Transactivation of the EGFR by cag+ Helicobacter pylori induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells

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Running title: H. pylori upregulates Egr-1

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Abbreviations: Egr-1, early growth response gene-1; EGFR, epidermal growth factor receptor; ERK, extracellular-regulated kinases; MAP kinases, mitogen-activated protein kinases; PAI, pathogenicity island; cag, cytotoxin associated genes; PMA, Phorbol-12-myristate-13-acetate; MOI, Multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; PBS, phosphate buffered saline; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase.

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ABSTRACT

*Helicobacter pylori*, in particular *cag* strains, have been shown to enhance gastric epithelial cell proliferation *in vivo*, an effect that likely contributes to gastric carcinogenesis. Egr-1 is a crucial regulator of cell growth, differentiation and survival, which are known to play a role in carcinogenesis and cancer progression. The aims of this study were to: 1) examine whether *H. pylori* could upregulate Egr-1 in gastric epithelial cell lines; 2) determine whether there was a differential response to infection with different strains; 3) examine the role of the *cag* pathogenicity island in this process and 4) elucidate the molecular mechanisms leading to Egr-1 upregulation. In this study, we show that infection of AGS cells with *cag* *H. pylori* resulted in a rapid (1-2 hours), but transient increase in Egr-1 mRNA and protein levels, whereas co-culture with *cag*-isolates did not elicit this response. Furthermore, two independent *cagE*- isogenic mutants of *H. pylori* also demonstrated an impaired ability to upregulate Egr-1. Upregulation of Egr-1 protein was inhibited by the ERK1/2 inhibitor PD98059 and overexpression of dominant negative MEK1 downregulated Egr-1 luciferase reporter gene activity. Treatment of AGS cells with EGFR kinase inhibitors PD153035 and AG1478 resulted in a reduction in *H. pylori*-mediated Egr-1 upregulation, demonstrating that EGFR transactivation plays a role in this early cellular process. Our findings show that *cag* *H. pylori* cause rapid induction of Egr-1 in gastric epithelial cells, which may contribute to *H. pylori*-mediated pathogenesis.

KEY WORDS: Pathogenic, bacteria, cancer, stomach, MAP kinases.
INTRODUCTION

*Helicobacter pylori* is a gastric pathogen that infects over half the world’s population. This gram-negative bacterium colonizes the epithelial layer of the stomach and induces a state of chronic inflammation that can lead to the development of life-threatening diseases including gastric and duodenal ulceration and distal gastric adenocarcinoma [1-3]. The interaction between *H. pylori* and gastric epithelial cells results in the activation of multiple signalling cascades such as MAP kinase pathways [4-6], and also the activation of transcription factors such as NF-κB and AP-1, which have been shown to be important in regulating the host inflammatory response [7-9].

*H. pylori* strains can be broadly divided into cag+ strains or cag- strains depending on whether or not they possess a 40kb region of genes known as the cag pathogenicity island (cag PAI)[10]. The genes that comprise the cag PAI encode for a type IV secretion system, which acts a molecular syringe to deliver the immunodominant CagA protein into the cytosol of the host cell [11-14]. In addition to this delivery of peptidoglycan to host cells by the bacterial type IV secretion has also been demonstrated [15]. *H. pylori* strains that possess the cag PAI have been found to induce more severe gastritis and augment the risk of developing peptic ulcer disease and distal gastric cancer [14]. Furthermore, it has been shown that *H. pylori* cag+ strains selectively enhance proliferation and attenuate apoptosis of epithelial cells in vivo compared to cag- strains [16,17]. Differences have also been observed in the abilities of cag+ and cag- *H. pylori* strains to activate various signalling cascades in infected gastric epithelial cell lines [4,8].

Early growth response gene-1 (Egr-1) is 80-82kDa transcription factor also known as zif268, NGFI-A, Krox24 and TIS8. It is rapidly and transiently induced by a number of extracellular stimuli including growth factors, cytokines, and injury-related stimuli [18]. Egr-1 is functionally implicated in numerous critical biological processes including inflammation, cell proliferation, cell differentiation, vascular wound response, and cancer progression [18]. Interestingly, Egr-1 levels have been found to be elevated in gastric cancer tissues and it has been suggested that Egr-1 may play an important role in carcinogenesis and cancer progression in the stomach [19].

In this study we set out to examine whether *H. pylori* could induce an increase in Egr-1 in gastric epithelial cells, and the mechanism regulating this effect. We also examined whether there were differences between strains in their ability to upregulate Egr-1, and in particular, to assess the importance of the cag pathogenicity island in this process.

MATERIALS AND METHODS

Cell culture and reagents

AGS gastric epithelial cells (American Type Culture Collection, Rockville MD) were maintained as previously described [7]. Cell culture experiments were carried out using 6, 12, 24 well or 100mm polypropylene tissue culture plates (Corning Costar, Cambridge, MA). All experiments were carried out using confluent monolayers unless otherwise stated. Phorbol-12-myristate-13-acetate (PMA) and pharmacological inhibitors AG1478, SB203580, PD98059, PD153035, SP600125 and actinomycin D were all obtained from Calbiochem (La Jolla, CA.).
H. pylori strains, clinical isolates and isogenic mutants

H. pylori were cultured and prepared as previously described [20]. Unless otherwise stated, experiments were performed using the cagA+/vacA+ H. pylori strain 43504 (American Type Culture Collection, Rockville MD). Isogenic H. pylori mutants lacking the cagE or cagA gene were studied together with their parental cag+, toxigenic wild type strain 60190 [21]. A second set of H. pylori isogenic mutants lacking either the cagE or cagA gene were also studied along with their parental cag+, toxigenic wild type strain J166. Mutants and clinical isolates J44 (cag-, vacA s1a m2), J238 (cag+, vacA s1a m2), J68 (cag-, vacA s2 m2), 107A (cag-, vacA s2 m2), and J166 (cag+, vacA s1b m1) were obtained from Vanderbilt University Campylobacter and Helicobacter Laboratory (Nashville, TN). All clinical isolates were obtained from patients with gastritis except for J166 which was from a patient with duodenal ulcer disease.

Western blotting

AGS cells were grown on 12 well plates and maintained in serum-free medium for 24 hours prior to the experiment to avoid cell stimulation by serum growth factors. H. pylori were added in serum-free medium, and co-cultured for varying lengths of time (Multiplicity of infection (MOI) 20:1; bacteria: cell). At the end of the experiment, monolayers were washed 3 times with phosphate buffered saline (PBS) and lysed. Samples were loaded onto an 8% SDS-PAGE gel, and transferred to nitrocellulose membranes (BIO-RAD, Hercules, CA.). Western blotting analysis was carried out using Egr-1 and ERK2 antibodies from Santa Cruz biotechnology, (Santa Cruz, CA), an actin antibody from Sigma-Adrich (St. Louis, MO) and a phosphospecific-ERK1/2 (Cell Signaling Biotechnology, Beverly, MA).

Analysis of Egr-1 and GAPDH mRNA levels using real-time RT-PCR (Taqman assay)

Total RNA was isolated from AGS cells using guanidine isothiocyanate-phenol-chloroform extraction. RNA (1μg) was reversed transcribed using random hexamer primers and Moloney murine leukaemia virus transcriptase, as previously described [22], and the resulting cDNA was stored at –80ºC.

Egr-1 and GAPDH mRNA levels were determined using real-time PCR with a GeneAmp 5700 sequence detection system (ABI/Perkin-Elmer). cDNA was incubated for 2 minutes at 50ºC, denatured for 10 minutes at 95ºC, and then subjected to 40 cycles of annealing at 55ºC for 20 seconds, extension at 60ºC for one minute followed by denaturation at 95ºC for 15 seconds. The gene specific primers used were Egr-1 sense primer, 5’CCCCTCGGATCCCTTTCCT 3’; Egr-1 antisense primer, 5’CAGCATCATCTCCTCCAGTT 3’; GAPDH sense primer, 5’GACCACGTCCATGCACTCA 3’; and GAPDH antisense primer, 5’CATCACGCCACAGTTCCC 3’. To detect amplicons generated using the gene specific primers, dual labelled fluorogenic (Taqman) probes containing FAM (at the 5’ end) and TAMRA (at the 3’ end) were synthesized (Sigma-Genosys, The Woodlands, Texas, USA). The Taqman probes used were: Egr-1, 5’ACTCGCACCATTGCCACACTACC 3’ and GAPDH, 5’ACCCAGAAGACTGTGAGCTGGCCC 3’. Egr-1 levels in each sample were
normalized to GAPDH expression, and the relative change in mRNA level was expressed as fold induction compared to untreated cells using the $-\Delta\Delta CT$ method.

**Electrophoretic Mobility Shift Assay (EMSA)**

Confluent 100mm plates of AGS cells were exposed to *H. pylori* over a 4-hour time course and nuclear extracts prepared as previously described [7]. Double-stranded oligonucleotides containing consensus binding sites for Egr were obtained commercially (Santa Cruz Biotechnology). The oligonucleotides were end-labelled using T4 polynucleotide kinase in the presence of [α-32P]ATP, then purified on a Sephadex G-25 spin column (Boehringer Mannheim, Indianapolis, IN). Binding reactions (20 µl) contained 0.1 ng (~15,000 cpm) of double-stranded probe, 5–12 µg of extracted protein, 2 µg of poly(dI-dC) (Pharmacia, Piscataway, NJ), 10 mmol/L 2-ME, and 1% Ficoll. After first incubating the protein extracts for 10 minutes at room temperature, the radiolabeled probe was added. After an additional 30 minutes at room temperature, reaction mixtures were then loaded on a nondenaturing 4.5% polyacrylamide gel in 0.2 mol/L glycine, 25 mmol/L Tris-HCl, and 1 mmol/L EDTA. The gel was run, dried, and exposed to autoradiography film for 6–18 h at -80°C with an intensifying screen. Supershift analyses were performed with anti-Egr-1 and anti Sp-1 (Santa Cruz Biotechnology) to confirm the identity of the bound protein. The antibodies were added to the DNA probe at the start of the 30-min incubation. In some experiments, binding specificity was also determined by competition with excess unlabeled probe.

**Transient transfection of AGS cells with an Egr-1 luciferase reporter**

AGS cells were transfected using Transfast (Promega) according to the manufacturer's instructions. Briefly, cells were plated 24 hours before transfection at a density of 3x10^5/well on a twelve-well tissue culture dish (Corning Costar). The next day the cells were transfected with 1.5µg of Egr-1 reporter gene DNA (kindly provided by Dr. William Aird, Beth Israel Deaconess Medical Center, Boston, MA). Thirty hours post transfection, media was removed and cells were incubated with serum-free medium for 16 hours to reduce background prior to stimulation with *H. pylori* or PMA (positive control) for 4 hours. Cells were then lysed and reporter activity measured in each sample using a Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. In some experiments a plasmid construct encoding a dominant negative MEK1 (kindly provided by Dr. Sang Hoon Rhee, Beth Israel Deaconess Medical Center, Boston, MA) or vector alone was co-transfected with the Egr-1 construct. All assays were performed in triplicate, and a single representative experiment is shown. Data are expressed as mean values + S.E.

**Statistical Analyses**

Statistical analyses were performed using Sigma-Stat for Windows version 2.0 (Jandel Scientific Software, San Rafael, CA). Analysis of variance followed by protected t tests were used for intergroup comparisons, except where otherwise stated.
RESULTS

Early growth response gene-1 (Egr-1) is upregulated in *H. pylori*-infected gastric epithelial cells

Early growth response gene-1 (Egr-1) is an immediate early gene and transcription factor that can be rapidly upregulated by numerous stimuli. Increased levels of Egr-1 can lead to changes in a number of biological processes such as proliferation, inflammation, and cancer progression [18]. To determine whether *H. pylori* (cag+ ATCC strain 43504) could upregulate Egr-1 we infected AGS cells over a 4-hour time course with the bacteria and then examined whole cell lysates with an Egr-1 antibody. As shown in Figure 1A, *H. pylori* upregulated Egr-1 after 1 hour, this was maximal at 2 hours, and levels returned to baseline by 4 hours. Identical results to those seen with AGS cells were also seen with another gastric epithelial cell line, MKN-28 (data not shown).

To demonstrate that the Egr-1 upregulated in *H. pylori*-infected gastric epithelial cells is capable of binding to Egr-1 target genes, we isolated nuclear extracts from cells infected with *H. pylori* over 4-hour time course and performed an electrophoretic mobility shift analysis (EMSA) using an Egr consensus sequence. As shown in Figure 1B nuclear extracts from untreated cells show little Egr-1 binding. After infection with *H. pylori* for 2-hours, maximal binding occurs which is again reduced by 4 hours. To determine whether Egr-1 was binding to the probe, a supershift experiment was also performed. Nuclear extracts prepared from AGS cells were incubated with either an antibody to Egr-1 or, Sp1 as a control. The complex only underwent a supershift in the presence of the Egr-1 antibody (Figure 1B, 5th lane, supershift indicated by arrow); in contrast no supershift was observed with the Sp1 antibody. These results confirm that Egr-1 was present in the complex binding to our Egr consensus sequence.

*H. pylori* infection results in increased Egr-1 mRNA expression and increases the activity of an Egr-1 luciferase reporter construct

We next examined whether infection of AGS cell with cag+ *H. pylori* could increase Egr-1 mRNA expression. Analysis of cDNA by real-time PCR revealed a 22-fold increase in Egr-1 mRNA levels at the 2-hour time point, which returned back to baseline levels by 6 hours (Figure 2A). These findings demonstrate that infection of AGS cells with *H. pylori* results in a rapid, but transient increase in Egr-1 mRNA levels, consistent with the Egr-1 protein data presented in Figure 1. We examined whether infecting AGS cells with *H. pylori* could induce activation of an Egr-1 luciferase reporter construct. AGS cells transfected with an Egr-1 reporter construct were treated with either *H. pylori* or our positive control (PMA) for 4-hours and luciferase activity of the cells was then measured. Infecting AGS cells with *H. pylori* resulted in a 2.9-fold increase in reporter activity while treatment with PMA resulted in a 4.7-fold increase above control (Figure 2B).

Egr-1 upregulation is mediated through contact between the bacterium and the cell

Next we tested whether soluble factors produced by the bacterium played a role in Egr-1 upregulation, or whether contact between bacterium and cell was necessary for induction using a Transwell system. We found that by preventing direct contact between
AGS cells and the bacteria, upregulation of Egr-1 was completely prevented, suggesting that contact, and not soluble factors, plays a role in this process (Figure 3).

**Egr-1 is differentially upregulated by cag+ and cag- strains of H. pylori.**

Our group and others have previously shown that cag+ and cag- strains of *H. pylori* are capable of differentially activating signal transduction pathways in gastric epithelial cells [4-6]. To determine whether Egr-1 was also preferentially upregulated by cag+ versus cag- strains of *H. pylori*, we infected AGS gastric epithelial cells with a panel of 3 cag+ and 3 cag- strains of *H. pylori* for 2-hours. Whole cell lysates were then analyzed by Western blotting using an Egr-1 specific antibody. As shown in Figure 4A, cag+ strains of *H. pylori* were capable of inducing Egr-1 production at 2 hours, however, in AGS cells infected with cag- strains, this induction did not occur. In addition, we also examined the upregulation of Egr-1 in AGS cells infected with each of the 6 strains over a 6-hour course. Figure 4B shows a complete 6 hour time course for the J166 cag+ strain and the J68 cag- strain, again demonstrating an increase in Egr-1 levels (3.2 fold) at 2 hours occurring with infection with the cag+ strain, but not the cag- strain. Since Egr-1 upregulation is known to be regulated via activation of the ERK1/2 signalling pathway [23] we also analysed the blots for increases in ERK1/2 phosphorylation. Our findings (figure 4B) show that increased ERK1/2 phosphorylation (2.4 fold at 2 hours), appeared to correlate with the increases seen in Egr-1 levels.

**Egr-1 upregulation by H. pylori is dependent on an intact cag secretion system, but not CagA**

Previously we and other investigators have shown that the cag pathogenicity island of *H. pylori* plays an important role in activating various signal transduction pathways within gastric epithelial cells [4-6]. To determine the importance of the cag secretion system to the upregulation of Egr-1 by *H. pylori*, we compared an isogenic cagE- mutant of *H. pylori* to its parental strain. The cagE gene has previously been found to be essential to the functioning of the type IV secretion system. For this experiment we infected AGS cells with wild type *H. pylori* strain J166 or its cagE- isogenic mutant over a 6-hour time course. Lysates were then collected and analyzed by Western blotting to determine the level of Egr-1 present in the cells. As shown in Figure 5A, the cagE-mutant had an impaired ability to upregulate Egr-1 protein levels compared to the parent strain. Analysis of Egr-1 mRNA levels by real-time PCR also revealed that the cagE-mutant was much less efficient in upregulating Egr-1 mRNA levels (~10 fold less after 2 hours of infection) than its wild type counterpart. ERK1/2 phosphorylation was also upregulated by the wild type strain, but not the cagE- mutant at the 2-hour time point, again correlating with the increase in Egr-1 levels. These findings were confirmed using cagE- isogenic mutant derived from parental strain 60190 (data not shown).

We next examined whether CagA participated in *H. pylori*-mediated Egr-1 upregulation. Cells were incubated with wild type *H. pylori* strain 60190 or a cagA-isogenic mutant over a 6-hour time course and their ability of to upregulate Egr-1 protein was examined. As shown in Figures 5B the cagA- mutant, unlike the cagE- mutant had a similar ability to upregulate Egr-1 levels compared to the parental strain. These findings indicate that induction of Egr-1 in AGS cells, although requiring the participation of the type IV secretion system, is not dependent upon translocation of CagA. Again ERK1/2
phosphorylation was examined, and appeared to correlate with increases in Erg-1 levels. These results were also confirmed using our second set of isogenic mutants derived from \textit{cag}+ strain 60190 (data not shown).

**ERK1/2 activation is required for Egr-1 upregulation in \textit{H. pylori}-infected gastric epithelial cells**

We and other investigators have previously reported that \textit{H. pylori} can activate numerous signal transduction pathways including the ERK1/2, p38 and JNK MAP kinase pathways [4-6,24]. Therefore we investigated whether MAP kinase signalling pathways were involved in Egr-1 upregulation. AGS cells were treated with a number of pharmacological inhibitors, including the ERK1/2 inhibitor PD98059 (25 \( \mu \text{M} \)), p38 inhibitor SB203580 (10 \( \mu \text{M} \)), and the JNK inhibitor SP600125 (50 \( \mu \text{M} \)). Only the ERK1/2 inhibitor significantly decreased (69%) Egr-1 protein levels (Figure 6A). Inhibitors of p38 and JNK were unable to prevent Egr-1 upregulation in \textit{H. pylori}-infected cells (data not shown). These findings indicate that ERK1/2 phosphorylation in part is required for \textit{H. pylori} mediated Egr-1 upregulation in AGS cells. In order to confirm our pharmacological data that ERK1/2 was involved in this process we utilized a dominant negative MEK1 construct, which was cotransfected into our cells along with our Egr-1 luciferase reporter construct. As shown in Figure 6B, overexpression of dominant negative MEK1 resulted in a 40% and 75% reduction in basal and \textit{H. pylori} stimulated Egr-1 luciferase reporter activity respectively compared with cells co-transfected with the Egr-1 reporter and vector control.

**Egr-1 upregulation in \textit{H. pylori} can be down-regulated by blocking epidermal growth factor receptor (EGFR) transactivation**

We have previously identified EGFR transactivation as one mechanism leading to the regulation of the ERK1/2 phosphorylation by \textit{H. pylori} [20]. We therefore examined whether blockade of EGFR kinase activity could similarly reduce \textit{H. pylori}-stimulated Egr-1 protein levels. Cells were pretreated with the EGFR kinase PD153035 (5 \( \mu \text{M} \)) for 30 minutes and then infected with \textit{H. pylori} over a 4-hour time course. Egr-1 protein levels were then examined by Western blotting. As shown in Figure 7, EGFR kinase inhibitor treatment resulted in a partial blockade (~50%) of \textit{H. pylori} mediated Egr-1 upregulation. Similar data was also obtained with EGFR kinase inhibitor AG1478 (1\( \mu \text{M} \)). These findings indicate that EGFR transactivation plays a role in \textit{H. pylori} mediated Egr-1 upregulation in AGS gastric epithelial cells.

**DISCUSSION**

Previous studies have shown that infection with \textit{H. pylori} is associated with increased gastric epithelial cell proliferation [16]. Increased cell turnover is an important risk factor for increased mutations and the progression to cancer. Egr-1 is a transcription factor that is able to regulate numerous genes involved in the proliferative response, which include metalloproteinases, growth factors and their receptors [18]. Therefore Egr-1 upregulation may be an important event in \textit{H. pylori}-associated cancer progression.
In this study we show that infection of gastric epithelial cells with \textit{H. pylori} results in a rapid, but transient increase in Egr-1, both at the level of mRNA and protein. Although the upregulation of Egr-1 in gastric epithelial cells by \textit{H. pylori} has previously been described by one other group [25], our study extends these observations in several important ways. We show differences in induction of Egr-1 between \textit{cag+} and \textit{cag-} \textit{H. pylori}, demonstrate the requirement for an intact pathogenicity island and show that \textit{H. pylori}-mediated EGFR transactivation, and ERK1/2 activation are upstream of Egr-1 upregulation.

One of the more significant findings of this study is that \textit{cag+} strains of \textit{H. pylori} are more potent in their ability to upregulate Egr-1 than \textit{cag-} strains. Furthermore, \textit{H. pylori} that have a non-functioning type IV secretion system due to mutation of the \textit{cagE} gene also produce a weaker activation of Egr-1. In previous studies it has been shown that infection of gastric epithelial cells with either \textit{H. pylori} \textit{cag+} and \textit{cag-} strains results in divergent responses. Signal transduction pathways such as the MAP kinase cascades, activation of transcription factors such as NF-\kappaB and upregulation of other early response genes such as \textit{c-fos} are all preferentially induced by \textit{cag+} strains [4,5,8]. Infection with \textit{cag+} strains similarly results in the upregulation of genes involved in the immune response, cell turnover and apoptosis. This may explain why individuals infected with \textit{cag+} strains are more likely to develop atrophic gastritis and distal gastric cancer than those infected with \textit{cag-} strains [14].

We and others have shown that \textit{H. pylori} are capable of causing transactivation of the EGFR via metalloproteinase dependent cleavage of HB-EGF [20,26]. We have previously found that \textit{cag-} strains of \textit{H. pylori} are less able to induce transactivation of the EGFR than their \textit{cag+} counterparts [20]. Likewise mutants with disrupted type IV secretion systems are less able than their parental strain to induce EGFR transactivation. This in turn affects the bacteria’s ability to induce early ERK1/2 phosphorylation, an event we have determined is necessary for Egr-1 upregulation. It has been found that ERK1/2 phosphorylation can also be mediated by soluble secreted factors [27] that are independent of the \textit{cag} pathogenicity island. Our transwell experiments suggest that these soluble factors do not participate in the rapid Egr-1 upregulation observed in our model system.

Infection of our cells with \textit{cagA-} mutants resulted in a similar pattern of Egr-1 induction to that of the wild type strain at early time points. However, later time points (>6hrs) were not examined in this study. Several groups have reported that CagA interacts with numerous host proteins, including Grb2, which results in a sustained activation of the ERK1/2 MAP kinase pathway up to 24 hrs [28,29]. This may potentially lead to Egr-1 upregulation, thus we cannot rule out the possibility that CagA may have effects on Egr-1 upregulation at later stages.

In our \textit{in vitro} model \textit{H. pylori}-induced Egr-1 upregulation appears to be short-lived. This is a typical activation pattern seen with other stimuli including growth factors, hormones and neurotransmitters [18]. Egr-1 gene regulation is tightly controlled by two transcriptional co-factors termed NFGFI-A binding proteins 1 and 2 (NAB1, NAB2), [18]. It has been demonstrated that NAB2 gene expression is itself regulated by Egr-1, therefore Egr-1 controls its own activity through a negative feedback loop [30]. Interestingly, Egr-1 mRNA expression and protein production have been found to be significantly higher in gastric cancer tissues than in normal mucosa [19]. We speculate
that since *H. pylori* chronically infects the stomach, sometimes for many decades, constant re-exposure of the epithelium to the bacterium may cause frequent cellular activation and upregulation of Egr-1. Moreover, Egr-1 has also been identified as a key transcriptional regulator of the EGFR [31] and other growth factors and their receptors [32]. Thus its continual activation over time may contribute to gastric epithelial cell hyperproliferation a known risk factor for gastric carcinogenesis.

In addition to being upregulated in gastric cancer Egr-1 has been found to play a particularly important role in prostate and breast cancer [33,34]. Egr-1 expression levels are elevated in human prostate carcinomas in proportion to grade and stage and in two models of prostate cancer, prostate cancer progression was found to be significantly delayed in mice lacking Egr-1 [34]. In a recent publication by Mitchell *et al*, DNAzymes targeting the Egr-1 inhibited human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth [33]. Collectively, these findings together with the data presented in this study, suggest that Egr-1 may play an important role in *H. pylori*-mediated carcinogenesis.

**GRANTS**

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**FOOTNOTE**

Competing interests: None.
FIGURE LEGENDS

Figure 1. Early growth response gene-1 (Egr-1) is upregulated in *H. pylori* infected gastric epithelial cells

Figure 1A, quiescent AGS cells were treated with *H. pylori cag+* ATCC strain 43504 (MOI 20:1) for the indicated times. Equal amounts of cell extracts were used to determine the levels of Egr-1 as described in MATERIALS AND METHODS. The upper panel shows Egr-1 levels present in cell lysates, the lower panel actin, which acts as a control for loading. The results are representative of 3 independent experiments. Figure 1B, serum-starved 100mm plates of confluent AGS cells infected with *H. pylori cag+* ATCC strain 43504 (MOI 20:1) over a 4-hour time course. Nuclear extracts were made and electrophoretic mobility gel shifts were performed using a radiolabeled Egr-1 consensus sequence as described in MATERIALS AND METHODS. A supershift analysis to confirm the complex contained Egr-1 was also was performed. Nuclear extracts from AGS cells infected with *H. pylori* for 2 hours were incubated with excess cold probe or antibodies against Egr-1 and Sp1 then subjected to EMSA. Supershift with Egr-1 antibody can be seen in the 5th lane as indicated by the arrow.

Figure 2. *H. pylori* infection results in increased Egr-1 mRNA expression

Figure 2A, infection of AGS cells with *H. pylori* results in an increased in Egr-1 mRNA expression over a 6-hour time-course as determined by real-time PCR. Data are normalized to GAPDH levels and results are expressed as fold induction above the unstimulated control. Figure 2B, *H. pylori* infection upregulates Egr-1 luciferase reporter activity. Cells were transfected for 48 hours with an Egr-1 luciferase reporter construct. Cells were then treated with media alone (control), *H. pylori cag+* ATCC strain 43504 (MOI 20:1) or PMA (10nM) for 4 hours. Luciferase activity was then measured in cell lysates. Values are expressed as mean ± SE (n = 3). *** P<0.001 vs. unstimulated control.

Figure 3. Egr-1 upregulation is mediated through contact between the bacterium and the cell

Serum-starved AGS cells were exposed directly to *H. pylori* or co-cultured with *H. pylori* separated by a 0.1µm filter that prevented direct contact between the bacteria and the epithelial cells. The experiment was performed over a 6-hour time course and Egr-1 levels were analyzed as described in MATERIALS AND METHODS. The upper panel shows Egr-1, the lower panel actin, which acts as a loading control. The results are representative of 3 independent experiments.

Figure 4. Egr-1 is differentially upregulated by *cag+* and *cag-* strains of *H. pylori*

Figure 4A, serum-starved AGS cells were treated with a panel of 3 *cag+* or 3 *cag-* strains of *H. pylori* for 2 hours. The levels of Egr-1 protein present in the cell lysates was determined by Western blotting as described in MATERIALS AND METHODS. The upper panel shows Egr-1 levels present in cell lysates, the lower panel shows levels of actin, which acts as a loading control. The results are representative of 3 independent experiments. Figure 4B, quiescent AGS cells were infected with a *cag+* (J166) or *cag-
(J68) strain of *H. pylori* over a 6-hour time-course. Lysates were analysed for Egr-1, phosphorylated ERK1/2 and ERK2 by Western blotting.

**Figure 5. Egr-1 upregulation by *H. pylori* is dependent on an intact cag pathogenicity island**

Figure 5A and 5B, serum-starved AGS cells were treated with a *H. pylori* cag+ strain J166 or its isogenic cagE−, or cagA− mutants, over a 6 hour time course. Western blotting was used to determine the levels of Egr-1 present in the cell lysates as described in MATERIALS AND METHODS. Upper panel shows Egr-1 levels present in cell lysates, the second panel phosphorylated ERK1/2 and the lower panel ERK2, which acts as a control for loading.

**Figure 6. The ERK1/2 signalling pathway is upstream of Egr-1 upregulation in *H. pylori* infected gastric epithelial cells**

Figure 6A, quiescent AGS cells were pretreated with 25 µM of the MEK1 inhibitor PD98059 for 30 minutes and then co-treated with *H. pylori* cag+ ATCC strain 43504 (MOI 20:1) over a 6-hour time course. Western blotting was used to determine the levels of Egr-1 present in the cell lysates as described in MATERIALS AND METHODS. The upper panel shows Egr-1 levels present in cell lysates, the lower panel actin, which acts as a control for loading. The results are representative of 3 independent experiments. Figure 6B, cells were co-transfected with an Egr-1 luciferase reporter and a dominant MEK1 overexpression construct or control DNA. Cells were then treated with media alone (control), *H. pylori* cag+ ATCC strain 43504 (MOI 20:1) for 4 hours. Luciferase activity was then measured in cell lysates. Values are expressed as mean ± SE (n=3). *** P<0.001 control vs. control + dominant negative MEK1. ** P<0.01 *H. pylori* vs. *H. pylori* +dominant negative MEK1.

**Figure 7. EGFR transactivation plays a role in *H. pylori* induced Egr-1 upregulation**

AGS cells were pretreated with EGFR kinase inhibitor PD153035 (5 µM) for 30 minutes then infected with *H. pylori* over a 4-hour time course. Egr-1 protein levels were analyzed by Western blotting as described in MATERIALS AND METHODS. Top panel shows Egr-1 levels, the lower panel actin, which acts as a control for loading. The results are representative of 3 independent experiments.
REFERENCES


Figure 1A.

![Figure 1A](image)

Figure 1B.

![Figure 1B](image)
Figure 2A.

![Graph showing Egr-1 mRNA induction over time with H. pylori infection. The x-axis represents hours of infection with H. pylori, and the y-axis represents the fold induction compared with control. There is a significant increase in Egr-1 mRNA levels at 2 hours.]

Figure 2B.

![Graph showing Egr-1 luciferase activity in response to different treatments. The x-axis represents different treatments: Control, H. pylori, and PMA, and the y-axis represents luciferase activity. There is a significant increase in luciferase activity in response to H. pylori and PMA treatments compared to the control.]
Figure 3.

*H. pylori* in contact with cells. *H. pylori* separated from cells using a 0.1 µm transwell.

<table>
<thead>
<tr>
<th>Egr-1</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Egr-1 band" /></td>
<td><img src="image" alt="Actin bands" /></td>
</tr>
</tbody>
</table>

0 2 4 6 0 2 4 6 Hours
Figure 4A.

<table>
<thead>
<tr>
<th></th>
<th>cag+ strains</th>
<th>cag- strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43504</td>
<td>J166 J238</td>
</tr>
<tr>
<td></td>
<td>J44 J68</td>
<td>107A</td>
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</table>

Egr-1

Actin

2 hours infection

Figure 4B.

<table>
<thead>
<tr>
<th></th>
<th>J166 (cag+)</th>
<th>J68 (cag-)</th>
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</thead>
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<tr>
<td>Egr-1</td>
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<tr>
<td>p-ERK1/2</td>
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<tr>
<td>ERK2</td>
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</tbody>
</table>

0 2 4 6 2 4 6 hours
Figure 5A.

![Western Blot](image1.png)

Figure 5B.

![Western Blot](image2.png)
Figure 6A.

Egr-1

Actin

0 2 4 6 0 2 4 6 Hours

H. pylori

H. pylori + PD98059

Figure 6B.

Egr-1 luciferase activity

Control  H.pylori  Control+ dn-MEK1  H. pylori dn-MEK1

***  ***

**
Figure 7.

<table>
<thead>
<tr>
<th></th>
<th>H. pylori</th>
<th>H. pylori + PD153035</th>
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<td>Egr-1</td>
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<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 1 2 4 1 2 4 Hours
Transactivation of the EGFR by \textit{cag+ Helicobacter pylori} induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells

Sarah Keates, Andrew C. Keates, Sheuli Nath, Richard M Peek and Ciaran P. Kelly

\textit{Gut} published online April 29, 2005

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