The CagA protein of *Helicobacter pylori*, which is injected from the bacteria into bacteria-attached gastric epithelial cells, is associated with gastric carcinoma. CagA is tyrosine-phosphorylated by Src family kinases, binds the SH2 domain-containing SHP-2 phosphatase in a tyrosine phosphorylation-dependent manner, and regulates its enzymatic activity. We established AGS human gastric epithelial cells that inducibly express wild-type or a phosphorylation-resistant CagA, in which tyrosine residues constituting the EPIYA motifs were substituted with alanines. Upon induction, wild-type CagA, but not the mutant CagA, elicited strong elongation of cell shape, termed the “hummingbird” phenotype. Time-lapse video microscopic analysis revealed that the CagA-expressing cells exhibited a marked increase in cell motility with successive rounds of elongation-contraction processes. Inhibition of CagA phosphorylation by an Src kinase inhibitor, PP2, or knockdown of SHP-2 expression by small interference RNA (siRNA) abolished the CagA-mediated hummingbird phenotype. The morphogenetic activity of CagA also required Erk activation. Thus, SHP-2 acts as a positive regulator of Erk activity in AGS cells. CagA prolonged duration of Erk activation in response to serum stimulation. Conversely, inhibition of SHP-2 expression by siRNA abolished the sustained Erk activation. However, little is known about the molecular mechanisms by which *H. pylori* exerts the pathogenesis.

A subset of *H. pylori* produces a 130- to 145-kDa immunodominant protein called cytotoxin-associated gene A antigen (CagA) (8). CagA is encoded by the *cag* gene, which is located at one end of a 40-kb DNA segment called the *cag* pathogenicity island (*cag* PAI). The *cag* PAI contains 31 putative genes, some of which encode components of a bacterial type IV secretion system (9–11). Recent studies have indicated that *cag*A-positive *H. pylori* strains are associated with severe gastric inflammation, and they are thus considered to be more virulent than *cag*A-negative strains (3, 12, 13). Epidemiological studies have further shown a positive relationship between *cag*A-positive *H. pylori* and gastric carcinoma (2, 8, 14).

Upon direct contact with gastric epithelial cells, *H. pylori* delivers CagA into host cells through its type IV secretion system (15–19). The injected CagA then associates with the plasma membrane and undergoes tyrosine phosphorylation by Src family of protein-tyrosine kinases (20, 21). In CagA-injected cells, phosphorylation of the membrane-localized CagA is followed by a strong elongation of cell shape, characteristically referred to as the “hummingbird” phenotype (15).

Tyrosine phosphorylation of CagA occurs at the unique Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present in the C-terminal region (8, 20–22), and this phosphorylation is thought to play an important role in the biological activity of CagA in gastric epithelial cells. To elucidate the mechanism by which CagA exerts pathogenic effects, we investigated cellular proteins that bind to tyrosine-phosphorylated but not non-phosphorylated CagA, and identified a Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase SHP-2 (22). The CagA-SHP-2 complex has recently been found in *in vivo* human gastric mucosa (23), suggesting that the protein interaction plays a role in the pathogenesis of *cag*A-positive *H. pylori* infection. The CagA-SHP-2 complex formation involves phosphorylated tyrosine in the EPIYA motif of CagA and the SH2 domains of SHP-2 (22, 24). Notably, CagA proteins in most Western
Role of SHP-2 in Morphogenetic Activity of H. pylori CagA

H. pylori isolates have an EPIYA-containing 34-amino acid sequence that variably duplicates. As a result, the number of EPIYA motifs in CagA varies among different Western H. pylori isolates (8, 25, 26). Intriguingly, Western CagA having a greater number of repeatable EPIYA motifs exhibits stronger activity to bind SHP-2 and to induce the hummingbird phenotype (24). In contrast, prevalent East Asian CagA proteins possess a significantly diverged EPIYA-containing sequence (26, 27), which binds SHP-2 with higher affinity than those of most if not all Western CagA species (24).

SHP-2 and its orthologues, Drosophila Corkscrew and Caenorhabditis elegans PTP-2, play a positive role in transduction of signals relayed from receptor-type protein-tyrosine kinases (28–32). The functions of SHP-2 in signal transduction requires its phosphatase activity. Also, SHP-2 plays an important role in the regulation of cell morphology and motility (33–36). Recently, Grb2 and c-Met have been reported to form physical complexes with CagA, although their interactions are independent of CagA phosphorylation (37, 38). These observations collectively suggest that the bacterial protein functions as a docking/scaffolding protein that recruits and deregulates multiple signaling molecules in the injected host cells (39).

We established human gastric epithelial cells in which wild-type or a phosphorylation-resistant CagA was inducibly expressed under the control of a tetracycline-regulated (tet-on) system. Using the CagA-inducible cells, we found that tyrosine phosphorylation of CagA is an essential prerequisite for induction of the hummingbird phenotype (39). In experiments using kinase inhibitors, cells were treated with 5 μM PP2 for 2 h or 20 μM U0126 for 5 h before harvest. Time-dependent change of cell morphology was monitored by time-lapse video microscopy (Olympus IX71 Bioimaging system).

Establishment of Stable Transfectants—Human AGS gastric epithelial cells were cultured in RPMI 1640/10% fetal calf serum (FCS). To establish the tet-on system, AGS cells (1 × 10⁶ cells/100-mm dish) were first transfected with 20 μg of pTet-On (Clontech), which expresses reverse tetracycline-dependent transactivator (rtTA) regulator protein, by using the calcium phosphate method. At 12 h after transfection, cells were cultured in RPMI 1640/10% FCS supplemented with 500 μg/ml G418 (Clontech). Cells resistant to G418 were selected and single-cell cloned by limiting dilution. Clone T71-2 showing the highest rtTA activity was subjected to a second round of transfection with 20 μg of the expression plasmid, in which the gene encoding HA-tagged wild-type or a phosphorylation-resistant or HA-tagged phosphorylation-incompetent CagA were carried by the tetracycline-responsive elements (22). The transfected cells were selected by 150 μg/ml hygromycin B (Sigma). Drug-resistant colonies were then picked up and were examined for induction of CagA. Positive cells were single-cell cloned by limiting dilution. To induce CagA, cells were incubated with RPMI 1640/10% FCS containing doxycycline (Dox) at a final concentration of 5 μg/ml. In experiments using kinase inhibitors, cells were incubated with 5 μM PP2 for 2 h or 20 μM U0126 for 5 h before harvest.

Cell Scatter Assay—Cells were seeded at 1 × 10⁶ cells in 60-mm dishes and were incubated in RPMI 1640/10% FCS for 48 h in the absence of Dox. After the additional incubation with or without 5 μg/ml Dox for 60 h, colonies were observed by microscopy.

Transient Transfection and Infection—AGS cells and monkey COS-7 cells were cultured in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% FCS. For transient transfection, 30 μg of plasmids was transfected into AGS cells (1.8 × 10⁶ cells/100-mm dish) by using 30 μl of LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. COS-7 cells (1.5 × 10⁶ cells/100-mm dish) were transfected with 20 μg of plasmids by using the calcium phosphate method as described previously (22). Morphology of the AGS cells was examined at 17 h after transfection. Cells showing the hummingbird phenotype were counted in five different fields in three dishes (the area of one field = 0.25 mm²).

In experiments using U0126 or U0124, cells were incubated with the reagent at various concentrations for 1 h before microscopic analysis. In experiments using other kinase inhibitors, cells were treated with 5 μM PP2 for 2 h, 20 μM SB203580 for 1 h, 20 μM SP600125 for 1 h, or 40 μM LY294002 for 2 h before analysis. For H. pylori infection with AGS cells, standard strain NCTC11637 or its isogenic strain lacking cagA gene was passaged on 5% sheep blood agar (Nippon BD Biosciences) by incubation in an atmosphere consisting of 5% O₂, 15% CO₂, and 80% N₂ for 2–4 days at 37 °C. Bacteria were then cultured in Brucella broth (Difco) supplemented with 5% FCS under the same conditions for 12–24 h at 37 °C with agitation. AGS cells (2 × 10⁶ cells/100-mm dish) cultured at 12 h post seeding were infected with H. pylori at a multiplicity of infection of 50 for 5 h.

Immunoprecipitation and Immunoblotting—AGS cells and COS-7 cells were harvested at 36 h after transfection and lysed in lysis buffer as described previously (22). Cell lysates were treated with the appropriate antibody, and immune complexes were trapped on protein A- or protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to SDS-8% PAGE except experiments to detect Erk1 (12.5% PAGE), Grb2 (13.5% PAGE), and Ras mutants (13.5% PAGE). Recombinant proteins were transferred to a polyvinylidene difluoride (Millipore) were soaked in solutions of primary antibodies and then visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences).

Analysis of Erk Phosphorylation—AGS or G11 cells (2 × 10⁶) were transfected with pSUPER-rtTA and 5 μM doxycycline for 5 h. After the additional incubation with 5 μM PP2 for 2 h, cells were harvested and lysed in lysis buffer as described previously (22). Cell lysates were treated with the appropriate antibody, and immune complexes were trapped on protein A- or protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to SDS-8% PAGE except experiments to detect Erk1 (12.5% PAGE), Grb2 (12.5% PAGE), and Ras mutants (15.5% PAGE). Recombinant proteins were transferred to a polyvinylidene difluoride (Millipore) were soaked in solutions of primary antibodies and then visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences)
sample buffer at the indicated times. The samples were subjected to Western blotting analysis with anti-ERK1 and anti-phospho-ERK antibodies. Intensities of chemiluminescence on the immunoblotted filter were quantitated using a luminescent image analyzer LAS1000 (Fujifilm).

RNA Interference—Synthetic small interference RNA (siRNA) probes of SHP-2 (5’-GAAUAUGCGCAUGCGUGTT-3’ and 5’-CAGCGAU- GACCCCAUAUUCCTT-3’) were purchased from Dharmacon. Twenty picomoles of SHP-2 siRNA and/or 4 μg of SHP-2 expression vector were transfected into AGS cells (1.2 × 10^6 cells/35-mm dish) by using 5 μl of LipofectAMINE 2000 reagent. After 36 h of treatment of cells with siRNA, the cells were transfected again with CagA or control empty vector (5 μg) by using 5 μl of LipofectAMINE 2000 reagent. The morphants of the AGS cells was examined at 17 h after transfection. The clone W1804 cells were transfected with 20 pmol of SHP-2 siRNA by using LipofectAMINE 2000 at 12 h post seeding. At 12 h after transfection, Dox was added to the culture and the cells were incubated for additional 48 h.

RESULTS

Establishment of Gastric Epithelial Cells That Inducibly Express CagA—Infection of cagA-positive H. pylori in human AGS gastric epithelial cells induces elongation of cell shape, referred to as the hummingbird phenotype (15). We previously demonstrated that transient expression of the cagA gene in AGS cells induced morphological changes that are similar to the hummingbird phenotype (22). To elucidate the mechanism by which CagA induces morphological change, we generated a series of AGS-derived, stable transfectant clones that express HA-tagged, wild-type CagA, derived from H. pylori strain NCTC11637, or HA-tagged, phosphorylation-resistant CagA (PR CagA), in which the tyrosine residues constituting the five EPIYA motifs were substituted with alanines, under the control of a tetracycline-inducible (tet-on) gene expression system (Fig. 1A). Among these clones, wild-type CagA clone W1804 and PR cagA clone C065 were arbitrarily chosen for further experiments. In this tet-on system, wild-type and PR CagA proteins were detectable by Western blotting as early as 6 h after induction with a tetracycline derivative, doxycycline (Dox). In W1804 cells, the level of wild-type CagA oscillated after induction, showing peaks at 36 and 60 h. We reproducibly obtained the same results in three independent experiments. In the case of C065 cells expressing PR CagA, the protein level peaked at ~36 h after induction and was maintained at the peak level thereafter (Fig. 1D). Hence, a specific decrease in wild-type CagA level at 48 h after induction may be a phenomenon related to tyrosine phosphorylation of CagA. The induced wild-type CagA underwent tyrosine phosphorylation and formed a physical complex with SHP-2 in AGS cells (Fig. 1, A–C). In contrast, no tyrosine phosphorylation of PR CagA was detectable in the clone C065 (Fig. 1, A and B). As a control experiment, AGS cells were infected with H. pylori strain NCTC11637. At 5 h after infection, cells were harvested and lysates prepared were subjected to immunoprecipitation with anti-CagA. As was the case with transfectant CagA, CagA injected by the bacteria formed a physical complex with SHP-2 in AGS cells, excluding the possibility that SHP-2 binds transfectant CagA but not injected CagA (Fig. 1D).

CagA Provokes Morphological Change—Induction of wild-type CagA but not PR CagA was followed by a change in cell morphology that was indistinguishable from the hummingbird phenotype induced by transient CagA expression in AGS cells (Fig. 1E). Here we designate “hummingbird cells” as those having one or more protrusions, the length of the protrusion being more than 2-fold of cell diameter. Hummingbird cells were observed as early as 24 h after induction of wild-type CagA. At 48 h of wild-type CagA induction, ~7–8% of the cells exhibited the hummingbird phenotype. Further incubation of cells with Dox, however, did not increase the number of hummingbird cells. The weak phenotype may be primarily due to the level of CagA induced in the AGS stable transfectant, which was significantly lower than that expressed in AGS cells by transient transfection of cagA gene or cagA-positive H. pylori infection.

Establishment of AGS cells that conditionally express CagA enabled us to study time-dependent changes in cell morphology following CagA induction. Using live cell time-lapse microscopy, we found that induction of CagA caused a marked increase in cell migration: a fraction of cells initiated to move from one place to another while showing successive elongation (i.e., the hummingbird phenotype) and contraction of cell shape (Fig. 2, arrows). After several rounds of the elongation-contraction processes, some cells became detached from the dish. The observed dynamic feature of cell shape may also explain why only a fraction of the cells exhibited the hummingbird phenotype despite continuous expression of CagA.

Essential Role of Tyrosine Phosphorylation of CagA in Both the Morphogenetic Change and Cell Scattering—The results of previous studies have indicated that the activity of CagA to induce the hummingbird phenotype is dependent on its tyrosine phosphorylation (18, 20–22, 41). On the other hand, a recent report indicated that CagA is capable of inducing cell scattering regardless of its phosphorylation status (37). This prompted us to examine whether CagA phosphorylation is indeed an essential requirement for cell elongation by CagA.

CagA is phosphorylated by the Src family of protein-tyrosine kinases (20, 21). Treatment of cagA-positive H. pylori-infected AGS cells with PP2, a specific inhibitor of Src family kinases, suppressed cell scattering (20). Accordingly, we examined the effect of PP2 on the induction of hummingbird cells by CagA (Fig. 3A). Treatment of the wild-type CagA-expressing AGS cells with PP2, which did not affect levels of induced CagA, potently inhibited tyrosine phosphorylation of CagA and strongly suppressed induction of hummingbird cells (Fig. 3A). The same results were also obtained by treatment of AGS cells transiently expressing cagA gene with PP2 (Fig. 3B). As a control, we expressed the membrane-targeting, constitutively active SHP-2, which induces a hummingbird-like morphology in AGS cells (22) and found that PP2 did not block the morphological change induced by the mutant SHP-2. This suggests the possibility that PP2 inhibited the action of cell machinery generally involved in cell elongation (Fig. 3C). Thus, tyrosine phosphorylation of CagA is an essential prerequisite for the morphological transformation of gastric cells by CagA. Furthermore, the result reinforces the idea that SHP-2 is a downstream effector of CagA in induction of the hummingbird phenotype (see also below).

It was recently reported that a phosphorylation-resistant CagA mutant in which the tyrosine residues constituting the five EPIYA motifs were substituted with the conserved amino acid phenylalanine was biologically as active as wild-type CagA in inducing cell elongation and scattering of AGS cells (37). We therefore generated the same CagA mutant, PR CagA-YF, and expressed it in AGS cells by transient transfection of cagA gene or cagA-positive H. pylori infection. Establishment of AGS cells that conditionally express CagA enabled us to study time-dependent changes in cell morphology following CagA induction. Using live cell time-lapse microscopy, we found that induction of CagA caused a marked increase in cell migration: a fraction of cells initiated to move from one place to another while showing successive elongation (i.e., the hummingbird phenotype) and contraction of cell shape (Fig. 2, arrows). After several rounds of the elongation-contraction processes, some cells became detached from the dish. The observed dynamic feature of cell shape may also explain why only a fraction of the cells exhibited the hummingbird phenotype despite continuous expression of CagA.

Essential Role of Tyrosine Phosphorylation of CagA in Both the Morphogenetic Change and Cell Scattering—The results of previous studies have indicated that the activity of CagA to induce the hummingbird phenotype is dependent on its tyrosine phosphorylation (18, 20–22, 41). On the other hand, a recent report indicated that CagA is capable of inducing cell scattering regardless of its phosphorylation status (37). This prompted us to examine whether CagA phosphorylation is indeed an essential requirement for cell elongation by CagA.

CagA is phosphorylated by the Src family of protein-tyrosine kinases (20, 21). Treatment of cagA-positive H. pylori-infected AGS cells with PP2, a specific inhibitor of Src family kinases, suppressed cell scattering (20). Accordingly, we examined the effect of PP2 on the induction of hummingbird cells by CagA. Treatment of the wild-type CagA-expressing AGS cells with PP2, which did not affect levels of induced CagA, potently inhibited tyrosine phosphorylation of CagA and strongly suppressed induction of hummingbird cells (Fig. 3A). The same results were also obtained by treatment of AGS cells transiently expressing cagA gene with PP2 (Fig. 3B). As a control, we expressed the membrane-targeting, constitutively active SHP-2, which induces a hummingbird-like morphology in AGS cells (22) and found that PP2 did not block the morphological change induced by the mutant SHP-2. This suggests the possibility that PP2 inhibited the action of cell machinery generally involved in cell elongation (Fig. 3C). Thus, tyrosine phosphorylation of CagA is an essential prerequisite for the morphological transformation of gastric cells by CagA. Furthermore, the result reinforces the idea that SHP-2 is a downstream effector of CagA in induction of the hummingbird phenotype (see also below).

It was recently reported that a phosphorylation-resistant CagA mutant in which the tyrosine residues constituting the five EPIYA motifs were substituted with the conserved amino acid phenylalanine was biologically as active as wild-type CagA in inducing cell elongation and scattering of AGS cells (37). We therefore generated the same CagA mutant, PR CagA-YF, and expressed it in AGS cells. We also expressed another phosphorylation-resistant CagA mutant, PR CagA, which was made by replacing the tyrosine residues with alanines, as previously described. Neither of the CagA mutants underwent tyrosine phosphorylation nor bound SHP-2 in AGS cells. Furthermore, these CagA mutants were incapable of inducing elongated cell shape in the cells (Fig. 3D). The observations support the conclusion that the morphogenetic activity of CagA depends on its tyrosine phosphorylation.

2 H. Higashi, R. Tsutsumi, and M. Hatakeyama, unpublished observation.
Next, to investigate the relationship between CagA phosphor-
ylation and cell scattering, we performed a cell scatter assay
using CagA-inducible AGS cells (Fig. 3E). Upon induction of
wild-type CagA in the colony made of W1804 cells, dissociation
and scattering of individual cells from colonies were observed.
Furthermore, a fraction of the scattered cells exhibited the
hummingbird phenotype. On the other hand, the parental
T71-2 cells as well as the PR CagA-inducible C065 cells were
in incapable of inducing cell scattering in the presence of Dox. Thus, CagA elicits not only cell elongation but also cell migration in a tyrosine phosphorylation-dependent manner.

Requirement of SHP-2 for CagA Activity—We previously demonstrated that tyrosine-phosphorylated CagA specifically binds to and thereby activates SHP-2 phosphatase, whereas the phosphorylation-resistant CagA mutant was incapable of doing so (Fig. 3D) (22).

To investigate whether SHP-2 is an essential CagA target involved in induction of the morphological transformation, we decided to inhibit SHP-2 expression in AGS cells. To do so, we used SHP-2-specific small interference RNA (siRNA) to silence the expression of SHP-2 by RNA interference. Treatment of AGS cells with siRNA for 24 h resulted in strong reduction of SHP-2 levels to as low as 20% of that of non-treated cells, without showing any sign of cell damage. CagA was subsequently expressed in the siRNA-treated AGS cells by either induction (Fig. 4A) or transient transfection (Fig. 4B). In both cases, SHP-2 silencing potently inhibited the activity of CagA to induce hummingbird cells (Fig. 4, A and B). This inhibitory effect of siRNA on CagA was cancelled by ectopic re-expression of SHP-2 (SHP-2-RR-Myc), which was insensitive to siRNA as a result of a silent mutation introduced into the SHP-2 cDNA (Fig. 4C). As a control, treatment of the SHP-2-knockdown AGS cells with phorbol 12-myristate 13-acetate (PMA) induced an elongated cell morphology as previously reported (42) (Fig. 4D). Hence, reduced SHP-2 activity does not inhibit the action of cell machinery generally involved in cell elongation. From these observations, we concluded that SHP-2 is a cellular component essentially required for the morphological transformation of gastric epithelial cells by CagA. We also note here that treatment of cells with siRNA neither affected expression levels nor tyrosine phosphorylation levels of CagA (Fig. 4, A and B). The finding excludes a possibility that CagA is a substrate of SHP-2.

CagA-SHP-2 Complex Does Not Contain Grb2—CagA was recently reported to bind Grb2, although the interaction was independent of CagA tyrosine phosphorylation (37). Because Grb2 can also bind to SHP-2 (43, 44), we wondered if CagA, SHP-2, and Grb2 are capable of forming a heterotrimeric complex in mammalian cells. To investigate this possibility, we constructed SRo promoter-driven mammalian expression vectors for Myc-tagged SHP-2 and Myc-tagged Grb2, and expressed one or both of them together with the CagA expression vector in COS-7 cells (Fig. 5). As was the case with AGS cells, CagA was efficiently tyrosine-phosphorylated in COS-7 cells and formed a physical complex with Myc-tagged SHP-2 (Fig. 5, lanes 4 and 11). On the other hand, a heterodimeric complex between CagA and Grb2 was not detected when the two proteins were co-expressed (Fig. 5, lanes 5 and 12). In a triple transfection experiment, in which Myc-Grb2 and Myc-SHP-2 were expressed in COS-7 cells in comparable amounts as examined by anti-Myc immunoblotting (Fig. 5, lane 9), CagA immunoprecipitation co-precipitated SHP-2 but not Grb2 (Fig. 5, lane 2). From these observations, we concluded that CagA does not bind Grb2 either directly or indirectly through SHP-2 under the condition in which CagA-SHP-2 interaction is easily detectable. Thus, the interaction between CagA and Grb2 is much weaker than that between CagA and SHP-2, if existed.

Requirement of Ras-independent Erk Activity in the Morphogenetic Activity of CagA—SHP-2 has been suggested to function upstream, downstream or at a parallel level of Ras in the mitogen-activated protein kinase (MAPK) cascade (45, 46). Accordingly, we next investigated whether extracellular signal-regulated kinase (Erk), a growth-related MAPK whose activity is known to be regulated by SHP-2 as well as Ras (30, 31), is involved in cell elongation induced by CagA-SHP-2 interaction. To this end, we treated CagA-expressing AGS cells with U0126, a specific MEK inhibitor, and examined its effect on induction of hummingbird cells by CagA (Fig. 6, A–C). This MEK inhibitor did not have any effect on CagA expression or CagA phosphorylation in AGS cells but potently inhibited Erk activation as revealed by anti-phospho-ERK antibody. As shown in Fig. 6 (A and B), treatment of cells with U0126, but not treatment of cells with U0124, which is an inactive derivative of U0126, inhibited induction of hummingbird cells by CagA in a dose-dependent manner. This finding indicates that Erk activity is also necessary for the morphological change induced by CagA. In contrast to U0126, SB203580 and SP600125, which are specific inhibitors of p38 MAPK and c-Jun N-terminal kinase (JNK), respectively, did not have any effect on the CagA activity (Fig. 6C). Furthermore, treatment of the CagA-expressing AGS cells with the P13K inhibitor LY294002, which specifically inhibited PI3K activity as determined by Akt activation, had no effect on the morphological change induced by CagA. The find-
ing excluded the possibility of a role of PI3K in the morphological transformation by CagA (Fig. 6D).
Because Erk could be activated by either a Ras-dependent or Ras-independent mechanism (47–50), we investigated whether Ras was involved in the hummingbird phenotype by expressing the constitutively active c-H-RasVal12 (Ras V12) or the dominant-negative c-H-RasAsn17 (Ras N17). Whereas the amounts of the mutant Ras proteins expressed were sufficient to interfere with the endogenous Ras activity as measured by status of Erk1 or Raf1 phosphorylation, neither of the Ras mutants had a significant effect on the hummingbird phenotype induced by CagA in AGS cells (Fig. 7, A and B). The results argue against the role of Ras in the morphogenetic activity of CagA. Consistently, ectopic expression of wild-type or dominant-negative Grb2 (51) had no effect on the induction of hummingbird cells by CagA (Fig. 7C). From these observations, we concluded that the hummingbird phenotype requires Ras-independent Erk activity.

**Fig. 3. Suppression of the hummingbird phenotype by inhibition of CagA tyrosine phosphorylation.** A, clone W1804 cells treated with Dox; B, AGS cells transiently transfected with WT CagA expression vector; or C, AGS cells transiently transfected with Myr-SHP-2ΔSH2-Mye expression vector were incubated with or without 5 μM PP2 for 2 h before examination of cell morphology. Percentages of cells showing the hummingbird phenotype were indicated. Error bars indicate 2× S.D. Total cell lysates (TCL) of harvested cells were immunoblotted (IB) with anti-HA or anti-phosphorytyrosine (anti-pY) antibody. D, AGS cells were transfected with indicated CagA expression vector or control empty vector. The cells were harvested 36 h after transfection, and lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates (IP) and total cell lysates were immunoblotted with indicated antibody. The cell morphology was examined 17 h after transfection. Ratios of the hummingbird cells are indicated. Error bars indicate 2× S.D. E, W1804 cells, C065 cells, or T71-2 cells were cultured for 48 h in the absence of Dox. The cells were then treated with or without Dox for 60 h. Cell morphology and cell scattering were examined by microscopy.
Fig. 4. Requirement of SHP-2 for the hummingbird phenotype. A, clone W1804 cells were transfected with SHP-2 siRNA or mock transfected, and then treated with Dox for 48 h. The cell morphology was examined by microscopy. Total cell lysates (TCL) were prepared from the same cells and were immunoblotted (IB) with anti-SHP-2, anti-HA, or anti-phosphotyrosine (anti-pY) antibody. Cells showing hummingbird phenotype were counted. Error bars indicate 2× S.D. B and C, AGS cells were transfected with SHP-2 siRNA and expression vectors or control empty vector as indicated. The cell morphology was examined by microscopy before harvest. Cells showing hummingbird phenotype were counted. Error bars indicate 2× S.D. Total cell lysates were prepared and immunoblotted with indicated antibody. D, AGS cells or AGS cells transfected with SHP-2 siRNA were treated with 100 nM PMA. Following 6-h PMA treatment, cell morphology was examined by microscopy (upper). Total cell lysates were immunoblotted with anti-SHP-2 antibody (lower).

Fig. 5. Specific interaction of CagA with SHP-2 but not Grb2. COS-7 cells were transfected with WT CagA, SHP-2-Myc, and WT Grb2-Myc expression vectors or control empty vector as indicated and harvested 36 h after transfection. Lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates (IP) and total cell lysates (TCL) were then immunoblotted (IB) with anti-Myc, anti-HA, or anti-phosphotyrosine (anti-pY) antibody. The positions of WT CagA, SHP-2-Myc, and WT Grb2-Myc are indicated.
Fig. 6. Requirement of Erk activity for induction of the hummingbird phenotype. A, clone W1804 cells treated with or without Dox were incubated with 20 μM U0126 for 5 h before examination of cell morphology. Total cell lysates (TCL) from harvested cells were immunblotted (IB) with anti-HA, anti-phosphotyrosine (anti-pY), anti-phospho-ERK (anti-pERK), or anti-ERK1 antibody. Cells showing the hummingbird phenotype were counted. Error bars indicate 2× S.D. B and C, AGS cells transfected with WT CagA expression vector were incubated with U0126, U0124, SB203580, or SP600125 at indicated concentrations for 1 h before examination of cell morphology. Total cell lysates from harvested cells were immunblotted with indicated antibody. Cells showing the hummingbird phenotype were counted. Error bars indicate 2× S.D. D, AGS cells transfected with WT CagA or control empty vector were treated with 40 μM LY294002 for 2 h before examination of cell morphology. Percentages of cells showing the hummingbird phenotype were indicated. Error bars indicate 2× S.D. Total cell lysates of harvested cells were immunblotted with anti-HA, anti-phosphotyrosine, anti-phospho-Akt (anti-pAkt), or anti-Akt antibody.
Sustained Activation of Erk by CagA—The kinetic patterns of Erk activation are related to specific cellular events (52). In particular, sustained Erk activation is thought to be associated with cell morphogenesis (53, 54). We therefore decided to determine whether CagA modulates the duration of Erk activation. To this end, we investigated time-dependent changes in Erk activity after serum stimulation in AGS cells in the absence or presence of CagA. As shown in Fig. 8A, CagA did not modify the transient Erk activation but significantly prolonged duration of Erk activation. On the other hand, neither transient nor sustained Erk activations were affected by the presence of 5 μg/ml Dox in the parental T71-2 cells (data not shown). This observation links CagA to an intracellular signaling system regulating duration of Erk activation.

It has been reported that the cellular activity of SHP-2 is required for the sustained Erk activation and thereby for cell morphological change induced by growth factors such as hepatocyte growth factor (HGF). Hence, we next investigated the role of SHP-2 in the regulation of Erk activity in gastric epithelial cells. To do so, we established a stable SHP-2-knockdown cell line, G11, by transfecting an expression vector for SHP-2-specific siRNA into AGS cells (Fig. 8B). In the G11 cells, the level of SHP-2 was reduced to as low as 4% of that in parental AGS cells. Using the parental AGS and G11 cells, we examined the effect of SHP-2 on Erk activation. As shown in Fig. 8C, a reduced SHP-2 level abolished the sustained activation of Erk. The findings indicate that SHP-2 is required for maintaining Erk in its active form in gastric epithelial cells and therefore suggest that CagA positively modulates Erk activity through forming a complex with SHP-2.

**DISCUSSION**

In this study, we identified an important role of SHP-2 in the morphogenetic activity of *H. pylori* CagA in gastric epithelial...
cells. CagA, which is translocated from *H. pylori* into cells, is thought to play a crucial role in the pathogenesis of *cagA*-positive *H. pylori* infection (2, 3, 14). However, the molecular mechanism by which CagA deregulates intracellular signaling is not fully understood. The translocated CagA induces an elongated cell shape termed the hummingbird phenotype, which is concomitantly associated with an increase in cell motility recognized as the “scattering” phenotype. We found that

![Graph showing relative amounts of phosphorylated Erk1 in each sample, defining the value in a sample of W1804 cells (Dox−) at time 0 (A) or of AGS cells at time 0 (C) as 1. B, total cell lysates from the parental AGS cells and the SHP-2-knockdown G11 cells were immunoblotted with anti-SHP-2 antibody.](http://www.jbc.org/)

**Fig. 8.** CagA and SHP-2 prolong a term of sustained Erk activation. AGS, G11, or W1804 cells were serum-starved for 48 h and were re-stimulated with complete medium containing 10% FCS. The cells were harvested at indicated time points after stimulation, and total cell lysates (TCL) prepared were subjected to immunoblotting analysis (IB) with anti-ERK1 or anti-phospho-ERK (anti-pERK) antibody. Graphs show relative amounts of phosphorylated Erk1 in each sample, defining the value in a sample of W1804 cells (Dox−) at time 0 (A) or of AGS cells at time 0 (C) as 1. B, total cell lysates from the parental AGS cells and the SHP-2-knockdown G11 cells were immunoblotted with anti-SHP-2 antibody.
the morphogenetic activity of CagA requires SHP-2, which binds specifically to a tyrosine-phosphorylated form of CagA. Ras-independent Erk activity is also necessary for the CagA-induced hummingbird phenotype. We also found that CagA prolongs the duration of Erk activation and that this sustained Erk activation is mediated by SHP-2 in AGS cells. These findings indicate a pivotal role of SHP-2 in the CagA-dependent morphological transformation of gastric epithelial cells.

The translocated CagA protein is localized to the plasma membrane and undergoes tyrosine phosphorylation by Src family kinases in gastric epithelial cells (20, 21). The CagA phosphorylation sites are characterized by the presence of EPIYA motifs, the number of which varies from one strain to the next (24, 26). Tyrosine phosphorylation plays an important role in transmission of intracellular signals by regulating physical and functional interaction between upstream and downstream signal transducers (55–58). It is therefore reasonable to assume that CagA, upon tyrosine phosphorylation, deregulates one or more of the intracellular signaling pathways and thereby causes pathogenesis of cagA-positive H. pylori infection. This notion is strongly supported by the results of the present work demonstrating that inhibition of CagA tyrosine phosphorylation inactivates the morphogenetic activity of CagA. Furthermore, two distinct CagA mutants, both of which are totally resistant to tyrosine phosphorylation, were incapable of inducing the hummingbird phenotype in AGS cells. Taken together, the results of the present work provide compelling evidence for the requirement of CagA tyrosine phosphorylation in its morphology-transforming activity.

CagA has been reported to interact with a number of cellular proteins, including SHP-2, C-terminal Src kinase (Csk), Grb2, and c-Met hepatocyte growth factor receptor (22, 37, 38, 59). Among these, the results of our work indicate the importance of SHP-2 and Csk as relevant CagA targets, because they are the only known molecules that selectively bind the tyrosine-phosphorylated form of CagA. Upon complex formation, CagA activates Csk kinase activity, which in turn inhibits the activity of Src family kinases. Hence, CagA-Csk interaction is thought to be involved in a negative feedback regulation of CagA activity (59). As is the case of Csk, SHP-2 interacts with CagA via the typical SH2 domain-phosphotyrosine interaction (24). Through complex formation, SHP-2 is converted from the catalytically inactive form to the active form. Although the CagA-SHP-2 complex was originally demonstrated by transient expression of the cagA gene in AGS cells (22), it has also been detected in AGS cells infected with cagA-positive H. pylori (Fig. 1D) as well as in in vivo gastric mucosa from a patient with cagA-positive H. pylori infection (23). These findings exclude the possibility that the CagA-SHP-2 complex is detectable only when CagA is expressed by gene transfection. In this regard, Selbach et al. (60) reported that injected CagA failed to bind SHP-2 but that translocated CagA bound SHP-2 in AGS cells. In their study, bacteria-injected CagA possessed 3× EPIYA (Tigr strain-derived CagA; “A-B-C” type according to the classification by Higashi et al. (24)), whereas translocated CagA had 5× EPIYA (NCTC11637 strain-derived CagA; “A-B-C-C-C” type). We have demonstrated that the strength of activity of an individual CagA to bind SHP-2 is proportional to the number of EPIYA-C motifs (24). Accordingly, in their experimental setting, translocated CagA was much more potent than injected CagA in forming a complex with SHP-2. We have also shown that A-B-C type CagA is significantly less active in inducing the hummingbird phenotype than is A-B-C-C-C type CagA. Consistent with this, Backert et al. (41) reported that translocation and phosphorylation of the Tigr strain-derived CagA is necessary but not sufficient for induction of the morphological change in AGS cells. Possibly, a weaker CagA species may require additional signals to induce an elongated cell shape.

Our work provides compelling evidence that SHP-2 is an indispensable cellular target of CagA in induction of the hummingbird phenotype, reinforcing the notion of a critical role for the CagA-SHP-2 complex in the pathological activity of CagA. Notably, CagA recruits SHP-2 from the cytoplasm to the plasma membrane, where it activates SHP-2 phosphatase activity. Activated SHP-2 may then dephosphorylate substrates that are also located in close proximity to the membrane and thereby generate signals that lead to morphological changes. In this context, CagA is regarded as a bacterial protein that mimics mammalian docking/scaffolding molecules such as Gab or insulin receptor substrate family proteins, although it does not have any significant sequence homology with known mammalian proteins (39).

Signaling components lying downstream of the CagA-SHP-2 complex remain to be elucidated. Results of biochemical analyses of Gab-SHP-2 interaction as well as those of genetic studies on the Drosophila SHP-2 homologue Corkscrew indicate that SHP-2 is capable of activating Ras (61, 62). Consistently, SHP-2 has been reported to bind the Grb2-Sos complex (43, 44), the membrane localization of which results in the activation of the Ras/Raf/Mek/Erk pathway. However, based on the results of a series of experiments using dominant-negative and/or dominant-positive mutants, we conclude that Ras is not required for the hummingbird phenotype induced by the CagA-SHP-2 interaction. Meanwhile, Mimuro et al. (37) has recently reported that CagA binds Grb2, activates Ras, and induces the cell scattering phenotype in a manner independent of CagA tyrosine phosphorylation. However, we were not able to detect a physical interaction between CagA and Grb2 under the condition in which CagA-SHP-2 complex was easily detectable (Fig. 5). Furthermore, we found that tyrosine phosphorylation of CagA is an essential prerequisite for both the hummingbird phenotype and the cell scattering phenotype induced by CagA. At present, we cannot explain the discrepancies between our results and those reported by Mimuro et al. (37).

In epithelial cells treated with HGF, induction of morphological change and scattering requires SHP-2 recruitment to c-Met receptors through the scaffolding protein Gab. Maroun and co-workers (53) reported that a sustained activation of Erk by SHP-2 is necessary for cell morphological change in Madin-Darby canine kidney epithelial cells treated with HGF. In AGS cells, translocated CagA binds c-Met receptors upon treatment of cells with HGF, and this interaction enhances cell scattering by HGF (38). However, CagA is capable of inducing the hummingbird phenotype without HGF treatment. Accordingly, CagA appears to hijack the c-Met-dependent signaling pathway by deregulating its intracellular component, SHP-2. Upon complex formation with CagA, SHP-2 may provoke a series of biochemical events that are otherwise activated in response to HGF. Consistent with this notion, induction of morphological change by CagA requires Erk activity. In gastric epithelial cells, CagA prolongs the duration of Erk activation and SHP-2 is required for the sustained Erk activation. The results indicate that SHP-2 is involved in the positive regulation of Erk activity, which is necessary for induction of the hummingbird phenotype by CagA.

Consistent with our observation that the CagA activity is independent of Ras, sustained Erk activation has been reported to be regulated by a Ras-independent pathway, including Rap1, one of Ras-related proteins (49). Rap1 activates B-Raf and subsequently induces sustained Erk activation by a growth factor. Also, Rap1 regulates cell morphogenetic processes (63). Recently, Wessler et al. (64) reported that infection of cagA-
positive *H. pylori* activated the Rap1/B-Raf pathway but not the Ras/Raf-1 pathway in AGS cells. Therefore, the CagA–SHP-2 complex might activate the Rap1/B-Raf pathway and induce sustained Erk activation, which is associated with the morphological change. Notably, however, the conclusion that Ras is not involved in the morphogenetic activity of CagA does not necessarily mean that CagA has no relation to the Ras pathway. Rather, activation of Ras by the CagA–SHP-2 complex may provoke a dysregulated mitogenic response of gastric epithelial cells. In any case, identification of bona fide substrates for SHP-2 should enable elucidation of the molecular link between SHP-2 and Erk as well as other molecules that collectively deregulate intracellular signaling and thereby cause cell dysfunction. In this regard, Amieva and co-workers (65) recently reported that CagA recruits SHP-2 in close proximity to the tight junction and thus modifies the apical-junctional complex functions. The work suggests that molecules constituting the tight junction are potential candidates for SHP-2 substrates.

It is most likely that CagA exerts a variety of biological activities once delivered into gastric epithelial cells. Some of these activities may depend on tyrosine phosphorylation, whereas others may not. Deregulation of signals controlled by tyrosine phosphorylation is a hallmark of cancer (66). Also, morphological abnormality is closely associated with malignant transformation (67). It is therefore reasonable to assume that deregulation of intracellular signaling by CagA, which most likely mimics the actions of mammalian docking/scaffolding proteins, substantially contributes to the multistep carcinogenesis of gastric epithelial cells.

Acknowledgments—We thank Sean Egan and Jeff Settleman for cDNAs. We also thank Akiko Fujita for technical assistance.

REFERENCES

1. Huang, J. Q., Sridhar, S., Chen, Y., and Hunt, R. H. (1998) *Gastroenterology* 114, 1169–1179
2. Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyu, P. H., Steffermann, G. N., and Nomura, A. (1995) *Cancer Res.* 55, 2111–2115
3. Parsonnet, J., Friedman, G. D., Orentreich, N., and Vogelman, H. (1997) *Gastroenterology* 113, 579–586
4. Honda, S., Fujioka, T., Tokieda, M., Satoh, R., Nishizono, A., and Nasu, M. (1998) *Mol. Cell. Biol.* 18, 5225–5235
5. Danesh, J. (1999) *Am. J. Physiol.* 277, G1–G13
6. Danesh, J. (1999) *Antimicrob. Agents Chemother.* 43, 3735–3744
7. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Mira, E., Gomez-Mouton, C., Zhao, Z. J., Lacalle, R. A., and Manes, S. (1998) *Science* 280, 528–531
8. Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., Figura, N., and Rappuoli, R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5751–5755
9. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Mira, E., and Covacci, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 14648–14653
10. Akopyants, N. S., Clifton, S. W., Kersulyte, D., Crabtree, J. E., Youree, B. E., Feng, G. S., and Yu, D. H. (1998) *Mol. Cell. Biol.* 18, 515–528
11. Covacci, A., Telford, J. L., DelGiudice, G., Parsonnet, J., and Rappuoli, R. (1999) *Science* 284, 1328–1333
12. Kuipers, E. J., Perez-Perez, G. I., Meuwissen, S. G., and Blaser, M. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1777–1780
13. Peak, R. M., Jr., Miller, G. M., Tham, K. T., Perez-Perez, G. I., Zhao, X., Atherton, J. C., and Blaser, M. J. (1995) *Lab. Invest.* 73, 760–770
14. Bugg, M., Busatto, G., Casarso, M., Shiao, Y. H., Russo, V., Leandro, G., Avellini, C., Fabiano, A., Sidoni, A., and Covacci, A. (1999) *Cancer* 85, 2506–2514
15. Segal, E., Ch, J. A., Ch, J., Lo, J., Falkow, S., and Tompkins, L. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 14559–14564
16. Asahi, M., Arayama, D., Sozo, T., Hatakeyama, M. (2000) *Cell. Microbiol.* 2, 155–165
17. Choudary, P. V., Rabinovitch, R., Elbirt, A., Gassen, D. L., and Schlessinger, J. (1994) *Cell* 77, 81–91
18. Tada, M., Iwakura, K., Sakaguchi, S., Yamaguchi, G., and Nakamura, Y. (2000) *Science* 287, 2506–2509
19. Stein, M., Rappuoli, R., and Covacci, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 1333–1339
20. Bell, B. M., Moes, S., Hauch, C. R., Meyer, T. F., and Backert, S. (2002) *Cell Mol. Biol.* 48, 345–355
21. Wu, X., Schmahl, T., Hatakeyama, M. (2002a) *Cancer Metastasis Rev.* 21, 325–337
22. Fujita, A., Taniyama, K., Sasaki, N., and Schlemper, R. J. (2000) *Gastroenterology* 119, 2605–2612
Helicobacter pylori CagA Induces Ras-independent Morphogenetic Response through SHP-2 Recruitment and Activation

Hideaki Higashi, Akihiro Nakaya, Ryouhei Tsutsumi, Kazuyuki Yokoyama, Yumiko Fujii, Susumu Ishikawa, Megumi Higuchi, Atushi Takahashi, Yo Kurashima, Yasuhiro Teishikata, Shinya Tanaka, Takeshi Azuma and Masanori Hatakeyama

J. Biol. Chem. 2004, 279:17205-17216.
doi: 10.1074/jbc.M309964200 originally published online February 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309964200

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

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

This article cites 67 references, 36 of which can be accessed free at http://www.jbc.org/content/279/17/17205.full.html#ref-list-1