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RESEARCH ARTICLE

Helicobacter pylori CagA inhibits endocytosis of cytotoxin VacA in host cells

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SUMMARY

Helicobacter pylori, a common pathogen that causes chronic gastritis and cancer, has evolved to establish persistent infections in the human stomach. Epidemiological evidence suggests that *H. pylori* with both highly active vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA), the major virulence factors, has an advantage in adapting to the host environment. However, the mechanistic relationship between VacA and CagA remains obscure. Here, we report that CagA interferes with eukaryotic endocytosis, as revealed by genome-wide screening in yeast. Moreover, CagA suppresses pinocytic endocytosis and the cytotoxicity of VacA in gastric epithelial cells without affecting clathrin-dependent endocytosis. Our data suggest that *H. pylori* secretes VacA to attack distant host cells while injecting CagA into the gastric epithelial cells to which the bacteria are directly attached, thereby protecting these attached host cells from the cytotoxicity of VacA and creating a local ecological niche. This mechanism might allow *H. pylori* to balance damage to one population of host cells with the preservation of another, allowing for persistent infection.

INTRODUCTION

Helicobacter pylori is present in approximately half of the human population worldwide and often persists as a stomach infection throughout an infected individual's lifetime. During decades of persistent infection, *H. pylori* can cause gastric diseases such as chronic gastritis, peptic ulcers, and cancer (Blaser and Atherton, 2004; Fukase et al., 2008) that are, at least in part, caused by two well-known virulence factors: vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA). VacA is secreted from *H. pylori* and internalized into host cells by endocytosis; this toxin causes cell vacuolation, cell death, an increase in the permeability of gastric epithelium (Cover and Blaser, 1992; Papini et al., 1998; Galmiche et al., 2000), and gastric ulcers in vivo (Telford et al., 1994; Fujikawa et al., 2003). VacA is also reported to suppress host defense mechanisms (Molinari et al., 1998; Gebert et al., 2003). CagA is an effector protein that is injected into host cells through the type IV secretion system (T4SS); *cagA* and the components of T4SS are encoded by a 40 kb gene cluster known as the *cag* pathogenicity island (*cag*PAI) (Censini et al., 1996). Injected CagA is tyrosine-phosphorylated by Src family kinases, and it associates with various host proteins such as SHP2 and GRB2, resulting in functional and morphological changes in host cells (Backert and Selbach, 2008). It has been proposed that CagA activates a growth-factor-like response (Segal et al., 1999) and disrupts polarity (Amieva et al., 2003) in host cells, thus promoting the survival and motility of the gastric epithelial cells that provide the ecological niche for *H. pylori* (Rieder et al., 2005; Tan et al., 2009). CagA-activated signaling might also account for the pathogenic potential of *H. pylori* in diseases such as cancer. Indeed, *cag*PAI is often identified in strains isolated from patients with severe gastric disease, and CagA has been used as a marker of virulent strains (Blaser et al., 1995).

VacA proteins exhibit considerable sequence variation in the N-terminal signal peptide (s-region), with the two versions termed s1 and s2, and in the middle region (m-region), with versions termed m1 and m2. The s1-m1 VacA has strong vacuolating activity in a wide range of cell types, whereas s1-m2 VacA shows vacuolating activity in a more limited range of cell types. The s2 VacA is virtually nontoxic in cell culture experiments (Cover and Blanke, 2005). Interestingly, epidemiological data have revealed a strong correlation between *cagA* and the virulent type of *vacA*. Most of the strains with s1 VacA showing high vacuolating activity are also positive for CagA, whereas those with s2 VacA lacking vacuolating activity are mostly negative for CagA (Atherton et al., 1995; Van Doorn et al., 1999). Despite these strong correlations, *vacA* and *cag*PAI are physically far apart in the genome of *H. pylori* (Tomb et al., 1997), suggesting that a functional connection between VacA and CagA is responsible for the coincidence of s1 VacA and CagA. *H. pylori* seems to have evolved to maximize genetic diversity by mutation, recombination, and shuffling of the genetic information within the population as an adaptation mechanism (Blaser and Atherton, 2004). Considering the relatively large size of *cag*PAI (40 kb), the physical distance between the *vacA* and *cag*PAI loci, and the extensive genetic reshuffling observed in *H. pylori*, it is likely that there is a selective pressure that results in the concurrence of intact *cag*PAI and highly active VacA genotypes. Although reports have shown functional antagonism between VacA and CagA in host cells in the processes of NFAT (nuclear factor of activated T cells) signaling (Yokoyama et al., 2005), epidermal growth factor (EGF)

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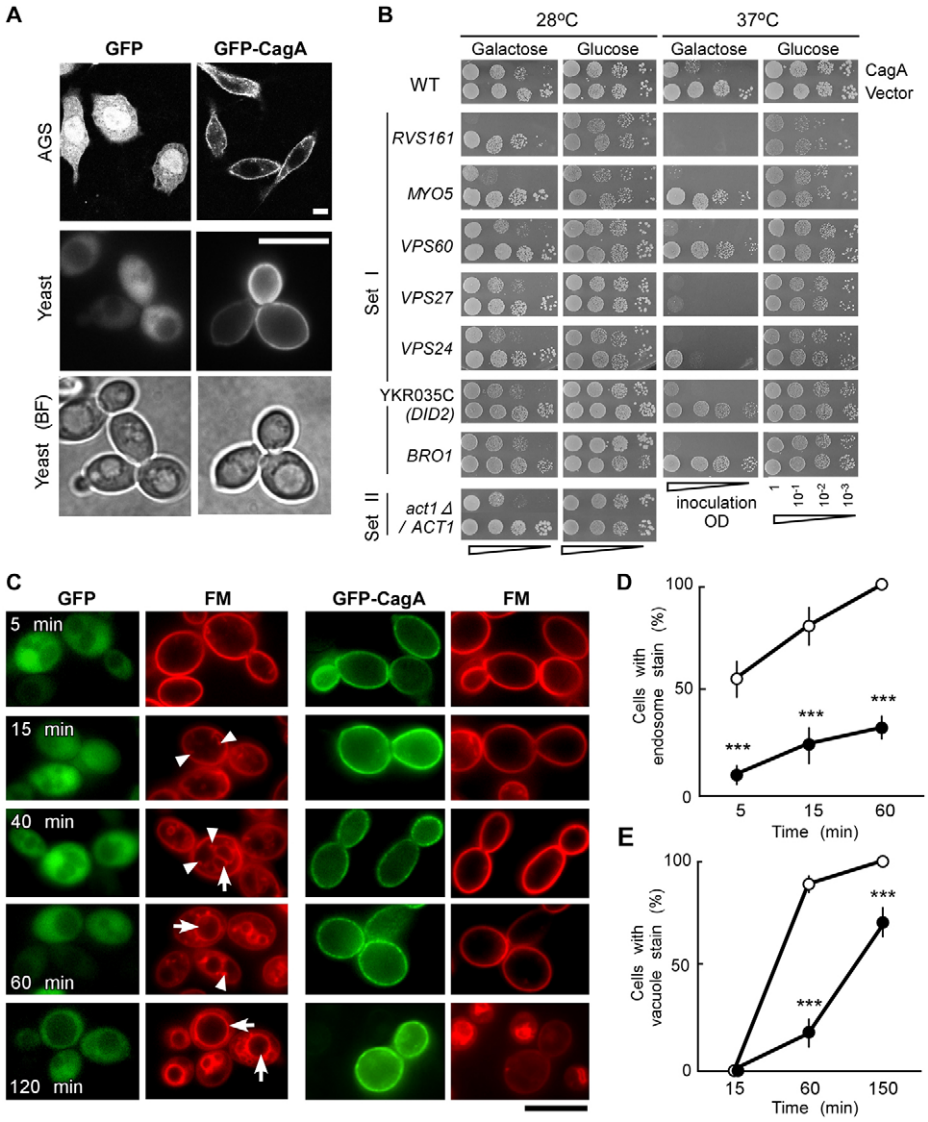

Fig. 1. Cellular localization of CagA and its effect on growth and endocytosis in yeast. (A) Localization of GFP-CagA or the GFP control in AGS cells or in yeast by fluorescence or bright-field (BF) microscopy 1 day after induction. Scale bars: 5 µm. (B) The effect of CagA on cell growth is shown for wild-type yeast or endocytosis-related yeast mutants as identified by screening using the homozygous (Set I) or heterozygous (Set II) deletion collections. Wild-type or mutant cells were spotted on galactose (CagA-inducing) or glucose (CagA-repressing) plates; the dilution series, as indicated by wedges at the bottom, started at 1 OD on the left, and the cell suspensions were diluted by a factor of ten progressing to the right. The plates were incubated at either 28°C or 37°C for 6 days. Yeast cells transformed with an empty vector served as a negative control. (C) The cellular localization and the effect of CagA on yeast endocytosis are shown by the fluorescent membrane dye FM4-64 (FM) and GFP. The cells were stained with FM4-64 on ice, and endocytosis was observed at the indicated time points after shifting the cells to 25°C. Yeast cells in the left two columns expressed GFP; those in the right two columns expressed GFP-CagA. Arrowheads indicate endosomes, and arrows indicate vacuoles. Scale bar: 5 µm. (D,E) Quantitative analysis of the endosomes (D) and vacuoles (E) shown in wild-type yeast expressing GFP (white circles) or GFP-CagA (black circles). Each point represents the average frequency ± s.e.m. from six independent observations of 20–60 cells each. ***P<0.001 compared with GFP-expressing cells.

receptor signaling (Tegtmeyer et al., 2008), and morphological change (Asahi et al., 2003; Argent et al., 2008), the underlying mechanism of the interplay between CagA and VacA remains obscure.

We performed genetic screening to identify the mechanism of action of CagA in eukaryotic cells, using the gene-knockout collection of *Saccharomyces cerevisiae* (Giaever et al., 2002) that has been utilized to elucidate the function of bacterial virulence factors such as IpgB2 and OspF of *Shigella* (Alto et al., 2006; Kramer et al., 2007). Here, we report that CagA inhibits endocytosis in yeast cells and human gastric epithelial cells. In the gastric epithelial cell line AGS, CagA specifically inhibited clathrin-independent pinocytic endocytosis, the mechanism by which VacA enters the host cells, without affecting clathrin-dependent endocytosis. Through this mechanism, CagA suppresses the internalization and the function of VacA in the host cells. Based on these findings, we propose that CagA works as a molecular shield against the deleterious effect of VacA in a subset of host epithelial cells, thus maintaining a favorable ecological niche for *H. pylori*.

RESULTS

Genome-wide screening of CagA function

In an effort to identify the biological process in eukaryotes that is disrupted by CagA, we chose a genetic screening system with a series of *Saccharomyces cerevisiae* strains that each carry a homozygous deletion of one of 4792 nonessential genes. The deficiency of a single gene in a yeast strain might compromise a specific biological process even though the corresponding strain is still viable. If CagA interferes with the specific biological process compromised by a particular deletion, the strain is expected to be hypersensitive to CagA toxicity. This screening is analogous to the synthetic lethality screening that uses double mutants, and therefore should identify the biological process compromised by CagA, but not necessarily the direct molecular targets of CagA.

A virulent East Asian type of the *cagA* gene (Azuma et al., 2004) derived from *H. pylori* CPY2052 was fused to the C-terminus of the green fluorescent protein gene to produce GFP-CagA. When expressed in AGS gastric epithelial cells (Fig. 1A), GFP-CagA

Table 1. Genes of yeast deletion strains with hypersensitivity to CagA

Set[a]	Gene[b]	CagA sensitivity[c] 28˚C	CagA sensitivity[c] 37˚C	Gene product (human orthologs[d])	Gene category[e]	Endocytosis-related classification[f]
–	Wild type	+	++	–	–	–
I	*MYO5*	+++	++++	Type I myosin (MYO1A-H)	TP, VT	Cortical patch
II	*ACT1*	++	NT	Actin (ACTB, ACTG)	TP, VT	Cortical patch
I	*RVS161*	++++	ND	Amphiphysin (AMPH1)	TP, VT	Cortical patch
I	*VPS27*	++	ND	ESCRT-0 complex (HRS/HGS)	TP, VT	Class E Vps
I	*VPS24*	++	ND	ESCRT-III complex (CHAMP3)	TP, VT	Class E Vps
I	*DID2*	++	+++	ESCRT-III complex (CHAMP1A, CHAMP1B)	TP, VT	Class E Vps
I	*VPS60*	++	++++	ESCRT-III complex (CHAMP5)	TP, VT	Class E Vps
I	*BRO1*	+	++++	Accessory of ESCRT complex (ALIX/AIP1/PDCD6IP, PTPN23)	TP, VT	Class E Vps
I	*BEM2*	++	+++	Rho GTPase-activating protein (ARFGAP22, ARFGAP24)	Others	–
I	*FET3*	+	+++	Ferro-O_2-oxidoreductase (–)	TP	–
I	*FLR1*	++	+++	Multidrug transporter activity (–)	TP	–
I	*HRK1*	++	+++	Protein kinase involved in ion homeostasis (–)	Others	–
I	*ROX1*	+	++++	Heme-dependent repressor of hypoxic genes (SOX18)	Others	–
I	*POP2*	++	+++	Exonuclease (CNOT7)	Others	–
I	*RAD51*	+	+++	Strand-exchange protein (RAD51A)	Others	–
I	*NUP188*	+	+++	Subunit of the nuclear pore complexes (–)	TP	–
I	*SET5*	++	+++	Zinc-finger protein (–)	UK	–
I	*ZUO1*	++	+++	Chaperone (DNJC2)	Others	–
I	*RPL43A*	++	+++	Large ribosome subunit (RPL37A)	Others	–

[a]Set of deletion strains. Set I: a set of 4792 strains with homozygous deletions in nonessential genes. Set II: a set of 1139 strains with heterozygous deletions in essential genes.

[b]Gene deleted in each deletion strain. *DID2* and *RPL43A* are partially deleted, because the corresponding strains were created by deleting the overlapping YKR035C and YPR044C, respectively, on the opposite strand.

[c]CagA sensitivity at Step 4 of Set I and at Step 3 of Set II in the screening process as described (supplementary material Fig. S2). ND, CagA sensitivity could not be determined because the cells did not grow on galactose plates even without induction; NT, not tested.

[d]Potential human orthologs, referred to in P-POD (http://ppod.princeton.edu/cgi-bin/ppod.cgi) or by Williams and Urbe (Williams and Urbe, 2007). A dash (–) indicates no known ortholog in mammals.

[e]Gene categories of biological processes determined using the SGD (http://www.yeastgenome.org) super gene ontology slim mapper tool of 'process'. Categories are indicated: TP, transport; VT, vesicle-mediated transport; others, other categories; UK, biological process unknown.

[f]Endocytosis-related gene product classification: cortical patch, proteins localized in the actin cortical patch where endocytic invagination begins; Class E Vps, Class E vacuolar protein sorting (Vps) proteins required to sort cargo proteins from early endosome to multivesicular body in late endosome.

localized to the cell periphery, underwent tyrosine phosphorylation, and induced cell elongation as previously reported (Segal et al., 1999), indicating that the GFP-fused CagA is functionally active. We expressed GFP-CagA or the GFP control in wild-type yeast under the control of the *GAL10* promoter, a galactose-inducible and glucose-repressible promoter. GFP-CagA localized to the periphery of the yeast cells, whereas GFP alone was distributed throughout the cytoplasm (Fig. 1A). GFP-CagA did not induce morphological changes in yeast (Fig. 1A), nor did it undergo tyrosine phosphorylation (supplementary material Fig. S1), probably because yeast does not have a Src-family kinase (Manning et al., 2002). Induction of CagA inhibited the growth of yeast moderately at 28°C and severely at 37°C (Fig. 1B, WT).

We next screened all 4792 deletion strains by expressing CagA in each strain; we identified 18 strains with hypersensitivity to the CagA-induced growth defects (Set I in Table 1; Fig. 1B; supplementary material Figs S2, S3; see Methods). Strikingly, half of the 18 genes identified are implicated in transport (*RVS161*, *MYO5*, *VPS60*, *NUP188*, *FET3*, *VPS27*, *VPS24*, *BRO1* and *DID2*) according to the gene ontology categories in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/). Five of these genes (*VPS60*, *VPS27*, *VPS24*, *BRO1* and *DID2*) encode Class E vacuolar protein

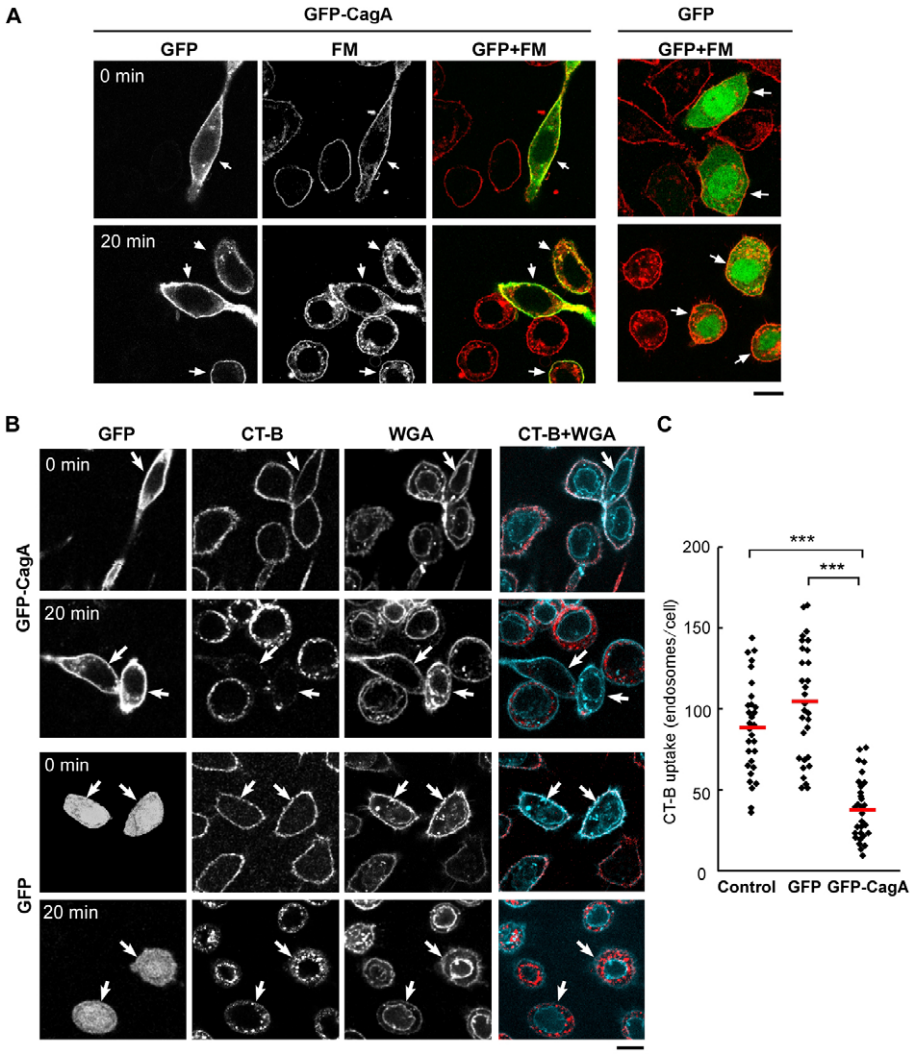

Fig. 2. Effects of CagA on endocytosis in AGS cells. (A) The effect of CagA on endocytosis is shown by staining AGS cells expressing GFP-CagA or GFP (green in merged images) with the membrane dye FM4-64FX (FM, red) on ice and fixing either before (0 minutes) or 20 minutes after the temperature shift to 37°C. Arrows indicate cells with GFP signal. (B) The effect of CagA on endocytosis is shown by visualizing fluorescently labeled CT-B (red) in AGS cells expressing GFP-CagA or GFP. The cells were incubated with CT-B on ice and fixed before (0 minutes) or 20 minutes after the AGS cells were shifted to 37°C. Cells were also stained with fluorescent WGA (cyan) to visualize the plasma and nuclear membranes. Arrows indicate cells with GFP signal. Scale bar: 5 μm. (C) Quantitative analysis is shown for the number of CT-B-positive endosomes per cell in cells without (Control) or with expression of GFP or GFP-CagA, 20 minutes after the temperature shift. The plasma membrane was stained with WGA to distinguish the cytoplasmic CT-B-positive endosomes (red) from those on the plasma membrane. The CT-B-positive endosomes were counted in the series of 1 μm z-sections in each cell; 30 cells were counted in each experimental group. Red bars indicate the median number of endosomes per cell. ***P<0.001.

sorting (Vps) proteins required for the formation of late endosomes and vesicular transport to vacuoles, the yeast equivalent of mammalian lysosomes (Katzmann et al., 2002; Williams and Urbe, 2007), whereas the *RVS161* and *MYO5* gene products are involved in the initial step of endocytosis (Kaksonen et al., 2006). We performed another set of screening with a series of heterozygous deletion strains for 1139 essential genes, resulting in the identification of a single strain with CagA hypersensitivity (Set II in Table 1 and Fig. 1B; supplementary material Fig. S2; see Methods). This strain harbored a heterozygous deletion of *ACT1*, an actin-encoding gene involved in endocytosis and vesicular transport (Kaksonen et al., 2006). Hence, eight of the 19 genes (Table 1) identified in our screening were associated with endocytosis pathways.

CagA expression inhibits endocytosis in yeast

To test the effects of CagA on yeast endocytosis more directly, we monitored endocytosis in wild-type yeast cells expressing either GFP or GFP-CagA by staining with a membrane-lipid probe, FM4-64. Cells expressing the control GFP construct readily formed endocytic vesicles (endosomes) upon shifting from 0°C to 25°C as shown by the FM4-64 staining (Fig. 1C, arrowheads). Within 60 minutes of the temperature shift, FM4-64 fluorescence was apparent in the membranes of vacuoles (Fig. 1C, arrows). By contrast, very few endosomes appeared in cells expressing GFP-CagA after 60 minutes, and vacuoles were only weakly stained (Fig. 1C). Quantitative analysis of the stained endosomes (Fig. 1D) and vacuoles (Fig. 1E) showed that GFP-CagA expression dramatically reduces the trafficking of FM4-64 from the plasma membrane to endosomes and ultimately to vacuoles. The low frequency of stained endosomes in GFP-CagA-expressing cells indicates that CagA inhibits an early stage of endocytosis in yeast, consistent with the localization of CagA at the plasma membrane.

CagA expression inhibits pinocytic endocytosis in AGS cells

The endocytosis machinery of yeast and mammalian cells share many homologous molecules. In fact, all the endocytic genes identified in our screening have mammalian orthologs (Table 1). We examined the endocytic activities of AGS cells expressing either GFP or GFP-CagA, using FM4-64FX as a membrane-lipid probe. In contrast to our observations in yeast, GFP-CagA did not have an obvious impact on the formation of FM4-64FX-positive endosomes in AGS cells (Fig. 2A). Mammalian cells possess multiple endocytic pathways that are distinguished from one another by the vesicle cargo and the dependence of the endocytic

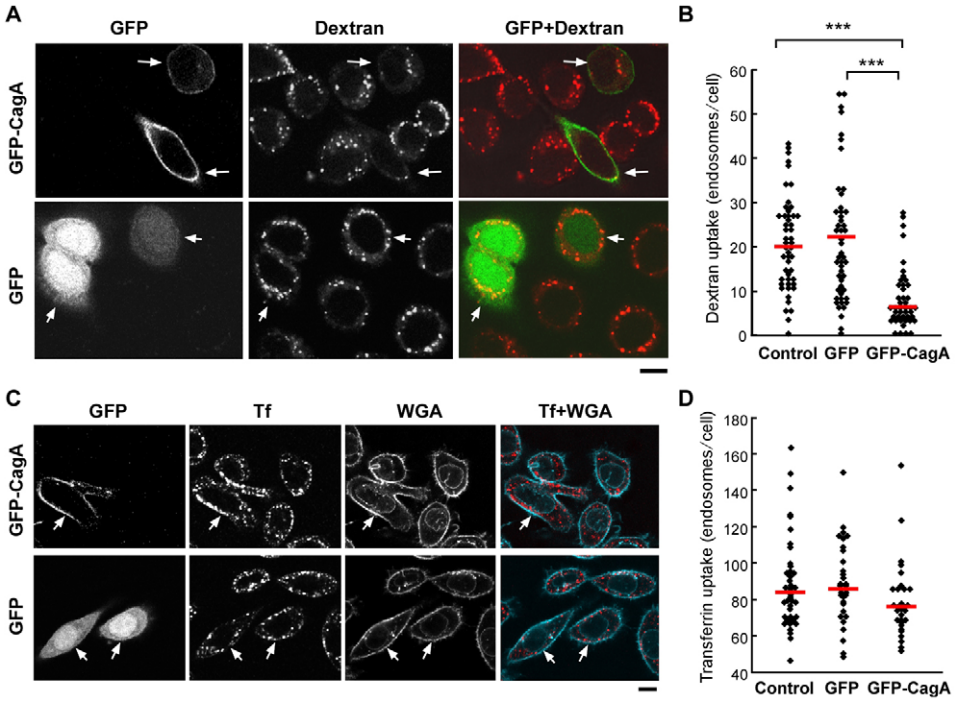

Fig. 3. Effect of CagA on the endocytosis of dextran and transferrin in AGS cells. (A) The effect of CagA on the endocytosis of dextran is shown by the fluorescently labeled dextran (red in the merged images) in AGS cells expressing GFP-CagA or GFP (green, arrows), before and 20 minutes after the temperature shift from 0°C to 37°C. (B) Quantitative analysis for the number of dextran-positive endosomes per cell in cells without (Control) or with expression of GFP or GFP-CagA, 20 minutes after the temperature shift. The dextran-positive endosomes were counted in the series of 1 μm z-sections in each cell; 40 to 50 cells were counted in each experimental group. Red bars indicate the median number of endosomes per cell. ***P<0.001. (C) The effect of CagA on endocytosis of transferrin (Tf) is shown by fluorescently labeled Tf (red in merged images) in AGS cells expressing GFP-CagA or GFP (green, arrows), before and 20 minutes after the temperature shift from 0°C to 37°C. The cells were also stained with WGA (cyan) in the same way as in Fig. 2B. Arrows indicate cells with GFP signal. (D) Quantitative analysis of Tf-positive endosomes assessed in the same way as in Fig. 2C, with WGA staining to distinguish cytoplasmic Tf-positive endosomes (red) from those on the plasma membrane. 30 to 40 cells were counted in each experimental group. Red bars indicate the median number of endosomes per cell. Scale bars: 5 μm.

mechanism on certain molecules, such as clathrin (Mayor and Pagano, 2007). We next examined the effect of CagA on the endocytosis of cholera toxin subunit B (CT-B), which is internalized by a clathrin-independent mechanism. Incubation of AGS cells with the fluorescently labeled CT-B at 0°C resulted in attachment of CT-B to the plasma membrane. Temperature shift from 0°C to 37°C caused rapid internalization of CT-B within 20 minutes, resulting in the formation of multiple CT-B-positive endosomes (Fig. 2B). Expression of GFP did not have an obvious impact on CT-B binding to the plasma membrane or subsequent endocytosis. Expression of GFP-CagA did not have an effect on the initial binding of CT-B to the plasma membrane; however, relative to the controls, AGS cells expressing GFP-CagA formed fewer CT-B-positive endosomes within the first 20 minutes after the temperature shift (Fig. 2C), in sharp contrast to the endocytosis observed using FM4-64FX. These results suggest that CagA might inhibit a subset of endocytic pathways in AGS cells (Fig. 2B,C).

CT-B is mainly internalized by a clathrin-independent endocytosis pathway characterized by fluid-phase-marker cargos (Mayor and Pagano, 2007). We examined the effect of GFP-CagA on the uptake of dextran, a fluid-phase marker (Sabharanjak et al., 2002). Expression of CagA significantly inhibited the formation of dextran-positive endosomes compared with the neighboring cells without GFP-CagA expression (Fig. 3A,B). GFP-expressing cells showed no difference in the formation of dextran-positive endosomes compared with nontransfected cells (Fig. 3A,B). We also examined endocytic activities of AGS cells using transferrin, a classical clathrin-dependent endocytosis cargo molecule (Fig. 3C,D). Consistent with the notion that CagA inhibits a subset of mammalian clathrin-independent endocytosis pathways, GFP-CagA did not cause an obvious delay in the uptake of transferrin. Taken together, these observations indicate that CagA inhibits clathrin-independent endocytosis of the fluid-phase marker dextran, without disrupting clathrin-dependent endocytosis of transferrin. In this study, we use the term pinocytic endocytosis, which is operationally defined by the internalization of the fluid-phase marker dextran, for the CagA-inhibited endocytosis pathway. Although this pinocytic endocytosis is poorly characterized and might be heterogeneous in nature (Mayor and Pagano, 2007), it has been proposed that it might represent a primordial endocytic pathway similar to yeast endocytosis (Kirkham and Parton, 2005).

CagA inhibits endocytosis of VacA

It has been reported that VacA is internalized into host cells by pinocytic endocytosis, as demonstrated by the co-localization of internalized VacA with the fluid-phase marker dextran (Gauthier

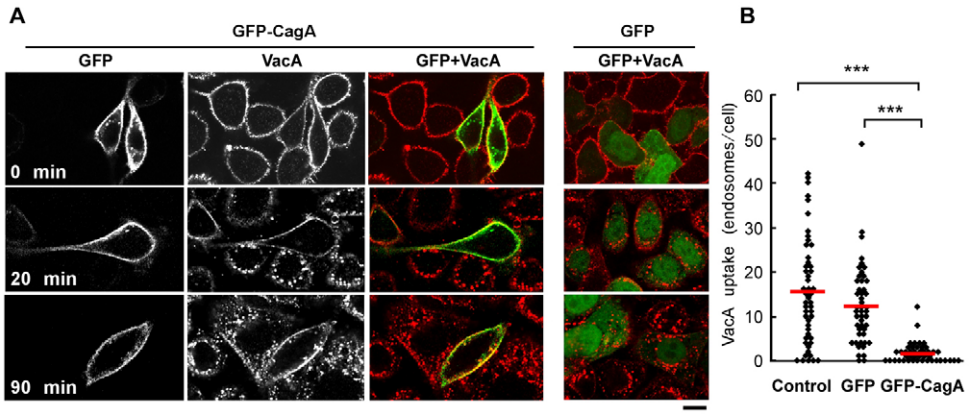

Fig. 4. Effect of CagA on VacA endocytosis in AGS cells. (A) Fluorescence images are shown for VacA (red in merged images) uptake in AGS cells expressing GFP-CagA or GFP (green). The cells were incubated with purified VacA for the indicated time after the temperature shift from 0°C to 37°C. Scale bar: 5 µm. (B) Quantitative analysis is shown for VacA-positive endosomes in the same way as in Fig. 2C (supplementary material Fig. S5). VacA-positive endosomes were counted in AGS cells without (Control) or with expression of GFP or GFP-CagA, 20 minutes after the temperature shift. 40 to 50 cells were counted in each experimental group. Red bars indicate the median number of endosomes per cell. ***$P<0.001$.

et al., 2005). Therefore, we examined whether CagA inhibits endocytosis of VacA. We incubated AGS cells expressing GFP or GFP-CagA with purified s1-m1 VacA and monitored endocytosis of VacA using anti-VacA antibodies and indirect immunofluorescence. Incubation of AGS cells with VacA at 0°C resulted in the attachment of VacA to the plasma membrane of GFP-expressing, GFP-CagA-expressing, and nontransfected cells (Fig. 4A; supplementary material Fig. S4). VacA began to enter the GFP-expressing and nontransfected cells 20 minutes after the temperature shift from 0°C to 37°C, as shown by the VacA-positive endosomes just beneath the plasma membrane. By contrast, VacA remained attached to the plasma membrane of GFP-CagA-expressing cells at 20 minutes (Fig. 4A; supplementary material Fig. S4). At 90 minutes, VacA was localized to numerous endosomes throughout the cytoplasm in GFP-expressing and nontransfected cells. By contrast, VacA remained mostly attached to the plasma membrane of the GFP-CagA-expressing cells, and only a few VacA-positive endosomes were observed in the cytosol of these cells (Fig. 4A; supplementary material Fig. S4). Quantitative data at 20 minutes clearly indicated that AGS cells expressing GFP-CagA formed significantly fewer VacA-positive endosomes compared with GFP-expressing or nontransfected cells (Fig. 4B). Most of the VacA-positive endosomes co-localized with dextran in AGS cells (supplementary material Fig. S5), consistent with a previous report (Gauthier et al., 2005) and our finding that CagA suppresses endocytosis of dextran. These observations indicate that CagA in AGS cells inhibits pinocytic endocytosis of VacA without affecting VacA attachment to the plasma membrane.

Recent studies suggested that ectopically expressed CagA might behave differently from native CagA injected by *H. pylori* through T4SS (Higashi et al., 2002; Amieva et al., 2003; Kwok et al., 2007). We therefore examined the effect of *H. pylori*-injected CagA on VacA endocytosis. We generated various isogenic deletion mutants of *H. pylori* for *cagA* (V+C–), *vacA* (V–C+) or both (V–C–) from wild-type *H. pylori* CPY2052, which produces a virulent-type s1-m1 VacA (supplementary material Fig. S6) and CagA (V+C+). Immunoblot analyses showed that deletion of either *vacA* or *cagA* did not affect the expression level of the other (Fig. 5A). Infection of AGS cells with the *cagA*$^+$ (C+) strains resulted in the appearance of tyrosine-phosphorylated protein in the infected cells that corresponded to the molecular weight of CagA (Fig. 5A), indicating the successful injection of CagA from *H. pylori* into AGS cells. We stained AGS cells for VacA 5 hours after the infection with either V+C+ or V+C– (Fig. 5B). VacA from V+C+ mostly stayed on the plasma membrane of AGS cells (Fig. 5B, arrowheads), and showed limited cytoplasmic vesicular staining, 5 hours after the infection. By contrast, AGS cells with V+C– infection showed strong cytoplasmic vesicular and vacuole membrane staining of VacA (Fig. 5B, arrows). We obtained similar results when we used the Western *H. pylori* strain NCTC11637, which possesses a Western-type *cagA* gene (Azuma et al., 2004) (supplementary material Fig. S7). These findings suggest that VacA stays on the plasma membrane in the presence of CagA at least for some time, whereas VacA rapidly enters host cells in the absence of CagA.

In the infection experiments described above, two processes take place at the same time: the uptake of *H. pylori*-secreted VacA by AGS cells and the accumulation of *H. pylori*-injected CagA in AGS cells. The CagA-mediated inhibition of endocytosis presumably depends on the accumulation of CagA in the host cells. This accumulation of intracellular CagA might take some time, and therefore CagA-mediated inhibition of VacA endocytosis might not be obvious at an early time point in the infection. To segregate these two processes, we first infected AGS cells for 2 days either with V–C+ to let *H. pylori* inject CagA into AGS cells or with V–C– as a negative control. Immunoblot analyses showed that infection of AGS cells with V–C+ resulted in the appearance of tyrosine-phosphorylated CagA, confirming the successful injection of CagA by V–C+ *H. pylori* under our experimental conditions (supplementary material Fig. S8). We then added gentamycin to eliminate *H. pylori*, and purified VacA to monitor VacA endocytosis (Fig. 5C). Immunostaining of VacA in AGS cells with V–C– infection showed VacA-positive endosomes as early as 1 hour after the addition of purified VacA (Fig. 5C, right panels). VacA staining was apparent on the membranes of cytosolic vacuoles (arrows) that

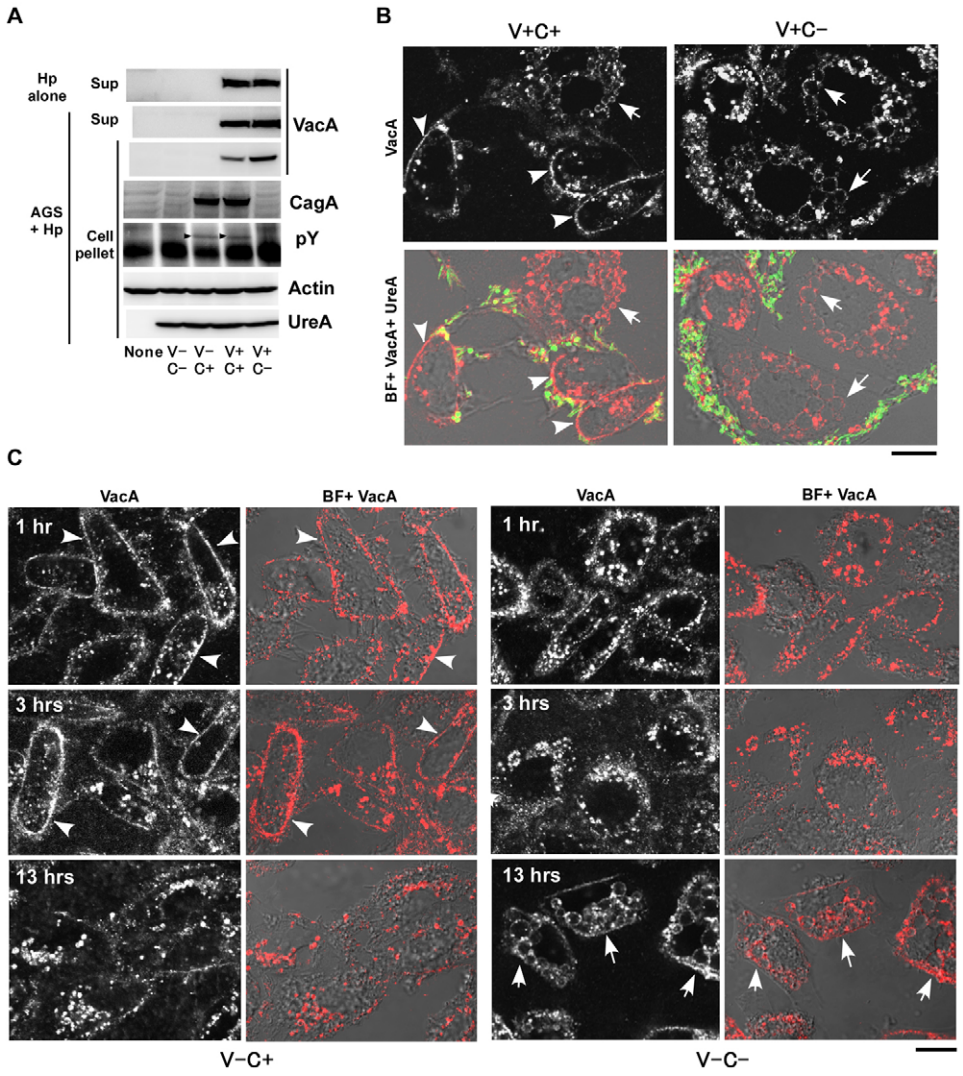

Fig. 5. Effect of *H. pylori*-injected CagA on VacA endocytosis in AGS cells.
(A) Immunoblot analyses are shown for the *H. pylori*-derived proteins in the infection experiments. Wild-type *H. pylori* CPY2052 (V+C+) and isogenic deletion mutants of *cagA* and *vacA* (V–C–), *vacA* (V–C+) and *cagA* (V+C–) were cultured without (Hp alone) or with AGS cells (AGS+Hp) for 5 hours. 'None' indicates AGS cells without *H. pylori*. The AGS+Hp cell pellets were analyzed by immunoblotting for VacA, CagA, phosphotyrosine (pY), actin and urease (UreA). The Hp and AGS+Hp-culture supernatants (Sup) were analyzed by immunoblotting for VacA. Arrowheads indicate the tyrosine-phosphorylated band corresponding to intracellular CagA. UreA and actin served as internal controls for the number of *H. pylori* and AGS cells, respectively. (B) VacA was visualized by immunostaining in AGS cells infected with V+C+ or V+C– *H. pylori* for 5 hours. VacA in AGS cells (red) was distinguished from that in *H. pylori* by co-staining with the *H. pylori*-specific UreA (green). (C) VacA (red in the bright-field images) was visualized by immunostaining at the indicated time after the addition of purified VacA (2 μg/ml) in AGS cells previously infected with V–C– or V–C+ *H. pylori* for 2 days. Arrowheads indicate VacA on the plasma membrane of AGS cells. Arrows indicate VacA on the vacuole membrane. Scale bars: 5 μm.

were formed 13 hours after the addition of purified VacA. By contrast, VacA staining in AGS cells with V–C+ infection showed the accumulation of VacA predominantly on the plasma membrane (Fig. 5C, left panels, arrowheads) at 1 hour and 3 hours after the addition of purified VacA. VacA then moved to endosomes by 13 hours. We also noticed that 13 hours after the addition of purified VacA, AGS cells infected with V–C+ showed much less vacuolation than the cells infected with V–C–. These observations indicate that native CagA, injected by *H. pylori*, inhibits VacA entry into the host cells, and might inhibit the VacA-mediated vacuolation of the host cells.

CagA inhibits VacA function in AGS cells

We next examined whether the inhibition of VacA endocytosis by CagA had any impact on the microbe-host interaction in terms of VacA function in host cells. We performed an assay of Neutral Red uptake to quantify vacuolation, the best-characterized VacA function, in infected cells. Infection of AGS cells with V+C+ *H. pylori* caused vacuolation as demonstrated by the high level of Neutral Red uptake. The V–-infected AGS cells showed low levels of Neutral Red uptake that were comparable to levels seen in the noninfected cells (Fig. 6A,B). Infection with V+C– *H. pylori* caused significantly more vacuolation than that with V+C+ *H. pylori*, and the difference remained significant during the observation period of 5 hours (Fig. 6B). To further analyze the effects of injected CagA on VacA functions, we infected AGS cells for 2 days with V–C+ to allow the bacteria to inject CagA into AGS cells or with V–C– as a negative control (Fig. 6C). We then eliminated the *H. pylori* with gentamycin and incubated the AGS cells with the VacA-containing culture supernatant of V+C+ *H. pylori* (VacA sup) to induce vacuolation (Fig. 6C, left). After treatment with VacA-containing *H. pylori* culture supernatant, AGS cells infected with V–C+ showed significantly lower vacuolation than those infected with V–C–. We obtained essentially identical results when we used purified VacA to induce vacuolation (Fig. 6C, right).

We also examined the effect of the injected CagA on the apoptosis-inducing activity of VacA in AGS cells. We infected AGS cells with V–C–, V–C+, V+C– or V+C+ *H. pylori* and determined apoptosis of AGS cells by nuclear condensation as detected by Hoechst 33342 staining (Fig. 6D,E). Infection with V+C+ *H. pylori* caused significantly more apoptosis than V–C+ (24.3±3.2% vs 17.4±3.5%, $P<0.05$), indicating the apoptosis was partly dependent

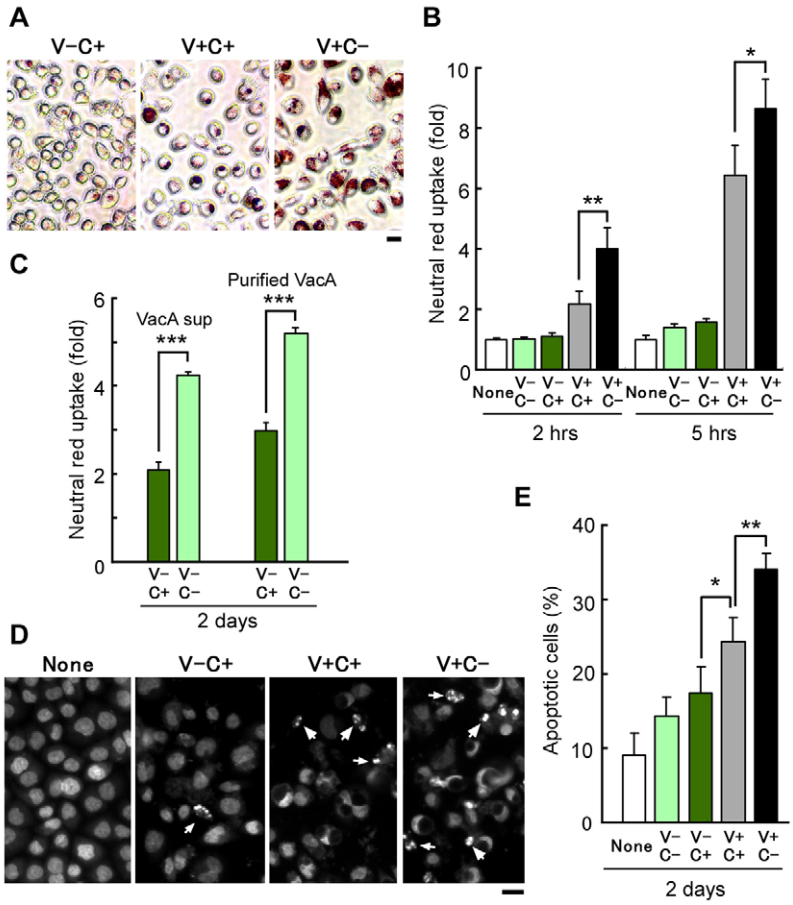

Fig. 6. Effect of *H. pylori*-injected CagA on the function of VacA. (A,B) The effect of *H. pylori*-injected CagA on the vacuolating activity of VacA is shown by the assays of Neutral Red uptake. Representative bright-field images (5 hours infection) (A) and quantitative analysis (B; 2 and 5 hours infection) of Neutral Red uptake are shown in AGS cells infected with the indicated *H. pylori* strains, followed by 16 hours culture with gentamycin to develop vacuoles. Scale bar: 10 μm. The value of Neutral Red uptake is expressed as the fold increase in uptake over the uptake in AGS cells without *H. pylori* (None), which is designated as 1. Error bars indicate s.e.m. from four independent observations. **P*<0.05, ***P*<0.01. (C) Quantitative vacuolation activity was assessed for VacA-containing *H. pylori* culture media (VacA sup) and purified VacA in AGS cells after 2 days infection with V–C– or V–C+. The value of the Neutral Red uptake was expressed as the fold increase over the uptake in the infected AGS cells without the addition of exogenous VacA. Error bars indicated s.e.m. from three independent observations. ****P*<0.001. (D,E) The effect of *H. pylori*-injected CagA was analyzed for the apoptosis-inducing activity of VacA. Apoptosis was detected as nuclear condensation made visible by Hoechst 33342 staining (D, arrows) and expressed as the percentage of apoptotic cells in AGS cells that had been infected with the indicated *H. pylori* strains for 2 days (E). Scale bar: 10 μm. Error bars indicate s.e.m. from four independent observations. **P*<0.05, ***P*<0.01.

on VacA, as previously reported (Kuck et al., 2001; Cover et al., 2003). Within the V+ strains, V+C– caused significantly more apoptosis than V+C+ (34.0±2.2% vs 24.3±3.2%, $P<0.01$), indicating that the presence of CagA suppresses the apoptosis-inducing function of VacA. Therefore, we concluded that *H. pylori*-injected CagA inhibits VacA entry to the cells and subsequent effects of VacA in AGS cells.

DISCUSSION

Genetic screening for CagA-hypersensitive strains in yeast resulted in the unexpected enrichment of genes encoding components of endocytic machinery, suggesting that CagA might interfere with endocytosis. Indeed, direct observation of endocytosis demonstrated that CagA significantly inhibited the early stage of endocytosis in yeast. Similarly, in AGS gastric epithelial cells, CagA inhibited the early stage of pinocytic endocytosis, a subset of the clathrin-independent endocytic pathways, without affecting clathrin-dependent endocytosis, probably reflecting the fact that mammalian cells have more-sophisticated endocytic pathways than yeast. Our findings indicate that CagA inhibits the pinocytic endocytosis of VacA into host cells and subsequent function of this cytotoxin. Because of this mechanism, CagA might suppress other endocytosis-dependent VacA functions in addition to the vacuolating and apoptosis-inducing activities of this multifunctional cytotoxin (Cover and Blanke, 2005).

It has been proposed that VacA allows *H. pylori* to obtain nutrients from host cells and to evade host-cell attacks by damaging them, thus facilitating colonization and/or persistent infection (Blaser and Atherton, 2004; Amieva and El-Omar, 2008). However, excessive damage to the gastric epithelium by VacA might be disadvantageous for *H. pylori*, because viable gastric epithelial cells produce a mucous layer, an essential ecological niche for *H. pylori* (Rieder et al., 2005; Amieva and El-Omar, 2008), and the epithelial cell surface provides a replicative niche for the bacteria (Tan et al., 2009). Therefore, *H. pylori* must have evolved ways of balancing the preservation of and the damaging of host cells in order to establish and maintain persistent infection (Blaser and Kirschner, 2007). Based on our findings, we propose that CagA works as a molecular shield against VacA in epithelial cells (supplementary material Fig. S9). When VacA-secreting *H. pylori* adheres to a host gastric epithelial cell, it injects CagA into that host cell; the CagA, in turn, protects that host cell from the excessive damage potentially caused by VacA. By contrast, host cells without CagA (epithelial cells without adherent *H. pylori*) and infiltrating immune cells are damaged by fully active VacA, allowing *H. pylori* to obtain nutrients from distant cells subjected to VacA-dependent damage and to fight host immune cells, while simultaneously maintaining its nearest gastric epithelial neighbors as viable host cells through injected CagA. This model provides a mechanistic explanation for the epidemiological linkage between CagA and the virulent-type VacA (Atherton et al., 1995; Van Doorn et al., 1999). Although the large *cag*PAI might be lost during chronic

infections in humans (Kersulyte et al., 1999; Suerbaum and Josenhans, 2007), highly active s1 VacA might provide a selection pressure that keeps *cag*PAI intact in the population. By contrast, *H. pylori* strains with less virulent s2 VacA would not experience such a strong selection pressure and are thus more prone to lose intact *cag*PAI because of the high rates of mutation and recombination in this pathogen (Blaser and Atherton, 2004; Suerbaum and Josenhans, 2007).

Apart from the inhibition of VacA function, CagA-mediated inhibition of pinocytic endocytosis would disturb the normal physiology of host cells, such as the turnover of cell-surface molecules. Indeed, it was recently reported that CagA sustains the cell-surface expression of EGF receptor by inhibiting its internalization (Bauer et al., 2009). We also observed that CagA inhibited the internalization of EGF receptor (supplementary material Fig. S10) as well as receptor tyrosine phosphatase α (RPTPα) (supplementary material Fig. S11), one of the VacA receptors. Notably, it has been reported that clathrin-independent endocytosis directs the EGF receptor to degradation, whereas clathrin-dependent endocytosis is essential for the sustained EGF signaling as this pathway recycles the EGF receptor back to the cell surface (Sigismund et al., 2008). Because CagA does not seem to interfere with clathrin-dependent endocytosis, it might selectively suppress the degradation of the EGF receptor without interfering with clathrin-dependent signaling. Inhibition of pinocytic endocytosis by CagA might also be involved in other effects of CagA, including the disruption of cell polarity and tight junctions (Backert and Selbach, 2008), as these host functions require normal endocytosis for their maintenance (Le et al., 1999; Yu and Turner, 2008). Whether CagA-mediated inhibition of pinocytic endocytosis is involved in these effects of CagA awaits further clarification of the molecular mechanisms by which CagA inhibits pinocytic endocytosis, a process that is poorly characterized at this time (Mayor and Pagano, 2007).

Interestingly, a functional linkage between VacA and CagA, complementary to our findings, has been reported. Specifically, highly active VacA suppresses CagA-induced elongation of AGS cells by inhibiting EGF receptor signaling (Tegtmeyer et al., 2008). We also observed that V–C+ *H. pylori* tended to cause more prominent cell elongation of AGS cells than V+C+ (data not shown). Very recently, Oldani et al. reported that forced expression of the C-terminal fragment of CagA interferes with the intracellular trafficking of VacA after endocytosis (Oldani et al., 2009). Combined with our findings, their results might suggest that CagA domain(s) outside of the C-terminal region might be required for the full suppression of VacA endocytosis. Nonetheless, their conclusion is consistent with ours in that CagA suppresses VacA-mediated cytotoxicity. Presumably, such functional antagonism would depend on the timing and the dynamic equilibrium of CagA accumulation and VacA uptake in host cells during chronic gastric infection with genetically heterogenous *H. pylori*. Therefore, the emerging theme is that these two major virulence factors are tightly coupled in their functions, and the balance of their activities is crucial for the successful adaptation of *H. pylori* to its host environment. Elucidation of the molecular mechanisms of CagA-mediated inhibition of pinocytic endocytosis will provide us with a better understanding of the biology of *H. pylori* and of the pathophysiology of the diseases associated with *H. pylori* infection, both of which are essential for developing new therapeutics and management strategies for *H. pylori*-associated diseases, including cancer.

METHODS

H. pylori and AGS cells

Helicobacter pylori strain CPY2052 was isolated from a gastric ulcer patient at Yamaguchi University Hospital, Japan (Tsuda et al., 1993). *H. pylori* strains CPY2052 and NCTC11637 were cultured on Brucella agar plates containing 5% or 10% fetal bovine serum (FBS) under 10% CO_2 at 37°C. AGS cells (ATCC CRL1739), which were derived from a human gastric adenocarcinoma, were grown in RPMI1640 medium containing 10% FBS (AGS medium) and transiently transfected with the indicated plasmids using FuGENE6 (Roche Diagnostics) for 1 day.

CagA gene and plasmids

The *cagA* gene was cloned from the genomic DNA of *H. pylori* strain CPY2052. The *cagA* fragment (GenBank accession numbers DQ091000 and AAZ23952 for cDNA and the deduced amino acid sequence, respectively) was inserted into the mammalian expression vector pEGFP-C1 (expression vector of GFP in AGS cells in the main text), producing plasmid pJ2022 (expression vector of GFP-CagA in AGS cells in the main text). For expression of CagA in yeast, the *cagA* gene from pJ2022 was inserted into p316GAL (Kawahata et al., 1999), producing plasmid pJ2082. The yeast-optimized GFP (yEGFP) cDNA (Cormack et al., 1997) from pK11 (a gift from Dr Susan C. Straley, University of Kentucky, USA) was inserted into the p316GAL to construct p316GAL-yEGFP (expression vector of GFP in yeast in the main text). The *cagA* gene from pJ2022 was subcloned into p316GAL-yEGFP to construct p316GAL-yEGFP-CagA, producing plasmid pJ2531 (expression vector of GFP-CagA in yeast in the main text). All of the constructs were confirmed by DNA sequencing at the Center for Gene Research, Yamaguchi University, Japan.

Yeast

The *Saccharomyces cerevisiae* BY4743 strain (*MAT***a***/MAT***a** *leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 his3Δ1/his3Δ1 met15Δ0/+ +/lys2Δ0*), the 4792 homozygous diploid deletion strains (#95401.H1R3) (described as Set I in this paper) and the 1139 heterozygous diploid deletion strains (#95401.H5R3) (Set II in this paper) were purchased from Research Genetics (AL, USA). Yeast cells were grown on standard YPD medium and dropout synthetic medium. One-by-one yeast transformation was performed as previously described (Chen et al., 1992). Transformed yeast cells were grown in uracil-dropout 2% raffinose (–U'Raf) liquid medium for 1 day at 28°C. 10 µl aliquots of yeast cell suspensions (with calculated OD_{600} of 1.0, 0.1, 0.01 and 0.001) were then spotted onto uracil-dropout medium plates containing 2% glucose (–U'Glc) or 2% galactose (–U'Gal) and incubated at either 28°C or 37°C for 3-6 days. For liquid culture, uracil-dropout 2% raffinose + 2% galactose (–U'Raf/Gal) medium was used. Synthetic complete medium containing glucose (SCGlc), galactose (SCGal) and raffinose (SCRaf) was used for the selection of deletion strains. Synthetic minimal medium containing only leucine, histidine and uracil (SM) was used for the confirmation of diploid strains.

Screening in yeast

Transformation of wild-type and deletion strains of *S. cerevisiae* was carried out with pJ2082 on 96-well plates using the *S. cerevisiae* Direct Transformation Kit (Kitagawa et al., 2007) (Wako, Osaka,

Japan). Transformed cells were selected with a 96-well-format picker (Genetyx), suspended in 25 µl of –U'Raf medium in 96-well plates, and cultured overnight at 28°C. The tenfold dilutions of these cultures were then spotted on –U'Gal plates and incubated at 28°C and 37°C for 5 days, and strains with lower growth rate than wild type were selected to obtain 124 strains of Set I and 28 strains of Set II (Step 1 of Set I and II; supplementary material Fig. S2). For further growth determination, strains grown in –U'Raf medium were spotted onto –U'Gal or –U'Glc plates as tenfold serial dilutions. The growth of each strain was given a score between 0 and 4, where 0 indicates that the original undiluted (OD 1.0) spot did not grow, 1 indicates the OD 1.0 spot grew but OD 0.1 spot did not, and 4 indicates the OD 0.001 spot grew. CagA-expressing strains with a growth score of 3 or less on –U'Gal plates were selected to obtain 94 strains from Set I and four strains from Set II (Step 2 of Set I and II). The 94 selected Set I deletion strains were grown without transformation at 28°C and 37°C on SCGal plates, and their growth was compared with that on SCGlc and SCRaf plates to assess their temperature sensitivities on galactose. Strains with slow growth on SCGal plates were eliminated, resulting in 72 strains from Set I (Step 3 of Set I). The growth score of the CagA-expressing strains on –U'Gal plates at day 4 was subtracted from that with the empty vector to obtain the CagA sensitivity score, which is expressed as –, +, ++, +++ and ++++ , as shown in Table 1, and the strains that scored ++ or more at 28°C and/or +++ or more at 37°C were selected to give a total of 23 strains from Set I and one strain from Set II (Step 4 of Set I and Step 3 of Set II). The remaining 23 strains of Set I were transformed with the uracil-complementing p316GAL vector, and the transformed strains were assessed for temperature sensitivity on –U'Gal at 28°C and 37°C to confirm strain quality following auxotrophic growth on SM medium. This step resulted in the elimination of two strains from Set I (Step 5 of Set I). The remaining 21 strains of Set I with the CagA-expressing plasmid were grown in 2 ml of –U'Raf or –U'Raf/Gal liquid media with vigorous shaking at 28°C or 37°C for 5 hours, and the protein expression level of CagA was monitored by comparing it with that of actin by immunoblotting. The CagA detection level was normalized using the actin level in each strain, where the calculated fold of the relative CagA:actin expression of each strain is 1 for wild type (supplementary material Fig. S3). If the strain value was more than 1.5-fold greater than the wild-type value at both 28°C and 37°C, we judged it as a high-CagA-expressing strain and eliminated it. After this process, 18 strains from Set I remained (Step 6 of Set I). The final 18 strains of Set I and one strain of Set II are indicated in Table 1.

Immunodetection

Proteins were detected by immunoblotting using antibodies for CagA (Austral Biologicals), phosphotyrosine (4G10, Upstate), actin (Santa Cruz Biotechnology), VacA (Yahiro et al., 1999) and UreA (Institute of Immunology).

Endocytosis assay

Endocytosis in yeast was assessed as reported previously (Vida and Emr, 1995). Briefly, the yeast strain BY4743 expressing either GFP or GFP-CagA was grown in 1 ml of –U'Raf liquid medium overnight, and a 1:20 dilution of the cell suspension was cultured in –U'Raf/Gal medium for 1 day. Cells were labeled with 40 µM FM4-64 (Invitrogen) in fresh medium for 30 minutes on ice. The cells were observed under a fluorescence microscope (Olympus) at 25°C. More than 30 cells expressing moderate levels of GFP or GFP-CagA from six independent colonies were counted to calculate the frequency of the indicated phenotypes.

For the endocytosis assay in mammalian cells, AGS cells expressing GFP or GFP-CagA were cultured on gelatin-coated cover slips, pre-chilled on ice with AGS medium supplemented with 5 mM HEPES (pH 7.4; HEPES medium) for 15 minutes. For the membrane-lipid probe FM4-64FX (Invitrogen), AGS cells were incubated with 4 µM FM4-64FX on ice for 30 minutes, followed by fixation immediately (0 minutes) or 20 minutes after the incubation at 37°C. For the CT-B-uptake assay, AGS cells were incubated with Alexa-Fluor-555–CT-B (Invitrogen; 1 µg/ml in HEPES medium) on ice for 30 minutes, followed by fixation immediately (0 minutes) or 20 minutes after incubation at 37°C. For the dextran-uptake assay, AGS cells were incubated with 70 kDa dextran conjugated with tetramethylrhodamine (Molecular Probes; 10 mg/ml in HEPES medium) on ice and incubated for 20 minutes at 37°C, followed by fixation for 2 hours for co-staining with VacA, or 5 hours for detecting dextran alone. For the VacA-uptake assay, AGS cells were incubated with 10 µg/ml (Fig. 2A) or 2 µg/ml (all other figures) of acid-activated s1-m1 VacA from ATCC49503 (Nakayama et al., 2004) in HEPES medium for 30 minutes on ice, washed with the HEPES medium, and fixed immediately (0 minutes), or 20 or 90 minutes after shifting to 37°C. 2 µg/ml and 10 µg/ml of VacA showed comparable results in endocytosis assays. VacA was detected by indirect immunofluorescence staining using VacA antibodies. Where indicated, fixed cells were stained by Alexa-Fluor-633-labeled wheat-germ agglutinin (Invitrogen; WGA, 10 mg/ml in PBS) for 30 minutes on ice before permeabilization to visualize the plasma and nuclear membranes. Fluorescence images were obtained using a confocal microscope (LSM5 Pascal and LSM510META, Carl Zeiss) at the Institute for Biomedical Research and Education, Yamaguchi University, Japan. For the quantitative endocytosis assay, *z*-series of optical sections of 1 µm were obtained from randomly chosen fields. The probed vesicles in the cytoplasm, as determined by the WGA staining, were counted manually in all optical sections in a given field. 30 to 50 cells, all from five to ten randomly chosen fields, were counted in each experimental group for the quantitative endocytosis assays.

H. pylori deletion mutants for *cagA* and/or *vacA*

The *cagA* gene was deleted in *H. pylori* strain CPY2052 or in NCTC11637 by homologous recombination using a targeting vector containing 0.8 kb of the 5′ region of *cagA*, the kanamycin-resistance cassette (*kan*) from pUC4K (GE Healthcare Biosciences), and 1.3 kb of the 3′ flanking region of *cagA*. The *vacA* gene was deleted using a targeting vector consisting of 0.7 kb of the 5′ flanking region of *vacA*, the chloramphenicol-resistance cassette (*cat*) (Wang et al., 1993), and 1.8 kb of the 3′ flanking region of *vacA*. The *cagA*- and *vacA*-deletion strains were selected on plates containing 5 µg/ml kanamycin or 8 µg/ml chloramphenicol, respectively. The *cagA*- and *vacA*-double-deletion mutant was obtained from the *cagA*-deletion mutant by additional transformation with the *vacA*-targeting vector. The identity of the *H. pylori* deletion mutants was confirmed by PCR and immunoblotting.

Infection of AGS cells with *H. pylori*

H. pylori strain CPY2052 or NCTC11637 and the isogenic mutants were cultured on Brucella agar plate containing 5% FBS under 10% CO_2 at 37°C for 1 day prior to the infection. Fresh *H. pylori* culture was suspended in AGS medium. AGS cells in 6-, 12- or 24-well plates were infected with the indicated strain of *H. pylori* at a multiplicity of infection of 100, and cultured at 37°C under 10% CO_2 for the indicated period. For infection longer than 1 day, culture medium and floating *H. pylori* were changed into fresh medium once a day. The protein expression levels were analyzed in the culture media and cell pellet by immunoblotting as indicated.

VacA

Purified s1-m1 VacA was prepared from the culture supernatant of *H. pylori* ATCC49503 as previously described (Nakayama et al., 2004). Purified VacA was stored at –80°C and acid-activated before use as described (de Bernard et al., 1995). The *vacA* genotype of *H. pylori* strain CPY2052 was determined by PCR using the primers as reported (Atherton et al., 1995). The Asian-specific middle region was determined using the specific primers as reported (Pan et al., 1998). VacA-containing culture supernatant of V+C+ *H. pylori* (VacA sup) was prepared fresh on the day of the experiment. Wild-type *H. pylori* CPY2052 was cultured in 12 ml of AGS medium (starting OD_{590} is 0.1) in 10 cm culture plates without shaking at 37°C, under 10% CO_2 for 4 hours. The cultured medium was centrifuged at room temperature; the resulting supernatant was filtrated with a 0.22 μm filter and used as the VacA sup.

Vacuolation assay

After 2 or 5 hours of co-culture of AGS cells and *H. pylori* (genotypes V–C–, V–C+, V+C– or V+C+), the medium containing the bacteria was replaced by AGS medium containing 60 μg/ml gentamycin and the cells were then incubated for a further 16 hours to develop vacuoles. To determine the effects of exogenously added VacA, AGS cells were infected with *vacA*-deleted *H. pylori* strains (genotype V–C+ and V–C–); the medium was then replaced by VacA sup with 60 μg/ml gentamycin and cells were incubated for 20 hours, or the medium was replaced by AGS medium containing 2 μg/ml purified VacA and 60 μg/ml gentamycin, and cells were incubated for 13 hours.

For both experiments, the quantitative vacuolation assay was performed using Neutral Red as previously described (Cover et al., 1991), with minor modification. Infected cells in a 24-well plate were incubated in 250 μl of PBS containing 0.05% Neutral Red and 0.3% BSA for 5 minutes and washed three times with 500 μl of PBS containing 0.3% BSA. Neutral Red was extracted with 250 μl of 70% ethanol plus 0.4% HCl, diluted tenfold with 70% ethanol, and quantified by absorption at 540 nm in cuvettes of 1 ml. The OD value observed for each mock infection of no *H. pylori* (None) was designated as 1 (OD_{540} is 0.06-0.08). For the assay of 2-day-infected AGS cells after exogenous VacA addition, the value for AGS cells infected with each strain with VacA addition was normalized by that without VacA addition as 1 (OD_{540} is 0.10-0.15). The assays were repeated in three or four wells for each treatment group.

Apoptosis assay

The AGS cells cultured in 24-well plates were infected with the indicated strains of *H. pylori* for 2 days. Hoechst 33342 (10 μM) was add to live infected cells for 10 minutes then fixed. Under the fluorescent microscope, more than 200 nuclei were counted from all the cells in four randomly chosen fields. The percentage of apoptotic cells (%) was calculated as the percentage of condensed nuclei in total counted nuclei. The averaged data from four fields represented each well. The assay was repeated in four wells for each treatment group.

EGFR and RPTPα internalization

For the assessment of EGFR internalization from the plasma membrane, AGS cells were incubated with antibody raised against the extracellular domain of EGFR (Santa Cruz Biotechnology; 0.25 μg/ml in HEPES medium) in serum-free medium for 30 minutes on ice, washed with ice-cold HEPES medium, and then incubated with EGF (Upstate Biotechnology; 100 ng/ml in HEPES medium)

TRANSLATIONAL IMPACT

Clinical issue

Stomach infection with the bacterium *Helicobacter pylori* affects billions of people worldwide. The infection, which is usually established during infancy and persists throughout the life of infected individuals, occasionally causes stomach diseases such as chronic gastritis, peptic ulcers and gastric cancers. *H. pylori* produces two main virulence factors: VacA, a toxin secreted into the extracellular space that enters host cells and causes cellular damage; and CagA, which for unknown reasons is directly injected into the gastric epithelial cells to which the bacteria are attached. *H. pylori* strains isolated from patients are genetically heterogeneous for these two factors. CagA is often identified in strains isolated from patients with severe gastric disease, and has thus been used as an indicator of virulent strains. Such CagA-positive strains almost always also express a toxic form of VacA, whereas CagA-negative strains usually express a nontoxic form of VacA. The nature of the relationship between these two virulence factors has been a long-standing enigma of *H. pylori*.

Results

By carrying out genome-wide screening in yeast, as well as experiments in gastric epithelial cells for CagA function, the authors show that CagA specifically inhibits the clathrin-independent pinocytic endocytosis pathway without affecting clathrin-dependent endocytosis. Because VacA enters into host gastric epithelial cells by pinocytic endocytosis, CagA inhibits the entry and function of VacA in the host cells, thereby protecting them from VacA-mediated toxicity. The authors also demonstrate that CagA inhibits the uptake of EGF receptor and RPTPα from the host plasma membrane, which depends, at least in part, on the pinocytic endocytosis pathway.

Implications and future directions

These data indicate that *H. pylori* strains that express toxic VacA must co-express CagA to maintain a balance between damage to and protection of the host gastric epithelial cells for their survival, as these cells constitute a crucial environmental niche for *H. pylori*. This functional antagonism might work as a selective pressure to maintain the evolutionary linkage between CagA and VacA in *H. pylori*. The capacity of CagA to inhibit endocytosis might also alter host cell signalling pathways, as endocytosis controls the amount of molecules at the plasma membrane and, in turn, the signals transduced by them. Therefore, CagA might contribute to changes in cancer-cell signaling pathways through modifying endocytosis in gastric epithelial cells infected with *H. pylori*. Understanding the function of CagA and VacA, and the nature of the functional relationship between them, will be essential for obtaining a clear understanding of the gastric diseases caused by *H. pylori* infection, including cancer.

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for 15 minutes; this was followed by fixation immediately (0 minutes) or 15 minutes after the transfer to 37°C. For the assessment of RPTPα internalization from the plasma membrane, AGS cells were incubated with antibody raised against the extracellular domain of RPTPα (Medical & Biological Laboratories; 4 μg/ml in HEPES medium) for 1 hour on ice, washed with ice-cold HEPES medium, then fixed immediately (0 minutes) or 20 minutes after the transfer to 37°C. The fixed cells were stained by Alexa-Fluor-633-labeled WGA, as described above ('Endocytosis assay'). Antibody-labeled EGFR and RPTPα were detected by the Alexa-Fluor-555-labeled secondary antibody (Invitrogen) after permeabilization of the cell membranes. For the quantitative internalization assay, the *z*-series of 1 μm optical sections were obtained for randomly chosen fields. The probed vesicles in the cytoplasm, as determined by the WGA staining, were counted manually in all optical sections in a given field. 30 to 40 cells were counted in each experimental group.

Statistics

Data are expressed as means ± s.e.m. unless otherwise stated. For the continuous values, statistical analysis was performed using Student's *t*-test, and values of $P<0.05$ were considered significant. For the distributive values of endocytosis assays, statistical analysis was performed using Mann-Whitney *U* test, and medians were used as representatives; here, values of $P<0.01$ were considered significant.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

H.A. and R.A. conceived and designed the experiments with J.K.A.; J.K.A. and Y.T. performed the experiments; J.K.A., H.A., Y.T. and R.A. analyzed the data; T.K., H.K., J.N., S.T., M.M., T.H. and R.A. contributed reagents, materials or analysis tools; J.K.A., H.A., T.H. and T.N. wrote the paper; K.N. provided advice on the project.

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