Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 9, Issue of March 1, pp. 6775–6778, 2002

Src Is the Kinase of the *Helicobacter pylori* CagA Protein *in Vitro* and *in Vivo**

Received for publication, December 21, 2001,
and in revised form, January 10, 2002
Published, JBC Papers in Press, January 11, 2002,
DOI 10.1074/jbc.C100754200

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The gastric pathogen *Helicobacter pylori* uses a type IV secretion system to inject the bacterial CagA protein into gastric epithelial cells. Within the host cell, CagA becomes phosphorylated on tyrosine residues and initiates cytoskeletal rearrangements. We demonstrate here that Src-like protein-tyrosine kinases mediate CagA phosphorylation *in vitro* and *in vivo*. First, the Src-specific tyrosine kinase inhibitor PP2 specifically blocks CagA phosphorylation and cytoskeletal rearrangements thereby inhibiting the CagA-induced hummingbird phenotype of gastric epithelial cells. Second, CagA is *in vivo* phosphorylated by transiently expressed c-Src. Third, recombinant c-Src and lysates derived from c-Src-expressing fibroblasts but not lysates derived from Src-, Yes-, and Fyn-deficient cells phosphorylated CagA *in vitro*. Fourth, a transfected CagA-GFP fusion protein is phosphorylated *in vivo* in Src-positive fibroblasts but not in Src-, Yes-, and Fyn-deficient cells. Because a CagA-GFP fusion protein mutated in an EPIYA motif is not efficiently phosphorylated in any of these fibroblast cells, the CagA EPIYA motif appears to constitute the major c-Src phosphorylation site conserved among CagA-positive *Helicobacter* strains.

Helicobacter pylori is a highly successful bacterial pathogen of the human stomach. Infection typically leads to a state of chronic inflammation that is frequently associated with diseases like chronic gastritis, peptic ulceration, and sometimes mucosa-associated lymphoid tissue lymphoma and adenocarcinoma of the stomach (1). Among several virulence determinants the *H. pylori* cytotoxin-associated pathogenicity island (*cag*PAI)[1] has gained special interest (2). Strains carrying *cag*-PAI are generally more virulent than strains lacking the island, which often resemble commensal bacteria more than pathogens. Whereas the function of most of the *cag*PAI genes is unknown, some share significant homology to *virB* and *virD* genes known from other Gram-negative pathogens like *Agrobacterium tumefaciens* and *Bordetella pertussis* (3). These genes encode components of so-called type IV secretion systems that can be regarded as molecular syringes injecting bacterial effector molecules into the cytoplasm of host cells. *H. pylori* has been shown to possess a functional type IV secretion system that transfers the bacterial CagA protein into gastric epithelial cells where it becomes tyrosine-phosphorylated (4–8). The *H. pylori* type IV secretion system is also implicated in the induction of proinflammatory cytokines (9–12).

CagA translocation and phosphorylation has been reported to induce rearrangements of the host cell actin cytoskeleton leading to an altered cell morphology, the so-called hummingbird or scattering phenotype (4, 13). The relevance of CagA tyrosine phosphorylation has been explored by site-directed mutagenesis. Bacteria carrying a *cagA* gene where tyrosine residue 972 (Tyr-972) of the C-terminal EPIYA repeat has been replaced by phenylalanine are unable to rearrange the host cell cytoskeleton (13). This indicated that phosphorylation of the CagA EPIYA motif might be critical for signaling to the actin cytoskeleton. Indeed, recent investigations have demonstrated that the cytoplasmic tyrosine phosphatase Shp-2 is specifically recruited to tyrosine-phosphorylated CagA and is involved in downstream signaling (14). However, the eukaryotic tyrosine kinase that mediates the initial phosphorylation of CagA has remained enigmatic. Here we show that members of the Src family are cellular CagA kinases both *in vitro* and *in vivo*. Recombinant c-Src is able to phosphorylate CagA *in vitro*. Moreover, specific pharmacological inhibition as well as genetic deletion of Src kinases inhibit the tyrosine phosphorylation of CagA in human fibroblast cells, whereas re-expression of c-Src in a Src-deficient cell re-establishes CagA tyrosine phosphorylation. Sequence analysis suggests that during the co-evolution of humans and *H. pylori* the CagA protein has evolved as a specific Src kinase substrate.

EXPERIMENTAL PROCEDURES

H. pylori Strains and Cell Culture—H. pylori strain P1 is a clinical isolate, and an isogenic Δ*cagA* knockout mutant was constructed (8). Cultivation of *H. pylori* was on horse serum agar plates under microairophilic conditions according to a standard procedure (8). AGS cells (ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line) were cultivated in 6-well tissue culture dishes using RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) for 2 days to reach monolayers of about 70% confluence. SYF cells were derived from c-*src*$^{-/-}$, c-*yes*$^{-/-}$, and c-*fyn*$^{-/-}$ triple knockout mouse embryos and SYF+c-*src* cells were obtained by stable transfection of SYF cells with mouse c-*src* (15). Both cell lines as well as NIH 3T3 cells were grown in tissue culture flasks in Dulbecco's modified Eagle's medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal bovine serum and 1% non-essential amino acids.

Inhibitor Studies—The tyrosine kinase inhibitors AG-370 (Biomol, Plymouth Meeting, PA), AG-1478, and PP2 (Calbiochem, San Diego, CA) were dissolved in Me_2SO and added to the cells immediately before infection. Controls were performed with Me_2SO alone. *H. pylori* were suspended in phosphate-buffered saline and added to AGS cells at a multiplicity of infection (MOI) of 100. After incubation for 5 h the cells were harvested in ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 (Sigma-Aldrich).

In Vitro CagA Phosphorylation—H. pylori (A_{550} = 0.9) were harvested in ice-cold kinase buffer (25 mM Hepes pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$, 1% Nonidet P-40, 5 mM dithiothreitol, 1 mM Na_3VO_4, COMPLETE protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany)) and lysed by 20 passages through a 20-gauche syringe. Five

* This work was supported by a grant from the Fonds der Chemischen Industrie (to T. F. M.) and by funding from the Bundesministerium für Bildung und Forschung (to C. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: *cag*PAI, cytotoxin-associated pathogenicity island; GFP, green fluorescent protein; PTK, protein-tyrosine kinase; EGFR, epidermal growth factor receptor.

units of recombinant human c-Src (Upstate Technologies, Lake Placid, NY) and 10 μCi of [γ-^{32}P]ATP (Amersham Biosciences, Inc.) were mixed with 30 μl of the lysate and incubated for 30 min at 30 °C. In a similar experiment, 1×10^7 SYF cells or SYF+c-*src* cells, respectively, were lysed in 1 ml of ice-cold kinase buffer as described above. 25 μl of cell lysates were incubated with 25 μl of *H. pylori* lysate and 10 μCi of [γ-^{32}P]ATP for 10 min. Reactions were stopped by addition of the appropriate amount of 4× reducing sample buffer (200 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 10% SDS, 0.4% bromphenol blue, 40% glycerol) and boiled for 5 min.

Transient Expression of CagA-GFP Fusion and Src Constructs—A C-terminal *cagA* fragment encoding EPIYA motifs and the Y972F mutant was cloned into the pEGFP-N1 expression vector as an N-terminal fusion to GFP as described (13). This fragment corresponds to positions 872–1000 of CagA from the TIGR strain (www.tigr.org/tdb/). Mouse c-*src* and constitutively active *src* in the mammalian retroviral expression vector pNeoMSV (16, 17). All constructs were transiently transfected into AGS, SYF, and SYF+c-*src* cells using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen). Cells were incubated at 37 °C under 5% CO_2 for 16 h and were harvested and subjected to SDS-PAGE.

Immunoblotting Experiments—Western blots were probed with a monoclonal phosphotyrosine-specific antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA), a monoclonal anti-Src antibody (18), anti-Src antibody GD11 (Upstate Technologies), or polyclonal anti-c-Yes and anti-c-Fyn antibodies (Santa Cruz Biotechnology). As a control, we used a monoclonal anti-α-tubulin antibody (Sigma-Aldrich). GFP expression was analyzed with a polyclonal anti-GFP antibody (MoBiTec, Göttingen, Germany). CagA was detected with a polyclonal rabbit anti-CagA antibody (6).

RESULTS AND DISCUSSION

Two bacterial virulence proteins, CagA and Tir from the human pathogen *H. pylori* and enteropathogenic *Escherichia coli* (EPEC), respectively, undergo tyrosine phosphorylation. Phosphorylation of both proteins interferes with host cell signaling events and induces rearrangements of the actin cytoskeleton (4, 13, 14, 19). Although the initial tyrosine phosphorylation step is crucial, the eukaryotic kinases that phosphorylate both Tir and CagA have not yet been identified. Here, we have analyzed the phosphorylation of the *H. pylori* CagA protein by a combination of *in vivo* and *in vitro* experiments, which show that Src is the kinase.

In general, protein-tyrosine kinases (PTKs) are thought to bind and phosphorylate their substrates depending on the amino acid sequence surrounding the respective tyrosine residue (20, 21). For example, receptor-tyrosine kinases prefer acidic amino acids at the −1 position and bulky lipophilic side chains at the +1 position. On the other hand, the Src family PTKs target tyrosines between an N-terminal lipophilic and a C-terminal small or acidic amino acid (20). We have previously reported that Tyr-972, but not Tyr-122, Tyr-899, or Tyr-918 of CagA from the TIGR *H. pylori* strain, is essential for phosphorylation (13). Homology searches of this motif revealed that Tyr-972 is well conserved in so-called Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence repeats that were first reported to be a major cause of CagA size variation (22). Among 27 CagA variants that were selected from the NCBI data base, EPIYA repeats were present in all proteins but at different numbers (Fig. 1*A*). The majority of CagA sequences had at least three EPIYA motifs whereas some contained up to five repeats, indicating that this motif is probably of general importance. To reveal if this motif can be recognized by specific kinases, we aligned the EPIYA sequence with consensus target sequences of eukaryotic tyrosine kinases. The EPIYA motif has isoleucine at the −1 position and the small amino acid alanine at the +1 position, which is closely related to the Src phosphorylation consensus motif EEIY(G/E) (Fig. 1*B*), suggesting that CagA may be a substrate for tyrosine kinases of the Src family. Interestingly, Asahi *et al.* (5) reported that epidermal growth factor receptor (EGFR) tyrosine kinase can also phosphorylate CagA *in vitro*. However,

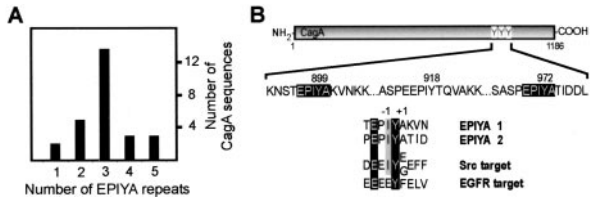

FIG. 1. **EPIYA repeats in the CagA protein are potential Src phosphorylation sites.** *A,* CagA proteins from different *H. pylori* strains typically contain several EPIYA repeats in the C terminus. A protein-protein BLAST search against *H. pylori* was performed with the query sequence EPIYA (word size, 2; expect value, 20,000) using the non-redundant data base at the NCBI (www.ncbi.nlm.nih.gov/). This study revealed 27 CagA sequences with at least 1100 amino acids. The number of EPIYAs varied from 1 to 5 repeat units. *B,* schematic presentation of the CagA protein from TIGR strain 26695 (www.tigr.org/tdb/) and the position of the EPIYA repeats. The *numbers* under the tyrosines represent the position in the complete CagA protein sequence. An alignment of the EPIYA repeats with Src and EGFR target sequences (20) is shown.

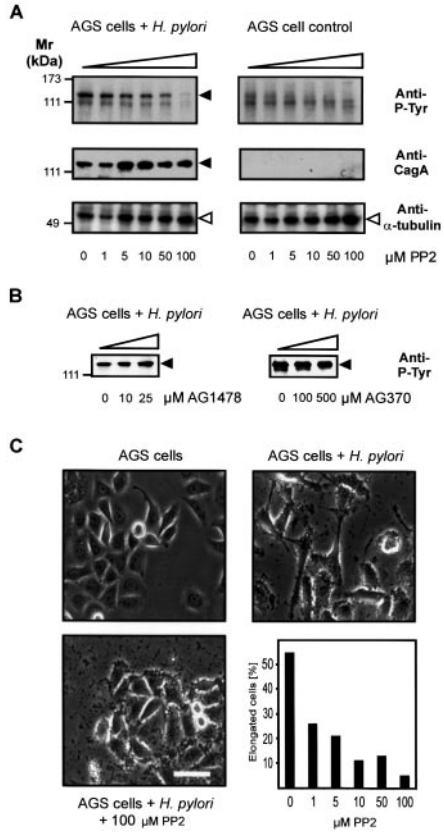

FIG. 2. **The Src kinase inhibitor PP2 prevents CagA phosphorylation and the hummingbird phenotype in AGS cells.** AGS cells were infected with *H. pylori* in the presence of tyrosine kinase inhibitors. CagA tyrosine phosphorylation was analyzed with phosphotyrosine-specific antibodies. Treatment of cells with PP2 reduced CagA phosphorylation in a dose-dependent manner (*A*, *upper panels*). PP2 had no general toxic effects as it did not significantly alter protein tyrosine phosphorylation in uninfected cells. Blots were reprobed with anti-CagA (*middle panel*) and anti-α-tubulin (*lower panel*) antibodies as controls. Two specific inhibitors of EGF/PDGF receptor tyrosine kinases, AG1478 and AG370, did not affect CagA phosphorylation (*B*). PP2 inhibits the CagA-dependent cellular phenotype (*C*). AGS cells infected with wild-type *H. pylori* are elongated and spindle-shaped (hummingbird phenotype) as compared with non-infected controls. Treatment of infected cells with PP2 prevented induction of the cellular phenoytpe in a dose-dependent manner. *Black arrowheads,* CagA; *open arrowheads*, α-tubulin; *bar,* 20 μm

the EPIYA motif shows only weak homology to the EGFR consensus sequence (Fig. 1*B*).

To determine whether Src family PTKs are important for

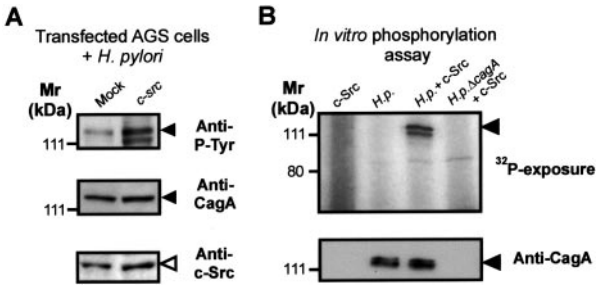

FIG. 3. **CagA can be phosphorylated by c-Src *in vivo* and *in vitro*.** *A, in vivo* phosphorylation of CagA. AGS cells were transfected with a vector encoding wild-type c-Src followed by infection with *H. pylori*. CagA phosphorylation (*closed arrowhead*) is increased by c-Src overexpression (*upper panel*). Anti-CagA and anti-c-Src (*open arrowhead*) blots were performed as controls. *B*, CagA *in vitro* phosphorylation by c-Src. Incubation of *H. pylori* lysate with recombinant human c-Src and [γ-^{32}P]ATP in an *in vitro* kinase reaction resulted in specific CagA phosphorylation (*arrowhead*). CagA phosphorylation is neither detected in lysate from an *H. pylori* Δ*cagA* mutant incubated with c-Src nor in wild-type *H. pylori* lysate incubated without c-Src.

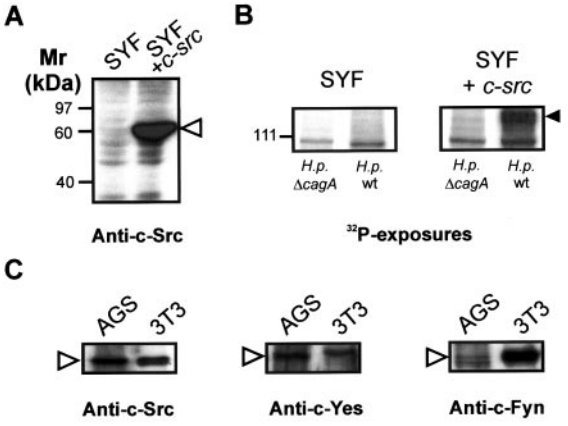

FIG. 4. **Src-dependent phosphorylation of CagA.** To investigate the role of Src family kinases for CagA phosphorylation we used a fibroblast cell line derived from c-*src*$^{-/-}$, c-*yes*$^{-/-}$, and c-*fyn*$^{-/-}$ triple knockout mouse embryos (*SYF*). Proteins from these cells were first probed with the monoclonal anti-Src antibody demonstrating the absence of Src in SYF cells (*A*). Lysates from these cells did not phosphorylate CagA in an *in vitro* kinase reaction. However, lysate from SYF cells that re-express c-Src induces strong CagA phosphorylation (*B*). Expression of the Src family members c-Src, c-Yes, and c-Fyn in AGS gastric epithelial cells were determined by immunoblotting (*C*). 3T3 fibroblasts served as a control.

CagA phosphorylation, we infected AGS gastric epithelial cells in the presence of PP2, a Src-specific tyrosine kinase inhibitor (23). After 5 h, whole cell lysates were prepared and analyzed by anti-phosphotyrosine and anti-CagA immunoblotting. Treatment with PP2 reduced CagA phosphorylation in a dose-dependent manner (Fig. 2*A*, *upper panel*). Even at low concentration (1 μM) PP2 reduced CagA phosphorylation significantly, and CagA phosphorylation was not detectable at 100 μM PP2. Treatment of uninfected AGS cells did not significantly affect tyrosine phosphorylation of most host proteins, demonstrating that PP2 inhibits a specific subset of kinases rather than blocking tyrosine phosphorylation in general (Fig. 2*A*, *upper panel*). To demonstrate that equal amounts of CagA and host cell proteins are present, the blots were reprobed with an anti-CagA antibody (*middle panel*) or anti-α-tubulin antibody (*lower panel*), respectively. Furthermore, treatment of infected AGS cells with tyrphostins AG1478 (24) or AG370 (25) as specific inhibitors of EGFR and platelet-derived growth factor receptor (PDGFR) tyrosine kinases, respectively, had no effect on the phosphorylation of CagA during infection even at high doses (Fig. 2*B*). Thus, CagA

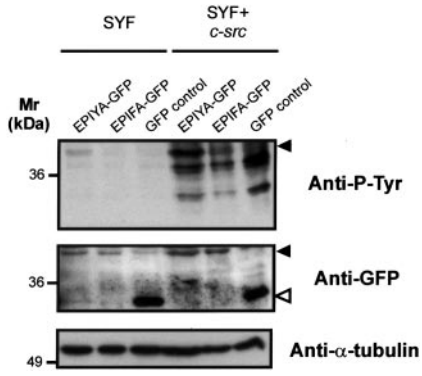

FIG. 5. **CagA-GFP fusion proteins are only phosphorylated in Src-expressing cells.** A CagA fragment containing the EPIYA motif was fused to GFP and expressed in SYF cells and SYF cells that stably re-express c-Src (*SYF+c-src*). The CagA-GFP fusion protein (*EPIYA-GFP*, *black arrowheads*) is not significantly phosphorylated in SYF cells (*upper panel*) but becomes strongly phosphorylated in SYF+c-*src* cells. When the tyrosine of the EPIYA motif was replaced by phenylalanine (*EPIFA*) tyrosine phosphorylation of the CagA-GFP fusion protein (*EPIFA-GFP*) was not observed in SYF and was strongly reduced in SYF+c-*src* cells. The GFP control (*open arrowhead*) did not become tyrosine-phosphorylated. Expression of all constructs was determined with an anti-GFP antibody (*middle panel*) and was checked for equal protein loading with an anti-α-tubulin antibody (*lower panel*).

is phosphorylated *in vivo* by a PP2-sensitive host cell kinase that is different from EGFR and PDGFR.

CagA tyrosine phosphorylation has been implicated in signaling events leading to the hummingbird phenotype in AGS cells (4, 13, 14). Thus, we analyzed the effect of PP2 on the induction of phenotype changes in infected AGS cells. In the absence of inhibitor, more than 55% of the infected cells were significantly elongated and displayed a spindle-shaped morphology (Fig. 2*C*). In the presence of 1 μM PP2 the appearance of this phenotypic alteration was reduced to 25% of the cell population. At 100 μM PP2, infected cells did not show the cellular phenotype, and the number of elongated cells dropped to less than 5% (Fig. 2*C*). This result verifies very recent reports (13, 14) demonstrating that CagA tyrosine phosphorylation is necessary to induce downstream signaling leading to the hummingbird phenotype.

In the micromolar range, PP2 is regarded as a specific Src family PTK inhibitor (23). However, the possibility that the inhibitory effect of PP2 on CagA phosphorylation in AGS cells may be due to a partial inhibition of other cellular tyrosine kinases cannot be excluded. To verify that Src PTK activity has an impact on CagA phosphorylation in AGS cells we transfected these cells with a c-Src construct, infected them with *H. pylori*, and analyzed CagA phosphorylation by immunoblotting (Fig. 3*A*, *upper* and *middle panels*). CagA phosphorylation in AGS cells overexpressing c-Src was substantially increased when compared with control AGS cells expressing only endogenous Src levels. An even stronger increase in CagA phosphorylation was obtained when AGS cells were transfected with constitutively active Src (data not shown). Therefore, increasing Src family PTK activity (Fig. 3*A*, *bottom*) strongly enhances CagA phosphorylation in AGS cells, indicating that c-Src is an *in vivo* kinase of this protein. Interestingly, the ability of activated Src variants to induce changes in the cellular morphology has been well documented in other cell types such as fibroblasts (26, 27).

To confirm that CagA can function as a c-Src substrate we performed *in vitro* kinase assays using recombinant human c-Src incubated with lysates from wild-type and CagA-deficient *H. pylori* in the presence of [γ-^{32}P]ATP. The lysates were resolved by SDS-PAGE, and CagA phosphorylation was detected by autoradiography (Fig. 3*B*, *upper panel*). In a parallel exper-

iment, these samples were blotted and probed with an anti-CagA antibody (Fig. 3*B*, *lower panel*). Strong phosphorylation of CagA was detected when c-Src was co-incubated with lysate from wild-type *H. pylori*. Bacterial lysate without c-Src was unable to phosphorylate CagA. As expected, incubation of c-Src with an isogenic *H. pylori* Δ*cagA* knockout mutant did not result in CagA phosphorylation. These results demonstrate that CagA serves as a c-Src substrate *in vitro*.

To further investigate the role of Src family PTKs for *in vivo* CagA phosphorylation we employed fibroblasts derived from c-*src*$^{-/-}$, c-*yes*$^{-/-}$, and c-*fyn*$^{-/-}$ triple knockout mouse embryos (SYF cells) (15). As a control, SYF cells stably re-expressing c-Src were used (SYF+c-*src*) (Fig. 4*A*). Because *H. pylori* was unable to translocate CagA into these cells, we first prepared cell lysates and performed *in vitro* CagA phosphorylation assays as described above. Whereas SYF cell lysates did not support CagA phosphorylation, lysates prepared from SYF+c-*src* cells strongly induced CagA tyrosine phosphorylation (Fig. 4*B*, *arrowhead*). Reprobing of the blot with an anti-CagA antibody showed identical CagA amounts present (data not shown). This result indicated that cellular Src family PTKs (in particular Src, Yes, or Fyn) are essential for CagA phosphorylation. To determine which of these Src family members are expressed in AGS gastric epithelial cells we probed whole cell lysates with antibodies to c-Src, c-Yes, and c-Fyn (Fig. 4*C*). NIH 3T3 fibroblasts were used as a positive control (*right lanes*). We found that c-Src and c-Yes are strongly expressed in AGS cells whereas c-Fyn expression was very low (Fig. 4*C*, *arrowheads*). This indicated that the Src family members c-Src and/or c-Yes might be major kinases of CagA *in vivo*.

To prove that Src family kinases mediate CagA phosphorylation *in vivo*, we fused a 15-kDa C-terminal CagA fragment containing the EPIYA motifs to green fluorescent protein (EPIYA-GFP, see Fig. 1*B*). In addition, we generated a construct in which Tyr-972 of the C-terminal EPIYA repeat has been replaced by phenylalanine (EPIFA-GFP). These constructs were transiently expressed in SYF and SYF+c-*src* cells, and phosphorylation of the EPIYA/EPIFA-GFP fusion proteins was determined by immunoblotting (Fig. 5, *upper panel*). Expression levels of the constructs were determined using an anti-GFP antibody (Fig. 5, *middle panel*). To assure that equal amounts of protein were present in each lane the blot was reprobed with an anti-α-tubulin antibody. Significantly, we observed a very faint band for phosphorylated wild-type EPIYA-GFP protein in SYF cells (only after long exposure times) and no signal for the EPIFA-GFP construct. However, SYF+c-*src* cells revealed strong phosphorylation of the wild-type EPIYA-GFP fusion construct and a faint band for the phosphorylated EPIFA-GFP protein. Densitometric analysis of CagA-GFP expression and tyrosine phosphorylation levels revealed that SYF+c-*src* cells induced more than 30-fold stronger phosphorylation of the CagA-GFP fusion protein than SYF cells. Thus, Src family kinases contribute to the majority of CagA phosphorylation *in vivo,* and c-Src re-expression in SYF cells is sufficient to mediate CagA tyrosine phosphorylation *in vivo*. Moreover, Tyr-972 of the distal EPIYA repeat of CagA from the TIGR strain appears to be the major Src kinase phosphorylation site within the CagA sequence.

CagA is regarded as an important *H. pylori* virulence factor that induces a transformed phenotype upon translocation into gastric epithelial cells. A recent report has shown that the phosphatase Shp-2 binds CagA in a phosphotyrosine-dependent manner and plays a role in downstream signaling (14). Here, we identify Src family kinases as additional host cell proteins that specifically interact with CagA. Inhibition of Src with the specific inhibitor PP2 prevents phosphorylation of CagA and CagA-dependent cytoskeletal rearrangements. As phosphorylation precedes virtually all effects induced by CagA, phosphorylation of CagA by Src-like PTKs is likely to be the initial event critically involved in CagA-induced host cell signaling. Furthermore, we identified a conserved EPIYA motif in CagA as a specific Src target site. CagA sequences from other *H. pylori* isolates contain varying numbers of EPIYA repeats that could allow for multiple phosphorylation events. Given that CagA phosphorylation is essential for modulating cellular functions, *H. pylori* would potentially be able to modify the effects of CagA by varying the number of EPIYA repeats. It is tempting to speculate that the number of phosphorylation sites present in CagA correlates with the degree of pathogenic effects induced during infection with *H. pylori*.

Acknowledgments—We thank Julia Blass and Christian Sommer for excellent technical assistance. We thank Antonello Covacci and Stefano Censini (Chiron, Siena, Italy) for providing an anti-CagA antiserum, Tony Hunter (Salk Institute, La Jolla, CA) for the c-Src expression construct, and Phil Soriano (FHCRC, Seattle, WA) for the SYF cells.

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Src Is the Kinase of the *Helicobacter pylori* CagA Protein *in Vitro* and *in Vivo*

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J. Biol. Chem. 2002, 277:6775-6778.
doi: 10.1074/jbc.C100754200 originally published online January 11, 2002
