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Helicobacter pylori Protein HP0175 Transactivates Epidermal Growth Factor Receptor through TLR4 in Gastric Epithelial Cells*[S]

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The pathophysiology of *Helicobacter pylori*-associated gastroduodenal diseases, ulcerogenesis, and carcinogenesis is intimately linked to activation of epidermal growth factor receptor (EGFR) and production of vascular endothelial growth factor (VEGF). Extracellular virulence factors, such as CagA and VacA, have been proposed to regulate EGFR activation and VEGF production in gastric epithelial cells. We demonstrate that the *H. pylori* secretory protein, HP0175, by virtue of its ability to bind TLR4, transactivates EGFR and stimulates EGFR-dependent VEGF production in the gastric cancer cell line AGS. Knock-out of the *hp0175* gene attenuates the ability of the resultant *H. pylori* strain to activate EGFR or to induce VEGF production. HP0175-induced activation of EGFR is preceded by translocation of TLR4 into lipid rafts. In lipid rafts, the Src kinase family member Lyn interacts with TLR4, leading to tyrosine phosphorylation of TLR4. Knockdown of Lyn prevents HP0175-induced activation of EGFR and VEGF production. Tyrosine-phosphorylated TLR4 interacts with EGFR. This interaction is necessary for the activation of EGFR. Disruption of lipid rafts with methyl β-cyclodextrin prevents HP0175-induced tyrosine phosphorylation of TLR4 and activation of EGFR. This mechanism of transactivation of EGFR is novel and distinct from that of metalloprotease-dependent shedding of EGF-like ligands, leading to autocrine activation of EGFR. It provides new insight into our understanding of the receptor cross-talk network.

Receptor-tyrosine kinases are among key cell surface receptors that transduce external signals through the membrane to regulate biological processes, such as cell proliferation, differentiation, and survival. Within this group, the epidermal growth factor receptor (EGFR)[3] family is closely associated with the pathophysiology of cancer. EGFR is mutated or overexpressed in a wide variety of epithelial tumors (1–3). The classical pathway of activation of the EGFR occurs through binding of a ligand to its extracellular domain and subsequent autophosphorylation of two receptor molecules (4). Ligands such as epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF) are synthesized as membrane-spanning molecules that are proteolytically cleaved to become active. Ligand binding initiates the formation of activated dimers/oligomers that undergo autophosphorylation of tyrosine residues in the cytoplasmic tail. This serves as the trigger for the recruitment of adaptor proteins and the initiation of signaling cascades within the cell. Mutations of EGFR, such as deletion of 801 bp encoded by exons 2–7 (5), result in a truncated receptor lacking 267 amino acid residues in the extracellular domain. This receptor shows ligand-independent, constitutive activity associated with increased tumorigenicity of tumors *in vivo* (6, 7). The paradigm for transactivation of EGFR has been the G protein-coupled receptor (GPCR)-mediated pathway (8). GPCR-dependent activation of matrix metalloproteases leads to cleavage of pro-HB-EGF and release of the mature growth factor, which then goes on to activate the EGFR (9–11). *Helicobacter pylori* is the causative agent of superficial gastritis, and infection with *H. pylori* increases the risk of adenocarcinoma of the stomach (12).

Vascular endothelial growth factor (VEGF) is a key factor regulating host angiogenesis in *H. pylori*-linked gastric malignancy (13, 14). However, the underlying mechanisms regulating the induction of VEGF by *H. pylori* are incompletely understood. Soluble, secreted factors of *H. pylori*, such as CagA, VacA, and BabA, have been reported to elicit a variety of host molecules involved in remodeling of the gastric epithelium (15). Signaling through the EGFR family of receptor tyrosine kinases regulates production of VEGF (16). Reports have documented the role of *H. pylori* in transactivation of the EGFR (17). Here we demonstrate an unexpected role of a secreted Toll-like receptor (TLR) 4-interacting protein HP0175 of *H. pylori* in effecting TLR4-dependent transactivation of the EGFR, leading to production of VEGF. The activation of EGFR does not involve the classical metalloprotease-dependent transactivation involving the shedding of HB-EGF or autocrine/paracrine EGF-mediated activation of EGFR. Our results brings to light a novel element of the cellular receptor cross-talk network.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S5.

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[3] The abbreviations used are: EGFR, EGF receptor; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF; MBCD, methyl-β-cyclodextrin; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA.

EXPERIMENTAL PROCEDURES

Reagents—Anti-EGFR, anti-phosphotyrosine, and EGF were from Cell Signaling Technology (Beverly, MA). Antibodies against Lyn, TLR4, and protein A/G (plus)-agarose, Lyn siRNA, EGFR siRNA, and control siRNA were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-flotillin was from BD Biosciences; neutralizing TLR4 antibody was from Imgenex (Bhubaneswar, India); the tyrosine kinase peptide substrate Raytide, LFM-A13, AG1478, GM6001, NS398, PP2, piceatannol, and protease inhibitors were from EMD Biosciences (San Diego, CA); and methyl-β-cyclodextrin (MBCD), luciferin, anti-FLAG antibody, and M2-FLAG-agarose were from Sigma. Fugene 6 and anti-Myc antibodies were from Roche Applied Science, and neutralizing EGF and HB-EGF antibody were from R&D Systems (Minneapolis, MN).

Plasmids—TLR4 and its dominant-negative mutant (encoding amino acids 1–643) lacking the TIR domain have been described (18). Mutants of hTLR4 were generated by site-directed mutagenesis based on the principle of overlap extension PCR using pEF-BOS-TLR4 as template. The first round of PCR was performed with primer pairs a and b for one reaction and primer pairs c and d for the second reaction. The second round of PCR was performed using the products of the first round as templates, and primers a and d. Primers b and c are depicted in supplemental Table S1. Primers a and d were 5′-GATCTCGAGAAAACCAGTGAGGATGATGC-3′ and 5′-GATGGATCCGATAGATGTTGCTTCCTGCC-3′, respectively. The final products were cloned between the XhoI and BamHI sites of pEF-BOS.

Cell Culture and Infections—The human gastric epithelial cell line AGS was obtained from the National Centre of Cell Science (Pune, India) and maintained in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Transfections were routinely carried out using Fugene 6 (Roche Applied Science) according to the manufacturer's protocol. HP0175 was purified as described earlier (19).

H. pylori strains were grown as described (19) and incubated with AGS or HEK293 cells at a multiplicity of infection 50–100 on culture plates. Strains 80A and 18A were *cag*$^-$ and *cag*$^+$ clinical isolates, respectively, obtained from the National Institute of Cholera and Enteric Diseases, Kolkata. These strains have been described previously (20).

VEGF Enzyme-linked Immunosorbent Assay—Cells (0.3×10^5/assay) were seeded into 96-well plates. After treatments, culture supernatants were collected and assayed for VEGF using the VEGF enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer's instructions.

Reverse Transcription-PCR—Total RNA was prepared from AGS cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. A total of 100 ng of RNA was reverse transcribed using the Titanium One-Step reverse transcription-PCR kit (BD Biosciences). The primers 5′-CTGCTGTCTTGGGTGCATTG-3′ (sense) and 5′-TTCACATTTGTTGTGCTGTAG-3′ (antisense) were used to amplify 378 bp of *vegf* mRNA. Glyceraldehyde-3-phosphate dehydrogenase was amplified using the primers 5′-CCA TCA ATG ACC CCT TCA TTG ACC-3′ (sense) and 5′-GAA GGC CAT GCC AGT GAG CTT CC-3′ (antisense) to generate a 604-bp product. The PCR conditions for *vegf* mRNA were denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 68 °C for 1 min for 30 cycles.

Luciferase Reporter Assay—The *vegf* promoter (−2018 to +50) was amplified by PCR using the primer pair 5′-ATAGGTACCTCTACAGCTTGTGCTCCT-3′ (sense), and 5′-ATAAAGCTTCCCCCAGCGCCACGACCT-3′ (antisense). The product was cloned into the vector pGL2 Basic (Promega) harboring the promoterless luciferase gene, using asymmetric KpnI and HindIII sites (underlined). AGS cells (2×10^5/assay) were transfected with the luciferase reporter construct along with β-galactosidase construct using Fugene 6. Transfected cells after treatment were lysed in buffer containing 25 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, 4 mM EGTA, 10% glycerol, 20 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin and centrifuged, and the supernatant was assayed for luciferase activity using luciferin as substrate.

EGFR and Lyn Kinase Assays—Cells after treatment were lysed in buffer containing 50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% (v/v) Triton X-100, 10% glycerol, 40 mM sodium β-glycerophosphate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (buffer A). Lysates were clarified by centrifugation. For EGFR kinase assays, EGFR was immunoprecipitated with anti-EGFR antibody. Immunoprecipitates were washed three times with buffer A and twice with kinase buffer (20 mM HEPES, pH 7.2, 3 mM NaCl, 2 mM $MnCl_2$, 24 μM EDTA, 50 μM sodium orthovanadate, 0.15 μM β-mercaptoethanol). Beads were resuspended in 50 μl of kinase buffer containing 100 ng of the synthetic peptide RLIEDNEYTARG (sc-3049; Santa Cruz Biotechnology), and 20 μM [γ-^{32}P]ATP (20 Ci/mmol). After incubation at 30 °C for 30 min, the incorporation of phosphates in the peptide was determined by spotting on p81 phosphocellulose paper. Lyn kinase assays were similarly performed using the peptide substrate Raytide.

Study of the Interaction of TLR4 with EGFR—Cells were transfected with FLAG-TLR4 (or its mutants). After treatments, cells were lysed in buffer A, followed by clarification of the supernatant by centrifugation and incubation with anti-FLAG antibody (1:100) overnight at 4 °C. Protein A-agarose was subsequently added and kept for another 3 h. Immunoprecipitated proteins were boiled in Laemmli buffer, separated by SDS-PAGE, and immunoblotted with anti-EGFR antibody to detect coimmunoprecipitated EGFR.

Analysis of Tyrosine Phosphorylation of TLR4—AGS cells were transfected with FLAG-TLR4 constructs as described above. After treatments, cells were lysed, immunoprecipitated with anti-FLAG antibody, and immunoblotted using anti-phosphotyrosine antibody.

Transfection of Small Interfering RNAs (siRNAs)—Cells were transfected with EGFR or Lyn or control siRNA (Santa Cruz Biotechnology) according to the manufacturer's protocol. Silencing of either EGFR or Lyn kinase was confirmed by Western blotting of cell lysates with anti-EGFR or anti-Lyn antibody.

Isolation of Lipid Rafts and Western Analysis—AGS cells (2×10^6/assay) after treatment were lysed at 4 °C in 1.5 ml of TBS containing 1% Triton X-100, protease inhibitors, and 1 mM EDTA and subjected to a sucrose flotation gradient. After 30 min at 4 °C, the extract was mixed with an equal volume of 80% (w/v) sucrose, overlaid with 30% (w/v) sucrose and 5% sucrose, and subjected to ultracentrifugation in an AH-629 rotor (Sorvall) at 27,000 rpm for 3 h at 4 °C. Fractions were collected as described (21), immunoprecipitated with 1 μg of anti-EGFR or anti-TLR4 or anti-flotillin antibody, boiled in Laemmli buffer, and subjected to analysis by Western blotting using the same antibodies.

RESULTS

HP0175 Induces VEGF Production in Gastric Epithelial Cells—In our effort to define in detail the repertoire of molecules responsible for VEGF expression, we tested the effect of HP0175, a soluble TLR4-interacting protein previously characterized by us (19). Unless stated otherwise, recombinant HP0175 purified from *Escherichia coli* was used for all studies. Here we show that exogenous HP0175 stimulates VEGF release from AGS cells. The HP0175-induced release of VEGF was time- and concentration-dependent (Fig. 1, *A* and *B*). Recombinant HP0175 purified from HEK293 cells also induced VEGF production (Fig. 1*B*, *last bar*), ruling out the possibility that endotoxin contamination was responsible for the observed effect of the recombinant protein. VacA has been identified as one of the soluble factors of *H. pylori* responsible for VEGF release from epithelial cells (13). HP0175-mediated release of VEGF was comparable with that elicited by VacA (data not shown). The role of HP0175 was supported by the observation that an isogenic strain inactivated in *hp0175* showed significantly reduced VEGF-inducing ability, which was restored upon complementation with the *hp0175* gene (Fig. 1*C*). HP0175 induced *vegf* transcription and activated VEGF promoter-driven luciferase expression in a time-dependent manner (supplemental Fig. S1, *A* and *B*, respectively). VEGF promoter-driven luciferase expression was diminished in a mutant inactivated in *hp0175* (Fig. 1*D*) and restored upon complementation with *hp0175*, supporting the notion that HP0175 is an important bacterial factor that elicits *vegf* production in *H. pylori*-challenged gastric epithelial cells.

HP0175-mediated VEGF Production Is Dependent on TLR4 and EGFR—We next attempted to delineate the signaling events associated with exogenous HP0175-mediated production of VEGF. Taking into account the fact that HP0175 interacts with TLR4, we tested the role of TLR4 in HP0175-dependent VEGF production. Treatment of AGS cells with neutralizing antibodies against TLR4 inhibited HP0175-induced VEGF release (Fig. 1*E*). However, VacA-mediated VEGF release from AGS cells occurred in a TLR4-independent manner (data not shown). Cells transfected with dominant negative TLR4 also showed attenuation of HP0175-induced *vegf* promoter-driven luciferase expression (Fig. 1*F*). This suggested a role of TLR4 in VEGF production in this instance. In view of the fact that VEGF production is associated with activation of EGFR (13), we tested the possible involvement of EGFR in HP0175-induced VEGF production. Knockdown of EGFR by RNA interference (supplemental Fig. S1*C*) attenuated HP0175-driven VEGF production (Fig. 1*G*) and VEGF promoter-driven luciferase activation (Fig. 1*H*).

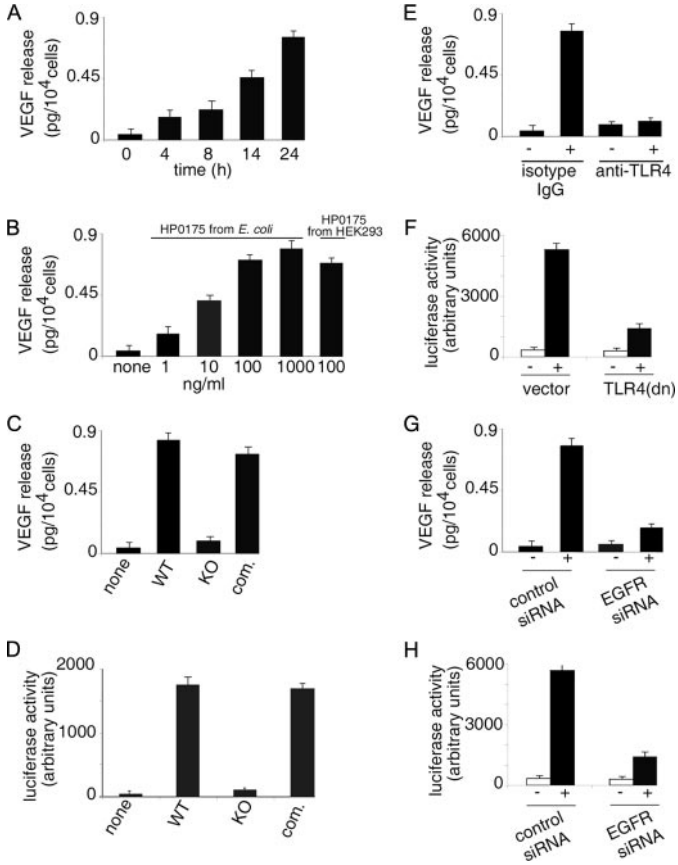

FIGURE 1. **HP0175 stimulates VEGF production in AGS cells.** Cells were left untreated (*none*) or treated with HP0175 (1 μg/ml) for different periods of time (*A*) or for 24 h with different concentrations of HP0175 (*B*) purified from *E. coli* (*A* and *B*) or from HEK 293 cells (*B*, *last bar*). In separate sets of experiments, cells were either pretreated with neutralizing antibody against TLR4 or isotype IgG (*E*) or transfected with control or EGFR-specific siRNA (*G*). *C*, cells were treated with wild type *H. pylori* or the *hp0175*-inactivated mutant (knock-out; *KO*) or knock-out complemented with *hp0175* (*com.*) for 24 h. In *A–C*, *E*, and *G*, the release of VEGF was measured in the supernatant using the VEGF enzyme-linked immunosorbent assay kit according to the manufacturer's protocol. *D*, in order to measure *vegf* promoter activation, cells were transfected with *vegf* promoter luciferase reporter and β-galactosidase construct before incubation with wild type *H. pylori* or the *hp0175*-inactivated mutant (*KO*) or knock-out complemented with *hp0175* (*com.*). *F*, in separate experiments, cells were transfected either with empty vector or with dominant-negative TLR4 (*TLR4(dn)*) along with *vegf* promoter luciferase reporter and β-galactosidase construct followed by incubation without (−) or with (+) HP0175 (1 μg/ml) for 5 h. *H*, cells were transfected with control or EGFR siRNA prior to transfection with *vegf* promoter luciferase reporter construct and treatment as described for *F*. In *D*, *F*, and *H*, luciferase was measured as described under "Experimental Procedures." Results represent the means ± S.D. of three separate experiments.

HP0175 Activates EGFR in a Metalloproteinase-independent Manner—The observation that both TLR4 and EGFR were involved in HP0175-driven VEGF production, provided the motivation to test the possibility that cross-talk between TLR4 and EGFR leads to activation of EGFR. This is the focus of the present report. Considering that HP0175-mediated VEGF production was dependent on EGFR, we tested whether exogenous HP0175 activates EGFR in AGS cells. HP0175 activated EGFR in a time- and dose-dependent manner (Fig. 2, *A* and *B*). Activation of EGFR was inhibited by the EGFR inhibitor AG1478

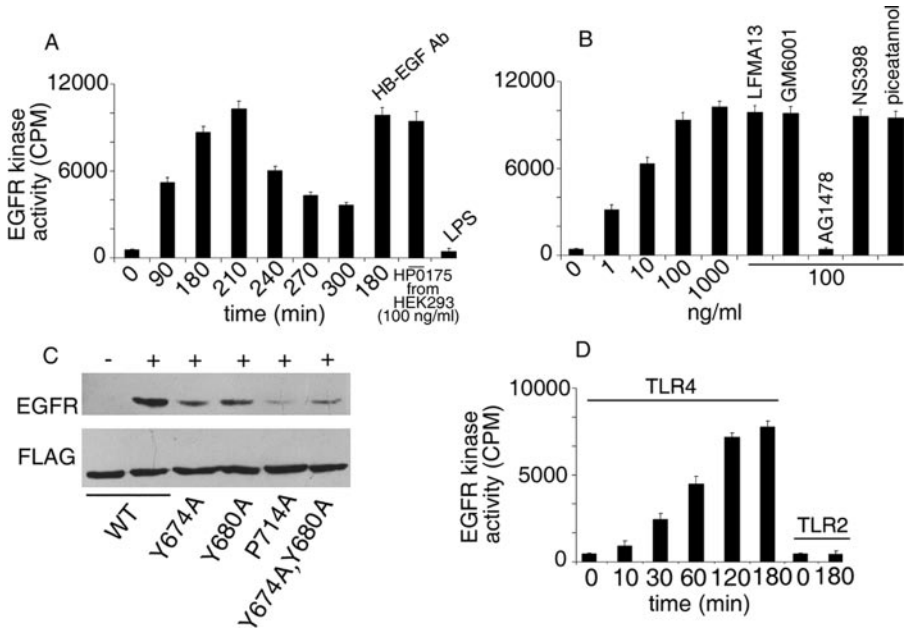

FIGURE 2. **HP0175-stimulated EGFR kinase activity and interaction of EGFR with TLR4.** Cells were treated with HP0175 (1 μg/ml) for different periods of time (*A*) or at different concentrations for 3 h (*B*). Where indicated, cells were treated either with neutralizing anti-HB-EGF (*A*) or with different inhibitors (*B*) prior to treatment with HP0175. In *A*, cells were also treated separately with HP0175 (1 μg/ml) purified from HEK 293 cells or with lipopolysaccharide (1 μg/ml) for 3 h. In all cases, cells were lysed and immunoprecipitated with anti-EGFR antibody, and kinase activity was measured as described under "Experimental Procedures." *C*, in order to study the interaction of EGFR with TLR4, cells were transfected with FLAG-tagged TLR4 (wild type (*WT*)) or the indicated mutants and treated without (−) or with (+) HP0175 (1 μg/ml) for 60 min. Cells were lysed and immunoprecipitated with M2-FLAG-agarose, followed by Western blotting with anti-EGFR antibody. The blot was reprobed with anti-FLAG antibody. *D*, HEK 293 cells (in 24-well plates) were transfected either with TLR4 or TLR2 as indicated. Cells were treated with HP0175 (1 μg/ml), and EGFR kinase activity was measured as described for *A*. Results in *A*, *B*, and *D* represent the means ± S.D. of three separate experiments. The blot in *C* is a representative of three separate experiments.

(Fig. 2*B*). HP0175 purified from HEK293 cells also activated EGFR (Fig. 2*A*). A previous report (17) has suggested that shedding of HB-EGF by the action of matrix metalloproteinases is linked to *H. pylori*-mediated activation of EGFR. We therefore tested whether HP0175 was activating such a pathway. Neither neutralizing HB-EGF antibody (Fig. 2*A*) nor GM6001 (batimastat, a metalloproteinase inhibitor) (Fig. 2*B*) inhibited HP0175-driven activation of EGFR. As a control, neutralizing HB-EGF antibody was capable of inhibiting HB-EGF-elicited EGFR kinase activity (supplemental Fig. S1*D*). As expected, HP0175-induced VEGF production was also not inhibited (data not shown). Neutralizing EGF antibody did not inhibit HP0175-induced VEGF release, whereas EGF-induced VEGF release was inhibited by this antibody (supplemental Fig. S1*E*). This suggested the likelihood of HP0175 inducing a novel transactivation mechanism. The ability of the intact bacterium to activate EGFR was diminished in a mutant inactivated in *hp0175* (supplemental Fig. S1*F*) and restored upon complementation with *hp0175*. EGFR kinase activation by *H. pylori* was abrogated when AGS cells were pretreated with neutralizing TLR4 antibody but not when cells were pretreated with neutralizing HB-EGF antibody or with GM6001 (supplemental Fig. S1*F*). These results provided support to the view that TLR4-dependent transactivation of EGFR plays an important role in *H. pylori*-induced signaling in gastric epithelial cells.

HP0175 Drives the Interaction between TLR4 and EGFR and Activation of EGFR—Co-immunoprecipitation studies were performed to determine whether HP0175 treatment of AGS cells triggered interaction of EGFR with TLR4. EGFR was detected in lysates of HP0175-stimulated FLAG-TLR4-transfected AGS cells captured with FLAG antibody but not in control lysates (Fig. 2*C*) or in cells stimulated with an irrelevant His-tagged protein (data not shown). HP0175 expressed in HEK293 cells was also found to stimulate the interaction of TLR4 with EGFR (data not shown). HEK293 cells are naturally TLR4- and TLR2-negative. Expression of TLR4 led to activation of EGFR kinase in a time-dependent manner in response to challenge with HP1075 (Fig. 2*D*). TLR2-transfected HEK293 cells did not show EGFR kinase activation in response to HP0175 (Fig. 2*D*), indicating the specificity of the observed effects. Lipopolysaccharide, a TLR4 ligand, was unable to induce EGFR kinase activity or VEGF release from AGS cells (Fig. 2*A*, *last bar*), indicating that the effect was specific for HP0175.

HP0175 Signaling Triggers Activation of Lyn Kinase—Activation of EGFR involves Src-dependent phosphorylation of EGFR (4). Among the members of the Src family, AGS cells express c-Src and Lyn (22). We evaluated the role of Src family kinases in HP0175-driven signaling events by measuring HP0175-mediated VEGF release and EGFR kinase activation. Both VEGF promoter-driven luciferase activation (supplemental Fig. S1*B*) and EGFR kinase activation (Fig. 3*A*) were inhibited by pretreatment with the Src family kinase inhibitor, PP2. Knockdown of Lyn kinase by RNA interference (supplemental Fig. S1*G*), inhibited HP0175-induced VEGF promoter-driven luciferase (Fig. 3*B*) as well as EGFR activation (Fig. 3*C*). HP0175-driven phosphorylation of Lyn on tyrosine residues occurred in a time-dependent manner (Fig. 3*D*). However, Lyn kinase was not affected by knockdown of EGFR (supplemental Fig. S2*A*). HP0175-induced Lyn kinase activity and tyrosine phosphorylation of Lyn were inhibited in AGS cells transfected with dominant negative TLR4 (Fig. 3, *E* and *F*, respectively) and Lyn kinase activity was inhibited in cells pretreated with neutralizing antibodies against TLR4 (supplemental Fig. S2*B*), arguing in favor of an essential role of TLR4 in Lyn activation. It therefore appeared that the binding of HP0175 to TLR4 facilitates binding of Lyn to its intracellular domain and subsequent activation of Lyn. In monocytic cells, the tyrosine kinase Syk has been reported to associate with TLR4 (23). We did not observe association of Syk with TLR4 after treatment of AGS cells with HP0175 (data not shown). The ability of the intact bacterium to activate Lyn kinase was diminished in a

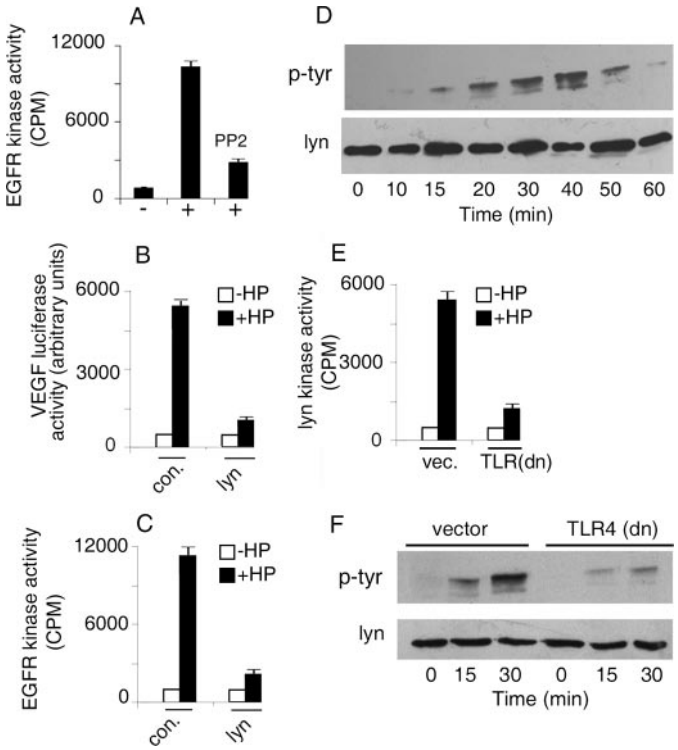

FIGURE 3. **HP0175 activates Lyn kinase.** *A*, AGS cells were left untreated or were pretreated with the Src kinase inhibitor PP2 for 30 min, followed by incubation without (−) or with (+) HP0175 for 3 h and measurement of EGFR kinase activity. Cells were transfected with either control (*con.*) or with Lyn-specific (*lyn*) siRNA and treated without or with HP0175, followed by measurement of VEGF luciferase (*B*) or EGFR kinase (*C*) activity. *D*, AGS cells were treated with HP0175 for different periods of time, lysed, and immunoprecipitated with anti-Lyn antibody, followed by Western blotting with anti-phosphotyrosine antibody. *E*, cells were transfected either with empty vector (*vec*) or TLR4 (*dn*), followed by incubation without or with HP0175 for 40 min. Cells were lysed and immunoprecipitated with anti-Lyn antibody, and the immunoprecipitate was used to measure Lyn kinase activity, as described under "Experimental Procedures." *F*, lysates were prepared from transfected cells treated with HP0175 (as in *E*) for different periods of time and immunoblotted with anti-phosphotyrosine antibody. Blots were reprobed with anti-Lyn antibody to ensure equal loading in all lanes. Results in *A–C* and *E* represent the means ± S.D. of three separate experiments. Blots are representative of three separate experiments. HP0175 was used at a concentration of 1 μg/ml.

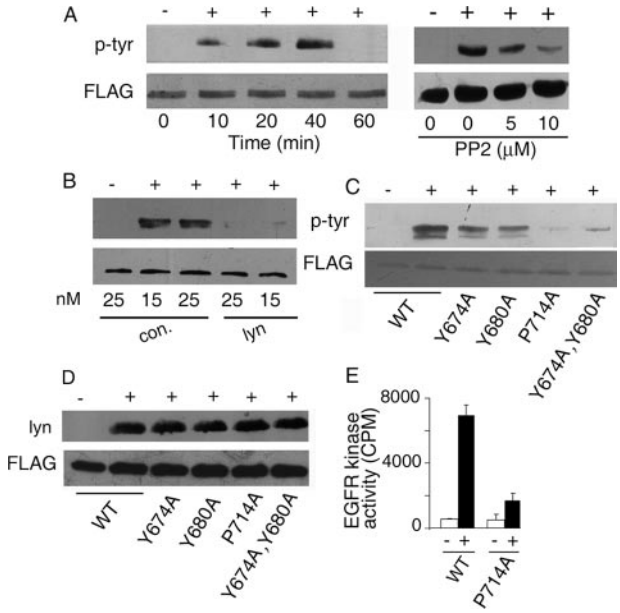

FIGURE 4. **HP0175 activates Lyn-mediated TLR4 phosphorylation and EGFR kinase.** *A*, AGS cells were transfected with wild type (*WT*) FLAG-tagged TLR4 or its mutants as indicated (*A–E*). *A*, cells transfected with WT-TLR4 were left untreated or treated with PP2 at different concentrations, followed by treatment without (−) or with (+) HP0175 for the indicated time periods (*A*) or for 40 min (*C–E*). *B*, cells were transfected with either control siRNA (*con.*) or Lyn siRNA (*lyn*), followed by treatment without (−) or with (+) HP0175. Cells were lysed after treatments. For *A–D*, lysates were immunoprecipitated with M2 FLAG-agarose, and Western blotting was performed with anti-phosphotyrosine antibody (*A–C*) or with anti-Lyn antibody (*D*). Blots were reprobed with anti-FLAG antibody to ensure equal loading. *E*, EGFR kinase activity was measured as described in the legend to Fig. 2*A*. Results in *E* represent the means ± S.D. of three separate experiments. Blots are representative of three separate experiments. HP0175 was used at a concentration of 1 μg/ml.

mutant inactivated in *hp0175* and restored upon complementation with *hp0175* (supplemental Fig. S3*A*).

Lyn Kinase Phosphorylates TLR4—TLR4 phosphorylation on tyrosine residues has been reported to be involved in TLR4-dependent signaling events (24). HP0175 induced TLR4 phosphorylation on tyrosine residues in a time-dependent manner (Fig. 4*A*). The Src kinase inhibitor PP2 inhibited tyrosine phosphorylation of TLR4 in HP0175-treated cells in a dose-dependent manner (Fig. 4*A*). Knocking down of Lyn using Lyn-specific siRNA abrogated the tyrosine phosphorylation of TLR4 (Fig. 4*B*) and its interaction with EGFR (supplemental Fig. S3*B*). Pretreatment of cells with piceatannol, an inhibitor of Syk kinase or with LFM-A13, an inhibitor of Bruton's tyrosine kinase, did not affect tyrosine phosphorylation of TLR4 (data not shown). Neither LFM-A13 nor piceatannol affected HP0175-induced EGFR kinase activation (Fig. 2*B*). Tyr^{674} and Tyr^{680} have been identified as phosphorylatable tyrosine residues of TLR4 (24), whereas Pro^{714} is crucial for signaling downstream of TLR4. Mutation of either Tyr^{674} or Tyr^{680} or both led to partial inhibition of HP0175-dependent tyrosine phosphorylation of TLR4, whereas the P714A mutation showed complete inhibition of TLR4 phosphorylation on tyrosine residues (Fig. 4*C*). These results suggested that as in the case of lipopolysaccharide signaling, these residues were probably important in HP0175-mediated signaling events. The role of HP0175 in mediating *H. pylori*-induced TLR4 tyrosine phosphorylation was confirmed by challenging AGS cells with bacteria inactivated in *hp0175*. Tyrosine phosphorylation of TLR4 and its interaction with EGFR were abrogated in the knock-out strain but restored upon complementation with *hp0175* (supplemental Fig. S4, *A* and *C*). Although HP0175 induced tyrosine phosphorylation of TLR4 in AGS cells, VacA, which like HP0175, elicits VEGF release, did not do so (supplemental Fig. S4*B*). We tested interaction of TLR4 with Lyn in response to treatment of AGS cells transfected with different FLAG-tagged constructs of TLR4. Lyn could be coimmunoprecipitated with TLR4 (Fig. 4*D*). This interaction was not affected in cells transfected with tyrosine phosphorylation-defective mutants of TLR4 (Fig. 4*D*), suggesting that tyrosine phosphorylation of TLR4 is not necessary for its interaction with Lyn.

Phosphorylation of TLR4 Is Required for EGFR Transactivation—In order to explore the role of tyrosine phosphorylation of TLR4 in its interaction with EGFR, AGS cells were transfected with FLAG-tagged TLR4 (P714A) (which is defective in tyrosine phosphorylation), challenged with HP0175, followed by immunoprecipitation with anti-FLAG and probing of the immunoprecipitates with anti-EGFR antibody. Interaction

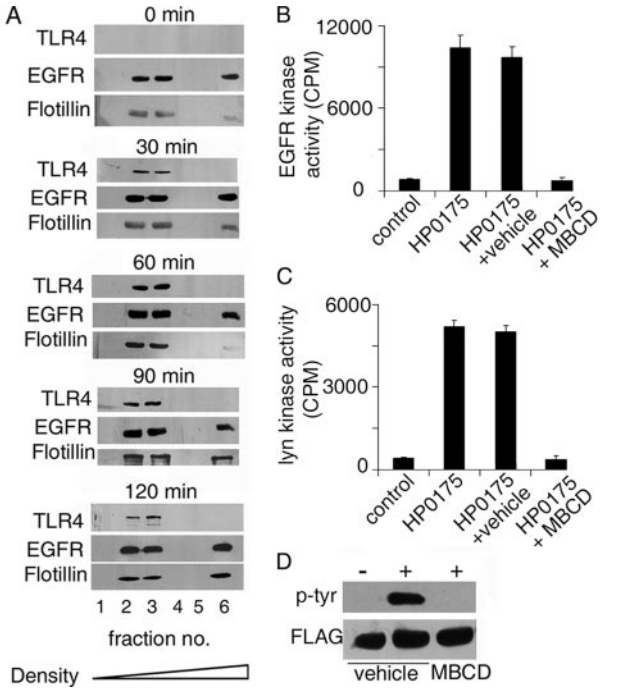

FIGURE 5. **Involvement of lipid rafts in HP0175-mediated signaling in AGS.** *A*, cells grown on 35-mm tissue culture plates were treated with HP0175 (1 μg/ml) for different periods of time (as indicated) and lysed. The Triton X-100 extract was fractionated by density gradient centrifugation as described under "Experimental Procedures," and fractions were immunoblotted with anti-TLR4 or anti-EGFR or anti-flotillin (raft marker) antibody. *B* and *C*, AGS cells were left untreated or pretreated with either MBCD or vehicle followed by incubation without (control) or with HP0175. Cell lysates were used to measure EGFR (*B*) or Lyn (*C*) kinase activity, as described under "Experimental Procedures." *D*, cells were transfected with FLAG-TLR4, followed by pretreatment with vehicle or MBCD. Cells were incubated without (−) or with (+) HP0175, lysed, immunoprecipitated with M2-FLAG-agarose, and immunoblotted with phosphotyrosine antibody. HP0175 was used at a concentration of 1 μg/ml.

of TLR4 with EGFR was compromised in the Tyr^{674} or Tyr^{680} or the Tyr^{674}/Tyr^{680} mutants. On the other hand, it was negligible for the P714A mutant (Fig. 2*C*). Tyrosine phosphorylation of TLR4 therefore appeared to be a prerequisite for its interaction with EGFR. Consistent with these observations, EGFR kinase activity was attenuated in cells transfected with the aforesaid mutant of TLR4 (Fig. 4*E*) as well as after knockdown of Lyn kinase by RNA interference (supplemental Fig. S1*H*).

HP0175 Induces Translocation of TLR4 into Rafts—Translocation of signaling receptors to lipid rafts is a well documented event governing spatiotemporal control of signaling events (25). Pretreatment of AGS cells with MBCD, which specifically depletes cholesterol and disrupts lipid raft organization, led to an abrogation of HP0175-induced VEGF promoter-driven luciferase production (supplemental Fig. S1*B*), suggesting a role of lipid rafts in the signaling events triggered by HP0175. We observed that a fraction of EGFR is constitutively present in lipid rafts of AGS cells (Fig. 5*A*), an observation made in Chinese hamster ovary cells as well (26). On the other hand, TLR4 translocated into rafts in a time-dependent manner after challenge with HP0175 (Fig. 5*A*) and was observed in rafts up to 120 min. Pretreatment of cells with MBCD prevented the activation of EGFR and Lyn kinases (Fig. 5, *B* and *C*, respectively) as well as the tyrosine phosphorylation of TLR4 (Fig. 5*D*), supporting the contention that Lyn-mediated tyrosine phosphorylation of TLR4 requires the presence of TLR4 in lipid rafts.

DISCUSSION

H. pylori ranks among the most successful microbes to have colonized the human stomach (27–29). Only a proportion of infected individuals develop ulcers, gastric adenocarcinoma, or gastric lymphoma (27, 30). Due to its role as an angiogenic factor, VEGF plays an important role in *H. pylori*-associated gastroduodenal diseases (31). VEGF is overexpressed in human gastric adenocarcinomas (32). On the one hand, it is involved in the reconstruction of normal mucosal architecture required during healing. On the other hand, VEGF also promotes gastric carcinomas by supporting tumor-associated angiogenesis. Considering the importance of VEGF, we initiated studies to determine whether the TLR4-interacting, secreted antigen HP0175 previously characterized by us (19) is involved in modulating VEGF production in gastric epithelial cells.

We observed that HP0175 elicited VEGF release in a TLR4-dependent manner. Elevated expression of TLR4 has been reported in gastric biopsy samples associated with *H. pylori* infection compared with uninfected samples (33), suggesting a probable involvement of TLR4 in *H. pylori*-associated pathophysiology. It was therefore of obvious relevance to understand the mechanism of HP0175-induced TLR4-dependent VEGF release. In view of the reports of involvement of EGFR in VEGF production (34, 35), here we focused our attention on understanding whether EGFR was involved in VEGF release and whether cross-talk between TLR4 and EGFR could be the trigger for HP0175-mediated VEGF production. RNA interference knockdown of EGFR supported the view that EGFR was involved in HP0175-mediated VEGF production. Most interestingly, we observed that exogenous HP0175 was capable of triggering EGFR activation. Intestinal homeostasis has been reported to be associated with TLR4-dependent up-regulation of cyclooxygenase-2 (36). Our results using the cyclooxygenase-2 inhibitor NS398 ruled out the involvement of cyclooxygenase-2 in HP0175-dependent EGFR kinase activation (Fig. 2*B*). Using intact bacteria, we also demonstrate that TLR4-dependent EGFR activation occurs irrespective of the status of the cag pathogenicity island (supplemental Fig. S1*I*). This contradicts the observations of Keates *et al.* (37), who have reported that infection of AGS cells by *H. pylori* is associated with EGFR transactivation and up-regulation of EGFR expression, an effect limited to cag^+ strains. According to Keates *et al.* (17), *H. pylori*-mediated EGFR activation is dependent on shedding of HB-EGF, an observation that does not agree with the findings of this study. In this connection, it is to be pointed out that Keates *et al.* have demonstrated phosphorylation of EGFR on tyrosine residues as the only evidence of EGFR transactivation, unlike in our study, where the kinase activity of EGFR has been measured. Several mechanisms of transactivation of EGFR have been reported. The GPCR protease-activated receptor-2 is capable of transactivating EGFR in the gastric cancer cell lines AGS and MKN28 (38). This mechanism is dependent on the activation of Src and is brought about by the action of EGF-like ligands that are cleaved and released by matrix metalloproteinases. Another GPCR, platelet-activating factor receptor, is involved in bacte-

rial lipoteichoic acid-mediated activation of EGFR, again involving a metalloprotease, ADAM10 (39). Several other reports have also demonstrated that activation of metalloproteases cleaves ligands involved in autocrine stimulation of EGFR (40, 41). Using an inhibitor of matrix metalloproteinases, we do not find evidence of involvement of matrix metalloproteinase(s) in HP0175-mediated activation of EGFR. In A431 carcinoma cells, HSP70-mediated association of TLR4 with EGFR and subsequent activation of EGFR was reported (42), although a detailed mechanism was not explored. Our report brings to light an unexpected novel mechanism of EGFR activation discussed below.

Sucrose density gradient centrifugation provides evidence for the localization of TLR4 with the lipid raft marker flotillin, suggesting that the binding of HP0175 to TLR4 triggers the translocation of TLR4 to lipid rafts (supplemental Fig. S5). This is a step that we recognize as a prerequisite for EGFR activation, since MBCD prevents activation of EGFR. Among eight members of the Src kinase family tested, AGS cells have been reported to express c-Src, Lyn, and c-Fgr (22). We further demonstrate that the translocation of TLR4 to lipid rafts facilitates its interaction with Lyn, followed by Lyn-dependent phosphorylation of TLR4 on tyrosine residues. Mutants of TLR4 incapable of undergoing Lyn-dependent phosphorylation on tyrosines could bind to Lyn but failed to activate EGFR. The detailed mechanism of TLR4-dependent EGFR activation requires further exploration. In light of the report by Evdonin *et al.* (42), it would be interesting to test whether the tyrosine phosphorylation of TLR4 is also required for TLR4-dependent EGFR activation in A431. A clinical isolate lacking the complete *cag* pathogenicity island is still capable of activating EGFR (supplemental Fig. S1*I*). In addition, pretreatment with neutralizing antibodies against TLR4 abrogates EGFR activation by *H. pylori* irrespective of the status of the *cag* pathogenicity island (data not shown). These results provide the basis of our contention that TLR4-dependent transactivation of EGFR induced by HP0175 probably plays a role in the pathophysiology of ulcerogenesis and/or carcinogenesis.

Disruption of epithelial tight junctions by the interaction of translocated CagA with the scaffolding protein Zo-1 (43) and VacA-mediated phosphorylation of GPCR kinase interactor 1 (44) could facilitate binding of EGF ligands to the EGFR. Overexpression of metalloproteases involved in ectodomain shedding of membrane proteins has been reported in the gastric mucosa of *H. pylori*-infected patients (45) This suggests that ADAM-dependent transactivation of the EGFR is likely to be of pathophysiological relevance. However, our findings suggest that *H. pylori* has multiple mechanisms of transactivating the EGFR. These findings are of importance in the context of enriching our understanding of cross-talk between EGFR and other cell surface receptors. Our previous studies had indicated that HP0175 induces apoptosis in AGS cells (19). It is intriguing that the same protein also triggers release of the angiogenic factor VEGF. Our preliminary, unpublished observations[4] suggest that HP0175 triggers both HIF-1α (which promotes VEGF production) and p53 (which is proapoptotic), with different time kinetics of induction of the two proteins. The temporal course of synthesis and turnover of these two proteins probably decides the outcome on system behavior. These processes are being investigated in detail now. The observations made *in vitro* on AGS, a gastric cancer-derived epithelial cell line underscore the importance of pursuing further experimentation *in vivo* on the role of the described signaling pathway in ulcerogenesis and/or carcinogenesis.

[4] S. Basu, S. K. Pathak, G. Chatterjee, S. Pathak, J. Basu, and M. Kundu, unpublished observations.

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