Mammalian Pragmin regulates Src family kinases via the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif that is exploited by bacterial effectors

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Several pathogenic bacteria have adopted effector proteins that, upon delivery into mammalian cells, undergo tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) or EPIYA-like sequence motif by host kinases such as Src family kinases (SFKs). This EPIYA phosphorylation triggers complex formation of bacterial effectors with SH2 domain-containing proteins that results in perturbation of host cell signaling and subsequent pathogenesis. Although the presence of such an anomalous protein interaction suggests the existence of a mammalian EPIYA-containing protein whose function is mimicked or subverted by bacterial EPIYA effectors, no molecule that uses the EPIYA motif for biological function has so far been reported in mammals. Here we show that mammalian Pragmin/SgK223 undergoes tyrosine phosphorylation at the EPIYA motif by SFKs and thereby acquires the ability to interact with the SH2 domain of the C-terminal Src kinase (Csk), a negative regulator of SFKs. The Pragmin–Csk interaction prevents translocation of Csk from the cytoplasm to the membrane and subsequent inactivation of membrane-associated SFKs. As a result, SFK activity is sustained in cells where Pragmin is phosphorylated at the EPIYA motif. Because EPIYA phosphorylation of Pragmin is mediated by SFKs, cytoplasmic sequestration of Csk by Pragmin establishes a positive feedback regulation of SFK activation. Remarkably, the Helicobacter pylori EPIYA effector CagA binds to the Csk SH2 domain in place of Pragmin and enforces membrane recruitment of Csk and subsequent inhibition of SFKs. This work identifies Pragmin as a mammalian EPIYA effector and suggests that bacterial EPIYA effectors target Pragmin to subvert SFKs for successful infection.

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Results

Exploration of Mammalian EPIYA-Containing Proteins. To seek mammalian EPIYA effectors, we focused on *H. pylori* CagA, an archetypical bacterial EPIYA effector (Fig. S1) (3, 7). CagA contains variable numbers of EPIYA motifs in its C-terminal region. On the basis of their flanking sequences, four distinct EPIYA segments (EPIYA-A, -B, -C, and -D segments), each of which contains a single EPIYA motif (EPIYA-A, -B, -C, and -D motifs), have been described (7). Upon tyrosine phosphorylation, EPIYA-A or -B motif serves as a docking site for Csk, whereas EPIYA-C or -D motif serves as a binding site for SHP2. Because *H. pylori* has been associated with humans for more than 58,000 y (17), incorporation of multiple EPIYA motifs into CagA may have given a selective advantage to *H. pylori* in adapting in the human stomach during a long period of coexistence. This in turn raises the idea that human cells possess a protein(s) with a functional EPIYA motif, which is used or evaded by *H. pylori*. A search of the human proteome with the National Center for Biotechnology Information BLAST program led to the identification of only six proteins that have a perfect EPIYA sequence (Table S1). These candidates contained Pragmin (also known as SgK223), a cytoplasmic pseudokinase originally isolated as a downstream effector of Rnd2, a Rho family GTPase predominantly expressed in neurons (18). Rnd2-associated Pragmin stimulates RhoA activity and thereby induces cell contraction. However, expression of Pragmin is not limited to neuronal cells, indicating that the protein has a more general role. Also notably, the EPIYA sequence is perfectly conserved among mammalian Pragmin orthologs, indicating that it has an important role in the function of Pragmin. In addition to the EPIYA motif, Pragmin possesses a pseudokinase domain, which does not seem to have intrinsic kinase activity, in the C-terminal region, although the function remains unknown (Fig. S2). We hypothesized that mammalian EPIYA effectors, if they exist, should have a scaffold/adopter function involved in intracellular signal transduction. Because many pseudokinases have been known to act as scaffolds or adaptors in the cells (19), we chose Pragmin for further analysis in this study.

Tyrosine Phosphorylation of the Pragmin EPIYA Motif. To elucidate the function of the Pragmin EPIYA motif, we sought to construct a mammalian expression vector for Pragmin. Because of the technical difficulty in cloning a human Pragmin cDNA into plasmid vectors, we used a pCMV-based mammalian expression vector for Myc-epitope-tagged wild-type rat Pragmin (Myc-Pragmin) (18). The rat and human Pragmins show 76% and 79% identities at the amino acid and nucleotide levels, respectively. A cDNA encoding a rat Pragmin mutant (Pragmin-Y391F) having a tyrosine-to-phenylalanine substitution at residue 391 in the EPIYA motif, which corresponds to Y411 in human Pragmin, was also generated and, after Myc-epitope tagging, was cloned into the pCMV vector (Fig. S2). The Pragmin expression vectors were transiently transfected into AGS human gastric epithelial cells. In cells, both ectopically expressed Pragmin and Pragmin-Y391F were localized to the cytoplasm, indicating that the Y391F substitution did not influence the intracellular distribution of Pragmin (Fig. 14).

We first wished to know whether Pragmin is tyrosine-phosphorylated at the EPIYA motif. In AGS cells, Myc-Pragmin was efficiently tyrosine-phosphorylated, whereas Myc-Pragmin-Y391F was much less phosphorylated, as determined by anti-phosphotyrosine immunoblotting (Fig. 1B). Hence, Pragmin undergoes tyrosine phosphorylation by endogenous kinases, and the EPIYA motif is the major if not the only site of Pragmin phosphorylation. In this regard, mammalian Pragmins contain more than 20 tyrosine residues in addition to one in the EPIYA motif. Some of those tyrosines may also undergo tyrosine phosphorylation, although to a much lesser extent than that at the EPIYA site. Next, we sought to identify tyrosine kinases that mediate Pragmin phosphorylation. Because *H. pylori* CagA is tyrosine-phosphorylated at EPIYA motifs by host SFKs (4, 7), we treated AGS cells expressing Myc-Pragmin with PP2, a specific inhibitor of SFKs, for 4 h before harvest and found that the PP2 treatment abolished tyrosine phosphorylation of Myc-Pragmin (Fig. 1C). Conversely, coexpression of c-Src markedly increased the level of Myc-Pragmin tyrosine phosphorylation (Fig. 1D). These results indicated that SFKs, which are constitutively activated in AGS cells, mediate EPIYA phosphorylation of Pragmin in the cells.

To investigate the physiological relevance for the tyrosine phosphorylation of Pragmin, we next tested whether the phosphorylation status of Pragmin is modified in response to external stimuli. To this end, AGS cells transiently transfected with the Myc-Pragmin or Myc-Pragmin-Y391F vector were cultured in the absence of serum for 24 h. The serum starvation substantially reduced the level of Pragmin tyrosine phosphorylation (Fig. 1E), indicating that decreased tyrosine kinase activity in serum-starved cells resulted in reduced tyrosine phosphorylation of Pragmin. Serum-starved AGS cells were then stimulated with EGF for 6 h. EGF treatment potently increased the level of tyrosine phosphorylation of Myc-Pragmin (Fig. 1E). In contrast, Myc-Pragmin-Y391F was hardly tyrosine-phosphorylated in serum-starved AGS cells before and after EGF treatment. Of note, EGF-dependent

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Tyrosine phosphorylation of Pragmin at the EPIYA motif. (A) AGS cells were transfected with a Myc-tagged wild-type Pragmin (Myc-Pragmin) or Myc-tagged Pragmin-Y391F (Myc-Pragmin-Y391F) vector and were stained with an anti-Myc antibody (green). Nuclei are visualized by DAPI staining (blue). (Scale bar, 10 μm.) (B) AGS cells were transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector. Total cell lysates (TCLS) were immunoprecipitated with an anti-Myc antibody, and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. Anti-pY, anti-phosphotyrosine antibody. (C) AGS cells transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector were treated with or without 10 μM PP2, a SFK inhibitor. Total cell lysates were subjected to immunoblotting with the indicated antibodies. (D) Lysates of AGS cells transfected with a Myc-Pragmin and/or c-Src vector were subjected to immunoblotting with the indicated antibodies. (E) AGS cells transiently transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector were treated with 100 ng/mL EGF for 6 h in the presence or absence of 100 nM PP2. Cell lysates were subjected to immunoblotting with the indicated antibodies.
tyrosine phosphorylation of Myc-Pragmin was not inhibited by treatment of cells with 100 nM PP2, the concentration of which almost completely suppressed SFK activity in cells (Fig. 1E and Fig. S3) (20). This observation indicated that the ligand-activated EGF receptor itself or a non-SFK kinase(s) lying downstream of the EGF receptor also phosphorylates the EPIYA motif of Pragmin.

**Pragmin Specifically Binds to Csk in an EPIYA Phosphorylation-Dependent Manner.** The above-described observations prompted us to search for a protein that can specifically interact with the Pragmin EPIYA motif in a tyrosine phosphorylation-dependent manner. During the course of this study, we noticed that the sequence distal to the Pragmin EPIYA motif (EPIYAESAK) is similar to that located downstream of the CagA EPIYA-B motif (EPIYAOVAK), to which the Csk SH2 domain binds in an EPIYA phosphorylation-dependent manner (6). We thus transiently transfected a FLAG-tagged Csk (Csk-FLAG) vector together with a Myc-Pragmin or HA-tagged CagA (CagA-HA) vector into AGS cells and found that Csk immunoprecipitation coprecipitated Myc-Pragmin or CagA-HA (Fig. 2A, Left). Reciprocally, Myc-Pragmin immunoprecipitation coprecipitated Csk-FLAG as well as endogenous Csk (Fig. 2A, Right). On the other hand, Myc-Pragmin-Y391F immunoprecipitation failed to coprecipitate Csk (Fig. 2A). Inactivation of the SH2 domain of Csk by a point mutation (S109C) also abolished the ability of Csk to coprecipitate Pragmin (Fig. 2B). Given these, we next focused on endogenous Pragmin and found that it was tyrosine-phosphorylated in two human gastric epithelial cell lines, AGS and MKN28 (Fig. 2C). Interaction of endogenous Csk with endogenous Pragmin was confirmed by reciprocal communiprecipitation using an anti-Csk or anti-Pragmin antibody in both AGS and MKN28 cells (Fig. 2D). The Pragmin-Csk complex was also detected in a normal human gastric epithelial cell line, GES-1 (Fig. 2D). These results indicated that Pragmin associates with Csk via the tyrosine-phosphorylated EPIYA motif of Pragmin and the SH2 domain of Csk.

To investigate whether the Pragmin EPIYA motif can display promiscuous specificity for multiple SH2 domains like bacterial EPIYA motif, we transiently coexpressed Pragmin and one of the SH2-containing proteins (SHP1, Grb7, c-Abl, CrkII, Grb2, and PI3-kinase p85 subunit), which were reported to interact with the EPIYA motifs of CagA (16), in AGS cells and examined their interaction with Pragmin through a sequential immunoprecipitation and immunoblotting technique. The results of the experiment revealed that Pragmin bound none of these SH2 domain-containing proteins (Fig. S4), indicating that the tyrosine-phosphorylated EPIYA motif of Pragmin is highly specific to the SH2 domain of Csk. It has been reported that SH2-binding specificity is primarily determined by three to five residues C-terminal to the phosphotyrosine (15). Considering the similarity of residues C-terminal to the EPIYA motif of Pragmin (EPIYAESAK) with that of the CagA EPIYA-B motif (EPIYAOVAK), degenerated SH2-binding specificity of the CagA EPIYA-B motif may be due to the difference in residues at +2 and +3 positions from the phosphotyrosine.

**Elevated SFK Activity in Cells Expressing Pragmin.** Csk inactivates SFKs by phosphorylating a consensus tyrosine residue near the C terminus (Y530 and Y527 in human and chicken c-Src, respectively) (21). Upon tyrosine phosphorylation, Y530 engages in intramolecular interaction that locks SFKs in an inactive conformation. To elucidate the biological consequences of Pragmin-Csk interaction, we investigated the activation status of SFKs in cells with or without Pragmin expression. To this end, we used an anti-Phospho-Src (Y530) antibody, which recognizes an inactive form of SFKs, and an anti-Phospho-Src (Y416) antibody, which reacts with an active form of SFKs. In AGS cells expressing Myc-Pragmin, the level of Y530-phosphorylated c-Src was decreased, whereas the level of active c-Src was increased (Fig. 3). Such changes in the level of c-Src phosphorylation were not observed in AGS cells expressing Myc-Pragmin-Y391F. Conversely, inhibition of endogenous Pragmin expression in AGS cells by specific siRNA reduced the level of active c-Src. Taken together, these results
indicated that Pragmin potentiates kinase activity of SFKs upon complex formation with Csk (Fig. 3B).

In addition to SFK activation, cells expressing Myc-Pragmin, but not Myc-Pragmin-Y391F, displayed a morphological change, which was characterized by a polygonal cell shape with multiple protrusions (Fig. 3C and Fig. S5A). The morphogenetic activity of Pragmin was inhibited by treatment of cells with PP2. Similar morphological change was also induced by ectopic expression of c-Src in AGS cells, and inhibition of Pragmin expression by specific siRNA attenuated the c-Src-mediated morphological change (Fig. 3D and Fig. S5B). The overall levels of protein tyrosine phosphorylation in cells expressing c-Src were also reduced after inhibition of Pragmin expression (Fig. 3E). These results collectively indicated that Pragmin provokes cell morphological transformation by potentiating SFK kinase activity.

**Colocalization of Pragmin and Csk in the Cytoplasm.** Csk is localized predominantly in the cytoplasm because it lacks fatty acid acylation domain for anchoring to the plasma membrane (21, 22). Given that all SFKs are anchored to the inner plasma membrane via myristoylation/palmitoylation, Csk needs to translocate from the cytoplasm to the membrane to inhibit SFKs. Membrane-recruitment of Csk is in most cases mediated via interaction with membrane-associated proteins such as Cbp (Csk-binding protein)/PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) and caveolin-1 via the SH2, SH3, and/or kinase domain of Csk (22). We therefore wished to know whether subcellular localization of Csk is altered upon complex formation with Pragmin. First, as a control experiment, we expressed Csk in AGS cells alone or together with CagA, which has been shown to interact with Csk and thereby recruit it to the plasma membrane (6). In the absence of CagA, Csk was broadly distributed to the cytoplasm (Fig. 4A). In the presence of CagA, however, Csk was translocated to the membrane, where it was colocalized with CagA (Fig. 4B). We then examined the effect of Pragmin on Csk localization. In AGS cells, both Csk and Pragmin were localized in the cytoplasm when singly expressed, and coexpression of Pragmin and Csk in the same cell did not alter cytoplasmic localization of Csk (Fig. 4C). These observations indicate that, in contrast to the CagA–Csk complex, the Pragmin–Csk complex keeps Csk in the cytoplasm, preventing SH2 domain-mediated interaction of Csk with other molecules.

**H. pylori CagA Inhibits the Pragmin-Csk Interaction.** The finding that Pragmin specifically binds to the SH2 domain of Csk in a tyrosine phosphorylation-dependent manner prompted us to investigate the effect of *H. pylori* CagA, which also binds to the Csk SH2 domain upon EPIYA phosphorylation, on the Pragmin–Csk

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**Fig. 3.** Activation of c-Src in cells expressing Pragmin. (A) AGS cells were transfected a Myc-Pragmin, Myc-Pragmin-Y391F, or CagA-HA vector. Cell lysates prepared were subjected to immunoblotting with the indicated antibodies: anti-pSrc(416), anti-Phospho-Src (Y416) antibody; anti-pSrc(530), anti-Phospho-Src (Y530) antibody. (B) AGS cells were transfected with Pragmin-specific siRNA. Cell lysates prepared were immunoblotted with the indicated antibodies. (C) AGS cells were transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector. Morphology of transfected cells was analyzed by microscope. (Scale bars, 10 μm.) (D) AGS cells treated with or without the Pragmin-specific siRNA were transfected with a c-Src vector. Cells were subjected to morphological investigation using microscopy. (Scale bars, 10 μm.) (E) AGS cells treated with or without the Pragmin-specific siRNA were transfected with a c-Src vector. Cell lysates were subjected to immunoblotting with the indicated antibodies. Anti-pY, anti-phosphotyrosine antibody.

**Fig. 4.** Subcellular localization of Pragmin-Csk complex. (A) AGS cells transfected with a Csk-FLAG vector were stained with an anti-FLAG antibody. (B) AGS cells cotransfected with Csk-FLAG and CagA-HA vectors were double-stained with anti-FLAG (Csk; green) and anti-HA (CagA; red) antibodies. (C) AGS cells cotransfected with Csk-FLAG and Myc-Pragmin vectors were double-stained with anti-FLAG (Csk; green) and anti-Myc (Pragmin; red) antibodies. Nuclei (blue) were visualized by DAPI staining. (Scale bars, 10 μm.)
complex. To this end, AGS cells were transiently cotransfected with Pragmin and Csk vectors in the presence or absence of a CagA vector, and the amount of the Pragmin–Csk complex formed in cells was examined. As a result, expression of CagA not only led to generation of the CagA–Csk complex but also substantially reduced the level of the Pragmin–Csk complex (Fig. 5A). In this regard, Pragmin has been reported to activate RhoA GTPases (18). Additionally, H. pylori CagA is capable of stimulating RhoA (23). However, inhibition of RhoA activity by Clostridial C3 transferase did not influence the level of the Pragmin–Csk complex in cells, arguing against an active role of RhoA in the Pragmin–Csk interaction (Fig. S6). To further elucidate the pathophysiological relevance of the above-described observations, we next performed an H. pylori infection experiment in AGS cells and found that infection with the H. pylori cagA-positive strain, but not that with the isogenic cagA-negative strain, reduced the level of the endogenous Pragmin–Csk complex, which was concomitantly associated with the appearance of the CagA–Csk complex (Fig. 5B). From these observations, we concluded that H. pylori CagA inhibits Pragmin–Csk interaction while simultaneously forming a complex with Csk. Because both H. pylori CagA and Pragmin commonly bind to the same SH2 domain of Csk upon EPIYA-tyrosine phosphorylation, it was thought that direct competition between CagA and Pragmin for Csk binding underlies the CagA-mediated inhibition of Pragmin–Csk complex formation.

Discussion

We demonstrated in this work that mammalian Pragmin undergoes tyrosine phosphorylation at the EPIYA motif by SFKs or in response to EGF stimulation. Upon EPIYA phosphorylation, Pragmin acquires the ability to interact with the SH2 domain of Csk. The Pragmin–Csk interaction is inhibited by H. pylori CagA, which also binds to the Csk SH2 domain in an EPIYA-dependent manner. Consistent with our present work, a comprehensive proteomic study by Zhang et al. (24) also revealed human Pragmin to be one of 76 proteins that are tyrosine-phosphorylated in response to EGFR stimulation. They further confirmed by mass spectrometry analysis that Pragmin was tyrosine-phosphorylated on Y411 at the EPIYA motif, which corresponds to Y391 in rat Pragmin.

The EPIYA motif is evolutionarily conserved throughout mammalian Pragmin orthologs and is also present in Xenopus Pragmin, indicating that the sequence plays an important role in the function of Pragmin. Now, the present work revealed a hitherto unidentified role of Pragmin in the regulation of SFKs, composed of nine members of Src-related nonreceptor tyrosine kinases that are membrane-tethered via lipid modification. Csk phosphorylates the C-terminal inhibitory tyrosine residue of SFKs and thereby inactivates their kinase activity. To accomplish this task, Csk must be translocated from the cytoplasm to the membrane, a process mediated by various membrane-associated proteins such as Cbp/PAG and caveolin-1 that, upon tyrosine phosphorylation, bind to the Csk SH2 domain (21, 22). We show that Pragmin is localized to the cytoplasm and, through complex formation, Pragmin anchors Csk in the cytoplasm, preventing its relocation to the membrane and subsequent inactivation of SFKs. Because SFKs phosphorylate the EPIYA motif of Pragmin, SFKs positively regulate their own kinase activity by sequestering Csk via EPIYA-phosphorylated Pragmin. Also notably, Pragmin is phosphorylated at the EPIYA motif by the activated EGF receptor or its downstream kinases distinct from SFKs. This finding indicates the presence of a positive feedback regulation that ensures sustained SFK activation in cells stimulated with a growth factor such as EGF (Fig. S7). Recently, Leroy et al. (25) reported that ectopic expression of c-Src in human colon carcinoma cells induces phosphorylation of a cluster of tyrosine kinases and pseudokinases that include Pragmin. They also showed that, upon tyrosine phosphorylation on Y391 (the EPIYA motif), Pragmin stimulates c-Src activity in colon carcinoma cells, although the underlying mechanism was not known (25). The results of the study by Leroy et al. are consistent with those of the present work and can be fully explained by the positive feedback regulation of SFKs via the Pragmin–Csk interaction.

It has been proposed that bacterial EPIYA motifs act as promiscuous master keys that can interact with and therefore simultaneously disturb a number of mammalian SH2 domain-containing proteins (16). For bacteria, the versatile binding properties may be beneficial in exerting virulence. On the other hand, such a master key function may not be allowed in mammals in which intracellular signaling circuits are subjected to strict regulation. Consistent with this idea, mammalian proteomes are significantly depleted of EPIYA-like sequences (16). In the present study we found that Pragmin EPIYA binds to Csk in an EPIYA phosphorylation-dependent manner. However, in contrast to bacterial EPIYA motifs, which are characterized by promiscuous SH2 binding, the Pragmin EPIYA motif did not exhibit diverse SH2-binding activity. Because SH2-binding specificity that is defined by three to five residues C-terminal to the phosphorylated tyrosine (15), degenerated SH2 binding in bacterial EPIYA effectors could be attributable to subtle differences within these particular residues. The molecular basis that confers promiscuous SH2 binding on bacterial EPIYA effectors obviously warrants further investigation.

Pragmin potentiates SFK activity by sequestrating Csk, an inhibitor of SFKs, to the cytoplasm. On the other hand, H. pylori-delivered CagA, which competes with Pragmin for Csk binding, inhibits Pragmin–Csk complex formation. Furthermore, CagA recruits Csk to the membrane upon complex formation, where Csk inactivates SFKs. The effect of CagA on SFKs (inhibition) is therefore opposite to that of Pragmin (activation). Because EPIYA tyrosine phosphorylation is required for full activation of CagA virulence (2, 3, 7), feedback inhibition of SFK activity by CagA may be beneficial for H. pylori in preventing excess CagA virulence, as previously proposed (6). Also notably, in epithelial cells, SFKs are required for the induction of antibacterial peptides such as defensins (26) and are involved in the activation of NF-κB in response to bacterial infection (27). It is therefore intriguing to speculate that CagA-mediated SFK inhibition enables H. pylori to evade innate defense mechanisms of host epithelial cells.

In the immune system, SFKs regulate antigen receptor signaling in T and B lymphocytes (28), mediate cytokine receptor signaling in various hematopoietic lineage cells (29), and promote bacterial phagocytosis by macrophages (30). Thus, sustained SFK activation, which is ensured by the Pragmin–Csk interaction, may play
an important role in priming antibacterial immune responses. In turn, aberration in the SFK activity in immune cells might result in a wide array of immune dysfunctions that help bacteria to achieve immune evasion. In this regard, we and others have previously suggested that H. pylori CagA could also be delivered into immune cells that have migrated to the inflammatory region of gastric mucosa (31, 32). This raises the idea that, upon delivery into immune cells, CagA suppresses SFKs to dampen host immune systems.

Given that SFKs comprise nine members, Csk must be an ideal target for bacteria to collectively inhibit these SFK members. The EPIYA motifs of B. henselae BepD and BepF, which are delivered into human endothelial cells, also bind to Csk upon tyrosine phosphorylation (16). H. ducreyi inhibits macrophase-mediated phagocytosis, and this inhibition requires an H. ducreyi EPIYA effector, Lspa (13, 33). By analogy with CagA, it is possible that EPIYA-phosphorylated Lspa binds Csk to inhibit SFKs in macrophages. Simultaneous development of such EPIYA effectors in different bacteria may be an excellent example of “genetic convergence,” in which EPIYA motif-containing proteins were incidentally selected as bacterial EPIYA effectors, most probably because of the special advantage in perturbing host cell functions, such as those mediated by SFKs for successful bacterial infection.

Here we identified a mammalian EPIYA effector, Pragmin. Expression of a prototypic EPIYA effector protein that acts as a scaffold/adaptor in mammalian signal transduction may give further insights into the mechanisms underlying generation and evolution of bacterial EPIYA effectors during the long and highly balanced degree of coevolution of bacteria with mammalian hosts.

Materials and Methods

Expression Vectors. The pCMV, pSp655S, and pCDNA-derived mammalian expression vectors for Pragmin, Csk, and H. pylori CagA are described in SI Materials and Methods.

Bacteria. AGS cells were infected with H. pylori NCTC11637 or its isogenic strain lacking the cagA gene (ΔcagA) for 5 h at a multiplicity of infection of 200 before harvest.

Cell Culture and Transfection. AGS and MKN28 human gastric carcinoma cells were cultured in RPMI medium 1640 supplemented with 10% FCS. GE-1 human normal gastric epithelial cells were cultured in DMEM containing 10% FCS. Transient transfection was performed according to standard protocol using Lipofectamine 2000 reagent (Invitrogen).

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting were performed as previously described (5, 6). Refer to SI Materials and Methods for a detailed description. Intensity of protein band was quantitated by a luminene image analyzer.

Immunofluorescence Microscopy. Cells fixed with 3% paraformaldehyde were treated with primary antibodies and were then visualized with Alexa Fluor-conjugated secondary antibodies, as previously described (5, 6). Images were acquired using a confocal microscope.

Data Analysis. All of the DNA and protein sequences were retrieved from the National Center for Biotechnology database (www.ncbi.nlm.nih.gov) and were analyzed using the BLAST search program. BioEdit and Weblogo were used to align and display protein sequences (http://weblogo.berkeley.edu). ClustalW and Treeview (http://align.genome.jou) were applied to build and view phylogenetic trees.

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**Supporting Information**

**SI Materials and Methods**

**Expression Vectors.** A pCMV-based mammalian expression vector for Myc-tagged rat Pragmin was kindly provided by Dr. M. Negishi, (Kyoto University, Kyoto, Japan). Myc-tagged Pragmin-Y391F, in which tyrosine-391 was substituted with phenylalanine, was generated from the rat Pragmin cDNA by site-directed mutagenesis. A pSP65SRα-based mammalian expression vector for the HA-tagged wild-type CagA protein has been described previously (1, 2). Rat Csk cDNA was FLAG-tagged and was cloned into pSP65SRα vector. A pcHA3-derived mammalian expression vector for FLAG-tagged Grb7 was a gift from Dr. A. Villa-lobo (Instituto de Investigaciones Biomedicas, Madrid, Spain). To disable the SH2 domain of Csk, serine-109 of Csk was substituted with cysteine (Csk-S109C-FLAG) by site-directed mutagenesis. Duplex siRNAs for human Pragmin/SgK223 and luciferase were synthesized by Greiner Bio One. Nucleotide sequences for the human Pragmin-specific siRNA used were sense (5′-GUCA-CAGGCAAGAUAGATTT-3′) and antisense (5′-UUCAUUUCUUGGCCUCGUAGCTT-3′). Nucleotide sequences for the luciferase-specific siRNA used as a control were sense (5′-CGUA-CCGGGAUAACUUCGATT-3′) and antisense (5′-UCGAAG-UAUUCCGCUAGCTT-3′). The siRNAs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) at a final concentration of 100 pmol/mL.  

**Bacteria.** *Helicobacter pylori* NCTC11637 strain and its cagA-defective isogenic strain (ΔcagA) have been reported previously (3). AGS cells were infected with *H. pylori* NCTC11637 or the isogenic ΔcagA strain for 5 h at a multiplicity of infection (MOI) of 200 before harvest.  

**Antibodies and Reagents.** Anti-Csk polyclonal antibody C-20 (Santa Cruz Biotechnology), anti-Myc monoclonal antibody 9E10, anti-SHP-1 polyclonal antibody C-19 (Santa Cruz Biotechnology), anti-Grb2 polyclonal antibody C-23 (Santa Cruz Biotechnology), anti-c-Abl polyclonal antibody K-12 (Santa Cruz Biotechnology), anti-CrkII monoclonal antibody (Transduction Laboratories), and anti-P13K (subunit p85α) polyclonal antibody Z-8, anti-FLAG monoclonal antibody (M2) (Sigma-Aldrich), and anti-SHP2 polyclonal antibody C-18 (Santa Cruz Biotechnology) were used as primary antibodies for immunoblotting and immunoprecipitation. Anti-HA polyclonal antibody Y-11 (Santa Cruz Biotechnology), HA-epitope-specific monoclonal antibody 12CA5, anti-Pragmin antibody A302-675A (Bethyl Laboratories), anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology), anti-c-Src monoclonal antibody 36D10 (Cell Signaling), anti-Phospho-Src (Y416) polyclonal antibody (Cell Signaling), anti-Phospho-Src (Y530) polyclonal antibody (Santa Cruz Biotechnology), anti-RhoA antibody Sc-179 (Santa Cruz Biotechnology), anti-CagA polyclonal antibody (Austral Biologicals), and anti-Actin polyclonal antibody C-11 (Santa Cruz Biotechnology) were used as primary antibodies for immunoblotting. Normal mouse or rabbit IgG (IgG) was purchased from Santa Cruz Biotechnology. RhoA inhibitor, Clostridial C3 transferase, was purchased from Cytoskeleton. EGF was purchased from Sigma. Src family protein kinase inhibitor PP2 was purchased from Calbiochem.  

**Cell Culture and Transfection.** AGS and MKN28 human gastric carcinoma cells were cultured in RPMI medium 1640 supplemented with 10% FCS under standard condition. Cells were transiently transfected with expression vectors using Lipofectamine 2000 reagent (Invitrogen) as previously described (1) and were harvested at 36 h after transfection. GES-1 human normal human gastric epithelial cells were cultured in DMEM supplemented with 10% FCS. To inhibit tyrosine kinase activity, cells were incubated with 10 μM PP2 for 4 h or 100 nM PP2 for 6 h. To inhibit RhoA activity, cells were treated with cell-permeable C3 transferase from *Clostridium botulinum* at the final concentration of 0.5 μg/mL for 12 h.  

**Immunoprecipitation and Immunoblotting.** Cells were lysed as described previously (1, 2). Cell lysates were incubated with antibodies, and immune complexes were trapped on protein G-Sepharose beads. Total cell lysates and immunoprecipitates were subjected to SDS/PAGE, and separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membrane filters were treated with a primary antibody (1:1,000) and then with a secondary antibody (1:10,000). Proteins were visualized by using Western blot chemiluminescence reagent (PerkinElmer Life Science). Intensity of protein band was quantitated by a luminescent image analyzer (LAS-4000; FUJIFILM).  

**Immunofluorescence Microscopy.** Cells were fixed with 3% paraformaldehyde at 20 h after transfection. Cells were then treated with the indicated antibodies and were visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen). The nuclei were stained with DAPI (Wako). Images were acquired using a confocal microscope system (TCS-SPE; Leica).  

**Cell Morphological Analysis.** AGS cells (1.2 × 10⁵ cells) were transiently transfected with expression vectors using Lipofectamine 2000 reagent. Morphology of AGS cells was examined at 20 h after transfection.  

**Data Analysis.** All of the DNA and protein sequences were retrieved from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) and were analyzed using the BLAST search program. BioEdit and WebLogo were used to align and display protein sequences (http://weblogo.berkeley.edu). ClustalW and TreeView (http://align.genome.jp/) were applied to build and view phylogenetic trees.

**Fig. S1.** Glu-Pro-Ile-Tyr-Ala (EPIYA)/EPIYA-like sequences present in bacterial EPIYA effectors. (A) Sequences surrounding each of the EPIYA motifs (highlighted in red) in bacterial EPIYA effectors reported to date are shown. Consensus sequence spanning the EPIYA motif is shown at bottom. (B) Tree of bacterial EPIYA effectors, based on the 15-aa sequences shown in A.

**Fig. S2.** Schematics of rat Pragmin and Pragmin-Y391F used in this work. Pragmin contains a pseudokinase domain, which does not have a kinase catalytic activity, in its C-terminal region. Pragmin-Y391F was made by replacing tyrosine-391, which constitutes the EPIYA motif, with nonphosphorylatable phenylalanine. Both wild-type Pragmin and Pragmin-Y391F were N-terminally tagged with the Myc epitope.

**Fig. S3.** Effect of 100 nM PP2 on the kinase activity of c-Src. AGS cells were treated with 100 nM PP2 for 6 h before harvest. Cell lysates were subjected to immunoblotting with an anti-c-Src antibody, anti-Phospho-Src (416) antibody [anti-pSrc(416)], which specifically reacts with the active form of c-Src, and anti-Actin antibody.
Fig. S4. SH2-binding specificity of Pragmin. (A) AGS cells were transfected with the indicated vectors. Total cell lysates (TCLs) were immunoprecipitated with an anti-SHP1 antibody, and the immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and SHP1. The CagA-SHP1 interaction is shown as a positive control of the experiment. (B) AGS cells were transfected with the indicated vectors. Total cell lysates were immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and Grb7-FLAG. The CagA-Grb7 interaction is shown as a positive control of the experiment. (C) AGS cells were transfected with the indicated vectors. Total cell lysates were immunoprecipitated with an anti-c-Abl antibody, and the immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and c-Abl. The CagA-c-Abl interaction is shown as a positive control of the experiment. (D–F) AGS cells were transfected with a Myc-Pragmin vector together with a CrkII vector (D), Grb2 vector (E), or PI3-kinase (PI3K) p85 vector (F). Total cell lysates were immunoprecipitated with an anti-CrkII antibody (D), anti-Grb2 antibody (E), or anti-PI3K p85 antibody (F). The immunoprecipitates were subjected to immunoblotting with the indicated antibodies. There was no interaction between Myc-Pragmin and CrkII (D), between Myc-Pragmin and Grb2 (E), or between Myc-Pragmin and PI3K p85 (F). The CagA-Csk interaction (D), the CagA-SHP2 interaction (E), and the CagA-Grb7 interaction (F) are shown as positive controls of the transfected protein-protein interaction, which were simultaneously performed in each of the transfection experiments (D–F).
Fig. S6. Effect of RhoA inhibition on complex formation of Pragmin with Csk. (A) AGS cells transfected with Myc-Pragmin and Csk-FLAG vectors were treated with Clostridial C3 transferase, a specific inhibitor of Rho GTPases, for 12 h before harvest. Total cell lysates (TCLs) were immunoprecipitated with an anti-Myc antibody. Total cell lysates and immunoprecipitates (IPs) were subjected to immunoblotting with the indicated antibodies. Values shown are relative ratios of Csk that bound to Pragmin (Csk/Pragmin ratios), which were calculated from the quantitation data of protein bands using a luminescent image analyzer. The positions of RhoA (*) and ADP ribosylated (i.e., inactivated) RhoA (**) are indicated. (B) AGS cell lysates were immunoprecipitated with an anti-Pragmin antibody. Total cell lysates and immunoprecipitates were subjected to immunoblotting with the indicated antibodies. Values shown are relative ratios of Csk that bound to Pragmin (Csk/Pragmin ratios), which were calculated from the quantitation data of protein bands using a luminescent image analyzer. The positions of RhoA (*) and ADP ribosylated (i.e., inactivated) RhoA (**) are indicated.

Fig. S5. Magnification of cell morphology shown in Fig. 3 (main text). (A) AGS cells were transfected with a Myc-Pragmin or Myc-Pragmin-Y391F vector. Morphology of transfected cells was analyzed by microscope. (B) AGS cells treated with or without the Pragmin-specific siRNA were transfected with a c-Src vector. Cells were subjected to morphological investigation using microscopy. (Scale bars, 10 μm.)
Fig. S7. A proposed model for the positive feedback regulation of SFKs by Pragmin. Basally activated SFKs in unstimulated cells phosphorylate a small fraction of Pragmin molecules, which reduce the amount of membrane-associated Csk below a certain level to keep the basal SFK activity. Stimulation of cells with EGF increases the amount of Pragmin that is phosphorylated at the EPIYA motif, which sequesters Csk in the cytoplasm and thereby prevents translocalization of Csk to the membrane. As a result, SFKs are kept in their active forms and further phosphorylate Pragmin at the EPIYA motif, giving rise to the formation of a positive feedback loop that ensures sustained SFK activation, which promotes cell proliferation as well as cell motility.

Table S1. List of human EPIYA-containing proteins

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>EPIYA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig heavy chain variable region (anti-vaccinia virus)</td>
<td>VDPEDGEPIYAEKFGGR</td>
</tr>
<tr>
<td>General transcription factor TFIIIE, α subunit</td>
<td>RFNEQIEPIYALLRETE</td>
</tr>
<tr>
<td>Solute carrier family 2/facilitated glucose transporter member 3</td>
<td>KDAGVQEPIYATIGAGV</td>
</tr>
<tr>
<td>Transmembrane protein 218</td>
<td>LIHYVLEPIYAKPLHSY</td>
</tr>
<tr>
<td>Pragmin/SgK223</td>
<td>REATQPEPIYAESTKRK</td>
</tr>
<tr>
<td>Coiled-coil domain-containing protein 146</td>
<td>KDEKDQEPIYAIIVPTIN</td>
</tr>
</tbody>
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