Neisseria gonorrhoeae PLD directly interacts with Akt kinase upon infection of primary, human, cervical epithelial cells

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Summary

Neisseria gonorrhoeae secretes a phospholipase D (NgPLD), which augments complement receptor 3 (CR3)-mediated invasion of cervical epithelial cells. To elucidate the signalling pathways triggered with gonococcus CR3-engagement and the putative function of NgPLD in these events, we analysed the contribution of the phosphoinositide-Akt pathway to cervical infection. Our data indicated that Akt plays a critical role in cervical infection. Inhibition of myosin light chain kinase, PtdIns(4,5)P₂, and Akt functions resulted in decreased gonococcus invasion of primary, human, cervical epithelial cells as well as Akt kinase activity. Akt activity was similarly impaired when cervical cells were challenged with NgPLD-mutant gonococci. Conversely, the PI3-kinase inhibitor, LY294002, enhanced gonococcal invasion of, and Akt activity within, primary cervical cells. We demonstrated that NgPLD directly binds to the Akt PH domain and can compete with a natural Akt ligand, PtdIns(3,4,5)P₃, for Akt binding. Collectively, our data suggested that NgPLD augments gonococcus invasion of cervical epithelia by interacting with Akt kinase in a PI3-kinase-independent manner, which results in subversion of normal cervical cell signalling.

Introduction

Neisseria gonorrhoeae is an exclusive human pathogen, which causes the disease gonorrhea. The gonococcus is highly human-adapted and has developed variable mechanisms of pathogenesis that are, in part, dependent upon the site of infection. In a cooperative manner, both gonococcal and cervical constituents contribute to the processes allowing progressive cervical infection (Edwards et al., 2002). Our previous studies indicate that, upon infection of primary, human, cervical epithelial (pex) cells, gonococci release a subset of proteins that aid in complement-receptor type 3 (CR3)-mediated membrane ruffling and invasion of the cervical epithelial cell (Edwards et al., 2002; 2003). Release of these bacterial products occurs in a contact-dependent manner (Edwards et al., 2003). Interestingly, however, gonococci do not possess a type three secretion system. A type four secretion system, which is encoded on a genetic island that is present in some strains of N. gonorrhoeae, was recently described (Dillard and Seifert, 2001); however, current data suggest that it also does not appear to play a role in protein secretion triggered with cervical infection (J.L. Edwards and M.A. Apicella, unpubl. data). The secretion mechanism used by gonococci to release these proteins upon infection of pex cells remains to be elucidated. Sustained secretion is dependent on complement deposition upon the gonococcal surface followed by the cooperative binding to the I-domain region of CR3; however, I-domain binding is sufficient to allow transient, low-level secretion (Edwards et al., 2003). We identified and characterized one of these secreted products as a phospholipase D (NgPLD) homologue (Edwards et al., 2003). Upon comparison with wild-type gonococci, a PLD-mutant strain exhibited: (i) the decreased ability to adhere to and invade pex cells, (ii) the impaired ability to recruit CR3 to the host cell surface with cervical challenge and (iii) while mutant bacteria are capable of some cytoskeletal rearrangement of the host cervical cell, they are incapable of eliciting full membrane ruffles (Edwards et al., 2003). Collectively, these data suggest that NgPLD might modulate host cell signalling events required for successful, progressive, cervical infection.

Akt (also known as protein kinase B) is a serine/threonine kinase that exhibits wide tissue distribution, although isoform-specific functions may occur in some cell types (for review see Chan et al., 1999; Song et al., 2005). Akt activation is a sequential process initiated by PI3-K-independent phosphorylation on Thr450, which does not contribute to its kinase activity but may serve as a signal.
indicating proper protein folding (Chan et al., 1999). Akt is then translocated to the plasma membrane by a process that is dependent on its pleckstrin homology (PH) domain and that may be modulated by chaperone or chaperone-like proteins [e.g. calponin homology domain integrin-linked kinase binding protein, CH-ILKBP (Fukuda et al., 2003)] or molecular complexes. Binding of a phosphoinositide to the Akt PH domain is thought to induce a conformational change that subsequently unMASKs and allows Thr308 (for Akt1, Thr309 and Thr305 for Akt2 and 3 respectively) phosphorylation by phosphoryositide-dependent protein kinase (PDK) 1. A second phosphorylation event occurs on Ser473 (Ser474 and Ser472 for Akt2 and 3 respectively) by an unidentified kinase (or kinase complex) that has arbitrarily been designated as PDK2. Serine phosphorylation is mediated by integrin-linked kinase (ILK) (Galetic et al., 1999; Laine et al., 2000). The PH domain of PDK1 also binds phosphoinositides; however, this interaction is believed to bring Akt and PDK1 in proximity to allow subsequent Akt phosphorylation because phosphoinositide binding does not affect PDK1 activity (Komander et al., 2004). Each step of the Akt activation pathway additively or synergistically contributes to full activity leading to diverse cellular functions. The ability of Akt to inhibit apoptosis as well as regulate the cell cycle, gene transcription, glucose and nutrient uptake and metabolism, endocytosis and vesicular trafficking, makes this kinase an attractive target by which pathogens could subvert normal host cell function. Indeed, several bacterial and viral pathogens have developed mechanisms to trigger Akt signalling pathways (Wilkowski et al., 2001; Yilmaz et al., 2004; Huang et al., 2005; Kierbel et al., 2005; Knodler et al., 2005; Lee et al., 2005).

Bacterial and human PLDs can bind to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) (Kusner et al., 2003). PtdIns(4,5)P$_2$ plays a critical role in regulating the actin cytoskeleton and membrane trafficking. Upon receptor stimulation local cellular levels of PtdIns(4,5)P$_2$ increase with PtdIns(4,5)P$_2$ being enriched in large, plasma membrane structures, e.g. lamellipodia and ruffles, and bacterial contact sites. Recently, it is demonstrated that the localized increase in PtdIns(4,5)P$_2$ may result from increases in total membrane constituents derived from membrane folds, although local PtdIns(4,5)P$_2$ biosynthesis also occurs (Huang et al., 2004). These studies show that PtdIns(4,5)P$_2$ is enriched in large (greater than several micrometres in size), plasma membrane structures that can span the depth of a cell and that are termed PtdIns(4,5)P$_2$-rich plasma membrane patches (PRPMPs) (Huang et al., 2004). PRPMPs are much larger than, and are distinct from, membrane rafts (Huang et al., 2004). PRPMPs define, and are exclusively colocalized with, regions of concentrated myosin, actin polymerization, ruffling and endocytosis (Huang et al., 2004). Therefore, the proposed function of PRPMPs is to drive membrane ruffling, as little-to-no ruffling occurs at areas void of PRPMPs (Huang et al., 2004). Engagement of CR3 on pex cells by wild-type gonococci triggers membrane ruffling, and signalling events mediating these processes appear to be modulated by NgPLD because ruffling is not observed on pex cells challenged with a PLD-mutant strain. Of interest is that both Akt (Skaletz-Rorowski et al., 2003) and PDK1 (Currie et al., 1999) localize within membrane ruffles. One study demonstrates that stimulation of endothelial cells with simvastatin results in Akt localization at discrete sites within the plasma membrane, whereas Akt is uniformly distributed in cells stimulated with vascular endothelial growth factor (Skaletz-Rorowski et al., 2003). These authors suggest that Akt resides within lipid-raft-like structures that coalesce into ‘super-rafts’ that comprise a large proportion of the plasma membrane (Skaletz-Rorowski et al., 2003), an event that appears to be stimulus-dependent.

Currently, there are no data defining putative effector molecules mediating membrane ruffling triggered by CR3 engagement in epithelial cells. Additionally, few studies have examined signal transduction in primary epithelial cell cultures. Our data indicate that NgPLD activity is associated with cytosolic- and membrane-enriched cervical cell fractions, and it enters these cells non-specifically with macropinocytosis of invasive gonococci (Edwards et al., 2003). We have now expanded these studies to identify host constituents that play a role in NgPLD-mediated signal transduction events leading to productive gonococcal infection of primary, human, cervical epithelial cells. Collectively, our data strongly suggest that N. gonorrhoeae subvert cervical cell signalling pathways by competing with PtdIns(3,4,5)P$_3$ (and possibly other phosphoinositides) for Akt binding. Akt activation augments CR3 recruitment to the cervical cell surface in a PtdIns(4,5)P$_2$ and myosin light chain kinase (MLCK)-dependent manner and plays a further role in gonococcus invasion of, and survival within, the host cervical cell.

**Results**

The phosphoinositide-Akt pathway plays an important role in gonococcal infection of primary, human, cervical epithelial cells

To further delineate signalling events triggered with gonococcus-induced CR3 engagement, we wanted to determine the involvement of the phosphoinositide-Akt pathway in cervical infection because cellular effects mediated by this pathway include cytoskeletal rearrangements, e.g. membrane ruffling, which is an important event in gonococcal cervical infection. To begin our analysis we performed association (i.e. adherence and invasion) and
invasion (i.e. gentamicin survival) assays in which pex cells were challenged with wild-type or PLD-mutant gonococci in the presence and absence of neomycin, which binds PtdIns(4,5)P₂ and inhibits its activity; LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3-K); and an Akt inhibitor (Table 1). Neomycin addition to invasion assays impaired the ability of wild-type bacteria to adhere to and to invade pex cells, but association and invasion levels were not further decreased for the 12913PLD mutant (Table 2A), suggesting a role for PtdIns(4,5)P₂ in cervical adherence and invasion by wild-type goncoccci. Conversely, inhibition of PI3-K activity using LY294002 had no effect on the ability of wild-type bacteria to associate with pex cells but did impair the ability of PLD-mutant gonococci to associate with these same cells. Furthermore, LY294002 enhanced the invasive ability of wild-type gonococci but further reduced invasion levels for mutant gonococci (Table 2A). Wild-type and mutant bacteria were both significantly and severely impaired in their ability to invade cervical epithelia in the presence of Akt inhibitor (Table 2A), suggesting a critical role for this protein in gonococcal invasion of cervical epithelial cells. However, Akt inhibition resulted in the decreased association of only wild-type, not mutant, bacteria with pex cells.

The observed decrease in the ability of wild-type bacteria to associate with pex cells in the presence of neomycin and Akt inhibitor exhibited a NgPLD-dependence because association levels were not further decreased in the absence of NgPLD. We have shown previously that the decreased ability of PLD-mutant gonococci to associate with pex cells can be correlated with the inability of PLD-mutant bacteria to recruit CR3 to the cervical surface. Consequently, to confirm these data and to determine if PtdIns(4,5)P₂ and Akt play a role in CR3 trafficking, we performed an enzyme-linked immunosorbant assay (ELISA) in the presence and absence of neomycin, LY294002 and Akt inhibitor (see Table S1 in Supplementary material). Consistent with the data outlined above, the addition of neomycin and Akt inhibitor to wild-type-infected pex cells resulted in decreased surface levels of CR3 to a level comparable to that measured for the PLD mutant-infected and uninfected cells when compared with the absence of either inhibitor. In contrast, the presence or absence of either inhibitor in studies performed with uninfected or PLD mutant-infected cells yielded CR3 surface levels that were not significantly different. Similarly, LY294002 had no effect on CR3 surface expression under any of the conditions assayed, suggesting that the decreased ability of mutant bacteria to associate with pex cells in the presence of this pharmacological agent was a function of their ability to invade or survive within pex cells, and not to receptor availability or their inability to adhere to these cells.

Akt activation is commonly thought to be dependent on the role of PI3-K in PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ formation; however, PI3-K-independent mechanisms are also reported (Konishi et al., 1997; Moule et al., 1997; Sable et al., 1997; Filipa et al., 1999; Cenni et al., 2003; Perez-Garcia et al., 2004). Our above data indicated that increased wild-type, but decreased PLD-mutant, gonococcal invasion occurs in the absence of PI3-K activity. Conversely, in a previous study, we demonstrate that inhibition of PI3-K activity using high (300 nM) concentrations of wortmannin significantly impairs the ability of gonococci to invade pex cells (Edwards et al., 2000). To resolve this apparent paradox, we repeated gonococcal association and invasion assays in the presence and absence of a panel of PI3-K inhibitors and LY303511, a LY294002 structural analogue that does not prohibit PI-3K activity and, thus, serves as negative control for LY294002 (Table 1). Similar data to those described were obtained from these studies (Table 2B). LY294002 impaired the

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**Table 1.** Pharmacological agents used in the described studies.

| Agent          | Target and/or site of action                          | Reference               |
|----------------|------------------------------------------------------|-------------------------|
| Akt inhibitor  | Akt kinase                                           | Hu et al. (2000)        |
| Akt inhibitor IV | Akt activation pathway downstream of PI₃ synthesis | Kau et al. (2003)       |
| Akt inhibitor V | Akt activation pathway, does not involve PI3-K or PDK1 | Yang et al. (2004)      |
| Akt inhibitor VII | Akt activation pathway, binds Akt PH domain         | Hiromura et al. (2004)  |
| LY294002       | PI-3 kinase                                          | Vitahos et al. (1994)   |
|                | Casine kinase-2                                      | Davies et al. (2000)    |
| LY303511       | Casine kinase-2                                      | Knight et al. (2004)    |
| ML-7           | Myosin light chain kinase                            | Davies et al. (2000)    |
| Neomycin       | PtdIns(4,5)P₂                                        | Kristof et al. (2005)   |
| Quercetin      | PI-3 kinase                                          | Saitoh et al. (1987)    |
|                | Myosin light chain kinase                            | Gabev et al. (1989)     |
|                | PI-4 kinase                                          | Matter et al. (1992)    |
| TBB            | Casine kinase-2                                      | Hagiwara et al. (1988)  |
| Wortmannin     | PI-3 kinase                                          | Nishioka et al. (1989)  |
|                | Myosin light chain kinase                            | Szyszka et al. (1995)   |
|                | Mitogen-activated protein kinase                     | Arca et al. (1993)      |
|                |                                                      | Nakanishi et al. (1992) |
|                |                                                      | Ferby et al. (1996)     |

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The cervical epithelial cell lysates. Data given were obtained from three trials performed in triplicate.

Association and invasion assays were performed as outlined in the text. Values given are the mean (variance) in which the percent association with the absence of, the indicated pharmacological agent as outlined in the text. NA, not applicable.

Table 2. Per cent association and invasion of pex cells by N. gonorrhoeae strains 1291 and 1291ΔPLD in the presence and absence of select signalling inhibitors.

|                | Mean association (%) | Mean Invasion (%) |
|----------------|-----------------------|-------------------|
|                | (± σ) P-value         | (± σ) P-value     |
|                | 1291 Wild-type | 1291ΔPLD | 1291 Wild-type | 1291ΔPLD |
| A              |                       |                   |                   |           |
| No inhibitor   | 29.35 (2.03)   | 15.30 (0.92)   | 2.71 (0.22)     | 0.29 (0.03) |
| Neomycin       | 12.62 (0.40)   | 15.13 (1.22)   | 0.56 (0.04)     | 0.31 (0.03) |
| LY294002       | 27.85 (1.41)   | 7.35 (0.36)    | 4.78 (0.03)     | 0.03 (0.01) |
| Akt inhibitor  | 13.17 (0.78)   | 16.29 (1.35)   | 0.03 (0.01)     | 0.03 (0.01) |
|                | < 0.05        | > 0.75        | < 0.05          | < 0.05     |
| B              |                       |                   |                   |           |
| No inhibitor   | 28.27 (1.10)   | 15.34 (0.83)   | 2.94 (0.13)     | 0.32 (0.02) |
| LY294002       | 27.44 (1.55)   | 7.77 (0.17)    | 4.65 (0.24)     | 0.03 (0.01) |
| LY303511       | 27.68 (0.55)   | 16.27 (1.29)   | 2.97 (0.12)     | 0.29 (0.02) |
| Wortmannin     | 15.22 (0.52)   | 14.62 (0.44)   | 0.21 (0.04)     | 0.04 (0.01) |
| Quercetin      | 17.64 (1.49)   | 14.23 (0.62)   | 0.29 (0.02)     | 0.16 (0.02) |
|                | < 0.05        | > 0.50        | < 0.05          | < 0.05     |
| C              |                       |                   |                   |           |
| No inhibitor   | 26.69 (1.14)   | 15.58 (0.22)   | 2.78 (0.24)     | 0.30 (0.01) |
| TBB            | 28.48 (1.95)   | 15.06 (0.28)   | 2.70 (0.09)     | 0.31 (0.03) |
| ML-7           | 13.11 (1.56)   | 15.62 (0.70)   | 0.44 (0.02)     | 0.10 (0.01) |
|                | < 0.05        | > 0.50        | < 0.05          | < 0.05     |

Association and invasion assays were performed as outlined in the text. Values given are the mean (variance) in which the per cent association and invasion were determined as a function of the original inoculum and the number of colony-forming units formed with subsequent plating of the cervical epithelial cell lysates. Data given were obtained from three trials performed in triplicate. P-values were determined using a Kruskal–Wallis k-sample analysis of variance calculated for the association and invasion of pex cells by gonococci in the presence of, upon comparison with the absence of, the indicated pharmacological agent as outlined in the text. NA, not applicable.

The ability of PLD-mutant, but not wild-type, bacteria to associate with pex cells, whereas LY303511 had no effect on the ability of either of these bacteria to associate with or to invade pex cells (Table 2B). As observed in the previous assay, LY294002 impaired the ability of mutant gonococci, but enhanced the ability of wild-type gonococci, to invade pex cells (Table 2B). Wortmannin and quercetin impaired the ability of wild-type bacteria to associate with pex cells, but they had no effect on the association of mutant gonococci with pex cells. However, invasion levels were decreased for both wild-type and mutant gonococci in the presence of quercetin or (reduced concentrations, 10 nM) of wortmannin. In concentrations sufficient to inhibit PI3-K activity LY294002, LY303511, wortmannin and quercetin can also impair the activity of other kinases (Table 1). To determine if the respective invasive ability of wild-type or of mutant gonococci could be attributed to the absence of PI-3K activity or alternatively to the activity of casein kinase-2 (CK-2) and/or MLCK, we extended our association and invasion assays to examine the potential effect of 4,5,6,7-tetrambromo-2-azabenzimidazole (TBB) and ML-7 addition (Table 2C). These data showed that CK-2 inhibition by TBB had no effect on association or on the invasive ability of wild-type or mutant bacteria; however, inhibition of MLCK with ML-7 significantly impaired the invasive ability of both wild-type and mutant bacteria (Table 2C). Conversely, only wild-type bacteria were impaired in their ability to associate with pex cells in the presence of ML-7.

Decreased wild-type and mutant gonococcal invasion in the presence of wortmannin, quercetin and ML-7 suggests that signalling pathways augmenting cervical invasion by these bacteria are dependent on MLCK activity. The finding that LY303511 and TBB had no significant effect on wild-type or mutant invasion, while LY294002 exhibited a converse effect on wild-type and mutant invasion levels, indicates that gonococcus-activated signalling pathway(s) occur independently of CK-2, and that the data obtained with the use of LY294002 could potentially be attributed to the loss of (or reduced) PI3-K activity. Collectively, these data further suggest that while Akt kinase, a downstream effector of PtdIns(3,4,5)P3, is crucial to gonococcal invasion, activation of PI3-K may not be the preferred pathway for wild-type bacteria, which exhibit
N. gonorrhoeae directly binds cervical Akt kinase

Increased Akt phosphorylation occurs with gonococcal infection in the absence of PI3-K activity

Akt activation occurs as a sequential process involving phosphoinositide binding followed by two sequential phosphorylation events. We captured Akt in wild-type gonococci-infected pex cells that were untreated or treated with LY303511, LY294002. Akt inhibitor, Akt inhibitor IV, wortmannin, quercetin, ML-7, or TBB to determine if Akt became phosphorylated in our cell culture model of cervical gonococcal disease. To allow a semi-quantitative comparison of Akt phosphorylation, equal loading of the immunoprecipitates was confirmed by stripping Western blots and staining the membranes to visualize total Akt loaded into each well and on each gel, as outlined above (data not shown). As evidenced by the increased intensity of an approximate 55–60 kDa band(s) on Western blots probed with phospho-Akt-specific antibodies, LY294002 resulted in increased Akt phosphorylation upon comparison with LY303511 addition or the absence of either agent. However, Akt phosphorylation was readily evident under all of these conditions examined (Fig. 1A). In some Western blots the presence of this single, intense band could be deciphered to actually be composed of two bands, suggesting that multiple Akt isoforms (Akt1, 60 kDa and Akt2 and/or 3, 55 kDa) are present and phosphorylated in gonococci-infected pex cells (Fig. 1). Akt phosphorylation occurred early (30 min post infection) during the infection and remained constant over the time-course assayed. However, the presence of additional bands in LY294002-treated cell immunoprecipitates increased with the length of time that the infection was allowed to progress (Fig. 1A). A band consistent with the molecular mass of Akt dimer formation was evident at 30 and 90 min post infection, the presence of this band decreased slightly by 3 h post infection. A band of a molecular mass consistent with Akt trimer formation became evident by 3 h post infection. These data might suggest that Akt dimeric complexes formed at earlier (30 and 90 min) times during infection were present as Akt trimeric complexes at 3 h post infection; however, the presence of Akt heterooligomeric complexes cannot be ruled out. An additional band of approximately 75–80 kDa was also evident at 30 and 90 min post infection, and this band increased in its intensity by 3 h post infection (Fig. 1A). This band was only faintly visible in the presence of PI3-K activity (Fig. 1A, C and D) and was not visible when Akt phosphorylation (i.e. Akt activity) was decreased (Fig. 1B–D). This peptide may mediate the observed covalent interaction of Akt with itself and/or with other molecules. In simultaneous, parallel studies Akt phosphorylation was severely decreased in the presence of two Akt inhibitors (Fig. 1B), which served as negative controls for the assay. Similarly, Akt phosphorylation was decreased in the presence of wortmannin, quercetin (Fig. 1C) and ML-7 (Fig. 1D) upon comparison with the absence of either of these agents, while comparable lev-
els of Akt phosphorylation were observed between the presence of and the absence of TBB (Fig. 1D). Akt phosphorylation was not observed in immunoprecipitates obtained from uninfected (control) pex cells untreated or treated with any of the chemical agents examined (Fig. 1A–D). Collectively, these data provide further support for the argument that MLCK plays a role in gonococcal pex infection and potentially in Akt activation in pex cells and that PI3-K activation may not be the preferred pathway for Akt activation during wild-type gonococcal infection.

Akt kinase is activated during gonococcal infection of pex cells

Our data strongly suggest, but do not directly demonstrate that Akt activity is required for successful gonococcal infection of pex cells. To confirm the data described above and to directly assess Akt activity in cervical infection we performed semi-quantitative Akt kinase assays in uninfected and strain 1291 gonococci-infected pex cells in the presence and absence of Akt inhibitor, Akt inhibitor VII, neomycin, ML-7, wortmannin, quercetin, LY294002, TBB and LY303511. Comparable Akt activity was demonstrated in gonococci-infected pex cells treated with LY303511 and TBB compared with that observed in untreated, infected cells, as indicated by the presence of an approximate 30 kDa band on Western blots probed with an anti-phospho-GSK-3α/β (Ser21/9) antibody (provided in the assay kit) (Fig. 2A). Akt activity was increased in the presence of LY294002 despite comparable amounts of Akt in each of the respective conditions assayed (Fig. 2A and B). Conversely, Akt activity was severely decreased in infection studies performed in the presence of both Akt inhibitors, neomycin, ML-7, wortmannin and quercetin (Fig. 2A). Akt activity was not observed in uninfected pex cells or in infected cells when the GSK-3 peptide was omitted from the kinase assay or when the anti-Akt antibody was omitted from the initial immunoprecipitation step, demonstrating the specificity of the assay. These results are consistent with the above-described data, and they provide further support for a role for Akt inhibition and to directly assess Akt activity in cervical infection we performed semi-quantitative Akt kinase assays in uninfected control pex cells untreated or infected with wild-type bacteria, as indicated by the presence of functional NgPLD in wild-type bacteria could function to initiate Akt activation by directly interacting with Akt, thereby bypassing the conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3. To examine this idea, we first performed a Far-Western blot analysis of separated 1291 and 1291ΔPLD gonococcal cell lysates (Fig. 3A). Membranes were incubated with Akt1 and then probed to determine if this kinase bound to gonococcal constituents. Far-Western blots probed with anti-Akt antibody demonstrated that Akt bound to a gonococcal peptide possessing a molecular mass similar to NgPLD (55 kDa) (Fig. 3A). This peptide was present in wild-type gonococcal cell lysates, but it was absent in lysates obtained from 1291ΔPLD bacteria. To confirm these data Akt and Akt complexes were captured by immunoprecipitation from wild-type gonococci-infected pex cell lysates and probed for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B). We confirmed these data by performing the converse experiment in which anti-PLD immune sera 1307 was used to capture NgPLD and NgPLD-immune complexes. An anti-phospho-Akt antibody was then used to probe for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B).

Collectively, our data indicate that Akt plays a critical role in gonococcal infection of pex cells and that infection with wild-type bacteria is augmented in the absence of (or reduced) PI3-K activity. Conversely, the absence of PI3-K activity impairs the ability of PLD-mutant gonococci to invade these same cells. This led us to wonder if the presence of functional NgPLD in wild-type bacteria could function to initiate Akt activation by directly interacting with Akt, thereby bypassing the conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3. To examine this idea, we first performed a Far-Western blot analysis of separated 1291 and 1291ΔPLD gonococcal cell lysates (Fig. 3A). Membranes were incubated with Akt1 and then probed to determine if this kinase bound to gonococcal constituents. Far-Western blots probed with anti-Akt antibody demonstrated that Akt bound to a gonococcal peptide possessing a molecular mass similar to NgPLD (55 kDa) (Fig. 3A). This peptide was present in wild-type gonococcal cell lysates, but it was absent in lysates obtained from 1291ΔPLD bacteria. To confirm these data Akt and Akt complexes were captured by immunoprecipitation from wild-type gonococci-infected pex cell lysates and probed for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B). We confirmed these data by performing the converse experiment in which anti-PLD immune sera 1307 was used to capture NgPLD and NgPLD-immune complexes. An anti-phospho-Akt antibody was then used to probe for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B).

NgPLD directly interacts with Akt kinase

Collectively, our data indicate that Akt plays a critical role in gonococcal infection of pex cells and that infection with wild-type bacteria is augmented in the absence of (or reduced) PI3-K activity. Conversely, the absence of PI3-K activity impairs the ability of PLD-mutant gonococci to invade these same cells. This led us to wonder if the presence of functional NgPLD in wild-type bacteria could function to initiate Akt activation by directly interacting with Akt, thereby bypassing the conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3. To examine this idea, we first performed a Far-Western blot analysis of separated 1291 and 1291ΔPLD gonococcal cell lysates (Fig. 3A). Membranes were incubated with Akt1 and then probed to determine if this kinase bound to gonococcal constituents. Far-Western blots probed with anti-Akt antibody demonstrated that Akt bound to a gonococcal peptide possessing a molecular mass similar to NgPLD (55 kDa) (Fig. 3A). This peptide was present in wild-type gonococcal cell lysates, but it was absent in lysates obtained from 1291ΔPLD bacteria. To confirm these data Akt and Akt complexes were captured by immunoprecipitation from wild-type gonococci-infected pex cell lysates and probed for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B). We confirmed these data by performing the converse experiment in which anti-PLD immune sera 1307 was used to capture NgPLD and NgPLD-immune complexes. An anti-phospho-Akt antibody was then used to probe for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B). We confirmed these data by performing the converse experiment in which anti-PLD immune sera 1307 was used to capture NgPLD and NgPLD-immune complexes. An anti-phospho-Akt antibody was then used to probe for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B). We confirmed these data by performing the converse experiment in which anti-PLD immune sera 1307 was used to capture NgPLD and NgPLD-immune complexes. An anti-phospho-Akt antibody was then used to probe for the presence of NgPLD by Western blotting. A band consistent with the molecular mass of NgPLD was present in infected cell anti-Akt immunoprecipitates that was not present in uninfected control immunoprecipitates (Fig. 3B).
N. gonorrhoeae directly binds cervical Akt kinase

NgPLD directly binds Akt by binding to the Akt PH domain

A Akt–NgPLD interaction was demonstrated by incubating primed supernatants, prepared as described in Experimental procedures, in Akt-lined 96-well microtitre plates. NgPLD is secreted by gonococci upon cervical infection and therefore is present in supernatants obtained from wild-type infection studies but is absent in supernatants obtained from 1291ΔPLD infections and from uninfected cervical cells (Figs S1 and S2). ELISA performed using anti-NgPLD antibody1307 revealed high-level Akt-NgPLD association occurred in wells to which the primed wild-type supernatants (WtSup) were added as evidenced by precipitation of the o-phenylenediamine dihydrochloride peroxidase substrate (Fig. 4). The colorimetric substrate precipitate was substantially lower in wells to which the primed mutant (MutSup) or primed control supernatants (ConSup) were added (Fig. 4). Similarly, negative control wells in which the primary or secondary antibody was omitted from the assay revealed only background levels of the colorimetric substrate precipitate (Fig. 4). Collectively, these data indicate that NgPLD, a secreted bacterial product, directly interacts with host Akt during cervical N. gonorrhoeae infection.

NgPLD directly binds Akt kinase. Assays were performed in duplicate on three separate occasions and as described in the text by incubating WtSup (WT), MutSup (ΔPLD), or ConSup (Con) in Akt1-coated microtitre plates. The data shown were obtained from a single, representative experiment; minimal variability occurred between each assay. In each assay the amount of Akt-bound NgPLD exceeded detection limits (i.e. absorbance of 3 at 490 nm) at dilutions less than 1:8 in wells incubated with WtSup, which is represented by the double slash marks on the corresponding line of the graph.

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was found to be statistically different \( (P < 0.05 \text{ for each comparison}) \). The addition of inhibitor IV, which acts down-stream of PtdIns(3,4,5)P_3 biosynthesis (Fig. 5B), to infection studies severely impaired the ability of gonococci to invade pex cells compared with the absence of inhibitor (Fig. 5C). Inhibitor V, which acts independently of PI3-K or PDK1 (Fig. 5B), also decreased gonococcal invasion but to a lesser degree than did inhibitor IV (Fig. 5C).

Inhibitor VII, which binds directly to the Akt PH domain (Fig. 5B), resulted in the greatest decrease in gonococcal invasion (Fig. 5C). Collectively, these data suggested that the PH domain plays a critical role in modulating gonococcal invasion because (i) the target sites of inhibitor function potentially overlap early in the Akt activation pathway (e.g. prior to phosphorylation, oligomerization) (Fig. 5) and (ii) inhibition resulting from inhibitor binding to the PH domain resulted in the greatest degree of decreased gonococcal invasion.

To confirm these data and to directly determine if gonococcal PLD directly binds to the PH domain of Akt we modified and repeated the above-described Akt–NgPLD ELISA by including or excluding from each assay Akt inhibitor VII, which would potentially prohibit an Akt–NgPLD interaction if binding occurred through the PH domain. ELISA demonstrated that NgPLD directly associated with Akt in wells incubated with WtSup in the absence of the inhibitor, but in the presence of inhibitor this interaction was severely decreased (Fig. 6A). Parallel studies performed using ConSup (Fig. 6B) or MutSup (Fig. 6C) resulted in only background levels of precipitation of the colorimetric substrate, and there was no difference between the presence of and the absence of the inhibitor. Blank wells and wells in which the primary or secondary antibody was omitted from each assay resulted only in precipitation of background levels of the colorimetric substrate (Fig. 6A–C). Collectively, these
data strongly indicate that gonococcal NgPLD directly interacts with cervical cell Akt kinase through its PH domain and this interaction augments progressive gonococcal infection.

NgPLD competes with PtdIns(3,4,5)P$_3$ for binding to the Akt PH domain, in vitro

3-Phosphoinositides are the preferred substrate for Akt binding. These lipid second messengers can bind to the PH domain of Akt initiating a signalling cascade leading to Akt kinase activity. Therefore, we wanted to determine if NgPLD could bind to Akt in the presence of PtdIns(3,4,5)P$_3$, which could potentially inhibit NgPLD PH domain-binding, in vivo. To determine if NgPLD could competitively bind to Akt in the presence of its natural ligand, we performed an ELISA in which variable concentrations of PtdIns(3,4,5)P$_3$ were added to 1× WtSup and ConSup in Akt-lined microtitre plates. Alternatively, in the same assay, 10 nM PtdIns(3,4,5)P$_3$ was incubated in the presence of 10-fold serial dilutions of WtSup and ConSup. Levels of Akt-bound NgPLD and PtdIns(3,4,5)P$_3$ were then measured. Incubation of WtSup with increasing concentrations of PtdIns(3,4,5)P$_3$ resulted in the decreased ability to detect NgPLD, whereas no significant difference was observed with the use of ConSup in the absence of, compared with the presence of, increasing concentrations of the PtdIns(3,4,5)P$_3$ competiter (Table 3). Similarly, wells incubated with PtdIns(3,4,5)P$_3$ in the presence of increasingly dilute WtSup competiter resulted in the increased binding of PtdIns(3,4,5)P$_3$ compared with the addition of less dilute WtSup (Table 3). No significant difference was observed in PtdIns(3,4,5)P$_3$ binding among wells incubated with ConSup. Wells in which the primary or secondary antibody were omitted, or blank wells, revealed only background levels of precipitation of the colorimetric substrate. These data indicate that NgPLD can compete with a natural ligand, PtdIns(3,4,5)P$_3$, for Akt binding, in vitro.

Exogenous PLD rescues Akt activity

The data described above demonstrate that NgPLD can directly bind to the Akt PH domain and that it can compete with PtdIns(3,4,5)P$_3$, a natural ligand for Akt in vivo, for Akt PH domain binding, in vitro. To determine the biological significance of this interaction we again performed an Akt kinase assay in which pex cells were challenged with wild-type bacteria or with PLD-mutant bacteria in the presence or absence of commercially available Streptomyces spp. PLD (SsPLD) or WtSup or MutSup. Akt kinase activity was minimal in 1291×PLD gonococci-infected cells compared with wild-type-infected cells (Fig. 7A); however, the addition of WtSup, which serves as an exogenous source of NgPLD, partially rescued Akt kinase activity in PLD-mutant-infected pex cells (Fig. 7A). Conversely, the addition of MutSup or SsPLD to mutant-infected cells did not alter Akt activity (Fig. 7A). These findings are illustrated by the respective increased or decreased intensity of the band on Western blots corresponding to phosphorylated GSK-3 despite the presence of equivalent amounts of total Akt under each condition assayed (Fig. 7B). Akt kinase activity was not observed in un-

| [PtdIns(3,4,5)P$_3$] add$^*$ | 1× Primed supernatant ± PtdIns(3,4,5)P$_3$ Mean (σ) $P$ |
|-----------------------------|-------------------------------------------------|
|                             | 0 1 pM 100 pM 10 nM                             |
| Wild-type Sup (NgPLD+)      | 2.409 1.303 0.622 0.234                         |
| (NgPLD–)                    | (0.20) NA (0.18) 0.019 (0.11) 0.011 (0.06) 0.002|
| Con Sup                     | 0.152 0.124 0.151 0.133                         |
| (NgPLD–)                    | (0.03) NA (0.02) 1.0 (0.01) 1.0 (0.01) 1.0     |

10 nM PtdIns(3,4,5)P$_3$ ± primed supernatant

| Sup dilution | 0 0.01× 0.1× 1× |
|--------------|----------------|
| Wild-type Sup| 2.241 1.151 0.568 0.245 |
| (NgPLD+)     | (0.14) NA (0.06) 0.008 (0.09) 0.007 (0.02) 0.003 |
| Con Sup      | 2.243 2.223 2.303 2.351 |
| (NgPLD–)     | (0.12) NA (0.11) 1.0 (0.16) 1.0 (0.19) 1.0 |

Assays were performed as described in the text. Values given are the mean (variance) obtained from three separate trials performed in triplicate. $P$-values were determined using a Student’s $t$-test calculated for the mean binding of NgPLD or PtdIns(3,4,5)P$_3$ to Akt1 in the presence of compared with the absence of serial dilutions of PtdIns(3,4,5)P$_3$ or WtSup (NgPLD+) or ConSup (NgPLD–) respectively. Under all conditions assayed, the absorbance detected for no primary, no secondary and blank negative control wells were within the following range: 0.041 ± 0.01–0.034 ± 0.01; $P$-values were < 0.001 for each of these assay conditions. NA, not applicable.

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NgPLD augments Akt activity and that this function is not conserved in SsPLD. This observation is consistent with our previous studies that show that NgPLD shares some, but not all, functional properties with other bacterial PLDs.

Collectively, the above-described data demonstrate that NgPLD binds to the Akt PH domain and that this interaction plays a role in gonococcal association and invasion of primary cervical cells by recruiting CR3 to the cervical cell surface and by promoting gonococcal invasion/intracellular survival. These processes also exhibit a dependence on PtdIns(4,5)P_2 and MLCK, but occur independently of PI3-K activation. Conversely, in the absence of functional NgPLD activity, signalling events mediating gonococcal invasion appear to require PI3-K, and although Akt activity is decreased, it is not absent. CR3-mediated gonococcal adherence to, and invasion of, pex cells requires both opsonic (i.e., iC3b) and non-opsonic (pilus and porin) interactions with the I-domain of this receptor. This led us to wonder if pilus- and porin-mediated engagement of the CR3 I-domain was sufficient to trigger low-level Akt activity, as integrin (e.g., CR3) activation results in both outside-in and inside-out signalling events. That is, do pilus and porin initiate Akt-mediated, outside-in signalling leading to increased receptor affinity and avidity, which is then followed by a NgPLD–Akt-mediated inside-out signalling event that augments gonococcal intracellular survival and potentiates progressive cervical infection? To determine if CR3 engagement contributes to Akt activity we again performed an Akt kinase assay in which Akt was captured in wild-type and uninfected pex cells that were incubated in the presence and absence of anti-iC3b, -CD11b, or -CD18 antibodies, or alternatively, purified gonococcal pilus and/or porin, recombinant I-domain, WtSup, δPLDSup, or Akt inhibitor (Fig. 8). Consistent with the data above, Akt activity was observed in assays performed with 1291 wild-type gonococci-infected pex cells. Akt activity was decreased slightly in infected cells in the presence of anti-iC3b, -CD11b, or -CD18 antibodies, or alternatively, purified gonococcal pilus and/or porin, recombinant I-domain, WtSup, δPLDSup, or Akt inhibitor (Fig. 8). Consistent with the data above, Akt activity was observed in assays performed with 1291 wild-type gonococci-infected pex cells. Akt activity was decreased slightly in infected cells in the presence of anti-iC3b antibody, which inhibits gonococcal binding to the I-domain of CR3. The addition of pilus and/or porin to uninfected pex cells resulted in low-level Akt activity, which was decreased in the presence of the CR3 I-domain competitor. However, the addition of pilus, porin and WtSup to uninfected pex cells resulted in...
a further increase in Akt activity, which was not observed with the use of ∆PLDSup. Engagement of the CR3 alpha subunit, which contains the I-domain, with an anti-CD11b antibody also resulted in a low level of Akt activity, which was not observed with the use of an antibody to the CD18, CR3 beta subunit. Similarly, and consistent with data described above, Akt activity was not observed in uninfected pex cells nor was it observed in gonococci-infected cells treated with Akt inhibitor or when the anti-Akt antibody was omitted from the initial immunoprecipitation step as well as when the GSK-3 peptide substrate was omitted from the kinase assay. Comparable amounts of Akt were present under all the conditions assayed (Fig. 8B). Collectively these data indicated that pils and porin (and possibly iC3b) binding to the CR3 I-domain contribute to Akt activity upon gonococcal infection of cervical epithelial cells and that NgPLD has an additive effect on this activity.

Discussion

We have provided evidence demonstrating that CR3-mediated, gonococcal infection of primary cervical cells triggers Akt activation and that these signalling events also require the activities of MLCK and PtdIns(4,5)P2 as well as NgPLD, a secreted gonococcal protein. Our data also indicated that PI3-K activation is not the preferred pathway by which wild-type gonococci induce invasion of the cervical epithelium. Although Akt activity is predominately documented to require the formation of PtdIns(3,4,5)P3, mediated by the action of PI3-K, PI3-K-independent Akt activation is also reported. Engagement of CR3 by gonococci triggers membrane ruffling and bacterial internalization within cervical epithelia. Recent studies show that Akt and PDK1 are localized within membrane ruffles and that Akt resides within 'super-rafts' (Currie et al., 1999; Sakletz-Rorowski et al., 2003), which may potentially be the PRPMPs described by Huang et al. (2004). PRPMPs define areas of ruffling and are further proposed to augment the interaction of PtdIns(4,5)P2 with downstream effectors. Several studies show that bacterial PLDs can regulate PtdIns(4,5)P2 biosynthesis and actin polymerization. In terms of NgPLD-mediated Akt activation, formation of PRPMPs potentially induced by gonococcus cervical infection may mediate the recruitment of Akt to distinct sites within the cervical cell membrane where the lower affinity of the Akt and the PDK1 PH domains for PtdIns(4,5)P2 (upon comparison with PtdIns(3,4)P2 and PtdIns(3,4,5)P3) could be competitive (Bottomley et al., 1998). A similar idea is proposed by Currie et al. (1999) who suggest that, although PDK1 exhibits a lower binding affinity for PtdIns(4,5)P2 than PtdIns(3,4)P2 or PtdIns(3,4,5)P3, the binding affinity for PtdIns(4,5)P2 is comparable to that of Akt for PtdIns(3,4)P2 or PtdIns(3,4,5)P3; therefore, the relative physiological abundance of PtdIns(4,5)P2 compared with 3-phosphoinositides may allow PtdIns(4,5)P2 binding. James et al. (1996) also suggest that the differential binding affinity of Akt for different phosphoinositides may not be sufficient to allow preferential binding in vivo to 3-phosphoinositides in view of the relative cellular abundance of PtdIns(4,5)P2. Furthermore, although the production of PtdIns(3,4,5)P3 at the plasma membrane does occur at localized sites, its presence is only transient (Jannney and Lindberg, 2004).

Our initial studies using primary cervical cells show that vinculin, talin, α-actinin, ezrin and myosin accumulate and colocalize with gonococci before the formation of membrane ruffles and bacterial internalization within macropinosomes (Edwards et al., 2000). Collectively, these proteins link the cytoplasmic tail of integrin receptors to actin filaments and effector proteins in a PtdIns(4,5)P2-dependent manner, thus serving as bridging molecules critical to activating integrin function (Longhurst and Jennings, 1998; Martel et al., 2001; Jannney and Lindberg, 2004; Niggli, 2005). We subsequently discovered that gonococcus-induced ruffling and invasion of cervical cells are mediated by the cβ3i integrin (i.e. CR3) (Edwards et al., 2001). A rapid increase in PtdIns(4,5)P2 occurs in response to integrin engagement (Longhurst and Jennings, 1998). Integrin engagement can also result in PtdIns(3,4)P2 production without the production of PtdIns(3,4,5)P3 (Bantfic et al., 1998a,b; Vanhaesebroeck and Waterfield, 1999), demonstrating that integrin-mediated cell stimulation and subsequent signal transduction do not necessarily result in or require PtdIns(3,4,5)P3. This is exemplified by the invasion of intestinal epithelial cells by the bacterium, Yersinia, which is mediated by β1-integrins and which is impaired in the absence of PtdIns(4,5)P2 (Pizarro-Cerda and Cossart, 2004). These findings underscore the importance of PtdIns(4,5)P2 to integrin activation and function and provide one explanation for the increased ability of gonococci to adhere to and to invade pex cells in the absence of PI3-K activity.

Our data demonstrating that NgPLD can competitively bind to the Akt PH domain in the presence of PtdIns(3,4,5)P3 together with the facts that PLDs bind actin and PtdIns(4,5)P2 (Kusner et al., 2003) as well as that Akt can directly interact with actin leading to increased Akt phosphorylation (Kusner et al., 2003), might suggest that NgPLD functions to mediate cervical cell signalling events by bringing Akt (and PDK1) in proximity to CR3 within the plasma membrane. We present data revealing Akt homo- and/or heterooligomer formation and increased Akt activity in the absence of PI3-K activity, which would result in more available PtdIns(4,5)P2. These data further hint at a covalent interaction mediated by an unknown peptide. It is proposed that oligomerization contributes to Akt activity and may be facilitated by the accu-
mulation of Akt and/or Akt binding proteins at the plasma membrane (Chan et al., 1999). Therefore, the PI3-K-mediated production of PtdIns(3,4,5)P$_3$ may inhibit gonococcal invasion in two ways: (i) increased competition for NgPLD for Akt binding and (ii) decreased availability of PtdIns(4,5)P$_2$ to mediate CR3 function. These ideas are further supported by our data showing that gonococcal adherence and invasion as well as Akt activation are decreased in the presence of the PtdIns(4,5)P$_2$ inhibitor, neomycin. It could also be argued that reduced cellular NADPH oxidase activity (Yamamori et al., 2004) or that impaired phagosomal maturation and acidification (seen with gonococcal infection) (Booth et al., 2003) observed in the absence of PI3-K activity could explain the increased invasion/intracellular survival observed during our studies using LY294002; however, these findings do not account for the parallel increases in Akt phosphorylation and Akt activity that we observed with the use of this inhibitor in our studies.

Akt di/trimer formation occurs non-covalently through the interaction of Akt PH domains, and it is shown that this interaction is highly specific in that the PH domain of Akt1 does not bind the PH domain of Akt2 (Datta et al., 1995). However, the Akt PH domain can interact with protein substrates as well as non-substrate proteins that potentially modulate Akt activity (Brazil et al., 2002; Fukuda et al., 2003; Ahn et al., 2004). Binding of proteins to the PH domain may mimic the Akt structural change required for phosphorylation and/or maintain Akt in an active conformation (Datta et al., 1995; Brazil et al., 2002; Ahn et al., 2004). The ability of NgPLD to compete with PtdIns(3,4,5)P$_3$ for Akt1 binding in vitro could imply that similar functions exist for NgPLD in Akt activation. Although a PI3-K-dependent role for eukaryotic PLD in mediating Akt signalling is demonstrated (Lim et al., 2003; Yamada et al., 2004; Banno et al., 2005; Li and Malik, 2005), a role for bacterial PLDs may prove to be isoform-specific. [We previously demonstrate that human PLD1 and PLD2 are not upregulated nor is their activity increased upon gonococcal infection of pex cells (Edwards et al., 2003).] We were able to partially rescue Akt activity in pex cells infected with PLD-mutant bacteria by the addition of primed wild-type supernatants (NgPLD$^+$) but not with primed PLD-mutant supernatants (NgPLD$^-$) or exogenous SsPLD. In another study, the addition of exogenous Streptomyces chromofuscus PLD to Chinese Hamster ovary cells results in Akt activation in a phosphatidic acid- and PI3-K-dependent manner (Nozawa, 2002), demonstrating that for this (secreted) bacterial PLD, modulation of Akt activity occurs indirectly.

The above-described ideas imply that gonococcal-induced Akt activation as well as invasion/intracellular survival. However, we did not directly assay PI3-K activity and therefore cannot rule out that inhibition (or stimulation) of another effector molecule is responsible for the observed increased gonococcal invasion/intracellular survival and Akt activation in the presence of LY294002, although CK-2 does not appear to be responsible. We also cannot rule out the production of PtdIns(3,4)P$_2$ by PI4-K-mediated phosphorylation of PtdIns(3)P. In immortal cell lines PI3-Ks are found to be ubiquitously expressed (Ho et al., 1997; Arcaro et al., 1998; Rozycka et al., 1998), which is most likely attributable to PIK3CA, the gene encoding the p110 PI3-K catalytic subunit, commonly serving as a genetic ‘hot-spot’ for mutation and gene amplification in many human malignancies (Wymann and Pirola, 1998; Ma et al., 2000; Osaki et al., 2004). In non-malignant cells and tissue PI3-K isoforms exhibit a distinct distribution. By in situ hybridization and Western blotting, El Sheikh et al. (2003) were unable to detect the presence of the PI3-K class IA p85 regulatory subunit or class II PI3-Ks C2α or C2β in ecto- or endocervical cells or tissue. These data might indicate that within the cervical epithelium either these enzymes are expressed at a very low level (below the level of the detection methods used) or that other PI3-Ks are expressed (e.g. PI3-KC2γ or novel PI3-Ks). Upon viewing preliminary microarray analysis of RNA isolated from our primary cervical cell system, we also found that message for class I and II enzymes were absent (PI3-KC2γ was not present on the microarray); however, a ‘Vsp34-like’ (class III enzyme) was present as were two additional messages that are ‘moderately similar’ to PI3-Ks (J.L. Edwards and M.A. Apicella, unpubl. data). Therefore, we cannot disregard the possibility that unconventional PI3-K isoforms exist in primary cervical cells, which are refractory to the actions of known PI3-K inhibitors, as is demonstrated for PI3K2α (Domin et al., 1997; Knight et al., 2004). Of interest is that fallopian tube epithelia and epithelia of the male urogenital tract exhibited high levels of PI3-K (El Sheikh et al., 2003). These cells and tissues do not express CR3 (Edwards et al., 2001) and gonococcal infection of these anatomical sites is characterized by an acute inflammatory response. In contrast, gonococcal cervical infection is frequently asymptomatic (Densen et al., 1982; Densen, 1989; Hook and Handsfield, 1999; Sparling, 1999). Recently it was reported that one function of Salmonella-induced Akt engagement is to downregulate the inflammatory response initiated by PI3-K activation upon epithelial infection (Huang et al., 2005). These data might suggest that the absence of a pro-inflammatory response, which is observed for I-domain-dependent, CR3-mediated endocytosis, is regulated by Akt effector function.

While this manuscript was in preparation, Lee et al. (2005) reported that gonococcal infection of A-431 cells...
(a vulvular, epidermal carcinoma cell line) resulted in PilT (the pilus retraction motor)-mediated Akt activation in a PI3-K-dependent manner. Increased PI3KCA also leads to increased Akt phosphorylation in immortal cells (Ebert et al., 2000; Singh et al., 2002; Harris et al., 2003), and aberrant activation of the PI3-K–Akt pathway is frequently associated with a malignant state (Osaki et al., 2004). Because A-431 cells are hypertriploid, it is not unreasonable to suggest that the PI3-K–Akt pathway may reside in an active state in these cells, which is then amplified with gonococcal infection. Indeed, basal Akt activity is observed in uninfected cells in these studies and upon viewing the data presented, there appeared to be significant Akt activity in kinase assays performed with wild-type bacteria, PilT mutant bacteria and inducible-mutant bacteria both in the presence and absence of the inducer (Lee et al., 2005). Whether Akt activity observed in studies performed with the PilT mutant can be attributed to NgPLD cannot be determined. However, these authors do note that PilT-mediated, PI3-K-dependent invasion is responsible for only approximately 40% of total gonococcal invasion of A-431 cells (Lee et al., 2005). Salmonella-induced Akt activation in HeLa cells occurs by two mechanisms; one is dependent on secretion of the SigD/SopB phosphatase by a type three secretion system (Steele-Mortimer et al., 2000) while the other requires viable bacteria but is SigD/SopB- and secretion system-independent (Huang et al., 2005). Sustained secretion of NgPLD is dependent on complement deposition upon the gonococcal surface and I-domain binding, although I-domain binding alone is sufficient to allow an initial, transient release of secreted products. I-domain-containing integrins are present on some immortal cell lines where they can serve as pilus receptors (Edwards and Apicella, 2005). In the absence of a complement source, however, it is unlikely that A-431 cells allow sustained secretion of NgPLD, but gonococcus protein secretion has not been examined using A-431 cells. The ability of gonococci to activate Akt very early in the infection process, when invasion levels are low, suggests that pilus and porin (plus minute amounts of NgPLD) initiate Akt activation in primary cervical cells. However, it is also possible that modification of the host cell membrane by NgPLD allows entry of this enzyme into the host cell before bacterial entry or that NgPLD phosphatidylycholine cleavage results in membrane curvature. Changes in membrane composition, and thus membrane curvature, could stimulate actin assembly, as is demonstrated in platelets, or could result in protein clustering/recruitment (Janmey and Lindberg, 2004). We were unable to detect Akt activity in uninfected, pex cells; however, Akt activation was not completely absent upon infection of these cells with PLD-mutant gonococci. We attributed this low-level activity to pilus and porin binding to the CR3 I-domain, which is then augmented by NgPLD. Therefore, it cannot be distinguish if the differences observed between our studies and those described by Lee et al. (2005) are the result of differences in the experimental materials and methods used, or if PilT and NgPLD, in conjunction with pilus and porin, both contribute to Akt activation.

Akt-dependent signalling pathways augmenting wild-type gonococcal invasion of, and/or its intracellular survival within, pex cells do not rely on the production of PtdIns(3,4,5)P3, as invasion levels are increased in the absence of PI3-K activity. Studies performed using CEACAM3- and CEACAM1-expressing HeLa cells also showed that gonococcal invasion/intracellular survival is enhanced in the absence of PI3-K activity; however, in these studies the use of wortmannin or LY294002 both resulted in increased invasion/intracellular bacterial survival (Booth et al., 2003). We attributed the ability of wortmannin and quercetin to inhibit gonococcal invasion levels to the absence of MLCK activity because both of these inhibitors can also inhibit MLCK and because use of a MLCK-specific inhibitor yielded similar data to that obtain with the use of wortmannin and quercetin. Although the concentrations of wortmannin used herein are thought to be below the level at which MLCK inhibition occurs, it should be noted that the pex cells used in these studies exhibit selective, increased sensitivity versus immortal cell lines to many pharmacological agents used to inhibit signalling effectors. Additionally, it should also be kept in mind that, although 300 nM wortmannin is required to inhibit MLCK in 3 min (Nakanishi et al., 1992), these same studies demonstrate that longer incubation periods (i.e. ≥ 30 min) with lower concentrations of wortmannin are sufficient to inhibit MLCK activity (Nakanishi et al., 1992). We pretreated pex cell monolayers with wortmannin for 1 h before the initiation of gonococcal infection, and wortmannin was maintained in the infection medium throughout the time-course of cervical cell challenge. Previous studies show that Rho and MLCK activation enhance myosin–actin interactions (Kawkitinarong et al., 2004) and that MLCK activity resulting in Ca2+ influxes plays a critical role in inducing contractile forces (Nakanishi et al., 1992) and in modulating cytoskeletal rearrangements required for pseudopod formation and phagocytosis in monocytoic cells (Tran et al., 2001). These findings are consistent with our previous published data demonstrating a role for Rho, myosin, Ca2+ and membrane ruffling leading to macropinocytosis of gonococci during infection of pex cells (Edwards et al., 2000; 2001). We also show previously that PLD-mutant gonococci fail to elicit membrane ruffling and do not cluster on the surface of pex cells, which could be an indicator of the lack of CR3 clustering (Edwards et al., 2003). Integrin clustering is required for endocytosis and is mediated by the contractile ability of myosin (Longhurst and Jennings, 1998). It is also of interest that protein kinase A (PKA)-mediated increases...
in cellular Ca\(^{2+}\) levels trigger activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase, which under some circumstances can directly phosphorylate Akt Thr308 (i.e. PDK1- and PI3-K-independent Akt activation) (Datta et al., 1999; Morisco et al., 2005) and that LY294002 treatment of smooth muscle cells leads to a significant increase in PKA activity (Komalavilas et al., 2001). While LY294002 can inhibit myosin contractile forces, it does not inhibit MLCK-mediated myosin light chain phosphorylation (Komalavilas et al., 2001). Collectively, these data might provide an additional/alternative explanation for the increased ability of gonococci to survive within pex cells in the presence of LY294002 and perhaps suggest a role for PKA in gonococcal-induced Akt activation.

Several microorganisms subvert Akt signalling pathways to augment infection and invasion. We provide evidence demonstrating that NgPLD, a secreted gonococcal product, competitively binds to the Akt PH domain. To our knowledge this is the first demonstration of a PI3-K-independent mechanism of Akt activation by a microorganism and the direct interaction of a bacterial product with this kinase. Our studies highlight the importance for PtdIns(4,5)P\(_2\) in CR3-mediated gonococcal infection of primary cervical cells and indicated a role for MLCK in these processes. However, many questions remain to be addressed. Integrin-linked kinase associates with the cytoplasmic tails of \(\beta\)-integrins (Hannigan et al., 1996) and modulates integrin-induced cytoskeletal rearrangements (Sakai et al., 2003). This kinase also directly (Galetic et al., 1999) or indirectly (Lynch et al., 1999) functions in Akt Ser473 phosphorylation. CH-ILKBP interacts with ILK and recently is shown to be required for Akt translocation to the plasma membrane in HeLa cells (Fukuda et al., 2003). It will, therefore, be of interest to determine if these and/or other integrin-associated effector molecules facilitate gonococcus-induced Akt activation. It also remains to be determined what the downstream consequences of Akt activation are with regard to gonococcal invasion. These and other unanswered questions will be the focus of future studies.

**Experimental procedures**

**Cell culture, bacteria and infection studies**

Surgical biopsies derived from the ecto- and the endocervix that were used to seed primary cervical epithelial (pex) cell systems were procured and maintained as described previously (Edwards et al., 2000) in defined keratinocyte serum-free medium (Life Technologies, Rockville, MD). Pharmacological agents used, as described in the studies outlined below, were not cytotoxic (viability greater than 95%) at the indicated concentrations as determined by trypan blue exclusion (pex cells) or by counting colony-forming units of wild-type and mutant gonococci incubated in the presence of compared with the absence of each reagent.

**Neisseria gonorrhoeae strains** 1291 (Dudas and Apicella, 1988) and 1291ΔPLD (Edwards et al., 2003) were used in the infection studies described herein. Infection studies were performed as previously described using a multiplicity of infection of 100 (Edwards et al., 2000). Pex cells were challenged with gonococci for variable time-periods (as noted); uninfected, control, cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected cell monolayers were subsequently harvested for kinase, immunoprecipitation, or quantitative invasion assay analyses as described previously and as briefly outlined below. Where indicated, antibodies were added to infection studies at a concentration of 10 \(\mu\)g ml\(^{-1}\) and they included anti-IC\(_3\)b neotigent (Quidel; San Diego, CA), anti-CD11b I-domain (antibody Bear1, Immunotech; Marseille, France), or anti-CD18 (antibody iB4; Calbiochem, La Jolla, CA). In total, 10 U ml\(^{-1}\) SsPLD (Sigma; St. Louis, MO) or 10 ng ml\(^{-1}\) recombinant I-domain (generously provided by E. Brown, University of California, San Francisco, CA) and/or purified *N. gonorrhoeae* strain MS11 pilus or porin (generously provided by M. Blake, CBER, FDA, Bethesda, MD) were included or omitted from each assay, as noted. Pharmacological inhibitors of specific signal transduction effector molecules were included in or excluded from infection studies, and they included neomycin (50 \(\mu\)M; Sigma); 1.5 \(\mu\)M Akt inhibitor; 1 \(\mu\)M Akt inhibitor IV, Akt inhibitor V, or Akt inhibitor VII; 10 \(\mu\)M LY294002 or LY303511; 10 nM wortmannin; 3 \(\mu\)M quercetin; 1 \(\mu\)M TBB, or 300 nM ML-7 (all obtained from Calbiochem). Cervical cell monolayers were pre-incubated (1 h, 37°C) with each, respective, pharmacological agent before the addition of gonococci. Our attempts to produce recombinant NgPLD have not been successful; therefore, where indicated, cervical cells were pretreated with 25 \(\mu\)M cycloheximide (MP Biomedical; Aurora, OH) to inhibit their protein synthesis. Following gonococcal challenge, infection supernatants were harvested, immediately transferred to ice, and gonococci were removed by centrifugation and by filtration of the supernatant through a 0.22 \(\mu\)m low protein-binding syringe filter to produce, uninfected (control), wild-type, or ΔPLD-mutant ‘primed’ supernatants. Primed supernatants were then filtered through Centricon YM-100 centrifugal filter units (Millipore Corporation; Bedford, MA) [molecular weight cut-off (MWCO) greater than 100 kDa]. The filtrate from this spin was then centrifuged through Centricon YM-30 centrifugal filter units (MWCO greater than 30 kDa). Filter-retained secreted gonococcal products were collected with a 1/10 volume of 1 M Tris (pH 7.4) to yield a 10× concentrated suspension of secreted gonococcal products each possessing a molecular mass between 30 kDa and 100 kDa. NgPLD possesses an approximate molecular mass of 55 kDa, and therefore, it is present in supernatants obtained from wild-type infection studies, but it is absent in supernatants collected from uninfected, control, cells or from cells challenged with the 1291ΔPLD mutant (see Figs S1 and S2). A 1/10 volume of the concentrated supernatant was added where indicated to infection studies or microtitre plates to yield a 1× final solution.

**Neisseria gonorrhoeae invasion of primary, human, cervical cells**

Pex cell monolayers were infected with wild-type or PLD-mutant gonococci as outlined above. The ability of gonococci to invade pex cells was quantitatively determined using stan-
dard gentamicin-resistance assays, performed as described previously (Edwards et al., 2000) and in which chemical additives were included in or excluded from the assay. Infections were allowed to proceed for 90 min before gentamicin treatment. Association assays were performed in a similar manner with the exception that gentamicin treatment was omitted. Per cent association (adherence and invasion) and invasion of *N. gonorrhoeae* 1291 or 1291ΔPLD in the presence or absence of experimental additives was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. A Kruskal–Wallis non-parametric analysis of variance was used to determine the statistical significance of the invasion assays described above.

**Determination of CR3 surface expression on primary cervical cells**

The level of CR3 on the surface of pex cells was quantitated as described by Edwards et al. (2003). Briefly, pex cells were seeded in 96-well microtitre plates and allowed to grow to confluence. Cervical cells were pre-incubated with neomycin, Akt inhibitor, or LY294002, as described above, and subsequently challenged with wild-type or PLD-mutant gonococci for 3 h, or they were left uninfected. Chemical agents were maintained in the cultures throughout the course of the infection. The infections were then terminated, and the cells were fixed with 2% paraformaldehyde. Immunoassays were then performed according to standard ELISA protocols using the H5A4 anti-CD11b (i.e. CR3, Developmental Studies Hybridoma Bank; University of Iowa, Iowa City, IA) primary and peroxidase-conjugated secondary antibodies. Absorbance of the o-phenylenediamine dihydrochloride peroxidase substrate was determined spectrophotometrically at 490 nm. Primary antibody was omitted from one well, and the secondary antibody was omitted from a second well, which served as controls for non-specific binding and endogenous peroxidase activity respectively.

**Immunoprecipitation, Western, and Far-Western blot analyses**

Confluent pex cells monolayers in 35 mm tissue culture-treated dishes were challenged with wild-type or PLD-mutant *N. gonorrhoeae* as described above, or they were left uninfected (0 min). The infection was allowed to proceed for 30, 60, or 90 min or for 3 h and subsequently terminated by immediately transferring the dishes to ice, removing the infection supernatant and rinsing the cell monolayer thrice with ice-cold phosphate-buffered saline (PBS). Immunoprecipitation was performed as described by Wen et al. (2000) using anti-NgPLD immune sera, 1307, or anti-Akt polyclonal antibody H-136 (Santa Cruz Biotechnology, Santa Cruz, CA) to capture immune complexes. Antibody H-136 recognizes Akt1, 2, and 3 of human origin. Antibody 1307 recognizes the gonococcal PLD active site (see Figs S1–S3 in Supplementary material). The ability of gonococcal constituents to bind Akt was determined by Far-Western blot analysis in which 1291 wild-type and 1291ΔPLD bacterial lysates were prepared by suspending bacteria in lysis buffer [1× PBS, 0.1% SDS, 1× protease inhibitor cocktail (Sigma)] at a concentration of 10^7 ml^-1. The bacterial suspensions were vortexed and then pulled through a 22-gauge syringe to ensure cell disruption. Pex immunoprecipitates and gonococcal lysates were separated on 4–12% denaturing polyacrylamide gradient gels and transferred to immobilin-P membranes (Millipore, Bedford, MA). Far-Western blot analysis was performed on separated, membrane-bound, gonococcal lysates by allowing membranes to incubate [room temperature (RT)] overnight, with rotation, with 10 ng of Akt1 kinase (Calbiochem) before standard Western blot analysis using anti-Akt antibody C-20 (Santa Cruz), which recognizes Akt1 and Akt 2 and 3 with less avidity. For coimmunoprecipitation assays, Western blotting was performed according to standard protocols using the 1307 anti-NgPLD immune sera or the anti-phospho-Akt antibodies p-Akt1/2/3 (Thr308) (Santa Cruz) or pS727Akt (Promega, Madison, WI). For semi-quantitative analysis of Akt phosphorylation, each set of Western blots were incubated with the anti-phospho-Akt antibodies pT308 (Rockland, Gilbertsville, PA) or p-Akt1/2/3 (Thr308) after which the membranes were stripped overnight (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol, with rotation, RT) and then re-probed using the anti-phospho-Akt antibodies pS727Akt or p-Akt1/2/3 (Ser473)-R. To ensure equal loading, membranes were stripped again and stained with GelCode Blue (Pierce) or re-probed using anti-Akt antibodies C-20 or H-136. The anti-Akt antibodies pT308 and p-Akt1/2/3 (Thr308) recognize Akt only when phosphorylated on a critical threonine residue (Thr308 for Akt1); antibody pS727Akt and p-Akt1/2/3 (Ser473)-R recognize a critical serine residue (Ser473 for Akt1) in its phosphorylated state. Following (Far-)Western blotting, antibody-labelled Akt kinase and Akt and NgPLD immunocomplexes were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

**Akt kinase assay**

Confluent pex cell monolayers were left untreated or they were treated (37°C, 1 h) with 50 μM neomycin, 1.5 μM Akt inhibitor, 1 μM Akt inhibitor VII, 10 μM LY294002, 10 μM LY303511, 1 μM TBB, 300 mM ML-7, 10 mM wortmannin, or 3 μM quercetin before infection with wild-type gonococci. Alternatively, in separate assays, cell monolayers were then left uninfected or they were challenged with 1291 wild-type or 1291ΔPLD bacteria for 2 h. As noted, 10 U ml^-1 SePLD; primed wild-type or ΔPLD infection supernatants; anti-iC3b, -CD11b, or -CD18 antibody; porin; pilus; and/or l-domain were added to pex cell monolayers simultaneously with bacteria. Infections were terminated by immediately transferring dishes to ice, removing the infection medium and rinsing the cells thrice with ice-cold PBS. Immunoprecipitation and assessment of Akt kinase activity were performed using the Akt Kinase Assay Kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's directions. Alternatively (because the above assay kit was unavailable), for studies designed to look at CR3 engagement, kinase assays were performed according to the protocol described by Cell Signaling Technology with the exception that anti-Akt C-20AC agarose-conjugated antibody (Santa Cruz) was used to capture Akt, the kinase reaction was carried out using GSK-3α peptide substrate (37 kDa; BioVision, Mountain View, CA) and Western blotting was performed with the phospho-GSK-3α/β (Ser21/9) antibody (Cell Signaling Technology). Negative controls for each assay included the omission of the immobilized Akt antibody or the GSK-3 protein substrate. To ensure equal loading, membranes were

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Akt-NgPLD ELISA

Akt1 kinase (5 ng or 10 ng) was used to coat 96-well microtitre plates. Microtitre plates were lined with 10 ng of Akt kinase in initial studies to determine NgPLD Akt adherence. Subsequent studies that were designed to examine the effect of Akt inhibitor VII or PtdIns(3,4,5)P3 on NgPLD Akt binding were performed using microtitre plates that were lined with 5 ng of Akt1 kinase to reduce binding levels to those within the range of the detector limits (i.e. A405 < 3). For either assay, wells were rinsed with PBS and blocked (30 min, RT) with PBS-0.25% bovine serum albumin-0.05% Tween-20 following Akt adherence. To determine NgPLD Akt binding, 100 μl of a 1× concentration of uninfected control, wild-type, or ∆PLD mutant primed infection supernatants (ConSup, WtSup and MutSup, respectively, prepared as noted above) was added to the first of nine wells containing 100 μl of dilution buffer for each preparation tested. Negative controls included the omission of the primary and/or the secondary antibody from the ELISA procedure and/or the omission of Akt from designated wells (blank). The supernatants were serially diluted (twofold) in each subsequent well to a final dilution of 1:512. Where indicated, Akt inhibitor VII was added to each well to a 10 nM final concentration following the addition of each respective supernatant. To determine if PtdIns(3,4,5)P3 could compete with NgPLD for Akt binding, 1× primed wild-type or uninfected control supernatants were each added to 24 wells of a microtitre plate in the absence of or in the presence of 1 pm, 100 pm, or 10 nM PtdIns(3,4,5)P3 (Cayman Chemical, Ann Arbor, MI). In total, 10 nM PtdIns(3,4,5)P3 was added to 48 additional wells in the absence of and in the presence of 0.1×, 0.1×, or 1× primed wild-type or uninfected control supernatants. Three wells were used for each condition assayed, three additional wells were used as negative controls, and each assay was performed in triplicate. For each ELISA described above, microtitre plates were incubated for 2 h at 4°C after which the supernatants were removed, each well was washed six times with PBS-0.05% Tween-20, and subsequently incubated (1 h, RT) in blocking buffer. ELISAs were then performed according to standard protocols using the anti-NgPLD immune sera, 1307, or anti-PtdIns(3,4,5)P3 monoclonal antibody, RC6F8 (Echelon Biosciences, Salt Lake City, UT) and the appropriate peroxidase-conjugated secondary antibody. Absorbance of the o-phenylene-diamine dihydrochloride peroxidase substrate was determined spectrophotometrically at 490 nm.

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