Phosphatidylinositol 3-Kinases in Carcinoembryonic Antigen-related Cellular Adhesion Molecule-mediated Internalization of *Neisseria gonorrhoeae*

James W. Booth‡§¶, David Telio‡§¶, Edward H. Liao, Shannon E. McCawi, Tsuyoshi Matsumoto**, Sergio Grinstein‡ ¶¶, and Scott D. Gray-Owen‡§¶¶

From the ‡Division of Cell Biology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and the ¶Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5S IA8, Canada, and **Takeda Chemical Industries Ltd., Osaka, 540-8645 Japan

*Neisseria gonorrhoeae* can be internalized by mammalian cells through interactions between bacterial opacity-associated (Opa) adhesins and members of the human carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family. We examined the role of phosphatidylinositol 3-kinases (PI3Ks) in gonococcal invasion of epithelial cell lines expressing either CEACAM1 or CEACAM3. CEACAM3-mediated internalization, but not that mediated by CEACAM1, was accompanied by localized and transient accumulation of the class I PI3K product phosphatidylinositol 3,4,5-trisphosphate at sites of bacterial engulfment. Inhibition of phosphatidylinositol 3-kinases reduced CEACAM3-mediated uptake but, paradoxically, led to an increase in intracellular survival of bacteria internalized via either CEACAM1 or CEACAM3, suggesting additional roles for PI3K products. Consistent with this finding, the class III PI3K product phosphatidylinositol 3-phosphate accumulated and persisted in the membrane of gonococcal phagosomes after internalization. Inhibition of PI3K blocked phagosomal acquisition of the late endosomal marker lysosome-associated membrane protein 2 and reduced phagosomal acidification. Inhibiting phagosomal acidification with concanamycin A also increased survival of intracellular gonococci. These results suggest two modes of action of phosphatidylinositol 3-kinases during internalization of gonococci: synthesis of phosphatidylinositol 3,4,5-trisphosphate is important for CEACAM3-mediated uptake, while phosphatidylinositol 3-phosphate is needed for phagosomal maturation and acidification, which are required for optimal bacterial killing.

Gonorrhea is typified by a urethral or cervical exudate that predominantly consists of polymorphonuclear neutrophils with intracellular and extracellular associated *Neisseria gonorrhoeae*. Gonococci interact with various human cell types, including mucosal epithelia, endothelia, and immune cells, through a number of adhesins. Most colony opacity-associated (Opa) protein variants mediate tight bacterial attachment by binding to various members of the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family. Four of these, CEACAMs 1, 3, 5, and 6, can act as receptors for bacteria expressing these adhesins. CEACAM1 is the most broadly distributed, being expressed in a variety of tissues and cell types where it can support bacterial invasion into and trafficking across epithelial layers (1), entry into primary endothelial cells (2), and engulfment by professional phagocytes (3). In contrast, CEACAM3 is expressed exclusively by neutrophils and supports the efficient non-opsonic uptake of Opa-expressing bacteria by a process reminiscent of that mediated by phagocytic Fc receptors (4). This CEACAM3-dependent uptake may constitute an important component of host innate immune defense against gonorrhea.

While binding of Opa-expressing bacteria to CEACAM receptors can lead to internalization of bacteria into the host cell, the molecular mechanisms underlying this uptake are poorly understood. The fate of gonococci after internalization and host cell factors that influence that fate are also unclear. Phosphatidylinositol 3-kinases (PI3Ks) play a key role in signaling downstream of numerous receptors and in orchestrating a range of membrane traffic events (5–7). These kinases generate phosphoinositides bearing phosphate on the 3-position of the inositol ring. The resulting lipid second messengers can be recognized by a variety of effecter proteins, leading to changes in membrane and cytoskeletal dynamics (8). Of note, PI 3-kinases have been implicated in bacterial invasion of epithelial cells by *Listeria monocytogenes* (9) and *Helicobacter pylori* (10). In contrast, invasion of epithelial cells by *Salmonella typhimurium* is PI3K-independent (11). PI3K-mediated uptake is also required for efficient phagocytosis of antibody-coated particles by macrophages, a process of engulfment that is similar in several respects to some forms of bacterial invasion (12, 13).

Given the importance of PI 3-kinases in numerous instances of membrane and cytoskeletal remodeling and our limited un-

---

* This research was funded in part by the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Both authors contributed equally to this work.

† Supported by a fellowship from the Canadian Cystic Fibrosis Foundation.

‡‡ Current holder of the Pitblado Chair in Cell Biology at the Hospital for Sick Children.

¶¶ Supported by a New Investigator Award from the Canadian Institutes of Health Research. To whom correspondence should be addressed: Dept. of Medical Genetics and Microbiology, University of Toronto, 4381 Medical Sciences Bldg., 1 King’s College Circle, Toronto, Ontario M5S IA8, Canada. Tel.: 416-946-5307; Fax: 416-978-6885; E-mail: scott.gray.owen@utoronto.ca.

** The abbreviations used are: Opa, opacity-associated; CEACAM, carcinoembryonic antigen-related cellular adhesion molecule; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PIH, pleckstrin homology; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIP2, phosphatidylinositol 3,4-phosphate; GFP, green fluorescent protein; LAMP-1, lysosome-associated membrane protein 1; LAMP-2, lysosome-associated membrane protein 2; ITAM, immunoreceptor tyrosine-based activation motif.
understanding of the mechanisms of Opa-dependent gonococcal uptake into human cells, we investigated whether PI 3-kinases are involved in uptake of gonococci via CEACAM receptors. To this end, we used human epithelial cells stably transfected with individual CEACAM receptors, a model system that has been used successfully before (3, 4). By expressing single CEACAM receptors in isolation, the reconstituted system circumvents complications arising from the co-expression of multiple CEACAM receptors in native cells. It also has the advantage that the cells are amenable to transfection, whereas primary human cells such as neutrophils are refractory to such manipulations.

Our results reveal major differences in phosphoinositide dynamics during gonococcal internalization depending on the CEACAM receptor used. In addition, distinct 3-phosphoinositol lipids are involved at different stages during gonococcal internalization and the subsequent maturation of the gonococcal phagosome.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids—**Wortmannin and LY294002 were from Calbiochem. TRITC and FITC were from Molecular Probes. Gentamicin was from Bioshop Canada Inc. (Burlington, Ontario, Canada). GC agar and Isovitalex were from BD Biosciences. Concanamycin A was from Sigma. Anti- gonococcal antibody UTR01 was prepared as described previously. Anti-LAMP-2 antibody H4B4 was from the Developmental Studies Hybridoma Bank (University of Iowa). Cy3- and Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Alexa488- and Texas Red-conjugated secondary antibodies and Alexa633-phalloidin were from Molecular Probes. Plasmids encoding chimeras of green fluorescent protein (GFP) with the PH domain of Akt, with two tandem FYVE domains, and with the PX domain of p40

**Cell Culture and Bacterial Growth—**HeLa cells stably expressing CEACAM1 and CEACAM3 have been described previously (3). HeLa-CEACAM1 and HeLa-CEACAM3 cells were grown in RPMI 1640 medium with 10% fetal calf serum. Transient transfections of these cells were performed with FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions with overnight incubations for plasmid expression. *N. gonorrhoeae* N313, a non-piliated strain expressing the CEACAM-binding Opa,

**Gentamicin Resistance Assays—**HeLa-CEACAM1 or HeLa-CEACAM3 cells were infected with gonococci for 30 min. Bars indicate the fraction of total associated bacteria that are internalized. White bars show untreated controls; black bars show cells treated with 100 nM wortmannin. Results for HeLa-CEACAM1 and HeLa-CEACAM3 were from separate experiments performed in triplicate (CEACAM1) or quadruplicate (CEACAM3); bars show means ± S.E. with >30 cells counted in each sample. The number of total associated bacteria per cell is as follows: CEACAM1 control, 12 ± 2; CEACAM1 treated with wortmannin, 11 ± 3; CEACAM3 control, 11 ± 3; CEACAM3 with wortmannin, 17 ± 6. Similar results were obtained for CEACAM3 with 100 μM LY294002.

**FIG. 1.** Inhibition of PI 3-kinases causes enhanced recovery of bacteria in gentamicin resistance assays. Gentamicin resistance assays were performed with HeLa-CEACAM1 or HeLa-CEACAM3 cells as described under Experimental Procedures. A and B, infection of HeLa-CEACAM1 or HeLa-CEACAM3 cells was performed with (black bars) or without (white bars) treatment of cells with 100 nM wortmannin. C and D, infection was performed with (black bars) or without (white bars) treatment of cells with 50 μM LY294002. Means ± S.E. of triplicate samples from individual experiments are shown. Similar effects of PI3K inhibition were seen in four independent experiments. cfu, colony-forming units.

**FIG. 2.** Inhibition of PI 3-kinases does not stimulate bacterial uptake. HeLa-CEACAM1 or HeLa-CEACAM3 cells were infected with gonococci for 30 min. Bars indicate the fraction of total associated bacteria that are internalized. White bars show untreated controls; black bars show cells treated with 100 nM wortmannin. Results for HeLa-CEACAM1 and HeLa-CEACAM3 were from separate experiments performed in triplicate (CEACAM1) or quadruplicate (CEACAM3); bars show means ± S.E. with >30 cells counted in each sample. The number of total associated bacteria per cell is as follows: CEACAM1 control, 12 ± 2; CEACAM1 treated with wortmannin, 11 ± 3; CEACAM3 control, 11 ± 3; CEACAM3 with wortmannin, 17 ± 6. Similar results were obtained for CEACAM3 with 100 μM LY294002.

**References**

1. S. E. McCaw, J. Schneider, E. H. Liao, W. Zimmermann, and S. D. Gray-Owen, manuscript in preparation.
measuring total bacterial association, lysis with saponin was done without prior gentamicin treatment.

**Microscopic Analysis of Invasion**—For microscopy assays, infections were performed as for the gentamicin assays with the following exceptions. HeLa-CEACAM cells were grown on glass coverslips. In experiments for scoring efficiency of uptake, as well as in some of the experiments visualizing GFP probes, the cells were washed three times with RPMI 1640 medium after centrifugation of bacteria to remove loosely adherent bacteria. After centrifugation (and washing when it was performed), cells were incubated with bacteria at 37 °C for various times from 20 min to 3 h. Also a 10-fold lower number of bacteria were added to HeLa-CEACAM1 cells in order to obtain levels of bacterial binding to the cells comparable to that seen with the HeLa-CEACAM3 cells. After infection the cells were washed two times with phosphate-buffered saline + 1 mM CaCl₂, 1 mM MgCl₂ and then fixed in 4% paraformaldehyde. Fixed coverslips were processed for microscopy as follows. Extracellular bacteria were labeled by incubating with polyclonal rabbit anti-gonococcal antibody UTR01 and then Alexa488-anti-rabbit antibody (or, in experiments where GFP constructs were expressed, Cy2-anti-rabbit). Cells were then permeabilized in 0.4% Triton X-100 for 15 min after which they were incubated with UTR01 as before and then with Cy3-goat anti-rabbit; in experiments where scoring of uptake was performed, Alexa633-phallolidin was also included at this step to label actin to facilitate localization of the HeLa cells. Cells were then washed three times in phosphate-buffered saline and mounted using Dako fluorescent mounting medium. All incubations with antibodies were preceded by three washes in phosphate-buffered saline and a 10-min incubation in 0.2% bovine serum albumin to block, and all antibody labeling steps were performed for 1 h at room temperature in the presence of 0.2% bovine serum albumin. For experiments with LAMP-2 labeling, bacteria were detected either by prelabeling with FITC or with UTR01 and Cy3-anti-rabbit, and LAMP-2 was detected with antibody H4B4 and either Texas Red- or Alexa488-conjugated anti-mouse secondary antibodies. Slides were analyzed by confocal microscopy using a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss, Inc.) with a 100× oil immersion objective and the conventional laser excitation and filter sets or by fluorescence microscopy using a Leica DMIRE microscope equipped with a Hamamatsu Orca ER camera controlled by OpenLab v.2.2.5 digital imaging software (Improvision).

For experiments with live cells, bacteria were labeled with TRITC before infection. Confocal stacks of z-slices were acquired at successive time points during infection. Fluorescence intensity was quantified by measuring the mean fluorescence intensity of the 50 brightest pixels in a region of interest containing the phagosome; this was then divided by measuring the mean fluorescence intensity of the 50 brightest pixels in a region of interest containing the phagosome; this was then divided by the average of the mean fluorescence intensities of two separate reference regions of cytoplasm to correct for effects of photobleaching.

**pH Measurements**—Gonococci were prelabeled by incubating in 12 μg/ml FITC in phosphate-buffered saline, pH 8.5, with gentle shaking in the dark for 30 min. Bacteria were then washed in 1% bovine serum albumin to quench residual FITC; and the bacteria were used to infect HeLa-CEACAM3 cells. After 30 min of infection, coverslips were moved into a Leiden chamber and placed on a 37 °C heated stage of a Leica DMIRE/E inverted fluorescence microscope. pH measurements were performed as described previously (19). Briefly, images were acquired using Metafluor software (Universal Imaging) with excitation at either 440 or 490 nm. pH was estimated from the ratio of fluorescence intensity at 440 and 490 nm. For each field acquired, the pH of the extracellular buffer was rapidly changed between 6.15 and 7.35; internal bacteria could be identified retrospectively as those for which the fluorescence intensities did not change significantly upon change of the buffer. pH measurements were performed over a period of 20 min. Calibration was then performed in situ with nigericin and buffers of defined pH values from 5.75 to 7.35. A minimum of 50 bacteria was analyzed at each pH to construct the calibration curves. For determining significance of differences of means, two-tailed Student’s t tests were used.

**RESULTS**

**Inhibition of PI3K Increases Bacterial Recovery in Gentamicin Assays**—We first assessed bacterial invasion of transfected epithelial cells by means of standard gentamicin resistance assays. Transfected HeLa cells stably expressing either CEACAM1 or CEACAM3 were infected with the gonococcal strain N313 expressing the CEACAM-specific Opa57. After allowing for internalization, adherent extracellular bacteria were killed by treatment with the cell-impermeant antibiotic gentamicin. Host cellular membranes were then solubilized to release internal bacteria that were plated on GC agar, and the resulting colonies were counted. To obtain an indication of whether PI 3-kinases are required for bacterial internalization, uptake assays were also performed after pretreatment of the HeLa cells with PI3K inhibitors.

Surprisingly we observed a substantial increase in the number of N. gonorrhoeae colonies recovered after gentamicin treatment when the HeLa-CEACAM3 cells were treated with the PI3K inhibitor wortmannin (Fig. 1A). Similar results were obtained with LY294002, a drug that inhibits PI3Ks by a distinct mechanism (Fig. 1C). Wortmannin and LY294002 also typically increased the recovery of gonococci following their infection of HeLa-CEACAM1 cells, although the amplitude of the effects was somewhat more variable (ranging from an insignificant to a 5-fold increase observed in independent experiments). No difference in the number of total (intracellular plus extracellular) bacteria associated with either of the HeLa cell lines was observed between cells that were untreated or treated with either inhibitor (Fig. 1, B and D).
The unexpected finding that bacterial recovery was increased after treatment with the inhibitors could be interpreted to indicate that impairment of PI3K stimulates bacterial uptake. To test this notion and to obtain an independent measure of bacterial internalization, we directly observed bacterial invasion by fluorescence microscopy. CEACAM-transfected HeLa cells were infected and then fixed and processed for immunofluorescence. Extracellular and internal bacteria were distinguished by immunostaining with an anti-gonococcal antibody before and after permeabilization of the host cells, respectively. Notably, as illustrated in Fig. 2, inhibition of PI3K did not increase the efficiency of uptake mediated by either CEACAM1 or CEACAM3. In fact, for HeLa-CEACAM3 cells, PI3K inhibition significantly lowered the proportion of associated bacteria that became internalized (Fig. 2, \( p < 0.014 \)).

The results in Figs. 1 and 2 indicate that PI3K inhibition leads to no change or a decrease in bacterial uptake as judged by direct visualization yet results in an increase in bacterial recovery in gentamicin assays. While these results appear at first sight contradictory, it should be noted that gentamicin resistance assays reflect a combination of two factors. For bacteria to be recovered as colony-forming units, they must not only be internalized by the eukaryotic cell to avoid contact with gentamicin, but they must also survive in the intracellular milieu until the time of host cell lysis. In this regard, it is noteworthy that a sizable fraction of the bacteria associated with the HeLa cells is seen to be internalized by microscopy (Fig. 2), but only a small fraction of the total cell-associated bacteria is recoverable as gentamicin-resistant colony-forming units at longer times (compare scales of axes in Fig. 1, A and C versus B and D). This implies that extensive death of intracellular gonococci is occurring over time. We hypothesized that the divergent observations made using the two assay systems result from PI3-kinases being involved in multiple steps during bacterial internalization and killing. PI3K activity appears to be required for optimal initial uptake of bacteria via CEACAM3. However, in the absence of PI3K activity, survival of those bacteria that do become internalized via either CEACAM receptor must be enhanced, leading to a greater recovery of gentamicin-resistant bacteria.

Phosphatidylinositol 3,4,5-Trisphosphate (PIP \(_3\) ) Accumulates Locally during CEACAM3-mediated Uptake—In further considering possible roles for PI3-kinases during bacterial invasion, it is important to note that several classes of PI3Ks are inhibited by LY294002 and wortmannin, and the activity of these different enzymes can result in synthesis of distinct 3-phosphoinositides. Class I PI3Ks in vivo use mainly phosphatidylinositol 4,5-bisphosphate as a substrate to generate PIP\(_3\), whereas class III PI3Ks generate predominantly phosphatidylinositol 3-phosphate (PI3P) using phosphatidylinositol as a substrate (20). These phosphoinositide species perform distinct functions in cell signaling and may be differentially involved in bacterial invasion and phagosome maturation.

A major advance in studies of phosphoinositide metabolism has been the recent development of probes consisting of GFP fused to protein domains that bind with high affinity to specific phosphoinositides (21). Using such probes, localized accumulation of specific phosphoinositide second messengers can be evaluated with high spatial and temporal resolution. It was thus demonstrated that engagement of Fc receptors can lead to highly localized accumulation of PIP\(_3\), presumably via activation of a class I PI3K (22). CEACAM3, but not CEACAM1, contains an ITAM similar to those found in immune receptors.
like the Fcγ receptor. It was therefore conceivable that a class I PI3K might be differentially involved in uptake mediated by these two distinct CEACAM receptors. To analyze this possibility, HeLa cells expressing CEACAM1 or CEACAM3 were transiently transfected with a plasmid expressing the PH domain of Akt, which binds with high affinity to PIP3, fused to GFP (Akt-PH-GFP). The Akt-PH-GFP probe localizes to the cytoplasm and nucleus in uninfected cells (data not shown). However, upon infection of HeLa-CEACAM3 cells with gonococci, we observed a striking accumulation of the probe at sites where bacteria bound to the cell surface (Fig. 3, A and B). Akt-PH-GFP was particularly concentrated in pseudopod-like extensions of the plasma membrane extending around the bacteria (Fig. 3, A inset). The accumulation of Akt-PH-GFP was abolished upon inhibition of PI3K by wortmannin (Fig. 3, C and D), confirming that the probe reports the distribution of 3-phosphoinositides. The main product of PI3K sensed by the probe is likely PIP3, although phosphatidylinositol 3,4-bisphosphate, which binds to the probe in vitro, may contribute to the signal. Notably, in contrast to the observations in HeLa-CEACAM3 cells, no noticeable accumulation of Akt-PH-GFP at sites of bacterial entry was observed in HeLa-CEACAM1 cells (Fig. 3, E and F). Thus, activation of localized synthesis of PIP3 is specific to the ITAM-containing CEACAM3 receptor.

Accumulation of Akt-PH-GFP in HeLa-CEACAM3 cells was rarely seen on the membrane of phagosomes containing fully internalized bacteria. The paucity of Akt-PH-GFP in sealed phagosomes suggests that PIP3 accumulation is a transient phenomenon during bacterial uptake. To better appreciate the kinetics of PIP3 accumulation, the dynamics of Akt-PH-GFP localization were followed in living HeLa-CEACAM3 cells during bacterial infection (Fig. 4). Accumulation of the probe occurred while bacteria were bound to the cell surface. The intensity peaked at roughly the time of phagosomal closure as estimated from reconstructions in the xz and yz planes of stacks of confocal images (data not shown). Following internalization of the bacteria, the fluorescence intensity on the formed phagosome rapidly declined until no enhancement over the cytoplasmic intensity could be observed. Quantitation of a representative experiment is shown in Fig. 4G. Thus, localized PIP3 accumulation is an early and transient event during CEACAM3-mediated internalization of bacteria.

PI3P Accumulates in Phagosomes Containing Gonococci—As discussed above, the enhanced recovery of gentamicin-resistant bacteria after inhibition of PI3K is not due to an increase in internalization, implying an increase in bacterial survival inside the cells. The early and transient appearance of PIP3 during uptake mediated by CEACAM3, but not CEACAM1, makes it unlikely that this 3-phosphoinositide is regulating bacterial survival within the cell, which was enhanced by PI3K.

Fig. 5. PI3P accumulates on gonococcal phagosomes. HeLa-CEACAM3 (A–I) or HeLa-CEACAM1 (J–L) cells were transfected with phox-GFP (A–F) or 2-FYVE GFP (G–I) and infected with gonococci for 60–75 min (A–F) or 30 min (G–I). A, D, G, and J show GFP fluorescence. B, E, H, and K show total bacteria. C, F, I, and L show external bacteria. In D–F, cells were treated with 100 nM wortmannin. Arrows indicate internalized bacteria that do not label with phox-GFP after wortmannin treatment. Bars, 5 μm.
inhibitors in both CEACAM1- and CEACAM3-expressing cells. An alternate PI3K product that may be involved in regulating bacterial survival after internalization is the singly phosphorylated PI3P. This lipid is produced in vivo primarily through action of class III PI 3-kinases and is localized predominantly in endosomal compartments (6). To test the possible involvement of PI3P in bacterial invasion and intracellular traffic, two probes that specifically bind to this phospholipid were used. These probes consist of GFP fused to either the PX domain of the p40phox subunit of the NADPH oxidase (phox-GFP) or to two tandem repeats of the FYVE domain from EEA1 (early endosome autoantigen 1; 2-FYVE-GFP). Both have been used previously as probes of PI3P localization (15, 16). First HeLa-CEACAM3 cells were transiently transfected with phox-GFP. In uninfected cells, the probe localizes to the cytoplasm as well as to small vesicular structures, reflecting the existence of PI3P in endosomal compartments. These cells were then infected with gonococci. In contrast to the early, transient accumulation of Akt-PH-GFP, phox-GFP was primarily seen on phagosomes that specifically bind to this phospholipid were used. These probes consist of GFP fused to either the PX domain of the p40phox subunit of the NADPH oxidase (phox-GFP) or to two tandem repeats of the FYVE domain from EEA1 (early endosome autoantigen 1; 2-FYVE-GFP). Both have been used previously as probes of PI3P localization (15, 16). First HeLa-CEACAM3 cells were transiently transfected with phox-GFP. In uninfected cells, the probe localizes to the cytoplasm as well as to small vesicular structures, reflecting the existence of PI3P in endosomal compartments. These cells were then infected with gonococci. In contrast to the early, transient accumulation of Akt-PH-GFP, phox-GFP was primarily seen on phagosomes (Fig. 5, A–C). On occasion, it did appear to localize also to phagosomes that had not yet closed in which bacteria were still accessible to labeling with external antibodies; however, such recruitment occurred only occasionally and was less intense (data not shown). In contrast, virtually all intracellular bacteria were found to be in phagosomes that accumulated phox-GFP (Fig. 5, A–C). The acquisition of phox-GFP appeared to be long-lived as phagosomes were still labeled with the probe several hours after infection (data not shown). As expected, phox-GFP localization to phagosomes was abolished by wortmannin treatment (Fig. 5, D–F). Similar persistent accumulation on gonococcal phagosomes was seen for the 2-FYVE-GFP probe, verifying the presence of PI3P (Fig. 5, G–I). We further examined the kinetics of PI3P accumulation by following the phox-GFP probe during infection in living HeLa-CEACAM3 cells (Fig. 6). These experiments indicated that phox-GFP probe was rapidly acquired shortly after the apparent closure of the phagosome and persisted for long periods of time (>1 h).

In infected HeLa-CEACAM1 cells, phox-GFP and 2-FYVE-GFP were also found to accumulate on gonococcal phagosomes (Fig. 5, J–L, and data not shown). Unlike phagosomes formed by CEACAM3-expressing cells, which were uniformly labeled, only some of the apparently internalized bacteria were positive for the probes in the HeLa-CEACAM1 cells. It is unclear whether this reflects the existence of two subpopulations of CEACAM1 phagosomes or a heterogeneous kinetics of acquisition and/or loss of PI3P.

**PI3K Activity Is Required for Phagosomal Maturation**—The appearance of PI3P on the bacteria-containing phagosome suggests that this phosphoinositide plays a role in the maturation process that confers microbicidal properties to the phagosome. To test whether inhibition of class III PI3Ks affects phagosomal maturation, we measured the acquisition by phagosomes of the late endosomal and lysosomal marker LAMP-2. While LAMP-1 is cleaved by the IgA1 protease that is secreted by N. gonorrhoeae, LAMP-2 is not targeted by this enzyme and thus remains a reliable marker (23, 24). An hour after infection, the majority of phagosomes in HeLa-CEACAM1 cells are positive for LAMP-2, indicating fusion with endosomal compartments (Fig. 7, A and B). Remarkably, in cells treated with PI3K...
inhibitors, acquisition of this marker is substantially reduced (Fig. 7, C–E).

While LAMP-2 acquisition provides an indication of the extent of fusion of the phagosome with late endosomal/lysosomal compartments, it is unclear whether this protein plays any role in bacterial killing. We therefore sought to obtain a separate functional measure of the killing capacity of the phagosomes containing gonococci. An important aspect of phagosomal maturation in professional phagocytes that is believed to contribute to the bacteriostatic or bactericidal character of the phagosome is the acidification of its lumen. Thus, we investigated whether acidification of the gonococci-containing phagosome is a PI3K-dependent process. Gonococci were covalently labeled with a pH-sensitive dye and allowed to invade HeLa-CEACAM3 cells. The pH of phagosomes containing internalized bacteria was then determined by ratiometric imaging of bacterial fluorescence inside live HeLa cells. In control cells, after 30 min of internalization bacteria were found to reside in an acidified compartment with a mean pH of 5.85 (Fig. 8A). In wortmannin-treated cells, phagosomes also acidified, but the acidification was significantly attenuated (mean pH value of 6.18, p < 3.5 × 10⁻⁵). Thus, PI3K activity is required for optimal acidification of the gonococcal phagosome.

These results raised the possibility that the inability of phagosomes to acidify fully contributes to the increased gonococcal survival in cells treated with PI3K inhibitors. We therefore tested whether direct inhibition of acidification could similarly promote intracellular survival. To this end, cells were treated with concanamycin A, an inhibitor of the vacuolar (H⁺)-ATPases. As illustrated in Fig. 8, the number of viable bacteria recovered in gentamicin resistance assays was significantly enhanced upon drug treatment. Microscopic analysis of invasion confirmed that concanamycin A treatment does not enhance internalization per se (data not shown), indicating that the increased bacterial recovery must have resulted from improved intracellular survival. Consistent with the model that inhibition of PI 3-kinase activity favors gonococcal survival by impairing acidification of the bacteria-containing phagosome, we found that the effects of LY294002 and concanamycin A on bacterial recovery were not additive (Fig. 8B). In fact, treatment with a combination of LY294002 and concanamycin A led to a lower recovery of bacteria than that in cells treated with concanamycin A alone, as would be expected if LY294002 inhibits bacterial internalization via CEACAM3, while having little or no positive effect on survival beyond that already achieved with concanamycin A. Thus, PI3K-dependent phagosomal acidification appears to be an important factor in determining the extent of gonococcal killing by the host cells.

**DISCUSSION**

Our results reveal distinct roles for two types of PI3K during CEACAM-mediated internalization and elimination of *N. gonorrhoeae* by human cells. PIP₃, locally accumulates during uptake via CEACAM3 but not CEACAM1. This accumulation is reminiscent of that seen during Fcγ receptor-mediated phagocytosis by macrophages (22). CEACAM3, but not CEACAM1, contains in its cytoplasmic domain an ITAM, similar to those found in Fc receptors, that is involved in signaling cytoskeletal changes underlying CEACAM3-mediated uptake (4, 25). Our results demonstrate that binding of Opa-expressing bacteria to CEACAM3 receptors also induces localized recruitment and/or activation of a class I PI 3-kinase and suggest that the activity of this kinase is required for optimal uptake. Inhibition of PI3K does not, however, lead to a complete block in uptake of gonococci. Previous experiments with Fcγ receptor-mediated engulfment demonstrated that the dependence of phagocytosis on PI3K activity was not absolute, decreasing in direct proportion with the size of the particles being engulfed (12). Interestingly when IgG-opsonized beads of 1-μm diameter, the approximate size of gonococci, were used, Fc receptor-mediated phagocytosis was also only partially inhibited by blockade of PI3K (12) as seen here for CEACAM3-mediated uptake of bacteria.
Gentamicin resistance assays are routinely used to measure bacterial internalization into mammalian cells. The discrepancies we observed between invasion measured by such assays and that measured by direct visualization underscore the fact that gentamicin assays must be interpreted cautiously as they require intracellular survival of the bacteria for final detection. This caveat is particularly important in cases like that of gonococci in our system in which extensive intracellular death of bacteria is apparent.

Little is known about the intracellular life of gonococci. They are capable of performing Opa-dependent transcytosis across epithelial cell layers (26), a process that is presumably important in bacterial colonization of the submucosa (27). Transepithelial migration occurs through an intracellular route and takes many hours to occur (26). Clearly then at least some bacteria must survive sufficiently long periods to emerge from the basolateral side of the epithelial cells. In the HeLa-CEACAM cells, bacteria do not appear to replicate in the intracellular compartment; rather the number of viable intracellular bacteria steadily declines over several hours (data not shown). This stands in contrast to bacteria that actively replicate within either epithelial or phagocytic cells, such as Salmonella spp. It should be noted, however, that prolonged survival of even a low percentage of internalized gonococci may be a crucial factor in determining the outcome of infection. Therefore, it is important to understand the factors that influence the intracellular fate of these bacteria. Our results imply that PI3K activity is an important factor in the maturation of the gonococcal phagosome, most likely by virtue of its generation of PI3P on phagosomal membranes. The role of PI 3-kinases in affecting intracellular survival of other pathogens is poorly understood. Mycobacteria promote their survival within macrophages by arresting phagosome maturation at an early stage, and this may be attributed to inhibition of class III PI3Ks by mycobacterial products (28). However, the role of PI3K in mycobacterial survival has not been directly investigated. The role of PI3K in intracellular survival of Salmonella is controversial. In one report, inhibition of PI3K had no effect on intracellular replication of bacteria (29), whereas in another report, replication of Salmonella was enhanced when assessed following several hours after internalization (30). Thus, further analysis of the role of phosphoinositides in survival of intracellular bacteria will be important.

A striking finding in our study was the persistence of PI3P in the membrane of phagosomes formed upon ingestion of gonococci by HeLa-CEACAM3 cells. PI3P accumulated on the phagosomes for at least several hours after bacterial uptake. This observation stands in sharp contrast to the results reported for phagosomes formed by Fc receptor-mediated phagocytosis or for Salmonella-containing vacuoles formed in epithelial cells. In both of these cases, PI3P accumulated only transiently, largely disappearing within 30 min (16, 29, 31, 32). This suggests basic differences in the process of maturation of the gonococcal phagosomes. These differences may reflect interference with phagosomal maturation induced by bacterial factors, e.g. the porin PorB (33) or the secreted endopeptidase that cleaves LAMP-1 (24), or may be due to factors specific to signaling through CEACAM receptors. Studies to address these issues will help to reveal how such factors influence the intracellular processing of this important pathogen.

Acknowledgments—We thank Dr. Keith Ireton for helpful advice throughout this work and Drs. Michael Yaffe and Tobias Meyer for kindly providing the phox-GFP and Akt-GFP constructs.

REFERENCES
1. Dehio, C., Gray-Owen, S. D., and Meyer, T. F. (1998) Trends Microbiol. 6, 489–495
2. Muenzner, P., Dehio, C., Fujisawa, T., Achtman, M., Meyer, T. F., and Gray-Owen, S. D. (2000) Infect. Immun. 68, 3681–3687
3. Gray-Owen, S. D., Lorenzen, D. R., Haude, A., Meyer, T. F., and Dehio, C. (1997) Mol. Microbiol. 26, 971–980
4. Billker, O., Popp, A., Brinkmann, V., Wenig, G., Schneider, J., Caron, E., and Meyer, T. F. (2002) EMBO J. 21, 560–571
5. Cantley, L. C. (2002) Science 296, 1655–1657
6. Simonsen, A., Wurmser, A. E., Emr, S. D., and Stenmark, H. (2001) Curr. Opin. Cell Biol. 13, 485–492
7. Corvera, S. (2001) Traffic 2, 859–866
8. Cullen, P. J., Cozier, G. E., Banting, G., and Mellor, H. (2001) Curr. Biol. 11, R882–R893
9. Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaeu, H., Kasuga, M., and Cassart, P. (1996) Science 274, 780–782
10. Kwok, T., Backert, S., Schwarz, H., Berger, J., and Meyer, T. F. (2002) Infect. Immun. 70, 2108–2120
11. Mecsas, J., Raupach, B., and Falkow, S. (1998) Mol. Microbiol. 28, 1269–1281
12. Cox, D., Tseng, C. C., Bjeck, G., and Greenberg, S. (1999) J. Biol. Chem. 274, 1240–1247
13. Arakli, N., Johnson, M. T., and Swanson, J. A. (1996) J. Cell Biol. 135, 1249–1260
14. Haugh, J. M., Codazzi, F., Teruel, M., and Meyer, T. (2000) J. Cell Biol. 151, 1269–1280
15. Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., Cantley, L. C., and Yaffe, M. B. (2001) Nat. Cell Biol. 3, 675–684
16. Vieira, O. V., Botelho, R. J., Rameh, L., Brachmann, S. M., Matsuo, T., Davidson, H. W., Schreiber, A., Backer, J. M., Cantley, L. C., and Grinstein, S. (2001) J. Cell Biol. 155, 19–25
17. Kunsch, E. M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T. F. (1993) EMBO J. 12, 641–650
18. Gray-Owen, S. D., Dehio, C., Haude, A., Grunert, F., and Meyer, T. F. (1997)
19. Demaurex, N., Romanek, R., Rotstein, O., and Grinstein, S. (1998) in Cell Biology: A Laboratory Handbook (Celis, J., ed) 2nd Ed, pp. 380–386, Academic Press, San Diego
20. Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Kats, R., Driscoll, P. C., Waskolski, R., Parker, P. J., and Waterfield, M. D. (2001) Annu. Rev. Biochem. 70, 585–602
21. Balla, T., and Varnai, P. (2002) Science’s STKE http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2002/125/pl3
22. Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T., and Grinstein, S. (2001) J. Cell Biol. 153, 1369–1380
23. Hauck, C. R., and Meyer, T. F. (1997) FEBS Lett. 405, 86–90
24. Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlson, S. R., Enns, C., and So, M. (1997) Mol. Microbiol. 24, 1083–1094
25. Chen, T., Bolland, S., Chen, I., Parker, J., Pantelic, M., Grunert, F., and Zimmermann, W. (2001) J. Biol. Chem. 276, 17413–17419
26. Wang, J., Gray-Owen, S. D., Knorre, A., Meyer, T. F., and Dehio, C. (1998) Mol. Microbiol. 30, 657–671
27. McGee, Z. A., Stephens, D. S., Hoffman, L. H., Schlech, W. F., III, and Horn, R. G. (1983) Rev. Infect. Dis. 5, 8708–8714
28. Pratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S., and Deretic, V. (2001) J. Cell Biol. 154, 631–644
29. Scott, C. C., Cuellar-Mata, P., Matao, T., Davidson, H. W., and Grinstein, S. (2002) J. Biol. Chem. 277, 12770–12776
30. Steele-Mortimer, O., Brumell, J. H., Knodler, L. A., Meresse, S., Lopez, A., and Finlay, B. B. (2002) Cell Microbiol. 4, 43–54
31. Elson, C. D., Anderson, K. E., Morgan, G., Chilvers, E. R., Lipp, P., Stephens, L. R., and Hawkins, P. T. (2001) Curr. Biol. 11, 1631–1635
32. Pattni, K., Jepson, M., Stenmark, H., and Banting, G. (2001) Curr. Biol. 11, 1636–1642
33. Mosleh, I. M., Huber, L. A., Steinlein, P., Pasquali, C., Gunther, D., and Meyer, T. F. (1998) J. Biol. Chem. 273, 35332–35338
Phosphatidylinositol 3-Kinases in Carcinoembryonic Antigen-related Cellular Adhesion Molecule-mediated Internalization of Neisseria gonorrhoeae

James W. Booth, David Telio, Edward H. Liao, Shannon E. McCaw, Tsuyoshi Matsuo, Sergio Grinstein and Scott D. Gray-Owen

J. Biol. Chem. 2003, 278:14037-14045.
doi: 10.1074/jbc.M211879200 originally published online February 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211879200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 15 of which can be accessed free at http://www.jbc.org/content/278/16/14037.full.html#ref-list-1