ADHERENCE OF THE HUMAN pathogen, Helicobacter pylori, to host cells elicits strong cellular and humoral immunological responses, including secretion of the proinflammatory cytokine IL-8, which is known to play a pivotal role in inflammatory responses to H. pylori infection (20, 28, 29). The outer inflammatory protein (OipA) and cag pathogenicity island (PAI) are H. pylori virulence factors thought to be involved in gastric IL-8 induction (26, 31). OipA is a member of a large outer membrane protein family and also acts as an adhesin (27). The cag PAI encodes a type IV secretion system that injects CagA and possibly other gene products into host cells (2). Injected CagA undergoes tyrosine phosphorylation by Src and Abl family kinases, resulting in alterations in host signaling pathways, actin stress fiber formation, and cell elongation, referred to as the “hummingbird phenotype,” as well as production of inflammatory mediators (15, 16, 18, 23). CagA alone is not sufficient to induce the hummingbird phenotype, since both cag PAI-positive and cag PAI-negative strains have been reported to induce actin stress fiber formation in gastric epithelial cells (1, 19). We recently showed that OipA and the cag PAI both activate site-specific phosphorylation of focal adhesion kinase (FAK), phosphatidylinositol 3-0H kinase (PI3K), and protein kinase B (Akt) (21, 22). Because OipA appears to be a major factor involved in actin stress fiber formation and cell phenotype, we hypothesized that both OipA and the cag PAI act in conjunction to regulate the signaling pathways involved in the regulation of actin stress fiber formation and cell phenotype.

The actin cytoskeleton is involved in diverse biological processes, including cell adhesion, motility, and cell survival, and is typically regulated by activation of cell surface receptors such as integrin or epidermal growth factor receptor (EGFR), coordination of phosphorylation/dephosphorylation of interacting proteins, cycles of actin polymerization/dem polymerization, and redistribution of actin stress fibers (12–14). We recently reported that H. pylori-induced EGFR activation resulted in tyrosine (Y) phosphorylation of FAK, Src, and PI3K, or serine phosphorylation of Akt, leading to actin stress fiber formation and IL-8 production (21, 22). However, the identification of downstream signaling partners mediating EGFR, FAK, Src, or PI3K/Akt signaling involved in H. pylori-induced phenotypic changes and cytokine production remained unknown.

Paxillin, a downstream effector of nonreceptor tyrosine kinases FAK and Src, is a focal adhesion-associated multidomain adaptor protein that localizes at focal adhesion sites [i.e., sites of cell contact with the extracellular matrix (ECM)] and provides structural links between the ECM and polymerized actin filaments (stress fibers). Cell attachment to the ECM or cell spreading induce paxillin phosphorylation primarily on Src homology 2-binding tyrosine residues Y31 and Y118 to integrate intracellular signaling, resulting in regulation of cell shape, adhesion, migration, and motility (3, 4, 24). It was recently reported that H. pylori-induced activation of paxillin Y118 through β1-integrin-dependent signaling plays a role in cell motility and cell scattering; in contrast, abrogating paxillin expression blocks H. pylori-induced cell motility in gastric cancer cells (17). However, the roles of EGFR, FAK/Src, or PI3K/Akt in regulating H. pylori-induced paxillin phosphorylation, actin stress fiber formation, and hummingbird phenotype in relation to the effects of the cag PAI or OipA were not clear.

Tabassam FH, Graham DY, Yamaoka Y. Paxillin is a novel cellular target for converging Helicobacter pylori-induced cellular signaling. Am J Physiol Gastrointest Liver Physiol 301: G601–G611, 2011. First published July 14, 2011; doi:10.1152/ajpgi.00375.2010.—Paxillin is involved in the regulation of Helicobacter pylori-mediated gastric epithelial cell motility. We investigated the signaling pathways regulating H. pylori-induced paxillin phosphorylation and the effect of the H. pylori virulence factors cag pathogenicity island (PAI) and outer inflammatory protein (OipA) on actin stress fiber formation, cell phenotype, and IL-8 production. Gastric cell infection with live H. pylori induced site-specific phosphorylation of paxillin tyrosine (Y) 31 and Y118 in a time- and concentration-dependent manner. Activated paxillin localized in the cytoplasm at the tips of H. pylori-induced actin stress fibers. Isogenic oipA mutants significantly reduced paxillin phosphorylation at Y31 and Y118 and reduced actin stress fiber formation. In contrast, cag PAI mutants only inhibited paxillin Y118 phosphorylation. Silencing of epidermal growth factor receptor (EGFR), focal adhesion kinase (FAK), or protein kinase B (Akt) expression blocks EGFR, FAK/Src, or phosphatidylinositol 3-kinase (Akt) expression by small-interfering RNAs or inhibiting kinase activity suppressed IL-8 production. OipA-induced IL-8 production was FAK- and Src-dependent, although a FAK/Src-independent pathway for IL-8 production also existed. We hypothesized that both OipA and the cag PAI act in conjunction to regulate the signaling pathways involved in the regulation of actin stress fiber formation and cell phenotype.

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elucidated. Here, we tested the hypothesis that *H. pylori*-induced EGFR, FAK/Src, and PI3K/Akt signaling converge to activate paxillin to regulate actin stress fiber formation, cell phenotype, and IL-8 production.

**EXPERIMENTAL PROCEDURES**

**Reagents.** Phosphospecific affinity-purified polyclonal antibodies for paxillin (Y31 and Y118), FAK (Y397) or Src Y418, mouse anti-paxillin, and polyclonal and purified anti-FAK were purchased...
from Biosource (Camarillo, CA). Anti-CagA antiserum was described previously (8). Anti-CagA antibody was purchased from Austral Biologicals (San Ramon, CA). Phosphoeyosine mouse monoclonal antibody (mAb), affinity-purified polyclonal antibodies for Akt (Ser473), horseradish peroxidase-conjugated goat anti-rabbit IgG (H&L), anti-mouse IgG, and the LumiGLO reagent chemiluminescent substrate detection system were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-β-actin mAb, Ponceau S solution, protease inhibitor cocktail, and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Selective F-actin probes Alexa Fluor 488 or 594 phalloidin, 4',6-diamidino-2-phenylindole (DAPI), and a SlowFade Anti-fade kit were obtained from Molecular Probes (Eugene, OR). All chemical inhibitors were obtained from Calbiochem (San Diego, CA). Mammalian FAK small-interfering RNA (siRNA) expression plasmid or EGFR siRNA/siAB assay kits were purchased from Upstate Cell Signaling Solution (Lake Placid, NY). A mammalian SignalSilence Akt siRNA kit was purchased from Cell Signaling Technology. Immunoprecipitation reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture.** The human gastric epithelial cancer cell lines AGS (American Type Culture Collection, Manassas, VA), MKN28, and MKN45 (both from Riken Bank, Tsukuba, Japan) were grown at 37°C and 5% CO2 in RPMI 1640 medium supplemented with peroxynitrite, streptomycin, and 10% FBS. Cells were seeded at a density of 1 × 10^5 cells/well in six-well plates or 5 × 10^4 cells in 10-cm dishes or were grown on glass cover slips in 10% FBS. Cells were serum-starved and cultured in antibiotic-free medium overnight before experiments. Cells at 80% confluence were left untreated in RPMI 1640 medium or were cocultured with *H. pylori* for specified times or multiplicity of infection (MOI) as described in the legends for Figs. 1–7. Cyto-toxicity of gastric epithelial cancer cells using chemical inhibitors was measured by CytoTox-ONE homogeneous membrane integrity assay (Promega, Madison, WI) according to the manufacturer’s instructions.

**H. pylori.** Functional oipA-positive/cag PAI-positive *H. pylori* strains TN2GF4, ATCC43504, and 26695 and their isogenic oipA mutants and cag PAI mutants were used (7, 31). *H. pylori* TN2GF4 was isolated from a Japanese gastric ulcer patient and has been reported to cause gastric cancer in Mongolian gerbils (25). *H. pylori* were cultured on brain heart infusion agar plates containing 7% horse blood for 24–36 h at 37°C under microaerophilic conditions. The bacteria were collected and suspended in PBS, and its density was estimated using spectrophotometry (A600) and by microscopic observation.

**Immunoblotting and immunoprecipitation.** AGS cells were cocultured with *H. pylori* at the specified MOI or were cocultured at an MOI of 100 for specified times as indicated in the legends for Figs. 1–7. Protein extraction and immunoblotting were performed using standard techniques. For immunoprecipitation analysis, equivalent amounts of protein from the control or infected samples were incubated with antibodies for 2 h at 4°C and then collected with Protein A-Sepharose 4 Fast Flow or Protein G-Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ). The precipitates were washed three times with lysin buffer and one time with PBS, subjected to 5% SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies as described in the text. Semiquantitative analyses were performed by quantifying the scanned X-ray films using Image J 1.36 software (http://rsbweb.nih.gov/ij/) from the National Institutes of Health. Results are expressed as means ± SE. Statistical analyses were performed using the Mann-Whitney Rank Sum test and the paired t-test depending on the data set of concern using the statistical software SigmaStat 3.01 (Ashburn, VA). *P < 0.05* was established as statistically significant.

**Immunofluorescence microscopy.** AGS cells grown on cover slips were left uninfected or infected with *H. pylori* for 1 h at an MOI of 100 as described in the legends for Figs. 1–7. Cells were fixed in 3.7% formaldehyde in PBS (pH 7.6), and paxillin phosphorylation was visualized by incubating overnight with the indicated phosphospecific antibodies (10–30 μg/ml) as described (21). Cells were washed and incubated with anti-rabbit FITC-conjugated anti-rabbit IgG secondary antibody and with selective F-actin probe Alexa Fluor 488 for hummingbird phenotype or 594 phalloidin to visualize F-actin polymerization and actin stress fiber formation. Nucleic acid was counterstained with DAPI (300 nM). The cover slips were washed and mounted on slides using the SlowFade Antifade kit (Molecular Probes) as recommended by the manufacturer to prevent rapid photo bleaching. The images were acquired using filters appropriate for FITC, Alexa Fluor 594 phalloidin, and DAPI. Uninfected or infected cells were subjected to identical culture, fixation, staining, and microscopy conditions using fluorescence microscopy (Olympus, America, Melville, NY). Representative images of each sample were taken at least in triplicate and were captured at ×1,000 magnification or as indicated in the legends for Figs. 1–7.

![Fig. 1. Helicobacter pylori-induced paxillin phosphorylation.](http://ajpgi.physiology.org/)

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**Figure 1.** Helicobacter pylori-induced paxillin phosphorylation. A: whole cell lysates from uninfected AGS cells or cells infected with wild-type *H. pylori* for 1 h at a multiplicity of infection (MOI) of 25–200 were prepared and immunoblotted as described in experimental procedures. Changes in tyrosine phosphorylation were assessed using the indicated phosphospecific paxillin antibodies or total paxillin antibody for paxillin content, and β-actin antibody was used as an internal control. B: whole cell lysates from uninfected AGS cells or cells infected with wild-type *H. pylori* at an MOI of 100 for 5–60 min were immunoblotted with indicated paxillin antibodies. A and B: for quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed as fold increases compared with those of mock-infected control cells. Data are presented as average values ± SE. At least 3 independent cocultures were performed. *P < 0.05* and **P < 0.01** vs. mock-infected control cells. C: whole cell lysates from uninfected AGS cells or cells infected for 30 min to 4 h with wild-type *H. pylori* at an MOI of 100 were immunoblotted with indicated paxillin antibodies. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells at 30 min. Data are presented as average values ± SE. At least 3 independent cocultures were performed. At each time point, the phosphorylation levels were significantly higher in infected cells than in uninfected cells (P < 0.01). D: whole cell lysates from uninfected MKN28 cells and MKN45 cells infected with wild-type *H. pylori* for 1 h at an MOI of 100 were immunoblotted with indicated paxillin antibodies. Blots were reprobed with paxillin antibody for total paxillin content, and β-actin antibody was used as the internal control. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. At least 3 independent cocultures were performed. The phosphorylation levels were significantly higher in infected cells than in uninfected cells (P < 0.01). E: whole cell lysates from uninfected AGS cells or cells infected with wild-type *H. pylori* for 1 h at an MOI of 25–200 were prepared and immunoblotted as described in experimental procedures. Changes in tyrosine phosphorylation were assessed using the indicated phosphospecific paxillin antibodies or total paxillin antibody for paxillin content, and β-actin antibody was used as the internal control. F: whole cell lysates from uninfected AGS cells or those infected with wild-type *H. pylori* at an MOI of 100 for 7.5–120 min with immunoblotted with the indicated paxillin antibodies. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. At least 3 independent cocultures were performed. *P < 0.05* and **P < 0.01** vs. mock-infected control cells.
siRNA transfection and silencing EGFR, FAK, or Akt protein expression. Cells were grown in six-well plates for 24 h to reach ~60% confluence on the day of transfection and were transfected with control siRNA or siRNA for EGFR, FAK, or Akt using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or transfection reagent provided in the kit according to the manufacturer’s instructions. Briefly, for each transfection, siRNAs or 5 μL of transfection reagent were diluted in 200 μL of Opti-MEM (Invitrogen). Diluted siRNA transfection reagent mix was incubated for 15 min at room temperature for complex formation. For subsequent transfection, complexes were added to each well and incubated for 4 h, followed by incubation with 20% FBS. The cells were cultured for 1–4 days posttransfection in normal growth medium. Before infection with H. pylori, cells were suspended overnight in serum-free medium and infected with H. pylori at an MOI of 100 for 1 h for investigating the downstream effectors by immunoblotting or 18 h for IL-8 production as indicated in the legends for Figs. 1–7.

Determination of IL-8 production. In vitro IL-8 production was measured as described previously (30). Briefly, gastric epithelial cells (~5 × 10⁶/mL) were plated on 24-well plates and cultured for 2 days. Serum-starved cells were preincubated with or without Src inhibitor (PP2) for 1 h, followed by H. pylori infection at an MOI of 100 for 18 h. Culture supernatants were collected and assayed for IL-8 production by an enzyme-linked immunosorbent assay according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Results are expressed as means ± SE. Statistical significance was determined using the Mann-Whitney Rank Sum test or paired t-test using statistical software SigmaStat 3.01 depending on the data set of concern. *P < 0.05 was accepted as statistically significant.

RESULTS

H. pylori-induced paxillin phosphorylation. We first investigated whether gastric epithelial AGS cells infected with live H. pylori would induce the phosphorylation of paxillin. H. pylori TN2GF4 induced tyrosine phosphorylation of both paxillin Y31 and Y118 in AGS cells in a concentration-dependent manner (Fig. 1A). Paxillin was phosphorylated at the lowest MOI tested, an MOI of 25. Total paxillin levels remained unchanged during the observation periods (Fig. 1A). At an MOI of 100, H. pylori induced phosphorylation of paxillin Y31 and Y118 in a time-dependent manner without affecting total paxillin levels (Fig. 1B). Maximal phosphorylation levels were observed between 1 and 2 h postinfection followed by a reduction after 4 h postinfection (Fig. 1C). Late-phase H. pylori infection (e.g., for 24 h) resulted in downregulation of paxillin phosphorylation (data not shown). Similar results were obtained using other gastric epithelial MKN28 or MKN45 cells (Fig. 1D) and with different H. pylori strains (Fig. 1E and F). Therefore, we used AGS cells and H. pylori TN2GF4 at an MOI of 100 in subsequent experiments.

H. pylori-induced activated FAK associates with paxillin. H. pylori is known to induce tyrosine phosphorylation of FAK (9, 21). To test whether phosphorylated FAK associated with activated paxillin, a putative substrate of FAK, we immunoprecipitated FAK in cell lysates from uninfected or H. pylori-infected cells, followed by immunoblotting with antibodies specific for paxillin Y31, Y118, or for total paxillin. As early as 15 min postinfection, paxillin was found to be associated with FAK (Fig. 2A). Maximal association between FAK and paxillin was observed 2 h postinfection and declined after 4 h. However, FAK-paxillin signaling complexes were not detected with normal rabbit IgG or protein A-Sepharose beads alone (data not shown). To determine if paxillin remained phosphorylated during complex formation, whole cell lysates from uninfected cells or H. pylori-infected cells were immunoprecipitated with phosphotyrosine antibody, followed by immunoblotting with phosphospecific paxillin Y118 antibody (Fig.

**Fig. 2.** H. pylori-induced focal adhesion kinase (FAK) interacts with activated paxillin. A: whole cell lysates from uninfected AGS cells or those infected with wild-type H. pylori at an MOI of 100 for indicated times were immunoprecipitated with an FAK antibody. Immune complexes were analyzed using SDS-PAGE and immunoblotted (IB) using phosphospecific paxillin tyrosine (Y) 31 or Y118 antibodies. The same blots were reprobed with anti-paxillin antibody to visualize total paxillin. Data are representative of 3 separate experiments. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. The phosphorylation levels were significantly higher in infected cells than in uninfected cells (*P < 0.01). B: whole cell lysates from uninfected AGS cells or those infected with wild-type H. pylori at an MOI of 100 for 2 h were immunoprecipitated (IP) with phosphotyrosine (pTyr) antibody and immunoblotted using phosphopaxillin Y118 antibody or reprobed with paxillin antibody. Data are representative of 3 separate experiments. IP, immunoprecipitation.
H. pylori-induced paxillin Y118 phosphorylation was observed 2 h postinfection, consistent with paxillin remaining phosphorylated during the period of FAK-paxillin complex formation. These results are consistent with the hypothesis that the H. pylori-induced FAK-paxillin signaling complex regulates downstream signaling pathways.

Abrogating FAK expression inhibits H. pylori-induced paxillin phosphorylation and actin stress fiber formation. H. pylori-induced FAK activation is known to play a role in actin stress fiber formation and cell phenotype (21). To confirm that H. pylori-induced paxillin phosphorylation was a consequence of FAK activation, we abrogated FAK expression in AGS cells using a FAK-specific siRNA or using nontargeted control siRNA plasmid (negative control). FAK expression was reduced to >90% following expression of a FAK-specific siRNA and was associated with inhibition of FAK Y397 phosphorylation despite H. pylori infection (Fig. 3A). H. pylori-induced tyrosine phosphorylation of paxillin Y31 and Y118 was not inhibited by the negative control siRNA. However, FAK knock down resulted in inhibition of H. pylori-induced paxillin Y31 and Y118 phosphorylation without affecting the total cellular paxillin content (Fig. 3A).

We used immunofluorescence microscopy to examine the role of FAK expression on the regulation of paxillin phosphorylation, actin stress fiber formation, and cell phenotype. AGS cells were transfected with either the negative control siRNA plasmid or with the FAK siRNA plasmid for 96 h, followed by H. pylori infection or mock infection for 1 h. H. pylori-infected cells transfected with the control siRNA plasmid showed enhanced phosphorylation of paxillin Y118, F-actin polymerization, and actin stress fiber formation relative to mock-infected cells (Fig. 3B). Phosphorylated paxillin colocalized along the tips (leading edges) of long and enlarged actin filaments (Fig. 3B). These bundles of actin filaments (stress fibers) were typically found in the cytoplasm of H. pylori-infected AGS cells, possibly related to phosphorylation of paxillin. This cellular stressor resulted in alignment of the actin filaments to form stress fibers. However, transfection of AGS cells with FAK siRNA not only reduced paxillin phosphorylation but also essentially abolished F-actin polymerization and actin stress fiber formation (Fig. 3B). Immunofluorescence data showed that 96 h after FAK siRNA transfection, >90% of cells had undetectable FAK expression (data not shown). These results confirmed the involvement of FAK in mediating H. pylori-induced paxillin phosphorylation, actin stress fiber formation, and cell phenotype.

Inhibiting Src and PI3K kinase activity reduced H. pylori-induced paxillin phosphorylation. We recently showed that H. pylori-induced activation of EGFR, FAK, Src, and PI3K/Akt is...
involved in cell phenotype and cytokine production (21, 22). We evaluated the roles of Src or PI3K kinase activity in the regulation of paxillin since these signaling pathways are potential candidates for *H. pylori*-mediated intracellular signaling regulation of actin cytoskeleton reorganization and cytokine induction. AGS cells were pretreated with 10 or 20 μM of chemical inhibitors, PI3K (LY-294002) or Src (PP2), followed by *H. pylori* infection at an MOI of 100 for 1 h (Fig. 4A). Immunoblot analyses showed that, compared with uninfected cell, *H. pylori* infection induced Src Y418 and paxillin phosphorylation at Y31 and Y118 sites. Preincubation of AGS cells with 10 μM PI3K inhibitor markedly inhibited *H. pylori*-induced paxillin Y31 and Y118 phosphorylation. Preincubation of AGS cells with 10 μM Src inhibitors also inhibited *H. pylori*-induced paxillin Y31 and Y118 phosphorylation (~50% reduction at both sites), but 20 μM was required to reduce phosphorylation of paxillin at Y31 and Y118 to nearly basal levels (Fig. 4A) without influencing the total levels of paxillin (Fig. 4A). We also showed that 10 or 20 μM Src inhibitor blocked Src Y418 phosphorylation, whereas the PI3K inhibitor was unable to block *H. pylori*-induced Src phosphorylation, confirming the specificity of Src inhibitor or location of Src as upstream of PI3K (Fig. 4A). Cytotoxicity was not observed with 10 or 20 μM of both inhibitors. These results support the hypothesis that the phosphorylation of the Src→PI3K signaling cascade plays a crucial role in *H. pylori*-mediated paxillin phosphorylation.

Reduced expression of Akt inhibited *H. pylori*-induced paxillin phosphorylation. To further examine the role of PI3K, an upstream regulator of Akt, we investigated the effect of the targeted knock down of Akt expression on *H. pylori*-induced paxillin phosphorylation. Transfection of the control siRNA plasmid did not affect *H. pylori*-induced Akt Ser473 or paxillin Y118 phosphorylation while targeted depletion of Akt expression followed by *H. pylori* infection for 1 h resulted in inhibition of Akt Ser473 and paxillin Y118 phosphorylation without affecting total paxillin levels (Fig. 4B). The result suggests that Akt is located upstream of paxillin and that Akt kinase activity regulates paxillin phosphorylation.

Inhibition of EGFR expression or loss of kinase activity downregulates *H. pylori*-induced paxillin phosphorylation. *H. pylori* infection upregulates expression, phosphorylation, and transactivation of EGFR in gastric epithelial cells (6, 22). To elaborate the role of EGFR kinase activity in regulation of paxillin activation, AGS cells were pretreated with 1 μM of EGFR inhibitor (AG1478), followed by infection with *H. pylori* at an MOI of 100 for 1 h. Immunoblot analyses showed that inhibiting EGFR tyrosine kinase activity followed by *H. pylori* infection reduced tyrosine phosphorylation of paxillin Y31 and Y118 without affecting the total paxillin level during infection time (Fig. 4C). To further confirm the role of EGFR, known to regulate FAK, Src, or PI3K signaling, we used EGFR-specific siRNA to abrogate EGFR expression in AGS cells. EGFR expression was reduced to >90% following EGFR-specific siRNA and was associated with inhibition of EGFR phosphorylation despite *H. pylori* infection. Transfection of the control siRNA plasmid did not affect *H. pylori*-induced paxillin Y118 phosphorylation, whereas EGFR knock down by EGFR-specific siRNA blocked *H. pylori*-induced paxillin Y118 phosphorylation to basal levels, indicating that EGFR plays an essential role in *H. pylori*-mediated paxillin phosphorylation (Fig. 4D). Overall, we concluded that *H. pylori*-induced EGFR activation through possible involvement of FAK/Src and PI3K signaling regulates paxillin activation and downstream signaling, consequently playing a leading role in altering cellular phenotype and cellular functions.

Role of *cag* PAI or OipA in paxillin phosphorylation and actin stress fiber formation. To test the hypothesis that *H. pylori* virulence factors *cag* PAI or OipA participate in regulation of paxillin and actin stress fiber formation, we compared the effect of wild-type *H. pylori* TN2GF4 with its *cag* PAI and *oipA* isogenic mutants in relation to paxillin phosphorylation (Fig. 5A). Interestingly, infection with the *cag* PAI and *oipA* mutants produced different patterns of paxillin phosphorylation. Compared with wild-type infection, the *cag* PAI mutants were associated with ~80% reduction in phosphorylation of paxillin Y118, without affecting the phosphorylation levels of paxillin Y31 (Fig. 5A). In contrast, phosphorylation of both paxillin Y31 and Y118 was reduced significantly following infection with the *oipA* mutants compared with infection with wild-type *H. pylori*, whereas total paxillin levels remained unchanged regardless of the virulence factor during the tested infection time. These results were confirmed using isogenic mutants for the *cag* PAI or *oipA* from parental strains ATCC43504 and 26695 (Fig. 5A).

Immunofluorescence microscopy further confirmed that phosphorylation of paxillin Y118 but not Y31 was reduced following infection with the *cag* PAI mutants (Fig. 5, B and C). Following infection with the *oipA* mutants, both paxillin phosphorylation and actin stress fiber formation almost disappeared, consistent with the notion that, during the early phase of infection, OipA plays a major role in paxillin phosphorylation, modulation of actin stress fiber formation, and actin cytoskeletal reorganization, leading to phenotypic changes.

Role of paxillin phosphorylation in *H. pylori*-induced hummingbird phenotype. To determine the role of the continued impact of *H. pylori*-induced paxillin phosphorylation on cell phenotype, we used phosphapaxillin Y118 antibody, actin probe Alexa Flour 488 phalloidin, and immunofluorescence microscopy. Cell elongation or the hummingbird phenotype was not observed in uninfected AGS cells (Fig. 6A). In contrast, cell elongation and formation of the hummingbird phenotype were observed at 4 h postinfection and was apparent 6 h postinfection (Fig. 6, C and D). *H. pylori*-induced paxillin phosphorylation was localized along elongated projections, suggesting a role for phosphorylated paxillin in the *H. pylori*-induced hummingbird phenotype. These findings suggest that activation of paxillin Y118 plays an important role in cell elongation and the hummingbird phenotype.

Role of Src, Akt, and FAK in *H. pylori*-induced IL-8 production. To clarify the opposing role of the *cag* PAI and *oipA* mutants in regulating cellular functions, we investigated the effects of FAK or Src activation in regulating wild-type *H. pylori* or *cag* PAI and *oipA* mutants’ regulated IL-8 production. We had already examined the effects of EGFR in regulating *H. pylori* and reported that both *cag* PAI and OipA are involved in EGFR-related IL-8 production (22). We previously showed that IL-8 levels reached maximal levels 18–21 h after *H. pylori* infection at an MOI of 100 and then plateaued until 30 h, regardless of the *H. pylori* strain (30, 31). Therefore, we cocultured *H. pylori* with AGS cells at an MOI of 100 for 18 h to measure IL-8 production.
Fig. 4. Role of phosphatidylinositol 3-kinase (PI3K), Src, or epidermal growth factor receptor (EGFR) in *H. pylori*-induced paxillin phosphorylation. 

A: AGS cells were pretreated for 1 h with 10 or 20 μM PI3K [LY-294002 (LY)] or Src (PP2) inhibitors, followed by infection with wild-type *H. pylori* (MOI of 100) for 1 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotted with indicated phosphospecific Src or paxillin antibodies. The same blots were reprobed with total paxillin. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. At least 3 independent cocultures were performed. In *H. pylori*-infected cells, the phosphorylation levels were decreased significantly in cells without each inhibitor compared with those with inhibitors (PI3K inhibitor, *P* < 0.01 for 10 and 20 μM; Src inhibitor, *P* < 0.05 for 10 μM and *P* < 0.01 for 20 μM).

B: equal amounts of total cell lysate from AGS cells transfected with nontargeted siRNA with or without *H. pylori* infection or cells transfected with protein kinase B (Akt)-specific siRNA for 72 h followed by infection with wild-type *H. pylori* for 1 h were analyzed with immunoblot analysis, using phosphospecific Akt Ser473 or paxillin Y118 and total paxillin or β-actin antibodies. Data representative of 3 separate experiments are presented. For quantification, the density of phosphospecific paxillin Y118 was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. In *H. pylori*-infected cells, the phosphorylation levels were decreased significantly in cells transfected with Akt siRNA compared with those transfected with control Akt (*P* < 0.01).

C: AGS cells were pretreated for 1 h with 1 μM EGFR inhibitor (AG1478), followed by infection with wild-type *H. pylori* (MOI of 100) for 1 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotted with indicated phosphospecific or total paxillin antibodies. For quantification, the density of phosphospecific sites was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. At least 3 independent cocultures were performed. In *H. pylori*-infected cells, the phosphorylation levels were decreased significantly in cells without EGFR inhibitor compared with those with inhibitors (*P* < 0.01). D: AGS cells were transfected with negative control siRNA or with EGFR-specific siRNA for 72 h, followed by infection with or without wild-type *H. pylori* for 30 min. Equal amounts of proteins from whole cell lysates were subjected to immunoblotting with phosphospecific EGFR or paxillin Y118 antibodies. The same blots were reprobed with paxillin for total contents or with β-actin antibodies to verify equal loading. At least 3 independent cocultures were performed. For quantification, the density of phosphospecific paxillin Y118 was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. In *H. pylori*-infected cells, the phosphorylation levels were decreased significantly in cells transfected with EGFR siRNA compared with those transfected with control EGFR (*P* < 0.01).
Fig. 5. Effect of isogenic mutants on paxillin phosphorylation and actin stress fiber formation. A: AGS cells were left uninfected or infected with wild-type *H. pylori*, cag pathogenicity island (PAI) mutants, or outer inflammatory protein (*oipA*) mutants for 1 h at a MOI of 100, and whole cell lysates were subjected to immunoblot analyses, using indicated phosphopaxillin antibodies. Blots were reprobed with a paxillin antibody to detect total paxillin, and 5-H9252-actin antibody was used as the internal control. At least 3 independent cocultures were performed. For quantification, the density of phosphospecific paxillin Y118 was normalized to that of total paxillin, and the levels were expressed in terms of fold increase compared with those of mock-infected control cells. Data are presented as average values ± SE. *P < 0.05 and **P < 0.01 vs. wild-type *H. pylori*-infected cells.

B and C: for immunofluorescence analysis, AGS cells were grown on cover slips and left uninfected or infected with wild-type *H. pylori*, cag PAI mutants, or *oipA* mutants for 1 h at an MOI of 100. These cells were stained to detect actin stress fibers and activated paxillin Y31 (B) or Y118 (C). Next, the cells were incubated with FITC-conjugated anti-rabbit IgG secondary antibody (green), Alexa Fluor 594 phalloidin (red), and DAPI (blue). All figures represent merged images with ×1,000 magnification. The scale bar shows 10 μm. At least 3 independent cocultures were performed. The representative images shown with *H. pylori* infection are, on average, observed in >80% of cells examined, whereas <10% of cells that were mock treated were infected.
We used pharmacological inhibitors of Src (PP2) to block Src kinase activity, followed by infection with *H. pylori* or its *cag* PAI and *oipA* mutants. Increasing concentrations of Src inhibitors resulted in the dose-dependent suppression of *H. pylori*-mediated IL-8 production. Pretreatment of AGS cells with 20 μM Src inhibitor suppressed IL-8 production by nearly 70% (Fig. 7A). Infection with the *cag* PAI mutant reduced nearly 80% of IL-8 production compared with the IL-8 production after wild-type infection, whereas preincubation with 20 μM Src inhibitor followed by infection with the *cag* PAI mutants further inhibited IL-8 production to nearly basal levels, indicating that *cag* PAI at least partly required Src-dependent signaling while Src-independent signaling pathways are also required for inhibition of IL-8 induction to the basal levels. The *oipA* mutants reduced IL-8 production to 50%, and preincubation with 20 μM Src inhibitor, followed by *oipA* mutants, did not affect IL-8 production, suggesting that OipA-dependent IL-8 production required Src kinase activity involvement. We previously showed that the *oipA*cag PAI double mutant could not induce IL-8 production (31). Taken together, these results suggest that collective inhibition of *cag* PAI and host Src kinase activity will reduce *H. pylori*-mediated IL-8 production.

To further clarify the role of FAK expression on *H. pylori*-regulated IL-8 production, we abrogated FAK expression using respective siRNA or using nontargeted control siRNA plasmid (negative control), followed by infection with *H. pylori*. In the presence of control siRNA, wild-type *H. pylori* induced IL-8 production; however, knock down of FAK resulted in a significant reduction in *H. pylori*-induced IL-8 production (Fig. 7B). These results suggest that FAK through possible involvement of paxillin is involved in regulating *H. pylori*-induced IL-8 production.

In the presence of negative control siRNA, the *cag* PAI mutant reduced IL-8 production to nearly 85%, whereas, in the presence of FAK siRNA, infection of cells with the *cag* PAI mutant further inhibited IL-8 production to nearly basal levels, indicating that FAK-independent signaling pathways are involved in *cag* PAI-regulated IL-8 production. The *oipA* mutant reduced IL-8 production to 50% of the levels induced with wild-type *H. pylori* infection, regardless of the presence of FAK siRNA. These results suggest that OipA regulates FAK involvement in IL-8 production, whereas an FAK-independent pathway for IL-8 production also exists, and the *cag* PAI may be involved in this pathway.

**DISCUSSION**

*H. pylori*-induced signaling pathways play an important role in regulating cellular function, the hummingbird phenotype, and cell motility (for review, see Ref. 11). *H. pylori* infection of the gastric mucosa results in a chronic immunological and inflammatory response that is linked to the development of gastric ulcers and gastric adenocarcinoma. Our results showed that *H. pylori*-induced EGFR, FAK/Src, or PI3K/Akt signaling pathways play an important role in paxillin activation, cell phenotype, and cytokine production. Furthermore, we found that *H. pylori*-infected gastric epithelial cells formed signaling complexes with FAK and paxillin. The roles of FAK- and paxillin-binding sites in FAK-paxillin complex regulation of the signaling pathways and their effects on morphologic outcome or IL-8 induction in gastric epithelial cells remain unclear, and studies are underway. We propose that paxillin functions as a novel host cellular target to regulate *H. pylori*-mediated EGFR, FAK/Src, and PI3K/Akt signaling, resulting in cytoskeletal remodeling and the hummingbird phenotype. In addition, it is partially involved in IL-8 production from gastric epithelial cells. Abrogating EGFR, FAK, or Akt expression by using specific siRNA downregulated *H. pylori*-induced paxillin phosphorylation and confirmed the role of EGFR, FAK, and
We showed that changes in intracellular signaling and actin cytoskeletal reorganization were linked with H. pylori virulence factors, the cag PAI and OipA. Infection with oipA mutants was associated with a reduction in both paxillin Y31 and Y118 phosphorylation and failure of actin stress fiber formation. These results suggest that OipA acts as a cornerstone in H. pylori-mediated activation of paxillin, actin cytoskeletal reorganization, and changes in cell phenotype. Therefore, we speculate that OipA being an outer membrane protein interacts with cell surface receptors such as EGFR to regulate actin cytoskeletal dynamics. These results confirm and extend our previous observations that OipA plays a major role in H. pylori-mediated site-specific activation of FAK and actin stress fiber formation (21). In contrast, infection with the cag PAI mutant was associated with a reduction in paxillin Y118 but not Y31 phosphorylation. Interestingly, the cag PAI mutants inhibited paxillin Y118 phosphorylation without affecting actin stress fiber formation, suggesting that site-specific activation of paxillin Y31 is a major regulator of actin stress fiber formation. These results further our prior observations that the cag PAI mutants do not influence the activation of five out of six tyrosine phosphorylation sites for FAK (Y397, Y576, Y577, Y861, and Y925, but not Y407) and have less effect on stress fiber formation than oipA mutants (21). These results are also consistent with previous studies showing that neither the cag PAI nor CagA is involved in the early phases of actin stress fiber formation (10, 19). However, they differ from a recent report by Snider et al. (17) suggesting that H. pylori-induced cell motility in AGS cells is a cag PAI-dependent mechanism. However, our studies are in agreement that activation of paxillin Y118 is cag PAI dependent. Snider et al. did not investigate the roles of the cag PAI on paxillin Y31 activation; the reason for the difference in results regarding the cag PAI on cell motility remains unknown. One difference in experimental design was that Snider et al. used cagE mutants as a marker of defective cag PAI function, whereas we used entire cag PAI deletion. Microarray data have shown that gene expression patterns in AGS cells cocultured with cagE mutants differ from those in cells cocultured with strains carrying a deletion of the entire cag PAI (5). A cag PAI-encoding TFSS injected with CagA undergoes tyrosine phosphorylation at the EPIYA motifs by host Src family kinases after 2 h of infection, and we observed paxillin phosphorylation as early as 7.5 min, which reached the maximum level 2 h following live H. pylori infection. Therefore, we suggest that OipA-initiated paxillin phosphorylation and actin stress fiber formation might be further influenced by CagA phosphorylation, its binding to unknown host proteins, and alterations in cellular function leading to the hummingbird phenotype. Finally, Snider et al. examined the late phase of infection, whereas we examined the early phase. Our data indicated that infection with cag PAI mutants for 1 h did not play a role in paxillin-mediated events such as actin stress fiber formation. It is, therefore, likely that both cag PAI and OipA cooperate in complete activation of paxillin and regulation of different related cellular functions.

Release of IL-8 from H. pylori-infected gastric epithelial cells is a key determinant of neutrophilic inflammation. We showed that pretreatment of cells with pharmacologic inhibitors of Src inhibited H. pylori-induced IL-8 production in an OipA-dependent manner. FAK knock down by siRNA also inhibited H. pylori-induced IL-8 production in an OipA-depen-
dent manner. Overall, OipA plays a major role in Src/FAK-paxillin-mediated IL-8 induction. However, because IL-8 production is predominantly regulated by cag PAI, the roles of Src and FAK signaling pathways might not be the major pathways of IL-8 induction. We previously showed that inhibition of EGFR and PI3K suppresses H. pylori-mediated IL-8 production both in cag PAI- and OipA-dependent manners (22). Overall, EGFR and PI3K/Akt pathways are mainly involved in IL-8 induction, possibly via paxillin activation.

In summary, EGFR-, FAK/Src-, and PI3K/Akt-regulated tyrosine phosphorylation of paxillin is an intercellular target in the H. pylori-host interaction that targets focal adhesion proteins and regulates downstream signaling, leading to actin stress fiber formation and phenotypic changes. We speculate that H. pylori-mediated paxillin activation functions as a novel host cellular target to regulate H. pylori-mediated EGFR, FAK/Src, and PI3K/Akt signaling. We propose that H. pylori-mediated phosphorylation of the paxillin site Y31 plays a major role in the initiation of signaling pathways, leading to actin stress fiber formation and adhesion dynamics while phosphorylation of the paxillin site Y118 might predominantly regulate cell motility and IL-8 production in part.

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DISCLOSURES

The views expressed are solely those of the authors and do not necessarily represent the official views of the VA or NIH.

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