a role in the balance between host-cell survival and cell death as a mediator of apoptosis. Akt phosphorylates many targets that modulate apoptotic function (Chan *et al.*, 1999; Datta *et al.*, 1999). Thus, we hypothesized that Akt activation by GBS is a mechanism that contributes to bacterial persistence inside the host cell, by inhibiting apoptotic events. This has recently been demonstrated to occur in the infection of epithelial cells by *S. typhimurium* (Knodler *et al.*, 2005), in which the SopB protein of *S. typhimurium* activates Akt and prevents caspase-3-mediated apoptosis (Knodler *et al.*, 2005). Thus, it was a natural progression to assay for modulation of GSK-3 phosphorylation in response to GBS infection. GSK-3 is a multifunctional serine/threonine kinase that acts downstream of Akt in the PI3K/Akt signalling pathway (Embi *et al.*, 1980). GSK-3 was the first target of Akt to be identified (Cross *et al.*, 1995), and while GSK-3 was first acknowledged for its role in glycogen metabolism, it has been found subsequently to

behave as a somewhat promiscuous molecular switch that is activated by diverse stimuli and regulates numerous cellular processes and pathways, including regulation of several transcription factors.

There are two GSK-3 isoforms, GSK-3 α (51 kDa) and GSK-3 β (47 kDa), which are ubiquitously expressed and highly conserved in mammalian cells, although they are encoded by distinct genes. GSK-3 is somewhat unusual in that it is regulated mainly by inhibition, rather than by activation. It has been demonstrated that PI3K activation of Akt results in phosphorylation of both GSK-3 α and GSK-3 β (Cross *et al.*, 1995); this phosphorylation inhibits GSK-3 activity, leading to dephosphorylation of GSK-3 substrates.

A role for GSK-3 in host-cell survival has been identified by Pap & Cooper (1998), who found that overexpression of active GSK-3 induces apoptosis, and that inhibition of GSK-3 (mediated by a dominant-negative mutation) prevents apoptosis downstream of PI3K inhibition. In our investigation, we observed that GBS infection of HeLa cells results in phosphorylation (inactivation) of both the α and the β isoform of GSK-3. This phosphorylation occurs quickly after infection with GBS, and persists for at least 60 min after infection. The induction of pro-survival signals in the host epithelial cell led us to wonder if infection by GBS would be sufficient to protect HeLa cells from apoptosis induced by the pharmacological agent CPT. We found that GBS infection protects HeLa cells from CPT-induced apoptosis, and that this effect lasts for at least 18 h post-infection.

Modulation of the balance between host-cell survival and host-cell death is a popular strategy for survival utilized by bacterial pathogens. One example is induction of apoptosis in phagocytic cells, such as macrophages, to avoid bacterial killing. Another approach is to avoid or delay apoptosis in an attempt to seek refuge from the immune system of the host, or gain access to privileged sites. The fact that a range of intracellular organisms are able to modulate host-cell apoptosis indicates that this function has been highly conserved through evolution, suggesting that it is deliberate and likely to be important in the pathogenic process.

Early investigations of GBS invasion found that GBS were able to persist inside human umbilical vein endothelial cells (HUVEC) and A549 cells for 8 h post-infection, and although they did not replicate, the number of intracellular GBS did not decline (Gibson *et al.*, 1993; Rubens *et al.*, 1992). Similar results have been recorded in later studies that investigated the ability of GAS and GBS to invade cultured cells. Greco *et al.* (1995) observed that GBS remain viable in HeLa, HEp-2 and HUVEC cells for 24 h after infection, and again observed that while the GBS do not replicate, they also do not decrease in number. In contrast, there was a gradual decrease in intracellular GAS over the 24 h period, with no viable intracellular GAS found at 24 h post-infection in any of the three cell lines assayed (Greco *et al.*, 1995). The fact that GBS are able to

Fig. 7. Schematic diagram illustrating the proposed host-cell signalling cascade facilitating GBS invasion of epithelial cells. GBS invasion is a complex process that involves multiple host-cell signalling pathways and events. Solid arrows, signalling events characterized in this investigation; dashed arrows, possible signalling events and/or interactions.

induce pro-survival signals inside the HeLa cell suggests a possible mechanism for GBS persistence in epithelial cells for relatively long periods of time.

Ulett *et al.* (2005) demonstrated that GBS activates caspase-3 and -9 in macrophages at 48 h post-infection, leading to apoptosis. It is possible that while GBS protects HeLa cells from apoptosis at early time points (up to 24 h) post-infection, after longer periods have passed, apoptosis becomes activated. This is the subject of ongoing investigation.

Although in some situations GBS causes asymptomatic colonization of the host, the ability of GBS to invade epithelial cells is central to the ability of the organism to cause invasive disease, a process contributing to the clinical features of GBS infection. In the present study, we sought to characterize the GBS epithelial-cell invasion process. HeLa cells were used as the infection model to explore signal-transduction processes between GBS and epithelial cells involved in invasion. The interaction between GBS and host epithelial cells is obviously complex (Fig. 7), and several facets are still unclear, including the events that occur at the cell surface to trigger PI3K activation and the subsequent signalling cascade. The results from this

investigation illustrate that GBS both requires and activates the PI3K/Akt host-cell-signalling pathway during invasion.

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