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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

and its derivatives, ABccc, abCCC, and PR CagA-HA, were described previously (20, 21). ΔCCC, ΔBCCC, ΔACCC, and ΔAB CagA mutants were generated from WT CagA-HA by internal deletions of amino-acid residues 868-1042, 901-1042, amino-acid residues 868-900 and 941-1042, and amino-acid residues 868-940, respectively. A cDNA encoding mouse FAK was provided by Dr. Tadashi Yamamoto (University of Tokyo) and was C-terminal Flag-tagged (WT FAK-Flag). cDNAs encoding Y397A FAK-Flag (substitution of Tyr-397 with alanine), Y576A/Y577A FAK-Flag, Y397A/576A/577A FAK-Flag, Y407A/Y861A/Y925A FAK-Flag, Y576A FAK-Flag, Y577A FAK-Flag, Y397A/Y407A/Y576A/Y577A/Y861A/Y925A FAK-Flag, K454R FAK-Flag and K578E/K581E FAK-Flag were generated from WT FAK-Flag cDNA by the use of Chameleon site-directed mutagenesis kit (Stratagene). The cDNAs were cloned into pSP65SRα mammalian expression vector. Expression vectors for WT SHP-2-Myc, Myr-SHP-2ΔSH2-Myc and Flag-tagged WT CagA (WT CagA-Flag) were described previously (20, 21). A cDNA encoding Myc-tagged, catalytically inactive SHP-2 was made by replacing Cys-459 with serine residue (SHP-2 C/S-Myc) or by replacing Arg-465 with methionine residue (R465M SHP-2-Myc) by site-directed mutagenesis, and was inserted into the pSP65SRα vector.

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

Immunoprecipitation and immunoblotting. AGS cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% Brij-35, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor and 10 μg/ml aprotinin]. Cell lysates were treated with specific antibodies or control IgG, and immune complexes were trapped on protein A- or protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins transferred to poly (vinylidene difluoride) membrane filter (Millipore) were soaked in solutions of primary antibodies and then visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences).

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

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

In vitro phosphatase assay. To purify FAK, AGS cells were lysed in lysis buffer. Cell lysates were treated with anti-FAK antibody and immune complexes were trapped on protein A-Sepharose beads. To purify SHP-2, COS-7 cells were transfected with WT SHP-2-Myc expression vector, SHP-2 C459S-Myc expression vector or control empty vector, and were lysed in lysis buffer without Na_3VO_4. Cell lysates were treated with anti-Myc antibody, and immune complex were trapped on protein G-Sepharose beads. Immunoprecipitates were then washed with lysis buffer without Na_3VO_4 6 times and with assay buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl] 3 times. Beads were then mixed as indicated, and were incubated in assay buffer containing 2 mM dithiothreitol at 37°C. After 60 min incubation, enzyme reaction was terminated by addition of SDS-PAGE loading buffer. Reaction mixtures were subjected to SDS-PAGE, and then immunoblotted with indicated antibodies.

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

phosphorylation by CagA. Accordingly, we examined if ectopic SHP-2 is capable of altering the tyrosine phosphorylation level of FAK in cells. As shown in Fig. 4A, expression of a membrane-targeted, constitutively active SHP-2, Myr-SHP-2ΔSH2-Myc (21), resulted in a significant decrease in the level of tyrosine-phosphorylated FAK in AGS cells. The observation indicated that activated SHP-2 directly or indirectly decreases the level of FAK tyrosine phosphorylation. Accordingly, we decided to investigate the possibility that FAK is a direct target of SHP-2 phosphatase. It has been reported that introduction of mutations in the conserved amino-acid residues, Asp-425 and Cys-459, that are located in the catalytic center of the tyrosine phosphatase domain of SHP-2 stabilizes an SHP-2-substrate intermediate complex (1). Accordingly, we generated such a substrate-trapping mutant (DM SHP-2-Myc) and expressed it in AGS cells. Immunoprecipitation of DM SHP-2-Myc co-precipitated endogenous FAK much stronger than WT SHP-2 did (Fig. 4B). To rule out the possibility of interaction between SHP-2 and FAK other than enzyme-substrate interaction, we also examined a phosphatase-dead mutant of SHP-2 (R465M SHP-2) (25), which acts as a non-substrate-trapping SHP-2 mutant, and found that the interaction between FAK and R465M mutant was extremely weak (Fig. 4B). These findings indicated that SHP-2 forms an enzyme-substrate

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

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

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

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

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

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

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

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

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

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

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

Compartmentalization of active FAK at the tips of membrane protrusions in cells with the hummingbird phenotype. To further investigate the role of FAK in the morphogenetic activity of CagA, we examined subcellular localization of FAK in AGS cells expressing CagA (Fig. 8). In cells with the hummingbird phenotype, CagA was distributed throughout the cell membrane but was absent in the distal ends of the membrane protrusions. Anti-FAK staining showed that FAK was mostly localized to the cytoplasm but not the plasma membrane. Intriguingly, however, a fraction of FAK molecules were specifically enriched at the tips of the membrane protrusions. Staining of the cells with anti-FAK[pY^{576}], which recognizes the active form of FAK, revealed that active FAK molecules were present at the tips of the extensions. These observations indicate that active FAK molecules, which have escaped from CagA-activated SHP-2 and thus maintain their kinase activity, are specifically concentrated at the tips of the membrane protrusions in cells with the hummingbird phenotype.

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Focal adhesions are sites where integrin-mediated adhesion links the actin cytoskeleton. FAK localizes to focal adhesions via its C-terminal focal adhesion-targeting (FAT) domain. This FAT region contains binding sites for integrin-associated proteins such as paxillin and talin (39). Cell migration is not able to take place in the absence of focal adhesion turnover. Although FAK *per se* is not essential for the formation of focal adhesion complexes (24), studies in many laboratories have shown that FAK activation plays a crucial role in focal contact formation (13, 39). Recent studies have shown that FAK phosphorylates and activates the type I phosphatidylinositol phosphate kinase isoform-γ661 (PIPKIγ661), which is involved in the formation of focal adhesion sites (26). FAK also functions to promote the disassembly of focal contacts, in part by activating intracellular proteases such as calpain, promoting turnover of focal adhesions (10). Thus, the kinase enhances both assembly and disassembly of the complexes, and the two seemingly opposite functions may underlie

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

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

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

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FIGURE LEGENDS

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

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

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

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

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

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

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

FIG. 4. Dephosphorylation of FAK by SHP-2. (A) AGS cells were transfected with Myr-SHP-2ΔSH2-Myc expression vector or control empty vector. Cell lysates were prepared and immunoprecipitated with anti-FAK antibody or control IgG. Immunoprecipitates (IP, left, upper panel) and total cell lysates (left, lower panel) were immunoblotted (IB) with indicated

antibodies. Representative photographs from three separate experiments are indicated. Arrows indicate positions of FAK, pY-FAK and Myr-SHP-2ΔSH2-Myc. Quantitation expressed as the ratio of tyrosine phosphorylated FAK to total FAK from three separate experiments is summarized in the histogram on the right. Each value was calculated from the intensities of anti-pY and anti-FAK immunoblotting by using a lumino-image analyzer and defining the value in the absence of Myr-SHP-2ΔSH2-Myc as 1. Error bars indicate 2x SD. (B) AGS cells were transfected with 30 μg of Myc-tagged WT SHP-2 (WT SHP-2-Myc) expression vector, substrate-trapping mutant of SHP-2 (DM SHP-2-Myc) expression vector, non-substrate trapping mutant of SHP-2 (R465M SHP-2-Myc) expression vector or control empty vector. Total cell lysates were immunoprecipitated with anti-Myc antibody. Immunoprecipitates and total cell lysates were immunoblotted with anti-FAK or anti-Myc antibody. Arrows indicate positions of FAK, and SHP-2-Myc. (C) AGS cells were transfected with 5 μg of DM SHP-2-Myc expression vector or control empty vector together with 25 μg of WT CagA-HA expression vector or control vector. Cell lysates prepared were immunoprecipitated with anti-Myc antibody. Immunoprecipitates and total cell lysates were immunoblotted with anti-FAK, anti-Myc, anti-HA or anti-phosphotyrosine (anti-pY)

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

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

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

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

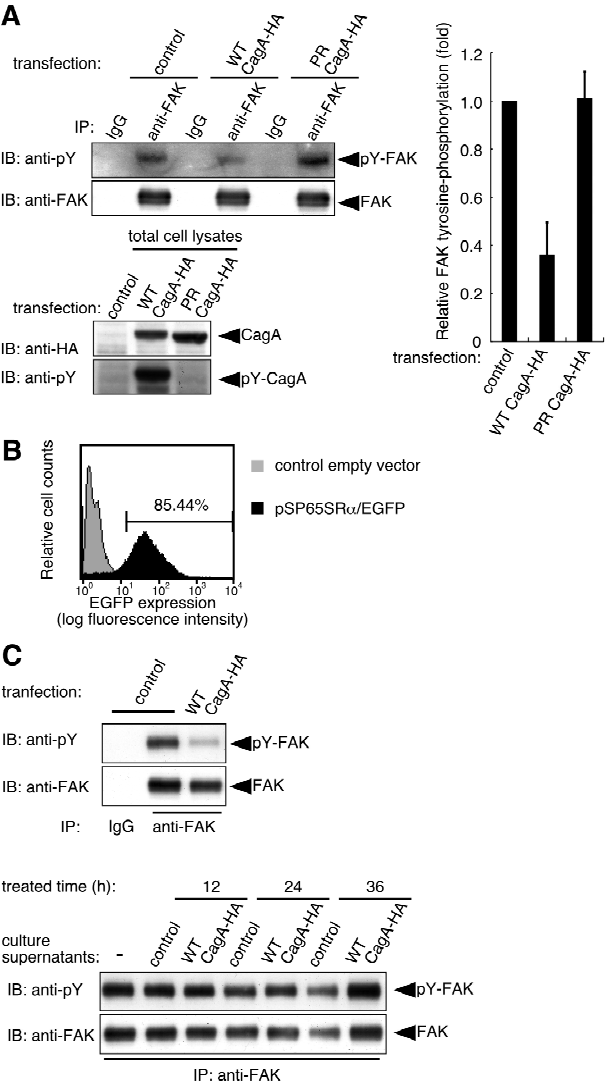
FIG. 6. FAK inhibition is involved in the induction of the hummingbird phenotype. (A) AGS cells were transfected with WT CagA-HA expression vector, FAK K578E/K581E-Flag expression vector and control empty vector in various combinations indicated. Cell morphology was examined 36 h after transfection by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

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

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

FIG. 8. Accumulation of FAK at the tips of protrusions in cells with the hummingbird phenotype. AGS cells transfected with WT CagA-Flag expression vector (B, C, and D), WT CagA-HA expression vector (F, G, H and I) or control empty vector (A and E) were stained with anti-Flag (green) (C, and D), anti-FAK (red) (A, B and D), anti-HA (red) (F, G, H and I) or anti-FAK[pY^{576}] (green) (E, F, G, H and I). Arrows indicate distal ends of membrane protrusions in cells with the hummingbird phenotype. Scale bars: 50 μm.

FIG. 1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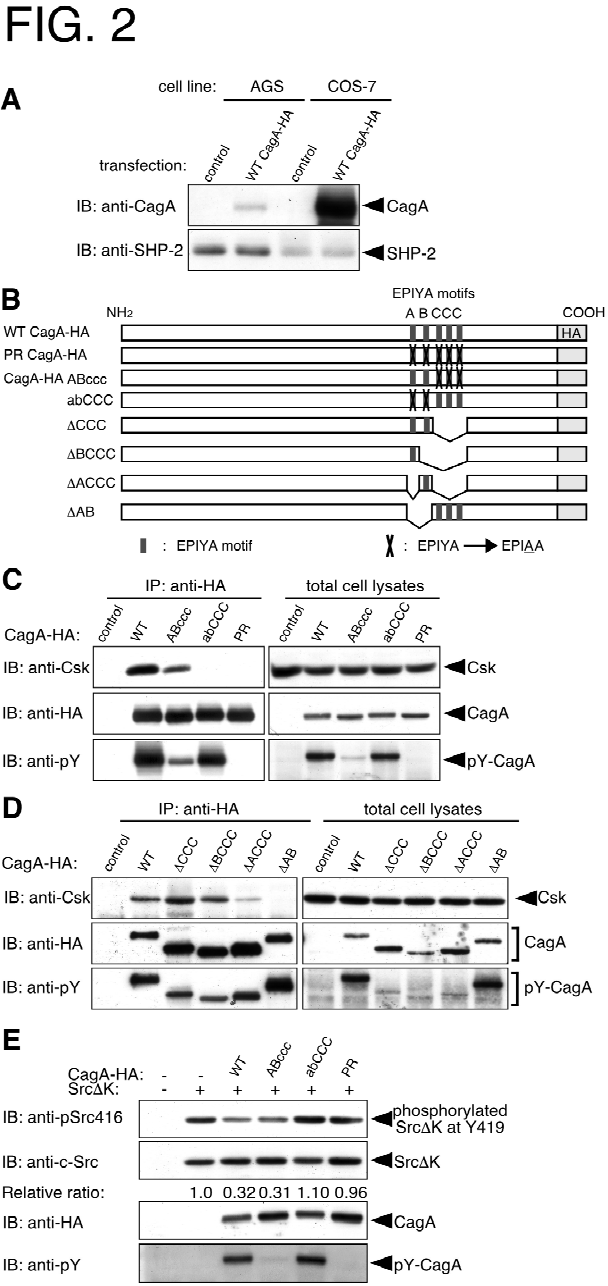
IP:
IgG
anti-FAK
IB: anti-pY
IB: anti-FAK
pY-FAK
FAK
total cell lysates
CagA
pY-CagA
IB: anti-HA
Relative FAK tyrosine-phosphorylation (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
B
Relative cell counts
85.44%
control empty vector
pSP65SRα/EGFP
EGFP expression
(log fluorescence intensity)
C
tranfection:
treated time (h):
12
24
36
culture supernatants:
-

FIG. 2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COS-7
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CagA
IB: anti-SHP-2
SHP-2
B
NH2
EPIYA motifs
A B CCC
COOH
WT CagA-HA
PR CagA-HA
CagA-HA ABccc
abCCC
ΔCCC
ΔBCCC
ΔACCC
ΔAB
HA
: EPIYA motif
: EPIYA → EPIAA
C
IP: anti-HA
total cell lysates
CagA-HA:
control
WT
ABccc
abCCC
PR
IB: anti-Csk
IB: anti-HA
IB: anti-pY
Csk
CagA
pY-CagA
D
IP: anti-HA
total cell lysates
CagA-HA:
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WT
ΔCCC
ΔBCCC
ΔACCC
ΔAB
IB: anti-Csk
IB: anti-HA
IB: anti-pY
Csk
CagA
pY-CagA
E
CagA-HA:
SrcΔK:
WT
ABccc
abCCC
PR
IB: anti-pSrc416
phosphorylated SrcΔK at Y419
IB: anti-c-Src
SrcΔK
Relative ratio:
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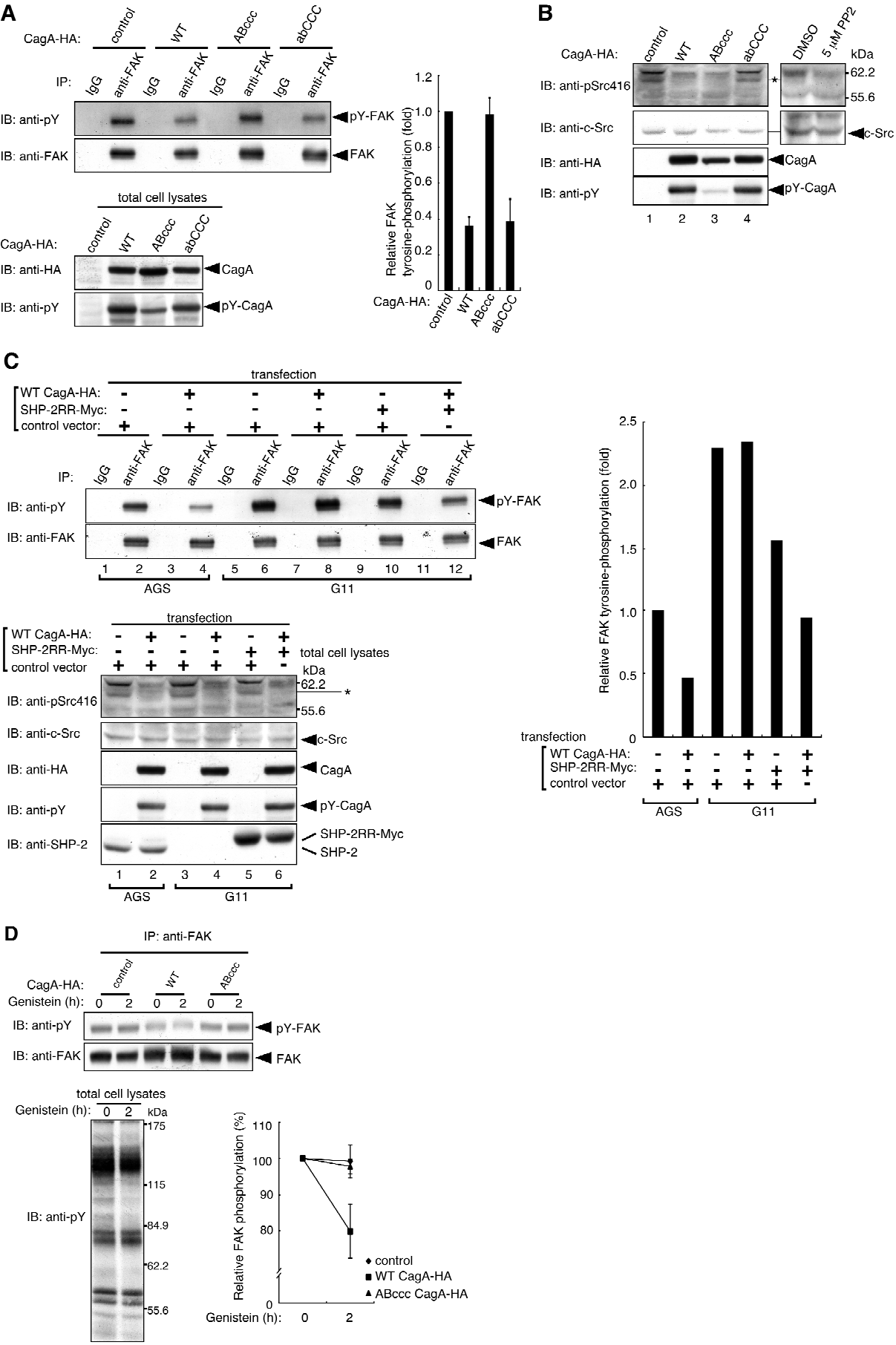
IB: anti-HA
CagA
IB: anti-pY
pY-CagA

FIG. 3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IP:
IgG
anti-FAK
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pY-FAK
IB: anti-FAK
FAK
total cell lysates
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
Relative FAK tyrosine-phosphorylation (fold)
B
DMSO
5 μM PP2
kDa
62.2
55.6
IB: anti-pSrc416
IB: anti-c-Src
c-Src
1 2 3 4
C
transfection
WT CagA-HA:
SHP-2RR-Myc:
control vector:
AGS
G11
IB: anti-SHP-2
SHP-2RR-Myc
SHP-2
Relative FAK tyrosine-phosphorylation (fold)
D
IP: anti-FAK
Genistein (h):
total cell lysates
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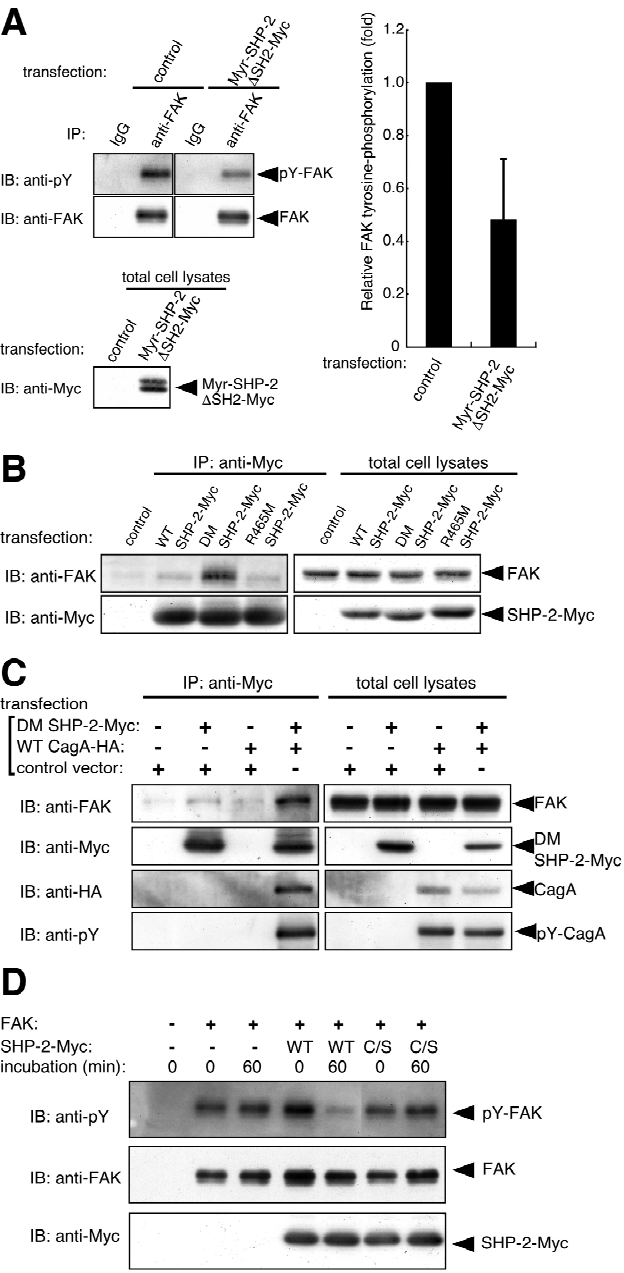
115
84.9
62.2
55.6
Relative FAK phosphorylation (%)
control
WT CagA-HA
ABccc CagA-HA

FIG. 4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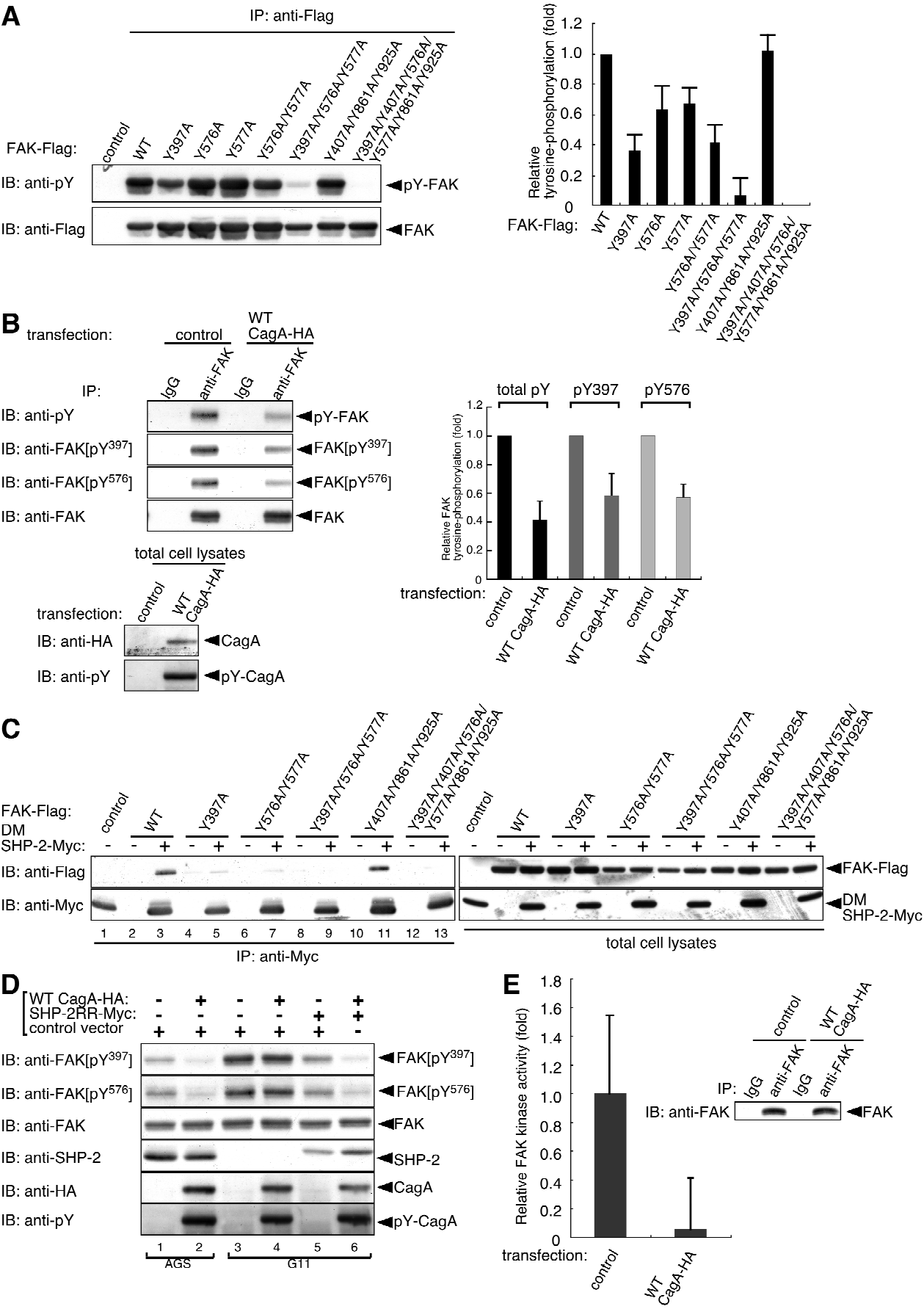
Myr-SHP-2 ΔSH2-Myc
IP:
IgG
anti-FAK
IgG
anti-FAK
IB: anti-pY
pY-FAK
IB: anti-FAK
FAK
total cell lysates
transfection:
control
Myr-SHP-2 ΔSH2-Myc
IB: anti-Myc
Myr-SHP-2 ΔSH2-Myc
Relative FAK tyrosine-phosphorylation (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
transfection:
control
Myr-SHP-2 ΔSH2-Myc
B
IP: anti-Myc
total cell lysates
transfection:
control
WT SHP-2-Myc
DM SHP-2-Myc
R465M SHP-2-Myc
control
WT SHP-2-Myc
DM SHP-2-Myc
R465M SHP-2-Myc
IB: anti-FAK
FAK
IB: anti-Myc
SHP-2-Myc
C
IP: anti-Myc
total cell lysates
transfection
DM SHP-2-Myc: - + - + - + - +
WT CagA-HA: - - + + - - + +
control vector: + + + - + + + -
IB: anti-FAK
FAK
IB: anti-Myc
DM SHP-2-Myc
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
D
FAK: - + + + + + +
SHP-2-Myc: - - - WT WT C/S C/S
incubation (min): 0 0 60 0 60 0 60
IB: anti-pY
pY-FAK
IB: anti-FAK
FAK
IB: anti-Myc
SHP-2-Myc

FIG. 5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FAK-Flag:
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WT
Y397A
Y576A
Y577A
Y576A/Y577A
Y397A/Y576A/Y577A
Y407A/Y861A/Y925A
Y397A/Y407A/Y576A/Y577A/Y861A/Y925A
IB: anti-pY
pY-FAK
IB: anti-Flag
FAK
Relative tyrosine-phosphorylation (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
FAK-Flag:
WT
Y397A
Y576A
Y577A
Y576A/Y577A
Y397A/Y576A/Y577A
Y407A/Y861A/Y925A
Y397A/Y407A/Y576A/Y577A/Y861A/Y925A
B
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IP:
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anti-FAK
IgG
anti-FAK
IB: anti-pY
pY-FAK
IB: anti-FAK[pY397]
FAK[pY397]
IB: anti-FAK[pY576]
FAK[pY576]
IB: anti-FAK
FAK
total cell lysates
transfection:
control
WT CagA-HA
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
total pY
pY397
pY576
Relative FAK tyrosine-phosphorylation (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
transfection:
control
WT CagA-HA
control
WT CagA-HA
control
WT CagA-HA
C
FAK-Flag:
DM
SHP-2-Myc:
control
WT
Y397A
Y576A/Y577A
Y397A/Y576A/Y577A
Y407A/Y861A/Y925A
Y397A/Y407A/Y576A/Y577A/Y861A/Y925A
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Y576A/Y577A
Y397A/Y576A/Y577A
Y407A/Y861A/Y925A
Y397A/Y407A/Y576A/Y577A/Y861A/Y925A
IB: anti-Flag
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IB: anti-Myc
DM SHP-2-Myc
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-Myc
total cell lysates
D
WT CagA-HA:
SHP-2RR-Myc:
control vector
IB: anti-FAK[pY397]
FAK[pY397]
IB: anti-FAK[pY576]
FAK[pY576]
IB: anti-FAK
FAK
IB: anti-SHP-2
SHP-2
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
1 2 3 4 5 6
AGS
G11
E
Relative FAK kinase activity (fold)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
transfection:
control
WT CagA-HA
control
WT CagA-HA
IP:
IgG
anti-FAK
IgG
anti-FAK
IB: anti-FAK
FAK

FIG. 6

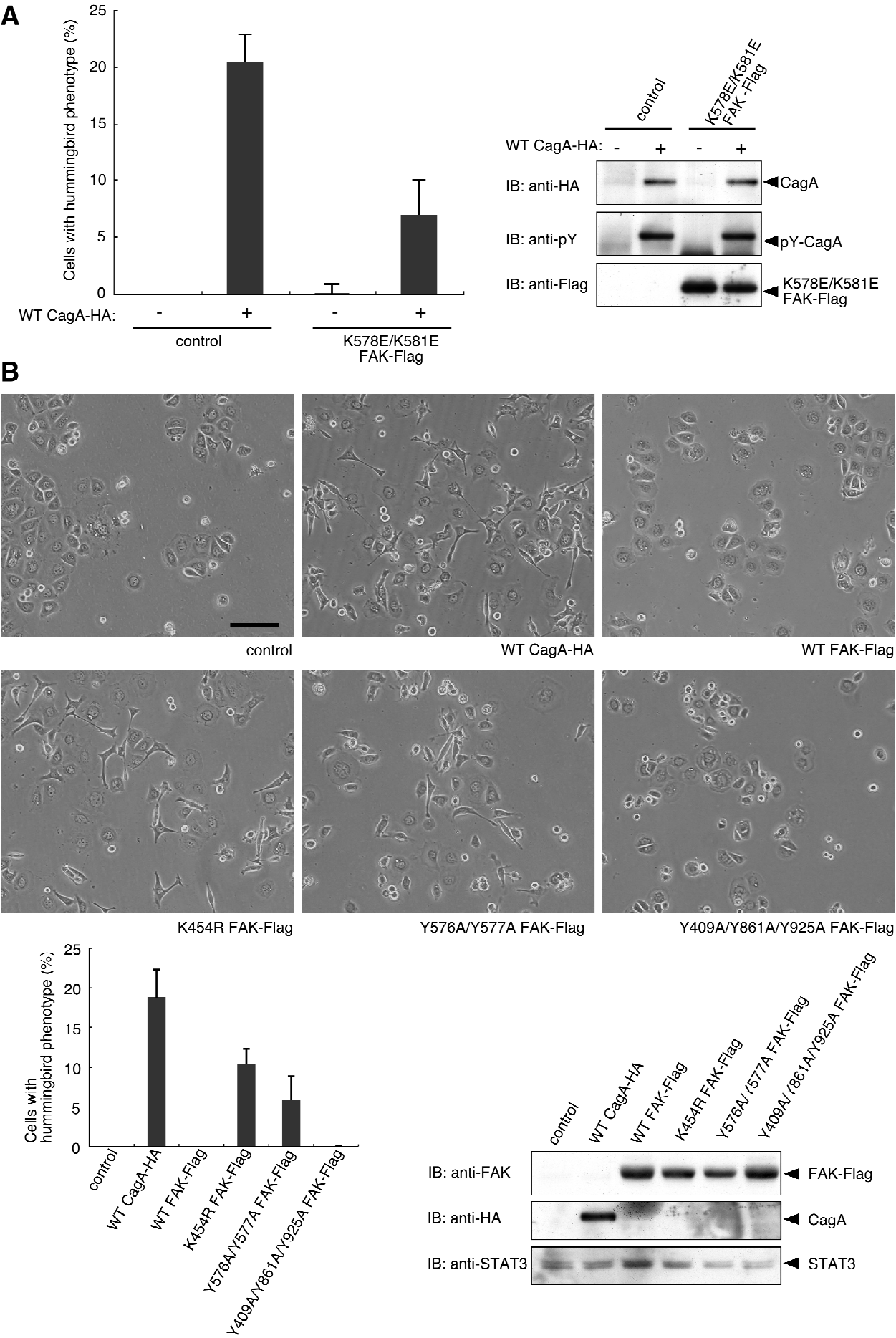

A
Cells with hummingbird phenotype (%)
25
20
15
10
5
0
WT CagA-HA: - + - +
control
K578E/K581E FAK-Flag
control
K578E/K581E FAK-Flag
WT CagA-HA: - + - +
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
IB: anti-Flag
K578E/K581E FAK-Flag
B
control
WT CagA-HA
WT FAK-Flag
K454R FAK-Flag
Y576A/Y577A FAK-Flag
Y409A/Y861A/Y925A FAK-Flag
Cells with hummingbird phenotype (%)
25
20
15
10
5
0
control
WT CagA-HA
WT FAK-Flag
K454R FAK-Flag
Y576A/Y577A FAK-Flag
Y409A/Y861A/Y925A FAK-Flag
control
WT CagA-HA
WT FAK-Flag
K454R FAK-Flag
Y576A/Y577A FAK-Flag
Y409A/Y861A/Y925A FAK-Flag
IB: anti-FAK
FAK-Flag
IB: anti-HA
CagA
IB: anti-STAT3
STAT3

FIG. 7

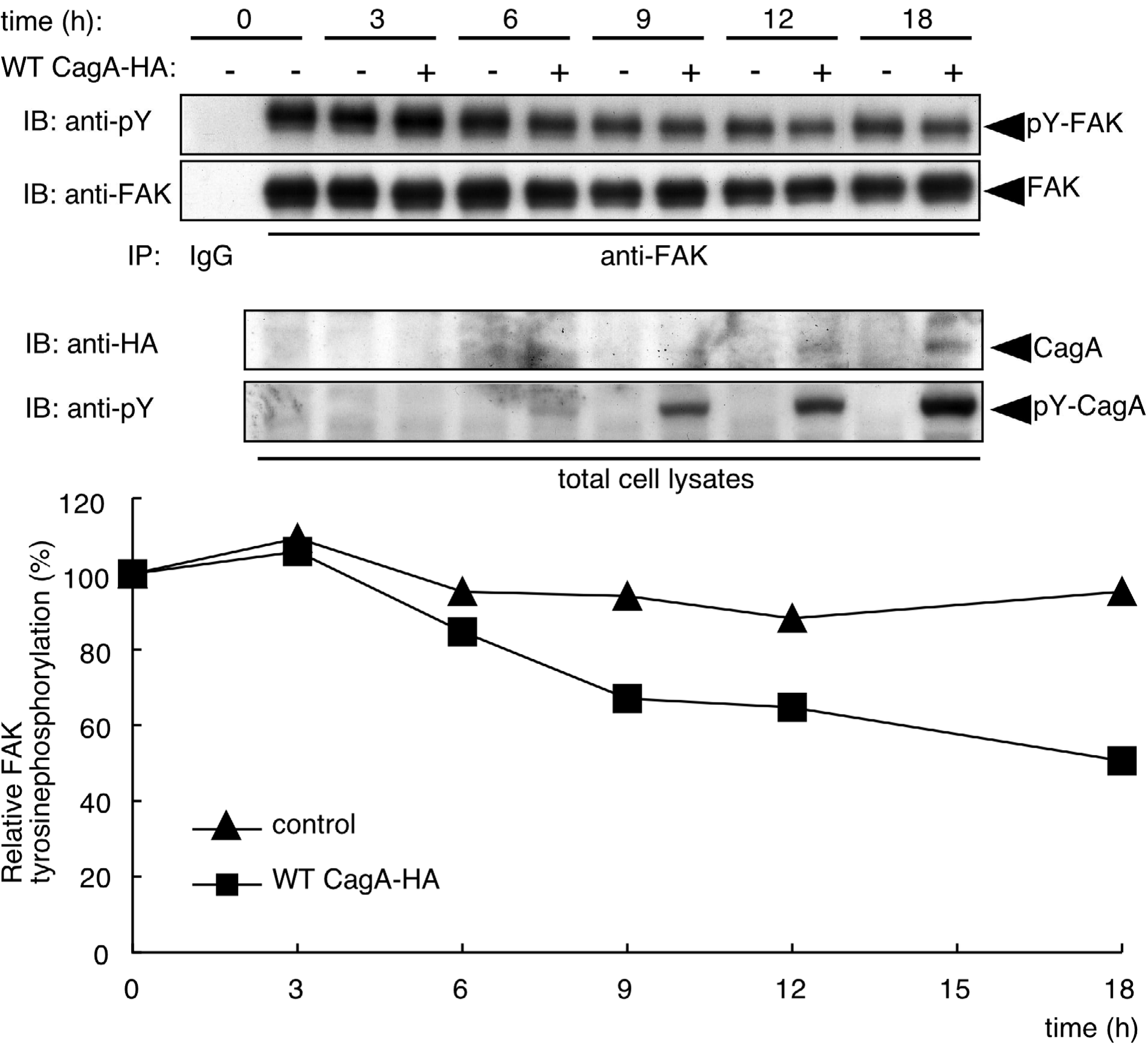
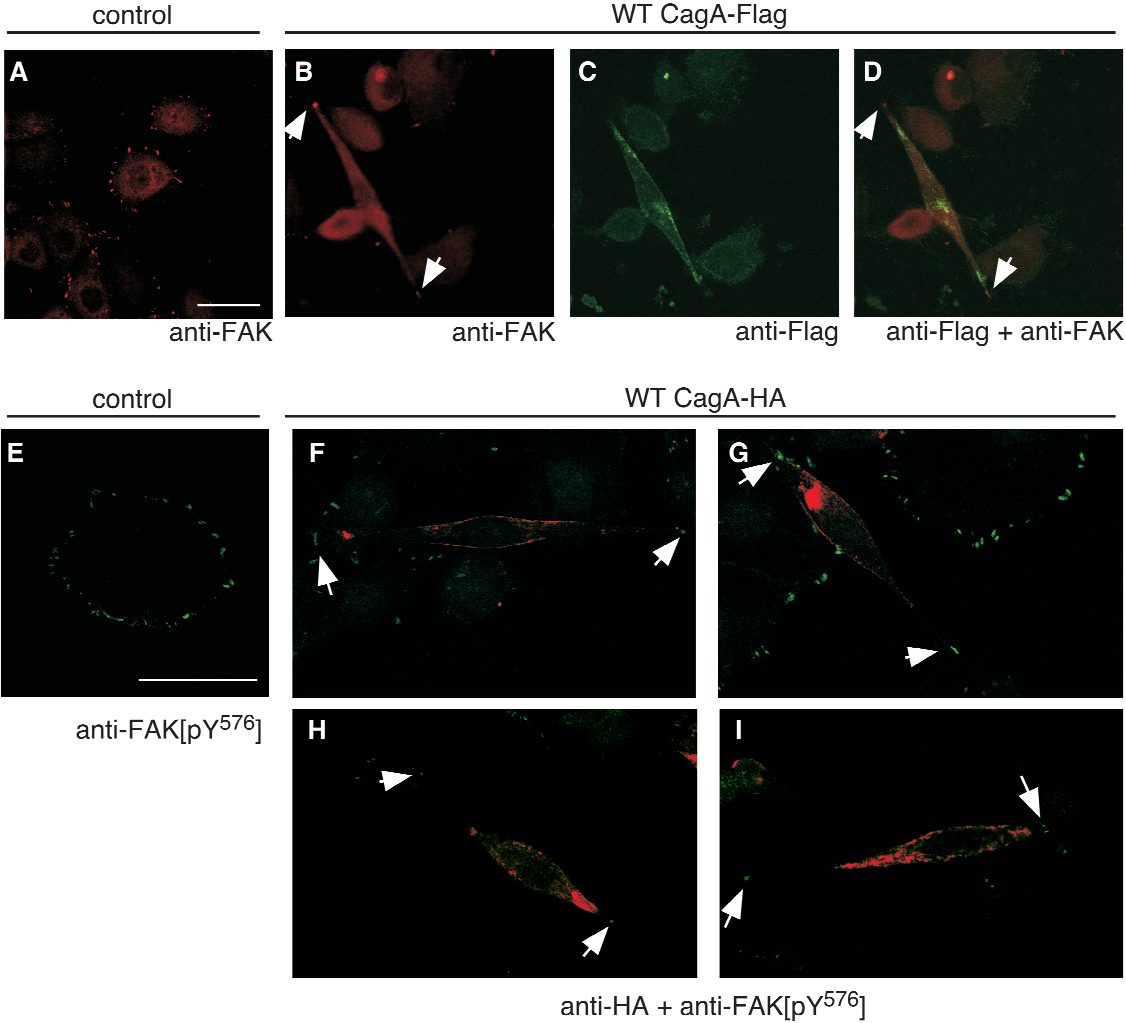

time (h):
0
3
6
9
12
18
WT CagA-HA:
- - - + - + - + - + - +
IB: anti-pY
pY-FAK
IB: anti-FAK
FAK
IP:
IgG
anti-FAK
IB: anti-HA
CagA
IB: anti-pY
pY-CagA
total cell lysates
Relative FAK tyrosinephosphorylation (%)
120
100
80
60
40
20
0
control
WT CagA-HA
0
3
6
9
12
15
18
time (h)

FIG. 8


control
WT CagA-Flag
A
B
C
D
anti-FAK
anti-FAK
anti-Flag
anti-Flag + anti-FAK
control
WT CagA-HA
E
F
G
H
I
anti-FAK[pY576]
anti-HA + anti-FAK[pY576]