MAJOR ARTICLE

Phosphorylation-Independent Effects of CagA during Interaction between *Helicobacter pylori* and T84 Polarized Monolayers

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To extend our knowledge of host-cell targets of *Helicobacter pylori*, we characterized the interaction between *H. pylori* and human T84 epithelial cell polarized monolayers. Transcriptional analysis by use of human microarrays and a panel of isogenic *H. pylori* mutants revealed distinct responses to infection. Of the 670 genes whose expression changed, most (92%) required the *cag* pathogenicity island (PAI). Although altered expression of many genes was dependent on CagA (80% of the PAI-dependent genes), expression of >30% of these host genes occurred independent of the phosphorylation state of the CagA protein. Similarly, we found that injected CagA localized to the apical surface of cells and showed preferential accumulation at the apical junctions in a phosphorylation-independent manner. These data suggest the presence of distinct functional domains within the CagA protein that play essential roles in protein targeting and alteration of host-cell signaling pathways.

Helicobacter pylori infects >50% of the world's population and exacts a tremendous medical burden because of its causative association with ulcer disease and gastric cancer [1]. The pathogenic potential of *H. pylori* is intricately linked to the presence of a pathogenicity island (PAI)–encoded type IV secretion apparatus that translocates the bacterial protein CagA into the eukaryotic cell [2, 3]. Once inside the host cell, CagA is phosphorylated by members of the Src family of tyrosine kinases on tyrosine residues present within ⩾1 EPIYA motif [4, 5]. CagA phosphorylation has been associated with aberrant host-cell signaling and striking morphological changes characterized by massive rearrangement of the actin cytoskeleton [3]. Recent work suggests that interaction of phosphorylated CagA with the host-cell tyrosine phosphatase SRC homology 2 phosphatase plays a major role in *H. pylori*–induced cellular changes [6].

A number of studies have attempted to expand our knowledge of host-cell targets of *H. pylori* using DNA microarrays (reviewed in [7]). Those studies relied primarily on the human gastric epithelial cell line AGS to assess host transcriptional changes that occur after infection with *H. pylori*. Although this cell line has been studied extensively, AGS cells do not polarize or form proper cell junctions and, hence, do not accurately mimic the gastric epithelial surface encountered by *H. pylori* in the human stomach. This caveat likely influences the changes previously identified after *H. pylori* infection, a fact that has been accentuated by the recent characterization of *H. pylori* interaction with Madin-Darby canine kidney (MDCK) polarized monolayers (PMs) [8]. *H. pylori* was shown to preferentially attach to and disrupt cellular apical-junction complexes and to colocalize with the tight-junction scaffolding protein zonula occludens (ZO)–1 [8].

We have characterized the interaction of *H. pylori* with the differentiated human intestinal epithelial cell

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line T84. Unlike AGS, T84 cells can be polarized to form a brush border and assemble tight junctions and desmosomes. These cells also secrete mucin and can be induced to form a mucus layer [9]. Temporal transcriptional profiling using a panel of wild-type *(wt)* and isogenic *H. pylori* mutants revealed infection-specific profiles that show many distinctions from nonpolarized cell profiles. In addition, expression of many of the host-cell signaling pathways that are regulated in a CagA-dependent manner are affected, regardless of the phosphorylation state of CagA.

MATERIALS AND METHODS

Bacterial and cell culture. The *H. pylori* strain used was G27-MA [10]. Isogenic mutants of this strain include Δ*cagA* [8], Δ*vacA* (*vacA* encodes a vacuolating cytotoxin [10]), ΔPAI [11], Δ*cagA*Δ*vacA* [10], and the nonphosphorylateable CagA mutants $cagA_{EPISA}$ [8] and $cagA_{EPISA}$Δ*vacA*. The $cagA_{EPISA}$Δ*vacA* strain was made by natural transformation of the Δ*vacA* construct [10] into the $cagA_{EPISA}$ strain [8]. For all experiments, the bacteria were grown in coculture with MDCK cells, as described elsewhere [10]. T84 cells (ATCC CCL-248) were maintained at 37°C and 5% CO_2 in a 1:1 mixture of Dulbecco's modified Eagle medium and HAM F12 (Gibco/BRL) supplemented with 5% fetal bovine serum. After infection with *H. pylori,* 10% Brucella broth was added to support bacterial viability. T84 monolayers were polarized on Transwell filters (Costar) for 16–20 days, and polarity was monitored by ^{3}[H]-inulin diffusion across the monolayer for 3 h [9]. Monolayers were infected at an MOI of 10 and lysed with Trizol after 2, 4, 8, 12, and 24 h. Total RNA was prepared as described elsewhere [12].

Immunofluorescence and confocal microscopy. Infected monolayers were fixed with 2% paraformaldehyde in 100 mmol/L phosphate buffer (pH 7.4), permeabilized with 1% saponin in 3% BSA, and stained with anti–*H. pylori* polyclonal antibody 67 [10]. Cell junctions were stained with anti–ZO-1 monoclonal antibody (Zymed Laboratories). CagA was stained with anti-CagA monoclonal antibody [8]. Bacteria and cells were visualized by confocal microscopy after staining with Alexa Fluor–coupled secondary antibodies (Molecular Probes). Slices (0.5-μm thick) were obtained by use of a Biorad confocal microscope, and reconstructed 3D images were assembled by use of Volocity software (version 3; Improvision).

Microarray hybridization and analysis. Detailed protocols for probe synthesis and microarray hybridization are available at http://cmgm.stanford.edu/pbrown/protocols. For each sample, 20 μg of total RNA was used for single-strand cDNA synthesis, labeling, and hybridization to a 43,000 element-spotted human cDNA microarray, as described elsewhere [13]. Array data were stored in the Stanford Microarray Database [14] and are available at http://genome-www5.stanford.edu. Data were filtered by omission of spots, with regression correlations <0.6, and by selection of genes whose $\log_2$ of red:green normalized ratio was >1 in 4 arrays. Only genes with >70% good data for all arrays were included for $\log_2$ transformation and analysis with CLUSTER and TREEVIEW software [15]. For some genes, changes in expression were observed as early as 2 h after infection. However, some genes showed changes only at the later time points. Statistical analysis to identify the most consistent and highly regulated genes was done by use of the significance analysis of microarrays algorithm (SAM) and Student's unequal variance 2-tailed *t* tests [16]. For statistical tests, data from the 5 arrays for a given strain were treated as a single class and were compared with the 5 arrays of the other class of interest. Only genes for which $P \leqslant .05$ or with a predicted false discovery rate of <10% were considered to be statistically significant. Enrichment of individual gene ontological categories was investigated by use of the GoMiner program [17] (available at http://discover.nci.nih.gov/gominer/).

RESULTS

H. pylori *infection–induced transcriptional response in T84 PMs.* Similar to AGS and MDCK cells, *H. pylori* delivers CagA to T84 cells and causes changes in cell shape in nonpolarized subconfluent monolayers; *H. pylori* causes scattering and elongation of T84 cells within 4 h after infection (data not shown). Because previous microarray studies used only nonpolarized cells, we attempted to identify additional host-cell targets by conducting transcriptional profiling of *H. pylori*–infected T84 PMs. We infected 16-day-old PMs with *wt H. pylori* (G27-MA) and with isogenic mutants containing disruptions in *cagA,* PAI, and *cagAvacA*. To confirm the biological reproducibility of our experiment, the *wt* infection was repeated and expanded to include isogenic mutations in *vacA,* $cagA_{EPISA}$, and $cagA_{EPISA}$ *vacA*. Harvested RNA was used for generation of probes that were hybridized to a 43,000-element human cDNA microarray in conjunction with a common reference prepared from uninfected T84 PMs.

Temporal changes in gene expression were observed as early as 2 h after infection. To define genes whose expression was altered by infection with *wt H. pylori,* we conducted Student's unequal variance 2-tailed *t* tests that compared the mock-infected samples from sets 1 and 2 with the *wt*-infected samples from both experiments. We identified 727 clones, representing 670 unique genes whose expression was different in infected monolayers (figure 1; a complete list of genes is provided as supplementary data, at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/).

Ontological classification of T84 transcriptional changes. To classify the 670 genes that were reproducibly changed after infection of T84 PMs with *wt H. pylori,* we used GoMiner, a

program that classifies genes into biological categories and calculates the probability of over- or underrepresentation within a particular group, compared with the master list, by use of the Fisher's exact test [17]. By use of this program, the most obvious class of regulated genes we identified included cytokines and other proinflammatory factors ($P = .007$). These included tumor necrosis factor (TNF)–α, TNF-α–induced protein 3 (TNFAIP3), the interleukin (IL)–1 receptor accessory protein (IL1RAP), NF-κB inhibitor α, IL-2 receptor γ chain (IL2RG), CD59, and chemokine ligands 2 and 20. IL2RG plays an important role in IL-2, IL-4, IL-7, IL-9, and IL-15 signaling. Of these, IL1RAP plays a crucial role in cytokine signaling; it is required for induction of acute-phase and proinflammatory proteins during infection and tissue damage via interaction with IL-1 at the IL-1 receptor. Of interest, both TNFAIP3 and NF-κB inhibitor α inhibit activation of NF-κB, which mediates *H. pylori*–related cytokine induction.

We also found a significant enrichment for components associated with cellular junctions ($P = .02$). Our analysis revealed increased expression of the syndecan binding protein, a component of the adherence junctions that connect the cytoskeletal filaments between cells and between cells and the extracellular matrix (ECM). We also found increased expression of the intercellular junctional components β-catenin, desmoglein, the cadherin family members desmoplakin and cadherin 5, gap junction–binding proteins 2 and 3, and the tight-junctional components Par3 and claudin 3 and 4. We observed decreased expression of α-catenin and cadherin 17.

We saw a strong enrichment for factors that interact with the host-cell cytoskeleton ($P = .01$). *H. pylori* has been shown to induce cytoskeletal rearrangement in numerous nonpolarized epithelial cell lines [18], and this rearrangement is accompanied by the accumulation of Arp3 at the site of bacterial attachment [19]. Arp3 exists as a protein complex with Arp2 and initiates the formation of actin-filament networks in response to intracellular signals. We found changes in expression of components that included multiple members of the Arp2/3 protein complex (capping protein α 1, coronin actin–binding protein, Wiskott-Aldrich syndrome, and radixin), intermediate filament components (plectin 1 and keratin 8 and 20), and cytoskeletal-associated proteins (actin-β, actin-related protein 3, plecstrin 2, villin 2, and tropomyosin 4).

Analysis with GoMiner also showed a strong enrichment of factors that play roles in apoptosis ($P = .05$). *H. pylori* is known to induce apoptosis in vitro in T84 cells [20] and in vivo in the gastric mucosa [21]. The identified genes play diverse roles in apoptosis; many function to promote apoptosis, whereas others function as apoptosis inhibitors. We saw increased expression of B cell CLL/lymphoma 10 (BCL10), myeloid cell leukemia sequence 1, the SRC homology domain 3–growth factor receptor–bound protein 2–like endophilin B1, baculoviral IAP repeat–containing proteins 2 and 3, immediate-early response 3, and serine/threonine kinase 3.

Virulence factor–dependent changes in gene expression. Next, we investigated the contribution of VacA, PAI, CagA, and specific CagA domains to the observed changes in T84 gene expression. Although we were unable to identify a VacA-dependent signature, we were able to identify PAI-dependent changes in gene expression. We adopted a reductionist approach to identify gene expression responses regulated by specific virulence factors. First, we used SAM to identify genes whose patterns of expression were significantly altered when we compared the mock-infected time course with that of the *H. pylori* strain that lacks the PAI (ΔPAI). This comparison revealed 64 clones, representing 60 unique genes whose changes in gene expression were PAI independent (provided as supplementary data available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/). Analysis with GoMiner revealed an enrichment of apoptosis-related genes ($P = .007$), including MYC, breast cancer 1 early onset, and THO complex 1. All 3 factors were down-regulated, suggesting that *H. pylori* may have the ability to suppress host-cell apoptosis independent of the presence of *cag*PAI.

Expression patterns of 610 of the 670 genes altered by *wt H. pylori* infection are PAI dependent, since those patterns are abrogated in the ΔPAI strain (figure 2 and supplementary data [available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/]). This represents 92% of the total number of altered genes and suggests that the major transcriptional response of T84 PMs to *H. pylori* infection is PAI mediated (figure 3). Next, we determined which of the PAI-dependent genes were CagA dependent. SAM analysis comparing the mock and Δ*cagA* data showed that expression patterns of 479 (79%) of the 610 genes were CagA dependent (figures 2 and 3). These CagA-dependent genes could be broken into a number of functional categories: genes with roles in cell adhesion (integrins α and β, the RET proto-oncogene, BH-protocadherin, laminin, desmocollin 2, and β-catenin), genes with roles in apoptosis (BCL10, baculoviral IAP repeat–containing [BIRC] 2, BIRC3, TNFAIP3, and myeloid cell leukemia 1), and genes known to play roles in cell junction formation (Gap junction–binding proteins 2 and 3, claudin 4, and Par3).

Induction of a host-cell transcriptional response by CagA independent of phosphorylation. CagA is phosphorylated at conserved EPIYA motifs by host-cell tyrosine kinases, and previous studies have suggested that this is crucial for alteration of downstream signaling pathways. We were therefore interested in identifying changes in gene expression with the nonphosphorylateable $cagA_{EPISA}$ mutant that were distinct from the functional CagA-dependent changes. By directly comparing the $cagA_{EPISA}$ with the mock-infected cells by use of SAM, we identified 151 (32%) of the 479 CagA-dependent genes whose regulation was phosphorylation independent (figures 2 and 3 and

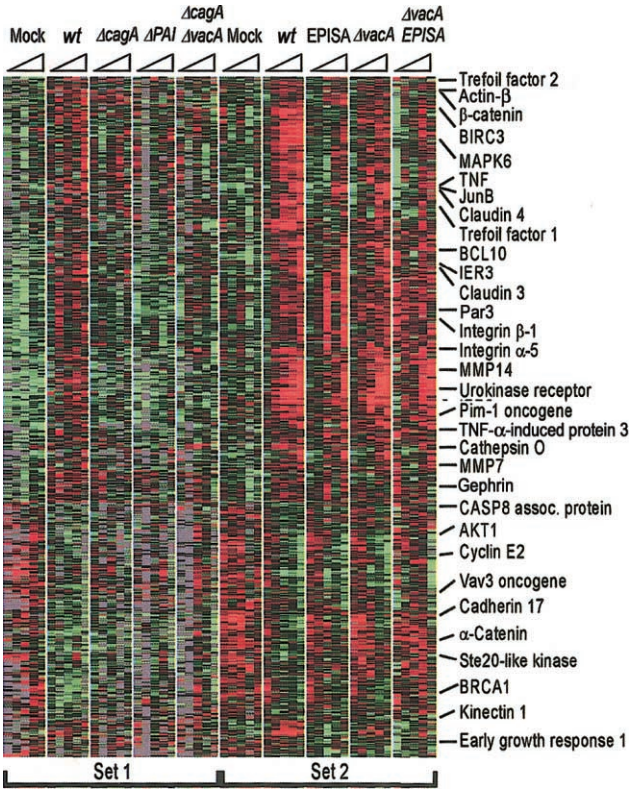

Figure 1. T84 transcriptional response to isogenic *Helicobacter pylori* strains. Monolayers were infected with the indicated strain of *H. pylori* for 2, 4, 8, 12, and 24 h (increasing time is represented by open triangles). Data from the 2 experiments are denoted by "Set 1" and "Set 2." Data are a measure of relative gene expression and represent the quotient of the hybridization of the fluorescent cDNA probe from each infected sample, compared with a reference pool. *Red* and *green,* high and low experimental sample:reference ratios, respectively. *Gray,* technically inadequate or missing data. A complete list of genes included in the cluster is available as supplementary data (available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/). AKT1, v-akt murine thymoma viral oncogene homolog 1; BCL10, B cell CLL/lymphoma 10; BIRC, baculoviral IAP repeat–containing; BRCA1, breast cancer 1 early onset; CASP8, caspase 8; Cyclin-dep., cyclin-dependent; IER3, immediate-early response 3; MAPK, mitogen-activated protein kinase; MMP7, metalloproteinase 7; TNF, tumor necrosis factor; *wt,* wild type.

supplementary data [available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/]). Of note, changes in expression of many genes that have been shown previously to require CagA phosphorylation were completely abrogated in the $cagA_{EPISA}$ mutant–infected cells, as would be expected. These include many components of the actin cytoskeleton, whose rearrangement has been shown to be CagA phosphorylation dependent in AGS cells [22].

The CagA phosphorylation–independent genes could be broken into a number of functional categories: factors with roles in cell adhesion and junction formation (α-catenin, Par3, urokinase-type plasminogen activator [uPA], and urokinase-type plasminogen activator receptor [uPAR]); genes with known roles in signaling (the ETS oncogene family member ELK1, cyclin-dependent kinase 7, IL-2 receptor γ, interferon-γ receptor 2, mitogen-activated protein kinase 6, and the Ras oncogene family member RAP1B); and genes known to act as apoptosis inhibitors (TNFAIP3, BIRC2, and BIRC3).

Phosphorylation-independent interaction of* H. pylori *with cell junctions and localization of CagA. Our laboratory has previously shown that *H. pylori* preferentially attaches to cell-cell junctions and colocalizes with the tight-junction scaffolding protein ZO-1 in MDCK cells [8]. This interaction causes disruption of the apical-junctional complex in a manner characteristic of many human diseases. Our finding that multiple junctional proteins were regulated in a CagA-dependent, but phosphorylation-independent, manner led us to investigate the localization of *H. pylori* on our T84 PMs. As with MDCK cells, *H. pylori* preferentially localized to cell junctions without requiring CagA phosphorylation; we saw similar localization with the $cagA_{EPISA}$ mutant (data not shown). In addition, we noted considerable redistribution of ZO-1 staining patterns (data not shown), suggesting that *H. pylori* also disrupts the apical-junc-

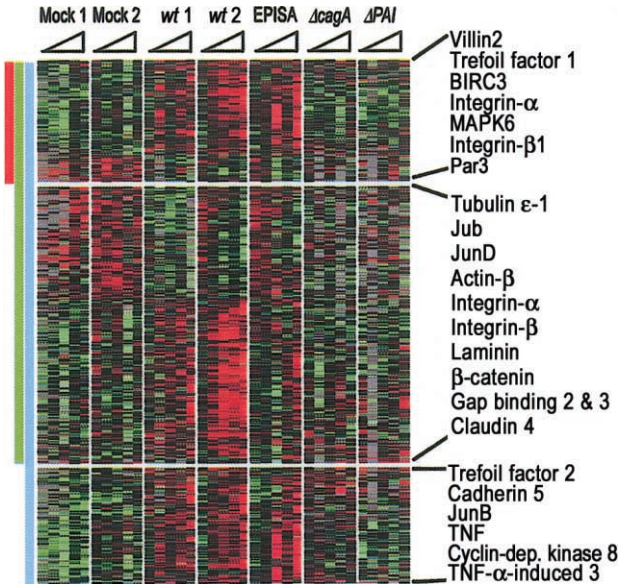

Figure 2. Virulence factor–dependent transcriptional responses. Monolayers were infected with the indicated strain of *Helicobacter pylori,* and data are represented as described in the legend to figure 1. The 663 clones found to be pathogenicity island (PAI) dependent are represented and indicated by the blue bar. These are further subdivided to CagA-dependent *(green bar)* and CagA-dependent but phosphorylation-independent *(red bar)* clones. *Red* and *green,* high and low experimental sample:reference ratios, respectively. *Gray,* technically inadequate or missing data. A complete list of genes included in the cluster is available as supplementary data (available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/). Cyclin-dep., cyclin-dependent; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; *wt,* wild type.

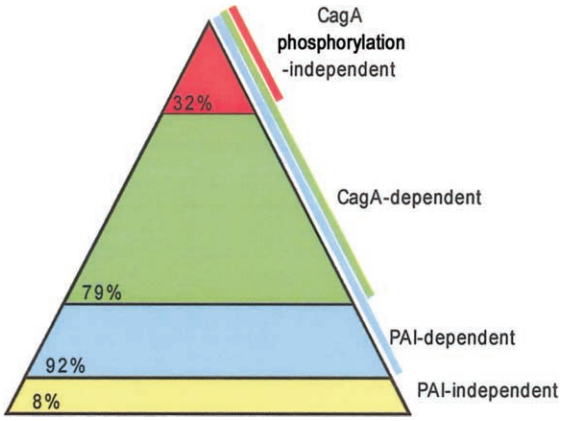

Figure 3. Schematic representation of the distribution of genes affected by *Helicobacter pylori* infection. The complete T84 727 clone data set is represented by the triangle as a whole. Expression of 8% of the genes are pathogenicity island (PAI) independent *(yellow),* whereas changes in the remaining 92% are PAI dependent *(blue bar).* The PAI-dependent data are further broken into virulence factor–dependent groups and show that, of the PAI-dependent genes, 79% are CagA dependent *(green bar).* Of these, 32% are phosphorylation independent.

tional complex in this model. To further characterize the role of phosphorylation on distribution of CagA, we investigated the localization of injected CagA in the T84 PMs. Immunofluorescent staining, followed by confocal microscopy, showed that *wt* CagA localization was enriched only in the subpopulation of T84 cells with attached bacteria (figure 4). Within these cells, CagA was associated with the apical membrane and enriched at the cell junctions. In addition, CagA staining was never seen in the cytoplasm or extending to the basolateral membrane of the monolayer. These staining patterns were indistinguishable for the $cagA_{EPISA}$ mutant (figure 4), suggesting that localization of CagA to the apical junctional surface of PMs occurs independent of the phosphorylation state of the protein.

Distinct T84 transcriptional response and AGS response. Finally, to place our observed transcriptional changes into a historical context with those previously observed in nonpolarized cells, we directly compared our T84 expression profiles to AGS profiles previously generated in our laboratory [23]. Because the AGS study used a different reference from the present work, we were unable to directly compare the absolute levels of expression of individual genes between the 2 studies. However, because both data sets directly compared *H. pylori*–infected with mock-infected cells, the patterns of expression of individual genes in response to infection could be analyzed. In addition, because our current array has 20,000 more elements than the one used in the original study, we were unable to recover information for all of our genes of interest. We were able to retrieve AGS data for 489 clones of the data set of 727 clones that we found to be changed in *H. pylori*–infected T84 PMs. Hierarchical clustering of both data sets and subsequent analysis revealed that, although many genes (>50%) showed

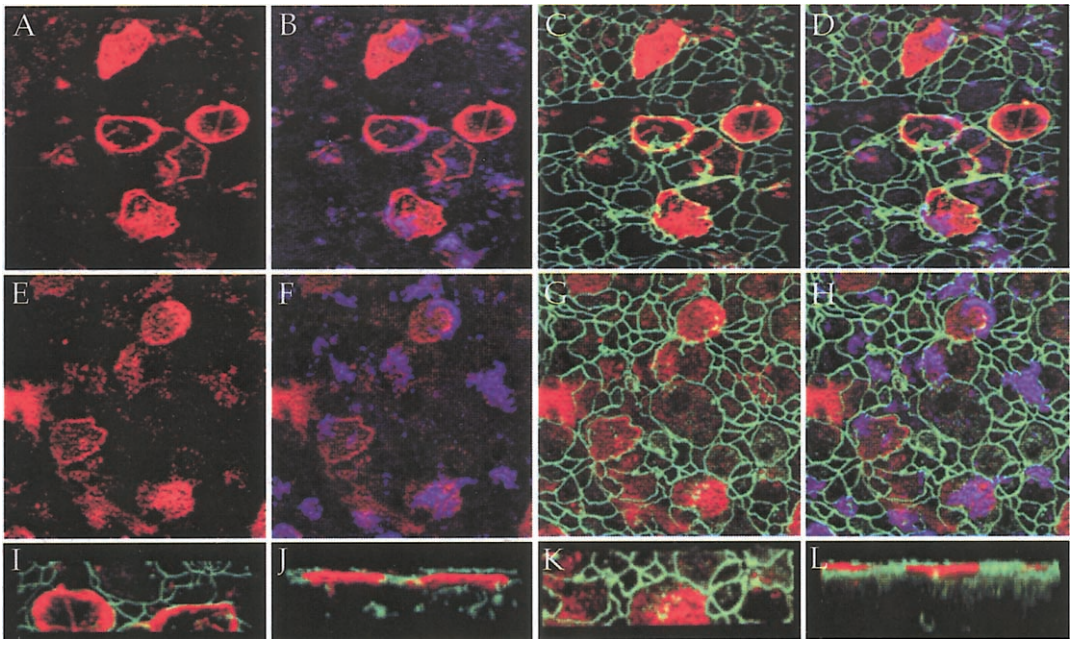

Figure 4. Confocal immunofluorescence z-projections of T84 polarized monolayers (PMs) infected for 24 h with wild-type *Helicobacter pylori (A, B, C,* and *D)* and the $CagA_{EPISA}$ mutant *(E, F, G,* and *H).* Infected PMs were stained with anti CagA *(red),* anti–ZO-1 *(green),* and anti–*H. pylori (blue)* antibodies. Various overlays are shown in the different panels. CagA localization is preferentially enriched at cell junctions *(A* and *E).* Enlarged cross-sections of CagA-positive cells from panels *C* and *G* are shown and represent wild-type and $CagA_{EPISA}$ *H. pylori*, respectively *(I* and *K).* These were rotated by 90° by use of Volocity software (version 3; Improvision) *(J* and *L)* to show the apical localization of the injected CagA protein.

similar patterns of expression in both cell lines, distinct clusters of genes whose patterns of expression were altered only in *H. pylori*–infected T84 PM were readily identifiable (see our supplementary data [available at http://cmgm.stanford.edu/falkow/whatwedo/supplementarydata/]). Included among these are cadherin 17, the ras homolog gene family member A, transforming growth factor (TGF)–β–inducible nuclear protein 1, FOS, tumor rejection antigen 1, NCK adaptor protein 1, carcinoembryonic antigen–related adhesion molecule 1 (CEACAM1), and the oncogenes Pim-1 and Vav3.

DISCUSSION

We conducted detailed transcriptional profiling of T84 PMs with *H. pylori* and defined isogenic virulence factor mutants by use of a system that closely mimics the gastric epithelium. This work represents the first global investigation of the response of PMs to *H. pylori* infection and shows distinct changes in host gene expression. We found T84-specific (CagA phosphorylation–dependent) alterations in expression of CEACAM1, which is a member of the cellular adhesion carcinoembryonic antigen (CEA) family. Altered expression of some members of this family seem to correlate with progression to cancer [24], and the significance of this family of proteins in *H. pylori* infection is evidenced by the results of a previous study in which increased expression of CEA10 was found to occur in *H. pylori*–infected animals displaying mild lymphocytic responses [13]. In addition, we found T84-specific (CagA phosphorylation–dependent) increased expression of the serine/threonine kinase Pim-1. Pim-1 expression has recently been shown to be increased in the glandular mucosa of ex-germ-free mice infected with *H. pylori* [25].

H. pylori–infected host cells show changes in expression of numerous junctional components. Deregulation of α- and β-catenin (PAI independent and CagA phosphorylation dependent, respectively) are of special interest, because they play roles in multiple forms of cancer [26–28]. Catenins link E-cadherin to the actin cytoskeleton; β-catenin binds the cytoplasmic domain of E-cadherin. However, excess nonstructural β-catenin associates with the adenomatosis polyposis coli (APC) multiprotein complex, which is a tumor suppressor and has been implicated in the development of colon cancer [29]. Normally, APC and excess β-catenin are targeted for degradation by phosphorylation by the WNT signaling pathway. However, when WNT is induced, excess β-catenin enters the nucleus and interacts with T cell factors (TCFs) or lymphocyte-enhancer factors that can then act as transcription factors. Target genes of the β-catenin/TCF complex include matrix metalloproteinase (MMP)–7 [30], MYC [31], JUN, and its downstream target, uPAR [32]. Of note, all of these genes were strongly up-regulated in our microarray study. This is the first suggestion that *H. pylori* infection may affect WNT signaling and lead to the nuclear accumulation of β-catenin. Preliminary evidence suggests that this is, indeed, the case, because *H. pylori*–infected T84 monolayers show diffuse β-catenin immunostaining patterns that partially colocalize with nuclear stains only in infected cell lines (S.H.E.-E. and D.S.M., unpublished data).

It is striking that the vast majority of host-cell changes (92%) in response to *H. pylori* are dependent on the presence of the PAI (figures 2 and 3). In fact, the majority of these are due to CagA, because only 21% of the PAI-dependent genes were CagA independent. Identification of a CagA-independent fraction of genes suggests that the mere presence of the type IV secretion apparatus, or translocation of additional unknown effectors, is sufficient to induce an effect on the host cell. However, CagA appears to play the major role among PAI genes in mediating host-cell signaling changes. Most importantly, we demonstrate that a nonphosphorylateable form of CagA induces statistically significant changes in expression of >30% of the genes shown to be CagA dependent. Other recent work also suggests that CagA can alter host-cell signaling pathways independent of phosphorylation. Previous work by Hirata et al. [33] and Umehara et al. [34] has shown that CagA regulation of serum response elements in HeLa cells and regulation of JAK/STAT signaling pathways in B lymphocytes, respectively, are independent of the phosphorylation state of CagA. In addition, Mimuro et al. have shown that nonphosphorylated CagA can interact with Grb2 and activate ERK signaling pathways [35].

For example, increased expression of Par3 is independent of CagA phosphorylation. Par3 plays a role in cell polarity in epithelial cells through the formation of tight junctions. The tight-junction–associated protein (JAM) is first recruited at the early stages of cell-to-cell contact. JAM contains a Par3-binding domain that is necessary for recruitment of Par3, Par6, and αPKC, all of which are essential for tight-junction formation. Differential regulation of Par3 is in keeping with the finding that *H. pylori* associates with the tight-junction proteins ZO-1 and JAM [8] and strengthens the suggestion that *H. pylori*–induced junctional dysfunction may contribute to the development and progression of disease.

uPA and uPAR were also both up-regulated in a phosphorylation-independent manner. uPA is a serine protease that converts plasminogen to plasmin, which then acts as a broad-spectrum protease to promote degradation of the ECM. Plasmin degradation of ECM factors is accomplished via activation of MMPs, 2 of which we found to be differentially regulated (MMP7 and MMP14). In addition to the enzymatic activity of the uPA-uPAR complex, uPAR regulates cell adhesion and migration via interaction with integrins. It is interesting to note that we found integrins αV and β to be strongly regulated in a phosphorylation-independent manner. Expression and activity of uPA-uPAR has been shown to positively correlate with invasiveness and metastatic properties of many tumors, and

expression of uPAR has been suggested as a prognostic biomarker for endometrial cancer [36]. Analysis of tissue biopsy specimens from *H. pylori*–infected individuals has shown increased expression of uPA in the antrum and corpus mucosa of patients with gastritis.

We found it striking that many different genes with predicted roles in apoptosis were regulated in the present study. Our virulence factor–dependent analysis showed that these factors could be broken down into 3 different groups: PAI independent, CagA phosphorylation dependent, and CagA phosphorylation independent. Analysis of these groups showed that all of the genes regulated in a PAI-independent manner are predicted to stimulate apoptosis and are strongly repressed by *H. pylori* infection. Delivery of the nonphosphorylateable form of CagA resulted in increased expression of the apoptosis inhibitors TNFAIP3, BIRC2, and BIRC3, suggesting that nonphosphorylated CagA actively suppresses apoptosis. When phosphorylated CagA was delivered, on the other hand, we saw increased expression of genes that promote apoptosis. Taken together, these data suggest that *H. pylori* possesses multiple pathways for the regulation of apoptotic pathways and that the balance between promotion and inhibition of apoptosis is a delicate one. *H. pylori* has the ability to down-regulate expression of apoptotic factors in a PAI-independent manner and to induce the expression of inhibitors of apoptosis by delivery of nonphosphorylated CagA. The ultimate phosphorylation of CagA and disruption of normal signaling pathways leads to the increased expression of apoptosis factors. This result suggests that the level of phosphorylated CagA found within a cell or the ability to modulate the delivery of a nonphosphorylateable form of CagA may play an important role in the ability of *H. pylori* to set up a dynamic equilibrium within the host that allows long-term, persistent infection. This is particularly interesting in light of the recent finding that, within a single human host, bacteria are found that have deleted the *cagA* gene [37] or have undergone intragenomic recombination, resulting in deletion of CagA phosphorylation sites [38]. Thus, bacteria may use genomic rearrangements as means of controlling the level of apoptosis seen within the human stomach.

Our finding that nonphosphorylateable CagA affects many host-cell signaling pathways likely has important clinical implications. It has long been accepted that *H. pylori* strains encoding CagA are much more likely to be associated with severe clinical disease manifestations [39, 40]. It is also now evident that this is due to the delivery of this protein to the host cell and its effects on host-cell signaling pathways [7]. CagA phosphorylation sites are encoded in a variable region located within the 3′ end of the gene, and variability in this region affects the degree of phosphorylation of the protein. What is currently unclear is the relationship between disease manifestation and the degree of phosphorylation of CagA; recent evidence suggests that the 2 may be intricately linked [41]. In vitro, we clearly see changes in host-cell signaling that are independent of phosphorylation, suggesting the presence of other mechanisms by which CagA effects host-cell signaling. Future epidemiological studies linking severity of clinical disease to delivery of CagA to the host cell, its ability to be phosphorylated, and the number of phosphorylation sites present within the protein should provide valuable information that will expand our understanding of the role of this protein in disease progression.

Finally, we found that injected CagA preferentially localizes to the apical membrane of T84 PMs and shows strong enrichment at cell-cell junctions. This occurs regardless of the phosphorylation state of CagA and is in keeping with the previous finding of Amieva et al. that nonphosphorylated CagA still has the ability to recruit the junctional-associated protein ZO-1 [8]. Our observation of CagA-dependent but phosphorylation-independent effects on host-cell signaling is likely due to the presence of several distinct functional domains of CagA. This view is also supported by the recent finding that transfection of the N-terminal or C-terminal portion of CagA into AGS cells results in markedly different phenotypes in terms of CagA localization and host-cell morphological changes (F. Bagnoli and M. Amieva, personal communication). Although it has been previously demonstrated that injected CagA associates with host-cell membranes and junctional components [4, 8], to our knowledge, the present study is the first to demonstrate that this association is specifically enriched at cell junctions in polarized epithelia and occurs only on the apical side of the monolayer. The basolateral exclusion of CagA suggests that it either associates with proteins that are specifically targeted to the apical surface or may contain an endogenous "sorting" signal that directs it to the apical surface of the host cell. Future studies investigating these ideas and identifying functional domains of the CagA protein and the role they play in host-cell signaling events will be of great interest and should greatly expand our understanding of this important virulence factor.

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