Role of Partitioning-defective 1/Microtubule Affinity-regulating Kinases in the Morphogenetic Activity of Helicobacter pylori CagA*

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Helicobacter pylori CagA plays a key role in gastric carcinogenesis. Upon delivery into gastric epithelial cells, CagA binds and deregulates SHP-2 phosphatase, a bona fide oncoprotein, thereby causing sustained ERK activation and impaired focal adhesions. CagA also binds and inhibits PAR1b/MARK2, one of the four members of the PAR1 family of kinases, to elicit epithelial polarity defect. In nonpolarized gastric epithelial cells, CagA induces the hummingbird phenotype, an extremely elongated cell shape characterized by a rear retraction defect. This morphological change is dependent on CagA-deregulated SHP-2 and is thus thought to reflect the oncogenic potential of CagA. In this study, we investigated the role of the PAR1 family of kinases in the hummingbird phenotype. We found that CagA binds not only PAR1b but also other PAR1 isoforms, with order of strength as follows: PAR1b > PAR1d > PAR1a > PAR1c. Binding of CagA with PAR1 isoforms inhibits the kinase activity. This abolishes the ability of PAR1 to destabilize microtubules and thereby promotes disassembly of focal adhesions, which contributes to the hummingbird phenotype. Consistently, PAR1 knockdown potentiates induction of the hummingbird phenotype by CagA. The morphogenetic activity of CagA was also found to be augmented through inhibition of non-muscle myosin II. Because myosin II is functionally associated with PAR1, perturbation of PAR1-regulated myosin II by CagA may underlie the defect of rear retraction in the hummingbird phenotype. Our findings reveal that CagA systemically inhibits PAR1 family kinases and indicate that malfunctioning of microtubules and myosin II by CagA-mediated PAR1 inhibition cooperates with deregulated SHP-2 in the morphogenetic activity of CagA.

Infection with Helicobacter pylori strains bearing cagA (cytotoxin-associated gene A)-positive strains is the strongest risk factor for the development of gastric carcinoma, the second leading cause of cancer-related death worldwide (1–3). The cagA gene is located within a 40-kb DNA fragment, termed the cag pathogenicity island, which is specifically present in the genome of cagA-positive H. pylori strains (4–6). In addition to cagA, there are ~30 genes in the cag pathogenicity island, many of which encode a bacterial type IV secretion system that delivers the cagA-encoded CagA protein into gastric epithelial cells (7–10). Upon delivery into gastric epithelial cells, CagA is localized to the plasma membrane, where it undergoes tyrosine phosphorylation at the C-terminal Glu-Pro-Ile-Tyr-Ala motifs by Src family kinases or c-Abl kinase (11–14). The C-terminal Glu-Pro-Ile-Tyr-Ala-containing region of CagA is noted for the structural similarity among distinct H. pylori isolates. Oncogenic potential of CagA has recently been confirmed by a study showing that systemic expression of CagA in mice induces gastrointestinal and hematological malignancies (15).

When expressed in gastric epithelial cells, CagA induces morphological transformation termed the hummingbird phenotype, which is characterized by the development of one or two long and thin protrusions resembling the beak of the hummingbird. It has been thought that the hummingbird phenotype is related to the oncogenic action of CagA (7, 16–19). Pathophysiological relevance for the hummingbird phenotype in gastric carcinogenesis has recently been provided by the observation that infection with H. pylori carrying CagA with greater ability to induce the hummingbird phenotype is more closely associated with gastric carcinoma (20–23). Elevated motility of hummingbird cells (cells showing the hummingbird phenotype) may also contribute to invasion and metastasis of gastric carcinoma.

In host cells, CagA interacts with the SHP-2 phosphatase, C-terminal Src kinase, and Crk adaptor in a tyrosine phosphorylation-dependent manner (16, 24, 25) and also associates with Grb2 adaptor and c-Met in a phosphorylation-independent manner (26, 27). Among these CagA targets, much attention has been focused on SHP-2 because the phosphatase has been recognized as a bona fide oncoprotein, gain-of-function mutations of which are found in various human malignancies (17, 18, 28). Stable interaction of CagA with SHP-2 requires CagA dimerization, which is mediated by a 16-amino acid CagA-multimerization (CM)2 sequence present in the C-terminal region of CagA (29). Upon complex formation, CagA aberrantly activates SHP-2 and thereby elicits sustained ERK MAP kinase activation that promotes mitogenesis (30). Also, CagA-activated SHP-2 dephosphorylates and inhibits focal adhesion kinase

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2 The abbreviations used are: CM, CagA-multimerization; HA, hemagglutinin; MAP, microtubule-associated protein; MARK, microtubule affinity-regulating kinase; m.o.i., multiplicity of infection; TER, transepithelial electrical resistance; MDCK, Madin-Darby canine kidney; siRNA, small interference RNA; PIPES, 1,4-piperazinediethanesulfonic acid; FAK, focal adhesion kinase.
FAK), causing impaired focal adhesions. It has been shown previously that both aberrant ERK activation and FAK inhibition by CagA-deregulated SHP-2 are involved in induction of the hummingbird phenotype (31).

Partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK) is an evolutionally conserved serine/threonine kinase originally isolated in C. elegans (32–34). Mammalian cells possess four structurally related PAR1 isoforms, PAR1a/MARK3, PAR1b/MARK2, PAR1c/MARK1, and PAR1d/MARK4 (35–37). Among these, PAR1a, PAR1b, and PAR1c are expressed in a variety of cells, whereas PAR1d is predominantly expressed in neural cells (35, 37). These PAR1 isoforms phosphorylate microtubule-associated proteins (MAPs) and thereby destabilize microtubules (35, 38), allowing asymmetric distribution of molecules that are involved in the establishment and maintenance of cell polarity.

In polarized epithelial cells, CagA disrupts the tight junctions and causes loss of apical-basolateral polarity (39, 40). This CagA activity involves the interaction of CagA with PAR1b/MARK2 (19, 41). CagA directly binds to the kinase domain of PAR1b in a tyrosine phosphorylation-independent manner and inhibits the kinase activity. Notably, CagA binds to PAR1b via the CM sequence (19). Because PAR1b is present as a dimer in cells (42), CagA may passively homodimerize upon complex formation with the PAR1 dimer via the CM sequence, and this PAR1-directed CagA dimer would form a stable complex with SHP-2 through its two SH2 domains.

Because of the critical role of CagA in gastric carcinogenesis (7, 16–19), it is important to elucidate the molecular basis underlying the morphogenetic activity of CagA. In this study, we investigated the role of PAR1 isoforms in induction of the hummingbird phenotype by CagA, and we obtained evidence that CagA-mediated inhibition of PAR1 kinases contributes to the development of the morphological change by perturbing microtubules and non-muscle myosin II.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—pSP65SRα-derived mammalian expression vectors for hemagglutinin (HA)-tagged East Asian CagA (CagA-ABD) and its phosphorylation-resistant mutant (CagA-abd) were described previously (29). CagA-ABD-ΔCM was generated from CagA-ABD by deleting the 16-amino acid CM sequence by site-directed mutagenesis as described previously (43). A pEF-His-A-derived mammalian expression vector for T7 epitope-tagged human PAR1/MARK2 was kindly provided by Dr. Shigeo Ohno (Yokohama City University, Japan). The cDNAs encoding human PAR1a/MARK3, PAR1c/MARK1, and PAR1d/MARK4 were purchased from OriGene Technologies. After adding a T7 epitope tag at the N terminus, the PAR1 cDNAs were inserted into pEF-His-A mammalian expression vector. PAR1b-K49R, PAR1b-S257I, and PAR1c-I275S were made by site-directed mutagenesis. Human Tau cDNA was tagged with FLAG epitope and inserted into the pcDNA3.1 mammalian expression vector.

**Recombinant Adenoviruses**—pAD/CMV/V5-DEST-derived adenovirus vectors for HA-tagged CagA-ABD and T7-tagged PAR1 were constructed using the ViraPower adenoviral expression system (Invitrogen) according to the manufacturer’s instruction. A recombinant adenovirus transducing β-galactosidase was used as a negative control. Recombinant adenoviruses were amplified in human HEK293A cells, and the virus titers were determined by counting the plaques of HEK293A cells.

**Cells, Transfection, and Transduction**—Monkey COS-7 cells and Madin-Darby canine kidney (MDCK) II cells were cultured
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Physiological interaction of CagA with PAR1/MARK isoforms. A, a quantitative analysis of CagA-PAR1 interaction. Left panel, a constant amount of HA-tagged CagA (CagA-ABD-HA) was mixed in vitro with a constant amount of T7-tagged PAR1 isoform (T7-PAR1). The protein mixtures were incubated at 4°C for 2 h and were then immunoprecipitated with an anti-HA antibody. The anti-HA immunoprecipitates (IP) and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with the indicated antibodies. Right panel, a constant amount of HA-tagged CagA (CagA-ABD-HA) was mixed in vitro with a constant amount of T7-tagged wild-type (WT) PAR1 or PAR1b-S257I. Lower panel, the amount of PAR1b-S257I that bound to CagA was quantitated and compared with that of wild-type PAR1b. The CagA binding activity of wild-type PAR1b was taken as 1. Error bars indicate mean ± S.E., n = 3. B, in vitro binding assay of CagA with PAR1c mutant. Upper panel, a constant amount of HA-tagged CagA (CagA-ABD-HA) was mixed in vitro with a constant amount of T7-tagged wild-type (WT) PAR1c or PAR1c-I275S. Lower panel, the amount of PAR1c-I275S that bound to CagA was quantitated and compared with that of wild-type PAR1c. The CagA binding activity of wild-type PAR1c was taken as 1. Error bars indicate mean ± S.E., n = 3.

in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. AGS human gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. COS-7 cells (1.5 × 10^6) were transfected with 20 μg of plasmids using the calcium phosphate method as described previously (16). AGS cells were seeded into 8-well Chamber well (1 × 10^5) cells/well; Nunc, Tokyo, Japan) and were transfected with plasmids (0.8 μg) using Lipofectamine 2000 (Invitrogen). MDCK II cells (1.5 × 10^5) were seeded into Transwell filter (12 mm diameter, 0.4 μm pore, Corning catalog number 3460) and transduced with recombinant adenoviruses at a multiplicity of infection (m.o.i.) of 240:1 for each adenovirus as described previously (19).

RNA interference—Duplex small interference RNAs (siRNAs) for human PAR1b were synthesized by Greiner Bio-One (Japan). Nucleotide sequences used were as follows: PAR1b-siRNA-535, sense, 5'-GGAAGAGGGCUAGCGUUAAAGA-3'; antisense, 5'-UUUACACGCUACCUUUCUCA-3'; PAR1b-siRNA-709, sense, 5'-GCAGAGGUUUGAAUUCGU-3'; antisense, 5'-UAUUAACCAUUCUCCGCAA-3'; luciferase-specific siRNA was used as negative control, sense, 5'-CGUACGCGAGAUAUCUUCG-3'; and antisense, 5'-UCGAAGGAAUUCGCUGACG-3'. siRNAs were transfected into cells with Lipofectamine 2000. Cells were harvested at 36 h after transfection, and knockdown efficiencies were investigated by immunoblotting.

Antibodies—Anti-HA monoclonal antibody (3F10, Roche Applied Science) and anti-T7 polyclonal antibody (M-21, Santa Cruz Biotechnology) were used for immunoprecipitation of HA-tagged CagA and T7-tagged PAR1, respectively. Anti-β-actin (C-11, Santa Cruz Biotechnology) was used to detect β-actin as a control. Anti-PAR1b antibody was a kind gift from Dr. Shigeo Ohno (Yokohama City University, Japan). Anti-S262-Tau antibody (Calbiochem catalog number 577814) and anti-FLAG antibody (M2, Sigma) were used to detect phosphorylated Tau and total Tau, respectively, in immunoblotting. Horseradish peroxidase-linked anti-mouse, anti-rat, and anti-rabbit IgG were purchased from GE Healthcare. Horseradish peroxidase-linked donkey anti-goat IgG was purchased from Santa
Cruz Biotechnology. Anti-HA (3F10 and 6E2, Cell Signaling), anti-β-tubulin (D-10, Santa Cruz Biotechnology), and antipaxillin (H-114, Santa Cruz Biotechnology) were used for immunostaining. Alexa Fluor-conjugated anti-rat, anti-mouse, and anti-rabbit antibodies and rhodamine-phalloidin were purchased from Invitrogen.

**In Vitro Protein Interaction Assay**—In vitro protein interaction assay was performed following the procedure described previously (43). In brief, COS-7 cells were singly transfected with the expression vector for HA-tagged CagA-ABD or T7-tagged PAR1 isoform. Cells were harvested at 36 h after transfection and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Brij-35) containing 2 mM Na$_3$VO$_4$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 10 μg/ml aprotinin (16). A constant amount of HA-tagged CagA-ABD expressed in COS-7 cells was mixed with a constant amount of each of the T7-PAR1 isoforms expressed in COS-7 cells. Total amount of proteins and volume were adjusted to the same level using lysates prepared from COS-7 cells transfected with a control vector. Mixed cell lysates were incubated at 4 °C for 2 h before performing immunoprecipitation.

**Immunoprecipitation and Immunoblotting**—Total cell lysates and anti-HA immunoprecipitates were subjected to SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membrane filters (Millipore), and the filters were incubated with a primary antibody (1:1000, 90 min at room temperature) and then with a secondary antibody (1:10,000, 45 min at room temperature). Proteins were visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences). Intensities of chemiluminescence on the immunoblotted filter were quantitated using a luminescence image analyzer (LAS-1000, Fujifilm) as described previously (29, 31).

**Transepithelial Electrical Resistance (TER)**—MDCK II cells (1.5 × 10$^5$) were seeded on a Transwell filter and cultured for 3 days to make a polarized monolayer. Polarized MDCK II cells were then transduced with adenovirus at the m.o.i. of 240:1 for each adenovirus. TER was measured every 24 h for 4 days using a Millicell-ESR epithelial voltohmmeter (Millipore). The specific TER value was calculated by subtracting the background value of the filter and the medium obtained from blank wells that had been cultured in parallel.

**Immunofluorescence**—AGS cells were fixed for 15 min with PHEM buffer, pH 6.9, containing 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl$_2$, 4% paraformaldehyde, 0.15% glutaraldehyde, and 0.2% Tween 20 (44). After blocking in phosphate-buffered saline with 0.6% Tween 20, 0.5% Na$_3$NO$_3$, and 5% bovine serum albumin for 1 h, cells were incubated with a primary antibody (1:200) for 2 h and then with Alexa Fluor-conjugated anti-rabbit, anti-mouse, or anti-rat antibody and rhodamine-phalloidin (1:200) for 1 h. Cells were washed three times for 5 min with phosphate-buffered saline containing 0.1% Tween 20, and slides were mounted with 90% glycerin in phosphate-buffered saline. All samples were observed by a confocal microscope (TCS-SPE, Leica). Images were acquired using the ×40 or ×63 objective in a 0.1- or 0.3-μm z-stake thickness according to the purpose.

**Quantitative Analysis of Focal Adhesions**—AGS cells were fixed and stained with an anti-paxillin antibody to visualize focal adhesions. Pictures were acquired on a fluorescence microscope on an 8-bit scale. Focal adhesions were quantified using ImageJ software (NIH, rsbweb.nih.gov). In brief, images obtained from the anti-paxillin staining were converted to binary, and objects with a size greater than 0.5 μm$^2$ were counted as focal adhesions. All images were background-subtracted prior to measurements. Nonspecific staining was removed manually.

**Statistics**—Statistical analyses for induction of the hummingbird phenotype by CagA in AGS cells were performed by Student’s t test. The effect of blebbistatin on the AGS cells were analyzed by χ$^2$ test. Error bars represent mean ± S.E. p < 0.05 was considered to be statistically significant.

**RESULTS**

**CagA Is a Universal Inhibitor of PAR1/MARK Isoforms**—In a previous study, we demonstrated that CagA specifically binds to the 27-amino acid stretch that is present in the C-terminal region of the PAR1b kinase domain (19). Interaction of CagA...
with PAR1b inhibits the kinase activity both in vitro and in vivo. Because CagA also interacts with other PAR1 isoforms via the CM sequence (19), it is a logical conclusion that CagA acts as a universal inhibitor of PAR1 kinases. To verify this, we generated pEF-based mammalian expression vectors for all of the human PAR1 isoforms (Fig. 1A). We also generated a kinase-dead PAR1b mutant, PAR1b-K49R, in which the lysine residue on the ATP-binding site in the kinase domain of PAR1b was replaced by an arginine residue (35, 42). We then expressed each of PAR isoforms together with the PAR1 kinase substrate, the Tau protein, in AGS human gastric epithelial cells in the presence or absence of CagA. Whereas the kinase-dead PAR1b-K49R failed to phosphorylate Tau, all the PAR1 isoforms induced Tau phosphorylation, which was inhibited by co-expression of CagA (Fig. 1B).

**Differential CagA Binding Activities among PAR1/MARK Isoforms**—To investigate the interaction of CagA with PAR1 isoforms in more detail, HA-tagged CagA (CagA-ABD-HA) was co-transfected with each T7-tagged PAR1 isoform (T7-PAR1a–d) into AGS cells. Cells were fixed at 24 h after transfection and immunostained with anti-HA or anti-T7 antibody and were visualized with Alexa Fluor-conjugated second antibodies. A, representative images of AGS cells expressing CagA (anti-HA, red) and/or PAR1 (anti-T7, blue). Bar stands for 30 μm. B, magnitudes for induction of the hummingbird phenotype by CagA were determined by the % ratio of hummingbird cells to the total CagA-positive cells. More than 600 CagA-positive cells were counted for each of three independent experiments. Student’s t test; error bars stand for mean ± S.E.; **, p < 0.01; n.s., not significant (n.s.) indicates p > 0.05. C, expression levels of CagA and PAR1 isoforms in transfected AGS cells; pSP65SRΔ empty vector was used as a negative control, and β-actin was used for the control of proteins loaded.

As noted above, the 27-amino acid CagA-binding sequence of PAR1b (residues 250–276) is highly conserved among the PAR1 isoforms (19). In particular, comparison of residues 250–268 of PAR1b, which have been reported to be indispensable for CagA interaction (43), with those of the corresponding regions in other PAR1 isoforms revealed that the identities of the amino acid sequences were 78–84% (Fig. 2B). In the CagA-binding regions of PAR1b (the strongest CagA interactor) and PAR1c (the weakest CagA interactor), Ser-257 of PAR1b and Ile-275 of PAR1c were physicochemically the most diverged amino acid residues. To determine whether the differences in CagA binding activity among PAR1 isoforms were because of the amino acid variations within the CagA-binding region, the PAR1b-S257I mutant in which Ser-257 of PAR1b was replaced by Ile and the PAR1c-I275S mutant in which Ile-275 of PAR1c was replaced by Ser were generated by site-directed mutagenesis. When compared with wide type PAR1, PAR1b-S257I showed a decreased activity for binding to CagA (Fig. 2C), whereas PAR1c-I275S showed an increased CagA binding activity (Fig. 2D). Thus, the difference in CagA binding activity among PAR1 isoforms is primarily attributable to the sequence variations in the CagA-binding regions.
nant adenoviruses (Fig. 3A). The strength of the tight junctions was then quantitatively evaluated by measuring TER of the polarized MDCK monolayer. As shown in Fig. 3B, all of the PAR1 isoforms, including PAR1c which bind CagA most weakly, were capable of counteracting the CagA activity to disrupt tight junctions to comparable levels. The results indicate that all of the human PAR1 family members are involved in the maintenance of tight junctions. In turn, CagA disrupts tight junctions by acting as a universal inhibitor of PAR1 kinases.

**Involvement of PAR1 Isoforms in Induction of the Hummingbird Phenotype by CagA**—Induction of the hummingbird phenotype by CagA is counteracted by elevated levels of PAR1b. This in turn indicates that CagA-PAR1b interaction and subsequent inhibition of PAR1b is important for induction of the hummingbird phenotype (7, 16). To determine whether PAR1b is the only PAR1 isoform that can inhibit the hummingbird phenotype, AGS cells were co-transfected with expression vectors for HA-tagged CagA and one of the T7-tagged PAR1 isoforms. At 24 h post-transfection, cells were stained with anti-HA and anti-T7 antibodies, and the percentages of hummingbird cells in CagA single-positive or CagA/PAR1 double-positive cells were calculated. In the morphological analysis, hummingbird cells were defined as cells with long and thin protrusions, the length of which is more than 2-fold the width of the cell body. Compared with CagA single-positive cells, CagA/PAR1 double-positive cells included a significantly decreased number of hummingbird cells, in which the length of the protrusion was also reduced (Fig. 4, A and B). In these PAR1-transfected cells, all of the PAR1 isoforms were capable of attenuating the CagA-induced hummingbird phenotype at equivalent levels (Fig. 4B), and the levels of CagA as well as PAR1 isoforms expressed were comparable (Fig. 4C). Hence, all of the PAR1 isoforms can counteract the morphogenetic activity of CagA. Thus, systemic inhibition of PAR1 isoforms by CagA must play a role in induction of the hummingbird phenotype.

**Effect of CagA on Subcellular Distribution of PAR1 Isoforms**—To elucidate the mechanism by which PAR1 inhibition contributes to the morphogenetic activity of CagA, we next investigated the intracellular distribution of PAR1 isoforms in the presence or absence of CagA. To do so, AGS cells were transfected with one of the T7-tagged PAR1, and subcellular distribution of the PAR1 isoforms was examined by using a confocal microscope. In AGS cells, all of the ectopically expressed PAR1 isoforms were diffusely distributed in the cytoplasm, as were the cases with Chinese hamster ovary (CHO) cells (35, 37) and HEK293 cells (38) (Fig. 5A). When CagA was co-expressed with one of the PAR1 isoforms, however, a significant fraction of PAR1 was translocated from the cytoplasm to the plasma membrane (Fig. 5B). Because CagA specifically localizes to the plasma membrane, this result indicates that the membrane-tethered CagA recruited PAR1 isoforms from the cytoplasm to the plasma membrane, where CagA inhibited the PAR1 kinase activity.

**Effects of PAR1 Isoforms on Microtubules and Microfilaments**—In mammalian cells, all of the PAR1 isoforms function as MARKs that phosphorylate MAPs, such as MAP2, MAP4, and Tau, and thereby regulate the dynamics of the microtubules (35, 37). Because cell morphology is determined by the cooperative action of microtubules and microfilaments (45, 46), it is possible that inhibition of PAR1 by CagA promotes the development of the hummingbird phenotype through perturbing microtubules. To test this possibility, we examined the role of PAR1 isoforms in microtubule organization...
of each PAR1 isoform on microtubules in gastric epithelial cells (Fig. 6A). In AGS cells that did not express ectopic PAR1, microtubules were radiated from the centrosome and reached the cell periphery upon staining with anti-β-tubulin antibody (Fig. 6A, see cells that are negative for anti-T7 staining). In contrast, in cells ectopically expressing one of the PAR1 isoforms, microtubules lost the radial appearance and primarily surrounded the nucleus, which was concomitantly associated with a decrease in anti-β-tubulin staining in the cell periphery (Fig. 6A, see cells that are positive for anti-T7 staining). Furthermore, ectopic PAR1-expressing cells became smaller and more rounded than nonexpressing cells. Consistent with results of a previous study using CHO cells (35, 37), this observation indicated that PAR1 kinases destabilize microtubules of AGS cells. We also visualized F-actin filaments in AGS cells with rhodamine-phalloidin staining (Fig. 6B). In AGS cells that did not express ectopic PAR1, F-actins were primarily organized into cortical actin bundles anchored to focal adhesions (Fig. 6B, see cells that are negative for anti-T7 staining). In cells expressing one of the PAR1 isoforms, however, the cortical F-actin bundles were markedly reduced (Fig. 6B, see cells that are positive for anti-T7 staining). Thus, PAR1 kinases not only regulate the dynamics of microtubules but also affect the reorganization of actin cytoskeletal system in AGS cells.

**Ablation of PAR1-mediated Microtubule Destabilization by CagA**—The above-described observations indicated that CagA counteracts the ability of PAR1 to destabilize microtubules upon physical complex formation. Accordingly, we next investigated the effect of CagA on the PAR1-regulated microtubule dynamics. In AGS cells co-expressing CagA and PAR1b, destabilization of microtubules by PAR1b was abolished (Fig. 7A). In these cells, the majority of CagA and PAR1b co-localized to the membrane as shown in Fig. 5B. AGS cells were next transfected with the T7-tagged PAR1b and HA-tagged CagA mutant, CagA-ABD-CM, that specifically lacks the PAR1-binding CM sequence (Fig. 7B) (43). Although the CagA mutant was still capable of localizing recruited PAR1b to the cell membrane nor abolished the ability of PAR1 to destabilize the microtubules (Fig. 7B). Furthermore, CagA-ABD-CM failed to induce the hummingbird phenotype as reported previously (43). These observations support the conclusion that CagA-mediated PAR1 inhibition and subsequent perturbation of microtubules are critically involved in the morphogenetic activity of CagA.
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To verify the above-described conclusion, we then knocked down PAR1b expression using siRNA in AGS cells (Fig. 8A). PAR1b knockdown per se induced morphological change in AGS cells, which was characterized by the appearance of multiple short protrusions (Fig. 8B). Expression of CagA in the PAR1b-knockdown cells elicited a dramatic increase in the number of hummingbird cells (Fig. 8, B and C). The observation consolidates that inhibition of PAR1 activity is critical in induction of the hummingbird phenotype by CagA. The conclusion was further supported by the experiment showing that, in contrast to the case of wild-type PAR1b, ectopic expression of a kinase-dead PAR1b, PAR1b-K49R, failed to inhibit induction of the hummingbird phenotype by CagA. Rather, PAR1b-K49R potentiated the hummingbird phenotype, probably due to inhibition of endogenous PAR1 by PAR1b-K49R, which may act as a dominant-negative PAR1 (Fig. 8D).

Involvement of Non-muscle Myosin II in the Hummingbird Phenotype Induced by CagA—The thin and long protrusions of the hummingbird phenotype are mechanistically due to retraction failure at the rear end of the moving cell (52). It has been reported that non-muscle myosin II, especially myosin IIB, is responsible for retraction of the rear tail (53–55). The fact that myosin II is physically and functionally associated with PAR1 (56) raises the possibility that inhibition of PAR1 by CagA may also be involved in the hummingbird phenotype through perturbing myosin II functions. To test this possibility, we investigated the effect of a specific myosin II inhibitor, blebbistatin (57), on induction of the hummingbird phenotype in AGS cells. Blebbistatin inhibits both the ATPase and gliding motility activities of vertebrate non-muscle myosin II without inhibiting myosin light chain kinase (MLCK). It rapidly disrupts the directed cell migration without affecting the assembly of microtubules (57). Treatment of AGS cells with 10 μM blebbistatin for 5 h induced short protrusions of cells (Fig. 10A), which are similar to those observed in the PAR1b-knockdown cells (Fig. 8B). Given this, we next transfected AGS cells with a CagA expression vector and treated cells with a suboptimal concentration of blebbistatin (1 μM) for 12 h, which on its own does not induce any morphological changes in AGS cells, before staining. The results of the experiment revealed that blebbistatin treatment significantly increased the number of cells with the hummingbird phenotype by CagA (Fig. 10, B and C). The length of protrusion in the hummingbird cell was also increased in the presence of blebbistatin (Fig. 10, B, D, and E). Thus, induction of the hummingbird phenotype by CagA was both quantitatively and qualitatively potentiated by inhibition of non-muscle myosin II. Although CagA-mediated SHP-2 deregulation and microtubule stabilization are essential for induction of the hummingbird phenotype (16, 58), perturbation of PAR1-regu-
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A

IB: anti-HA

IB: anti-PAR1b

IB: anti-β-Actin

B

Control-siRNA

PAR1b-siRNA

C

Hummingbird cells (%)

Control-siRNA

PAR1b-siRNA

PAR1b-siRNA

CagA-ABD-HA

D

Hummingbird cells (%)

Control

WT-PAR1b

PAR1b-K49R

CagA-ABD-HA

FIGURE 8. Effect of PAR1b knockdown on the cell morphology. A, PAR1b knockdown in AGS cells. AGS cells were transfected with two PAR1b-specific duplex small interference RNA (PAR1b-siRNA-525 and PAR1b-siRNA-709) or luciferase-specific siRNA (used as a negative control) in the presence or absence of CagA-ABD-HA. Cells were harvested at 36 h post-transfection, and PAR1b expression level was determined by a specific anti-PAR1b antibody. β-Actin was used as a loading control. A representative immunoblot (IB) of three independent experiments was shown. Both of the PAR1b-specific siRNAs specifically knocked down the PAR1b level more than 70%. B, effect of PAR1b knockdown on the morphology of AGS cells in the presence or absence of CagA. AGS cells were transfected with PAR1b-siRNA or control siRNA in the presence or absence of CagA-ABD-HA. Cells were re-seeded at 12 h after transfection, and cell morphology was investigated at 36 h post-transfection. Typical phase-contrast images were presented. C, effect of PAR1b knockdown on induction of the hummingbird phenotype by CagA. In the experiment presented in B, the percentage of hummingbird cells in each of siRNA-treated groups was determined by counting more than 500 cells from 5 randomly selected fields (10× objective). For Student’s t test, error bars represent mean ± S.E.; *, p < 0.05; **, p < 0.01; n = 3. D, effect of kinase-dead PAR1b-K49R on induction of the hummingbird phenotype by CagA. AGS cells were co-transfected with CagA-ABD-HA and wild-type PAR1b or kinase-dead PAR1b-K49R, and percentages of hummingbird cells in cells expressing CagA only (Control), CagA plus wild-type PAR1b, or CagA plus PAR1b-K49R were determined after double staining of cells with anti-PAR1b antibody and anti-HA antibody, which detects HA-tagged CagA. More than 500 CagA-positive cells were counted for each of three independent experiments. For Student’s t test, error bars indicate mean ± S.E.; *, p < 0.05; **, p < 0.01.

lated myosin II activity by CagA may underlie the cell-retraction defect, the characteristic of the hummingbird phenotype.

DISCUSSION

In this study, we found that H. pylori CagA interacts with the PAR1 kinases and inhibits the kinase activity, which in turn causes microtubule stabilization. Excessively stabilized microtubules then promote disassembly of focal adhesions, contributing to the reduced cell-substrate interaction and subsequent cytoskeleton systems in a redundant fashion. The actin cytoskeletal changes caused by PAR1 may be due at least in part to the functional interaction between microtubules and a RhoA-specific guanine nucleotide exchange factor such as GEF-H1 (61–63). GEF-H1 activity is suppressed when it binds to microtubules and is increased when it is released from microtubules (64). Accordingly, disruption of microtubules by PAR1 causes an increase in activity of GEF-H1, which in turn activates RhoA and thereby causes actin-cy-
toskeletal reorganization and myosin II-dependent contraction, which lead to cell rounding.

Deregulation of SHP-2 by CagA is critically involved in the hummingbird phenotype (16, 17, 29, 31). We previously reported that CagA-activated SHP-2 dephosphorylates FAK at its activating phosphorylation residues (Tyr-375, Tyr-575, and Tyr-577), which results in FAK inactivation (31). Down-regulation of FAK then impairs turnover of the focal adhesion, the site of clustered integrins and associated molecules that are linked to actin stress fibers (65–67), thereby promoting induction of the hummingbird phenotype. This study revealed that induction of the hummingbird phenotype requires systemic inhibition of PAR1 kinases in addition to deregulated SHP-2 (19, 43). How then is PAR1 involved in the hummingbird phenotype? Recent studies have shown that microtubules make direct contact with focal adhesions and promote their disassembly by delivering dynamin and ACF7, which trigger endocytosis of focal adhesion components such as integrins (51, 64). Thus, interaction of CagA with PAR1 isoforms impairs the ability of PAR1 to destabilize microtubules and thereby promotes disassembly of focal adhesions.

Mechanistically, the hummingbird phenotype is caused by a defect of rear retraction during cell movement (52). This morphological abnormality is reminiscent of that observed in cells lacking non-muscle myosin II (53, 54). Indeed, myosin II-mediated contraction is thought to generate force to retract the rears of migrating cells and to maintain a polarized cell shape (63, 64). We found in this study that inhibition of non-muscle myosin II potentiates induction of the hummingbird phenotype by CagA. Notably, Guo and Kemphues (56) reported a physical and functional interaction between PAR1 and non-muscle myosin II in establishing cell polarity in Caenorhabditis elegans zygotes, raising the possibility that PAR1 regulates myosin II functions through complex formation and/or phosphorylation and that CagA stimulates the formation of long protrusions, the hallmark of the hummingbird phenotype, by perturbing non-muscle myosin II via PAR1 inhibition.

This study revealed that microtubules and non-muscle myosin II are key structural elements underlying the morphogenetic activity of CagA. First, CagA subverts focal adhesions by inhibiting FAK through CagA-mediated SHP-2 activation and by stabilizing microtubules through CagA-mediated PAR1 inhibi-
tion. Second, CagA perturbs non-muscle myosin II and thereby prevents retraction of rear protrusions during cell movement. Depolarization of epithelial cells is associated with the conversion of apical-basolateral polarity to antero-posterior (front-back) polarity. The antero-posterior polarity, which is also regulated by PAR1 kinases, allows cells to migrate in a directional fashion (68). Focal adhesion assembly at the anterior front and dissolution at the posterior end are critical for migration in many adherent cells (55, 66, 69). Hence, the hummingbird phenotype may need to be understood in terms of the antero-posterior polarity defect caused by the CagA-PAR1 interaction.

Strong association of H. pylori cagA-positive strains with the development of gastric carcinoma indicates the importance of CagA in gastric carcinogenesis. Indeed, the oncogenic potential of CagA has recently been demonstrated by studies using cagA-transgenic mice (15). It has long been thought that the CagA-induced hummingbird phenotype is related to the oncogenic activity of CagA. Pathophysiological relevance for the hummingbird phenotype in gastric carcinogenesis has also been revealed by recent clinical observations that infection with H. pylori carrying CagA with stronger ability to induce the hummingbird phenotype is more closely associated with gastric carcinoma (20–23). The hummingbird phenotype requires CagA-mediated perturbation of SHP-2 and PAR1, deregulation of which are substantially involved in the oncogenic process. SHP-2 is a bona fide oncoprotein, gain-of-function mutations of which are associated with various human malignancies (28). Also, PAR1 plays an essential role in the development and maintenance of epithelial cell polarity, disruption of which is a hallmark of carcinoma. PAR1d have been suggested to be tumor suppressors of pancreatic carcinoma (70). Furthermore, inactivation of LKB1, an upstream activating kinase of PAR1, causes Peutz-Jeghers syndrome, which predisposes to the development of gastrointestinal tumors (71). Thus, molecular mechanisms underlying induction of the hummingbird phenotype by CagA may also be critically involved in in vivo tumorigenesis by CagA. Because hummingbird cells display elevated motility with reduced cell-cell

**FIGURE 10.** Effect of myosin II inhibitor, blebbistatin, on induction of the hummingbird phenotype by CagA. A, AGS cells were treated with blebbistatin (Bleb), a myosin II-specific inhibitor, at the final concentration of 10 μM for 5 h. B, AGS cells were transfected with an expressing vector for HA-tagged CagA (CagA-ABD) or control vector. At 12 h post-transfection, cells were treated with or without a suboptimal concentration of blebbistatin (1 μM) for 5 h. Cells were then fixed and immunostained with an anti-HA antibody (green). F-actins were visualized by staining cells with rhodamine-phalloidin (red). Merged images of anti-HA (green) staining and rhodamine-phalloidin (red) staining are presented. Bar represents 50 μm. C, induction of hummingbird cells by CagA in the presence or absence of 1 μM blebbistatin. Bar graphs show the percentages of hummingbird cells in 600 CagA-positive cells shown in B. Experiments were independently performed three times, and the data were analyzed by Student’s t test. Error bars represent mean ± S.E.; *, p < 0.05; **, p < 0.01. D, effect of myosin II inhibition on the length of protrusions in hummingbird cells. Box and whisker plots show the length of protrusions of hummingbird cells induced by CagA in the presence or absence of 1 μM blebbistatin shown in B. In the plots, a box with ends indicates the points for the first and third quartiles; horizontal line through the box shows the median point; the whiskers (lines) are from each end of the box to the smallest and largest values. For Student’s t test, *, p < 0.05, n = 60. E, percentages of hummingbird cells with extremely long protrusions (>129 μm) in cells expressing CagA-ABD with or without blebbistatin treatment are shown. χ² test, error bars represent mean ± S.E.; *, p < 0.05, n = 60.
and cell-substrate interactions, the morphogenetic activity of CagA may further contribute to invasion and metastasis of gastric carcinoma cells.

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