



# HOKKAIDO UNIVERSITY

Title	Focal Adhesion Kinase Is a Substrate and Downstream Effector of SHP-2 Complexed with Helicobacter pylori CagA
Author(s)	Tsutsumi, Ryouhei; Takahashi, Atsushi; Azuma, Takeshi et al.
Citation	Molecular and Cellular Biology, 26(1), 261-276 <a href="https://doi.org/10.1128/MCB.26.1.261-276.2006">https://doi.org/10.1128/MCB.26.1.261-276.2006</a>
Issue Date	2006-01
Doc URL	<a href="https://hdl.handle.net/2115/5960">https://hdl.handle.net/2115/5960</a>
Rights	Copyright © American Society for Microbiology
Type	journal article
File Information	MCB26_1.pdf



1 **FAK Is a Substrate and Downstream Effector of SHP-2**  
2 **Complexed with *Helicobacter pylori* CagA**

3

4 Running title: INHIBITION OF FAK BY CagA-ACTIVATED SHP-2

5

6 **Ryouhei Tsutsumi,<sup>1</sup> Atsushi Takahashi,<sup>1</sup> Takeshi Azuma,<sup>2</sup> Hideaki**  
7 **Higashi,<sup>1</sup> and Masanori Hatakeyama<sup>1\*</sup>**

8

9 *Division of Molecular Oncology, Institute for Genetic Medicine and*  
10 *Division of Chemistry, Graduate School of Science, Hokkaido University,*  
11 *Sapporo<sup>1</sup> and International Center for Medical Research and Treatment,*  
12 *School of Medicine, Kobe University, Kobe, Japan<sup>2</sup>*

13

14 \*Corresponding author. Mailing address for Masanori Hatakeyama: Division  
15 of Molecular Oncology, Institute for Genetic Medicine, Hokkaido  
16 University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan. Phone/Fax:  
17 81-11-706-7544. E-mail: [mhata@igm.hokudai.ac.jp](mailto:mhata@igm.hokudai.ac.jp)

18

19 Materials and Methods: 1010 words

20 Introduction, Results and Discussion: 6453 words

1        Infection with *cagA*-positive *Helicobacter pylori* (*H. pylori*) is  
2 associated with atrophic gastritis, peptic ulcer and gastric  
3 adenocarcinoma. The *cagA* gene product CagA is translocated from *H.*  
4 *pylori* into gastric epithelial cells and undergoes tyrosine  
5 phosphorylation by Src family kinases (SFKs). Tyrosine-phosphorylated  
6 CagA binds and activates SHP-2 phosphatase and the C-terminal Src  
7 kinase (Csk) while inducing an elongated cell shape termed the  
8 hummingbird phenotype. Here we show that CagA reduces the level of  
9 focal adhesion kinase (FAK) tyrosine phosphorylation in gastric  
10 epithelial cells. The decrease in phosphorylated FAK is due to  
11 SHP-2-mediated dephosphorylation of FAK at the activating  
12 phosphorylation sites, not due to Csk-dependent inhibition of SFKs,  
13 which phosphorylate FAK. Coexpression of constitutively active FAK  
14 with CagA inhibits induction of the hummingbird phenotype, whereas  
15 expression of dominant-negative FAK elicits an elongated cell shape  
16 characteristic of the hummingbird phenotype. These results indicate  
17 that inhibition of FAK by SHP-2 plays a crucial role in the  
18 morphogenetic activity of CagA. Impaired cell adhesion and increased  
19 motility by CagA may be involved in the development of gastric lesions  
20 associated with *cagA*-positive *H. pylori* infection.

1       *Helicobacter pylori* (*H. pylori*) is a Gram-negative micro-aerophilic  
2 bacterium that colonizes at least half of the world human population.  
3 Chronic infection with *H. pylori* is known to be a risk factor for the  
4 development of gastric diseases such as atrophic gastritis, peptic ulcer and  
5 distal adenocarcinoma of the stomach (14, 15, 23, 32, 52). The *cagA* gene is  
6 known as one of the virulence genes of *H. pylori*, and infection with  
7 *cagA*-positive *H. pylori* is associated with a high risk of gastric cancer (7,  
8 38, 42). The *cagA* gene encodes a 120~145-kDa immuno-dominant protein  
9 CagA, which is injected from the bacterium into a bacterium-attached  
10 gastric epithelial cell by the type IV secretion system (3, 5, 12, 34, 45, 49).  
11 Translocated CagA localizes to the inner surface of the plasma membrane  
12 and undergoes tyrosine phosphorylation, which is mediated by Src family  
13 kinases (SFKs) (46, 48).

14       Infection of gastric epithelial cells with *cagA*-positive *H. pylori*  
15 induces a unique elongated cell shape termed the “hummingbird phenotype”  
16 (45). We previously demonstrated that tyrosine-phosphorylated CagA  
17 specifically interacts with the SH2 domain-containing protein tyrosine  
18 phosphatase SHP-2 and stimulates the phosphatase activity. SHP-2 has been  
19 shown to function as a critical positive regulator of cell growth and cell  
20 motility (16, 31). The CagA-SHP-2 interaction is both essential and

1 sufficient for induction of the hummingbird phenotype (18, 19, 21).

2 CagA possesses multiple tyrosine phosphorylation sites, which are  
3 characterized by the presence of an EPIYA (glutamic  
4 acid-proline-isoleucine-tyrosine-alanine) motif. CagA proteins isolated  
5 from various *H. pylori* strains exhibit sequence polymorphism, especially in  
6 their C-terminal regions containing the EPIYA motifs. Most if not all of the  
7 CagA proteins of *H. pylori* isolated in Western countries possess conserved  
8 EPIYA-A and EPIYA-B sites followed by a Western CagA-specific site  
9 (EPIYA-C), which variably duplicates among Western isolates (in most  
10 cases 1-3 times) (20, 54, 55). Representative CagA species of *H. pylori*  
11 isolated in East Asian countries also possess EPIYA-A and EPIYA-B sites  
12 but not EPIYA-C. Instead, they possess an East Asian CagA-specific EPIYA  
13 site termed EPIYA-D. The EPIYA-C and EPIYA-D sites are major tyrosine  
14 phosphorylation sites of CagA, and they respectively constitute low-affinity  
15 and high-affinity binding sites for the SH2 domains of SHP-2. The strength  
16 of individual CagA to bind SHP-2 is correlated with the activity of CagA to  
17 induce the hummingbird phenotype (18, 20).

18 In addition to SHP-2, CagA also binds to the C-terminal Src kinase  
19 (Csk) in a tyrosine phosphorylation-dependent manner (51). Csk negatively  
20 regulates SFKs by specifically phosphorylating the inhibitory tyrosine

1 residue conserved among the C-terminal regions of SFKs (30, 36, 37). The  
2 CagA-Csk interaction potentiates the kinase activity of Csk and thereby  
3 downregulates SFKs. Since SFKs phosphorylate CagA, their inhibition by  
4 Csk results in the reduction of CagA phosphorylation and decreases the  
5 level of the CagA-SHP-2 complex. Hence, CagA-dependent Csk activation  
6 is considered as a negative feedback regulation that attenuates excess  
7 CagA-SHP-2 signaling (51).

8 In this study we found that, upon being complexed with and activated  
9 by CagA, SHP-2 dephosphorylates and inactivates focal adhesion kinase  
10 (FAK), a tyrosine kinase that regulates the turnover of focal adhesion spots  
11 (39, 44). We also found that inhibition of the FAK kinase activity induces  
12 an elongated cell shape characteristic of the hummingbird cell. The results  
13 indicate that FAK is a substrate and downstream target of SHP-2 involved  
14 in induction of the hummingbird phenotype by CagA. Deregulated cell  
15 adhesion by CagA, which is accompanied by increased cell motility, may  
16 play an important role in the pathophysiological activities of *cagA*-positive  
17 *H. pylori*.

## MATERIALS AND METHODS

1  
2       **Antibodies.** Anti-FAK polyclonal antibody (C-20) (Santa Cruz),  
3 anti-hemagglutinin (HA) monoclonal antibody (3F10) (Roche) and anti-Flag  
4 monoclonal antibody (M2) (Sigma-Aldrich) were used as primary antibodies  
5 for immunoblotting, immunoprecipitation and immunostaining. Anti-Myc  
6 monoclonal antibody (9E10) was used as primary antibodies for  
7 immunoblotting and immunoprecipitation. Anti-phosphotyrosine  
8 monoclonal antibody (4G10) (Upstate), anti-HA polyclonal antibody (Y-11)  
9 (Santa Cruz), anti-CagA polyclonal antibody HPP-5003-9 (AUSTRAL  
10 Biologicals), anti-Csk polyclonal antibody (C-20) (Santa Cruz),  
11 anti-Phospho-Src family (Tyr416) polyclonal antibody (anti-pSrc416) (Cell  
12 Signaling), anti-c-Src polyclonal antibody (N-16) (Santa Cruz), anti-SHP-2  
13 polyclonal antibody (C-18) (Santa Cruz), anti-FAK[pY<sup>397</sup>] phosphospecific  
14 polyclonal antibody (BioSource), and anti-STAT3 antibody (Cell Signaling)  
15 were used as primary antibodies for immunoblotting. Anti-FAK[pY<sup>576</sup>]  
16 phosphospecific polyclonal antibody (BioSource) was used as primary  
17 antibodies for immunoblotting and immunostaining. Normal rabbit IgG was  
18 purchased from Santa Cruz.

19       **Plasmids.** Expression vectors for HA-tagged, wild-type (WT) CagA  
20 derived from *H. pylori* NCTC11637 strain (WT CagA-HA, ABCCC type)

1 and its derivatives, ABccc, abCCC, and PR CagA-HA, were described  
2 previously (20, 21).  $\Delta$ CCC,  $\Delta$ BCCC,  $\Delta$ ACCC, and  $\Delta$ AB CagA mutants were  
3 generated from WT CagA-HA by internal deletions of amino-acid residues  
4 868-1042, 901-1042, amino-acid residues 868-900 and 941-1042, and  
5 amino-acid residues 868-940, respectively. A cDNA encoding mouse FAK  
6 was provided by Dr. Tadashi Yamamoto (University of Tokyo) and was  
7 C-terminal Flag-tagged (WT FAK-Flag). cDNAs encoding Y397A FAK-Flag  
8 (substitution of Tyr-397 with alanine), Y576A/Y577A FAK-Flag,  
9 Y397A/576A/577A FAK-Flag, Y407A/Y861A/Y925A FAK-Flag, Y576A  
10 FAK-Flag, Y577A FAK-Flag, Y397A/Y407A/Y576A/Y577A/Y861A/Y925A  
11 FAK-Flag, K454R FAK-Flag and K578E/K581E FAK-Flag were generated  
12 from WT FAK-Flag cDNA by the use of Chameleon site-directed  
13 mutagenesis kit (Stratagene). The cDNAs were cloned into pSP65SR $\alpha$   
14 mammalian expression vector. Expression vectors for WT SHP-2-Myc,  
15 Myr-SHP-2 $\Delta$ SH2-Myc and Flag-tagged WT CagA (WT CagA-Flag) were  
16 described previously (20, 21). A cDNA encoding Myc-tagged, catalytically  
17 inactive SHP-2 was made by replacing Cys-459 with serine residue (SHP-2  
18 C/S-Myc) or by replacing Arg-465 with methionine residue (R465M  
19 SHP-2-Myc) by site-directed mutagenesis, and was inserted into the  
20 pSP65SR $\alpha$  vector.

1        **Cell culture and transfection.** AGS human gastric epithelial cell and  
2 AGS-derived stable transfectant clone G11, in which SHP-2 was  
3 constitutively knocked down by *SHP-2*-specific siRNA (22), were cultured  
4 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).  
5 Expression vectors were transiently transfected into AGS cells by using  
6 Lipofectamine 2000 reagent (Invitrogen) as manufacturer's instruction. For  
7 immunoprecipitation,  $2 \times 10^6$  cells in a 100-mm dish were transfected with  
8 expression vectors and were harvested 36 h after the transfection. To  
9 investigate the role of tyrosine kinase activity in the level of FAK tyrosine  
10 phosphorylation, AGS cells were treated with 100  $\mu$ M of genistein  
11 (Calbiochem) for 2 h. For the analysis of cell morphology,  $6 \times 10^5$  cells in a  
12 100-mm dish were transfected with expression vectors. Twelve hours after  
13 transfection, cells were collected and split into three 35-mm dishes, and  
14 were cultured another 24 h. Cells showing the hummingbird phenotype were  
15 designated as those having one or more protrusions, the length of the  
16 protrusion being more than 2-fold of cell diameter (19). Cells were counted  
17 in 5 different  $0.25\text{-mm}^2$  fields in each of dishes (n=3). COS-7 cells were  
18 cultured in Dulbecco's modified Eagles medium (DMEM) supplemented  
19 with 10% FBS. Expression vectors were transiently transfected into COS-7  
20 cells by using calcium phosphate method as previously described (21).

1        **Immunoprecipitation and immunoblotting.** AGS cells were lysed in  
2 lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1%  
3 Brij-35, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM phenylmethylsulfonylfluoride, 10 µg/ml  
4 leupeptin, 10 µg/ml trypsin inhibitor and 10 µg/ml aprotinin]. Cell lysates  
5 were treated with specific antibodies or control IgG, and immune complexes  
6 were trapped on protein A- or protein G-Sepharose beads. Total cell lysates  
7 and immunoprecipitates were subjected to SDS polyacrylamide gel  
8 electrophoresis (SDS-PAGE). Proteins transferred to poly (vinylidene  
9 difluoride) membrane filter (Millipore) were soaked in solutions of primary  
10 antibodies and then visualized using Western blot chemiluminescence  
11 reagent (PerkinElmer Life Sciences).

12        **Quantitation of protein bands.** Intensities of chemiluminescence on  
13 the immunoblotted filter were quantitated with the use of a LAS-1000  
14 lumino-image analyzer (FUJIFILM), a high-sensitivity cooled CCD camera  
15 system, in which the light is converted into an electrical signal. The  
16 intensity of the image obtained by the CCD method is directly proportional  
17 to the light intensity, indicating that the digital image obtained by the CCD  
18 method is broader in dynamic range and has better linearity and is therefore  
19 more accurate than the X-ray film system in quantitative analysis. Each of  
20 the immunoblotting bands was quantitated by using the LAS-1000 analyzer

1 under the condition in which the intensity of the image obtained was  
2 proportional to the light intensity.

3 **In vitro phosphatase assay.** To purify FAK, AGS cells were lysed in  
4 lysis buffer. Cell lysates were treated with anti-FAK antibody and immune  
5 complexes were trapped on protein A-Sepharose beads. To purify SHP-2,  
6 COS-7 cells were transfected with WT SHP-2-Myc expression vector,  
7 SHP-2 C459S-Myc expression vector or control empty vector, and were  
8 lysed in lysis buffer without  $\text{Na}_3\text{VO}_4$ . Cell lysates were treated with  
9 anti-Myc antibody, and immune complex were trapped on protein  
10 G-Sepharose beads. Immunoprecipitates were then washed with lysis buffer  
11 without  $\text{Na}_3\text{VO}_4$  6 times and with assay buffer [50 mM Tris-HCl (pH 7.5),  
12 100 mM NaCl] 3 times. Beads were then mixed as indicated, and were  
13 incubated in assay buffer containing 2 mM dithiothreitol at 37°C. After 60  
14 min incubation, enzyme reaction was terminated by addition of SDS-PAGE  
15 loading buffer. Reaction mixtures were subjected to SDS-PAGE, and then  
16 immunoblotted with indicated antibodies.

17 **In vitro kinase assay.** Kinase activity of FAK was measured with a  
18 nonradioactive isotope solid-phase ELISA kit using the poly (Glu, Tyr) as  
19 substrate (Universal Tyrosine Kinase Assay Kit, TaKaRa). FAK was  
20 purified from AGS cells transfected with WT CagA-HA or control empty

1 vector by immunoprecipitation with an anti-FAK antibody.  
2 Immunoprecipitates were subjected to the *in vitro* kinase assay as  
3 manufacturer's instructions.

4 **Immunostaining.** AGS cells transfected with CagA expression vector  
5 or control empty vector were fixed with 3% paraformaldehyde and  
6 permeabilized with 0.1% Triton X-100. Cells were then treated with a  
7 primary antibody and were visualized with Alexa Fluor 546-conjugated  
8 anti-rabbit antibody, Alexa Fluor 488-conjugated anti-mouse antibody,  
9 Alexa Fluor 546-conjugated anti-rat antibody or Alexa Fluor  
10 488-conjugated anti-rabbit antibody (Invitrogen). Images were acquired  
11 using a confocal microscope system (Olympus).

## RESULTS

1  
2       **CagA reduces the level of FAK tyrosine phosphorylation.** Upon  
3 ectopic expression of *H. pylori* CagA, AGS human gastric epithelial cells  
4 elicit an elongated cell shape (hummingbird phenotype), elevated cell  
5 motility and a tendency to detach from the culture plate (19, 21). These  
6 observations suggested that CagA perturbs intracellular signaling that  
7 regulates cell adhesion as well as cell motility. To try to determine the  
8 mechanisms through which CagA exerts these biological actions, we  
9 examined the effect of CagA on the activity of focal adhesion kinase (FAK),  
10 a protein tyrosine kinase acting as an important regulator of focal adhesions  
11 that are involved in both cell morphology and cell motility (24, 39, 44).  
12 Since the FAK kinase activity is regulated by its tyrosine phosphorylation  
13 (8, 9, 39, 43, 44), we decided to first examine whether ectopic expression  
14 of CagA alters the tyrosine-phosphorylation status of FAK. To do so, we  
15 transiently transfected an expression vector for hemagglutinin (HA)  
16 epitope-tagged, wild-type CagA (WT CagA-HA) (21) or control empty  
17 vector into AGS cells, and the cell lysates prepared were  
18 immunoprecipitated with an anti-FAK antibody. Immunoblotting of the  
19 immunoprecipitates with an anti-phosphotyrosine antibody revealed that the  
20 level of tyrosine-phosphorylated FAK was significantly decreased when WT

1 CagA-HA was expressed (Fig. 1A). In our experiment, the transfection  
2 efficiency was approximately 85% in AGS cells (Fig. 1B). Thus, many if  
3 not all of the FAK proteins were present as hypophosphorylated or  
4 unphosphorylated forms in each of the CagA-expressing cells. Treatment of  
5 AGS cells with culture supernatants prepared from CagA-transfected AGS  
6 cells, which exhibited reduced FAK tyrosine phosphorylation (Fig. 1C, top  
7 panel), did not induce any changes in the level of FAK tyrosine  
8 phosphorylation (Fig.1C, bottom panel). The result ruled out possibility of  
9 involvement of paracrine mechanisms underlying the decrease in the level  
10 of FAK tyrosine phosphorylation. A decrease in the level of FAK tyrosine  
11 phosphorylation was also observed when CagA was expressed in another  
12 human gastric epithelial cell, MKN28 (data not shown). From these  
13 observations, we concluded that CagA reduces the level of FAK tyrosine  
14 phosphorylation in gastric epithelial cells.

15 CagA exerts its pathophysiological actions in both tyrosine  
16 phosphorylation-dependent and -independent manners (2, 4, 11, 21, 29, 51).  
17 Hence, we next examined whether the effect of CagA on FAK is dependent  
18 on CagA tyrosine phosphorylation. To do so, AGS cells were transfected  
19 with an expression vector for WT CagA-HA or phosphorylation-resistant  
20 (PR) CagA-HA, in which all the tyrosine residues that constitute the EPIYA

1 sites were replaced with non-phosphorylatable alanine residues. In contrast  
2 to WT CagA-HA, expression of PR CagA-HA did not alter the level of FAK  
3 tyrosine phosphorylation (Fig. 1A). The result indicated that decrease in  
4 FAK phosphorylation is dependent on tyrosine phosphorylation of CagA  
5 and suggested possible involvement of SHP-2 and/or Csk in the CagA  
6 activity because they are the only molecules known to specifically interact  
7 with tyrosine-phosphorylated CagA (21, 51).

8  
9 **CagA binds to SHP-2 and Csk through distinct EPIYA sites.** CagA  
10 could reduce the level of FAK tyrosine phosphorylation either by inhibiting  
11 FAK kinases or by activating FAK phosphatases. Since FAK is  
12 phosphorylated and activated by Src family kinases (SFKs) (8), CagA might  
13 inhibit FAK phosphorylation by repressing SFKs through Csk activation  
14 (51). Alternatively, CagA-activated SHP-2 might directly or indirectly  
15 dephosphorylate FAK (28, 57). We have previously shown that SHP-2  
16 specifically binds to the EPIYA-C site of Western CagA or the EPIYA-D  
17 site of East Asian CagA in AGS cells (20). We have also shown that CagA  
18 is capable of binding with Csk through either the EPIYA-A/B sites or  
19 EPIYA-C site in a tyrosine phosphorylation-dependent manner when the  
20 two proteins were co-expressed in COS-7 cells (51). Since the level of

1 transfected CagA in COS-7 cells was ~15-fold greater than that of  
2 transfected CagA in AGS cells (Fig. 2A), which is comparable to that in  
3 AGS cells infected with cagA-positive *H. pylori* (19), we wished to  
4 investigate the EPIYA sites involved in CagA-Csk interaction in AGS cells.  
5 To this end, we generated a series of EPIYA mutants from WT CagA-HA  
6 (ABCCC-type, Western CagA) (20, 21) as schematically summarized in Fig.  
7 2B. When expressed in AGS cells, the ABccc CagA mutant was found to  
8 undergo tyrosine phosphorylation, although the level was much less than  
9 that of WT or abCCC CagA-HA (Fig. 2C). This observation was consistent  
10 with our previous conclusion as well as conclusions by others that EPIYA-C  
11 is the prevalent tyrosine phosphorylation site of CagA (4, 20). In AGS cells,  
12 endogenous Csk was co-immunoprecipitated with WT or ABccc CagA-HA  
13 but not with abCCC CagA-HA or PR CagA-HA (Fig. 2C). Thus, the  
14 EPIYA-A/B sites are responsible for the CagA-Csk interaction in gastric  
15 epithelial cells. This conclusion was consolidated by the observation that  
16 Csk did not bind the  $\Delta$ AB CagA-HA mutant, which lacks EPIYA-A/B sites  
17 but retains 3 x EPIYA-C sites (Fig. 2D). Consistent with the fact that Csk  
18 has a single SH2 domain, the  $\Delta$ BCCC or  $\Delta$ ACCC CagA-HA deletion mutant  
19 was still co-precipitated with Csk (Fig. 2D), indicating that each of the  
20 EPIYA-A and EPIYA-B sites is independently capable of forming a

1 complex with Csk via the SH2 domain. From these observations, we  
2 concluded that CagA utilizes distinct EPIYA sites for interaction with  
3 cellular targets, Csk and SHP-2, in gastric epithelial cells; CagA binds Csk  
4 via the EPIYA-A or EPIYA-B site, whereas it binds SHP-2 via the EPIYA-C  
5 site. A decreased interaction of Csk with ABccc CagA-HA compared to that  
6 with WT CagA-HA (Fig. 2C) may be due to reduced tyrosine  
7 phosphorylation or structural alteration at the EPIYA-A/B sites caused by  
8 mutations introduced into the EPIYA-C sites.

9 The kinase activity of SFKs is regulated by the tyrosine  
10 phosphorylation at the autophosphorylation site (ex. Tyr-416 in chicken  
11 c-Src and Tyr-419 in human c-Src) and the C-terminal inhibitory  
12 phosphorylation site (Tyr-527 in chicken c-Src and Tyr-530 in human c-Src)  
13 of SFKs. For example, c-Src phosphorylated at Tyr-530 by Csk is  
14 enzymatically inactive, whereas c-Src phosphorylated at Tyr-419 is active.  
15 Thus, the effects of CagA and its derivatives on Csk activity were examined  
16 with the use of a kinase-dead c-Src, Src $\Delta$ K, which undergoes intermolecular  
17 autophosphorylation at Tyr-419 by endogenous c-Src and therefore acts as  
18 an indicator for the activity of c-Src in cells as described previously (51).  
19 Immunoblotting analysis using anti-pSrc416 antibody, which specifically  
20 detects autophosphorylation sites in active forms of SFKs, revealed that the

1 level of Src $\Delta$ K autophosphorylation was decreased when WT or ABccc  
2 CagA-HA was expressed in AGS cells (Fig. 2E). In contrast, expression of  
3 abCCC or PR CagA-HA had no effect on Src $\Delta$ K autophosphorylation in  
4 AGS cells. The results indicated that the activity of CagA to stimulate Csk  
5 and thereby to inactivate SFKs is dependent on the presence of the  
6 EPIYA-A/B sites of CagA to which Csk binds.

7  
8 **CagA-SHP-2 interaction is responsible for reduced FAK**  
9 **phosphorylation.** To investigate the mechanism by which CagA reduces the  
10 level of FAK tyrosine phosphorylation, we tried to determine which EPIYA  
11 sites are required for the CagA activity by expressing the CagA EPIYA  
12 mutants in AGS cells (Fig. 3A). The level of tyrosine-phosphorylated FAK  
13 remained unaffected by ectopic expression of the ABccc CagA-HA mutant,  
14 which binds and activates Csk but not SHP-2. On the other hand, the abCCC  
15 CagA-HA mutant, which binds and activates SHP-2 but not Csk, decreased  
16 the level of FAK tyrosine phosphorylation. Hence, the activity of CagA to  
17 reduce FAK tyrosine phosphorylation is dependent on the EPIYA-C site, to  
18 which SHP-2 binds, but is independent of EPIYA-A and EPIYA-B sites, to  
19 which Csk binds. This indicates that CagA-Csk interaction, which inhibits  
20 SFK activity, is not involved in the decrease in FAK tyrosine

1 phosphorylation by CagA. To consolidate this conclusion, we investigated  
2 SFK activities in cells expressing various CagA mutants with the use of the  
3 anti-pSrc416 antibody, which specifically recognizes active forms of SFKs.  
4 As shown in Fig. 3B, the anti-pSrc416 antibody detected two major bands,  
5 60-kDa and 62-kDa bands, whose phosphorylation levels were specifically  
6 decreased upon treatment with PP2, a specific inhibitor of SFKs. It has been  
7 reported that c-Src, Fyn, Lyn and Yes are involved in CagA  
8 phosphorylation in gastric epithelial cells (46, 48). From the molecular  
9 sizes, the 62-kDa band corresponded to Yes and the 60-kDa band  
10 corresponded to c-Src. Expression of WT CagA-HA and ABccc CagA-HA,  
11 both of which bind to and activate Csk, potently inhibited the SFK activity  
12 (Fig. 3B, lanes 2 and 3), whereas the abCCC CagA-HA mutant, which  
13 specifically binds to and activates SHP-2 but not Csk, failed to do so (Fig.  
14 3B, lane 4). Since the abCCC CagA-HA mutant totally retains the ability to  
15 decrease the level of FAK tyrosine phosphorylation (Fig. 3A), the results  
16 provide compelling evidence that decreased FAK tyrosine phosphorylation  
17 by CagA is independent of the CagA activity to inhibit SFK activity via  
18 CagA-Csk interaction.

19       Strict dependence of the CagA activity on FAK phosphorylation to the  
20 EPIYA-C site raised the possibility that CagA-SHP-2 interaction, which is

1 mediated by EPIYA-C, is involved in the biochemical event. Accordingly,  
2 we next examined the effect of CagA on FAK tyrosine phosphorylation in  
3 AGS-derived G11 cells, which stably express *SHP-2*-specific siRNA and  
4 thus show a marked reduction in SHP-2 expression (22). Notably, the level  
5 of FAK tyrosine phosphorylation was significantly elevated in G11 cells  
6 compared with the level in parental AGS cells (Fig. 3C, left, top panel,  
7 lanes 2 and 6; for quantitation, see right panel). Restoration of the SHP-2  
8 expression in G11 cells by expressing SHP-2RR-Myc (19), which is  
9 insensitive to *SHP-2*-specific siRNA, again decreased the level of FAK  
10 tyrosine phosphorylation (top panel, lanes 6 and 10). The observation  
11 indicated that SHP-2 is physiologically involved in the regulation of FAK  
12 tyrosine phosphorylation regardless of CagA. In contrast to the case with  
13 the parental AGS cells, expression of WT CagA-HA in G11 cells did not  
14 reduce the level of FAK tyrosine phosphorylation (left, top panel, lanes 2, 4,  
15 6, 8). Upon re-expression of SHP-2 in G11 cells, however, CagA was again  
16 capable of reducing the level of FAK tyrosine phosphorylation (left, top  
17 panel, lanes 10 and 12). From these observations, we concluded that SHP-2  
18 is required for the CagA-mediated decrease in FAK tyrosine  
19 phosphorylation.

20

1           **Inhibition of SFKs by CagA is independent of the reduced level of**  
2 **FAK phosphorylation.** In *in vitro* studies, SHP-2 has been shown to  
3 dephosphorylate the C-terminal inhibitory tyrosine residue of SFKs,  
4 although its activity has not been confirmed *in vivo* (40). More recently,  
5 Zhang et al. reported that SHP-2 functions upstream of Csk and SFKs via  
6 dephosphorylation of the adaptor protein PAG/Cbp in fibroblasts (58). In  
7 either case, the SHP-2 activity potentiates SFK activity, arguing against the  
8 idea that CagA-activated SHP-2 inhibits SFKs and thereby reduces the level  
9 of FAK tyrosine phosphorylation. Indeed, analysis using the anti-pSrc416  
10 antibody revealed that SFK activity was not decreased in G11 cells  
11 compared with that in parental AGS cells (Fig. 3C, left, bottom panel, top  
12 row, lanes 1 and 3). Furthermore, expression of WT CagA-HA in AGS or  
13 G11 cells resulted in the inhibition of SFK kinase activity (lanes 1-4). In  
14 contrast, ectopic expression of SHP-2 in G11 cells did not change the SFK  
15 activity in the absence (lanes 3 and 5) or presence of CagA (lanes 4 and 6).  
16 The results indicated that inhibition of SFK activity by CagA is mediated by  
17 CagA-Csk interaction and that SHP-2 does not play a major role in the  
18 regulation of SFK activity in gastric epithelial cells. Also notably,  
19 expression of CagA in G11 cells, which resulted in SFK inhibition  
20 regardless of the presence of SHP-2 (lanes 3-6), decreased the level of FAK

1 tyrosine phosphorylation only in the presence of SHP-2 (Fig. 3C, left, top  
2 panel, lanes 8 and 12). The observation further argues against the idea that  
3 inhibition of SFK activity by CagA causes reduced level of FAK tyrosine  
4 phosphorylation. To pursue this further, AGS cells were transfected with a  
5 control vector, WT CagA or ABccc CagA expression vector. At 12 h after  
6 transfection, cells were treated with genistein, a general protein tyrosine  
7 kinase inhibitor, and the rate of FAK dephosphorylation was determined.  
8 Whereas treatment of AGS cells with genistein for 2 h significantly  
9 inhibited the levels of tyrosine-phosphorylated proteins in the cells (Fig.  
10 3D, lower, left panel), it did not reduce the level of FAK tyrosine  
11 phosphorylation (Fig. 3D, upper panel; for quantitation, see right panel).  
12 Thus, tyrosine-phosphorylated FAK was fairly stable in the cells. In the  
13 genistein-treated cells, however, WT CagA but not ABccc CagA was still  
14 capable of reducing FAK tyrosine phosphorylation. Again, the result does  
15 not support the idea that inhibition of tyrosine kinase activities including  
16 those of SFKs is responsible for the reduced FAK tyrosine phosphorylation  
17 in cells expressing CagA.

18 .

19 **SHP-2 dephosphorylates FAK.** The above observations indicated a  
20 more direct role of SHP-2 in the reduced level of FAK tyrosine

1 phosphorylation by CagA. Accordingly, we examined if ectopic SHP-2 is  
2 capable of altering the tyrosine phosphorylation level of FAK in cells. As  
3 shown in Fig. 4A, expression of a membrane-targeted, constitutively active  
4 SHP-2, Myr-SHP-2 $\Delta$ SH2-Myc (21), resulted in a significant decrease in the  
5 level of tyrosine-phosphorylated FAK in AGS cells. The observation  
6 indicated that activated SHP-2 directly or indirectly decreases the level of  
7 FAK tyrosine phosphorylation. Accordingly, we decided to investigate the  
8 possibility that FAK is a direct target of SHP-2 phosphatase. It has been  
9 reported that introduction of mutations in the conserved amino-acid  
10 residues, Asp-425 and Cys-459, that are located in the catalytic center of  
11 the tyrosine phosphatase domain of SHP-2 stabilizes an SHP-2-substrate  
12 intermediate complex (1). Accordingly, we generated such a  
13 substrate-trapping mutant (DM SHP-2-Myc) and expressed it in AGS cells.  
14 Immunoprecipitation of DM SHP-2-Myc co-precipitated endogenous FAK  
15 much stronger than WT SHP-2 did (Fig. 4B). To rule out the possibility of  
16 interaction between SHP-2 and FAK other than enzyme-substrate interaction,  
17 we also examined a phosphatase-dead mutant of SHP-2 (R465M SHP-2)  
18 (25), which acts as a non-substrate-trapping SHP-2 mutant, and found that  
19 the interaction between FAK and R465M mutant was extremely weak (Fig.  
20 4B). These findings indicated that SHP-2 forms an enzyme-substrate

1 intermediate complex with FAK. Furthermore, co-expression of WT  
2 CagA-HA greatly increased the ability of DM SHP-2-Myc to bind FAK (Fig.  
3 4C), indicating that CagA-activated SHP-2 acquired the ability to form an  
4 enzyme-substrate intermediate complex with FAK. Next, we  
5 immunopurified tyrosine-phosphorylated FAK, WT SHP-2-Myc, and a  
6 catalytically inactive SHP-2 that was made by replacing Cys-459 with  
7 serine (SHP-2 C/S-Myc) (33) and performed an *in vitro* phosphatase assay  
8 of SHP-2 using *in vivo*-phosphorylated FAK as a substrate. The results of  
9 the assay revealed that WT SHP-2-Myc dephosphorylated FAK, whereas  
10 SHP-2 C/S-Myc did not (Fig. 4D). Based on these observations, we  
11 concluded that there is an enzyme-substrate relationship between SHP-2 and  
12 FAK.

13

14 **Dephosphorylation of activating phosphorylation sites of FAK by**  
15 **SHP-2.** There are six tyrosine-phosphorylation sites in FAK (39). FAK  
16 activation by an integrin signal induces tyrosine phosphorylation of FAK at  
17 Tyr-397, causing recruitment of SFKs to the motif surrounding the Tyr-397  
18 phosphorylation site. FAK-bound SFKs then facilitate maximal activation of  
19 FAK kinase activity through phosphorylation at Tyr-576 and Tyr-577 within  
20 the FAK kinase domain. Thus, tyrosine phosphorylation at Tyr-397, -576,

1 and -577 is required for full activation of the FAK kinase activity (8, 9, 39,  
2 43, 44). SFKs also phosphorylate Tyr-407, Tyr-861 and Tyr-925, and  
3 phosphorylated Tyr-925 becomes a binding site of Grb2 and thereby  
4 activates the Ras-MAP kinase pathway. In order to determine which  
5 tyrosine residues in FAK are phosphorylated in AGS cells, we generated a  
6 series of tyrosine-to-alanine mutants from WT FAK-Flag and expressed  
7 them in AGS cells. As expected, the  
8 Y397A/Y407A/Y576A/Y577A/Y861A/Y925A FAK-Flag mutant was not  
9 phosphorylated in AGS cells (Fig. 5A, left panel; for quantitation, see right  
10 panel). In contrast, the triple Y407A/Y861A/Y925A mutant was  
11 tyrosine-phosphorylated to a level comparable to that of WT FAK-Flag.  
12 These results indicated that FAK is phosphorylated at Tyr-397, -576 and  
13 -577, but not at Tyr-407, -861 or -925, in AGS cells. The conclusion was  
14 further supported by the findings that the Y397A, Y576A or Y577A mutant  
15 was less phosphorylated than was WT FAK-Flag and that the  
16 Y397A/Y576A/Y577A mutant was only slightly tyrosine-phosphorylated.  
17 The decrease in the level of FAK tyrosine phosphorylation was more than  
18 50% in the Y397A mutant but was less than 50% in the Y576A and Y577A  
19 mutants. The double Y576A/Y577A mutant showed reduction of tyrosine  
20 phosphorylation, almost equal to the sum of reductions shown in the Y576A

1 mutant and the Y577A mutant. The result is consistent with the notion that  
2 Y397 phosphorylation promotes phosphorylation at Y576 and T577. The  
3 finding that FAK is phosphorylated at Tyr-397, -576 and -577 also  
4 suggested that SHP-2 dephosphorylates activating phosphorylation sites of  
5 FAK and, by doing so, inhibits FAK kinase activity in the cells. Indeed,  
6 immunoblots of FAK with phospho-FAK-specific antibodies,  
7 anti-FAK[pY<sup>397</sup>] and anti-FAK[pY<sup>576</sup>], revealed that Tyr-397 and Tyr-576  
8 were phosphorylated in AGS cells and the levels of phosphorylation at  
9 Tyr-397 and Tyr-576 were reduced upon expression of WT CagA-HA (Fig.  
10 5B left, top panel; for quantitation, see right panel). Anti-FAK[pY<sup>577</sup>], a  
11 phospho-Y577 FAK-specific antibody, was insufficiently sensitive to  
12 directly identify FAK phosphorylation at Tyr-577 (data not shown). In  
13 accordance with these results, substrate trapping experiments revealed that  
14 the Y397A and Y576A/Y577A FAK-Flag mutants exhibited significantly  
15 reduced activities to form complexes with DM SHP-2-Myc (Fig. 5C, lanes 3,  
16 5, 7) and that the triple Y397A/Y576A/Y577A mutant did not bind to DM  
17 SHP-2-Myc (Fig. 5C, lane 9). On the other hand, the triple  
18 Y407A/Y861A/Y925A mutant bound to the substrate trapping mutant of  
19 SHP-2 to a level comparable to that of WT FAK-Flag (lane 11). These  
20 studies confirm that Tyr-397, Tyr-576 and Tyr-577 are major sites of FAK

1 tyrosine phosphorylation in AGS cells and that CagA-activated SHP-2  
2 dephosphorylates FAK at these sites. It should also be noted that FAK  
3 hyperphosphorylation in G11 cells caused by SHP-2-knockdown was also  
4 associated with increased levels of phosphorylation at Tyr-397 and Tyr-576  
5 (Fig. 5D, lanes 1, 3). Re-introduction of SHP-2 into G11 cells gave rise to  
6 reduced levels of tyrosine phosphorylation at Tyr-397 and Tyr-576, which  
7 were further decreased in the presence of CagA as expected (lanes 3-6).

8 To confirm that CagA-mediated FAK dephosphorylation causes  
9 inhibition of FAK activity, we performed an *in vitro* kinase assay of FAK  
10 prepared from AGS cells with or without CagA expression. As shown in Fig.  
11 5E, FAK prepared from WT CagA-HA-expressing AGS cells exhibited  
12 reduced kinase activity compared to the activity exhibited by FAK prepared  
13 from parental AGS cells. Accordingly, CagA inhibits the kinase activity of  
14 FAK in gastric epithelial cells.

15

16 **FAK dephosphorylation is both required and sufficient for**  
17 **induction of the hummingbird phenotype.** Since a constitutively active  
18 SHP-2 mutant induced an elongated cell shape in AGS cells when it was  
19 forced to tether the plasma membrane (21), dephosphorylation of FAK by  
20 SHP-2 was thought to be involved in the morphological changes induced by

1 CagA. To examine the relationship between FAK dephosphorylation and the  
2 hummingbird phenotype, we co-expressed CagA together with a  
3 constitutively active FAK mutant, in which two glutamic acid residues were  
4 introduced in the activation loop of the FAK kinase domain (K578E/K581E)  
5 (17), and examined the effect of the FAK mutant on the induction of  
6 hummingbird cells by CagA. As shown in Fig. 6A, the constitutively active  
7 FAK significantly inhibited the CagA activity to induce the hummingbird  
8 phenotype, indicating that downregulation of FAK kinase activity plays a  
9 role in induction of the hummingbird phenotype. To pursue this possibility  
10 further, we generated a kinase-dead mutant of FAK by replacing Lys-454  
11 with arginine. The resulting K454R FAK-Flag mutant acts as a  
12 dominant-negative mutant when expressed in relative excess to endogenous  
13 FAK in cells. If CagA induces the hummingbird phenotype by inhibiting  
14 FAK kinase activity, the dominant negative FAK molecule should mimic  
15 CagA and induce similar morphological changes. As expected, ectopic  
16 expression of K454R FAK-Flag, but not WT-FAK-Flag, in AGS cells  
17 resulted in the induction of an elongated cell shape that resembles the  
18 hummingbird phenotype induced by WT CagA-HA (Fig. 6B). We also  
19 examined a Y576A/Y577A FAK-Flag that mimics FAK dephosphorylated at  
20 Tyr-576 and Tyr-577 by SHP-2. Again, expression of the Y576A/Y577A

1 FAK-Flag mutant, but not the Y409A/Y861A/Y925A FAK-Flag mutant, in  
2 AGS cells gave rise to induction of cell elongation. These observations  
3 indicate that inhibition of FAK kinase activity by SHP-2 causes  
4 morphological changes in AGS cells that are characteristic of  
5 CagA-expressing cells. The relatively low frequency of induction of  
6 elongated cell shape by kinase-inactive FAKs may simply be due to  
7 inefficient inhibition of endogenous FAK activity by these  
8 dominant-negative FAK mutants in transient transfection experiments,  
9 although it is possible that additional intracellular pathways are required to  
10 effectively induce the hummingbird phenotype in addition to FAK  
11 inhibition.

12 To rule out the possibility that the reduced FAK tyrosine  
13 phosphorylation is a result, and not a cause, of the hummingbird phenotype,  
14 we also investigated time-course kinetics of FAK tyrosine phosphorylation  
15 in AGS cells transfected with WT CagA-HA. The results shown in Fig. 7  
16 indicate that decrease in FAK tyrosine phosphorylation was detectable 9 h  
17 after transfection, much earlier than induction of the hummingbird  
18 phenotype, which becomes visible approximately 18 h after transfection.  
19 The kinetic change is consistent with the conclusion that CagA-activated  
20 SHP-2 directly dephosphorylates FAK, arguing against the possibility that

1 cell skeletal changes that are associated with hummingbird cells cause the  
2 reduced level of FAK tyrosine phosphorylation.

3

4 **Compartmentalization of active FAK at the tips of membrane**  
5 **protrusions in cells with the hummingbird phenotype.** To further  
6 investigate the role of FAK in the morphogenetic activity of CagA, we  
7 examined subcellular localization of FAK in AGS cells expressing CagA  
8 (Fig. 8). In cells with the hummingbird phenotype, CagA was distributed  
9 throughout the cell membrane but was absent in the distal ends of the  
10 membrane protrusions. Anti-FAK staining showed that FAK was mostly  
11 localized to the cytoplasm but not the plasma membrane. Intriguingly,  
12 however, a fraction of FAK molecules were specifically enriched at the tips  
13 of the membrane protrusions. Staining of the cells with anti-FAK[pY<sup>576</sup>],  
14 which recognizes the active form of FAK, revealed that active FAK  
15 molecules were present at the tips of the extensions. These observations  
16 indicate that active FAK molecules, which have escaped from  
17 CagA-activated SHP-2 and thus maintain their kinase activity, are  
18 specifically concentrated at the tips of the membrane protrusions in cells  
19 with the hummingbird phenotype.

## DISCUSSION

1  
2 Among the various CagA-interacting molecules reported to date, only  
3 SHP-2 and Csk bind specifically to the tyrosine-phosphorylated form of  
4 CagA (2, 11, 18, 21, 29, 51). Upon complex formation, CagA stimulates  
5 their catalytic activities. Thus, SHP-2 and/or Csk may mediate some if not  
6 all of the phosphorylation-dependent CagA activities. Indeed, we have  
7 already shown that activation of SHP-2 by CagA is both essential and  
8 sufficient for induction of the hummingbird phenotype (19, 21). In this  
9 study, we demonstrated that CagA reduces the tyrosine phosphorylation  
10 level of FAK, a tyrosine kinase that plays a critical role in focal adhesion  
11 turnover, in a manner dependent on CagA phosphorylation. This decrease in  
12 FAK tyrosine phosphorylation could be explained by either CagA-Csk or  
13 CagA-SHP-2 interaction. In the former case, CagA-activated Csk inhibits  
14 SFK activity and thereby prevents SFK-dependent FAK phosphorylation. In  
15 the latter case, CagA-activated SHP-2 directly or indirectly  
16 dephosphorylates FAK. To investigate these two possibilities, we made use  
17 of the EPIYA sites of CagA. The present work revealed that in gastric  
18 epithelial cells Csk specifically binds to the EPIYA-A or EPIYA-B site,  
19 whereas SHP-2 has been shown to bind to the EPIYA-C site (20). In this  
20 regard, we previously reported that Csk is capable of binding CagA through

1 either the EPIYA-A/B sites or EPIYA-C site when they are overexpressed in  
2 COS-7 cells (51). The differences between the previous and present results  
3 may be due to different levels of CagA expression. Indeed, the level of  
4 transfected CagA in COS-7 cells was more than 15-fold greater than that of  
5 transfected CagA in AGS cells, which is comparable to the level of CagA  
6 transduced by infection with *cagA*-positive *H. pylori* (19). Given that  
7 SHP-2 and Csk bind to CagA in a mutually exclusive manner (data not  
8 shown), the interaction between Csk and the EPIYA-C site may be  
9 competitively inhibited by the high-affinity interaction between SHP-2 and  
10 the EPIYA-C site in AGS cells, where endogenous SHP-2 is in relative  
11 excess to CagA (21). On the other hand, in COS-7 cells, overexpression of  
12 CagA results in the accumulation of CagA proteins, which are  
13 phosphorylated at the EPIYA-C site but not bound to SHP-2 because of  
14 their relative excess to endogenous SHP-2 proteins. Such SHP-2-unbound  
15 CagA molecules may then bind to Csk via the EPIYA-A/B sites or EPIYA-C  
16 site in COS-7 cells. Accordingly, we consider that results obtained using  
17 AGS cells are more reflective of the pathophysiologically relevant  
18 situation.

19 The finding of requirement of the EPIYA-C site, but not the  
20 EPIYA-A/B sites, for the CagA activity to reduce FAK tyrosine

1 phosphorylation raised the possibility that CagA-activated SHP-2 is  
2 responsible for the biochemical event. It has been reported that SHP-2 can  
3 directly activate SFKs by dephosphorylating the C-terminal inhibitory  
4 tyrosine residue (40). More recently, Zhang et al. demonstrated that  
5 SHP-2-deficient fibroblasts exhibit reduced SFK activity and suggested that  
6 SHP-2 positively regulates SFK activity by controlling the ability of  
7 PAG/Cbp to recruit Csk to the membrane through PAG/Cbp  
8 dephosphorylation (58). Since both of the reported SHP-2 activities on  
9 SFKs result in the activation, they cannot explain the current observation  
10 that CagA-stimulated SHP-2 reduces the level of FAK tyrosine  
11 phosphorylation (8). Indeed, our present work shows that CagA expression,  
12 while activating SHP-2, inhibits rather than activates SFKs in gastric  
13 epithelial cells. This inhibition of SFK kinase activity by CagA was  
14 attributed to CagA-Csk interaction, but not to CagA-SHP-2 interaction,  
15 since the abCCC CagA mutant, which binds SHP-2 but not Csk, still  
16 retained the ability to reduce FAK tyrosine phosphorylation (Fig. 3A) but  
17 did not modify SFK activity (Fig. 3B). Accordingly, while SHP-2 is capable  
18 of activating SFKs either directly or through PAG/Cbp dephosphorylation  
19 (40, 58), this SHP-2 activity is counteracted by CagA-Csk interaction,  
20 which stimulates Csk and thereby inhibits SFKs independent of PAG/Cbp. It

1 should also be noted that expression of the abCCC CagA mutant, which  
2 binds SHP-2 but not Csk, or siRNA-mediated knockdown of SHP-2 did not  
3 alter the SFK kinase activity in gastric epithelial cells. Thus, the degree of  
4 involvement of SHP-2 in the regulation of SFK activity may be cell  
5 context-dependent. In this regard, there is also the possibility also exists  
6 that CagA sequesters SHP-2 away from its normal targets, leading to a  
7 paradoxical inactivation of SFKs, which results in the reduced level of FAK  
8 tyrosine phosphorylation. However, the results of our experiment using a  
9 general tyrosine kinase inhibitor indicate that inhibition of tyrosine kinase  
10 activities including those of SFKs in cells cannot mimic the CagA activity  
11 to reduce the level of FAK tyrosine phosphorylation. Furthermore, ABccc  
12 CagA, which binds Csk but not SHP-2, inhibits SFK activity, whereas  
13 abCCC CagA, which binds SHP-2 but not Csk, fails to do so. The results  
14 indicate that inhibition of SFK is mediated by CagA-activated Csk but not  
15 by sequestration of SHP-2 by CagA from its normal substrates. Given that  
16 ABccc CagA cannot reduce the level of FAK tyrosine phosphorylation, the  
17 results further suggest that SFK inhibition by CagA is independent of FAK  
18 dephosphorylation. In addition, SHP-2 knockdown, which may mimic  
19 abnormal sequestration of SHP-2 by CagA from its normal targets, does not  
20 inhibit SFK activity. Together with the observation that SFK activity is

1 efficiently inhibited by CagA even in SHP-2-knockdown cells, these results  
2 collectively rule out the possibility that CagA-SHP-2 interaction causes  
3 SFK inactivation, which results in reduction in the level of FAK tyrosine  
4 phosphorylation.

5       The above-described observations indicate that CagA-activated SHP-2  
6 is directly involved in the reduction in the level of FAK tyrosine  
7 phosphorylation. Indeed, the results of a series of present works support an  
8 enzyme-substrate relationship between SHP-2 and FAK. First, enhanced  
9 tyrosine phosphorylation of FAK is observed in SHP-2-knockdown cells.  
10 Second, overexpression of constitutively active SHP-2 reduces the level of  
11 FAK tyrosine phosphorylation. Third, FAK is dephosphorylated by SHP-2  
12 *in vitro*. Fourth, FAK specifically binds to the substrate-trapping mutant of  
13 SHP-2. From these observations, we concluded that FAK is an *in vivo*  
14 substrate of SHP-2. FAK is activated via autophosphorylation at Tyr-397,  
15 which is initiated by integrin activation. Upon phosphorylation, Tyr-397  
16 becomes a binding site for SFKs, which phosphorylate FAK at Tyr-576 and  
17 Tyr-577 to further activate FAK kinase activity. FAK is also reportedly  
18 phosphorylated at Y407, Y861 and Y925 (8, 9, 39, 43, 44). Among these  
19 FAK tyrosine residues, Tyr-397, Tyr-576 and Tyr-577 are selectively and  
20 constitutively phosphorylated in gastric epithelial cells and CagA-activated

1 SHP-2 dephosphorylates these tyrosine residues. Accordingly, CagA binds  
2 and activates SHP-2, which in turn dephosphorylates the activating  
3 phosphotyrosine residues and thereby inhibits FAK kinase activity.

4 It has been reported that tyrosine phosphorylation of FAK in response  
5 to integrin signaling was impaired in mouse embryonic fibroblasts rendered  
6 acutely deficient in SHP-2 (58). It has also been reported that the levels of  
7 FAK tyrosine phosphorylation in embryonic fibroblasts prepared from WT  
8 and *SHP-2*-knockout mice were comparable (57). The differences between  
9 those results and our results may be due to different cell types (fibroblasts  
10 versus epithelial cells) and/or different experimental systems (*SHP-2*  
11 knockout versus SHP-2 knockdown) employed. It should also be noted that  
12 SHP-2 is recruited to the membrane by receptor tyrosine kinase or a  
13 scaffolding/adaptor protein such as Gab in response to a growth factor,  
14 whereas it is translocated to the membrane by SHPS-1/SIRP-1 $\alpha$  in integrin  
15 signaling (35, 50). Accordingly, the effect of SHP-2 on FAK might differ  
16 depending on upstream molecules that recruit SHP-2 to the membrane.

17 Cells with the hummingbird phenotype show increased motility and  
18 exhibit a tendency to detach from the culture plate. Thus, CagA has been  
19 suspected to perturb intracellular signaling that regulates cell adhesion and  
20 cell movement in a tyrosine phosphorylation-dependent manner (19). In this

1 respect, FAK is a legitimate downstream target of CagA because it plays  
2 pivotal roles in cell adhesion and cell morphology as well as cell motility  
3 (39, 44). Two lines of evidence support the idea that reduced FAK activity  
4 plays a role in the morphogenetic activity of CagA. First, a constitutively  
5 active mutant of FAK (K578E/K581E), which has  
6 phosphorylation-independent enhanced kinase activity, inhibited induction  
7 of the hummingbird phenotype by CagA. Second, ectopic expression of  
8 kinase-dead FAK (K454R) or a dephosphorylated form of FAK  
9 (Y576A/Y577A) was capable of inducing cell elongation that resembles the  
10 hummingbird phenotype. In this regard, many studies have implicated FAK  
11 as a positive regulator of cell motility in response to integrin signaling (24,  
12 44). However, recent studies have also shown that downregulation of FAK  
13 activity plays an important role in growth factor-induced changes in cell  
14 morphology and cell movement. Lu et al. demonstrated that treatment of  
15 human A431 epidermal carcinoma cells with epidermal growth factor (EGF)  
16 elicits rapid tyrosine dephosphorylation and inhibition of FAK, which is  
17 associated with elongated cell shape and increased cell motility (27).  
18 Vadlamudi et al. reported that heregulin induces FAK dephosphorylation,  
19 which is also associated with increased migratory potential, in breast cancer  
20 cells (53). Both studies suggested that tyrosine phosphatases such as SHP-2

1 may be involved in dephosphorylation and inactivation of FAK in growth  
2 factor-stimulated epithelial cells. Yano et al. also reported that  
3 downregulation of FAK by siRNA resulted in increased cell migration, in  
4 association with the induction of aberrant large protrusions, in HeLa cells  
5 (56). These observations are consistent with results of the present study  
6 showing that inhibition of FAK by CagA-activated SHP-2 is involved in  
7 induction of hummingbird cells with elevated cell motility.

8 In the present study, approximately 20% of the AGS cells transfected  
9 with the CagA expression vector exhibited the hummingbird phenotype at  
10 36 h after transfection. The low frequency of the hummingbird phenotype  
11 compared to the high transfection efficiency (~85%) and significant  
12 reduction in the level of FAK tyrosine phosphorylation (~65%) can be  
13 explained as follows. First, the hummingbird phenotype is a rapid and  
14 dynamic cellular process that is associated with multiple rounds of  
15 extension and retraction of the protrusions (19). Thus, a single  
16 CagA-expressing AGS cell never stays in its elongated state. Second, the  
17 hummingbird phenotype may be induced only in a fraction of  
18 CagA-expressing cells whose FAK kinase activity is decreased to a level  
19 within certain ranges. More specifically, only CagA-expressing cells in  
20 which FAK kinase activity is inhibited but not totally lost might develop

1 the hummingbird phenotype. This idea is supported by the finding that a  
2 small amount of active FAK is present in cells with the hummingbird  
3 phenotype (see later discussion). Third, there may be other signaling  
4 pathways that participate to achieve maximal CagA response in inducing the  
5 hummingbird phenotype in addition to FAK inhibition.

6 Focal adhesions are sites where integrin-mediated adhesion links the  
7 actin cytoskeleton. FAK localizes to focal adhesions via its C-terminal  
8 focal adhesion-targeting (FAT) domain. This FAT region contains binding  
9 sites for integrin-associated proteins such as paxillin and talin (39). Cell  
10 migration is not able to take place in the absence of focal adhesion turnover.  
11 Although FAK *per se* is not essential for the formation of focal adhesion  
12 complexes (24), studies in many laboratories have shown that FAK  
13 activation plays a crucial role in focal contact formation (13, 39). Recent  
14 studies have shown that FAK phosphorylates and activates the type I  
15 phosphatidylinositol phosphate kinase isoform- $\gamma$ 661 (PIPKI $\gamma$ 661), which is  
16 involved in the formation of focal adhesion sites (26). FAK also functions  
17 to promote the disassembly of focal contacts, in part by activating  
18 intracellular proteases such as calpain, promoting turnover of focal  
19 adhesions (10). Thus, the kinase enhances both assembly and disassembly  
20 of the complexes, and the two seemingly opposite functions may underlie

1 the ability of FAK to regulate focal adhesion turnover. Accordingly,  
2 downregulation of FAK by CagA impairs the focal adhesion system,  
3 resulting in altered amounts and intracellular distribution of active focal  
4 adhesion sites. The decrease in the focal adhesion sites promotes  
5 detachment of CagA-expressing cells from the plate. Intriguingly, there still  
6 remains a small amount of active FAK molecules, which are specifically  
7 enriched at the tips of the membrane protrusions, in CagA-expressing cells  
8 with the hummingbird phenotype. This observation indicates that a specific  
9 compartmentalization of active FAK, which has escaped from  
10 CagA-stimulated SHP-2, may promote assembly of new focal adhesion  
11 complexes that generate precursor sites for membrane protrusions. Such a  
12 polarized localization of active FAK should also be important for a single  
13 cell to move from one place to another with a small number of focal  
14 adhesions. As a result, cells with the hummingbird phenotype may exhibit  
15 high motility while showing a net decrease in FAK tyrosine phosphorylation.  
16 Obviously, cytoskeletal molecules that are regulated by FAK, SHP-2 and/or  
17 SFKs should be involved in the morphogenetic activities of CagA in gastric  
18 epithelial cells. In fact, it has been suggested that dephosphorylation of  
19 cortactin plays a role in the development of elongated cell shape induced by  
20 CagA (47). We have also observed that expression of CagA in AGS cells

1 results in decreased tyrosine phosphorylation of paxillin (data not shown),  
2 which is phosphorylated and dephosphorylated by FAK and SHP-2,  
3 respectively (6, 41). Accordingly, molecules such as paxillin may play  
4 crucial roles in induction of the hummingbird phenotype by acting as  
5 downstream effectors of the CagA-SHP-2-FAK pathway.

6 Morphological transformation as well as increased motility of gastric  
7 epithelial cells induced by CagA may disrupt the normal architecture of  
8 gastric mucosa and enhance local inflammation by *H. pylori* infection in the  
9 stomach. Continuous mucosal damage caused by *cagA*-positive *H. pylori*  
10 would obviously stimulate epithelial cell turnover, increasing the chances  
11 for accumulation of genetic mutations that promote multistep gastric  
12 carcinogenesis.

13

14

#### ACKNOWLEDGMENTS

15 We thank Dr. Tadashi Yamamoto for *FAK* cDNA and Dr. Takashi  
16 Matozaki for helpful discussion. We also thank Siho Yamazaki, Yumiko  
17 Fujii and Yasuhiro Teishikata for technical support.

18 This work was supported by Grants-in-Aid for Scientific Research and  
19 a Grand-in-Aid for JSPS Fellows from the Ministry of Education, Culture,  
20 Sports, Science and Technology (MEXT) of Japan, by a grant from Princess

1 Takamatsu Foundation, and by a grant from Uehara Memorial Foundation.

## REFERENCES

- 1  
2 1. **Agazie, Y. M., and M. J. Hayman.** 2003. Development of an efficient  
3 "substrate-trapping" mutant of Src homology phosphotyrosine  
4 phosphatase 2 and identification of the epidermal growth factor receptor,  
5 Gab1, and three other proteins as target substrates. *J. Biol. Chem.*  
6 **278**:13952-13958.
- 7 2. **Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J.**  
8 **Nelson, and S. Falkow.** 2003. Disruption of the epithelial  
9 apical-junctional complex by *Helicobacter pylori* CagA. *Science*  
10 **300**:1430-1434.
- 11 3. **Asahi, M., T. Azuma, S. Ito, Y. Ito, H. Suto, Y. Nagai, M.**  
12 **Tsubokawa, Y. Tohyama, S. Maeda, M. Omata, T. Suzuki, and C.**  
13 **Sasakawa.** 2000. *Helicobacter pylori* CagA protein can be tyrosine  
14 phosphorylated in gastric epithelial cells. *J. Exp. Med.* **191**:593-602.
- 15 4. **Backert, S., S. Moese, M. Selbach, V. Brinkmann, and T. F. Meyer.**  
16 2001. Phosphorylation of tyrosine 972 of the *Helicobacter pylori* CagA  
17 protein is essential for induction of a scattering phenotype in gastric  
18 epithelial cells. *Mol. Microbiol.* **42**:631-644.
- 19 5. **Backert, S., E. Ziska, V. Brinkmann, U. Zimny-Arndt, A.**  
20 **Fauconnier, P. R. Jungblut, M. Naumann, and T. F. Meyer.** 2000.

- 1 Translocation of the *Helicobacter pylori* CagA protein in gastric  
2 epithelial cells by a type IV secretion apparatus. *Cell. Microbiol.*  
3 **2**:155-164.
- 4 6. **Bellis, S. L., J. T. Miller, and C. E. Turner.** 1995. Characterization of  
5 tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J.*  
6 *Biol. Chem.* **270**:17437-17441.
- 7 7. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M.**  
8 **Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995.  
9 Infection with *Helicobacter pylori* strains possessing *cagA* is associated  
10 with an increased risk of developing adenocarcinoma of the stomach.  
11 *Cancer Res.* **55**:2111-2115.
- 12 8. **Calalb, M. B., T. R. Polte, and S. K. Hanks.** 1995. Tyrosine  
13 phosphorylation of focal adhesion kinase at sites in the catalytic domain  
14 regulates kinase activity: a role for Src family kinases. *Mol. Cell. Biol.*  
15 **15**:954-963.
- 16 9. **Calalb, M. B., X. Zhang, T. R. Polte, and S. K. Hanks.** 1996. Focal  
17 adhesion kinase tyrosine-861 is a major site of phosphorylation by Src.  
18 *Biochem. Biophys. Res. Commun.* **228**:662-668.
- 19 10. **Carragher, N. O., M. A. Westhoff, V. J. Fincham, M. D. Schaller,**  
20 **and M. C. Frame.** 2003. A novel role for FAK as a protease-targeting

- 1 adaptor protein: regulation by p42 ERK and Src. *Curr. Biol.*  
2 **13**:1442-1450.
- 3 11. **Churin, Y., L. Al-Ghoul, O. Kepp, T. F. Meyer, W. Birchmeier, and**  
4 **M. Naumann.** 2003. *Helicobacter pylori* CagA protein targets the c-Met  
5 receptor and enhances the motogenic response. *J. Cell. Biol.*  
6 **161**:249-255.
- 7 12. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G.**  
8 **Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and R.**  
9 **Rappuoli.** 1993. Molecular characterization of the 128-kDa  
10 immunodominant antigen of *Helicobacter pylori* associated with  
11 cytotoxicity and duodenal ulcer. *Proc. Natl. Acad. Sci. USA*  
12 **90**:5791-5795.
- 13 13. **Damsky, C. H., and D. Ilic.** 2002. Integrin signaling: it's where the  
14 action is. *Curr. Opin. Cell Biol.* **14**:594-602.
- 15 14. **Danesh, J.** 1999. *Helicobacter pylori* infection and gastric cancer:  
16 systematic review of the epidemiological studies. *Aliment Pharmacol.*  
17 *Ther.* **13**:851-856.
- 18 15. **Dooley, C. P., H. Cohen, P. L. Fitzgibbons, M. Bauer, M. D.**  
19 **Appleman, G. I. Perez-Perez, and M. J. Blaser.** 1989. Prevalence of  
20 *Helicobacter pylori* infection and histologic gastritis in asymptomatic

- 1 persons. N. Eng. J. Med. **321**:1562-1566.
- 2 16. **Feng, G. S.** 1999. Shp-2 tyrosine phosphatase: signaling one cell or  
3 many. Exp. Cell. Res. **253**:47-54.
- 4 17. **Gabarra-Niecko, V., P. J. Keely, and M. D. Schaller.** 2002.  
5 Characterization of an activated mutant of focal adhesion kinase:  
6 'SuperFAK'. Biochem. J. **365**:591-603.
- 7 18. **Hatakeyama, M.** 2004. Oncogenic mechanisms of the *Helicobacter*  
8 *pylori* CagA protein. Nat. Rev. Cancer **4**:688-694.
- 9 19. **Higashi, H., A. Nakaya, R. Tsutsumi, K. Yokoyama, Y. Fujii, S.**  
10 **Ishikawa, M. Higuchi, A. Takahashi, Y. Kurashima, Y. Teishikata, S.**  
11 **Tanaka, T. Azuma, and M. Hatakeyama.** 2004. *Helicobacter pylori*  
12 CagA induces Ras-independent morphogenetic response through SHP-2  
13 recruitment and activation. J. Biol. Chem. **279**:17205-17216.
- 14 20. **Higashi, H., R. Tsutsumi, A. Fujita, S. Yamazaki, M. Asaka, T.**  
15 **Azuma, and M. Hatakeyama.** 2002. Biological activity of the  
16 *Helicobacter pylori* virulence factor CagA is determined by variation in  
17 the tyrosine phosphorylation sites. Proc. Natl. Acad. Sci. USA  
18 **99**:14428-14433.
- 19 21. **Higashi, H., R. Tsutsumi, S. Muto, T. Sugiyama, T. Azuma, M.**  
20 **Asaka, and M. Hatakeyama.** 2002. SHP-2 tyrosine phosphatase as an

- 1 intracellular target of *Helicobacter pylori* CagA protein. Science  
2 **295**:683-686.
- 3 22. **Higuchi, M., R. Tsutsumi, H. Higashi, and M. Hatakeyama.** 2004.  
4 Conditional gene silencing utilizing the *lac* repressor reveals a role of  
5 SHP-2 in *cagA*-positive *Helicobacter pylori* pathogenicity. Cancer Sci.  
6 **95**:442-447.
- 7 23. **Huang, J. Q., S. Sridhar, Y. Chen, and R. H. Hunt.** 1998.  
8 Meta-analysis of the relationship between *Helicobacter pylori*  
9 seropositivity and gastric cancer. Gastroenterology **114**:1169–1179.
- 10 24. **Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji,**  
11 **S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto.** 1995. Reduced  
12 cell motility and enhanced focal adhesion contact formation in cells  
13 from FAK-deficient mice. Nature **377**:539-544.
- 14 25. **Kontaridis, M. I., S. Eminaga, M. Fornaro, C. I. Zito, R. Sordella, J.**  
15 **Settleman, and A. M. Bennett.** 2004. SHP-2 positively regulates  
16 myogenesis by coupling to the Rho GTPase signaling pathway. Mol.  
17 Cell. Biol. **24**:5340-52.
- 18 26. **Ling, K., R. L. Doughman, A. J. Firestone, M. W. Bunce, and R. A.**  
19 **Anderson.** 2002. Type I gamma phosphatidylinositol phosphate kinase  
20 targets and regulates focal adhesions. Nature **420**:89-93.

- 1 27. **Lu, Z., G. Jiang, P. Blume-Jensen, and T. Hunter.** 2001. Epidermal  
2 growth factor-induced tumor cell invasion and metastasis initiated by  
3 dephosphorylation and downregulation of focal adhesion kinase. *Mol.*  
4 *Cell. Biol.* **21**:4016-4031.
- 5 28. **Manes, S., E. Mira, C. Gomez-Mouton, Z. J. Zhao, R. A. Lacalle,**  
6 **and C. Martinez-A.** 1999. Concerted activity of tyrosine phosphatase  
7 SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol.*  
8 *Cell. Biol.* **19**:3125-3135.
- 9 29. **Mimuro, H., T. Suzuki, J. Tanaka, M. Asahi, R. Haas, and C.**  
10 **Sasakawa.** 2002. Grb2 is a key mediator of *Helicobacter pylori* CagA  
11 protein activities. *Mol. Cell* **10**:745-755.
- 12 30. **Nada, S., M. Okada, A. MacAuley, J. A. Cooper, and H. Nakagawa.**  
13 1991. Cloning of a complementary DNA for a protein-tyrosine kinase  
14 that specifically phosphorylates a negative regulatory site of p60<sup>c-src</sup>.  
15 *Nature* **351**:69-72.
- 16 31. **Neel, B. G., H. Gu, and L. Pao.** 2003. The 'Shp'ing news: SH2  
17 domain-containing tyrosine phosphatases in cell signaling *Trends*  
18 *Biochem. Sci.* **28**:284-293.
- 19 32. **NIH Consensus Conference.** 1994. *Helicobacter pylori* in peptic ulcer  
20 disease. *J. Am. Med. Assoc.* **272**:65-69.

- 1 33. **Noguchi, T., T. Matozaki, K. Horita, Y. Fujioka, and M. Kasuga.**  
2 1994. Role of SH-PTP2, a protein-tyrosine phosphatase with Src  
3 homology 2 domains, in insulin-stimulated Ras activation. *Mol. Cell.*  
4 *Biol.* **14**: 6674-6682.
- 5 34. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R.**  
6 **Haas.** 2000. Translocation of *Helicobacter pylori* CagA into gastric  
7 epithelial cells by type IV secretion. *Science* **287**:1497-1500.
- 8 35. **Oh, E. S., H. Gu, T. M. Saxton, J. F. Timms, S. Hausdorff, E. U.**  
9 **Frevert, B. B. Kahn, T. Pawson, B. G. Neel, and S. M. Thomas.** 1999.  
10 Regulation of early events in integrin signaling by protein tyrosine  
11 phosphatase SHP-2. *Mol. Cell. Biol.* **19**:3205-3215.
- 12 36. **Okada, M., S. Nada, Y. Yamanashi, T. Yamamoto, and H. Nakagawa.**  
13 1991. CSK: a protein-tyrosine kinase involved in regulation of src  
14 family kinases. *J. Biol. Chem.* **266**:24249-24252.
- 15 37. **Okada, M., and H. Nakagawa.** 1989. A protein tyrosine kinase  
16 involved in regulation of pp60<sup>c-src</sup> function. *J. Biol. Chem.*  
17 **264**:20886-20893.
- 18 38. **Parsonnet, J., G. D. Friedman, N. Orentreich, and H. Vogelman.**  
19 1997. Risk for gastric cancer in people with CagA positive or CagA  
20 negative *Helicobacter pylori* infection. *Gut* **40**:297-301.

- 1 39. **Parsons, J. T.** 2003. Focal adhesion kinase: the first ten years. *J. Cell*  
2 *Sci.* **116**:1409-1416.
- 3 40. **Peng, Z. Y., and C. A. Cartwright.** 1995. Regulation of the Src  
4 tyrosine kinase and Syp tyrosine phosphatase by their cellular  
5 association. *Oncogene* **16**:1955-1962.
- 6 41. **Ren, Y., S. Meng, L. Mei, Z. J. Zhao, R. Jove, and J. Wu.** 2004. Roles  
7 of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src  
8 activation in response to epidermal growth factor. *J. Biol. Chem.*  
9 **279**:8497-8505.
- 10 42. **Rugge, M., G. Busatto, M. Cassaro, Y. H. Shiao, V. Russo, G.**  
11 **Leandro, C. Avellini, A. Fabiano, A. Sidoni, and A. Covacci.** 1999.  
12 Patients younger than 40 years with gastric carcinoma: *Helicobacter*  
13 *pylori* genotype and associated gastritis phenotype. *Cancer*  
14 **85**:2506-2511.
- 15 43. **Schaller, M. D., J. D. Hildebrand, J. D. Shannon, J. W. Fox, R. R.**  
16 **Vines, and J. T. Parsons.** 1994. Autophosphorylation of the focal  
17 adhesion kinase, pp125<sup>FAK</sup>, directs SH2-dependent binding of pp60<sup>src</sup>.  
18 *Mol. Cell. Biol.* **14**:1680-1688.
- 19 44. **Schlaepfer, D. D., S. K. Mitra, and D. Ilic.** 2004. Control of motile  
20 and invasive cell phenotypes by focal adhesion kinase. *Biochim.*

- 1 Biophys. Acta **1692**:77-102.
- 2 45. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999.  
3 Altered states: involvement of phosphorylated CagA in the induction of  
4 host cellular growth changes by *Helicobacter pylori*. Proc. Natl. Acad.  
5 Sci. USA **96**:14559-14564.
- 6 46. **Selbach, M., S. Moese, C. R. Hauck, T. F. Meyer, and S. Backert.**  
7 2002. Src is the kinase of the *Helicobacter pylori* CagA protein *in vitro*  
8 and *in vivo*. J. Biol. Chem. **277**:6775-6778.
- 9 47. **Selbach, M., S. Moese, R. Hurwitz, C. R. Hauck, T. F. Meyer, and S.**  
10 **Backert.** 2003. The *Helicobacter pylori* CagA protein induces cortactin  
11 dephosphorylation and actin rearrangement by c-Src inactivation.  
12 EMBO J. **22**:515-528.
- 13 48. **Stein, M., F. Bagnoli, R. Halenbeck, R. Rappuoli, W. J. Fantl, and A.**  
14 **Covacci.** 2002. c-Src/Lyn kinases activate *Helicobacter pylori* CagA  
15 through tyrosine phosphorylation of the EPIYA motifs. Mol. Microbiol.  
16 **43**:971-980.
- 17 49. **Stein, M., R. Rappuoli, and A. Covacci.** 2000. Tyrosine  
18 phosphorylation of the *Helicobacter pylori* CagA antigen after  
19 *cag*-driven host cell translocation. Proc. Natl. Acad. Sci. USA  
20 **97**:1263-1268.

- 1 50. **Tsuda, M., T. Matozaki, K. Fukunaga, Y. Fujioka, A. Imamoto, T.**  
2 **Noguchi, T. Takada, T. Yamao, H. Takeda, F. Ochi, T. Yamamoto,**  
3 **and M. Kasuga.** 1998. Integrin-mediated tyrosine phosphorylation of  
4 SHPS-1 and its association with SHP-2. Roles of Fak and Src family  
5 kinases. *J. Biol. Chem.* **273**:13223-13229.
- 6 51. **Tsutsumi, R., H. Higashi, M. Higuchi, M. Okada, and M.**  
7 **Hatakeyama.** 2003. Attenuation of *Helicobacter pylori* CagA·SHP-2  
8 signaling by interaction between CagA and C-terminal Src kinase. *J.*  
9 *Biol. Chem.* **278**:3664-3670.
- 10 52. **Uemura, N., S. Okamoto, S. Yamamoto, N. Matsumura, S.**  
11 **Yamaguchi, M. Yamakido, K. Taniyama, N. Sasaki, and R. J.**  
12 **Schlemper.** 2001. *Helicobacter pylori* infection and the development of  
13 gastric cancer. *N. Engl. J. Med.* **345**:784-789.
- 14 53. **Vadlamudi, R. K., L. Adam, D. Nguyen, M. Santos, and R. Kumar.**  
15 2002. Differential regulation of components of the focal adhesion  
16 complex by heregulin: role of phosphatase SHP-2. *J. Cell. Physiol.*  
17 **190**:189-199.
- 18 54. **Yamaoka, Y., T. Kodama, K. Kashima, D. Y. Graham, and A. R.**  
19 **Sepulveda.** 1998. Variants of the 3' region of the *cagA* gene in  
20 *Helicobacter pylori* isolates from patients with different *H.*

- 1 *pylori*-associated diseases. J. Clin. Microbiol. **36**:2258-2263.
- 2 55. **Yamaoka, Y., M. S. Osato, A. R. Sepulveda, O. Gutierrez, N. Figura,**  
3 **J. G. Kim, T. Kodama, K. Kashima, and D. Y. Graham.** 2000.  
4 Molecular epidemiology of *Helicobacter pylori*: separation of *H. pylori*  
5 from East Asian and non-Asian countries. Epidemiol. Infect. **124**:91-96.
- 6 56. **Yano, H., Y. Mazaki, K. Kurokawa, S. K. Hanks, M. Matsuda, and H.**  
7 **Sabe.** 2004. Roles played by a subset of integrin signaling molecules in  
8 cadherin-based cell-cell adhesion. J. Cell. Biol. **166**:283-295.
- 9 57. **Yu, D. H., C. K. Qu, O. Henegariu, X. Lu, and G. S. Feng.** 1998.  
10 Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration,  
11 and focal adhesion. J. Biol. Chem. **273**:21125-21131.
- 12 58. **Zhang, S. Q., W. Yang, M. I. Kontaridis, T. G. Bivona, G. Wen, T.**  
13 **Araki, J. Luo, J. A. Thompson, B. L. Schraven, M. R. Philips, and B.**  
14 **G. Neel.** 2004. Shp2 regulates SRC family kinase activity and Ras/Erk  
15 activation by controlling Csk recruitment. Mol. Cell **13**:341-355.

## FIGURE LEGENDS

1  
2 FIG. 1. CagA reduces the level of FAK tyrosine phosphorylation. (A)  
3 AGS cells were transfected with indicated CagA expression vector or  
4 control empty vector. Cell lysates were prepared and immunoprecipitated  
5 with anti-FAK antibody or control IgG. Immunoprecipitates (IP) and total  
6 cell lysates were immunoblotted (IB) with indicated antibodies. Anti-pY  
7 represents anti-phosphotyrosine. Positions of FAK, tyrosine-phosphorylated  
8 FAK (pY-FAK), CagA and tyrosine-phosphorylated CagA (pY-CagA) are  
9 indicated by arrows. Quantitation expressed as the ratio of  
10 tyrosine-phosphorylated FAK to total FAK from three separate experiments  
11 is summarized in the histogram on the right. Each value was calculated  
12 from the intensities of anti-pY and anti-FAK immunoblotting by using a  
13 lumino-image analyzer and defining the value in the absence of CagA as 1.  
14 Error bars indicate 2x SD. (B) AGS cells were transfected with EGFP  
15 expression vector or control empty vector. Cells were harvested 36 h after  
16 transfection and were subjected to flow cytometric analysis to calculate  
17 transfection efficiency. Percentage of EGFP-positive cells is indicated. (C)  
18 Culture supernatants were prepared from AGS cells transfected with WT  
19 CagA-HA expression vector or control empty vector 36 h after transfection.  
20 AGS cells were treated with the culture supernatants for indicated time

1 periods. Cell lysates were prepared from CagA-transfected AGS cells (top)  
2 or AGS cells treated with the culture supernatant (bottom), and were  
3 immunoprecipitated with anti-FAK antibody or control IgG.  
4 Immunoprecipitates were immunoblotted with indicated antibodies.  
5 Positions of FAK and pY-FAK are indicated by arrows.

6  
7 FIG. 2. CagA-Csk interaction is mediated by EPIYA-A or EPIYA-B site  
8 of CagA. (A) Lysates from AGS or COS-7 cells transfected with control or  
9 WT CagA-HA expression vector were immunoblotted (IB) with anti-CagA  
10 or anti-SHP-2 antibody. Arrows indicate positions of CagA and SHP-2. (B)  
11 Schematic views of HA-tagged WT CagA and its derivatives. (C) AGS cells  
12 were transiently transfected with indicated CagA expression vector or  
13 control empty vector. Total cell lysates were prepared and  
14 immunoprecipitated with anti-HA antibody. Immunoprecipitates (IP) and  
15 total cell lysates were subjected to immunoblotting with anti-Csk, anti-HA  
16 or anti-phosphotyrosine (anti-pY) antibody. Arrows indicate positions of  
17 Csk, CagA and tyrosine-phosphorylated CagA (pY-CagA). (D) AGS cells  
18 were transfected with indicated CagA expression vector or control empty  
19 vector. Cell lysates were prepared and were immunoprecipitated with  
20 anti-HA antibody. Immunoprecipitates and total cell lysates were

1 immunoblotted with indicated antibodies. Arrows indicate positions of Csk,  
2 CagA and pY-CagA. (E) AGS cells were transfected with indicated  
3 expression vector or control empty vector. Cell lysates were prepared and  
4 were subjected to immunoblotting with indicated antibodies. Positions of  
5 SrcΔK, CagA and pY-CagA are indicated. Relative ratios of phosphorylated  
6 SrcΔK at Y-419 are indicated. Each value was calculated from the  
7 immunoblotting data by using a luminescence image analyzer and defining  
8 the value in the absence of CagA as 1.

9  
10 FIG. 3. Involvement of CagA-SHP-2 interaction in the reduced FAK  
11 tyrosine phosphorylation by CagA. (A) AGS cells were transfected with  
12 indicated CagA expression vector or control empty vector. Cell lysates were  
13 prepared and immunoprecipitated with anti-FAK antibody or control IgG.  
14 Immunoprecipitates (IP) and total cell lysates were immunoblotted (IB)  
15 with indicated antibodies. Arrows indicate positions of FAK,  
16 tyrosine-phosphorylated FAK (pY-FAK), CagA and tyrosine-phosphorylated  
17 CagA (pY-CagA). Quantitation expressed as the ratio of tyrosine  
18 phosphorylated FAK to total FAK from three separate experiments is  
19 summarized in the histogram on the right. Each value was calculated from  
20 the intensities of anti-pY and anti-FAK immunoblotting by using a

1 lumino-image analyzer and defining the value in the absence of CagA as 1.  
2 Error bars indicate 2x SD. (B) Total cell lysates from AGS cells transfected  
3 with WT CagA-HA, ABccc CagA-HA, abCCC CagA-HA or control empty  
4 vector were immunoblotted with indicated antibodies (left). The asterisk  
5 indicates the anti-pSrc416-specific band that corresponds to c-Src in size.  
6 AGS cells were incubated with 5  $\mu$ M PP2 or 0.2% dimethylsulfoxid  
7 (DMSO) for 2 h before harvest and cell lysates were subjected to  
8 immunoblotting with indicated antibodies (right). Arrows indicate CagA,  
9 pY-CagA and c-Src. (C) AGS cells (lanes 1 to 4) or AGS-derived G11 cells  
10 (lanes 5 to 12), in which expression of SHP-2 was constitutively inhibited  
11 by siRNA, were transfected with WT CagA-HA expression vector,  
12 SHP-2RR-Myc expression vector and/or control empty vector as indicated.  
13 Total cell lysates were prepared and immunoprecipitated with anti-FAK  
14 antibody or control IgG. Immunoprecipitates (left, upper panel) and total  
15 cell lysates (left, lower panel) were immunoblotted with indicated  
16 antibodies. The asterisk indicates the anti-pSrc416-specific band that  
17 corresponds to c-Src in size. Positions of FAK, pY-FAK, c-Src, CagA,  
18 pY-CagA, SHP-2 and SHP-2RR-Myc are indicated. Quantitation expressed  
19 as the ratio of tyrosine phosphorylated FAK to total FAK is summarized in  
20 the histogram on the right. Each value was calculated from the intensities of

1 anti-pY and anti-FAK immunoblotting by using a lumino-image analyzer  
2 and defining the value in AGS cells without CagA as 1. (D) AGS cells were  
3 transfected with control, WT CagA-HA or ABccc CagA-HA expression  
4 vector and at 12 h after transfection were harvested or treated with 100 mM  
5 genistein for additional 2 h before harvest. Cell lysates prepared were then  
6 immunoprecipitated with anti-FAK antibody. Immunoprecipitates (upper  
7 panel) and total cell lysates (left, lower panel) were immunoblotted with  
8 indicated antibodies. Arrows indicate positions of pY-FAK and FAK.  
9 Quantitation expressed as the percentages of tyrosine-phosphorylated FAK  
10 to total FAK from three separate experiments is shown in the lower, right  
11 panel. Each value was calculated from the intensities of anti-pY and  
12 anti-FAK immunoblotting by using a lumino-image analyzer and defining  
13 the value without genistein treatment (0 h) as 100%. Error bars indicate 2x  
14 SD.

15

16 FIG. 4. Dephosphorylation of FAK by SHP-2. (A) AGS cells were  
17 transfected with Myr-SHP-2 $\Delta$ SH2-Myc expression vector or control empty  
18 vector. Cell lysates were prepared and immunoprecipitated with anti-FAK  
19 antibody or control IgG. Immunoprecipitates (IP, left, upper panel) and  
20 total cell lysates (left, lower panel) were immunoblotted (IB) with indicated

1 antibodies. Representative photographs from three separate experiments are  
2 indicated. Arrows indicate positions of FAK, pY-FAK and  
3 Myr-SHP-2 $\Delta$ SH2-Myc. Quantitation expressed as the ratio of tyrosine  
4 phosphorylated FAK to total FAK from three separate experiments is  
5 summarized in the histogram on the right. Each value was calculated from  
6 the intensities of anti-pY and anti-FAK immunoblotting by using a  
7 lumino-image analyzer and defining the value in the absence of  
8 Myr-SHP-2 $\Delta$ SH2-Myc as 1. Error bars indicate 2x SD. (B) AGS cells were  
9 transfected with 30  $\mu$ g of Myc-tagged WT SHP-2 (WT SHP-2-Myc)  
10 expression vector, substrate-trapping mutant of SHP-2 (DM SHP-2-Myc)  
11 expression vector, non-substrate trapping mutant of SHP-2 (R465M  
12 SHP-2-Myc) expression vector or control empty vector. Total cell lysates  
13 were immunoprecipitated with anti-Myc antibody. Immunoprecipitates and  
14 total cell lysates were immunoblotted with anti-FAK or anti-Myc antibody.  
15 Arrows indicate positions of FAK, and SHP-2-Myc. (C) AGS cells were  
16 transfected with 5  $\mu$ g of DM SHP-2-Myc expression vector or control empty  
17 vector together with 25  $\mu$ g of WT CagA-HA expression vector or control  
18 vector. Cell lysates prepared were immunoprecipitated with anti-Myc  
19 antibody. Immunoprecipitates and total cell lysates were immunoblotted  
20 with anti-FAK, anti-Myc, anti-HA or anti-phosphotyrosine (anti-pY)

1 antibody. Arrows indicate positions of FAK, DM SHP-2-Myc, WT CagA-HA  
2 and tyrosine-phosphorylated CagA (pY-CagA). (D) *In vitro* phosphatase  
3 assay of SHP-2. FAK immunopurified from AGS cells and SHP-2  
4 immunopurified from COS-7 cells expressing WT SHP-2-Myc or SHP-2  
5 C/S-Myc were mixed and incubated for 60 min. Reaction mixtures were  
6 then immunoblotted with anti-phosphotyrosine (anti-pY), anti-FAK or  
7 anti-Myc antibody. Arrows indicate positions of FAK,  
8 tyrosine-phosphorylated FAK (pY-FAK) and SHP-2-Myc.

9  
10 FIG. 5. CagA-SHP-2 dephosphorylates FAK at Tyr-397, Tyr-576 and  
11 Tyr-577. (A) AGS cells were transfected with indicated FAK-Flag  
12 expression vector or control empty vector. Cell lysates were  
13 immunoprecipitated with anti-Flag antibody. Immunoprecipitates (IP) were  
14 subjected to immunoblotting (IB) with anti-phosphotyrosine (anti-pY) or  
15 anti-Flag antibody. Arrows indicate positions of tyrosine-phosphorylated  
16 FAK (pY-FAK) and FAK (left). Quantitation of the data in the left panel is  
17 expressed as the ratio of tyrosine phosphorylated FAK to total FAK from  
18 three separate experiments (right). Each value was calculated from the  
19 intensities of anti-pY and anti-Flag immunoblotting by using a  
20 lumino-image analyzer and defining the value of WT FAK-flag as 1. Error

1 bars indicate 2x SD. (B) AGS cells were transfected with WT CagA-HA  
2 expression vector or control empty vector. Cell lysates were  
3 immunoprecipitated with anti-FAK antibody or control IgG.  
4 Immunoprecipitates and total cell lysates were immunoblotted with  
5 indicated antibodies. Arrows indicate positions of FAK, pY-FAK, FAK  
6 phosphorylated at indicated tyrosine residues, CagA and  
7 tyrosine-phosphorylated CagA (pY-CagA). Quantitation expressed as the  
8 ratio of tyrosine phosphorylated FAK to total FAK from three separate  
9 experiments is summarized in the histogram on the right. Each value was  
10 calculated from the intensities of anti-pY and anti-FAK immunoblotting by  
11 using a lumino-image analyzer and defining the value in the absence of  
12 CagA as 1. Error bars indicate 2x SD. (C) AGS cells were transfected with  
13 WT FAK-Flag or mutant FAK-Flag expression vector together with DM  
14 SHP-2-Myc expression vector or control empty vector as indicated. Cell  
15 lysates were prepared and immunoprecipitated with anti-Myc antibody.  
16 Immunoprecipitates and total cell lysates were then subjected to  
17 immunoblotting with anti-Flag or anti-Myc antibody. Arrows indicate  
18 positions of FAK-Flag and DM SHP-2-Myc. (D) AGS cells or G11 cells  
19 were transfected with WT CagA-HA expression vector, SHP-2RR-Myc  
20 expression vector and control empty vector as indicated combination. Total

1 cell lysates were immunoblotted with indicated antibodies. Arrows indicate  
2 positions of FAK, FAK phosphorylated at indicated tyrosine residues,  
3 SHP-2, CagA and pY-CagA. (E) FAK immunopurified from AGS cells  
4 transfected with WT CagA-HA expression vector or control empty vector  
5 was subjected to *in vitro* kinase assay. Relative kinase activities are  
6 indicated in the histogram defining the value in the absence of CagA as 1.  
7 Experiments were triplicates and error bars indicate 2x SD.  
8 Immunoprecipitates were immunoblotted with anti-FAK antibody.

9  
10 FIG. 6. FAK inhibition is involved in the induction of the hummingbird  
11 phenotype. (A) AGS cells were transfected with WT CagA-HA expression  
12 vector, FAK K578E/K581E-Flag expression vector and control empty vector  
13 in various combinations indicated. Cell morphology was examined 36 h  
14 after transfection by microscopy. Percentages of cells with the hummingbird  
15 phenotype are shown. Error bars indicate 2x SD (left). Transfected AGS  
16 cells were lysed and immunoblotted with indicated antibodies. Arrows show  
17 positions of CagA, tyrosine-phosphorylated CagA (pY-CagA) and FAK  
18 K578E/K581E-Flag (right). (B) AGS cells were transfected with WT  
19 CagA-HA, WT FAK-Flag, K454R FAK-Flag, Y576A/Y577A FAK-Flag,  
20 Y407A/Y861A/Y925A FAK-Flag expression vector or control empty vector.

1 Cell morphology was examined 36 h after transfection by microscopy. Scale  
2 bar indicates 0.2 mm. Percentages of cells with the hummingbird phenotype  
3 induced by WT CagA-HA or dominant-negative FAK are shown (left, lower).  
4 Error bars indicate 2x SD. Transfected AGS cells were lysed and  
5 immunoblotted with indicated antibodies. STAT3 is shown as a loading  
6 control. Arrows indicate positions of FAK mutants, CagA and STAT3. (right,  
7 lower).

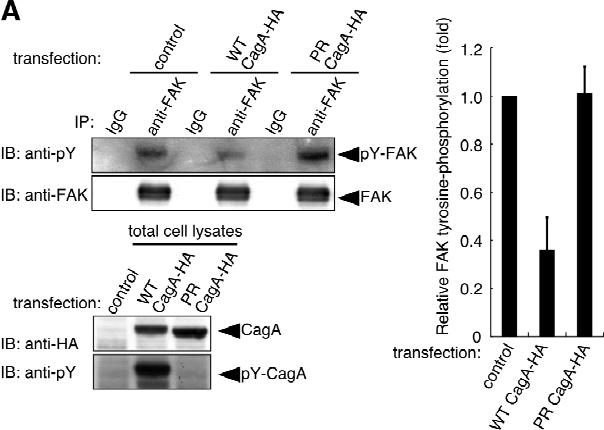
8  
9 FIG. 7. Kinetic changes in the level of FAK tyrosine phosphorylation  
10 by CagA. AGS cells transfected with WT CagA-HA expression vector or  
11 control empty vector were harvested at indicated time points after  
12 transfection. Cell lysates were immunoprecipitated with anti-FAK antibody  
13 or normal rabbit IgG. Immunoprecipitates (IP) and total cell lysates were  
14 immunoblotted with indicated antibodies. Arrows indicate positions of FAK,  
15 tyrosine-phosphorylated FAK (pY-FAK), and tyrosine-phosphorylated CagA  
16 (pY-CagA) (top). Quantitation expressed as the ratio of tyrosine  
17 phosphorylated FAK to total FAK is summarized in the graph on the bottom.  
18 Each value was calculated from the intensities of anti-pY and anti-FAK  
19 immunoblotting by using a luminescence image analyzer and defining the  
20 value in the untransfected AGS (time=0) as 100%.

1

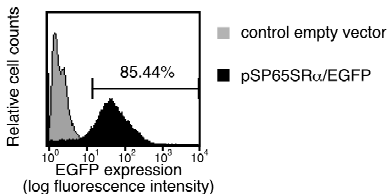
2        FIG. 8. Accumulation of FAK at the tips of protrusions in cells with the  
3 hummingbird phenotype. AGS cells transfected with WT CagA-Flag  
4 expression vector (B, C, and D), WT CagA-HA expression vector (F, G, H  
5 and I) or control empty vector (A and E) were stained with anti-Flag  
6 (green) (C, and D), anti-FAK (red) (A, B and D), anti-HA (red) (F, G, H  
7 and I) or anti-FAK[pY<sup>576</sup>] (green) (E, F, G, H and I). Arrows indicate distal  
8 ends of membrane protrusions in cells with the hummingbird phenotype.  
9 Scale bars: 50  $\mu$ m.

# FIG. 1

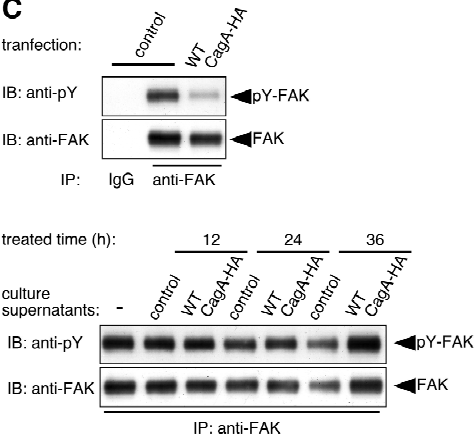
## A



## B

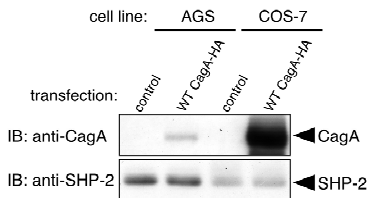


## C

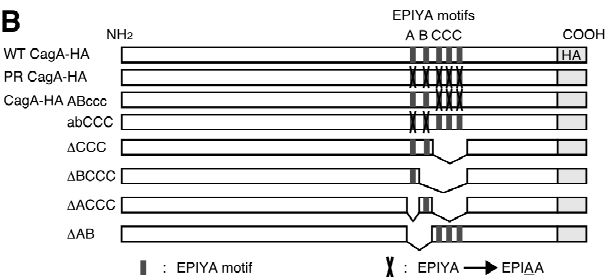


# FIG. 2

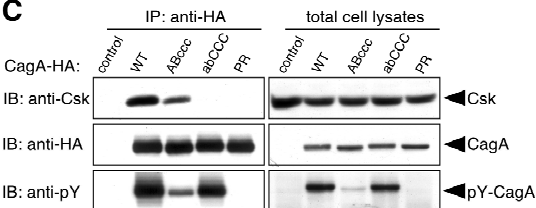
## A



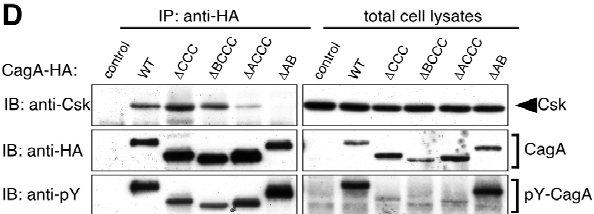
## B



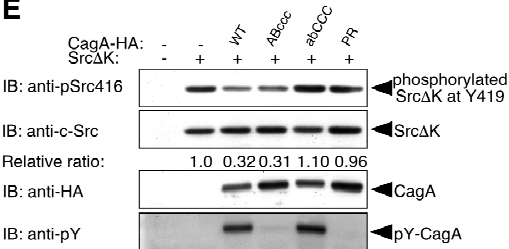
## C



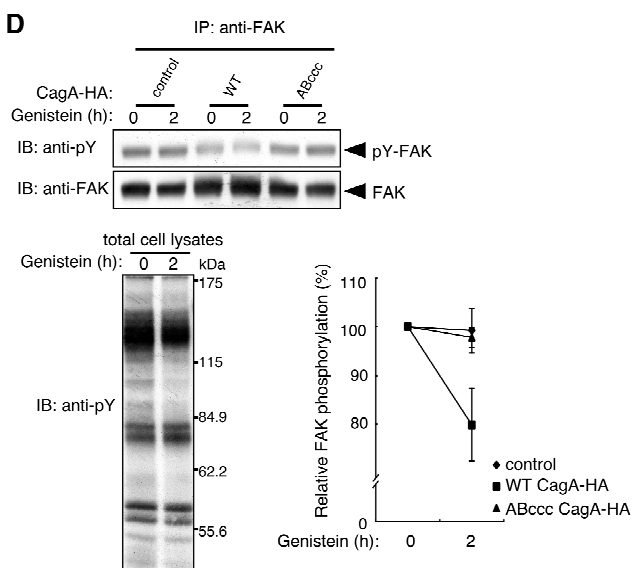
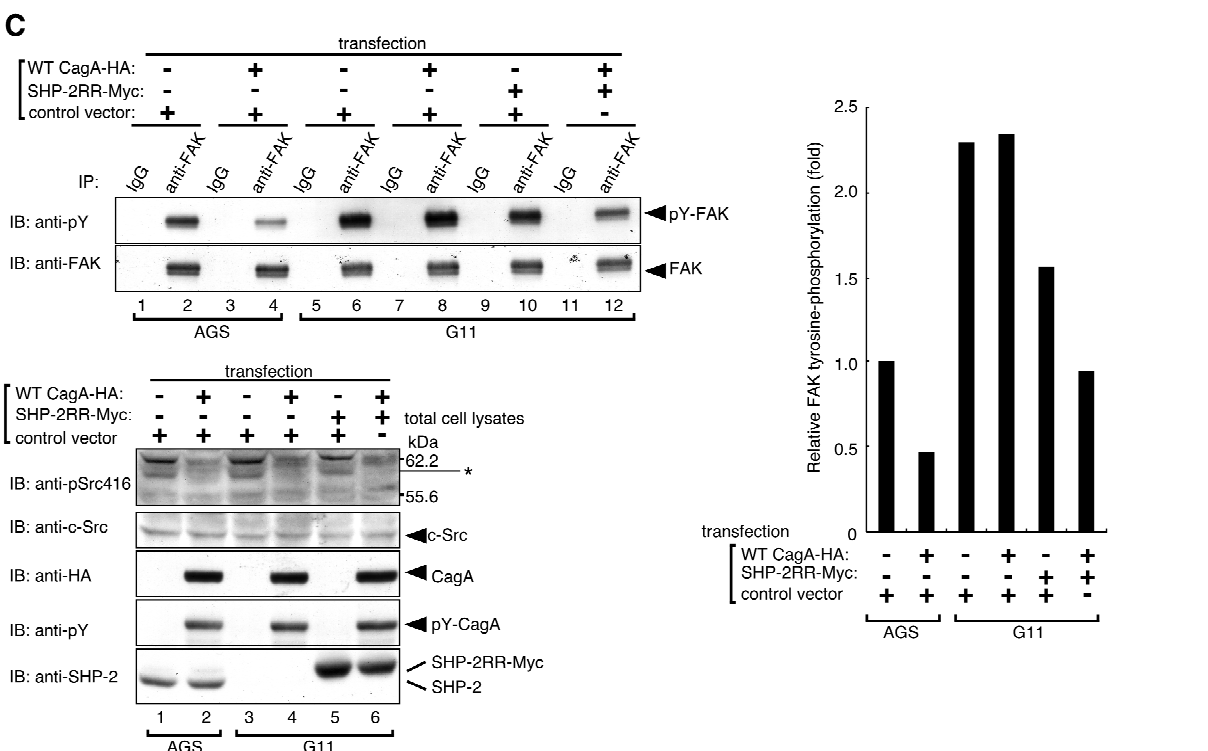
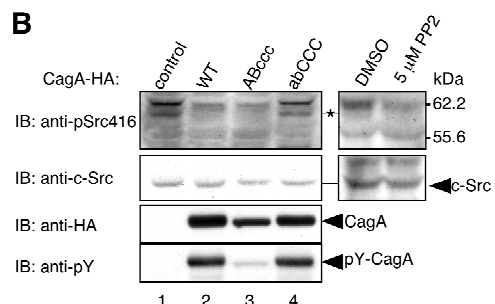
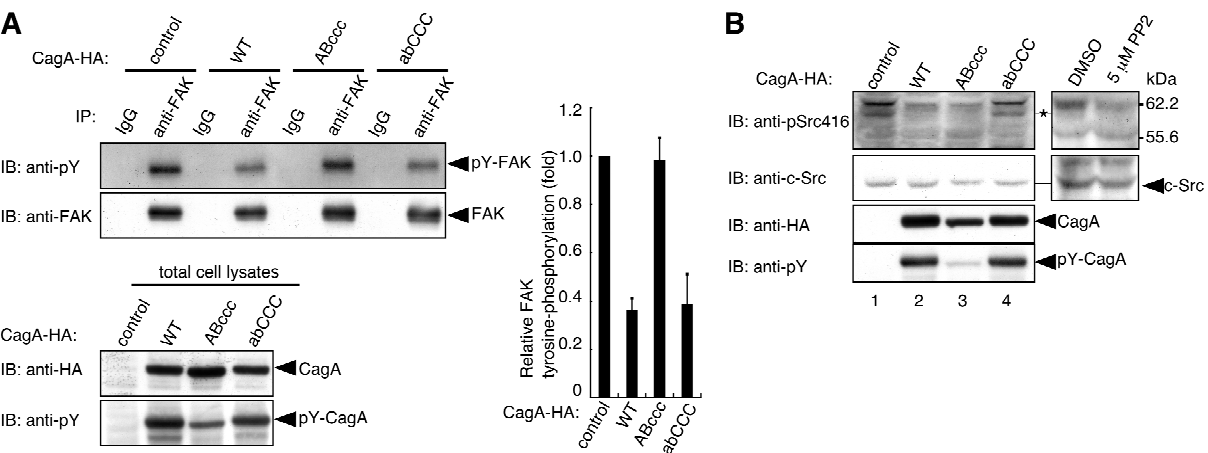
## D



## E

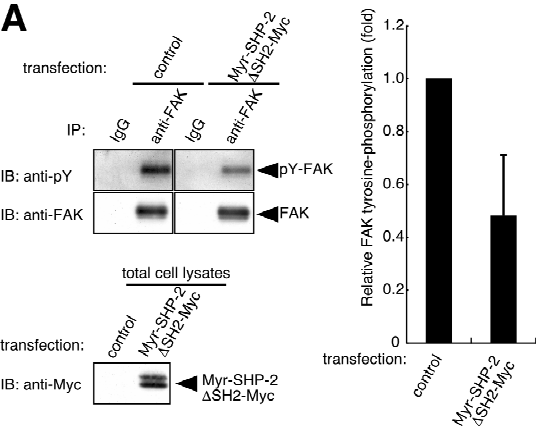


# FIG. 3

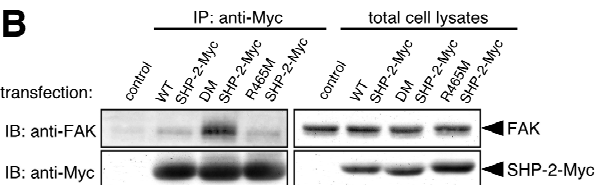


# FIG. 4

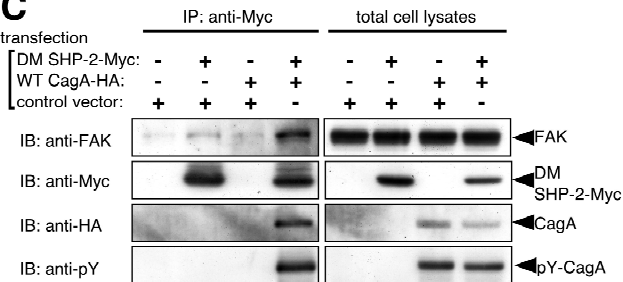
## A



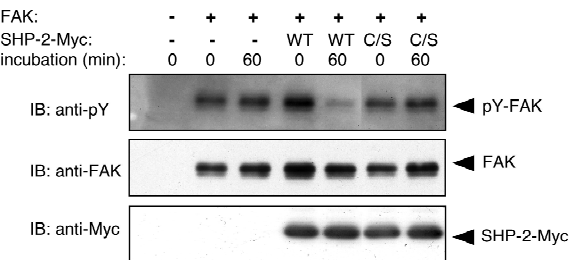
## B



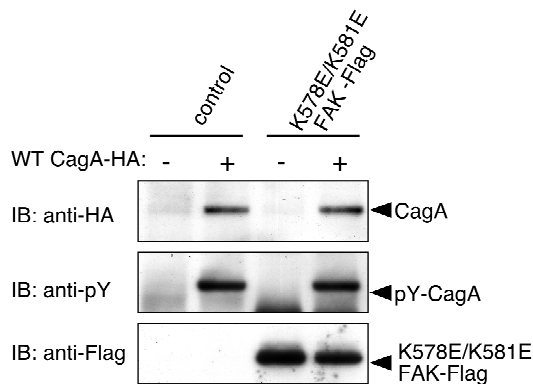
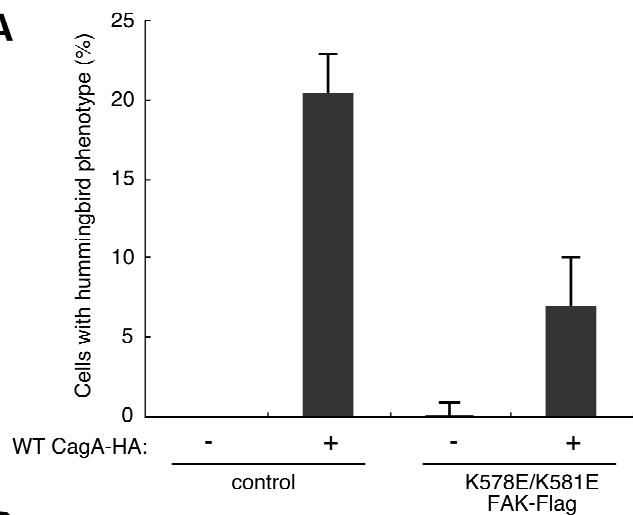
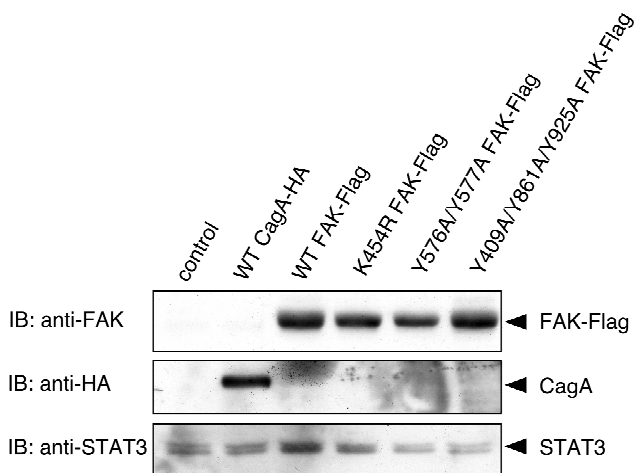
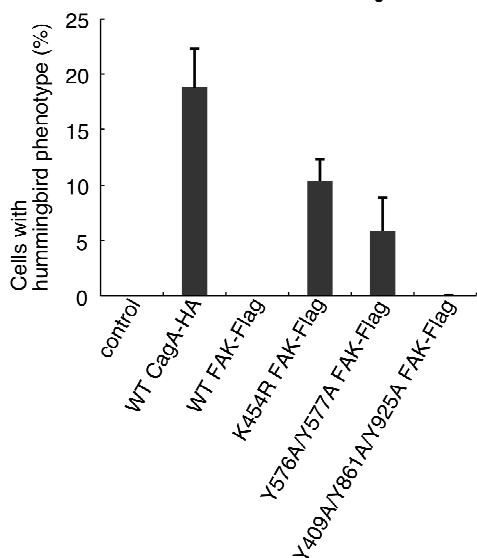
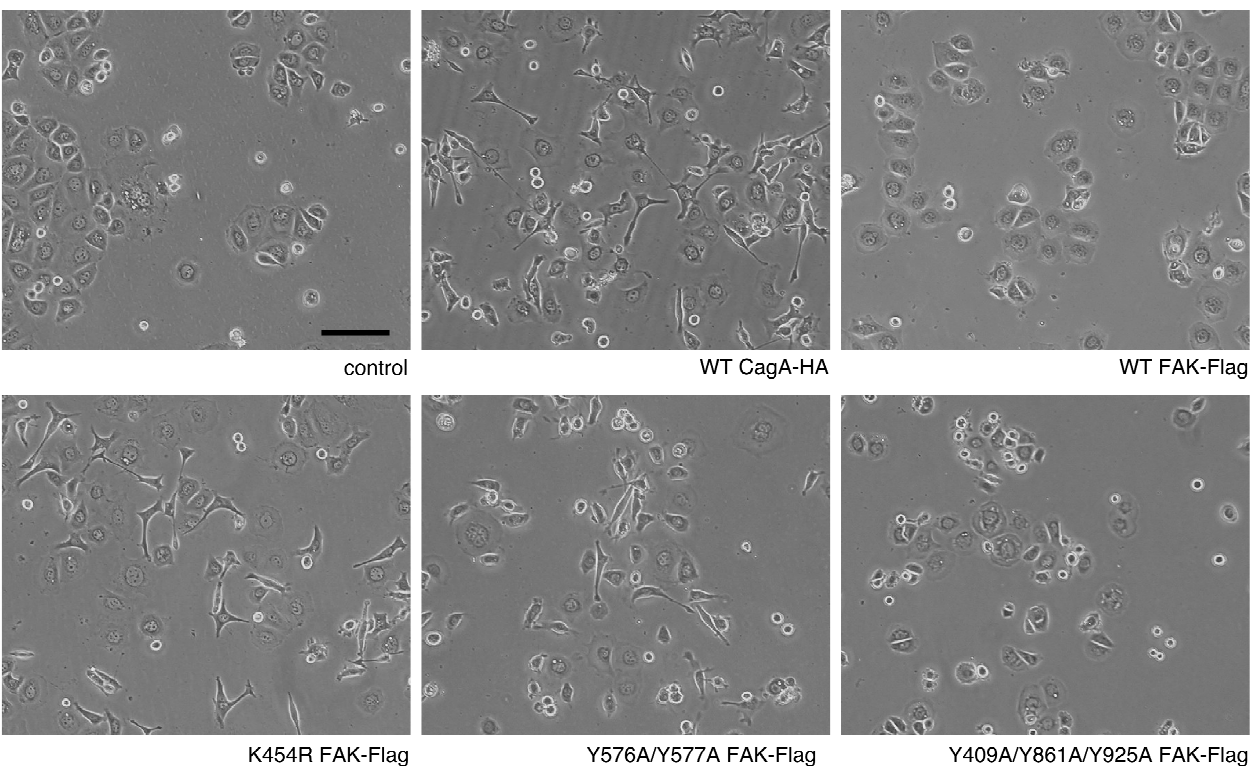
## C



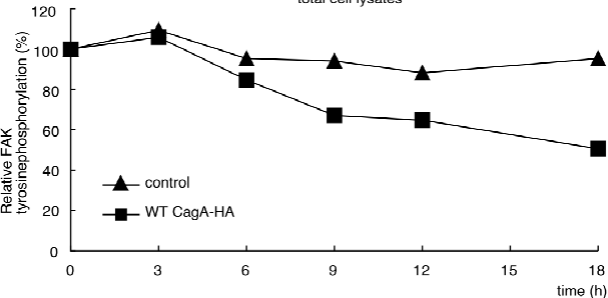
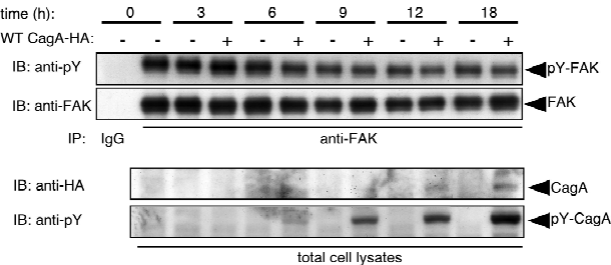
## D





**FIG. 6****A****B**

# FIG. 7



**FIG. 8**

