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HELICOBACTER PYLORI REGULATES CELLULAR MIGRATION AND APOPTOSIS BY ACTIVATION OF PI3K SIGNALING

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Abstract

Helicobacter pylori is the strongest identified risk factor for gastric adenocarcinoma. One *H. pylori* virulence constituent that augments cancer risk is the *cag* secretion system, which translocates CagA and peptidoglycan into host cells, eventuating in activation of signal transduction pathways. AKT is a target of phosphatidylinositol 3-phosphate kinase (PI3K) and is activated in gastric cancer, but the relationship between PI3K-AKT and *H. pylori*-induced cellular responses with carcinogenic potential remains unclear. We defined the molecular pathways mediating *H. pylori*-stimulated AKT activation and the biological consequences of these events in gastric epithelial cells. *H. pylori* enhanced PI3K-AKT signaling in a Src and EGFR-dependent manner, which was also mediated by a functional *cag* secretion system, and peptidoglycan. PI3K activation attenuated apoptosis in response to infection and was required for *H. pylori*-induced cell migration. These results indicate that PI3K-AKT signaling regulates pathophysiologic responses to *H. pylori* that may lower the threshold for carcinogenesis.

Keywords

PI3K; AKT; *Helicobacter*; cancer; migration; apoptosis

Introduction

Chronic gastritis induced by *Helicobacter pylori* persists for decades and increases the risk of gastric adenocarcinoma [1]. Although *H. pylori*-induced gastritis is the strongest known risk factor for gastric cancer, only a fraction of colonized individuals ever develop neoplasia, and enhanced cancer risk is mediated by strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity. The *cag* pathogenicity island (*cag* PAI) is a virulence locus present in approximately 60% of U.S. *H. pylori* strains [1], and strains that

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harbor the *cag* PAI (*cag*$^+$) significantly augment the risk for distal gastric cancer compared to strains that lack the *cag* island (*cag*$^-$) [1].

Several *cag* genes, such as *cagE*, encode components of a type IV secretion system that exports bacterial proteins into host cells. The terminal product of the *cag* island, CagA, is translocated into gastric epithelial cells following bacterial attachment [2,3]. CagA subsequently undergoes tyrosine phosphorylation by Src and Abl kinases, and phospho-CagA alters gastric cell morphology and aberrantly activates signaling molecules such as SHP-2 [4,5]. Unphosphorylated CagA can also exert effects within host cells such as alteration of cell polarity and activation of β-catenin, responses that have been implicated in carcinogenesis [6,7]. In addition to CagA, components of peptidoglycan can be translocated into host cells by the *cag* secretion system where they are sensed by the intracellular pattern recognition receptor Nod1, which activates NF-κB and induces production of pro-inflammatory cytokines such as IL-8 [8].

Signal transduction pathways activated in response to bacterial contact play an important role in *H. pylori* pathogenesis. Phosphatidylinositol 3-kinase (PI3K) is an integral component of a signal transduction pathway that regulates host cellular responses altered in tumorigenesis. PI3K signaling can be activated by ligand-dependent activation of receptor tyrosine kinases such as EGFR [9]. Src kinases, acting both downstream and upstream of EGFR, can also activate PI3K signaling [10]. PI3K activation results in stimulation of phosphatidylinositol-dependent kinase 1 (PDK1), a kinase that phosphorylates and activates AKT [11]. AKT mediates the downstream effects of PI3K by phosphorylating multiple targets that regulate diverse cellular functions including proliferation and survival. PI3K-AKT signaling is increased in gastric cancer specimens and enhanced levels of AKT phosphorylation correlate with advanced stages of disease [12]. Thus, PI3K is well positioned to regulate epithelial responses that may predispose to malignancies.

Cellular migration plays an important role in the invasive potential and metastatic growth of cancers and *H. pylori* can increase gastric epithelial cell migration, although the mechanisms required for this response are not clearly defined [13,14]. Of note, EGFR transactivation increases intestinal epithelial cell motility in a PI3K- and Src-dependent manner [15]. Cell survival is another response that is regulated by PI3K and AKT activation [16]. AKT-dependent phosphorylation of pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins [17] and pro-caspase 9 [18] attenuates apoptosis, thereby promoting cell survival and enhancing the susceptibility of cells to mutagenesis. Since *H. pylori* increases cell proliferation and attenuates apoptosis in humans and in rodent models of infection [19,20], we determined the ability of *H. pylori* to activate PI3K-AKT signaling in gastric epithelial cells and investigated the molecular pathways mediating these events to define potential tumor-promoting responses toward this pathogen.

Methods

Cell Culture and Reagents

AGS or MKN28 human gastric epithelial cells were grown in RPMI medium 1640 (GIBCO/BRL) with 10% FBS (Sigma) and 20 μg/ml gentamicin (GIBCO/BRL) under 5% CO_2 air at 37°C. Pharmacological inhibitors LY294002 (Cell Signaling Technology), AG1478 (Calbiochem), PP2 (Calbiochem), SU6656 (Calbiochem), AG1295 (Calbiochem), and STI-571 (LC Laboratories) were used at concentrations of 50 μM, 600 nM, 10 μM, 2μM, 50μM and 10μM, respectively. For Western immunoblot and flow cytometry analysis, AGS cells were plated at 5×10^5 cells/well in 6-well plates in 2 mL culture medium. For cell migration assays, 5×10^5 cells were plated in 35 mm culture dishes in 2 mL medium.

H. pylori strains

The *H. pylori cag*$^+$ rodent-adapted strain 7.13, the *cag*$^+$ clinical strain J166, or the *cag*$^-$ clinical isolate J68, were grown in *Brucella* broth with 5% FBS for 18 hours, harvested by centrifugation, and were added to gastric cells at a bacteria-to-cell ratio of 100:1. Isogenic *cagA*$^-$, *cagE*$^-$, and *slt*$^-$ null mutants were constructed within strain 7.13 by insertional mutagenesis using *aphA* and were selected with kanamycin (25 μg/ml) as described previously [21]. *H. pylori* were heat-killed by boiling at 100°C for 10 minutes, while *H. pylori* filtrates were prepared by passing broth supernatants through a 0.2 μM pore-size filter (Corning).

Western Blot Analysis

Gastric cell lysates were harvested in lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) including protease and phosphatase inhibitors (Sigma). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred to poly-vinylidene difluoride membranes (Pall). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST), incubated for 24 hours with a purified rabbit polyclonal anti-phospho-AKT antibody (1:1000 dilution; Cell Signaling), a rabbit polyclonal anti-total AKT antibody (1:1000 dilution; Cell Signaling), a monoclonal mouse anti-GAPDH antibody (1:2000 dilution; Santa Cruz Biotech), a mouse monoclonal anti-phospho-tyrosine-99 antibody (1:300 dilution; Santa Cruz Biotech), a rabbit polyclonal anti-CagA antibody (1:5000 dilution; Austral Biologicals), a rabbit polyclonal anti-phospho-Src family antibody (1:1000 dilution; Cell Signaling), a rabbit polyclonal anti-Src antibody (1:1000 dilution; Cell Signaling), a mouse monoclonal anti-phospho-EGFR (Tyr1068) antibody (1:1000; Millipore), a rabbit polyclonal anti-EGFR antibody (1:3000; Millipore), a rabbit polyclonal anti-phospho-Gab1 antibody (1:1000; Cell Signaling), or a rabbit polyclonal anti-Gab1 antibody (1:1000 dilution; Cell Signaling). Goat anti-rabbit (1:5000 dilution; Santa Cruz Biotech) or goat anti-mouse (1:5000 dilution; Santa Cruz Biotech) horseradish peroxidase-conjugated secondary antibodies were used followed by enhanced chemiluminescence detection following the manufacturer's instructions (Perkin Elmer). Immunoblots were quantified with the GeneTools Software (Syngene).

Flow Cytometry Analysis

AGS cells co-cultured with *H. pylori* were washed with PBS and harvested using 0.25% Trypsin/EDTA (GIBCO/BRL). Cells were collected by centrifugation and resuspended in binding buffer (10x: 0.1 M HEPES pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 5×10^5 cells/mL. Cells were stained with Annexin V-APC (BD Bioscience) and Propidium iodide/RNAse (BD Bioscience) and were analyzed by quantitative flow cytometry.

Apoptotic Resistance Assays

AGS cells were infected with *H. pylori* for 3 hours followed by incubation with 50 μM LY294002 for 1 hour. After a four hour exposure to 1 μM Staurosporine (Sigma-Aldrich), cells were harvested for Annexin V-flow cytometry analysis as described [22].

Cell Migration Analysis

Confluent AGS cell monolayers in plates coated with 2.5 μg human fibronectin (BD Bioscience) were pre-incubated with pharmacological inhibitors for one hour. Eight circular wounds were generated in each plate using a rotating silicon tip [23]. *H. pylori* was then added to the cells and wound images were taken at zero, six and sixteen hours post infection using Q-Capture Imaging Software. Areas were measured using Image J software (NIH).

Transient transfection of siRNA

AGS cells (2.5×10^5) in 6-well plates were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, transfection reagent (5.0 μl/well) was mixed with siRNA oligos (10 μl of 10 μM solution/well) in 500 μl Opti-MEM (Life Technologies). Cells were incubated with the transfection mixture for 24 hours, fresh medium was added, and bacterial co-cultures were performed 24 hours later.

Statistical Analysis

All experiments were performed on at least three independent occasions. Statistical analysis was performed by Student's t test and ANOVA using Prism Graph Pad. A P-value < 0.05 was defined as statistically significant.

Results

H. pylori activates PI3K-AKT signaling in gastric epithelial cells

We used a *cag*$^+$ *H. pylori* strain, 7.13, that reproducibly causes gastric cancer in rodent models, to determine whether *H. pylori* alters AKT activity. AGS cells were infected with strain 7.13 at a multiplicity of infection (MOI) of 100 or were exposed to medium alone. *H. pylori* strain 7.13 increased AKT phosphorylation compared to uninfected controls at each time point (Figure 1). Similar patterns of AKT activation were observed following infection of AGS cells with the *cag*$^+$ human clinical isolate J166 or infection of MKN28 human gastric epithelial cells with strains 7.13 or J166 (data not shown).

Inactivation of cagE abolishes AKT activation by H. pylori

We next investigated the role of bacterial factors in PI3K-AKT signaling events. Neither heat-killed bacteria nor soluble factors contained in *H. pylori* filtrates stimulated phosphorylation of AKT, indicating that viable *H. pylori* are required for AKT activation (Figure 2A, 2B). The *cag* secretion system encodes several proteins that affect cellular signaling after live *H. pylori* have bound host cells. To define the role of *cag* components in AKT activation, AGS cells were incubated with the *H. pylori cag*$^+$ strain 7.13 or its isogenic *cagA*$^-$ or *cagE*$^-$ null mutant derivatives. AKT activation was significantly decreased in cells incubated with the 7.13 *cagE*$^-$, but not the *cagA*-, mutant versus the wild-type strain (Figure 2C, 2D). Similarly, the *cag*$^-$ clinical isolate J68 failed to induce AKT phosphorylation (data not shown). These findings indicate that a functional *cag* secretion system, but not *cagA*, is required for induction of PI3K-AKT signaling.

Peptidoglycan is required for activation of AKT by H. pylori

In addition to CagA, peptidoglycan can be translocated by the *cag* secretion system, and can alter host signaling. Therefore, we examined the role of peptidoglycan in AKT activation using a 7.13 isogenic *slt* mutant. The *slt* mutant lacks the soluble lytic transglycosylase (slt) required for peptidoglycan turnover and release. We first established that inactivation of *slt* in strain 7.13 does not alter CagA translocation into host cells (Figure 3A). We then co-cultured AGS cells with wild-type strain 7.13 or the isogenic *slt*$^-$ mutant. Cells co-cultured with the *slt*$^-$ mutant contained significantly lower levels of phospho-AKT compared to cells infected by wild-type 7.13 (Figure 3B, 3C). These results indicate that peptidoglycan, in conjunction with a functional *cag* secretion system, is required for maximal AKT stimulation by *H. pylori*.

H. pylori-induced AKT activation is dependent on activation of PI3K, Src, and EGFR

AKT activation is regulated by many of the same constituents that are activated by *H. pylori* (e.g., EGFR, Src) [9,10,24,25]. As a prelude to defining the molecular pathways mediating *H. pylori*-induced AKT activation, we first confirmed that our prototype strain could activate

EGFR and Src, and also established the efficacy of PI3K, EGFR and Src inhibitors. *H. pylori* strain 7.13 induced phosphorylation of Src and EGFR in AGS cells (Figure 4A). AGS cell lysates were then assessed for phospho-AKT after stimulation with EGF, a potent inducer of AKT activation. Each inhibitor was sufficient to attenuate EGF-stimulated AKT activation (Figure 4B). AGS cells were then co-cultured with strain 7.13 in the absence or presence of the PI3K inhibitor LY294002 or the Src inhibitor PP2. *H. pylori* alone activated AKT by two hours (Figure 4C, 4D). As predicted, AKT activation in response to *H. pylori* was completely abolished by PI3K inhibition (Figure 4C, 4D). PI3K-dependent AKT activation was further confirmed using an independent PI3K inhibitor, Wortmannin (200nM) (data not shown).

H. pylori-induced activation of AKT was also dependent on Src, as treatment with the Src inhibitor PP2 blocked AKT activation (Figure 4C, 4D). The inhibitor PP2, however, can also exert activity against platelet-derived growth factor receptor (PDGFR) signaling as well as signaling initiated by c-Abl and c-kit [26]. Therefore, we repeated co-culture experiments in the presence or absence of specific inhibitors of these pathways. *H. pylori*-induced AKT activation was unchanged in the presence of PDGFR or c-Abl/c-kit inhibitors (Figure 4E, 4F), indicating that Src plays a role in microbial-induced activation of AKT.

EGFR transactivation can mediate PI3K and Src activation and this receptor can be transactivated by *H. pylori* (Figure 4A) [24]; therefore we next determined the role of EGFR in *H. pylori*-induced AKT activation. Co-culture of AGS cells with *H. pylori* in the presence of the EGFR kinase inhibitor AG1478 significantly reduced AKT phosphorylation to levels seen in uninfected controls (Figure 4C, 4D). However, the EGFR inhibitor AG1478 also inhibits FAK, a component of another PI3K-dependent pathway. Therefore, to rule out involvement of FAK, we determined whether *H. pylori* infection could stimulate Gab1 phosphorylation, an event dependent on EGFR transactivation. As shown in Figure 4G, co-culture with wild-type strain 7.13 induced Gab1 phosphorylation. The pattern of Gab1 phosphorylation mirrored AKT activation as *H. pylori* mutant strains that lacked *cagE* or *slt* failed to induce phosphorylation of Gab1 (Figure 4G). Finally, to more firmly implicate EGFR and Src signaling in these events, we co-cultured *H. pylori* strain 7.13 with AGS cells in the presence or absence of a Src family kinase inhibitor that does not activate PDGFR (SU6656) [27]. As demonstrated in Figures 4E and 4F, pre-incubation with SU6656 attenuated the ability of *H. pylori* to activate AKT. Collectively, these results indicate that transactivation of EGFR and Src activation are likely required for *H. pylori*-induced AKT activation.

PI3K signaling is required for cell migration in response to H. pylori

Colonic epithelial migration is dependent on PI3K and Src activation [28]. To determine if *H. pylori* promotes cell migration in a PI3K-dependent manner, AGS cells were treated with the PI3K inhibitor LY294002 and infected with *H. pylori*. Wounds were then induced and measured over time using time-lapse microscopy. Inhibition of PI3K did not significantly alter cell motility in uninfected cells (Figure 5A, 5B). *H. pylori* significantly increased wound healing compared to uninfected cells, but this was abolished by inhibition of PI3K (Figure 5A, 5B). Treatment of cells with EGFR (AG1478) and Src (PP2) inhibitors also blocked migration in response to *H. pylori* (Figure 5C), which mirrored results investigating the effects of *H. pylori* on AKT activation (Figure 4). Since PP2 can also inhibit PDGFR signaling, we repeated migration assays in the presence or absence of the specific PDGFR inhibitor AG1295. *H. pylori*-induced cell migration was unchanged in the presence of the PDGFR inhibitor (Figure 5C), indicating that Src likely plays a role in cell motility that is induced by *H. pylori*.

H. pylori and PI3K can also activate the small GTPase Rac [29,30], an important regulator of the migratory phenotype of cancer cells. Therefore, we next investigated whether Rac activation influenced *H. pylori*-induced wound closure by repeating wound-healing assays in the presence of a specific Rac1 inhibitor (NSC23766). Inhibition of Rac did not significantly

alter cell motility in uninfected cells (Figure 5D, 5E). Similar to results seen with PI3K inhibition, *H. pylori*-induced cell migration was completely abolished in the presence of Rac inhibition (Figure 5D, 5E). These results indicate that *H. pylori* promotes gastric epithelial cell migration via a PI3K, Src and Rac-dependent pathway, likely transduced by upstream signaling from EGFR transactivation.

H. pylori-induced cell migration is dependent on cagE and peptidoglycan

Having demonstrated that *cagE* and peptidoglycan are required for *H. pylori*-induced AKT activation, we next determined the role of these bacterial factors in cell migration. Similar to the patterns observed for AKT activation (Figure 2), cell migration was significantly decreased following infection with the *cagE*$^-$ or *slt*$^-$ mutants compared to wild-type 7.13 (Figure 6). These results indicate that PI3K signaling and *cag*-mediated peptidoglycan translocation mediate *H. pylori*-induced cell migration.

Activation of AKT by H. pylori attenuates apoptosis and promotes cell survival

Attenuation of *H. pylori*-induced cell migration by PI3K inhibition persisted to 16 hours (Figure 5), but this was accompanied by an increase in cellular detachment, indicative of cell death. Because AKT activation by PI3K attenuates apoptosis, we next determined the contribution of PI3K signaling to *H. pylori*-mediated apoptosis. AGS cells co-cultured with strain 7.13 in the absence or presence of the PI3K inhibitor LY294002 or vehicle control were stained with Annexin V and Propidium iodide for analysis of apoptosis using flow cytometry. As expected, treatment with the PI3K inhibitor alone induced a small population of uninfected cells into early apoptosis. Co-culture of AGS cells with *H. pylori* increased apoptosis, but this phenotype was significantly enhanced in the presence of the PI3K inhibitor (Figure 7A, 7B).

To more robustly demonstrate that PI3K-AKT signaling regulates *H. pylori*-induced cell survival, we transiently transfected AGS cells with scrambled or AKT-specific siRNA. Western blot analysis indicated that AKT expression was significantly reduced using AKT-specific, but not scrambled, siRNA (Figure 7C). *H. pylori* strain 7.13 was then co-cultured with AKT-deficient or wild-type control AGS cells and apoptosis was assessed using flow cytometry. Similar to results obtained using a chemical inhibitor of PI3K, inhibition of AKT significantly augmented the ability of *H. pylori* to induce apoptosis (Figures 7D, 7E). These data indicate that activation of PI3K-AKT promotes gastric cell survival in the presence of *H. pylori*.

A recent study demonstrated that *H. pylori* can not only induce apoptosis, but can also promote resistance to this phenotype in response to a known apoptosis-inducing agent, Staurosporine (Stsp) [22]. To extend our data implicating PI3K-AKT in cell survival, we evaluated the ability of PI3K to promote apoptotic resistance in *H. pylori*-infected AGS cells. Cells were infected with *H. pylori* strain 7.13 and then treated with Stsp to induce apoptosis. Inhibition of PI3K did not significantly alter apoptosis in uninfected cells exposed to Stsp (Figure 7F). AGS cells infected with *H. pylori* were more resistant to Stsp-induced apoptosis than cells pre-treated with medium alone (Figure 7F). However, inhibition of PI3K attenuated the anti-apoptotic activity of *H. pylori*, further supporting a role for *H. pylori*-induced PI3K signaling in promoting cell survival.

Discussion

PI3K is a host signaling molecule related to carcinogenesis. Our current experiments have shown that activation of PI3K-AKT can regulate microbially-induced carcinogenic responses by 1) demonstrating that *H. pylori* can activate AKT in gastric epithelial cells *in vitro*, 2) capitalizing on an *H. pylori* isogenic mutant system to demonstrate a requirement for

peptidoglycan translocation in AKT activation, 3) defining upstream signaling mediators of *H. pylori*-induced AKT activation and 4) combining transient inhibitor and gene silencing techniques with studies of epithelial responses that have carcinogenic potential (e.g. cell migration, survival). Collectively, these studies indicate that *H. pylori* coopts the PI3K-AKT signaling cascade, which, over prolonged periods of time, may lower the threshold for carcinogenesis.

In contrast to extensive literature invoking PI3K and AKT as tumorigenic molecules, few reports have examined the effects of bacterial pathogens on this signaling cascade. *Haemophilus influenzae* activates PI3K-AKT in epithelial cells, which then leads to a down-regulation of p38-MAPK activation [31]. *Salmonella* exploits PI3K in intestinal epithelial cells as an anti-inflammatory signal to reduce IL-8 production, which may contribute to the establishment of colonization in the intestine [32]. Our results suggest that induction of PI3K-AKT signaling by *H. pylori* requires a functional *cag* secretion apparatus and peptidoglycan, revealing a previously unrecognized effect of this *cag* island substrate, since the only defined role to date of *cag*-mediated peptidoglycan delivery is NOD1-dependent induction of IL-8 secretion [8]. In other cell systems, such as eosinophils, peptidoglycan has been shown to activate PI3K signaling and to regulate IL-8 production through Toll-like receptor (TLR) 2 [33]. However, further experiments are required to determine the precise mechanism through which PI3K is activated in *H. pylori*-infected gastric epithelial cells.

Hyperproliferation has been reproducibly demonstrated in *H. pylori*-infected tissue [34] and this is accompanied by decreased levels of apoptosis in colonized human and rodent gastric epithelium [19,20]. Several reports have demonstrated that one role of AKT is to inhibit the function of caspases, which induce apoptosis and cell-cycle arrest. In addition to PI3K activation, however, *H. pylori* activates other pathways that influence cell survival. For example, MEK/ERK activation in response to *H. pylori* has been shown to increase Mcl-1 levels, leading to apoptosis resistance [22]. The collective result of activation of these pathways is inhibition of apoptosis and increased cell proliferation, events that favor tumorigenesis.

In macrophages, *H. pylori* cag^+ strains activate PI3K, leading to actin polymerization and delayed phagocytosis [35]. Our current studies focused on epithelial cells demonstrate a dramatic reduction in *H. pylori*-induced cell migration in the presence of PI3K inhibitors, suggesting that PI3K may also mediate actin dynamics in gastric epithelial cells. We determined that cell migration was not affected by loss of CagA, but did require a functional type IV *cag* secretion system and peptidoglycan. Our results differ from studies demonstrating that CagA is required for a full motogenic response to *H. pylori* through its interactions with c-Met and subsequent MEK/ERK signaling [36]. We speculate that these differences may be due in part to the use of different strains as well as different techniques to assess cell migration. However, Al-Ghoul *et al.* have shown that *H. pylori* mutants that do not translocate CagA can still stimulate cell motility. These findings suggest that additional factors translocated by the type IV secretion system may affect *H. pylori*-dependent motility [13], and our results indicating that peptidoglycan is required to promote cell migration are consistent with these findings.

In summary, *H. pylori* induces PI3K-AKT signaling in gastric epithelial cells, which requires the *cag* secretion system and peptidoglycan as well as EGFR transactivation and Src activation in host cells. *H. pylori*-induced PI3K activation mediates cell migration and protection from apoptosis, two phenotypes related to carcinogenesis. Taken together, these data present insights into the pathogenic mechanisms underlying *H. pylori* infection.

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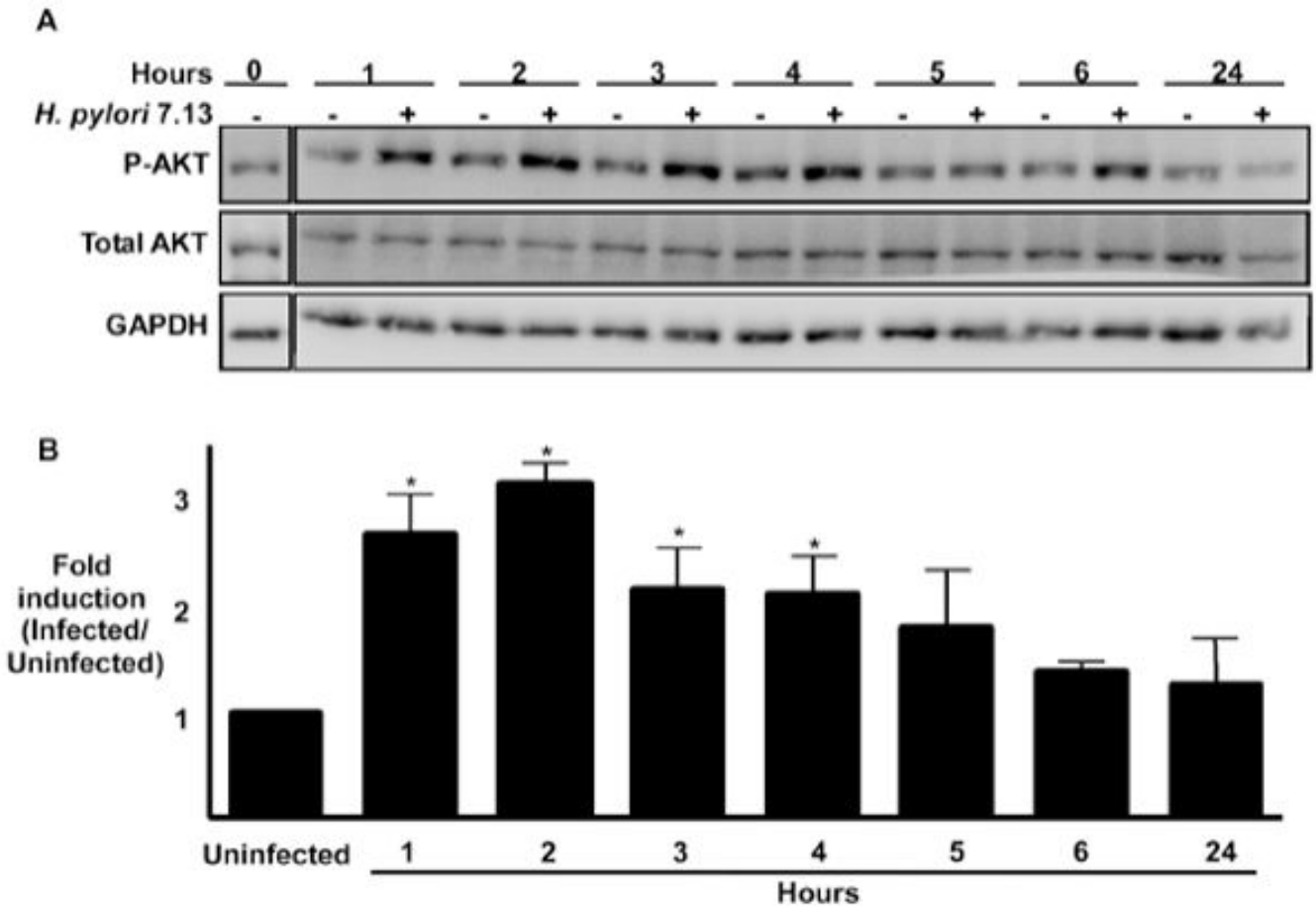

Figure 1. *H. pylori* induces AKT activation *in vitro* in a time-dependent manner

(A) AGS cells were co-cultured with the *H. pylori cag*$^{+}$ strain 7.13 at a bacteria/cell ratio of 100:1. One through twenty-four hours after incubation, whole cell lysates were harvested and subjected to Western blot analysis using an anti-phospho-AKT antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(B)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Levels of phospho-AKT were normalized to total AKT and levels were expressed as fold-induction of infected cells compared with uninfected cells at each time point. Error bars = SEM. **P* < 0.01 vs. uninfected control.

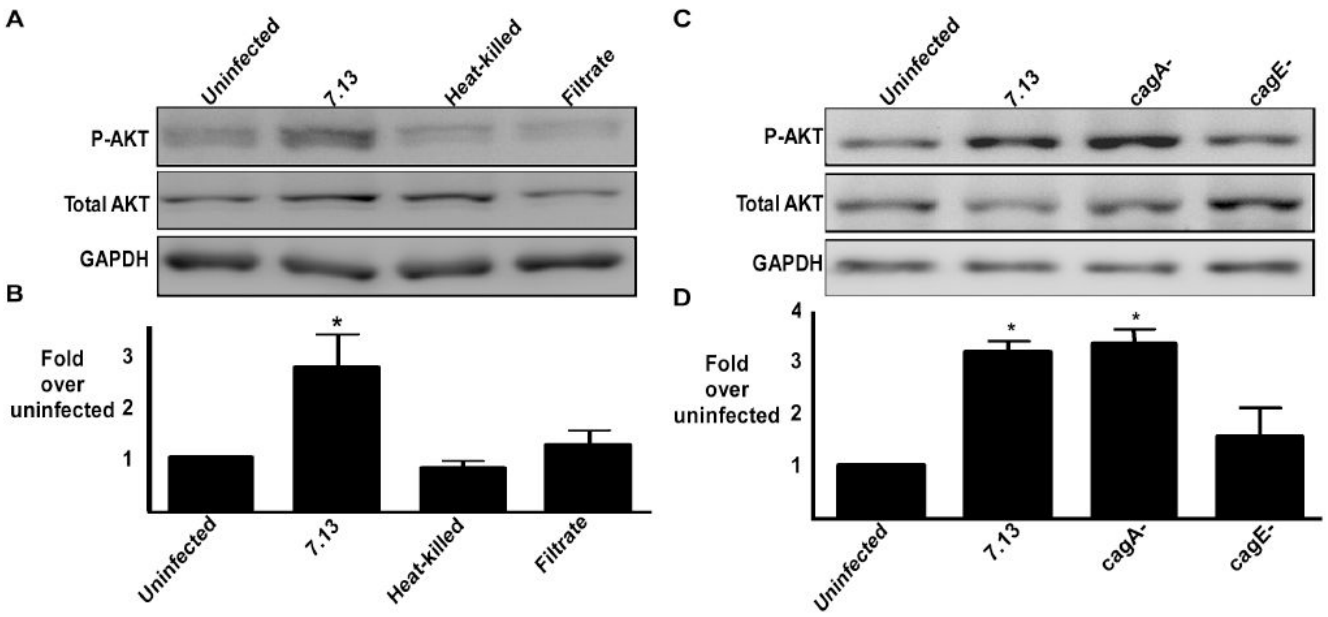

Figure 2. AKT phosphorylation by *H. pylori* is dependent on specific genes within the *cag* pathogenicity island

(A) AGS cells were incubated in the absence or presence of live *H. pylori* strain 7.13 at a bacteria/cell ratio of 100:1, heat-killed *H. pylori*, or *H. pylori* 7.13 filtrate for two hours. Whole cell lysates were subjected to Western blot analysis using an anti-phospho AKT antibody. Anti-total AKT blots served as normalization controls for AGS cell viability under different experimental conditions and anti-GAPDH blots served as loading controls. ***(B)*** Densitometric analysis of Western blots performed on 3 occasions. Error bars = SEM. **P* <0.04 vs. AGS cells alone. ***(C)*** AGS cells were cultured in the absence or presence of the *H. pylori cag*$^{+}$ strain 7.13 or its isogenic *cagA*$^{-}$ or *cagE*$^{-}$ null mutant derivatives at bacteria/cell ratios of 100:1. Two hours post infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-AKT antibody. A representative blot is shown. Western blots for total AKT served as normalization controls and Western blots for GAPDH served as loading controls. ***(D)*** Densitometric analysis of multiple Western blot repetitions performed on at least 5 occasions. Error bars = SEM. **P* < 0.002 vs. AGS cells alone.

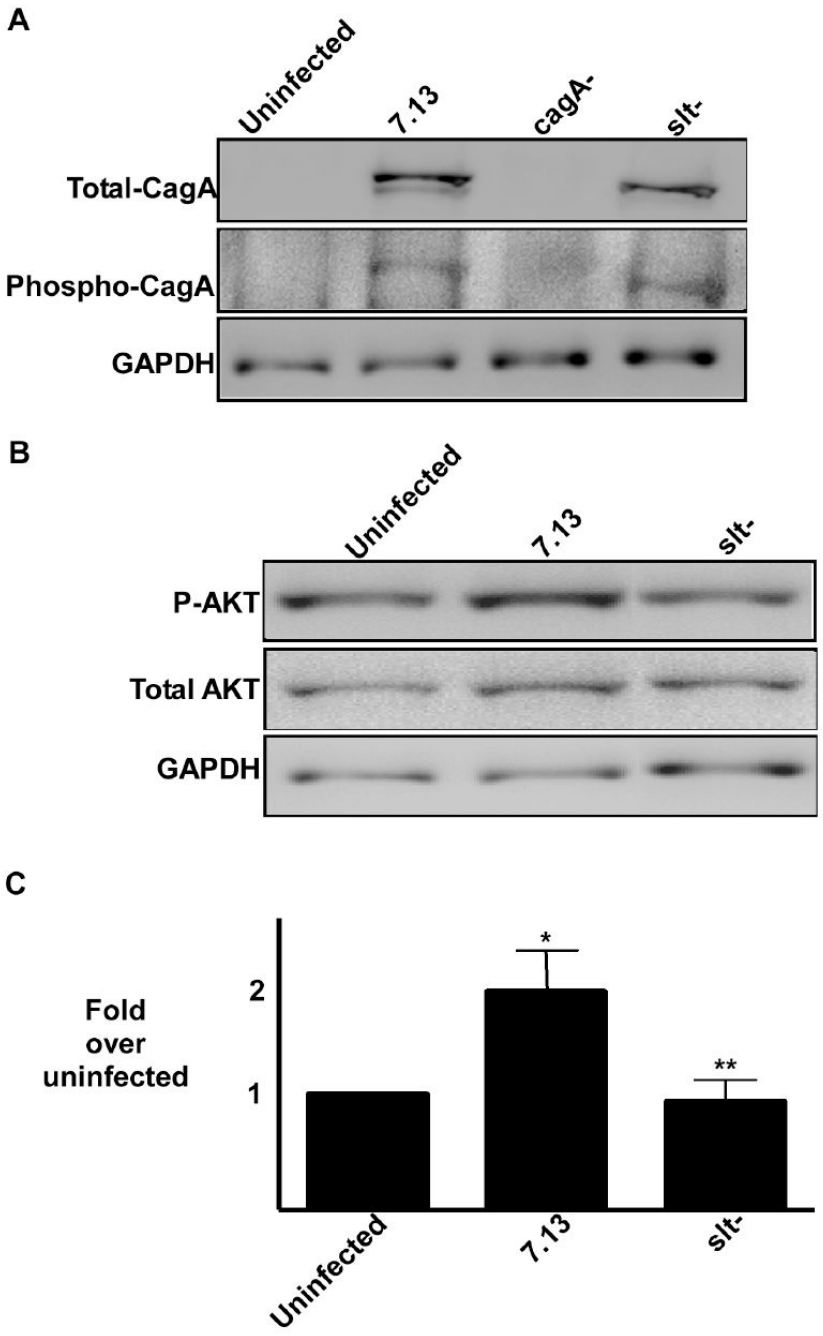

Figure 3. AKT phosphorylation by *H. pylori* is mediated by peptidoglycan

(A) AGS cells were cultured in the absence or presence of wild-type *H. pylori* strain 7.13 or its isogenic *cagA*$^-$ or *slt*$^-$ null mutant at a bacteria/cell ratio of 100:1. Two hours post infection, whole-cell lysates were subjected to Western blot analysis using an anti-phospho-tyrosine 99 antibody or an anti-CagA antibody. A representative blot is shown. Western blots for GAPDH served as loading controls. ***(B)*** *H. pylori* strain 7.13 or its isogenic *slt* null mutant derivative, were added to AGS cells at a bacteria/cell ratio of 100:1. Two hours after incubation, whole-cell lysates were subjected to Western blot analysis using an anti-phospho-AKT antibody. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH

served as loading controls. ***(C)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. **P* < 0.04 vs AGS cells alone; ***P* < 0.009 vs AGS cells incubated with wild-type *H. pylori*.

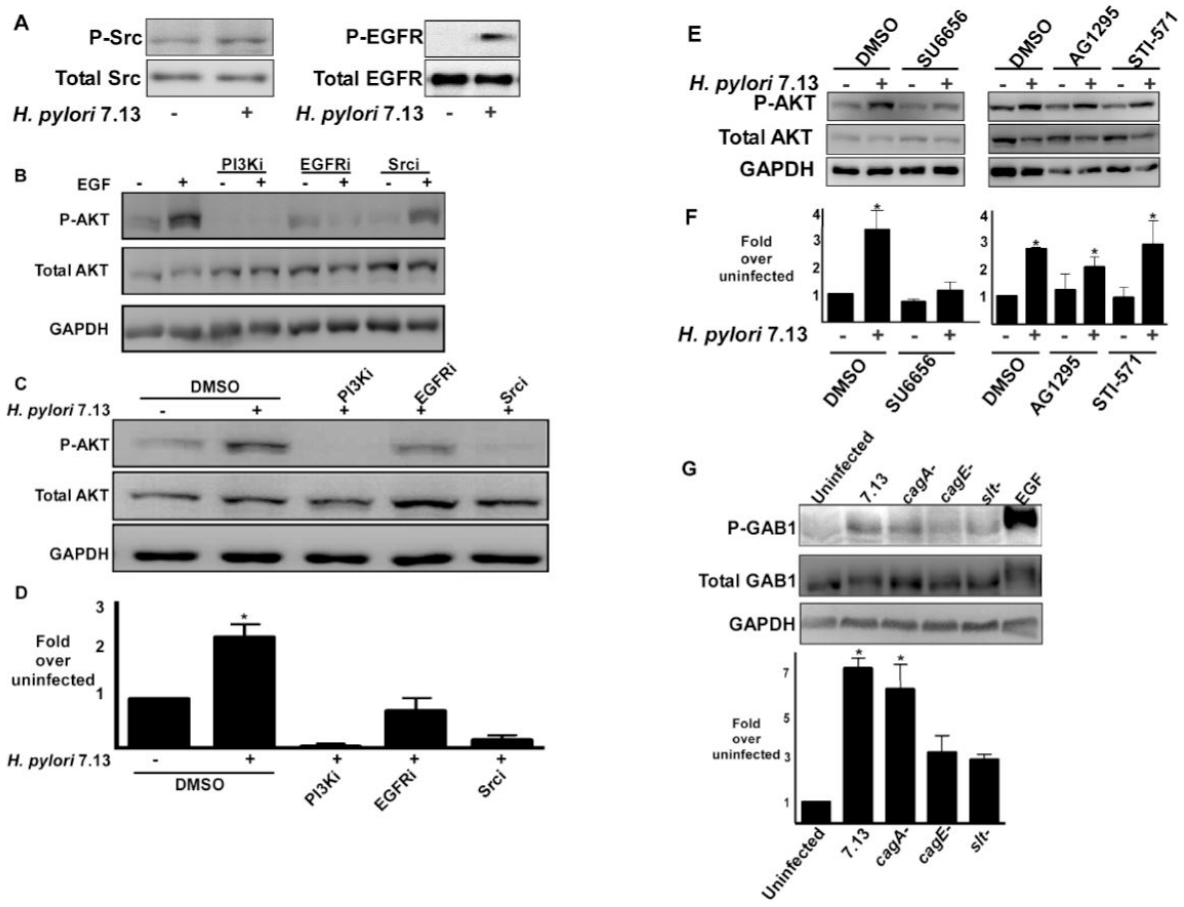

Figure 4. *H. pylori*-induced AKT phosphorylation in AGS cells is dependent on activation of PI3K, EGFR, and Src

(A) *H. pylori* strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1. Two hours post-infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-Src or an anti-phospho-EGFR antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total Src or EGFR served as normalization controls for AGS cell viability under different experimental conditions. ***(B)*** AGS cells were incubated with the PI3K inhibitor LY294002 (50 μmol/L), EGFR kinase inhibitor AG1478 (600 nmol/L), or Src inhibitor PP2 (10 μmol/L) for one hour prior to EGF exposure for 15 minutes. Levels of phospho- and total AKT were determined by Western blot analysis of whole cell lysates. ***(C)*** *H. pylori* strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1 in the absence or presence of vehicle alone (DMSO), or 50 μmol/L LY294002, 600 nmol/L AG1478, or 10 μmol/L PP2. Two hours post-infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-AKT antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(D)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. **P* < 0.0001 vs. AGS cells alone. ***(E)*** *H. pylori* strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1 in the absence or presence of vehicle alone (DMSO), or 2 μmol/L SU6656, 50 μmol/L AG1295, or 10 μmol/L STI-571. Two hours post-infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-AKT antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT

served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(F)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. **P* < 0.04 vs. AGS cells alone. ***(G)*** AGS cells were cultured in the absence or presence of the *H. pylori cag*$^{+}$ strain 7.13 or its isogenic *cagA*$^{-}$, *cagE*$^{-}$, or *slt*$^{-}$ null mutant derivatives at bacteria/cell ratios of 100:1. Two hours post infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-Gab1 antibody. EGF was used as a positive control for Gab1 phosphorylation and was added for 15 minutes. A representative blot is shown. Western blots for total Gab1 served as normalization controls and Western blots for GAPDH served as loading controls. Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions is shown below representative Western blot. Error bars = SEM. **P* < 0.02 vs. AGS cells alone.

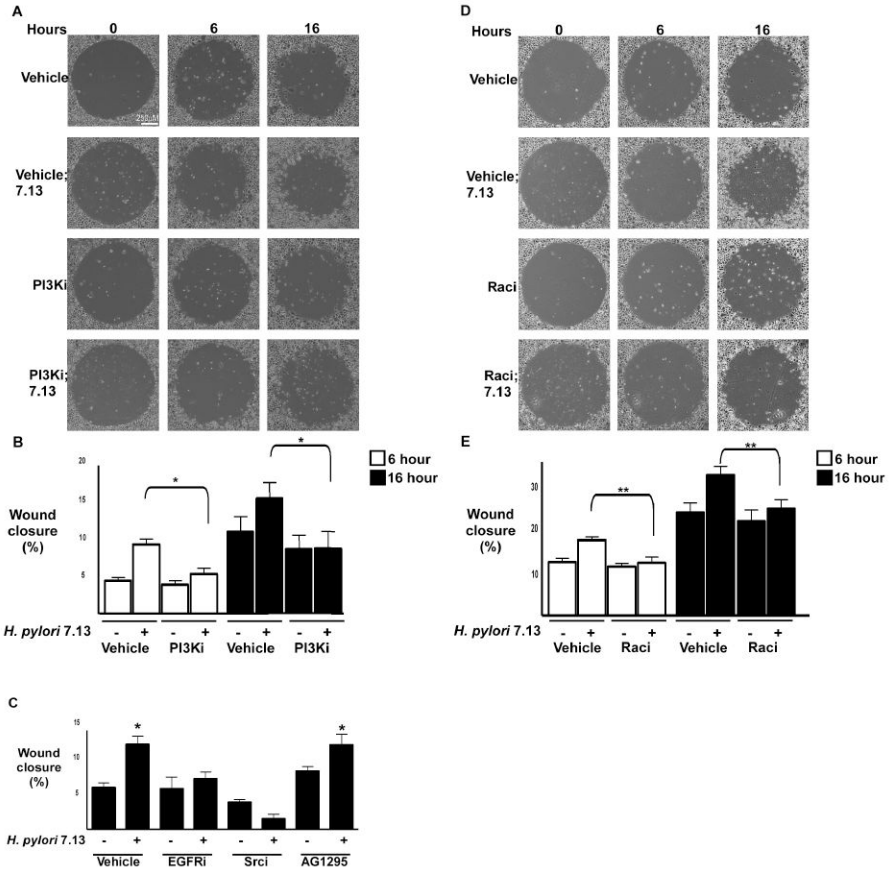

Figure 5. Activation of PI3K-AKT and Rac mediates *H. pylori*-induced cell migration

(A) AGS cells were grown to confluency and incubated with the PI3K inhibitor LY294002 (50 μM) or vehicle alone (DMSO) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero, six and sixteen hours post-infection. ***(B)*** Quantification of wound closure for each treatment group in experiments performed on at least 5 independent occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.04$ vs AGS cells infected with *H. pylori* strain 7.13 in the presence of the PI3K inhibitor LY294002 at both six and 16 hours. ***(C)*** AGS cells were grown to confluency and incubated with 600 nmol/L AG1478, 10 μmol/L PP2, or 50 μmol/L AG1295, or vehicle alone (DMSO) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero and six hours post-infection. Quantification of wound closure for each treatment group in experiments performed on at least 3 independent occasions is shown. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.005$ vs uninfected AGS cells alone or in the presence of AG1295. ***(D)*** AGS cells were grown to confluency and incubated with the Rac1 inhibitor NSC23766 (50 μM) or vehicle alone (water) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero, six and sixteen hours post-infection. ***(E)*** Quantification of wound closure for each treatment group in experiments performed on at least 5 independent occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. ** $P < 0.01$ vs AGS cells infected with *H. pylori* strain 7.13 in the presence of the Rac inhibitor NSC23766 at both six and 16 hours.

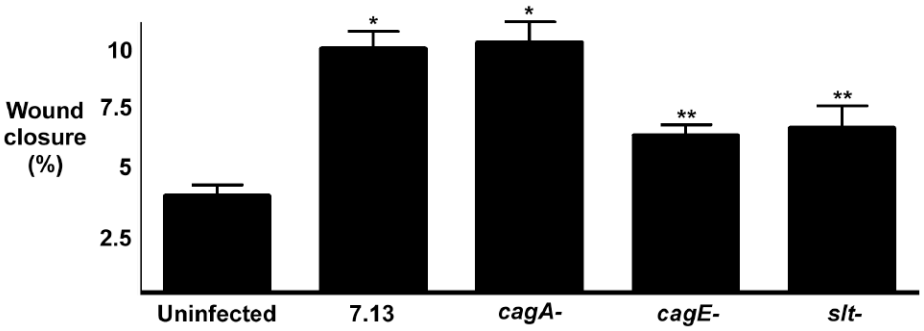

Figure 6. *H. pylori*-induced cell migration is dependent on the *cag* pathogenicity island and peptidoglycan

AGS cells were grown to confluency and a wound was introduced into the monolayer. Medium, *H. pylori* strain 7.13, or isogenic *cagA*$^-$, *cagE*$^-$, or *slt*$^-$ null mutant derivatives were then added at bacteria/cell ratios of 100:1. Wound areas were measured at time zero and six hours post-infection. Quantification of wound closure is shown for each treatment group in experiments performed on at least 3 occasions. Error bars = SEM. * $P < 0.007$ vs uninfected control; ** $P < 0.007$ vs AGS cells infected with *H. pylori* strain 7.13.

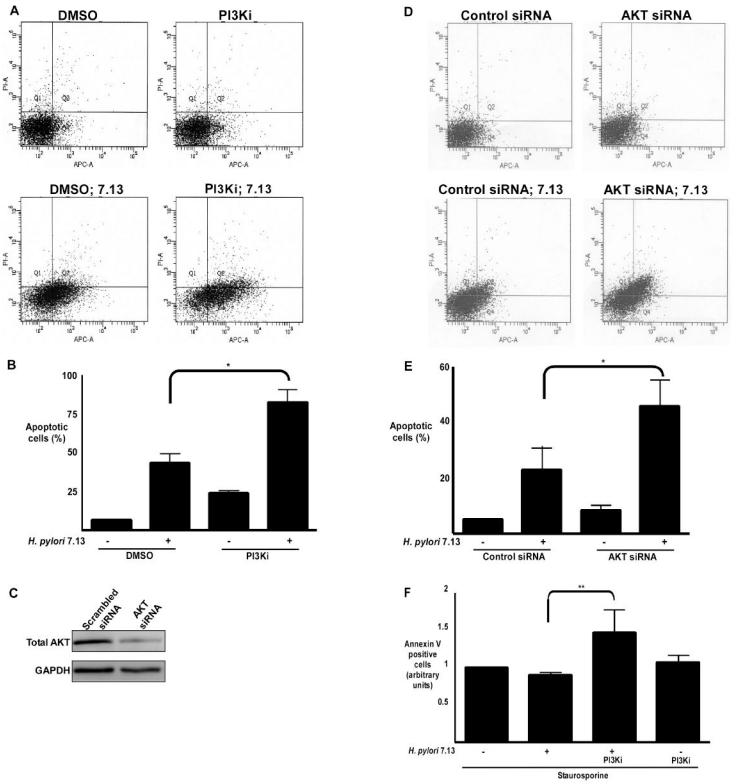

Figure 7. Activation of AKT by *H. pylori* promotes cell survival

(A) AGS cells were co-cultured with *H. pylori* strain 7.13 at a bacteria/cell concentration of 100:1, in the absence or presence of the PI3K inhibitor LY294002 (50 μM) or vehicle alone (DMSO) for 24 hours. Live cells were stained with Annexin V-APC and PI, and apoptosis was quantified by flow cytometry. The upper right quadrant represents late apoptosis, and the lower right quadrant represents early apoptosis. ***(B)*** Combined percentage of early and late apoptotic cells for experiments performed on at least 5 occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.005$ vs AGS cells infected with *H. pylori* strain 7.13 at MOI of 100:1 in the presence of vehicle alone. ***(C)*** AGS cells were transiently transfected with scrambled or AKT-specific siRNA, total protein was extracted and subjected to Western blot analysis using an anti-AKT antibody. ***(D)*** AGS cells transiently transfected with control or AKT-specific siRNA were co-cultured with *H. pylori* strain 7.13 at a bacteria/cell concentration of 100:1 for 24 hours. Live cells were stained with Annexin V-APC and PI, and apoptosis was quantified by flow cytometry. The upper right quadrant represents late apoptosis, and the lower right quadrant represents early apoptosis. ***(E)*** Combined percentage of early and late apoptotic cells for experiments performed on at least 3 occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.05$ vs AKT siRNA-treated AGS cells infected with *H. pylori* strain 7.13. ***(F)*** AGS cells were co-cultured with or without *H. pylori* strain 7.13 at a bacterial/cell concentration of 100:1, treated with LY294002 (50μM) or medium alone, and then exposed to Staurosporine (Stsp). Cells were then stained with Annexin V-APC and PI, and subjected to flow cytometry. (-), cells incubated without *H. pylori*. ** $P < 0.05$ vs AGS cells infected with *H. pylori* strain 7.13 in the presence of Stsp alone.