DELINEATION OF A NOVEL *HELICOBACTER PYLORI* RECEPTOR, DECAY-ACCELERATING FACTOR

By

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To my wonderful son Colin, for the best year of my life

and

To my best friend and beloved wife Nina, thank you for your support

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ORIGINAL PUBLICATIONS

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LIST OF ABBREVIATIONS

Abl..... Abelson Murine Leukemia viral oncogene homolog

ActD ..... Actinomycin D

AJ ..... Adherens-Junction

ANOVA.....Analysis of Variance

AP-1 .....Activator Protein 1

BabA ..... Lewis^{b} Blood Group Antigen Adhesin

BB ..... *Brucella* Broth

BCA..... Bicinchoninic Acid

BMDC .....Bone Marrow-Derived Cell

Cag .....Cytotoxin Associated Gene

CCP ..... Complement Control Protein

cDNA ..... Complementary DNA

CFU .....Colony-Forming Units

CHO ..... Chinese Hamster Ovary

CHX .....Cycloheximide

COX .....Cyclooxygenase

CREB ..... cyclic-AMP Response Element Binding Protein

Crk..... v-crk Sarcoma Virus CT10 Oncogene Homolog

Csk .....Carboxy-terminal Src Kinase

DAF..... Decay-accelerating factor

DNA ..... Deoxyribonucleic Acid

Dr ..... *E. coli* DAF binding adhesin

E-cadherin..... Epithelial cadherin

ELISA..... Enzyme-Linked Immunosorbant Assay

EPIYA ..... Glutamate-Proline-Isoleucine-Tyrosine-Alanine

ERK..... Extracellular Regulated Kinase

FBS..... Fetal Bovine Serum

GPI ..... Glycosylphosphatidylinositol

Grb2..... Growth Factor Receptor-Bound Protein 2

H&E ..... Hematoxylin and Eosin

HIV..... Human Immunodeficiency Virus

HLA..... Human Leukocyte Antigen

Hop..... Hypothetical Outer-membrane Protein

IHC..... Immunohistochemical

IKK..... IκB kinase

IL..... Interleukin

iNOS ..... Inducible Nitric Oxide Synthase

ISRE ..... Interferon-Stimulated Responsive Element

IκB..... Inhibitor of κB

JAM..... Junctional Adhesion Molecule

JNK ..... Jun-N Terminal Kinase

kDa ..... Kilodalton

L-Arg ..... L-Arginine

Le ..... Lewis

LPS ..... Lipopolysaccharide

mAb ..... Monoclonal Antibody

MALDI-TOF ..... Matrix-Assisted Laser Desorption Ionization, Time-of-Flight

MALT ..... Mucosa Associated Lymphoid Tissue

MAPK ..... Mitogen Activated Protein Kinase

MARK ..... Microtubule Affinity Regulated Kinase

MDCK ..... Madin-Darby Canine Kidney

MEK ..... MAP ERK Kinase

MKK ..... MAPK kinase

MKKK ..... MKK kinase

MNU ..... *N*-methyl-*N*-nitrosourea

MOI ..... Multiplicity of Infection

mRNA ..... Messenger RNA

MS ..... Mass Spectrometry

NapA ..... Neutrophil Activating Protein A

NF-κB ..... Nuclear Factor Kappa B

NO ..... Nitric Oxide

NOD ..... Nucleotide Oligomerization Domain

OipA ..... Outer Inflammatory Protein A

OMP ..... Outer Membrane Protein

PAGE ..... Polyacrylamide Gel Electrophoresis

PAI ..... Pathogenicity Island

PBS ..... Phosphate Buffered Saline

PBST ..... PBS plus Tween

PCR ..... Polymerase Chain Reaction

PNG ..... Peptidoglycan

PNH ..... Paroxysmal Nocturnal Haemoglobinuria

PTP ..... Protein Tyrosine Phosphatase

PVDF ..... Polyvinylidene Difluoride

RIPA ..... Radioimmunoprecipitation assay

RNA ..... Ribonucleic Acid

ROS ..... Reactive Oxygen Species

RT ..... Room Temperature

RT-PCR ..... Reverse Transcriptase Polymerase Chain Reaction

SabA ..... Sialic Acid-Binding Adhesin A

SDS ..... Sodium Dodecyl Sulfate

SEM ..... Standard Error of the Mean

SH2 ..... Src Homology Domain 2

SHP-2 ..... SH2-domain Containing-Tyrosine Phosphatase

siRNA ..... Small-interfering RNA

Src ..... v-src Sarcoma Viral Oncogene Homologue

TCF/LEF ..... T cell Factor/Lymphoid Enhancer Factor

TFSS ..... Type IV Secretion System

T_h ..... T helper

TJ ..... Tight-Junciton

TLR ..... Toll-like Receptor

TNF ..... Tumor Necrosis Factor

VacA ..... Vacuolating cytotoxin

WT ..... Wild-type

ZO-1 ..... Zona Occludens 1

CHAPTER I

INTRODUCTION

Helicobacter pylori

Helicobacter pylori is a Gram-negative, urease-, catalase-, and oxidase-positive curved bacillus that possess 4-5 sheathed polar flagella that are used for motility (**Figure 1**). *H. pylori* is one of the most genetically diverse bacteria known, with virtually every isolate being unique. *H. pylori* is specifically adapted for survival in its niche, the human stomach. Many strains of *H. pylori* express factors that have evolved to affect host cell signaling pathways, resulting in enhanced risk for pathogenicity. Approximately 50% of the world's population is colonized with *H. pylori*; however, only a subset of infected persons develop disease (226). Of infected individuals, 10% develop peptic ulcer disease, 1% develop gastric adenocarcinoma, and less than 0.1% develop mucosa associated lymphoid tissue (MALT) lymphoma. *H. pylori* infection is most commonly acquired at a young age and is thought to be passed from parent to child (72). Although infection can be found in all regions of the world, the prevalence of *H. pylori* colonization is higher in developing regions than in developed countries (69, 72). Risk factors for colonization with *H. pylori* include low socioeconomic status, household crowding, country of origin, and ethnicity (72, 291).

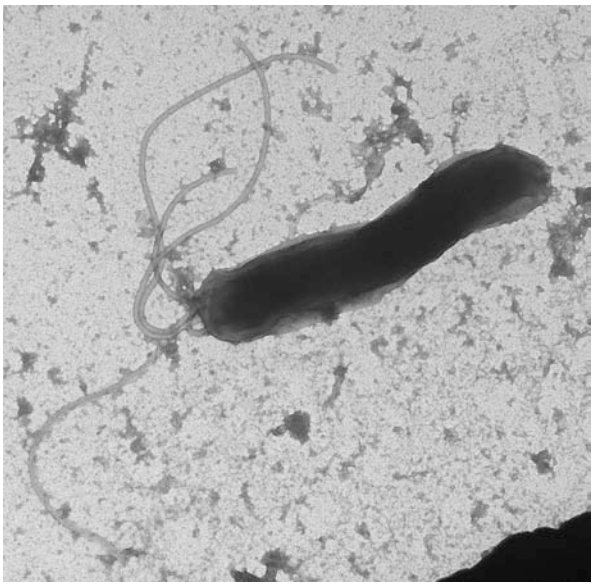

Figure 1. *Helicobacter pylori.* *H. pylori* is a gram-negative bacillus that has a characteristic curved rod shape. *H. pylori* possess 4-5 polar flagella that facilitate motility in the mucous gel layer above the gastric epithelium. Electron micrograph provided by and reprinted with the permission of Aime T. Franco.

Identification of *H. pylori* as an etiological agent for disease

H. pylori was identified in 1982 when Robin Warren and Barry Marshall made the seminal observation that curved bacillary bacteria were consistently found associated with foci of chronic inflammation in the stomach (312). While physicians had reported the presence of spiral bacteria associated with gastric tissue as early as 1938 and 1940 (60, 90), the prevailing dogma was that the stomach was a sterile organ, and any observed bacteria were likely contaminants from the mouth. A study by Palmer, reported in 1954, effectively ended the debate by demonstrating that in 1,140 gastric biopsy specimens, no spiral bacteria were found (222). However, the staining technique used in the Palmer study was not effective for visualizing *H. pylori*. When Warren and Marshall published their initial observations, many in the medical community were resistant to the notion that a bacterium could stably colonize the gastric mucosa and potentiate inflammation, resulting in gastric disease. However, Marshall was unwilling to cede to pressure from the medical community and performed the definitive experiment to fulfill Koch's postulates: drinking a culture of *H. pylori*, recording symptoms and documenting pathology via serial endoscopies, and then eliminating the infection with antibiotics (177). Eventually physicians realized the validity of Warren and Marshall's findings, and today, gastritis and peptic ulcers are treated with antibiotics. *H. pylori* infection has also now become an important model to study chronic inflammation and cancer. The impact on public health has been so significant that Warren and Marshall were awarded the 2005 Nobel Prize in Medicine or Physiology.

The role of *H. pylori* colonization in the genesis of gastric adenocarcinoma

Gastric adenocarcinoma is the second leading cause of cancer-related death in the world (226). Approximately 649,000 persons die from this malignancy each year and 5-year survival rates in the United States are <15% (38). Before *H. pylori* was known to be causally linked with gastric cancer, the pathologic progression of this disease had been well characterized. Two histologically distinct variants of gastric adenocarcinoma are predominant: diffuse-type and intestinal-type adenocarcinoma. Diffuse-type gastric cancer consists of individually infiltrating neoplastic cells that do not form glandular structures, while intestinal-type adenocarcinoma progresses through a series of histologic steps known as the Correa pathway, initiated by the transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis and intestinal metaplasia, and finally to dysplasia and adenocarcinoma (**Figure 2**) (37, 226, 273). Intestinal-type gastric cancer is more common among men (male:female ratio 2.1:1) and older patients (mean age 50.4 years for men and 47.7 for women) (39, 118). The reasons for this gender disparity are not clear. However, the duration of disease progression is likely due to the time required to accumulate mutations necessary for transformation.

H. pylori selectively colonizes gastric epithelium and induces persistent gastritis. Microbial persistence implies linkage in which signals of the colonizing organism affect signals of the host, and indeed, *H. pylori* has the ability to send and receive signals from gastric epithelium, allowing the host and bacteria to participate in a dynamic equilibrium.

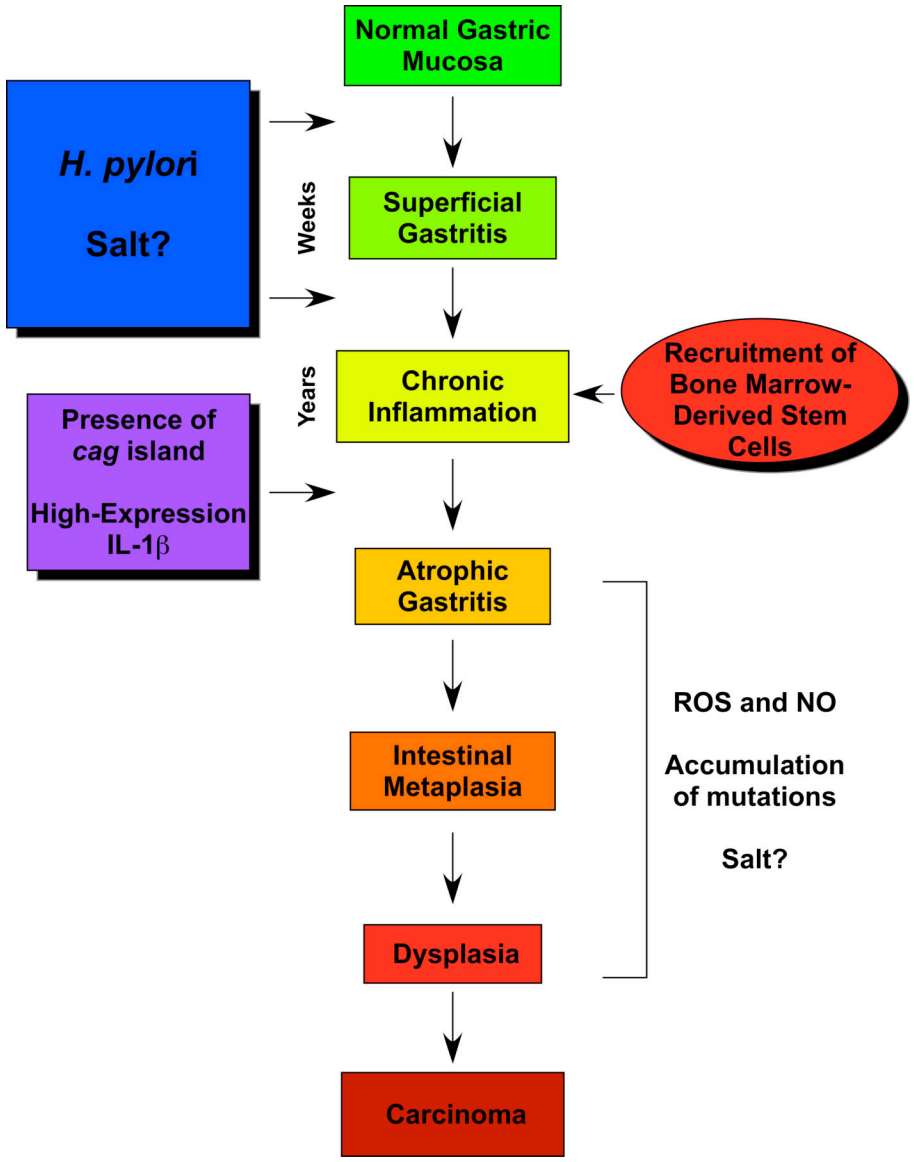

Figure 2. Progression to intestinal-type gastric adenocarcinoma. *H. pylori* colonization usually occurs during childhood and, over a period of days to weeks, leads to superficial gastritis. The presence of the *cag* pathogenicity island within infecting *H. pylori* isolates and host polymorphisms that promote high expression levels of the cytokine IL-1β augment the risk of development of atrophic gastritis, intestinal metaplasia, dysplasia and, eventually, gastric adenocarcinoma over the course of many years. The development of cancer has been attributed to the accumulation of mutations in DNA caused by chronic inflammation, recruitment and engraftment of bone marrow-derived cells, and an imbalance between epithelial cell proliferation and apoptosis. ROS, reactive oxygen species; NO, nitric oxide. Adapted from Peek and Blaser & Fox and Wang (86, 226).

However, there are biological costs to the long-term relationship between *H. pylori* and humans as chronic colonization confers a significantly increased risk of developing peptic ulcer disease, atrophic gastritis, intestinal metaplasia, and distal gastric adenocarcinoma (16, 37, 83, 109, 114, 132, 145, 149, 154, 190, 206, 224, 226, 234, 269, 272, 299, 314). For reasons that are not clearly understood, duodenal ulcer patients are not at increased risk for gastric cancer (115).

Based upon these data, the World Health Organization has classified *H. pylori* as a class I carcinogen for gastric cancer, and since virtually all infected persons have superficial gastritis, it is likely that the organism plays a causative role early in the progression to adenocarcinoma (**Figure 2**). A randomized controlled study determined that eradication of *H. pylori* significantly decreases gastric cancer risk in infected individuals without pre-malignant lesions (319), while another study demonstrated that eradication of *H. pylori* significantly reduces the presence of pre-malignant lesions (187), providing additional evidence that this organism influences early stages in gastric carcinogenesis. In addition, our laboratory has recently shown that in the Mongolian gerbil model of *H. pylori* infection and gastric cancer, early intervention with antibiotic therapy significantly reduced the development of gastric dysplasia and neoplasia (245). These studies support the idea that *H. pylori* eradication therapy can play an important role in gastric cancer prevention, but the stage in human disease at which intervention is most effective is still not clear. However, as with all disease related to chronic injury, the earliest intervention seems to be the most effective.

A tumor can be thought of as an atypical organ, possessing its own vasculature and mechanisms for self-propagation. Thus, it has been hypothesized that, like normal organs, a tumor may possess a stem cell population from which tumor cells originate (239, 258). Recently several groups have identified prospective cancer stem cells in tumors in different organs (4, 35, 76, 133, 251, 270, 271, 310). A commonality among these stem cells is that they all possess characteristics that are similar to peripheral stem cells. Peripheral stem cells are attractive candidates as tumor progenitor cells because they have characteristics that are necessary for sustained tumorigenesis such as the ability to temporarily bypass normal growth control programs, which under normal circumstances allows proliferation for wound healing and tissue replacement. Historically, the stem cell zone of the stomach has been defined as the area of highest density of proliferating cells, which are thought to contain both the true stem cells and the first few generations of rapidly proliferating daughter cells. This region has been considered the most likely candidate for the accumulation of mutations necessary for the development of neoplastic growth. However, one outcome of chronic inflammation is atrophy and loss of these specialized cells. Therefore, an additional population of stem cells may be necessary for the development of gastric cancer. A recent study by Houghton *et al.* elegantly demonstrated that in mice that had been transplanted with genetically labeled bone marrow and infected with *Helicobacter felis,* bone marrow-derived cells (BMDCs) homed to and repopulated the gastric mucosa and developed over time into cancer (131). These data have greatly shifted the model for the development of gastric cancer and provide a mechanism that helps explain the progression to cancer caused by chronic inflammation induced by *H. pylori*. The mechanism is thought to be one in which chronic inflammation

leads to injury, which over time progresses to depletion of gastric stem cell populations. Stem cell failure, in turn, leads to the recruitment and engraftment of BMDCs into areas previously populated by the stem cells, where the BMDCs functionally replace gastric stem cells. With ongoing inflammation and injury, BMDCs are exposed to a hostile tissue environment containing free radicals that damage DNA and cytokines that dysregulate cell systems, causing them to fail to regulate growth programs appropriately and progress instead through stages of metaplasia and dysplasia (86).

However, only a small percentage of colonized persons ever develop neoplasia, raising the hypothesis that enhanced cancer risk may be related to *H. pylori* strain differences, inflammatory responses governed by host genetic diversity, and/or specific interactions between host and microbial determinants. These observations, in conjunction with evidence from our laboratory and others, indicating that carriage of certain *H. pylori* strains is inversely related to the prevalence of Barrett's esophagus and esophageal adenocarcinoma (33, 59, 167, 301, 307), underscore the importance of identifying mechanisms that regulate biological interactions of these organisms with their hosts which promote gastric carcinogenesis. Results generated by such studies would permit physicians to more appropriately focus diagnostic and eradication strategies on targeted high-risk populations to optimize prevention of subsequent neoplastic events.

H. pylori and chronic inflammation

Infection with *H. pylori* results in the presence of chronic active gastritis, which is characterized by both chronic (lymphocytic) and active (neutrophilic) forms of inflammation (108, 178). *H. pylori* is one of several organisms that can elicit an inflammatory response that predisposes the host to neoplastic transformation. Some examples of such microorganisms that colonize mucosal surfaces or epithelial cells and increase the risk of cancer are Human papilloma virus, Hepatitis C and B viruses, Epstein-Barr Virus, and parasitic helminthes *Opisthorchis viverrini* and *Schistosoma haematobium* (123, 199).

H. pylori produce several bacterial factors that are proinflammatory, such as neutrophil activating protein (NapA), urease, and the *cag* secretion system (discussed in the next section). NapA is a cytosolic protein that forms a dodecameric structure capable of binding large quantities of iron, insuring that *H. pylori* can acquire enough iron for survival (329). However, NapA release by *H. pylori* autolysis has the side effect of eliciting an immune response consisting of neutrophils, monocytes, and mast cells (197). Urease is an enzyme that converts urea into ammonia and carbonate, which is further converted to carbon dioxide. The production of ammonia and carbon dioxide neutralizes the bacterial cytosol and acidic microenvironment surrounding the bacteria, which is necessary for successful colonization and prolonged survival (315). However, urease is also immunogenic and contributes to the immune response directed against *H. pylori* (105). Thus, *H. pylori* produces constituents that promote survival in the gastric niche,

but have the consequence of eliciting an immune response, which results in injury and disease for the human host.

Two topics of great interest are how *H. pylori* evade clearance by the inflammatory response and how the immune response is dysregulated during infection. *H. pylori* has co-evolved with humans for at least 58,000 years (74, 101), and, as a result, is highly adapted to survive in the harsh environment of the stomach and avoid elimination by the immune system. One constituent that contributes to survival is bacterial arginase encoded by *rocF*, which converts L-arginine (L-Arg) to L-ornithine and urea, which is then further metabolized to ammonia by *H. pylori* urease (185). The benefits conferred by arginase for *H. pylori* are two fold: neutralization of the local environment and resistance to innate immune killing. Macrophages and epithelial cells produce inducible nitric oxide synthase (iNOS) in response to *H. pylori,* which utilizes L-Arg as a necessary precursor molecule in nitric oxide (NO) synthesis (92, 104, 318). NO is a highly reactive molecule that is toxic to many bacterial pathogens, but which also has the negative effect of causing DNA damage in host cells, which contributes to the accumulation of mutations that promote oncogenesis (15, 78, 210). Macrophage-produced NO has been shown to kill *H. pylori in vitro* and is thought to play an important role *in vivo*. Thus, depriving cells of L-Arg through the production of bacterial arginase may enhance bacterial survival (29, 92, 104, 320).

Survival of *H. pylori* may also be promoted by evasion of innate immune pattern recognition receptors such as toll-like receptors (TLR). Activation of TLRs leads to the

initiation of proinflammatory signaling. TLR-5 is a toll-like receptor that detects flagella of many bacterial organisms as they come into contact with epithelial cells. Unlike the flagella of many other bacteria, *H. pylori* flagella do not activate TLR-5 (100). *H. pylori* LPS is at least 1000-fold less immunogenic than *Escherichia coli* LPS, which is recognized by TLR-4 (198). These mechanisms likely help to dampen the immune response to *H. pylori* and contribute to persistent infection. By surviving direct encounters with phagocytes and oxidative compounds, *H. pylori* are able to persist despite an inflammatory response induced by it own constituents, such as NapA, urease, and the *cag* secretion system.

While *H. pylori* is well-adapted for survival within the gastric niche, and avoids activating many components of the innate immune system, such as TLRs, Anderson *et al*. have shown that TLR-9 plays an important role in inducing inflammation in *Helicobacter*-infected mice (7). TLR-9 distinguishes bacterial DNA from mammalian DNA by recognizing hypomethylated CpG dinucleotides, eliciting a strong T_h1-like inflammatory response (116, 122). However, TLR-9 is not constitutively expressed on gastric epithelial cells, although it is expressed on epithelial cells in Peyer's patches in the small intestines (267). Recently Peyer's patches have been shown to play a critical role in recognizing both *H. pylori* and *H. felis* infection in mice and mediating the subsequent inflammatory response (151, 203). There is also evidence to suggests that Peyer's patches preferentially recognize the coccoid form of *H. pylori* (203), a nonculturable form that is induced under unfavorable conditions such as an anaerobic environment, increased oxygen tension, and long-term culture (240, 322). Thus, TLR-9 expressed on

cells in Peyer's patches may be a key mediator of inflammation induced by the coccoid form of *H. pylori*.

The *H. pylori cag* pathogenicity island and type IV secretion system

H. pylori strains isolated from different individuals are extremely diverse (5, 103, 250, 292), and studies have demonstrated that the genetic composition of isolates can change over time (137). Although this extraordinary diversity has slowed the search for bacterial determinants unambiguously associated with cancer, several genetic loci have been identified that augment risk for carcinogenesis.

The *cag* pathogenicity island is a 40 kB locus present in approximately 60% of *H. pylori* strains in the United States (2, 5, 32, 292). Although all *H. pylori* strains induce gastritis, strains harboring the *cag* island (cag^+) augment the risk for severe gastritis, atrophic gastritis, gastric ulcer disease, and distal gastric cancer compared to cag^- strains (**Figure 2**) (24, 42, 52, 53, 156, 223, 228, 229, 237, 248, 268, 294, 309). *Cag* genotype also influences the topography of colonization in the human stomach, as *H. pylori* cag^- strains are located predominately within the mucus gel layer, while disease-associated cag^+ strains are found immediately adjacent and frequently adherent to epithelial cells (30).

Several *cag* genes encode products that form a type IV secretion system, which acts as a molecular syringe to translocate effector molecules into host epithelial cells. Upon binding to host cells, a component of the secretion system (CagL) interacts with $\alpha_5\beta_1$

integrins, enabling the secretion system to deliver molecules into the cytoplasm of host cells (159). This system is known to deliver at least two bacterial factors into host cells. Components of bacterial cell wall peptidoglycan can be translocated by the *cag* secretion system into host cells where they are recognized by a component of innate immune system, the intracellular pattern recognition receptor NOD1 (306). In addition, the product of the terminal gene in the *cag* island, CagA, is delivered into host cells where it exerts numerous effects.

CagA

CagA is a 120-140 kDa protein that contains tyrosine phosphorylation motifs (glutamate-proline-isoleucine-tyrosine-alanine, EPIYA) within the carboxy-terminal variable region of the protein (**Figure 3**) (278). There are at least four phosphorylation motif regions that can be found in CagA, which are termed EPIYA-A, -B, -C, or -D (118). Each motif is distinguished by the amino acid sequence surrounding the EPIYA motif (118). Most variants of CagA contain an EPIYA-A and -B site, which are phosphorylated to a lesser degree, whereas the major -C and -D phosphorylation sites segregate to either Western or East-Asian strains respectively (118). Thus, the majority of *cag*$^{+}$ Western strains are CagA A-B-C and East-Asian strains are A-B-D strains. Additionally, the number of EPIYA-C regions vary to contain between 1-3 repeated copies among different strains (118). Following its injection into epithelial cells, CagA can affect a myriad of cell signaling pathways, which can be divided into either phosphorylation or non-phosphorylation-dependent signaling.

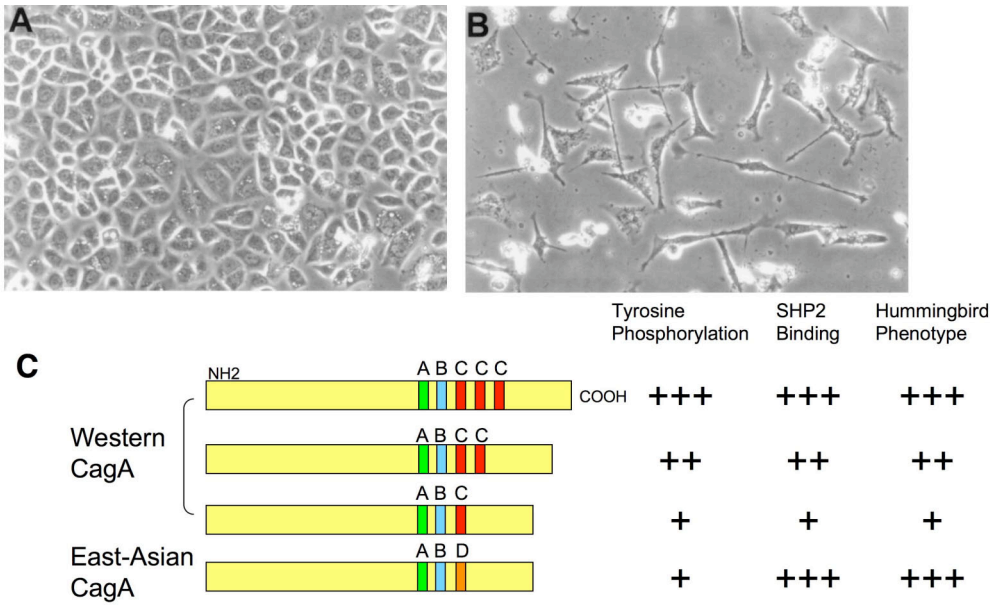

Figure 3. The influence of EPIYA-repeat polymorphisms on the pathophysiological activities of CagA. A.) Uninfected AGS gastric epithelial cells B.) *H. pylori* infected AGS cells demonstrating the characteristic cell elongation "hummingbird" phenotype. C.) EPIYA-C and EPIYA-D sites are the major tyrosine phosphorylation sites of Western and East-Asian CagA, respectively. Among Western CagA species, those having larger numbers of EPIYA-C exhibit stronger SHP-2 binding activity and greater ability to induce hummingbird like cells than those having less numbers of EPIYA-C sites. The EPIYA-D site of East-Asian CagA binds SHP-2 more strongly than does the EPIYA-C site of Western CagA. As a result, East-Asian CagA can induce hummingbird cells more intensely than Western CagA. The photographs in panels A and B were originally published by Segal *et al.* (256). Panel C is adapted from Hatakeyama and Higashi (119).

CagA can undergo tyrosine phosphorylation in the early stages of cellular infection by members of the Src family of kinases (Src, Fyn, Lyn, and Yes) and in the later stages by the protein tyrosine kinase Abl (**Figure 4**) (10, 13, 212, 256, 257, 279, 286). Phospho-CagA subsequently binds and activates at least three eukaryotic Src Homology 2 (SH2) domain containing proteins: the protein tyrosine phosphatase SHP-2, carboxy-terminal Src kinase (Csk), and the adaptor protein Crk (126, 283, 286, 297). These interactions lead to morphological changes that are reminiscent of unrestrained stimulation by growth factors, known as the "hummingbird" phenotype (**Figure 3B**) (10, 13, 125, 126, 212, 256, 257, 278, 279, 297). The hummingbird phenotype requires two successive events: 1) the induction of motility leading to cell scattering and 2) host cell elongation (196). Formation of the CagA-SHP-2 complex requires tyrosine phosphorylation of the EPIYA-C or -D sites in Western or East-Asian CagA proteins respectively. The intensity of SHP-2 signaling in Western strains directly correlates with an increased number of EPIYA-C motifs (**Figure 3)** (125). Interestingly, the EPIYA-D site of East-Asian strains perfectly matches the consensus high-affinity binding sequence for the SH2 domains of SHP-2 (118). As expected, the EPIYA-D site of East-Asian CagA exhibits stronger SHP-2 binding and greater morphogenetic activity than the EPIYA-C motif of Western CagA. The second SH2 domain-containing protein identified to interact with CagA is Csk. The interaction of phospho-CagA with Csk negatively regulates Src-family kinases by phosphorylating inhibitory tyrosine residue located in the carboxy-terminal regions (297). In this way, phospho-CagA induces a negative regulatory feedback loop that limits the activity of Src. The inhibition of Src reduces the amount of phosphorylated CagA present in the cell and subsequently attenuates CagA-SHP-2 signaling (297). Because sustained

activation of SHP-2 induces apoptosis in gastric epithelial cells, Csk mediated negative feedback may represent a regulatory mechanism that permits *cag*$^{+}$ *H. pylori* to persists for the lifetime of the host without causing serious damage in the majority of cases (297). Recently, phosphorylated-CagA was also found to interact with the Crk protein family in complex with Abl (283, 286). This interaction was determined to be important for the induction of the hummingbird phenotype, but the full implication of this observation has yet to be determined.

CagA also affects numerous pathways in a phosphorylation-independent manner (**Figure 4**). CagA interacts with the adaptor protein Grb2 and activates the Ras/MAPK cascade eventuating in ERK1/2 activation, which leads to cell scattering (194). Activation of ERK1/2 by CagA can also lead to the activation of NF-κB and the release of the proinflammatory cytokine IL-8 (26, 150). NF-κB activation requires the C-terminal region of the protein but is independent of phosphorylation status (26). Unmodified CagA also interacts with Par1, a MARK kinase that phosphorylates microtubule-associated proteins (249). The CagA/Par1 interaction inhibits Par1 kinase activity, coinciding with the disruption of apical-junctional complexes, perturbation of epithelial differentiation in polarized MDCK epithelial cells, and the induction of the hummingbird phenotype (6, 14, 249). Disruption of apical-junction complexes coincides with colocalization of CagA with the tight-junction proteins ZO-1 and JAM, and in some instances, recruiting these junctional proteins to ectopic sites of *H. pylori* binding (6). In addition, CagA induces the nuclear localization of the proto-oncogene β-catenin in a phosphorylation independent

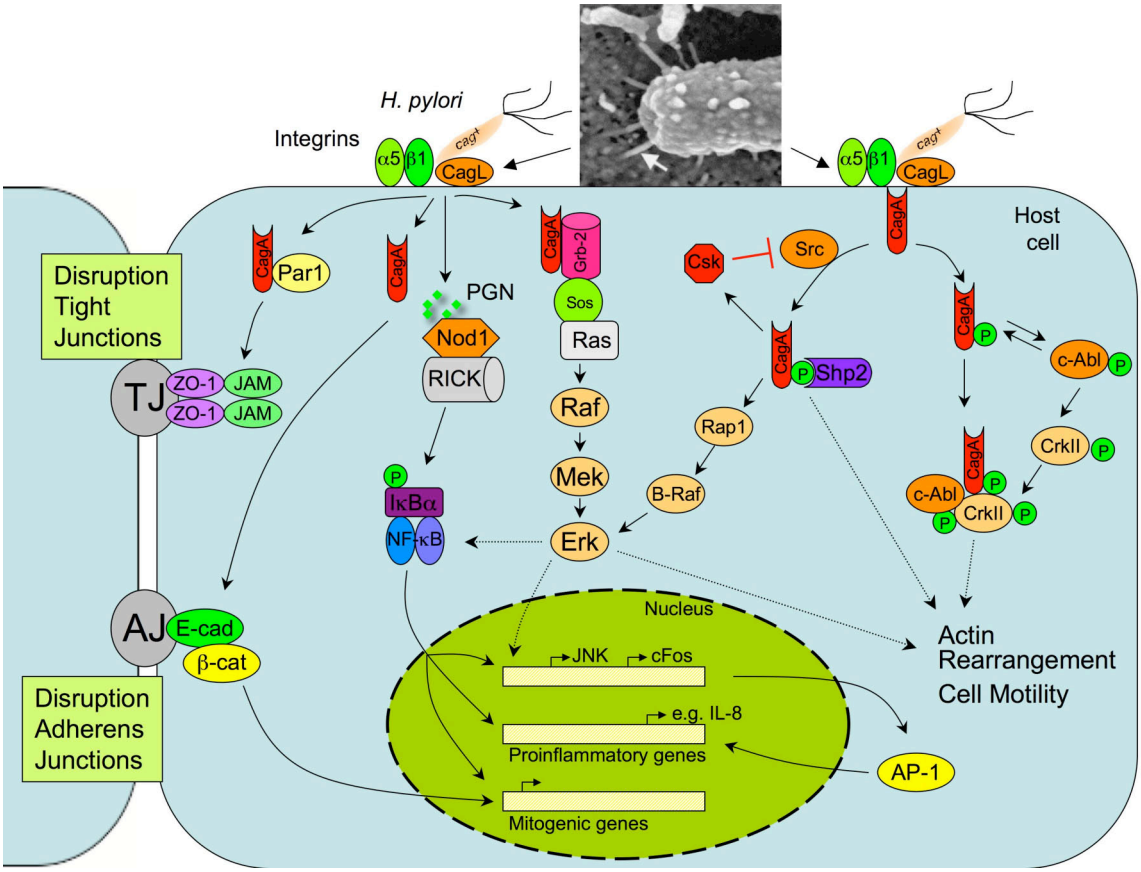

Figure 4. Type IV *cag* secretion system-mediated effects on the host cell. The *cag* secretion system (electron micrograph, white arrow) binds to the host cell via an interaction involving $\alpha_5\beta_1$ integrins and CagL. CagA is then translocated into the host cell and affects signaling pathways involved in proliferation, motility, actin-cytoskeletal rearrangements, disruption of cell-cell junctions, and proinflammatory responses. The secretion system also translocates components of the bacterial cell wall molecule peptidoglycan (PGN), which is recognized by the intracellular receptor Nod1, leading to the induction of proinflammatory signaling responses. TJ, Tight-Junctions; AJ, Adherens-Junctions. The electron micrograph was originally published by Rohde *et al.* (243). Figure adapted from Backert and Meyer (12).

manner (88). There are data to suggest that CagA induces nuclear β-catenin by binding E-cadherin and disrupting the β-catenin/E-cadherin interaction, thereby freeing β-catenin to localize to the nucleus (202). β-catenin then forms heterodimers with the TCF/LEF transcription factor to initiate transcription (88), and may activate target genes that are involved in transformation.

Indeed, a recent investigation by our laboratory has demonstrated that in the Mongolian gerbil model of *H. pylori* infection, gerbils infected with *cagA*$^-$ isogenic mutants developed no precancerous or cancerous lesions compared to gerbils infected with the wild-type strain in which tumors developed in at least 50% of infected animals by 12-16 weeks (89). In addition, Ohnishi *et al*. have recently demonstrated a direct causal link between CagA and gastrointestinal cancer by utilizing a transgenic mouse model of CagA expression (217). Transgenic mice that expressed wild-type CagA developed gastric epithelial hyperplasia by fours weeks of age and at 72 weeks some of the mice developed gastric polyps (8-22%) and adenocarcinomas (1-2%) of the stomach and small intestine (217). This study also strengthened the link between CagA phosphorylation and oncogenicity by demonstrating that in mice expressing a phosphoresistant-CagA, the development of gastric epithelial hyperplasia was almost completely attenuated and gastrointestinal cancer was not observed (217). While CagA plays an important role in inducing cell responses that lead to gastric cancer, the selective advantage that this protein provides for the bacteria is not well defined. However, the recent studies showing that CagA disrupts tight-junctions suggest that CagA may help to release nutrients into the local environment and/or allow the bacteria to invade into the epithelial mucosa, which

may contribute to the ability of *H. pylori* to persist for decades in the harsh niche of the stomach (6, 14, 283).

The vacuolating cytotoxin, VacA

An independent *H. pylori* locus linked with gastric cancer is *vacA*, which encodes a bacterial toxin known as the vacuolating cytotoxin (VacA) (41, 44, 235, 255, 289). *In vitro*, VacA induces the formation of intracellular vacuoles (163) and can also induce gastric epithelial cell apoptosis (43). The vacuolating activity of VacA has been extensively studied and is dependent on the formation of oligomeric VacA structures in the host cell membrane that exhibit anion-selective channel activity (56, 138, 285, 293). VacA has also been shown to suppress T and B cell activation-induced proliferation *in vitro* (25, 97, 281, 295), which may contribute to the longevity of *H. pylori* colonization by dysregulating the adaptive immune response. Unlike the *cag* island, *vacA* is present in virtually all *H. pylori* strains examined (11, 44); however, strains vary in cytotoxin activity due to variations in *vacA* gene structure. The regions of greatest diversity are localized near the 5' signal region of *vacA* (allele types s1or s2) and in the mid-region of *vacA* (allele types m1 or m2) (11, 302, 303). Functionally, s1-type strains are associated with vacuolating activity *in vitro* (11, 247), with the hydrophobic N-terminus of the toxin playing a vital role in this process (308, 326). In contrast, s2-type VacA is generally non-vacuolating due to the presence of a hydrophilic N-terminal 12-amino acid extension that blocks activity (161, 162, 181). Mid-region allele type determines the cell specificity of vacuolation by affecting toxin binding to epithelial cells, such that m1 forms cause vacuolation in a wider range of epithelial cell lines than m2 forms (140, 162, 221, 274).

H. pylori strains that possess a type s1/m1 *vacA* allele are associated with an increased risk of gastric cancer compared to s2/m2 strains (99, 169, 191, 192). A recent study by Rhead *et al.* identified a third polymorphic region of importance for VacA function, termed the intermediate (i) region, which is located between the signal and mid-regions (241). Two allelic variants of the i-region were identified (i1 and i2) (241). *H. pylori* s1/m1 strains were found to typically be i1-type and s2/m2 strains were i2-type (241). Interestingly, s1/m2 strains varied in their i-region status, and this affected vacuolation ability *in vitro*, with s1/i1/m2 strains inducing vacuolation, whereas s1/i2/m2 strains did not demonstrate vacuolating activity (241). These findings may have important implications for previous studies relating *vacA* mid-region type to disease because s1/m2 strains may have differed in i-region type and vacuolating activity.

Host factors that influence the propensity for development of gastric cancer

Although important, *H. pylori* constituents are not absolute determinants of carcinogenesis, which has highlighted the need to identify host factors that may be linked with gastric cancer. Cyclooxygenases catalyze key steps in the conversion of arachidonic acid to endoperoxide, a substrate for a variety of prostaglandin synthases that, in turn, catalyze the formation of prostaglandins and other eicosanoids (112). Prostaglandins regulate a diverse array of physiologic processes including immunity and maintenance of vascular tone (112). Three isoforms of cyclooxygenase have been identified, each possessing similar activities, but differing in expression characteristics. COX-1 and COX-3, a splice variant of COX-1, are expressed constitutively while COX-2 can be induced by

growth factors and pro-inflammatory cytokines (317). Up-regulation of COX-2 is a promoting event for colorectal carcinogenesis and COX-2 expression is increased in epithelial cells co-cultured with *H. pylori* (143, 244) and within gastric mucosa of *H. pylori*-infected individuals (92, 253). COX-2 expression is further increased within *H. pylori*-induced pre-malignant (atrophic gastritis and intestinal metaplasia) and malignant (adenocarcinoma) lesions (242, 282) and COX inhibitors have been shown to decrease the risk for distal gastric cancer (3, 79).

Polymorphisms within the human *IL-1β* or *TNF-α* gene promoters that are associated with increased expression of IL-1β or TNF-α (pro-inflammatory cytokines that have potent acid-suppressive properties) also heighten the risk for atrophic gastritis and gastric adenocarcinoma (65, 66, 94, 173). These relationships are only present among *H. pylori*-colonized persons, emphasizing the importance of host-environment interactions and inflammation in the progression to gastric cancer and consistent with these observational studies, *H. pylori* induces expression of each of these cytokines during co-culture with gastric epithelial cells *in vitro* (226). *H. pylori* strain characteristics further augment the risk of gastric cancer exerted by host genotype. Figuiredo *et al.* stratified infected subjects on the basis of both high-expression *IL-1β* polymorphisms and virulence genotypes of their infecting *H. pylori* strains (80). In persons with high-expression *IL-1β* alleles colonized by *H. pylori cag*$^{+}$ or *vacA* s1-type strains, the relative risks for gastric cancer were 25- and 87-fold over baseline, respectively (80), indicating that interactions between specific host and microbial determinants are biologically significant for the development of gastric cancer.

Salt intake is also an important risk factor for disease. An increased risk for the development of gastric cancer has been associated with high intake of dietary salt in epidemiological studies (296), and salt intake has also been positively associated with *H. pylori*-induced gastric cancer in animal models (208, 288). Salt has been shown to act synergistically and dose-dependently to promote the development of gastric adenocarcinoma in Mongolian gerbils treated with the carcinogen *N*-methyl-*N*-nitrosourea (MNU) (146, 208). In humans, mucosal damage induced by dietary salt intake may facilitate persistent infection by *H. pylori* (84). *H. pylori* gene expression can also be affected by salt concentration. Growth of *H. pylori* in media containing increasing concentrations of salt leads to increased translocation and phosphorylation of CagA *in vitro* (168). Thus, increased CagA activity in the gastric epithelium due to high salt concentrations may contribute to oncogenesis. These data suggest that there are several mechanisms working in tandem that increase cancer risk, but more research is needed to fully elucidate risk factors. However, evidence from studies on the association between dietary salt intake and the risk of gastric cancer indicates that dietary modifications that reduce salt are likely protective against gastric cancer (296).

H. pylori **contact-mediated cytokine release**

The presence of acute inflammatory components within *H. pylori*-infected mucosa suggests that soluble mediators capable of attracting polymorphonuclear cells, such as cytokines, are key regulators of disease. Compared with uninfected individuals, the

gastric epithelium from infected persons contains increased levels of IL-1β, IL-2, IL-6, IL-8, and TNF-α (50, 51, 75, 229, 323), and within this group, IL-8 has been studied most intensively as a mediator of pathogenesis. IL-8 is a potent neutrophil-activating chemokine that is secreted by gastrointestinal epithelial cells in response to pathogenic bacteria (64). IL-8 binds to the extracellular matrix and establishes a haptotactic gradient that directs inflammatory cell migration towards the epithelial cell surface (141, 182-184). Expression of IL-8 is increased within *H. pylori*-colonized gastric mucosa (49, 229) where it localizes to gastric epithelial cells (49). Further, levels of IL-8 are directly related to the severity of gastritis (229). *In vitro*, *H. pylori* stimulates IL-8 expression from gastric epithelial cells and these events are dependent upon an active interplay between viable bacteria and epithelial cells (48, 55, 264). Thus, a paradigm for the acute component of gastric inflammation is that contact between *H. pylori* and epithelial cells stimulates IL-8 secretion, which then regulates neutrophilic infiltration into the gastric mucosa.

Molecular regulation of *H. pylori*-induced IL-8 gene expression

The human IL-8 gene contains several motifs within its promoter region including binding sites for NF-κB, AP-1 (comprised of the binding elements c-*fos* and c-*jun*), and a recently identified novel element that is homologous to an interferon-stimulated responsive element (ISRE) (1, 31, 225, 324). NF-κB constitutes a family of transcription factors sequestered in the cytoplasm, whose activation is tightly controlled by inhibitory proteins termed IκB's (305). Multiple signals, including microbial contact, stimulate

phosphorylation of IκBα by IκB kinase ß (IKKß), which leads to proteasome-mediated degradation of phospho-IκBα, thereby liberating NF-κB to enter the nucleus where it regulates transcription of a variety of genes, including immune response genes (188). Mitogen-activated protein kinases (MAPK) are components of signal transduction networks that target transcription factors such as AP-1 and participate in a diverse array of cellular functions, including cytokine expression (96, 135, 254). MAPK cascades are organized in three-kinase tiers consisting of a MAPK, a MAPK kinase (MKK), and a MKK kinase (MKKK), and transmission of signals occurs by sequential phosphorylation and activation of components specific to a respective cascade. In mammalian systems, MAPK modules include ERK1/2, p38, and JNK (96, 135, 254). Contact between *H. pylori* and gastric epithelial cells *in vitro* results in brisk activation of NF-κB as well as p38, ERK1/2, and JNK, which is followed by increased IL-8 expression (1, 147, 174, 263, 324).

To define the bacterial factors involved in *H. pylori*-induced IL-8 secretion, our laboratory and others have demonstrated that *H. pylori cag*$^+$ strains selectively activate NF-κB, p38, ERK 1/2, and JNK in gastric epithelial cells (148, 189). However, these effects may not be completely dependent upon CagA translocation (**Figure 5**). Viala *et al.* demonstrated that components of cell wall peptidoglycan are delivered to the host cell cytoplasm by the *cag* secretion system and are then recognized by the intracellular pathogen-recognition receptor Nod1, which eventuates in NF-κB activation and the induction of IL-8 (306). An additional layer of complexity is added when one considers that maximal *H. pylori*-induced IL-8 gene transcription requires the presence of NF-κB,

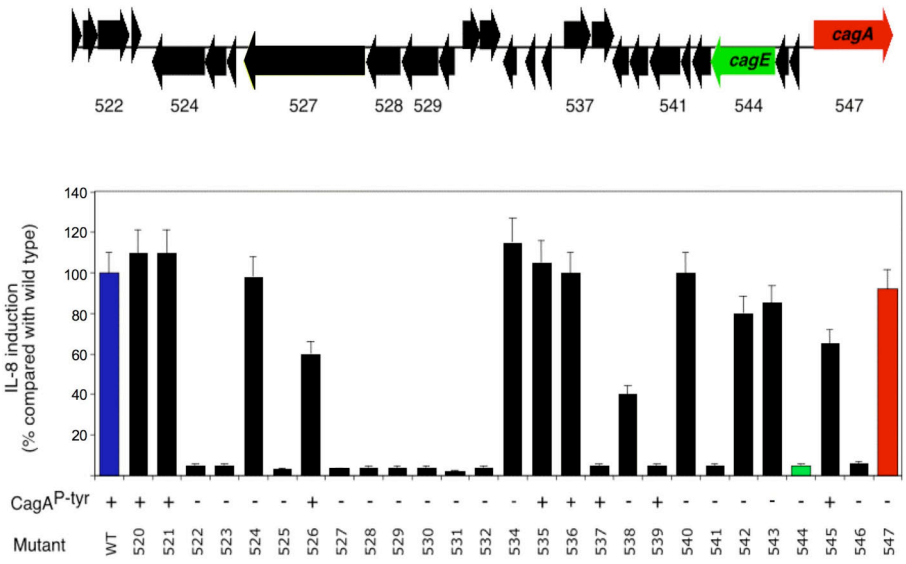

Figure 5. Translocation of CagA and induction of IL-8 in AGS cells is dependent upon numerous components of the *cag* pathogenicity island. Induction of IL-8 for individual isogenic *cag* island mutant strains of *H. pylori* is shown on the y-axis in comparison to the wild type (WT) strain 26695 in blue (100%). The ability of each wild type or mutant strain to translocate CagA into AGS cells as measured by CagA tyrosine phosphorylation (x-axis) is represented by (+) or (-). *cagA* and *cagE* are highlighted. Adapted from Fischer *et al.* (81).

AP-1, and ISRE elements, and that ISRE activation may be dependent upon an *H. pylori* outer membrane protein (OMP) exogenous to the *cag* island, OipA (324). However, the involvement OipA in cytokine release has not been universally accepted, as other investigators have reported that mutation of OipA does not significantly affect *H. pylori*-induced expression of IL-8 (62). Collectively, these data indicate that the inflammatory response to *H. pylori* likely involves multiple intracellular pathways converging on the IL-8 promoter, but the specific mechanisms that mediate IL-8 expression remain poorly defined. Investigations that define these pathways are critical since *H. pylori*-mediated host signaling is of central importance for understanding the inflammatory response to this pathogen, which if left untreated over decades, may progress to gastric cancer.

Rodent models of *H. pylori*-induced gastric adenocarcinoma

Rodent models have provided valuable insights into the host, bacterial, and environmental factors involved in gastric carcinogenesis. Long-term (>1 year) *H. pylori* infection of Mongolian gerbils can lead to inflammation-induced gastric adenocarcinoma, without the co-administration of known carcinogens (130, 214, 313, 331), and gastric cancer development in this model occurs in the distal stomach, as in humans. We have shown that various *H. pylori* wild-type and mutant strains colonize gerbils well (136, 231), allowing an examination of the role of virulence determinants on parameters of gastric injury. Recently our group has demonstrated that the gerbil-adapted *H. pylori* strain 7.13 can induce adenocarcinoma in 59% of challenged gerbils by 8-16 weeks post-innoculation (88). However, there are limitations to using this model. Mongolian gerbils

are outbred with undefined genetic backgrounds, which tend to increase the variability of responses to any stimulus. Compared with mice, gerbils are relatively poorly characterized and few reagents including antibodies or immune markers are available for detailed investigation. Finally, the ability to utilize inbred mice with defined genotypes as well as transgenic lines allows a more detailed analysis of host susceptibility to *H. pylori* virulence determinants and pathological consequences. Therefore, our laboratory and others have also utilized murine models of gastritis.

One host determinant that may influence the development of gastric cancer is gastrin. *In vitro*, gastrin stimulates gastric epithelial cell proliferation (139), and transgenic mice that over-express gastrin (INS-GAS mice) spontaneously develop gastric cancer, although this requires the virtual lifetime of the animal (2 years) (311). Concomitant infection with *H. pylori* or a related *Helicobacter* species, *H. felis*, accelerates this process (85, 87, 311), suggesting that persistently elevated gastrin levels synergistize with *Helicobacter* to augment the progression to gastric cancer.

One phenotypic difference between neoplasia that develops in INS-GAS mice compared to gerbils and humans is anatomic location. Even though *H. pylori*-infected gerbils and humans with hypergastrinemia and corpus-predominant gastritis often develop parietal cell loss similar to experimentally infected INS-GAS mice (157), most adenocarcinomas in gerbil or human tissue occur in the antrum (130, 313). In contrast, cancer in INS-GAS mice develops more frequently in the corpus (85, 87, 311); thus, although carcinogenesis in this model is likely regulated by the same host conditions (e.g. hypergastrinemia)

induced by *H. pylori* infection in humans, the most susceptible gastric site for disease is different. Collectively, these findings indicate that development of gastric cancer in humans is associated with features present in both *H. pylori*-infected gerbils (distal gastric adenocarcinoma), and mice (parietal cell loss and altered glandular differentiation). Therefore, these models likely are complementary, each contributing important information to our understanding of the events leading to transformation associated with *H. pylori* colonization.

H. pylori adherence and pathogenesis

Adherence of *H. pylori* to gastric epithelial cells is important for persistent colonization of the stomach. The gastric environment is hostile and *H. pylori* need to withstand a constant wave of peristalsis, and shedding and regeneration of the mucus gel layer. Contact between *H. pylori* and gastric epithelium is mediated by interactions between bacterial binding proteins known as adhesins and host proteins known as receptors. Though the majority of *H. pylori* in colonized hosts are free-living, approximately 20% bind to gastric epithelial cells (124). Adhesion by *H. pylori* to gastric epithelium is highly specific *in vivo* and when *H. pylori* is found in the duodenum, it only overlays islands of gastric metaplasia (321). Sequence analysis of the genomes from three completely sequenced *H. pylori* strains 26695, J99, and HPAG1 has revealed that an unusually high proportion of identified open reading frames are predicted to encode outer membrane proteins (OMPs) (5, 216, 292), many of which have been identified as

adhesins (36). This large repertoire of OMPs permits *H. pylori* to engage in a range of interactions with host cells, some of which play a role in pathogenesis (61, 275).

BabA is an OMP encoded by the strain-specific gene *babA2* that binds the Lewis^b (Le^b) histo-blood-group antigen on gastric epithelial cells (99, 134). BabA binding specificities reflect *H. pylori* strain adaptation to different glycosylation patterns that predominate in a particular host population and BabA-mediated Le^b binding can be altered by both bacterial phase variation and genetic recombination (275). Of interest, *babA2* and another gene encoding an outer membrane protein (Omp27) have been shown to co-vary with the presence of the *cag* island (134), and since evolutionary pressures tend to select for the co-inheritance of genes involved in common pathways, these observations raise the hypothesis that outer membrane proteins may act in conjunction with the *cag* secretion system to aberrantly alter epithelial cell responses. Consistent with this hypothesis, toxigenic *H. pylori* strains that possess *babA2* and *cagA* incur the highest risk for gastric cancer (99). The *H. pylori* adhesin SabA binds the sialyl-Lewis^x (sLe^x) antigen, which is an established tumor antigen and marker of gastric dysplasia (176). Gastric inflammation induced by *H. pylori* up-regulates the expression of sLe^x on epithelial cells, which amplifies interactions between this molecule and SabA.

Studies focused on adherence have also provided mechanistic insights into the topography of injury that develops within the stomach. Syder and colleagues studied bacterial colonization and inflammation in transgenic mice that lacked acid-secreting parietal cells (284). Mice were colonized with an *H. pylori* strain that expressed adhesins

that bind epithelial NeuAcα2,3Galß1,4 glycan receptors. In control mice, *H. pylori* exhibited tropism for gastric mucosa that did not contain parietal cells, and lymphocytic infiltration was found preferentially in this area. In mice having a genetic ablation of parietal cells, epithelial progenitor cells synthesized NeuAcα2,3Galß1,4 glycans, and this was accompanied by an expansion of bacterial colonization and lymphoid aggregates within the glandular epithelium (284). Collectively, these studies indicate that adherence of *H. pylori* to gastric epithelium likely plays an important role in the induction of inflammation and injury.

Several *H. pylori* proteins have been identified that are involved in adherence but have no known receptors. The outer membrane proteins AlpA and AlpB, which are encoded by the same operon, are involved in adherence (213). These genes are highly homologous, but both proteins are required for Alp mediated adherence (58, 211, 213). AlpAB binding to gastric epithelial cells differs from BabA-mediated binding, suggesting that a different receptor is involved (213). In addition, AlpAB is critical for successful colonization of both guinea pigs and mice, suggesting that AlpAB likely plays an important role in human disease (58, 170). Further, phase variation of *H. pylori* adhesins, such as BabA and SabA, suggests that interactions with gastric cells may vary among strains. Thus, identifying adhesin/receptor interactions and their roles in the *H. pylori* lifecycle are critical to understanding *H. pylori*-induction of injury and disease.

Decay-accelerating factor and gastric injury

Decay-accelerating factor (DAF/CD55) was first described in 1969 as an erythrocyte surface protein that regulates complement activation (128). DAF has since been shown to be a member of a family of regulators of complement activation proteins, and its primary function is to inactivate the C3/C5 convertases of the classical and alternative complement pathway by dissociating them into their constituent proteins (27). Inactivation of C3/C5 convertases protects cells from inadvertent complement-mediated lysis. DAF is a 70-80 kDa glycoprotein that contains five extracellular domains consisting of four contiguous complement control protein (CCP) domains followed by a serine/threonine rich heavily *O*-glycosylated C-terminal domain that elevates the molecule at the cell membrane surface where it is attached to the outer leaflet of the membrane by a glycosylphosphatidylinositol (GPI)-anchor (**Figure 6**) (260). DAF is highly expressed on cells that are exposed to serum such as endothelial cells and inflammatory cells. The clinical importance of DAF in underscored by its function in several human diseases including paroxysmal nocturnal hemoglobinuria (PNH) (195, 246), rheumatoid arthritis (287), systemic sclerosis and psoriasis (304), and ulcerative colitis (300). DAF also suppresses hyperacute and acute graft rejection in xenotransplantation (57, 328).

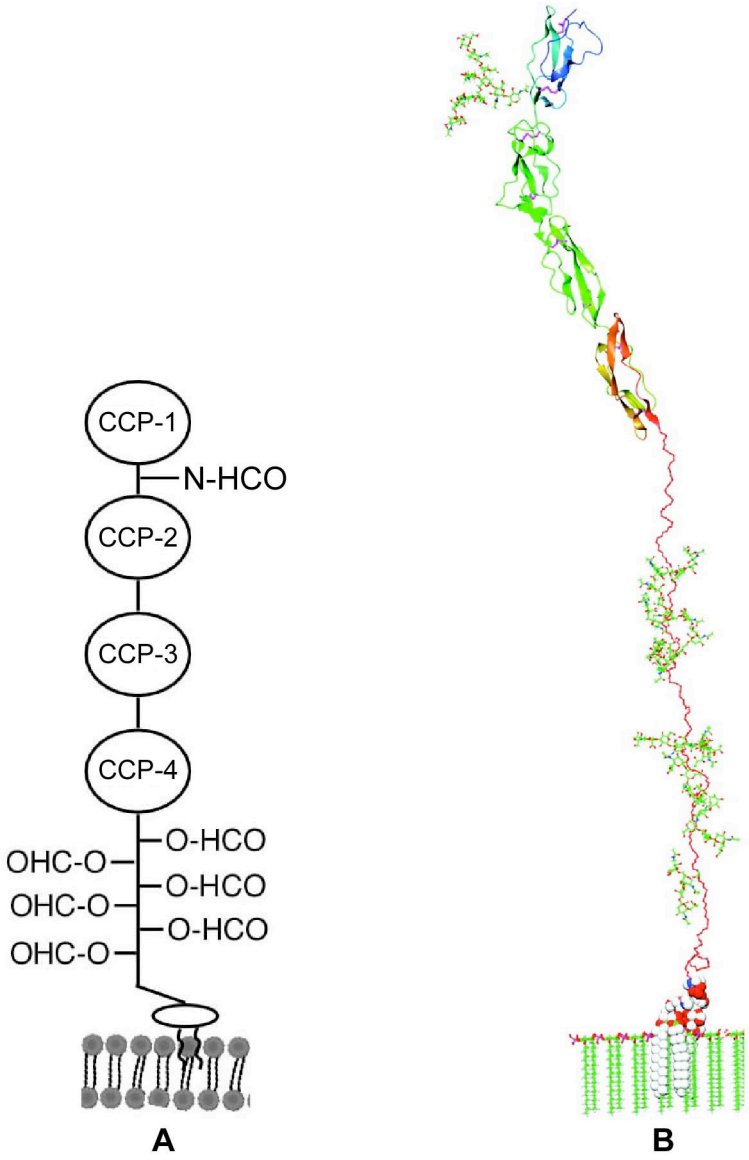

Figure 6. The structure of decay-accelerating factor (DAF). A.) DAF is a 70-80 kDa protein made up of four complement control protein (CCP) domains with a *N*-linked glycan located between CCP-1 and 2, and a heavily *O*-glycosylated serine/theronine rich stalk domain that is attached to the lipid bilayer by a GPI anchor. B.) Crystal structure of DAF originally published by Lukacik *et a*l. (172)

In addition to its role in preventing complement-mediated attack, DAF also functions as a cellular receptor for pathogenic organisms including uropathogenic diffusely-adhering *E. coli*, coxsackieviruses, echoviruses, and enteroviruses (17, 34, 111, 117, 120, 144, 207, 236, 260-262). A common property of these organisms is the ability to persist for prolonged periods within the host, a pattern that mirrors the chronicity of *H. pylori* infection. *E. coli* that express DAF-binding Dr adhesins cause chronic interstitial nephritis (259), while echoviruses and coxsackieviruses are associated with chronic fatigue syndrome and chronic dilated cardiomyopathy, respectively (179, 327). Thus, DAF is a receptor that is exploited by pathogens notable for their ability to induce chronic inflammation, injury, and disease.

DAF has also been shown to orchestrate epithelial pro-inflammatory responses to pathogens. Co-culture of T84 intestinal epithelial cells that express endogenous DAF with Afa/Dr diffusely adhering *E. coli* leads to activation of ERK1/2, p38, and JNK, which eventuates in IL-8 secretion (21, 22, 45), results that mirror the effects of *H. pylori* on gastric epithelial cells. *In vivo* data indicate that DAF may also mediate pathologic changes associated with *H. pylori* infection. Expression of DAF is increased within *H. pylori*-infected human gastric tissue compared to uninfected mucosa, and the intensity of DAF expression is directly related to *H. pylori* colonization density and the severity of inflammation (18, 238, 252). Increased DAF expression is present in gastric cancer precursor lesions such as intestinal metaplasia and gastric adenomas, and in gastric adenocarcinoma specimens compared to non-transformed gastric mucosa (152), suggesting that aberrant expression of DAF precedes the development of gastric cancer.

In vitro, expression of DAF can be induced by *H. pylori*-responsive pro-inflammatory cytokines such as IL-1ß and TNF-α (21, 22), and polymorphisms within the promoter regions of these cytokines confer differing risks for gastric cancer among *H. pylori*-infected persons (65, 66, 94, 173). Thus, there is strong evidence from *in vitro* and *in vivo* model systems that DAF may regulate pathologic outcomes that develop in response to *H. pylori*.

Summary and dissertation goals

Gastric adenocarcinoma is strongly associated with the presence of *H. pylori*, and both microbial and host factors influence the risk for carcinogenesis. Adherence of *H. pylori* to epithelial cells likely plays an important role in the development of gastric injury. DAF is a surface protein that can orchestrate pro-inflammatory responses and is over-expressed in both pre-malignant and malignant gastric lesions. Thus, DAF may mediate host responses related to inflammation and carcinogenesis within the context of *H. pylori* colonization, prompting us to hypothesize that *H. pylori*:DAF interactions contribute to pathogenesis. Molecular delineation of intracellular pathways activated by such host-microbial interactions will not only improve our understanding of *H. pylori*-induced carcinogenesis, but may also provide mechanistic insights into other malignancies that arise within the context of inflammatory states (e.g. ulcerative colitis and colon cancer).

Identification of DAF as an *H. pylori* receptor and its role in pathogenesis

DAF is a molecule that is induced in *H. pylori* associated human disease and is co-opted by several mucosal pathogens as a receptor. Previous studies have shown that the expression of DAF on gastric epithelial cells is localized to the luminal surface of gastric epithelial cells; thus, we hypothesized that *H. pylori* uses DAF as a receptor. In Chapter II, this hypothesis is investigated using an *in vitro* model of *H. pylori:*DAF interaction. The results from this study identify DAF as a novel *H. pylori* receptor. We then characterized the domains of DAF that are important mediators of *H. pylori* binding and provide evidence that DAF is a vital receptor for *H. pylori* induction of inflammation *in vivo* utilizing a murine genetic model of DAF deficiency.

The molecular regulation of DAF mediated by *H. pylori*

H. pylori induces significantly increased expression of DAF in gastric epithelial cell culture models of infection and increased DAF expression coincides with *H. pylori* infection in humans. These results raised the question as to whether the bacteria induce DAF through a specific mechanism or if exposure of the cells to the pathogen elicited a non-specific response to protect cells from complement attack during inflammation. Therefore, we sought to delineate the bacterial and host constituents that mediate DAF induction. In Chapter III, we demonstrate that *H. pylori* induces the transcriptional up-regulation of DAF. The cell signaling pathway by which *H. pylori* signals DAF up-regulation is identified and we demonstrate that a functional *cag* secretion system is necessary for DAF induction *in vitro* and in an *in vivo* murine model of infection. This work underscores the importance of dissecting *H. pylori* induced cell signaling events

and demonstrates a novel mechanism by which the *cag* secretion system induces the expression of a cognate cellular receptor, thereby implicating this virulence locus as mediating previously undescribed events that may increase the fitness of *H. pylori* within its gastric niche.

CHAPTER II

THE ROLE OF DECAY-ACCELERATING FACTOR AS A RECEPTOR FOR *HELICOBACTER PYLORI* AND A MEDIATOR OF GASTRIC INFLAMMATION

Summary

Persistent gastritis induced by *Helicobacter pylori* is the strongest known risk factor for peptic ulcer disease and distal gastric adenocarcinoma, a process for which adherence of *H. pylori* to gastric epithelial cells is critical. Decay-accelerating factor (DAF), a protein that protects epithelial cells from complement-mediated lysis, also functions as a receptor for several microbial pathogens. In this study, we investigated whether *H. pylori* utilizes DAF as a receptor and the role of DAF within *H. pylori*-infected gastric mucosa. *In vitro* studies showed that *H. pylori* adhered avidly to CHO cells expressing human DAF but not to vector control expressing cells. In *H. pylori*, disruption of the virulence factors *vacA*, *cagA*, and *cagE* did not alter adherence, but in DAF, deletion of complement control protein (CCP) domains 1-3 abolished binding. In cultured gastric epithelial cells, *H. pylori* induced transcriptional up-regulation of DAF, and genetic deficiency of DAF attenuated the development of inflammation among *H. pylori*-infected mice. These results indicate that DAF may regulate *H. pylori*-epithelial cell interactions relevant to pathogenesis.

Introduction

Helicobacter pylori induces an inflammatory response in the stomach that persists for decades and biological costs incurred by chronic infection include an increased risk for peptic ulceration, gastric adenocarcinoma, and non-Hodgkins lymphoma of the stomach (200, 226). However, most colonized individuals remain asymptomatic and increased disease risk is related to bacterial strain differences, epithelial responses governed by host diversity, and/or specific interactions between host and microbial determinants.

While the vast majority of *H. pylori* in colonized hosts are free-living, approximately 20% bind to gastric epithelial cells and this adherence is required for induction of injury. The *H. pylori cag* pathogenicity island encodes a type IV secretion system that, following adherence, translocates peptidoglycan and CagA into host cells (10, 13, 212, 257, 279, 306). CagA subsequently undergoes Src-dependent tyrosine phosphorylation and activates a eukaryotic phosphatase (SHP-2), eventuating in dephosphorylation of host cell proteins and cellular morphological changes (13, 126, 257, 278). Recently, CagA has also been shown to activate ß-catenin and induce NF-κB-mediated IL-8 release from gastric epithelial cells (26, 88). The presence of the *cag* island influences the topography of colonization, as *H. pylori* cag^- strains predominate within the mucus gel layer, while cag^+ strains are found immediately adjacent to epithelial cells (30). Concordant with these properties, *H. pylori* strains that harbor a functional *cag* island are associated with an increased risk for ulcer disease and gastric cancer compared to cag^- strains (226).

Another *H. pylori* locus linked with pathologic outcomes is *vacA*, which encodes a bacterial toxin (VacA) that induces vacuolation and apoptosis of epithelial cells (43, 163, 227). VacA binds to a unique receptor-type protein tyrosine phosphatase, PTPζ, a member of a family of receptor-like enzymes that regulate cellular proliferation, differentiation, and adhesion (93). Another virulence factor, an adhesin termed BabA (encoded by the *H. pylori* strain-selective gene *babA2*), binds the blood-group antigen $Lewis^b$ on gastric epithelial cell membranes (275), and *H. pylori* $babA2^+$ strains increase the risk for gastric adenocarcinoma (99). Finally, genetic ablation of parietal cells in mice induces gastric epithelial progenitor cells to synthesize NeuAcα2,3Galß1,4 glycan, which serves as a receptor for *H. pylori*, and this is accompanied by an expansion of bacterial colonization and inflammation within the glandular epithelium (215, 284). Collectively, these results indicate that dynamic and specific interactions between *H. pylori* and host receptors legislate pathologic outcome.

Decay-accelerating factor (DAF) is an intrinsic regulator of complement, which is attached to the outer leaflet of the cell membrane (260). It is a 70 kDa glycoprotein containing 4 contiguous 60 amino acid long repeats termed complement control protein repeats (CCPs) followed by a serine-threonine rich heavily *O*-glycosylated C-terminal domain that elevates the molecule at the membrane surface (260). DAF is membrane linked by a glycosylphosphatidylinositol (GPI)-anchor. DAF protects self cells from complement activation on their surfaces by dissociating membrane-bound C3 convertases that are required for cleaving C3 and initiating further propagation of the complement cascade.

Previous work has shown that DAF is utilized as a cellular receptor for several pathogenic organisms including uropathogenic diffusely-adhering *E. coli*, coxsackieviruses, echoviruses, and enteroviruses (17, 34, 111, 117, 120, 144, 207, 236, 260-262). Studies by other investigators have shown that expression of DAF is increased within *H. pylori*-infected human gastric tissue compared to uninfected mucosa, and this increase is directly related to the density of *H. pylori* colonization and severity of inflammation (18, 238, 252). It has also been shown that increased DAF expression is present in gastric cancer precursor lesions such as intestinal metaplasia and gastric adenomas, and in gastric adenocarcinoma specimens compared to non-transformed gastric mucosa (152), suggesting that aberrant expression of DAF precedes the development of gastric cancer. Since DAF is over-expressed within *H. pylori*-associated pre-malignant and malignant lesions, we investigated whether *H. pylori* utilizes DAF as a receptor *in vitro* and the role of DAF within *H. pylori*-infected gastric mucosa in order to define a potential pathogenic response towards this organism.

Experimental Procedures

Recombinant cell lines

Chinese hamster ovary (CHO) cell transfectant clones that stably express human DAF cDNA (DAF/A9), cDNA CCP deletion constructs (DAFΔCCP1/029-6B, DAFΔCCP2/043-7A, DAFΔCCP3/044-2D, DAFΔCCP4/054-5 x 4), a cDNA serine/threonine (S/T) rich region deletion construct (DAFΔS/T), a cDNA S/T region deletion construct containing an in-frame fusion of the cDNA encoding the amino-terminal region of DAF with the carboxy-terminus of HLA-B44 (DAFΔS/T + HLA), or vector alone were used as previously described (46). Cells were cultured at 37°C in Ham's nutrient mixture F-12 (GIBCO-BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 50 U of penicillin/ml, 50 μg of streptomycin/ml, 2 mM L-glutamine, nonessential amino acids, and 250 μg of G-418/ml when indicated. AGS (ATCC CRL 1739) or MKN28 (kindly provided by Dr. Robert Coffey, Vanderbilt University) human gastric epithelial cells were grown in RPMI 1640 (GIBCO-BRL) with 10% heat-inactivated FBS and 20 μg/ml gentamicin in an atmosphere of 5% CO_2 at 37°C.

Bacterial strains used *in vitro*

Experiments were performed with the *cag*$^+$ toxigenic *H. pylori* strain J166, as well as 8 additional (5 *cag*$^+$ toxigenic, 3 *cag*$^-$ non-toxigenic) well-characterized clinical strains. Clinical strains were selected from a larger population of isolates that have been previously described as part of an ongoing prospective study designed to study

mechanisms of *H. pylori* pathogenesis (227). Since we sought to analyze the importance of *H. pylori* genes related to disease, we selected strains that varied in *cag* status and toxin production. Isogenic *cagA*, *cagE*, and *vacA* null mutants were constructed within strain J166 by insertional mutagenesis, using *aphA* (conferring kanamycin resistance) as previously described (54, 227), and were selected on *Brucella* agar with kanamycin (25 μg/ml). *H. pylori* wild-type P1 and isogenic *alpAB* mutant strains and *alpAB* mutant constructs were a generous gift from the laboratory of Steffan Backert (Otto von Guericke Unviersity, Magdeburg, Germany). *H. pylori* wild-type G27 and *omp27*(*hopQ*) and *omp28*(*babA*) mutant strains were generous gifts of Nina Salama (Fred Hutchinson Cancer Research Center, Seattle, WA). The isogenic *oipA* null mutant was constructed in strain 7.13, G27, and J166 by insertional mutagenesis as previously described (325), using a chloramphenicol resistance gene cassette (*cat*), and recombinants were selected on *Brucella* agar containing chloramphenicol (10ug/mL). The isogenic *hp1501, hp0605,* and *hp0025* null mutants were constructed in strain 7.13 by insertional mutagenesis as previously described (227, 325). The isogenic *wbcJ* mutant was generated in strain J166 as previously described (186). As bacterial controls, *Campylobacter jejuni* strain 81176 and a Dr DAF adhesin negative, flagellated *Escherichia coli* strain (HB101) were also co-cultured with CHO cells (54).

Recombinant CHO cells expressing full-length DAF, its domain deletion mutants, or vector alone were seeded in 100 mm polypropylene tissue culture dishes (Nunc, Denmark) at 2.5 x 10^6 cells/dish and allowed to grow for 24 hours to subconfluency. *H. pylori* were grown in *Brucella* broth with 10% FCS for 18 h, harvested by centrifugation,

resuspended to a concentration of 1 x 10^{10} colony forming units (cfu)/ml (OD_{600}=1 equals 5.5 x 10^8 cfu/ml), and added to cells at a bacteria:cell ratio of 10:1 (136). Co-culture experiments with viable *H. pylori* were performed in antibiotic-free media with 10% FBS. For quantitative culture of adherent bacteria, *H. pylori*:CHO cell co-cultures were washed after 4 hours with 2 ml phosphate-buffered saline (PBS; pH 7.6) x 2 to remove non-adherent bacteria, and total cell extracts were harvested in 500 μl PBS using a rubber policeman as described (54). Three to six serial 10-fold dilutions (50 μl extract in 450 μl PBS) of 500-μl aliquots of cell extracts were cultured on 5% sheep blood agar plates, and incubated for 3 to 5 days under microaerobic conditions before *H. pylori* colonies were counted. Results are expressed as cfu/ml.

Immunofluorescence

CHO and gastric cells were plated in 4-well chamber slides at 5 x 10^4 cells/well and grown to subconfluency over 18-24 hours, and cells treated with or without *H. pylori* for 4 hours (MOI=100) were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, incubated in 0.1% PBST with 5% BSA for 1 hour, and then incubated with mouse monoclonal anti-DAF antibody 1H4 (1:100) (46) for 1 hour. For dual immunofluorescence, slides were stained with mouse monoclonal anti-DAF antibody 1H4 (1:100) and rabbit anti-*H. pylori* antibody (1:100, DakoCytomation). Slides were washed 3x in 0.1% PBST and then incubated with either goat anti-mouse Alexa Fluor 546-conjugated antibody (1:100; Molecular Probes) for single immunofluorescence or Alexa Fluor 488-conjugated goat anti-rabbit antibody and Alexa Fluor 546-conjugated goat anti-mouse antibody (1:100, Molecular Probes) for dual

immunofluorescence at room temperature for 1 hour. Nuclei were stained using DAPI. Slides were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and immunofluorescence was observed using a fluorescence microscope (113). For antibody inhibition studies, CHO cells were pre-incubated with anti-DAF monoclonal antibodies (1:100) 11D7 (directed against CCP domain 1), 1H4 (directed against CCP domain 3), 8D11 (directed against CCP domain 4) (46), and ascites fluid containing the monoclonal antibody IF7 (1:1000) (directed against CCP domain 2) (111) for 30 minutes prior to infection with *H. pylori.* Anti-DAF antibodies were provided by Douglas Lublin (Washington University, St. Louis).

Western analysis

Transfected CHO cells or gastric cells from *H. pylori*:AGS or *H. pylori*:MKN28 cell co-cultures were lysed in RIPA buffer (50 mM Tris, pH7.2; 150 mM NaCl; 1% Trioton X-100; 0.1% SDS) and protein concentrations were quantified by the Bradford assay (Pierce) (54). Proteins (20 μg) were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Ann Arbor, MI). DAF levels were measured in gastric cells by Western blotting using anti-DAF (1:1000, 1H4) antibodies (54). Primary antibodies were detected using goat anti-mouse (Santa Cruz) horseradish peroxidase-conjugated secondary antibodies and visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) according to the manufacturer's instructions.

Real-time PCR

MKN28 and AGS gastric epithelial cells were grown to confluence, serum-starved for 24 hours and then co-cultured with *H. pylori* for 2, 6, 12, and 24 hours (MOI=100). RNA was prepared from *H. pylori*:gastric cell co-cultures using TRIzol Reagent following the manufacturer's instructions (Invitrogen), and contaminating DNA was removed using the RNeasy RNA purification kit (Qiagen). Reverse transcriptase PCR was performed using TaqMan reverse transcription reagents (Applied Biosystems), which was followed by real-time quantitative PCR using the TaqMan Gene Expression Assay and a 7300 Real-Time PCR system (Applied Biosystems). *Daf* cDNA was quantitated using a *daf* TaqMan Gene Expressions primer set (Hs00167090_m1) purchased from Applied Biosystems and expression levels were normalized to levels of 18S rRNA (VIC labeled).

Mice, bacteria, and experimental infections

Daf1 knockout mice were developed as described previously (165). Briefly, the *Daf1* gene on one chromosome was inactivated by homologous recombination and Cre/*Lox*P-mediated deletion in murine GK129 embryonic stem cells. The recombined embryonic stem cells were microinjected into blastocytes, chimeras were generated, and the chimeric mice were then bred with the C57BL/6 strain. Eight- to 12-week knockout mice and wild-type C57BL/6 mice were used. All experiments were approved by the Case Western Reserve Institutional Animal Care and Use Committee. *Brucella* broth containing 2 x 10^7 cfu of the *H. pylori* rodent-adapted strain SS1 was used as inoculum and was delivered by gastric intubation as previously described (95).

Eight weeks post-challenge, mice were euthanized. At necropsy, linear strips extending from the squamocolumnar junction through proximal duodenum were fixed in 10% neutral-buffered formalin, paraffin-embedded, cut at 5 μM, and stained with hematoxylin and eosin. Indices of inflammation and injury in the gastric cardia, corpus, and antrum were scored on an ordinal scale from 0-5 in increments of 0.5 by a single veterinary pathologist blinded to treatment groups as previously described (95).

For quantitative culture, gastric tissue was homogenized, plated, and incubated under microaerobic conditions at 37^{o}C for 5-6 days as previously described (95). After verification by Gram's stain, urease, catalase and oxidase reactions, colonies were counted and comparisons between groups were based on the log cfu/gram of stomach tissue as described (95).

Small-interfering RNA

DAF was knocked down *in vitro* using small-interfering RNA (siRNA). *Daf* specific siRNA (Dharmacon Smartpool siGENOME cat# M-004573-00) was transiently transfected into MKN28 and AGS gastric epithelial cells. Fluorescently labeled siGLO Cyclophilin B siRNA (Dharmacon) was used as a positive control for mRNA knock-down and transfection efficiency. A siGLO RISC-Free siRNA (Dharmacon), which is chemically modified to inhibit RISC uptake and processing and does not target any known genes, was used as a control for transfection efficiency and non-specific effects of introducing siRNAs into cells. A siCONTROL non-targeting siRNA (Dharmacon), which is processed by RISC but does not target any known genes, were used as a

negative control for non-specific effects of siRNA. siRNA stocks were resuspended in 1x siRNA buffer (Dharmacon) to a concentration of 20 μM. AGS and MKN28 gastric epithelial cells were plated in 12-well plates at 1 x 10^5 and 2 x 10^5 respectively in 1 ml antibiotic free RPMI. Dharmafect 2 transfection reagent was used for transient transfection. siRNA (0.5 μl/well for 10 μM) was added to opti-MEM (50 μl/well) and in a separate tube Dharmafect 2 (2 μl/well) was added to opti-MEM (50 μl/well) and incubated at room temperature for 5 minutes. The 2 tubes were then mixed and incubated at room temperature for 20 minutes. 100 μl of transfection mixture was added to each well. *Daf* mRNA knock-down was assessed at 24 and 48 hours using real-time qRT-PCR. DAF protein levels were measured using Western immunoblotting. Once optimal conditions were identified for significantly reduce levels of DAF protein, cells were incubated with *H. pylori* strain J166 and isogenic *cagA* and *cagE* mutants as controls for 6 hours at an MOI of 100. CagA translocation and phosphorylation were assessed by Western immunoblotting using anti-CagA and anti-phosphotyrosine antibodies as previously described (257).

IL-8 enzyme-linked immunosorbant assay (ELISA)

Cell culture media was collected from cells that had been co-cultured with *H. pylori* strain J166 at a MOI of 100 and IL-8 induction was quantified using an Quantikine IL-8 ELISA kit (R&D Systems, Inc.) according to the manufactures instructions as previously described (113, 298).

Statistical analysis

The Mann-Whitney U test was used for statistical analyses of inter-group comparisons. Significance was defined as $p \leq 0.05$.

Results

Expression of human DAF increases cellular binding of *H. pylori in vitro*

To determine if DAF mediates *H. pylori* binding to host cells, we used CHO cells stably transfected with a human DAF cDNA or vector alone (**Figure 7A**) and co-cultured the transfectants with a well-characterized *H. pylori* strain, J166, which is easily transformable and binds well to gastric epithelial cells (230). We assessed binding by quantitative culture. Compared to cells lacking DAF, *H. pylori* strain J166 bound more avidly to DAF-expressing cells and recoverable colony-forming units (cfu) were >1 log-fold higher following only 4 hours of co-culture (**Figure 7B**).

To further assess DAF binding by *H. pylori*, we used immunofluorescence. For this, strain J166 was co-cultured with CHO cells that either did or did not express DAF. A significantly greater number of *H. pylori* adhered to DAF^+ versus DAF^- CHO cells (**Figure 7C**).

To insure that *H. pylori* binding to DAF was a specific interaction rather than a non-specific bacterial response of the cells to bacteria, we incubated DAF^+ and DAF^- CHO cells with equivalent numbers of non-diffusely adhering *E. coli* or *C. jejuni* for 4 hours and measured binding by quantitative culture. Adherence of *E. coli* or *C. jejuni* did not differ between CHO cells that either expressed or did not express DAF (**Figure 7B**). Thus, the DAF interaction specifically mediated adherence of *H. pylori* to host cells.

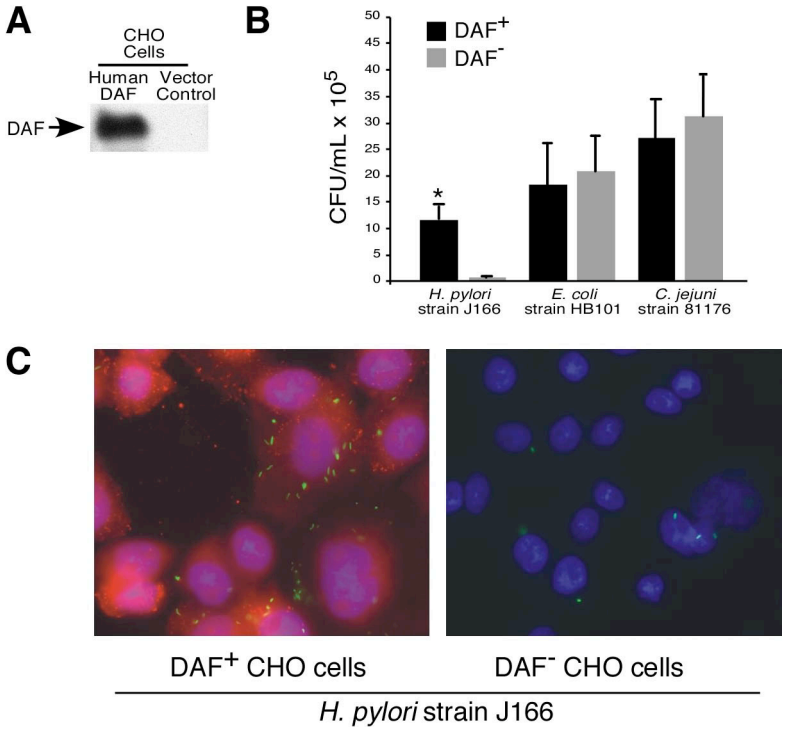

Figure 7. *H. pylori* strain J166 adheres significantly more avidly to CHO cells expressing full-length human DAF. A.) Western blot for human DAF using CHO cells transfected with either full-length human DAF or vector alone. B.) CHO cells transfected with either full-length DAF (DAF^+) or vector alone (DAF^-) were incubated with *H. pylori* strain J166, *E. coli* strain HB101, or *C. jejuni* strain 81176 (10:1 bacteria:epithelial cell ratio). Bacterial adherence was assessed using quantitative culture as described in Experimental Procedures. Error bars, SEM. *, $p<0.05$ versus infected DAF^- cells. C.) Distribution of *H. pylori* (green) and DAF (red) in CHO cells transfected with either full-length human DAF or vector alone was detected by immunofluorescence as described in Experimental Procedures.

Binding to DAF is independent of *H. pylori* virulence constituents encoded by the *cag* pathogenicity island or *vacA*

The *H. pylori cag* island and *vacA* induce epithelial responses that may lower the threshold for disease. Consequently, we examined the effects of these virulence determinants on binding of *H. pylori* to DAF-expressing cells. To do this, we co-cultured DAF^+ or DAF^- CHO cells with *H. pylori* wild-type cag^+ toxigenic strain J166 or isogenic *cagA*, *cagE*, or *vacA* null mutant derivatives. Compared to the parental wild-type strain, loss of *cagA* had no effect on binding to DAF (**Figure 8A**). Inactivation of *cagE* or *vacA* decreased the extent of bacterial binding compared to the wild-type strain; however, the level of reduction was similar in DAF-expressing and DAF-deficient CHO cells (**Figure 8A**). These results indicate that, although *cagE* and *vacA* may contribute to binding of *H. pylori* to host cells, these effects do not involve DAF.

The extent of *H. pylori* binding to DAF-expressing cells varies among a population of clinical isolates

Most persons infected with *H. pylori* cag^+ toxigenic strains remain asymptomatic, suggesting that additional microbial and/or host factors influence disease. Therefore, we next investigated DAF binding patterns among a population of clinical *H. pylori* isolates. Although absolute levels varied between different isolates, all 9 strains tested (6 cag^+ toxigenic, 3 cag^- non-toxigenic) displayed at least a 10-fold increase in binding affinity to DAF^+ versus DAF^- CHO cells (**Figure 8B**). Levels of binding did not segregate with *cag* genotype or toxigenicity, confirming the results from isogenic mutant experiments (**Figure 8A**).

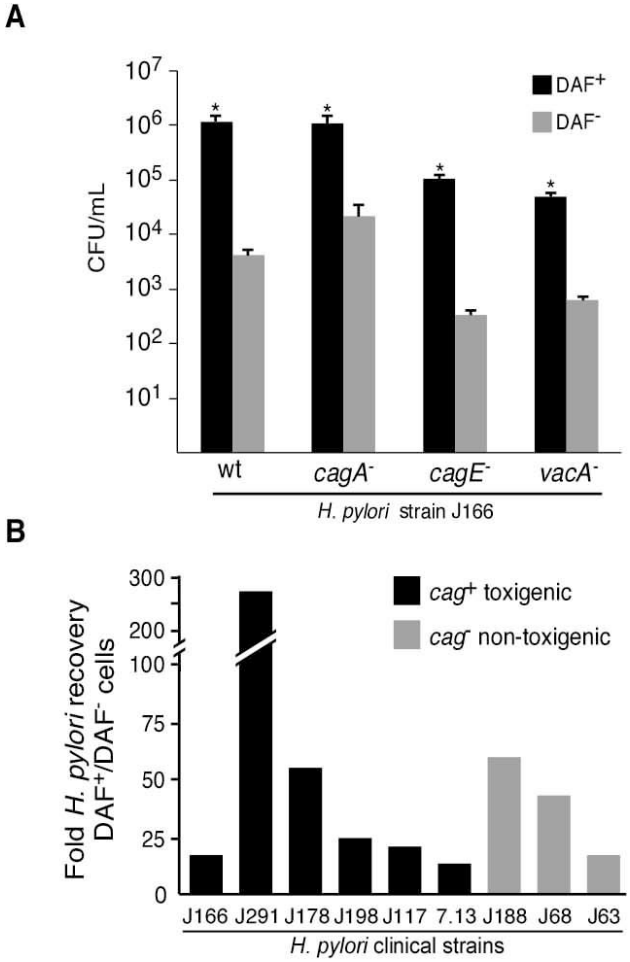

Figure 8. Adherence of *H. pylori* strain J166 to DAF-expressing cells is not mediated by the *cag* pathogenicity island or *vacA*. A.) CHO cells transfected with either full-length DAF (DAF^+) or vector alone (DAF^-) were cultured in the presence of the *H. pylori* cag^+ toxigenic strain J166 or isogenic *cagA*, *cagE*, or *vacA* null mutant derivatives at bacteria:cell ratios of 10:1. Adherence was assessed by quantitative culture. Error bars, SEM. *, $p<0.05$ versus infected DAF^- cells. B.) Adherence to DAF^+ or DAF^- CHO cells by *H. pylori* clinical isolates with varying *cag* island status and toxigenic phenotypes was assessed using quantitative culture. Results are expressed as a ratio of *H. pylori* recovered from DAF^+ versus DAF^- cells. A representative result of multiple repetitions performed on at least 2 occasions is shown.

The *H. pylori* interaction with DAF is not mediated by several known adhesins and outer-membrane proteins

To better understand the *H. pylori*:DAF interaction, we sought to identify the *H. pylori* adhesin that binds DAF. To do this we generated isogenic mutants in several well-known *H. pylori* adhesins (BabA, SabA, AlpAB), outer-membrane proteins that are involved in pathogenesis and may be associated with adherence (OipA and HopQ), and putative OMPs that have not been well characterized (Omp2, Omp32, and HefA). Because LPS antigenic modifications are associated with adherence, we also generated an isogenic *wbcJ* mutant that is defective in LPS modification and as a result does not present Le^X, Le^Y, or *O*-antigens on the bacterial cell surface (186, 198). Table 1 lists the isogenic mutants and the parental strains in which the mutants were generated. These wild-type strains and the isogenic mutants were tested for DAF adherence by co-culture with DAF^+ and DAF^- CHO cells and quantitative culture. Wild-type *H. pylori* strain P1 does not bind well to either DAF^+ or DAF^- CHO cells. Wild-type strains G27 and 7.13 do adhere significantly more avidly to the DAF^+ versus DAF^- cells. None of the isogenic mutants demonstrated a significant attenuation in DAF binding. These results indicate that the genes listed in Table 1 are not necessary for the *H. pylori* interaction with DAF.

Binding of *H. pylori* to CHO cells that express mutant DAF

DAF is composed of four CCPs, and microbial pathogens vary in their utilization of CCP domains for binding. Based on this, we next localized the sites on DAF that *H. pylori* utilize by studying CHO cells stably transfected with deletion mutants of each of the four

Table 1. *H. pylori* parental strains and isogenic mutants tested for adherence to DAF^+ vs DAF^- CHO cells in which no difference in DAF affinity was determined for WT vs. isogenic mutant

Null Gene	***H. pylori* strain(s)**
babA (omp28)	7.13, G27
sabA	7.13
alpAB	J166, P1
oipA	7.13, J166, G27
hopQ (omp27)	G27
wbcJ	J166
omp32 (*hp1501*)	7.13
hefA (*hp0605*)	7.13
omp2 (*hp0025)*	7.13

DAF CCP domains. Each deletion mutant expresses an amount of DAF at least equal to cells expressing wild-type DAF (46). Removal of CCP domains 1, 2 or 3 completely abolished *H. pylori* binding to DAF (**Figure 9**), whereas removal of CCP 4, the domain in closest apposition to the cell surface, resulted in an approximate 60-70% reduction in binding.

To further confirm the role of CCP domains for *H. pylori* binding, DAF^+ and DAF^- CHO cells were pre-treated with the DAF CCP-specific monoclonal antibodies (mAbs) 11D7, IF7, IH4, and 8D11 prior to infection with *H. pylori* strain J166. Pre-incubation with each individual mAb alone did not reduce *H. pylori* binding to DAF^+ cells (data not shown). However, pre-incubation with an equal mixture of the four CCP-specific mAbs completely blocked adherence of *H. pylori* to DAF-expressing cells (**Figure 9B**), confirming results using cells transfected with DAF CCP deletion mutants (**Figure 9A**).

DAF CCP domains are linked to a serine/threonine (S/T)-rich heavily *O*-glycosylated C-terminal domain that elevates the molecule at the membrane surface. Since *H. pylori* can bind to carbohydrate residues on cell surfaces (1,2), we determined the requirement for the DAF S/T region by utilizing a CHO cell transfectant clone that stably expresses a DAF cDNA construct containing a deletion of the S/T-rich domain, or a clone expressing a S/T deletion construct that attaches the four CCP domains to the unrelated non-complement protein HLA-B44. The latter construct is anchored by the transmembrane

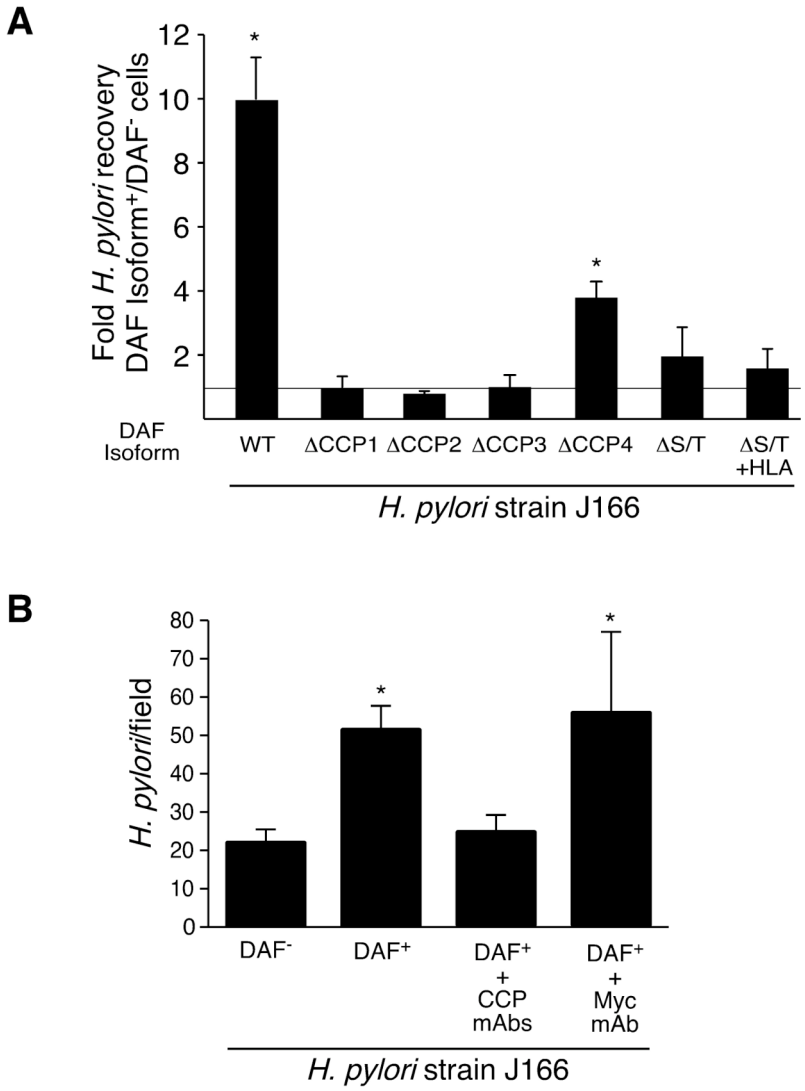

Figure 9. Adherence of *H. pylori* to DAF-expressing cells is mediated by multiple DAF domains. A.) Attachment of *H. pylori* strain J166 to CHO cells expressing either full-length DAF or individual CCP or S/T region deletion mutants. *H. pylori* strain J166 was co-cultured with CHO cells stably transfected with either full-length human DAF (DAF^+), vector alone (DAF^-), a series of deletion mutants that individually lack one of the four DAF CCP domains, a serine/threonine (S/T) rich region deletion mutant (ΔS/T), or a S/T rich region deletion mutant containing an in-frame fusion of the cDNA encoding the amino-terminal region of DAF with the carboxy-terminus of HLA-B44 (ΔS/T + HLA). Bacterial adherence was assessed using quantitative culture as described in Experimental Procedures and is expressed as a ratio of DAF^+:DAF^- cells. Therefore, a value of 1 represents baseline. Error bars, SEM. *, $p<0.05$ versus infected DAF^- cells. B.) Binding of *H. pylori* to CHO cells transfected with either full-length human DAF or vector alone in the presence or absence of an equal mixture of anti-DAF CCP-specific monoclonal antibodies 11D7, IF7, 1H4 or 8D11, or an irrelevant anti-Myc monoclonal antibody was detected by immunofluorescence as described in Experimental Procedures. Error bars, SEM. *, $p<0.05$ versus infected DAF^- cells.

and cytoplasmic domains of HLA-B44 and functions efficiently as a complement regulatory protein. Results from binding experiments using both of these S/T deficient DAF clones demonstrate that removal of the *O*-glycosylated region decreased *H. pylori* binding to DAF (**Figure 9A**), indicating that the S/T region does not simply function as a non-specific spacer for binding. These results indicate that *H. pylori* binding to DAF either involves all CCP domains, or is dependent on DAF's conformation in its intact state.

H. pylori induces DAF expression in human gastric epithelial cells

Since *H. pylori* is a human pathogen that selectively colonizes gastric epithelium, we investigated whether *H. pylori* alters DAF expression in human gastric epithelial cells. Real-time PCR analysis demonstrated that *H. pylori* induced DAF expression in MKN28 (**Figure 10A**) and AGS (data not shown) gastric epithelial cells beginning at 2 hours. Levels decreased to baseline by 24 hours post-inoculation. *H. pylori* co-culture mRNA changes reflected increased DAF protein expression since increases in levels were detected at 6 hours and DAF protein remained elevated for 24 hours (**Figure 10B**). Immunofluorescence staining confirmed the increased DAF expression on MKN28 gastric epithelial cells following co-culture with *H. pylori* (**Figure 10C**). These results indicate that the prototype *H. pylori* strain J166 induces transcriptional up-regulation of DAF in human gastric epithelial cells.

H. pylori-induced gastric inflammation is attenuated in the absence of DAF

To determine if the DAF binding is physiologically relevant within the context of *H. pylori*-induced inflammation *in vivo*, we utilized *Daf1*$^{-/-}$ mice and the rodent-adapted *H.*

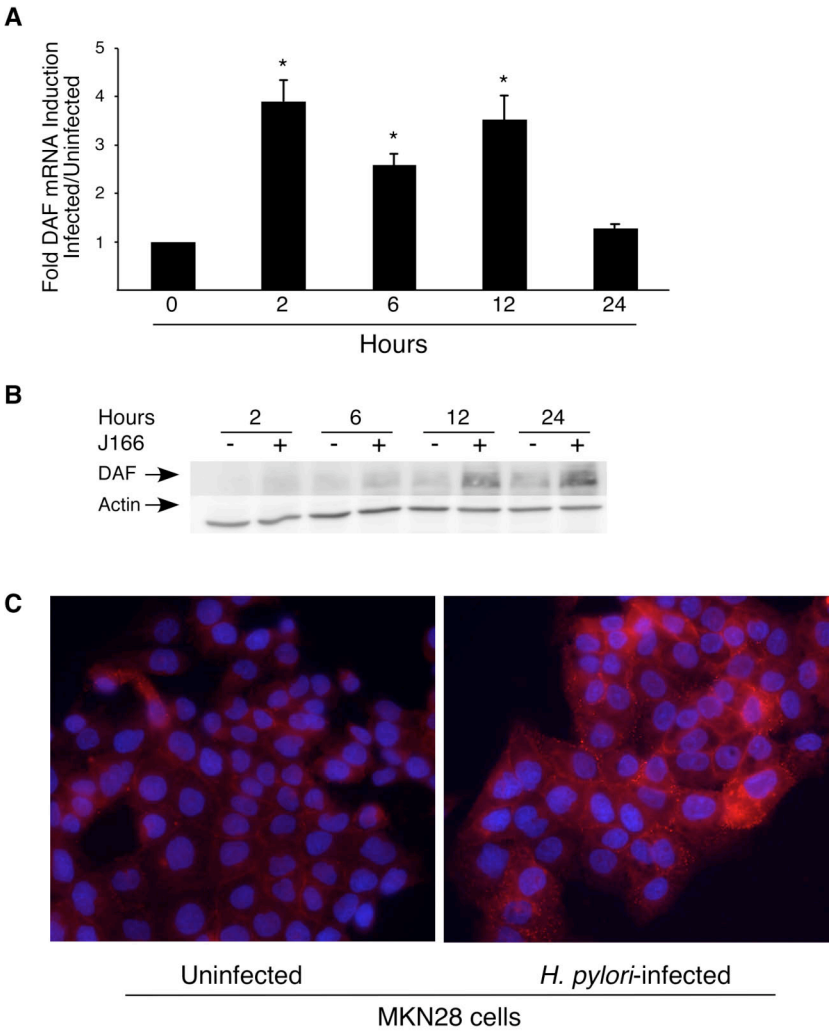

Figure 10. *H. pylori* strain J166 up-regulates DAF in gastric epithelial cells. A.) MKN28 gastric epithelial cells were cultured for 24 hours prior to incubation with *H. pylori* strain J166. Levels of *daf* mRNA were determined by real-time RT-PCR as described in Experimental Procedures and were normalized to corresponding levels of 18S rRNA. Results are expressed as fold increase in *daf* mRNA in *H. pylori*-infected versus uninfected samples. Error bars, SEM. *, $p<0.05$ versus uninfected cells at time 0. B.) MKN28 cells were cultured for 24 hours prior to incubation with *H. pylori* strain J166. Cell extracts harvested at different time-points were then used for Western blot analysis using an anti-DAF antibody as described in Experimental Procedures. (-), cells incubated with medium alone. A representative blot is shown. Anti-actin blots served as normalization controls for MKN28 cell viability under different experimental conditions. Equal protein loading was also determined by Fas green staining (not shown). C.) Distribution of DAF (red) in AGS gastric epithelial cells was detected by immunofluorescence following infection with medium alone (left panel) or *H. pylori* strain J166 (right panel) for 24 hours.

pylori strain SS1. We infected wild-type and *Daf1*$^{-/-}$ mice in 2 independent challenges and followed disease outcome. All mice challenged with *H. pylori* were successfully infected and there were no differences in colonization efficiency or density between wild-type and DAF deficient mice (**Figure 11A**).

Eight weeks post-challenge, there were few lesions in the stomachs of wild-type or *Daf1*$^{-/-}$ mice inoculated with broth alone (**Figure 11B**), whereas, as expected, all wild-type mice challenged with *H. pylori* developed gastritis. Inflammation was most extensive at the transition zones between the antrum or cardia and the corpus. Inflammatory cells within infected mucosa consisted of polymorphonuclear cells and large mononuclear cells in the lamina propria (**Figure 12**). In the lamina propria, the infiltrate separated and displaced the glands (**Figure 12**). Gastric pits were lengthened and were lined by less mature flattened epithelial cells with basophilic cytoplasm; mitotic figures were frequently identified. In the submucosa, edema often accompanied the cellular infiltrate (**Figure 12**).

In contrast to infected wild-type mice, in *H. pylori*-colonized DAF deficient mice, the intensity of inflammation was significantly attenuated ($p=0.013$; **Figures 11B, 12**). Moreover, there were no differences in severity of gastritis between uninfected and infected *Daf1*$^{-/-}$ mice ($p=0.53$; **Figure 11B**). These results thus indicate that DAF contributes to the ability of *H. pylori* to induce injury within the gastric niche.

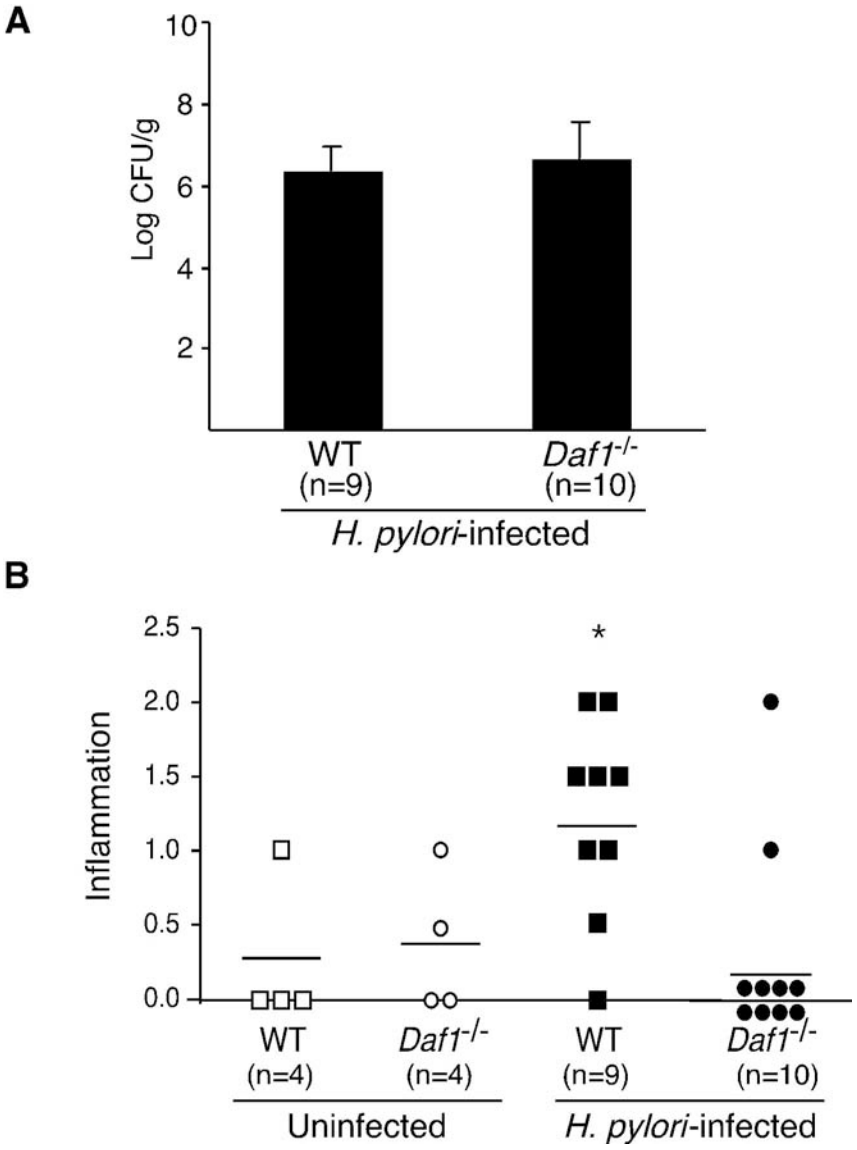

Fig. 11. DAF deficiency significantly attenuates inflammation, but not colonization density, in *H. pylori*-infected mice. A.) Wild-type (WT) or *Daf* $^{-/-}$ littermates were infected with *H. pylori* strain SS1 for 8 weeks in 2 independent experiments. Colonization density was determined by quantitative culture as described in Experimental Procedures and results are expressed as log cfu/gram of stomach tissue. Error bars, SEM. B.) Comparison of gastric inflammation in wild-type (WT) or *Daf* $^{-/-}$ mice infected with *H. pylori* strain SS1 or broth alone. Mucosal inflammation was determined by histologic testing, as described in Experimental Procedures, and scores are expressed as scatter-plots with mean values. *, p=0.013 versus *H. pylori*-infected *Daf* $^{-/-}$ mice.

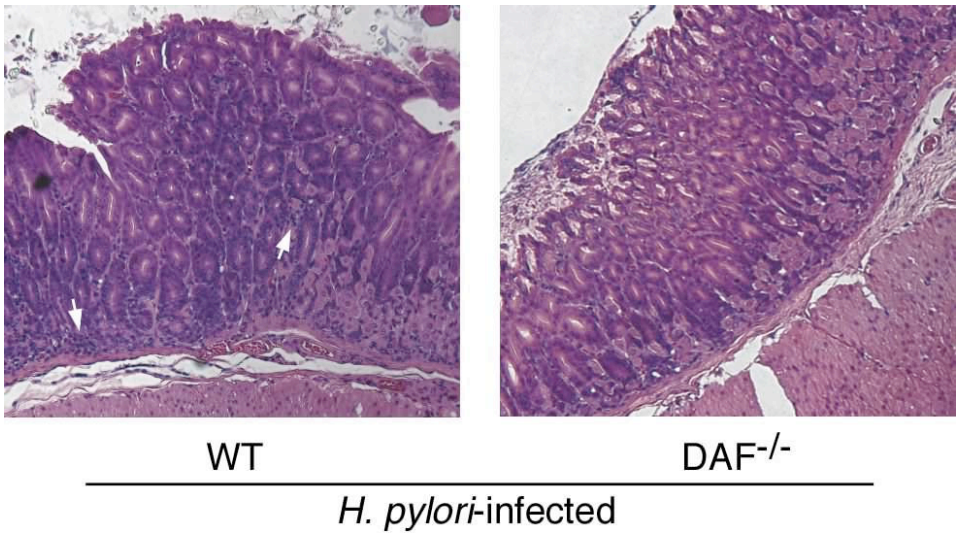

Figure 12. Development of inflammation and injury within the gastric corpus of *H. pylori*-infected wild-type, but not *Daf* $^{-/-}$ mice, 8 weeks post-inoculation. Representative hematoxylin and eosin stains are shown (original magnification x 40). Mild-moderate inflammation is present within the lamina propria of *H. pylori*-infected wild-type mice (left panel, arrows). In contrast, no significant inflammation or injury is present within gastric mucosa of *H. pylori*-infected *Daf* $^{-/-}$ mice (right panel).

DAF silencing does not affect CagA translocation or phosphorylation

We have shown that the inflammatory response initiated by *H. pylori* infection was significantly attenuated in *Daf* $^{-/-}$ mice; therefore, we sought to determine the pro-inflammatory signaling events that may be initiated by the interaction of *H. pylori* with DAF. An important mediator of *H. pylori* induced inflammation is expression of IL-8. While DAF is a GPI-anchored protein with no cytoplasmic signaling domain, it has been shown to associate with other signaling molecules such as the TLR-4 signaling complex as well as the Src family of protein tyrosine kinases (121, 232, 266, 277). Also, the *H. pylori* pathogenicity factor CagA is phosphorylated by the Src and Abl family of kinases upon translocation into host cells (257, 286). Therefore, we hypothesized that DAF may represent a novel signaling receptor that mediates the effects of the *cag* island.

To test this hypothesis, we silenced DAF *in vitro* using siRNA and examined the translocation and phosphorylation of CagA and levels of induced *H. pylori* induced IL-8. Transfection with 10 μM anti-DAF siRNA effectively reduced *daf* mRNA expression to 10-15% of control levels 24 hours post-transfection in both AGS (**Figure 13A**) and MKN28 cells (data not shown) and DAF protein was undetectable by western blot at 24 hours post-transfection. Therefore, we began co-culture of DAF knock-down cells with *H. pylori* 24 hours post-transfection. *H. pylori* strain J166 was co-cultured with both AGS and MKN28 cells for 6 hours and CagA translocation and phosphorylation were measured by western blot (**Figure 13B**). We found that knock-down of DAF had no effect on CagA translocation or phosphorylation, suggesting that DAF is not necessary for the delivery of CagA or subsequent Src/Abl mediated phosphorylation. In addition,

silencing of DAF had no effect on *cag* dependent *H. pylori* induction of IL-8 (**Figure 13C**).

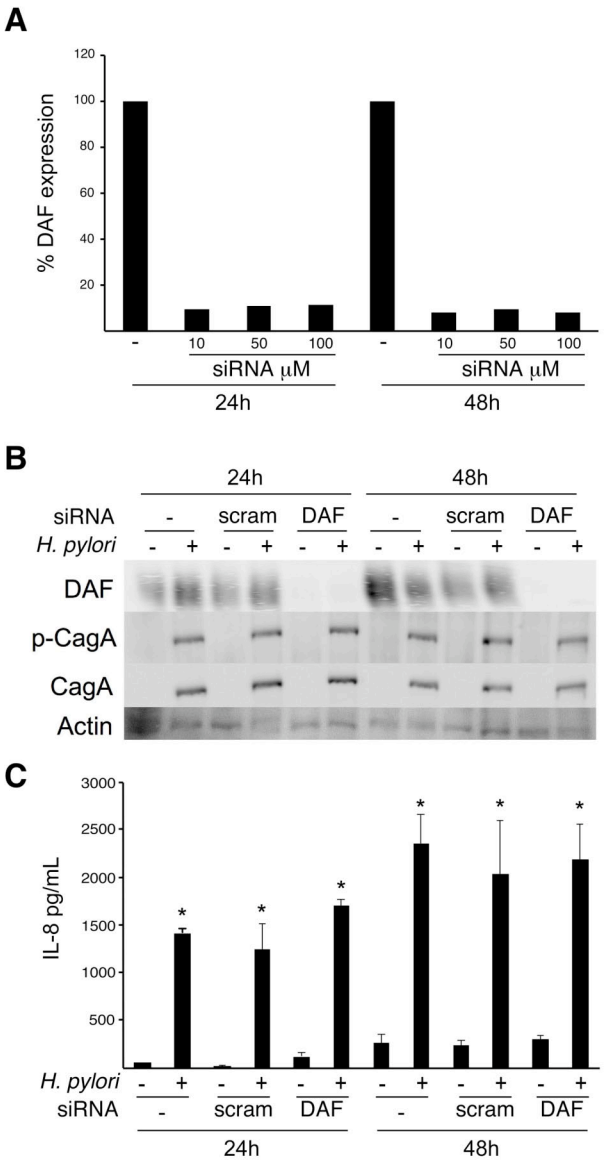

Figure 13. DAF is not a mediator of *in vitro* translocation/phosphorylation of CagA or the induction of IL-8. A.) AGS cells were transiently transfected with either 10, 50, or 100 μM scrambled control or DAF siRNA for 24 and 48 hours. Levels of *daf* mRNA were measured using real-time RT-PCR. Data are expressed as percent expression of untreated sample at the corresponding time point. B.) AGS cells were transiently transfected with 10 μM scrambled siRNA or DAF siRNA for 24 and 48 hours and then infected with the *H. pylori* strain J166 for 6 hours. Cell lysates were collected and analyzed using a western immunoblot. The blot was stained using anti-DAF, anti-phosho-CagA, anti-CagA, and anti-Actin antibodies. C.) Co-culture supernatants from control or *H. pylori* infected control and siRNA treated cells were subjected to an IL-8 ELISA. The experiments were repeated on 3 occasions. Error bars, SEM; *, $p \leq 0.05$.

Discussion

Colonization of humans by pathogenic bacteria is common, but disease occurs in only a fraction of infected persons. Our current experiments identify a new mechanism that may contribute to *H. pylori* pathogenesis. This insight was gained by 1) capitalizing on a recombinant cell model to demonstrate that the protein DAF serves as a receptor for *H. pylori*, 2) finding that *H. pylori* can induce DAF expression in a biologically relevant *in vitro* model of microbial:gastric epithelial cell interaction, 3) both confirming and mapping the components of DAF required for these effects, and 4) through the use of a *Daf1*$^{-/-}$ knockout mouse, documenting that the interaction is important for pathogenesis. Collectively, these studies indicate that *H. pylori* co-opts DAF as a receptor to induce disease.

The hallmark of the gastric inflammatory response to *H. pylori* is its capacity to persist for decades. This is in contrast to inflammatory reactions induced by other mucosal pathogens, such as *Salmonella*, that either resolve within days or progress to eliminate the host. Research to date has shown that *H. pylori* has evolved numerous strategies to facilitate its persistence within the stomach including limiting the bactericidal effects of pro-inflammatory molecules (104) and varying the antigenic repertoire of surface-exposed proteins (9). Adherence of *H. pylori* to gastric epithelial cells is also critical for colonization. According to our data, the ability of *H. pylori* to utilize DAF as a receptor contributes to the latter strategy and is consistent with the role that this molecule plays in other host:microbial interactions involving persistent pathogens. *E. coli* that express

DAF-binding Dr adhesins cause chronic interstitial nephritis (259). Echoviruses and coxsackieviruses that target DAF as a receptor are associated with chronic fatigue syndrome and chronic dilated cardiomyopathy, respectively (179, 327). The current studies focused on *H. pylori*:DAF interactions further implicate DAF as a receptor that is exploited by pathogens notable for their ability to induce chronic inflammation, injury, and disease.

Our *in vitro* results indicate that binding of DAF by *H. pylori* requires all of the CCP domains and the S/T-rich *O*-glycosylated region, a pattern that is distinct from those involved in DAF binding by other pathogens. For example, Dr-expressing *E. coli* require CCP2 and CCP3 (111, 117, 207). *E. coli* that express X adhesins, require CCP3 and CCP4 (26). Echovirus 7 utilizes CCPs 2-4 (34, 120) and coxsackieviruses A21 and B3 exploit CCP1 and CCP2, respectively (17, 144, 262). Another layer of complexity beyond the scope of this investigation is added when results from inhibition studies are considered. Anti-DAF antibodies that block cellular binding of coxsackievirus 21 reciprocally enhance binding of echovirus 7 (261), raising the possibility that ligand binding of one CCP domain affects binding of another CCP moiety within the same DAF molecule. Importantly, we have a unique in vitro model of bacterial:epithelial interactions using an *H. pylori* strain that is easily transformable in which to evaluate the individual and collective effects of each of these factors.

We were unsuccessful in identifying the *H. pylori* adhesin that binds DAF. However, *H. pylori* strain P1 may lack the adhesin needed for DAF binding, which may present an

opportunity to identify the adhesin by RNA microarray or proteomic comparison of strain P1 and a strain that binds well to DAF expressing cells such as J166. We have also determined that the DAF binding protein is likely a novel adhesin that may be represented by one of the poorly described *H. pylori* OMPs or hypothetical proteins.

Murine models have provided valuable insights into the host, bacterial, and environmental factors involved in *H. pylori*-induced gastric inflammation and injury. Using a *Daf1*$^{-/-}$ mouse, we now demonstrate that loss of DAF does not alter colonization but attenuates the inflammatory response to *H. pylori*. This may occur via more than one mechanism. Based on our *in vitro* data, DAF does not play a role in CagA translocation or phosphorylation and is not necessary for the induction of IL-8. Therefore, the immuno-modulatory role played by DAF is not likely due to *cag* PAI-mediated effects. Our available data so far indicate that *H. pylori* up-regulates DAF in gastric epithelial cells. This is consistent with reports from other investigators that DAF expression is increased within infected human gastric mucosa, where it localizes to the apical surface of gastric epithelial cells (18, 238, 252). One possibility raised by our findings is that in addition to direct bacterial stimulation, expression of DAF can be increased by *H. pylori*-induced pro-inflammatory cytokines such as IL-1ß and TNF-α, which are up-regulated in response to transepithelial migration of neutrophils (21, 22). DAF has also been recently identified as an apical epithelial ligand for polymorphonuclear cells that regulates the rate of neutrophil migration across apical epithelial membranes (160). Finally, since *H. pylori* binding to DAF involves its complement regulatory CCP2 and 3 domains, the binding might affect complement activation. All these questions will require further study. Thus, DAF

regulated by both *H. pylori* and host immune mediators is well-positioned to modulate the inflammatory response to this pathogen.

In conclusion, *H. pylori* binds avidly to cells expressing human DAF and this is mediated by DAF CCPs 1-4 and the *O*-glycosylated serine threonine rich C-terminal domain. *H. pylori* induces transcriptional up-regulation of DAF in gastric epithelial cells and *in vivo*, DAF deficiency decreases the intensity of inflammation in *H. pylori*-infected mice. Taken together, these data open a new avenue of investigation in pathogenic mechanisms underlying *H. pylori* infection.

CHAPTER III

REGULATION OF THE *HELICOBACTER PYLORI* CELLULAR RECEPTOR DECAY-ACCELERATING FACTOR

Summary

Chronic gastritis induced by *Helicobacter pylori* is the strongest known risk factor for peptic ulceration and distal gastric cancer, and adherence of *H. pylori* to gastric epithelial cells is critical for induction of inflammation. One *H. pylori* constituent that increases disease risk is the *cag* pathogenicity island, which encodes a secretion system that translocates bacterial effector molecules into host cells. Decay-accelerating factor (DAF) is a cellular receptor for *H. pylori* and a mediator of the inflammatory response to this pathogen. *H. pylori* induces DAF expression in human gastric epithelial cells; therefore, we sought to define the mechanism by which *H. pylori* up-regulates DAF and to extend these findings into a murine model of *H. pylori*-induced injury. Co-culture of MKN28 gastric epithelial cells with the wild-type *H. pylori cag*$^+$ strain J166 induced transcriptional expression of DAF, which was attenuated by disruption of a structural component of the *cag* secretion system (*cagE*). *H. pylori*-induced expression of DAF was dependent upon activation of the p38 mitogen-activated protein kinase pathway, but not NF-κB. Hypergastrinemic INS-GAS mice infected with wild-type *H. pylori* demonstrated significantly increased DAF expression in gastric epithelium versus uninfected controls or mice infected with an *H. pylori cagE*$^-$ isogenic mutant strain. These results indicate that *H. pylori cag*$^+$ strains induce up-regulation of a cognate cellular receptor *in vitro* and

in vivo in a *cag*-dependent manner, representing the first evidence of regulation of an *H. pylori* host receptor by the *cag* pathogenicity island.

Introduction

Helicobacter pylori induces an inflammatory response in the stomach that persists for decades and increases the risk not only for peptic ulceration, but also for gastric adenocarcinoma and non-Hodgkins lymphoma of the stomach (200, 226). Gastric adenocarcinoma is the second leading cause of cancer-related death in the world, and chronic gastritis induced by *H. pylori* is the strongest known risk factor for this malignancy (20, 23, 38, 70, 199, 226). However, only a fraction of infected persons ever develop cancer, underscoring the importance of defining mechanisms that regulate biological interactions between *H. pylori* and their hosts that promote transformation.

While the vast majority of *H. pylori* in colonized hosts are free-living, approximately 20% bind to gastric epithelial cells and adherence is important in the induction of injury (124). BabA is an outer-membrane protein (OMP) encoded by the strain-specific gene *babA2*, which binds the Lewis^b (Le^b) histo-blood-group antigen on gastric epithelial cells (99, 134). BabA binding specificities reflect *H. pylori* strain adaptation to different glycosylation patterns that predominate in a particular host population and BabA-mediated Le^b binding can be altered by both bacterial phase variation and genetic recombination (275). Another *H. pylori* adhesin, SabA, binds the sialyl-Lewis^x (sLe^x) antigen, which is an established tumor antigen and marker of gastric dysplasia (176). Gastric inflammation induced by *H. pylori* up-regulates the expression of sLe^x on epithelial cells, which amplifies interactions between this molecule and SabA.

We recently identified another *H. pylori* receptor, Decay-accelerating factor (DAF), that is up-regulated following bacterial contact (209). DAF is an intrinsic regulator of complement that is attached to the outer leaflet of the cell membrane by a GPI anchor (260). DAF protects cells from complement activation on their surfaces by dissociating membrane-bound C3 convertases that are required for cleaving C3 and further propagating the complement cascade. DAF can also be utilized as a cellular receptor by several pathogenic organisms associated with chronic inflammatory diseases, including uropathogenic diffusely-adhering *E. coli*, coxsackieviruses, echoviruses, and enteroviruses (17, 34, 111, 117, 120, 144, 207, 236, 260-262).

Expression of DAF is increased within *H. pylori*-infected human gastric tissue compared to uninfected mucosa, and the intensity of expression is directly related to the density of *H. pylori* colonization and severity of inflammation (18, 238, 252). We recently demonstrated that DAF influences the inflammatory response to *H. pylori* as infected DAF deficient mice developed significantly less severe inflammation compared to infected wild-type mice, suggesting that the interaction between *H. pylori* and DAF is important for pathogenesis (209).

In addition to host effectors that mediate injury, *H. pylori* constituents can also regulate pathogenic responses. Following adherence, *H. pylori* strains that possess a type IV secretion system (TFSS) encoded by the *cag* pathogenicity island (PAI), translocate CagA and components of peptidoglycan into host cells (10, 13, 212, 256, 257, 279, 306). CagA subsequently undergoes Src and Abl-dependent tyrosine phosphorylation and

activates a eukaryotic phosphatase (SHP-2), eventuating in dephosphorylation of host cell proteins and cellular morphological changes (13, 125, 126, 257, 278, 286). *H. pylori* peptidoglycan components delivered by the *cag* secretion system are recognized by the intracellular pattern recognition receptor NOD1, which initiates cell-signaling events including activation of NF-κB (306). *In vivo,* the presence of the *cag* island also influences the topography of colonization, as *H. pylori cag*$^-$ strains predominate within the mucus gel layer, while *cag*$^+$ strains are found immediately adjacent to epithelial cells (30). Compared to *cag*$^-$ strains, *H. pylori cag*$^+$ strains augment the risk for severe pathologic outcomes, such as peptic ulceration and gastric cancer (226). Since adherence likely plays a critical role in pathogenesis, we sought to delineate the host and bacterial factors that mediate *H. pylori* induction of DAF. We demonstrate that *H. pylori cag*$^+$ strains up-regulate DAF expression in gastric epithelial cells *in vitro* and *in vivo* in a *cag*-dependent manner, and that this induction is mediated by p38 MAP kinase activation.

Experimental Procedures

Reagents and constructs

Actinomycin D, cycloheximide, the p38 inhibitor SB203580, and the JNK1/2/3 inhibitor JNK inhibitor II were obtained from Calbiochem, while the MEK1/2 inhibitor PD98059 was obtained from Cayman Chemical. Mouse monoclonal anti-DAF antibodies IA10 (BD PharMingen) and MCA1614 (AbD Serotec) were used for Western analysis and immunohistochemistry respectively. The pNF-κB luciferase vector (Clontech) and pRL *Renilla* luciferase vector (Promega) were used for NF-κB luciferase studies. Dominant-negative mutant IκBα S32/36A and dominant-negative IKKβ K44A constructs were used for NF-κB inhibition studies (generous gifts of Dr. Andrew Neish, Emory University School of Medicine) (330).

Cell Culture

MKN28 human gastric epithelial cells (kindly provided by Dr. Robert Coffey, Vanderbilt University) were grown in RPMI 1640 (GIBCO-BRL) with 10% heat-inactivated FBS and 20 μg/ml gentamicin in an atmosphere of 5% CO_2 at 37^{o}C.

Bacterial strains

Experiments were performed with the *H. pylori cag*$^{+}$ strains J166 and 7.13 (88, 209). Isogenic *cagA, cagE,* and *cagM* null mutants were constructed by insertional mutagenesis, using *aphA* (conferring kanamycin resistance) as previously described (54, 227), and were selected on *Brucella* agar with kanamycin (25 μg/ml). Heat-killed *H.*

pylori were generated by heating the bacteria to 80°C for 10 minutes. *H. pylori* lysates were generated by sonication as previously described (100). Lysates were then sterilized using a 0.2μm pore size filter.

Western analysis

MKN28 gastric epithelial cells were grown to confluence, then cultured in serum-free medium for 24 hours and then co-cultured with *H. pylori* for specified times at a multiplicity of infection (MOI) of 100. *H. pylori*-infected and uninfected MKN28 cells were lysed in RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% Triton X-100; 0.1% SDS) and protein concentrations were quantified by the BCA assay (Pierce) (54). Proteins (30 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Ann Arbor, MI). DAF levels were examined in gastric cells by Western blotting using an anti-DAF (1:1000, IA10) antibody. Primary antibodies were detected using goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) according to the manufacturer's instructions. Western blots were imaged and band intensities were quantified using the ChemiGenius Gel Bio Imaging System (Syngene).

Real-time quantitative RT-PCR

MKN28 gastric epithelial cells were grown to confluence, then cultured in serum-free medium for 24 hours and then co-cultured with *H. pylori* for specified times (MOI=100). RNA was prepared from *H. pylori*:gastric cell co-cultures using the RNeasy RNA

purification kit (Qiagen) following the manufacturer's instructions. Reverse transcriptase PCR was performed using TaqMan reverse transcription reagents (Applied Biosystems), which was followed by real-time quantitative PCR using the TaqMan Gene Expression Assay and a 7300 Real-Time PCR system (Applied Biosystems). *Daf* and *gapdh* cDNA were quantified using a TaqMan Gene Expressions primer set purchased from Applied Biosystems. Fold induction of *daf* mRNA was determined from the threshold cycle values normalized for *gapdh* mRNA expression and was then normalized to the value derived from cells cultured with medium alone.

Transfections and Luciferase assay

MKN28 cells were transiently transfected using Fugene 6 reagent (Roche) per the manufacturer's instructions. For transfection in 24-well plates, MKN28 cells were plated at 1 x 10^5 cells/well. The expression constructs were used at the following concentrations per well: pNF-κB luciferase, 50 ng; pRL *Renilla* luciferase, 5 ng; pDN-IκBα, 50 ng; pDN-IKKβ, 50 ng. Sheared salmon sperm DNA was used to bring total DNA to 200 ng/well. Fugene 6 was aloud to come to room temperature. In separate tubes DNA and Fugene 6 (0.6 μl/well) were mixed with opti-MEM (10 μl/well) and incubated at room temperature for 5 minutes. DNA/opti-MEM and Fugene 6/opti-MEM mixtures were then mixed and incubated for 20 minutes at room temperature. Transfection mixtures were then added to cells (20 μl/well). Cells were allowed to incubate with the transfection mixture for 24 hours, then cultured in serum-free medium for an additional 24 hours, and then co-cultured with *H. pylori* strain J166 (MOI=100). Samples were assayed for

luciferase activity on a TD-20/20 Luminometer (Turner Designs) using the Dual Luciferase® reporter kit (Promega) according to the manufacturer's instructions.

Experimental animal infections

All procedures were approved by the Institutional Animal Care Committee of Vanderbilt University. Male INS-GAS transgenic mice on the FVB/N background, 6-8 weeks of age, were challenged with either sterile *Brucella* broth, wild-type *H. pylori* strain 7.13, or a 7.13 *cagE*$^-$ mutant by oral gavage as previously described (87). Mice were euthanized at 4, 12, and 24 weeks post-challenge. At necropsy, linear strips extending from the squamocolumnar junction through proximal duodenum were fixed in 10% neutral-buffered formalin, paraffin-embedded, and cut at 5 μM increments. Sections were then deparaffinized and DAF immunohistochemical (IHC) staining was carried out as previously described (88) using the anti-DAF antibody MCA1614 (Serotec). A single pathologist (Elizabeth Harris), experienced in murine pathology and blinded to treatment groups, scored DAF IHC staining on an ordinal scale from 0-4 by as previously described (265).

To assess colonization, gastric tissue was homogenized, plated, and incubated under microaerobic conditions at 37^{o}C for 5-6 days as previously described (87). Colonies were verified as *H. pylori* by Gram's stain, urease, catalase and oxidase reactions as described (87). Successful colonization was confirmed by IHC staining using an anti-*H. pylori* antibody.

Statistical analysis

An ANOVA one-way analysis of variance and the Tukey-Kramer post test were used for analysis of *in vitro* data. The Mann-Whitney U test of inter-group comparisons was used for analysis of *in vivo* data. Significance was defined as $p \leq 0.05$. All calculations were performed with the GraphPad Prism 4 statistical analysis software package (GraphPad Software, Inc).

Results

H. pylori induction of DAF is regulated at the transcriptional level

We previously demonstrated that *H. pylori* up-regulates DAF *in vitro* (209). To determine if DAF induction was transcriptionally or post-transcriptionally mediated, the *H. pylori* *cag*$^{+}$ strain J166 was co-cultured with MKN28 gastric epithelial cells that had been pretreated with either actinomycin D (inhibitor of transcription) or cycloheximide (inhibitor of translation). DAF protein expression was assessed after 24 hours of co-culture (**Figure 14**). Actinomycin D completely blocked DAF induction in response to *H. pylori* ($p<0.001$), and inhibition of translation by cycloheximide blocked DAF induction in a dose-dependent manner. Vehicle treated, *H. pylori*-infected cells expressed significantly more DAF than *H. pylori*-infected cells that had been pretreated with cycloheximide ($p<0.01$) and DAF expression in cycloheximide-treated, infected cells was not significantly higher than vehicle treated, uninfected control cells. These results indicate that up-regulation of DAF by *H. pylori* in human gastric epithelial cells is mediated at a transcriptional level.

H. pylori induction of *daf* requires viable bacteria

Bacteria can activate epithelial signaling pathways via multiple mechanisms. To determine if live *H. pylori* are necessary for *daf* induction or if inert bacterial components are sufficient, we incubated MKN28 cells with viable bacteria or with *H. pylori* that had either been heat-killed or lysed by sonication and assessed *daf* mRNA expression using real-time quantitative RT-PCR (**Figure 15**). Co-culture of cells with live *H. pylori*, as

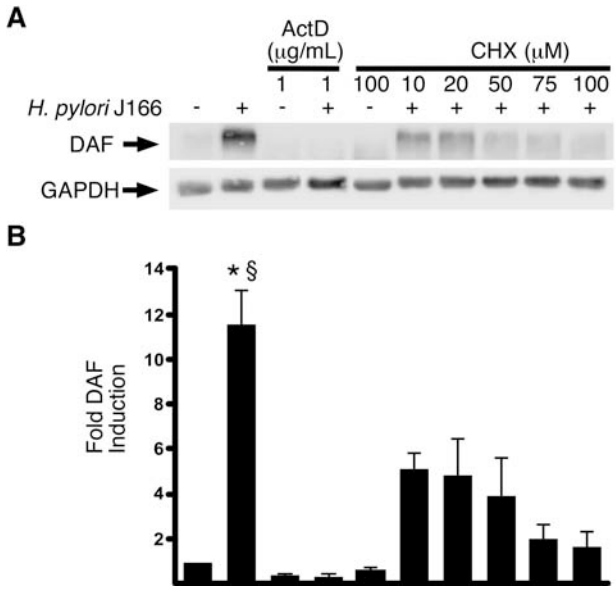

Figure 14. *H. pylori* induces the transcriptional up-regulation of DAF in gastric epithelial cells. MKN28 gastric epithelial cells were pretreated with either actinomycin D (ActD) or cycloheximide (CHX) at the indicated concentrations and then co-cultured with the *H. pylori cag*$^{+}$ strain J166 for 24 hours at an MOI=100. A.) Western blot analysis was performed using an anti-DAF antibody as described in "Experimental Procedures". (-), cells incubated with medium alone. A representative blot is shown. Anti-GAPDH blots served as normalization controls. B.) Densitometry represents data from 3 independent experiments, Error bars, SEM. *, $p<0.001$ J166 versus control cells or ActD treated cells. §, $p<0.01$ J166 versus CHX treated cells.

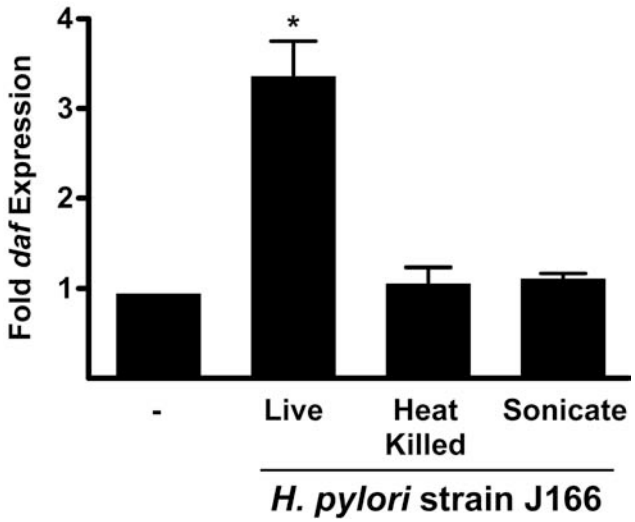

Figure 15. *H. pylori* induction of *daf* requires viable bacteria. MKN28 cells were incubated with medium alone (-), live, heat-killed, or sonicates of *H. pylori* strain J166 for 2 hours. Levels of *daf* mRNA were determined by real-time qRT-PCR as described in "Experimental Procedures" and were normalized to corresponding levels of *gapdh* mRNA. Results are expressed as fold increase in *daf* mRNA in *H. pylori*-infected versus uninfected samples. Error bars, SEM. *, $p<0.001$ versus uninfected cells.

expected, significantly induced *daf* mRNA. However, incubation with either heat-killed or sonicated *H. pylori* failed to induce expression of *daf*. These results indicate that induction of *daf* in gastric epithelial cells is dependent upon an active interplay with viable bacteria.

DAF induction is mediated by a functional type IV secretion system

The requirement for viable *H. pylori* to induce *daf* raised the possibility that bacterial components intimately involved in epithelial contact may mediate *daf* expression. The *cag* PAI encodes a bacterial TFSS that translocates effector molecules such as peptidoglycan and CagA into host cells following binding, thus affecting cell function. Therefore, we determined if *H. pylori*-mediated up-regulation of DAF is *cag* PAI dependent.

MKN28 cells were co-cultured with either wild-type *H. pylori* or isogenic *cagA* or *cagE* null mutant derivatives. Real-time qRT-PCR analysis demonstrated that co-culture with the *cagA*$^{-}$ mutant induced *daf* expression to levels similar to those induced by the wild-type strain (**Figure 16A**). However, co-culture with the *cagE*$^{-}$ mutant failed to induce *daf* and expression levels were no different than levels in uninfected cells. Western blot analysis confirmed that inactivation of *cagE* significantly attenuates the ability of *H. pylori* to induce DAF (**Figure 16B**). Experiments were also performed with an independent *H. pylori cag*$^{+}$ strain, 7.13, which readily infects animals and has been shown to induce gastric cancer in Mongolian gerbils and hypergastrinemic mice

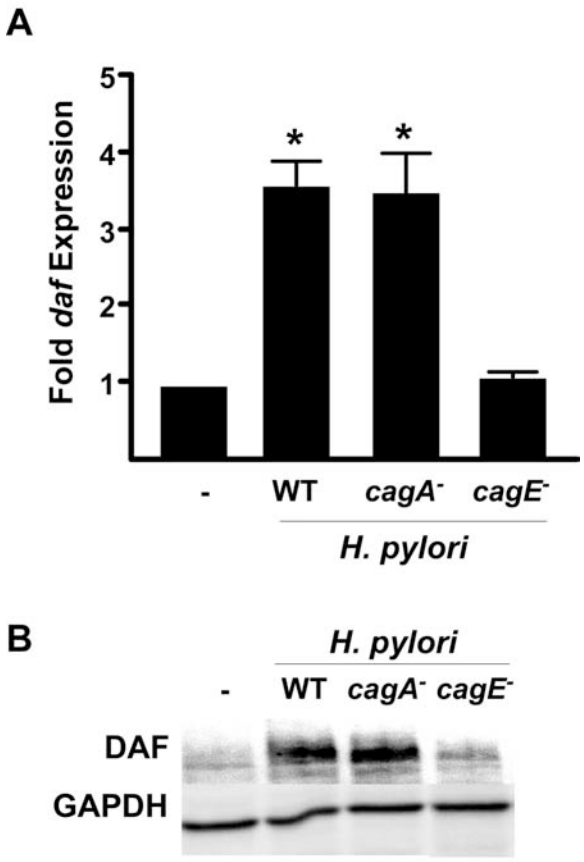

Figure 16. DAF induction is mediated by a functional *cag* secretion system, but not CagA. MKN28 cells were co-cultured with wild-type *H. pylori* strain J166 or isogenic *cagA*$^-$ or *cagE*$^-$ mutants at an MOI=100. A.) Levels of *daf* mRNA were determined by real-time qRT-PCR following 2 hours of co-culture and were normalized to corresponding levels of *gapdh* mRNA. Results are expressed as fold increase in *daf* mRNA in *H. pylori*-infected versus uninfected samples. Error bars, SEM. *, $p<0.05$ versus uninfected cells. B.) Cell extracts were used for Western blot analysis using an anti-DAF antibody. A representative blot of multiple repetitions performed on 3 occasions is shown. Anti-GAPDH blots served as normalization controls.

(87-89, 245). Similar to results obtained with strain J166, real-time qRT-PCR results showed that *daf* induction was dependent upon *cagE*, but not *cagA*. The importance of the *cag* secretion system was more rigorously confirmed by demonstrating that inactivation of another *cag* gene encoding a structural component of the TFSS (*cagM*) similarly attenuated *daf* expression (data not shown). These results indicate that *H. pylori* induction of DAF is dependent upon a functional *cag* secretion system, but not CagA *per se*.

<u>*H. pylori* induction of *daf* occurs via a NF-κB-independent pathway</u>

Activation of the transcription factor NF-κB by *H. pylori* is mediated by the *cag* secretion system (26, 47, 102, 127, 155, 171, 180, 201, 204, 306). The *daf* promoter contains a κB response element and activation of NF-κB leads to the up-regulation of DAF in response to pro-inflammatory stimuli (8, 73, 129, 290). To define the role of NF-κB in *H. pylori*-induced DAF expression, MKN28 cells were transiently transfected with constructs that express either a dominant-negative IκBα or dominant-negative IKKβ, as well as a NF-κB responsive luciferase reporter construct. As expected, *H. pylori* strain J166 significantly increased NF-κB mediated luciferase activity, which was abolished by the dominant-negative IκBα and IKKβ constructs (**Figure 17A**). However, inhibition of NF-κB had no effect on the ability of *H. pylori* to induce *daf* (**Figure 17B**).

<u>Activation of p38 mediates *daf* up-regulation by *H. pylori*</u>

Our group and others have shown that *H. pylori cag*$^{+}$ strains activate MAP kinases such as ERK, p38, and JNK in a *cag*-dependent manner (54, 148). Therefore, we investigated

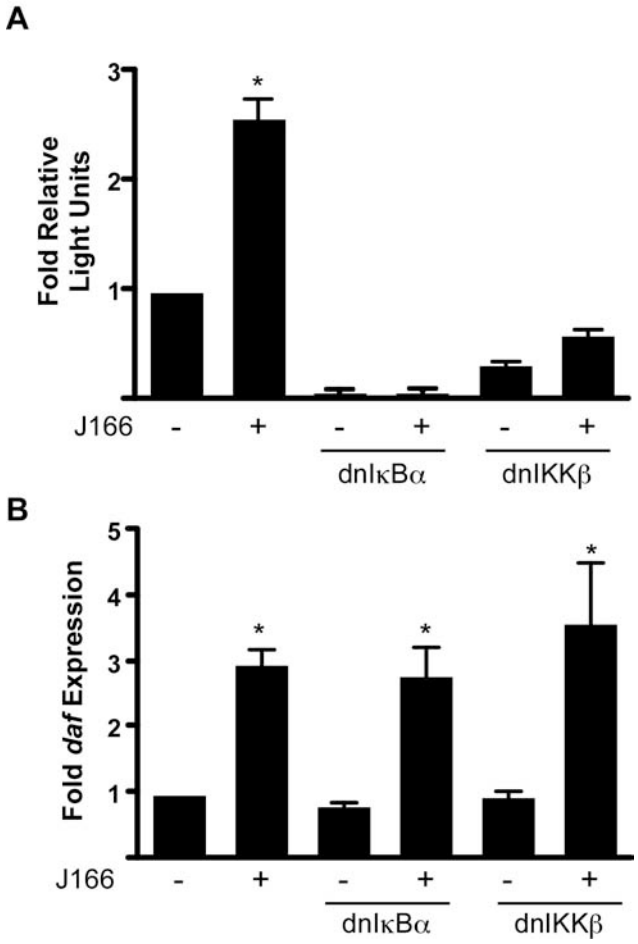

Figure 17. NF-κB is not required for *H. pylori* induction of *daf*. MKN28 cells were transiently transfected with a NF-κB responsive Luciferase reporter construct and either a dominant-negative IκBα (dn-IκBα) or a dominant-negative IKKβ (dn-IKKβ) expression construct. Cells were then co-cultured with *H. pylori* strain J166 at MOI=100. A.) NF-κB driven Firefly Luciferase activity was assayed on a luminometer after 6 hours of co-culture and normalized to *Renilla* Luciferase activity. Error bars, SEM. *, $p<0.001$ versus uninfected cells. B.) Levels of *daf* mRNA were determined by real-time qRT-PCR following 2 hours of co-culture with the *H. pylori* strain J166 and were normalized to corresponding levels of *gapdh* mRNA. Results are expressed as fold increase in *daf* mRNA in *H. pylori*-infected versus uninfected samples. Error bars, SEM. *, $p<0.05$ versus uninfected cells.

the role of these signaling molecules in the transcriptional up-regulation of *daf*. MKN28 cells were pretreated with inhibitors of MEK, p38, or JNK and then *daf* mRNA expression was quantified by real-time qRT-PCR. Inhibition of p38 blocked the induction of *daf* by *H. pylori* strains J166 (**Figure 18**) and 7.13 (data not shown), whereas inhibition of ERK had no effect. Inhibition of JNK resulted in slightly higher levels of *daf* than observed in the *H. pylori*-infected vehicle-treated control; however, this difference was not statistically significant. These data indicate that *H. pylori*-induced *daf* expression is mediated in a p38 MAPK-dependent manner.

Inactivation of a component of the *cag* secretion system attenuates *H. pylori* induction of DAF *in vivo*

To determine if the *in vitro* observations in MKN28 cells mirrored events within colonized gastric mucosa, DAF expression was assessed in transgenic hypergastrinemic INS-GAS mice. INS-GAS mice over-express gastrin and spontaneously develop gastric cancer, but this requires the virtual lifetime of the animal (311). Infection with *H. pylori* accelerates this process and closely models lesions found in human disease (85, 87, 311). Therefore, we infected INS-GAS mice with the *H. pylori* cag^+ strain 7.13, which readily infects rodents, and investigated DAF expression (87-89, 245).

Mice were challenged with *Brucella* broth alone, wild-type strain 7.13, or a 7.13 $cagE^-$ isogenic mutant for 4, 12, and 24 weeks. DAF expression was detected using immunohistochemistry and scored on an ordinal scale from 0-4 as previously described

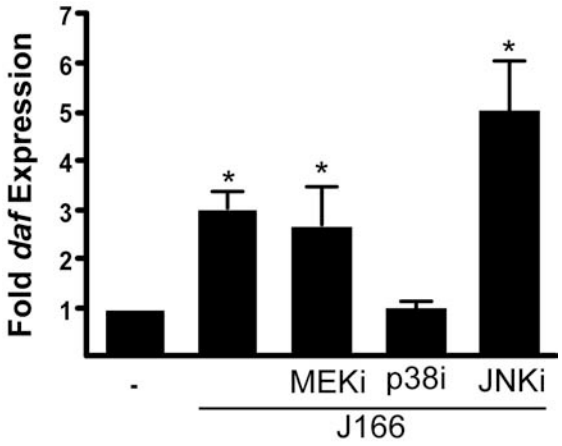

Figure 18. Activation of p38 is required for *daf* up-regulation by *H. pylori*. MKN28 cells were pretreated with pharmacological inhibitors of MEK1/2 (PD98095, 50 μM), p38 (SB203580, 10 μM), JNK (JNK inhibitor II, 10 μM), or vehicle control (DMSO) (-) for 30 minutes and then co-cultured with the *H. pylori* strain J166 at MOI=100. Levels of *daf* mRNA were determined by real-time qRT-PCR following 2 hours of co-culture and were normalized to corresponding levels of *gapdh* mRNA. Results are expressed as fold increase in *daf* mRNA in *H. pylori*-infected versus uninfected samples. Error bars, SEM. *, $p<0.05$ versus uninfected cells.

(265). DAF staining in uninfected mice was localized to stromal plasma cells, lymphocytes, and endothelial cells, with focal weak staining of surface foveolar epithelial cells (**Figure 19D**). There were no differences in DAF staining detected at 4 weeks post challenge among the groups. However, mice infected with wild-type *H. pylori* strain 7.13 for 12 weeks and 24 weeks demonstrated significantly more abundant DAF staining versus uninfected mice (**Figure 19A**). DAF staining was accentuated along the luminal surface in gastric epithelial cells that comprise the foveolar pits and was often accompanied by light diffuse cytoplasmic staining (**Figure 19B, inset**). The intensity of DAF staining in mice infected with the *cagE*$^-$ mutant (**Figure 19C**) was significantly attenuated compared to mice infected with wild-type *H. pylori* and was similar to uninfected mice. These *in vivo* findings recapitulate our *in vitro* data and confirm that a functional *cag* secretion system is required for *H. pylori*-mediated induction of DAF in gastric epithelial cells.

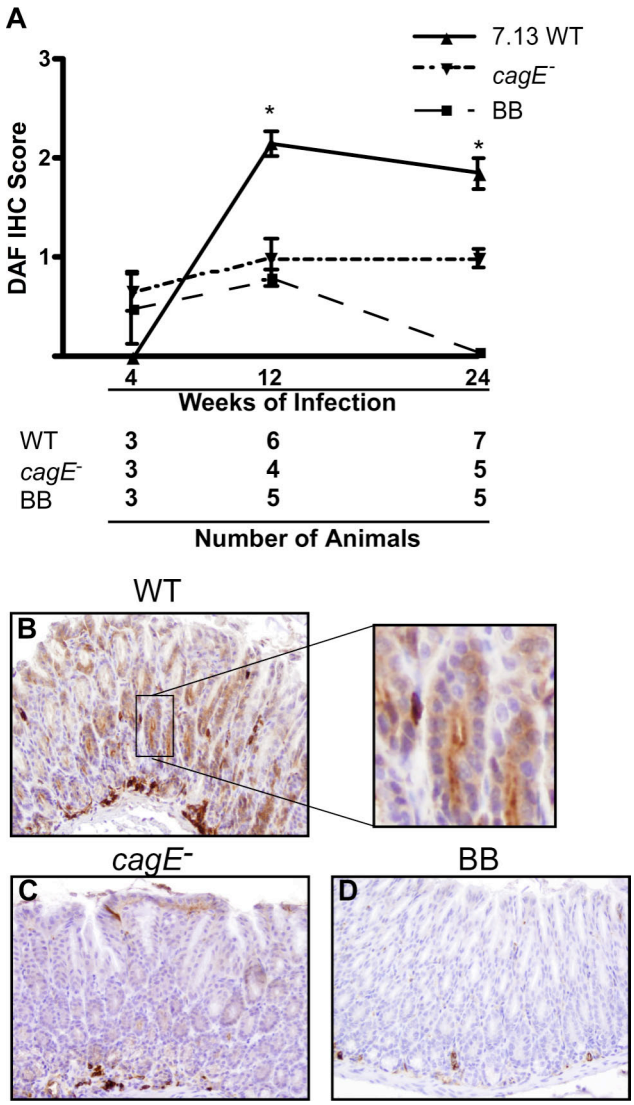

Figure 19. Inactivation of a component of the *cag* secretion system attenuates *H. pylori* induction of DAF *in vivo.* INS-GAS mice were challenged with *Brucella* Broth (BB) control, wild-type *H. pylori cag*$^{+}$ strain 7.13, or an isogenic 7.13 *cagE*$^{-}$ mutant for 4, 12, or 24 weeks. A.) Immunohistochemical staining of DAF was performed and scored on an ordinal scale from 0-4 by a single pathologist. Error bars, SEM. *, $p<0.05$ WT 7.13 vs. BB or *cagE*$^{-}$. (B-D) Representative DAF IHC stained sections from mice challenged for 12 wk with wild-type *H. pylori* strain 7.13 (B); 7.13 *cagE*$^{-}$ isogenic mutant (C); or *Brucella* broth alone (D). Magnification, 20x.

Discussion

Our results have demonstrated that 1) *H. pylori cag*$^{+}$ strains induce DAF expression in a *cag* PAI dependent manner that does not require CagA, 2) *H. pylori*-induction of *daf* is abolished by inhibition of p38, and 3) an *in vivo* model of *H. pylori*-induced gastritis recapitulates our *in vitro* observations by demonstrating a requirement for a functional *cag* secretion system to induce DAF in epithelial cells. Collectively, these data indicate that *H. pylori* utilizes the *cag* island to affect the expression of DAF, potentially increasing adherence capacity, which may be important for initial and chronic colonization of its host.

Increased pathologic outcomes have been associated with infection by *H. pylori cag*$^{+}$ strains, but the mechanism by which these strains increase disease risk is not completely understood. Several studies have highlighted the importance of the translocated effector protein CagA, which is responsible for aberrant activation of multiple signaling pathways. These include activation of β-catenin, SHP-2, and Grb-2, molecules that have been implicated in carcinogenesis. However, our results demonstrate that CagA is not required for increased expression of DAF.

Our finding that a functional *cag* secretion system is sufficient for *H. pylori*-mediated induction of DAF implicates additional bacterial factors that may be translocated into host cells leading to DAF induction. A candidate molecule for such induction is the bacterial cell wall component peptidoglycan. Peptidoglycan motifs that are recognized by

NOD1 are delivered into host cells via the *cag* secretion system and an important signaling event mediated by NOD1 is activation of NF-κB (306). However, while others have shown that DAF regulation is responsive to NF-κB activation by pro-inflammatory stimuli (8, 164), our data demonstrate that NF-κB activation is not necessary for DAF induction by *H. pylori*.

Listeria monocytogenes induces IL-8 secretion via NOD1 activation in a NF-κB and p38-dependent manner (219), and *H. pylori*-induced secretion of IL-8 is dependent upon p38 activation and NOD1 activation of NF-κB (1, 148, 263, 306). Activation of NOD1 can also induce activation of p38, and although the mechanism of this action remains unclear (280), there are several mechanisms by which p38 may promote increased DAF expression. Activation of p38 can transactivate the transcription factor CREB, which has previously been shown to transcriptionally up-regulate DAF in intestinal epithelial cells (73, 129, 290). Alternatively, *daf* mRNA transcript stability has been shown to be increased by activation of p38 in monocytic cell lines (91). Investigations into the mechanism by which p38 mediates DAF induction are currently ongoing in our laboratory.

Murine models provide valuable insights into host, bacterial, and environmental factors involved in *H. pylori*-induced gastric injury and inflammation. The INS-GAS model of gastritis has been used extensively for the study of *H. pylori*-induced inflammation and injury (85, 87, 153, 218, 276, 311). Utilizing this model, we have shown that the pattern of DAF up-regulation mirrors our *in vitro* studies; specifically, a functional *cag* secretion

system plays an active role in the induction of epithelial DAF. Since *H. pylori* cag^+ strains are found in closer juxtaposition to gastric epithelium than cag^- strains (30), our current results suggest that DAF may represent one of several receptors that are up-regulated during chronic inflammation and which contribute to the persistence of more virulent *H. pylori* strains.

In addition to its role in maintaining chronic inflammation during infection, DAF has also been shown to play a role in tumorigenesis. Increased expression of DAF by transformed cells has been linked with resistance to immune clearance (82, 98, 142). Increased DAF expression is present in gastric cancer precursor lesions such as intestinal metaplasia, gastric adenomas, and gastric dysplasia, suggesting that aberrant expression of DAF precedes the development of gastric cancer (152). Our results implicating the *cag* pathogenicity island in DAF up-regulation may also help to explain why persons infected with *H. pylori* cag^+ strains are at significantly increased risk for the development of gastric cancer versus those infected with cag^- strains.

In conclusion, *H. pylori* induces the transcriptional up-regulation of the cellular receptor DAF. DAF induction is mediated by the *cag* secretion system, but does not require the translocated effector protein CagA. DAF induction is also mediated by activation of p38 MAPK. *In vivo,* a functional *cag* secretion system is important for the induction of DAF by *H. pylori* in a murine model of gastritis. Collectively, these data have identified a novel mechanism by which *H. pylori* cag^+ strains may tightly regulate their interactions with gastric epithelial cells and lower the threshold for more severe disease.

CHAPTER IV

CONCLUSIONS AND FINAL REMARKS

Conclusions and future directions

Twenty-six years have passed since the discovery of *H. pylori* and its capacity to chronically colonize gastric mucosa. This discovery has greatly advanced the understanding and treatment of gastric diseases such as peptic ulcer disease, MALT lymphoma, and gastric cancer. The study of *H. pylori* has also enhanced our general understanding of diseases that are associated chronic inflammation. Elucidation into the function of the secreted bacterial cytotoxin VacA has offered new paradigms for the role of bacterial toxins in disease, furthering our understanding of T cell immunity and which interestingly, may have potential as a treatment for HIV infection (220). The results of studying CagA and the *cag* secretion system exemplify the pluralistic functions of *H. pylori* virulence factors in disease, as CagA has proven to be a multifunctional protein that affects many cell signaling pathways. CagA plays a significant role in the induction of gastric cancer (89) and has provided an important cell biology model system for the study of apoptosis, homeostasis, motility, transformation, and the function of cell junction associated proteins.

Disease associated with *H. pylori* infection is the result of chronic inflammation. *H. pylori* is uniquely adapted to the gastric niche, utilizing survival mechanisms that help the

bacteria avoid immune clearance. The fact that all *H. pylori* infections result in chronic inflammation indicates that inflammation is a necessary part of the *H. pylori* lifecycle; however, it not clear what advantage this may provide to the bacteria. Some have speculated that the resulting disruption to the epithelium releases nutrients necessary for survival of the bacteria. However, chronic inflammation has a well established role in the promotion of neoplastic transformation (40). Inflammation induced by *H. pylori*, in combination with the effects of pathogenicity factors, over time leads to tissue damage, erosion, and atrophy of the gastric mucosa, resulting in epithelial hyper-proliferation to replace cells that are destroyed. These cells are exposed to reactive oxygen species that damage DNA, and a cytokine milieu that dysregulates cell function. One seminal study in mice has shown that at an undefined point during the progression of disease, bone marrow-derived cells (BMDCs) engraft into the gastric glands, replacing the endogenous stem cell population that has been eliminated by inflammation. After accumulating mutations, BMDCs and their progenitors become resistant to apoptosis and anti-proliferative signals, leading to transformation and eventually invasive cancer. However, most infections do not result in cancer, underscoring the importance of defining the specific pathogenicity factors and host determinants that result in an environment conducive for cancer promotion.

Adherence of *H. pylori* is critical for the progression of disease and is also necessary for successful colonization of the host. *H. pylori* cannot translocate pathogenicity factors, such as CagA, without direct interaction with host cells. Therefore, the study of host cell receptors and bacterial adhesins is paramount for understanding *H. pylori* pathogenicity

and disease progression. When this project began, several host receptor/adhesin interactions had been identified. BabA and SabA are bacterial adhesins that bind Le^b and sLe^x antigens respectively (134, 176). *H. pylori* LPS is modified with *O*-glycans and Lewis antigens that are mediators of adherence (175). Several members of the hypothetical outer-membrane protein (Hop) family are thought to play an important role in adherence, and AlpA and AlpB have been shown to play a critical role in colonization of mice and guinea pigs, but have no known cognate receptors (58, 170). However, much remains to be discovered regarding *H. pylori* adherence and its pathological consequences.

The results of the work comprising this dissertation contribute to the understanding of *H. pylori* adherence to host cells. In Chapter II, we identified DAF as a novel *H. pylori* receptor, and demonstrated that the majority of the molecule is necessary for efficient *H. pylori* binding. Our results indicate that *H. pylori* interacts most avidly with CCP domains 1-3, or that the interaction is dependent upon the conformation of full-length DAF. We also showed that *H. pylori* up-regulates DAF through direct interaction with the epithelial cell, and defined the importance of DAF as a receptor *in vivo* by demonstrating that *H. pylori* infected $DAF^{-/-}$ mice exhibit significantly attenuated inflammation compared to levels seen in infected wild-type mice. Attenuated inflammation is not likely due to disruption of *cag*-mediated effects because *in vitro* silencing of DAF in human epithelial cells did not affect CagA translocation, phosphorylation, or the induction of IL-8. Thus, we have established that the *H. pylori* interaction with DAF affects inflammation, albeit through an undefined mechanism.

In Chapter III, we extended these observations and defined the mechanism by which *H. pylori* induces the expression of DAF. We demonstrated that *H. pylori* induces the transcriptional up-regulation of DAF via the *cag* type IV secretion system. However, the induction of DAF was independent of CagA translocation, implicating either translocated moieties of peptidoglycan or an unidentified translocated molecule. Another possibility is that *cag* binding to host cells activates receptor mediated intracellular signaling events. We also identified activation of the p38 MAPK pathway as a necessary component of DAF induction. This is analogous to other studies that have shown that *H. pylori* activates MAPK pathways through *cag* PAI dependent mechanisms (54, 148, 189); however, the specific *H. pylori* effector that is driving MAPK activation in the absence of CagA has yet to be defined. Others have shown that activation of the intracellular pattern recognition receptor NOD1 results in activation of p38, but the mechanism of this action remains unclear (280). Components of *H. pylori* peptidoglycan are translocated through the *cag* secretion system and result in NOD1 activation (306). Thus, *H. pylori* translocated peptidoglycan may induce DAF expression via NOD1 activation of p38, but additional work is required to confirm this hypothesis. Finally, we demonstrated that the *cag* secretion system is important for DAF induction *in vivo* using a mouse model of *H. pylori* pathogenesis, confirming the relevance of our finding that the *cag* secretion system mediates DAF induction by recapitulating the results in the significantly more complex environment of a host stomach.

Taken together these data suggest a novel mechanism of pathogenesis utilized by *cag*$^+$ strains (**Figure 20**). Others have observed that *H. pylori cag*$^+$ strains are more frequently

found in close contact with epithelial cells in the gastric mucosa (30). A potential explanation for this finding is that *H. pylori* cag^+ strains selectively induce DAF as a receptor, thereby allowing a more intimate interaction with host cells. We have demonstrated in Chapter II that DAF is an important mediator of *H. pylori* induced inflammation. Therefore, the increased oncogenic potential of cag^+ strains may be due in part to the pro-inflammatory *H. pylori*:DAF interaction. *H. pylori* adherence to gastric epithelial cells is a dynamic interaction mediated by several receptors and bacterial adhesins. Mahdavi *et al.* demonstrated that *H. pylori* binds sLe^x after the bacteria induces sLe^x up-regulation in host cells (176). A model was proposed in which *H. pylori* first binds a constitutively expressed receptor, such as Le^b, and then up-regulates other receptors, such as sLe^x, allowing a more robust interaction with the host cell (176). Our data suggest that DAF, much like sLe^x, may be regulated by *H. pylori* to adjust binding avidity much like a rheostat. However, the *H. pylori*:DAF interaction may be a more intricate component in bacterial pathogenesis than simply as a mediator of attachment. *H. pylori* is known to induce the formation and clustering of lipid rafts at points of attachment similar to those induced by *E. coli* (63), and *E. coli* mediate raft clustering through interactions with DAF (260). Therefore, DAF may represent a molecule involved in orchestrating *H. pylori*-directed raft formation and clustering, allowing increased avidity to the host cell and potentially mediating at least three other events involved in *H. pylori* pathogenesis: intracellular signaling, invasion, and tropism for cell-cell junctions.

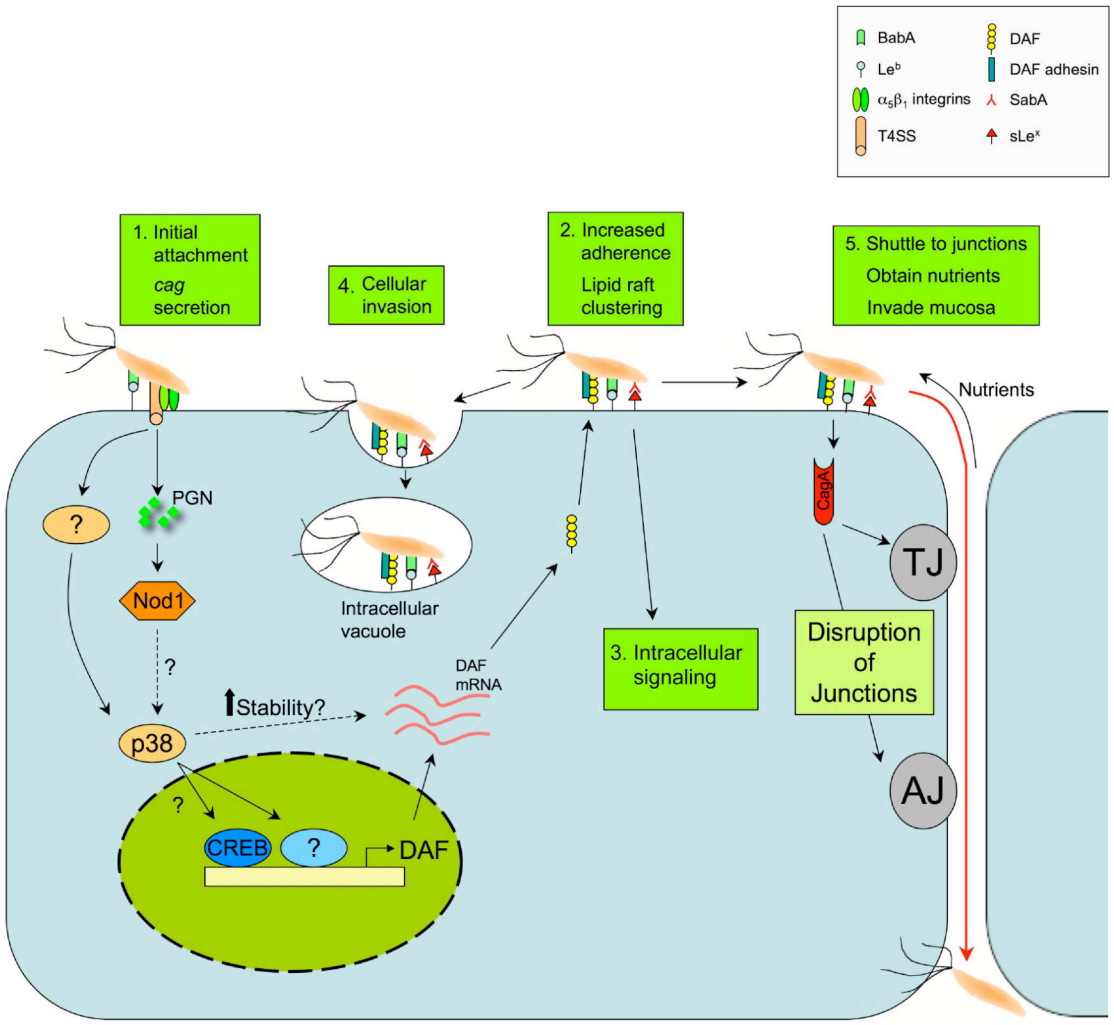

Figure 20. Proposed model of *H. pylori* pathogenic responses mediated by DAF based on our data and results from other investigators. 1.) *H. pylori* binding to the host cell is mediated initially by constitutively expressed receptors such as Le^b allowing the *cag* secretion system to activate p38 MAPK, leading to increased expression of DAF. 2.) *H. pylori* interacts more intimately with the host cell via induced receptors such as DAF and sLe^x, leading to the formation and clustering of lipid rafts. 3.) DAF mediates intracellular signaling. 4.) Raft clustering and DAF signaling mediate invasion into the host cell and survival in intracellular vacuoles. 5.) DAF mediates *H. pylori* tropism for cell-cell junctions, positioning the bacteria for the uptake of nutrients and invasion of the gastric mucosa, which is facilitated by CagA disruption of cell-cell junctions. PGN, peptidoglycan; TJ, tight-junction; AJ, adherens-junction.

Others have shown that pathogens that utilize DAF as a receptor can also activate intracellular signaling through DAF binding (21, 22, 45, 106). This suggests that there may be a parallel mechanism by which *H. pylori* initiates proinflammatory signaling via DAF binding (**Figure 20**). While DAF is a GPI-anchored protein that lacks a transmembrane or cytoplasmic domain, it has been shown to interact with other cell surface proteins that possess cytoplasmic signaling domains. These interactions allow DAF to activate signaling into the host cell, which may be an important component in two subsequent aspects of *H. pylori* pathogenesis: host cell invasion and disruption of cell-cell junctions.

H. pylori recruitment and formation of lipid rafts leads to the formation of pedestals and invaginations into the host cell, allowing the bacteria to invade and reside in intracellular vacuoles (63). *H. pylori* has classically been considered to be an extracellular pathogen. However, several studies have shown that *H. pylori* are found in intracellular sites within gastric epithelial cells *in vitro* and *in vivo* (28, 67, 68, 71, 158, 205, 233, 316). Similarly, Dr^+ *E. coli* strains bind DAF, leading to lipid raft recruitment and invasion of epithelial cells, allowing *E. coli* to reside in intracellular compartments (77, 106, 107, 110). Interestingly, these compartments appear similar to the compartments in which *H. pylori* are found (63). We hypothesize that DAF may play an important role in *H. pylori* invasion of gastric epithelial cells (**Figure 20**). To test this, DAF siRNA expression constructs (166) could be used to generate stable DAF knock-down cell lines to assess *H. pylori* invasion using a gentamicin protection assay and electron microscopy. If DAF is determined to be an important mediator of *H. pylori* epithelial invasion, this will

demonstrate a novel and important mechanism in *H. pylori* pathogenesis that may contribute to the persistence of the bacteria, evasion of the immune system, and might represent a therapeutic target.

A third possibility is that DAF plays a role in directing *H. pylori* localization to cell-cell junctions. Upon binding to epithelial cells, *H. pylori* co-localize with the tight-junction markers ZO-1 and JAM (6). This may allow the bacteria to acquire nutrients that are released by CagA-mediated disruption of the junctions and/or facilitate invasion of the gastric mucosa. Coxsackievirus has been shown to also specifically migrate to tight-junctions where the virus gains entry into the cell, and this trafficking is mediated by DAF (45). The virus orchestrates clustering of lipid rafts via DAF-mediated activation of Abl kinase, triggering Rac dependent actin rearrangements that direct virus movement to the tight-junctions (45). The similarities observed between *H. pylori* and coxsackievirus lead us to hypothesize that DAF may mediate *H. pylori*'s tropism for tight junctions (**Figure 20**). To test this, DAF could be knocked-down via siRNA and the number of *H. pylori* localized to junctional complexes could be quantified using immunofluorescence and transmission electron microscopy. If DAF does mediate *H. pylori* localization to tight-junctions, this would represent a novel mechanism for an important facet of *H. pylori* pathogenesis.

We have highlighted the importance of defining *H. pylori* adhesin/receptor interactions and identified DAF as a receptor for *H. pylori*. Therefore, another future extension of this work is to identify the bacterial adhesin(s) required for this interaction and define its

role in pathogenesis. The *H. pylori* genome has been estimated to encode a large number of outer membrane proteins, many of which may function as adhesins. Because of the large number of potential targets, an efficient methodology for quickly identifying DAF adhesins could be employed, such as proteomics. For example, *in vitro* protein interaction assays could be used to isolate *H. pylori* proteins that are found to interact with DAF. These proteins could then be identified using matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF MS). These experiments may identify a novel bacterial adhesin or class of adhesins that interact with DAF. This would be particularly exciting, as it would permit one to focus on a specific bacterial protein and isogenically inactivate the gene encoding this effector to perform more robust experiments *in vitro* and *in vivo*; thus, more clearly implicating DAF's role in pathogenesis.

In addition to identifying the DAF adhesin, defining the role of DAF in gastric cancer is also of great importance. Several studies have demonstrated that DAF provides a selective advantage for transformed cells by protecting against complement mediated lysis and DAF may also help to protect against other immune mediated killing mechanisms (193). Increased DAF expression parallels severity of cancer staging, and may contribute to the neoplastic potential of the cells through signaling events mediated by DAF (193). We have identified an important role for DAF in *H. pylori* pathogenesis using an *in vivo* mouse model in Chapter II; however, the importance of DAF in *H. pylori*-induced cancer remains to be determined. To define the role of DAF in an *in vivo* model of *H. pylori* induced cancer, we are in the process of backcrossing C57/BL6 DAF$^-$

$^{/-}$ mice onto the FVB/N INS-GAS background. Once crossed onto this background, the INS-GAS DAF$^{-/-}$ will be infected with the carcinogenic *H. pylori* strain 7.13 and monitored for the development of premalignant lesions as well as gastric cancer. It is likely that in the absence of DAF, the development of gastric cancer will be significantly delayed or completely attenuated because inflammation is a key mediator of *H. pylori*-induced cancer. The results of these experiments will also greatly enhance our understanding of the role of DAF *in vivo*.

Final Remarks

The discovery of *H. pylori* and the subsequent recognition of its role in human disease marked a paradigm shift in the way physicians view gastric diseases. This impact has been far-reaching and our understanding of the mechanisms of *H. pylori* pathogenesis has advanced rapidly. Constituents that contribute to disease have been identified in both humans and *H. pylori*, furthering the ability to identify those at increased risk for the development gastric diseases, such as ulceration or cancer. Bacterial survival factors have been identified that allow persistence of *H. pylori* for decades in the harsh niche of the stomach, and pathogenicity factors such as the *cag* PAI and the vacuolating cytotoxin have been discovered to have a multitude of functions, which are important to the determination of disease outcome. In addition, several cellular receptors have been identified that allow the bacteria to interact with host cells. This dissertation has outlined the identification and characterization of novel host receptor that is likely to play an important role in human disease.

A theme that emerges when one examines *H. pylori* pathogenesis is one of bacterial constituents resulting in injury and disease caused by induction of inflammation that develops in response to the microbial constituents. *H. pylori* urease and NapA are important for colonization and survival in the gastric niche, but are also proinflammatory (86). The *cag* secretion system plays a role in disrupting cell-cell junctions and normal cell function, which may induce nutrient release, but the *cag* secretion system also mediates the production of proinflammatory cytokines such as IL-8 and promotes neoplastic transformation (118). The *H. pylori*:DAF interaction may represent another factor in *H. pylori* pathogenicity that may have negative consequences for the host. One potential outcome is that bacterial binding to DAF interferes with the host cell's ability to protect itself from complement-mediated lysis. C3b is extensively deposited on the gastric epithelium during *H. pylori* infection (19), lending credence to this hypothesis. There are many avenues of investigation opened by the findings in this dissertation and more work is needed to define the full implications of the *H. pylori*:DAF interaction.

While there is still much to be elucidated regarding the significance of the *H. pylori*:DAF interaction in human disease, we have identified several important facets. Co-segregation of DAF affinity and *cag* PAI status was not identified in our experiments (Chapter II); however, we found that *cag* positive strains up-regulate DAF both *in vitro* and *in vivo* (Chapter III). We also found that DAF is an important mediator of inflammation *in vivo* (Chapter II), suggesting that part of the increased pathogenicity of *H. pylori cag* positive strains is due to the up-regulation and binding of DAF. The mechanism of DAF as a mediator of inflammation and its potential role in carcinogenesis is not clear. However,

DAF has recently been identified as an apical epithelial ligand for polymorphonuclear cells that regulates the rate of neutrophil migration across apical epithelial membranes (160). This may represent a mechanism by which DAF expression regulates the inflammatory response mediated against *H. pylori*. To define the role of DAF in *H. pylori*-induced gastric cancer, we have initiated studies to utilize INS-GAS DAF$^{-/-}$ mice. In addition, identification of the *H. pylori* DAF adhesin will provide a deeper understanding of the role of DAF in pathogenesis.

It is important to note that DAF serves as a receptor for many pathogens and that the subsequent chronic inflammation is the foundation upon which disease develops. With this body of work, we have added *H. pylori* to this list of pathogens. Our results support a role for DAF as a mediator of inflammation in addition to providing a means of interacting with the host cell. These data also suggest that DAF may play a key role in the development of gastric cancer. If so, DAF may represent a potential target of therapeutic benefit for treatment of *H. pylori*-associated disease and gastric cancer.

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