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Helicobacter pylori Strain-specific Genotypes and Modulation of the Gastric Epithelial Cell Cycle[1]

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ABSTRACT

***Helicobacter pylori cag*$^+$ strains enhance gastric epithelial cell proliferation and attenuate apoptosis *in vivo*, which may partially explain the increased risk of gastric cancer associated with these strains. The goals of this study were to identify specific *H. pylori* genes that regulate epithelial cell cycle events and determine whether these effects were dependent upon p53-mediated pathways. AGS gastric epithelial cells were cultured alone or in the presence of 21 clinical *H. pylori* isolates, *H. pylori* reference strain 60190, or its isogenic *cagA*$^-$, *picB*$^-$, *vacA*$^-$, or *picB*$^-$/*vacA*$^-$ derivatives. Coculture of *H. pylori* with AGS cells significantly decreased cell viability, an effect most prominent with *cag*$^+$ strains ($P < 0.001$ *versus* *cag*$^-$ strains). *cag*$^+$ strains significantly increased progression of AGS cells from G_1 into G_2-M at 6 h and enhanced apoptosis by 72 h. Compared with the parental 60190 strain, the *picB*$^-$ mutant attenuated cell cycle progression at 6 h ($P \leq 0.05$), and decreased apoptosis with enhanced AGS cell viability at 24 h ($P \leq 0.04$). The *vacA*$^-$ mutant decreased apoptosis and enhanced viability at later (48–72 h) time points ($P \leq 0.05$). Compared with the wild-type strain, the *picB*$^-$/*vacA*$^-$ double mutant markedly attenuated apoptosis and increased cell viability at all time points ($P \leq 0.05$). Furthermore, cocolonization with *H. pylori* had no significant effect on expression of p53, p21, and MDM2. The diminished AGS cell viability, progression to G_2-M, and apoptosis associated with *cag*$^+$ *H. pylori* strains were dependent upon expression of *vacA* and genes within the *cag* pathogenicity island. These results may explain heterogeneity in levels of gastric epithelial cell proliferation and apoptosis found within *H. pylori*-colonized mucosa.**

INTRODUCTION

Gastric colonization with *Helicobacter pylori* induces local inflammation in essentially all hosts, a persistent process that increases the risk of developing atrophic gastritis, intestinal metaplasia, and noncardia gastric adenocarcinoma (1–4). However, only a small percentage of persons carrying *H. pylori* develop neoplasia; enhanced cancer risk may be related either to differences in expression of specific bacterial products, to differences in host response to the bacteria, or to the interaction between host and microbe (5). The first strain-specific gene identified in *H. pylori* was *cagA* (6, 7), a component of the *cag* pathogenicity island (8, 9). Several genes contained within this island, such as *picB* (*cagE*), encode products that are homologues of type IV bacterial secretory proteins (8), and previous studies have shown that mutation in *picB* markedly reduces nuclear factor-κB induction (10) and interleukin 8 secretion (11) by gastric epithelial cells. Persons colonized with *H. pylori* strains that possess *cagA* (and thus the *cag* island) are at increased risk for developing severe gastritis, atrophic gastritis, and distal gastric cancer compared with persons harboring *cagA*$^-$ strains (12–16). The gene *vacA*, which encodes a secreted vacuolating cytotoxin, represents a second locus of heterogeneity among *H. pylori* strains. *H. pylori* strains of the *vacA* s1 subtype are usually strongly toxigenic in *in vitro* cell culture assays, and strains of this subtype almost always are *cagA*$^+$ (17); this strong association has been observed (18) despite the relative distance of *vacA* and the *cag* pathogenicity island on the *H. pylori* chromosome (19).

Host responses to *H. pylori* also may be important in affecting the threshold for carcinogenesis. Exposure of epithelial cells to *H. pylori* alters cell replication and apoptosis *in vitro* and *in vivo* (20–33). *H. pylori* colonization increases gastric mucosal apoptosis (20, 21, 23), but epithelial cell proliferation rates in gastric tissue from persons carrying *H. pylori* have been either reduced or enhanced in various studies (22, 25, 26, 27). One explanation for this variation is that induction of cell growth or death may be affected by specific bacterial characteristics (34). For example, we have reported recently that *cagA*$^+$ strains selectively enhance proliferation and attenuate apoptosis compared with *cagA*$^-$ strains (22); however, in another study, apoptosis was not related to strain variation (24).

Coincubation of gastric cells with *H. pylori* and either tumor necrosis factor α, IFN-γ, or receptor-activating Fas antibodies increases apoptosis compared with coincubation with *H. pylori* alone (28, 35). Coincubation with *H. pylori* also stimulates gastric epithelial cell apoptosis *in vitro* by binding to class II MHC receptors (36), suggesting that several signal transduction cascades may be affected by bacterial contact. One pathway that affects both cell growth and death is mediated by p53, which regulates cell cycle checkpoint function (37). Activation of p53 and subsequent induction of the cyclin-dependent kinase inhibitor p21 may either inhibit cell growth or stimulate apoptosis, depending on the specific cell type and/or growth conditions (37).

Therefore, we investigated the relationship of *H. pylori* genotype and epithelial cell cycle events. Our goals were to identify specific *H. pylori* constituents that regulate gastric epithelial cell cycle events and to determine whether these effects were dependent upon p53-mediated pathways. For these experiments, we used the AGS gastric epithelial cell line as our model system because the interaction between *H. pylori* and this cell line induces transcription of both specific bacterial (38) and host cell genes (10) relevant to inflammation. We report here that VacA and products of genes within the *cag* pathogenicity island have independent effects on cell viability and apoptosis, and that these events are not mediated by elevations in p53 or p21.

MATERIALS AND METHODS

H. pylori Clinical Isolates and Isogenic Mutant Strains

Experiments were performed with the *cagA*$^+$ *vacA* s1a *H. pylori* reference strain 60190 (ATCC 49503), as well as with 21 well-characterized clinical *H. pylori* strains (Table 1) isolated from patients undergoing upper endoscopy at the Nashville Department of Veterans Affairs Gastroenterology Clinic. The 21 clinical strains (10 *cag*$^+$, 11 *cag*$^-$; Table 1) used in this study were selected from a larger population of isolates that have been described previously as part

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Table 1 *Characteristics of 22 H. pylori isolates and relationship to cell cycle distribution after coculture with AGS gastric epithelial cells*

H. pylori strain designation	6-h % G_2M	72-h % sub-G_1	*cagA*[a] status	*vacA* allele[a]		Tox[b]
				Signal Region	Midregion	
Control AGS cells alone	42.3 ± 5	3.0 ± 2	NA[c]	NA	NA	NA
H. pylori strains that significantly increased proportion of cells in both 6-h G_2M and 72-h sub-G_1 compared with controls[d]						
166	56.3	16.2	+	s1b	m1	+
291	56.8	27.5	+	s1b	m1	+
9365	53.8	18.5	+	s1b	m1	+
178	57.8	23.1	+	s1a	m1	−
238	48.4	14.0	+	s1a	m1	+
60190	48.2	14.2	+	s1a	m1	+
H. pylori strains that significantly increased proportion of cells in either 6-h G_2M or 72-h sub-G_1 compared with controls[d]						
198	50.0	4.2	+	s1a	m2	−
117	43.6	13.1	+	s1a	m2	−
H. pylori strains that did not increase cells in either 6-h G_2M or 72-h sub-G_1 compared with controls[d]						
54	40.9	5.5	+	s1a	m1	+
939	44.6	2.5	+	s1a	m2	−
104	41.7	4.1	+	s1a	m1	+
9366	46.2	2.6	−	s2	m2	−
68	46.3	2.0	−	s2	m2	−
190	44.5	7.0	−	s2	m2	−
102	45.2	6.5	−	s2	m2	+
107	47.7	1.9	−	s2	m2	−
63	42.1	2.6	−	s2	m2	−
150	42.0	2.6	−	s2	m2	−
188	45.5	6.3	−	s2	m2	−
195	45.2	0.8	−	s2	m2	−
262	37.1	3.6	−	s2	m2	−
116	40.1	3.0	−	s2	m2	−

[a] *cagA* and *vacA* allele types determined by PCR of genomic DNA, as described previously.
[b] Toxigenicity determined by neutral red assay for HeLa cells, as described previously.
[c] NA, not applicable.
[d] $P < 0.05$ *versus* controls.

of an ongoing prospective study designed to study mechanisms of *H. pylori* pathogenesis *in vivo* (12, 22). Because we sought to analyze the importance of *H. pylori* virulence-related genes (*i.e., cagA, picB,* and *vacA*) in modulation of cell cycle events, we selected strains that varied in *cag* status and in *vacA* genotypes. Among the 11 cag^+ strains (10 clinical isolates and 60190), both s1a ($n = 8$) and s1b ($n = 3$) *vacA* signal sequence alleles were present (Table 1). All of the cag^- strains were *vacA* s2, consistent with the previously reported linkage dysequilibrium between *vacA* s2 and absence of *cagA* (17). Examination of this group of isolates allowed us to stratify strains by both *vacA* and *cagA* genotype, facilitating identification of specific bacterial components that regulate gastric epithelial cell cycle events. The four cag^+ strains used in viability and apoptosis experiments represented a subset of the total cag^+ population ($n = 11$) and varied in *vacA* signal sequence genotype. Two of the strains were *vacA* s1a (60190 and 178), and two were *vacA* s1b (166 and 291). For uniformity, the same four cag^+ strains used in survival experiments also were used in apoptosis studies. This allowed us to assess the influence of genes within the *cag* pathogenicity island, as well as differing *vacA* types, on epithelial cell cycle events. Procedures were approved by the Vanderbilt University and Nashville Department of Veterans Affairs Institutional Review Boards. All clinical strains underwent a maximum of five *in vitro* passages prior to incubation with gastric epithelial cells. To genotype *H. pylori* clinical isolates, genomic DNA was prepared (12), and PCR for *cagA* and *vacA* signal and midregion type was performed as described previously (12, 17).

Isogenic *vacA* and *picB* mutants were generated within *H. pylori* reference strain 60190 harvested at the same *in vitro* passage level. Two different *picB* null mutants were constructed by insertional mutagenesis, using either *cat*[3] (39) or *aphA* (conferring km; Ref. 40) genes. Briefly, the 5′ region of *picB*, containing a unique *Bgl*II site, was amplified from strain 60190 using the primers 5′-GAGCAAGAGGTTCAAAAGCGCC-3′ and 5′-CACCCCAC-CATGCATTTTTTCC-3′. The resultant PCR product was then cloned into pGem (Promega), and *cat* or *aphA* was subsequently cloned into the *Bgl*II site. *H. pylori* isogenic *picB* mutants were generated by natural transformation and allelic exchange using these plasmids, as described previously (39). *vacA* was inactivated in a similar fashion using either pCTB8:km (41) or pCTB5:CAT, which contains *cat* cloned into the unique *Bgl*II site of *vacA* in pCTB5 (41). Isogenic *picB* and *vacA* mutants were selected on *Brucella* agar with either chloramphenicol (10 μg/ml) or kanamycin (25 μg/ml). Isogenic *cagA* mutants of strain 60190 have been reported previously (42). To inactivate both *picB* and *vacA* in a single strain, the *picB*::km *H. pylori* isogenic mutant was transformed with pCTB5:CAT, whereas the *vacA*::km mutant was transformed with the plasmid containing *picB*::CAT. Double mutants were selected on *Brucella* agar containing kanamycin (25 μg/ml) and chloramphenicol (10 μg/ml). The presence of the *cat* or *aphA* cassettes within *vacA* and/or *picB* was confirmed by PCR-amplifying products of the expected size from bacterial chromosomal DNA, using specific primers.

H. pylori Culture

For coculture experiments with gastric epithelial cells, *H. pylori* were grown in *Brucella* broth with 5% FBS for 48 h, harvested by centrifugation (2000 × *g*), and resuspended in antibiotic-free RPMI 1640 with 10% FBS to a concentration of 1×10^8 cfu/ml. For all experiments, *H. pylori* were added to cells at a bacteria:cell concentration of 100:1, based on previous reports that *H. pylori* reproducibly induce apoptosis in AGS cells at this ratio (28, 29). For bacterial viability studies, serial 10-fold dilutions of supernatants removed from *H. pylori*:AGS cell cocultures were plated onto Trypticase soy agar with 5% sheep blood and incubated for 72 h under microaerobic conditions, as described previously (12). Bacteria were identified as *H. pylori* by urease and oxidase activity as well as by Gram's stain morphology, and colony counts were determined by an observer blinded to AGS cell cycle results.

Cell Culture

AGS human gastric epithelial cells (ATCC CRL 1739) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS and 20 μg/ml gentamicin in an atmosphere of 5% CO_2 at 37°C. Experiments were performed in antibiotic-free media with 10% FBS using T-150 flasks (Corning Costar, Cambridge, MA), 24- or 96-well polypropylene tissue culture plates (Nunc, Roskilde, Denmark), and chamber slides (four-well/slide; Lab-Tek; Nunc). AGS cells were studied because they possess wild-type *p53* (29), in contrast to KATO III and other gastric cell lines that have *p53* deletions and/or rearrangements, which may affect the response to signals that regulate cell cycle events (43). AGS cells were not serum starved and remained subconfluent during each assay, because we attempted to recapitulate events that occur in the native actively replicating gastric mucosa.

[3] The abbreviations used are: *cat* or CAT, chloramphenicol acetyltransferase; km, kanamycin resistance; FBS, fetal bovine serum; cfu, colony-forming unit(s).

Assessment of AGS Cell Viability

AGS cells were seeded to a subconfluent density of 5×10^4 cells/well in 24-well plates, incubated overnight, and washed with sterile PBS before inoculation with *H. pylori* (5×10^6 cfu/well; total volume 1.0 ml). Control cells were inoculated with RPMI 1640/10% FBS alone, and coincubation was performed up to 72 h in triplicate. At the end of each incubation, cell viability was determined in a hemacytometer by trypan blue exclusion (0.04% trypan blue; Life Technologies, Inc.) using phase-contrast microscopy.

Flow Cytometry

AGS cells were seeded into T-150 flasks at a subconfluent density of $0.6–1.2 \times 10^6$ cells/flask, incubated overnight, washed with PBS, and inoculated with either *H. pylori* at a concentration of 100:1 bacteria to cells or with RPMI/10% FBS alone. Coculture experiments were performed for 6–72 h, at which times control and treated cells were trypsinized, and 1.0×10^6 cells were aliquoted for flow cytometry. The remaining cells were processed for protein analysis by the Bradford assay (Bio-Rad). Cells were incubated with 20 μg/ml propidium iodide (Sigma), and DNA content was measured using a FACSCaliber (Becton Dickinson). Data were plotted using Cell Quest software (Becton Dickinson); 15,000 events were analyzed for each sample.

Assessment of Apoptosis

DNA-specific Fluorochrome Staining. AGS cells were cultured on chamber slides with or without *H. pylori* for 24–72 h, fixed with ice-cold methanol for 10 min, incubated with 1 μg/ml propidium iodide for 5 min, and evaluated by fluorescent microscopy. Nuclei with highly condensed and fragmented chromatin were considered apoptotic.

DNA Fragmentation ELISA. DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biochemicals, Indianapolis, IN) that detects nucleosomal fragments in cytoplasmic fractions of cells undergoing apoptosis but not necrosis. For these experiments, 5×10^3 AGS cells/well in 96-well plates were incubated subconfluently in triplicate with *H. pylori* (5×10^5 cfu/well) or media alone for 24 to 48 h and lysed, and supernatants after centrifugation were used for ELISA. Absorbance measured at 405 nM was compared between controls and *H. pylori*-cultured samples.

Western Analysis

Cells were lysed in Kinase Lysis Buffer [KLB; 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.1% NP40, 4 mM EDTA, 50 mM NaF, 0.1 mM NaV, 1 mM DTT, and the protease inhibitors: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 50 μg/ml phenylmethylsulfonyl fluoride (Sigma), and 200 μg/ml 4-(2-aminoethyl)-benzenesulfonylfluoride) (Calbiochem-Novabiochem Corp.)]. Total cell protein extracts were normalized for concentration by the Bradford assay (Bio-Rad) and 50 μg of protein separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were incubated with mouse monoclonal antibodies against p53 (PAb1801), p21 (EA10; Calbiochem, Oncogene Research Products), and MDM2 (Santa Cruz). Primary antibodies were detected using goat antimouse horseradish peroxidase-conjugated secondary antibody (Pierce) and visualized by the ECL detection system (Amersham Corp., Arlington Heights, IL), according to the manufacturer's instructions.

Statistics

Results are expressed as mean ± SE. The Mann-Whitney *U* test was used for statistical analyses of intergroup comparisons, whereas *vacA* subtypes, toxin production, and clinical outcome within the group of cag^+ strains were compared using the Mantel-Haenszel test. *P*s ≤ 0.05 were considered significant.

RESULTS

Viability of AGS Gastric Epithelial Cells Is Decreased after Coculture with *H. pylori* Cells. In various studies, *H. pylori* has been shown to either reduce or enhance gastric epithelial cell viability *in vitro* (28, 30–33). To determine whether *H. pylori* strain variation may account for the discordant results, we incubated AGS gastric epithelial cells with six well-characterized, minimally passaged clinical *H. pylori* isolates (three cag^+: 166, 291, and 178; three cag^-: 68, 9366, and 107) or a cag^+ toxigenic *H. pylori* reference strain (60190) and quantified cell viability at 24, 48, and 72 h after inoculation. AGS cells cocultured with *H. pylori* had significantly ($P < 0.05$) reduced viability compared with control AGS cells at each time point (Fig. 1). When survival was compared for cells cocultured with cag^+ or cag^- strains, cag^+ isolates significantly decreased AGS cell viability at each time point ($P < 0.001$ at 24, 48, and 72 h; Fig. 1). Because differences in bacterial viability among cag^+ compared with cag^- strains during the 72-h time course could have contributed to differences in AGS cell survival, we also quantitated bacterial viability at 24, 48, and 72 h using the seven *H. pylori* strains described above. There were no significant differences in *H. pylori* viability at any time point during AGS cell coculture among cag^+ and cag^- strains, and viable bacteria persisted for 72 h (data not shown). These findings indicate that coculture with *H. pylori*, particularly cag^+ strains, significantly reduces AGS cell viability and raise the hypothesis that differences in cell survival may be attributable to bacterial strain-specific alterations in gastric epithelial cell cycle events.

***H. pylori* Strains Differentially Alter Cell Cycle Events in Gastric Epithelial Cells *in Vitro*.** We next examined whether the *H. pylori*-induced reductions in AGS cell viability were associated with arrest of cells at a specific phase of the cell cycle or with increased apoptosis and whether these changes correlated with particular *H. pylori* genotypes. For these experiments, 21 minimally passaged clinical isolates (10 cag^+ and 11 cag^-; Table 1), as well as strain 60190, were incubated with AGS cells for 6–72 h, and the AGS cells were analyzed by flow cytometry. In preliminary experiments examining a smaller number of clinical strains (5 cag^+ and 5 cag^-), the effects of *H. pylori* on cell cycle distribution in this system were most prominent at 6 and 72 h (data not shown); therefore, we focused our analysis on events occurring between these time points.

Six h after incubating AGS cells with cag^+ strains, progression of cells from G_1 into G_2-M (50 ± 6%) increased compared with controls (42 ± 5%; $P = 0.07$; Fig. 2*A*). For AGS cells cocultured with cag^-

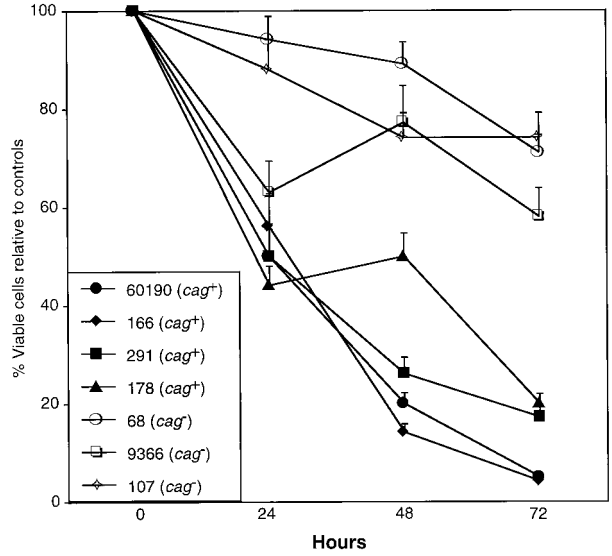

Fig. 1. Incubation with *H. pylori* cells decreases viability of gastric epithelial cells *in vitro*. AGS cells were grown alone or in the presence of *H. pylori* cells in RPMI 1640 supplemented with 10% FBS. *H. pylori* strains examined included cag^+ strains 60190, 166, 291, and 178 (*filled symbols*); and cag^- strains 68, 9366, and 107 (*open symbols*). Cell viability was assessed by trypan blue exclusion 24–72 h after inoculation, and results are expressed as percentages of viable cells relative to controls. Data represent means of three independent experiments; *bars*, SE.

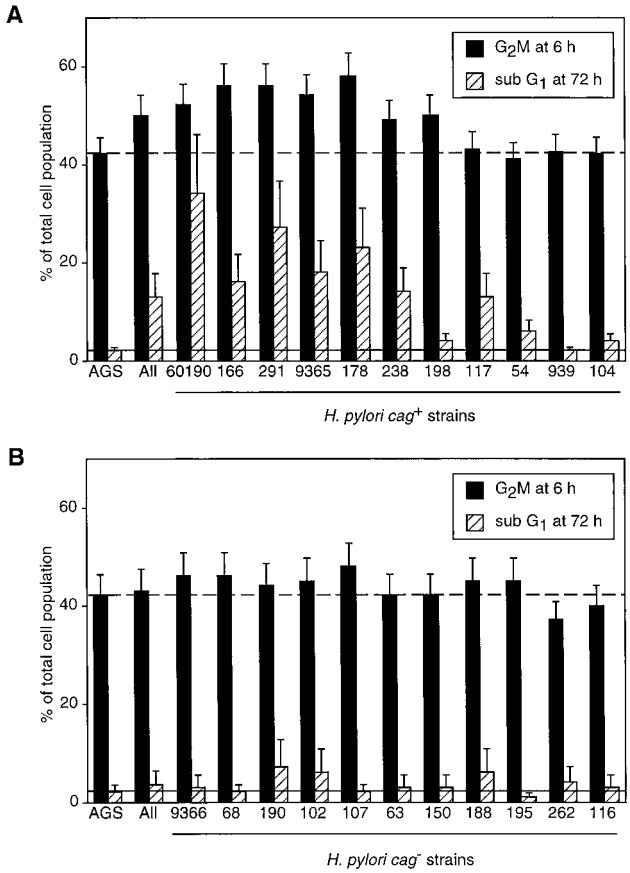

Fig. 2. Cell cycle distribution of AGS cells grown alone or in the presence of *cag*$^+$ ($n = 11$; *A*) or *cag*$^-$ ($n = 11$; *B*) *H. pylori* strains at 6 and 72 h after inoculation. Adherent cells were stained with propidium iodide and examined using flow cytometry. Data were plotted using Cell Quest software (Becton-Dickinson), and 15,000 events were analyzed for each sample. The proportion of AGS cells in G_2-M at 6 h and sub-G_1 at 72 h for controls, all *cag*$^+$ or *cag*$^-$ (*All*), and each individual strain is shown. Lines representing the percentage of control cells in G_2-M at 6 h (*dashed lines*) and sub-G_1 at 72 h (*straight lines*) are shown for reference. The Mann Whitney *U* test was used for statistical comparisons. *Bars*, SE.

strains, the proportion at 6 h that were in G_2-M did not differ from controls (44 ± 3% *versus* 42 ± 5%; $P = 0.37$; Fig. 2*B*). By 72 h, coincubation with *cag*$^+$ strains significantly increased the sub-G_1 AGS cell population compared with controls (13 ± 3% *versus* 3 ± 0.5%; $P = 0.03$; Fig. 2*A*). The sub-G_1 population among AGS cells coincubated with *cag*$^-$ strains was no different than for AGS cells alone ($P = 0.4$). Similarly, the proportion of AGS cells in sub-G_1 at 72 h was significantly greater in samples coincubated with *cag*$^+$ than with *cag*$^-$ strains ($P = 0.002$; Fig. 2).

However, although some *cag*$^+$ strains clearly affected cell cycle events, this property was not conserved among all isolates examined (Fig. 2*A*). Within the *cag*$^+$ group, 7 of 11 strains accelerated progression from G_1 into G_2-M at 6 h, and 6 (86%) of these 7 strains significantly enhanced movement into sub-G_1 by 72 h (Table 1). One additional *cag*$^+$ strain (117) was associated with the 72-h increase in sub-G_1 but failed to induce early (6 h) entry into G_2-M. The remaining 3 *cag*$^+$ strains as well as the 11 *cag*$^-$ strains were not associated with these changes, thus demonstrating a strong (91%) concordance between the early *H. pylori*-induced progression of cells into G_2-M and a later increase in the sub-G_1 population. Of interest, a higher percentage of AGS cells cocultured with *cag*$^-$ strains were in G_1 at 72 h (50 ± 5.6%) compared with *cag*$^+$ isolates (41 ± 9%; $P = 0.009$), suggesting that *H. pylori cag*$^-$ strains may preferentially induce G_1 growth arrest. These findings indicate that coincubation with certain *cag*$^+$ but not *cag*$^-$ strains increases progression of AGS cells into G_2-M at 6 h and sub-G_1 by 72 h, thereby resulting in the appearance of an enhanced population of cells with subdiploid DNA content.

To determine whether the enhanced sub-G_1 population represented apoptotic cells, we examined nuclear morphology in propidium iodide-stained AGS cells that were grown alone or in the presence of *H. pylori*. Coincubation with *H. pylori* strain 60190 induced morphological features consistent with apoptosis, including chromatin condensation, as well as nuclear segmentation compared with controls (data not shown).

To independently assess as well as quantitate apoptosis, we also measured the extent of DNA fragmentation present in cytoplasmic fractions of AGS cells after coincubation with *H. pylori*. Incubation of AGS cells with *H. pylori* cells significantly increased cytoplasmic oligonucleosomal fragments at both 24 and 48 h ($P < 0.001$ for each time point; Fig. 3), compared with AGS cells alone. Coculture with *cag*$^+$ isolates significantly increased nucleosomal release at both 24 and 48 h compared with *cag*$^-$ strains ($P < 0.001$ for each time point; Fig. 3). To determine whether induction of apoptosis might represent a nonspecific response to bacterial coculture, AGS cells also were incubated with equivalent numbers of *Escherichia coli*, *Shigella flexneri*, or *Campylobacter jejuni* for 24 h, and nucleosomal release was quantitated by ELISA. DNA fragmentation was no different in AGS cells coincubated with these bacterial species compared with AGS cells alone (data not shown), suggesting that in this system, *H. pylori* specifically stimulated apoptosis. In total, these data indicate that both *cag*$^+$ and *cag*$^-$ *H. pylori* strains can induce apoptotic changes in the epithelial cell, but that the effect is more pronounced in the presence of *cag*$^+$ strains.

***H. pylori*-induced Cell Cycle Progression and Apoptosis Is Not Mediated by Induction of p53, p21, or MDM2.** Next, we sought to determine whether the strain-specific *H. pylori*-induced modulation of AGS cell cycle correlated with activation of p53 signaling. To verify that the p53 signaling pathway was intact in AGS cells as reported previously (29), we treated cells with 5-fluorouracil or Adriamycin

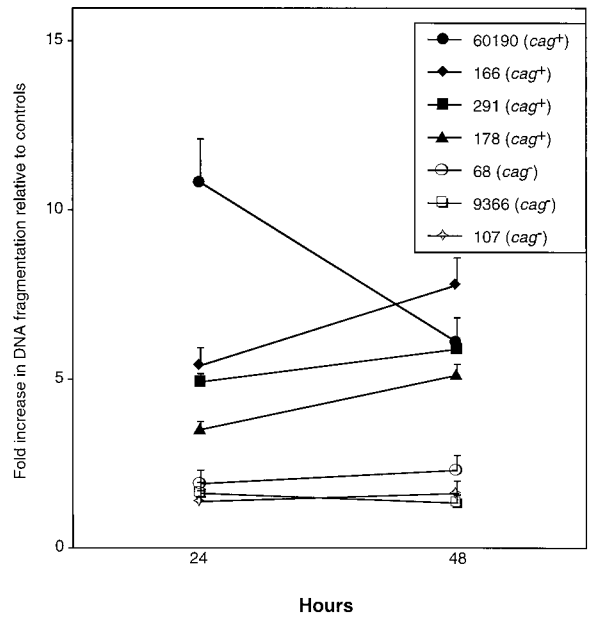

Fig. 3. Apoptosis in AGS cells after incubation with different *H. pylori* strains assessed by DNA fragmentation analysis. AGS cells (5×10^3/well) were incubated alone or in the presence of *H. pylori* (5×10^5/well) for 24–48 h, and DNA fragmentation was quantitated by ELISA. *H. pylori* strains examined included *cag*$^+$ strains 60190, 166, 291, and 178 (*filled symbols*); and *cag*$^-$ strains 68, 9366, and 107 (*open symbols*), and results are expressed as the levels of nucleosomal release relative to controls. *Bars*, SE.

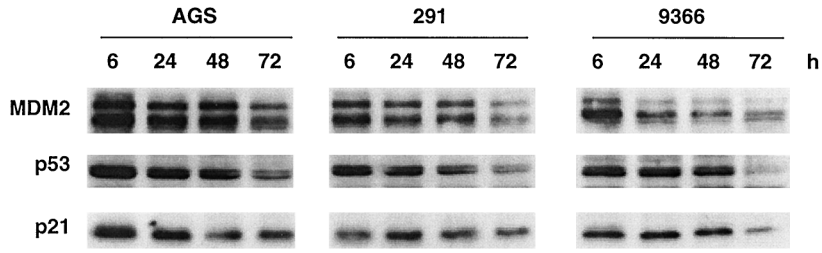

Fig. 4. Coincubation with *H. pylori* cells does not increase expression of p53, p21, and MDM2 in AGS cells. Protein lysates were prepared from control AGS cells or cells cocultured with *H. pylori* strains 291 (*cag*$^+$) or 9366 (*cag*$^-$). Proteins were resolved with monoclonal antibodies to p53, p21, and MDM2 as described in "Materials and Methods." Representative immunoblots from three independent experiments are shown.

and examined p53 and p21 protein levels. Exposure of AGS cells to either drug markedly elevated p53 and p21 protein levels in a time-dependent manner (data not shown), indicating that our model *in vitro* system could be used to analyze p53-mediated pathways.

AGS cells were cultured alone or in the presence of five clinical *H. pylori* strains, and expression of p53 and its downstream targets p21 and MDM2 was analyzed at sequential time points. The five strains examined were chosen from among the seven isolates studied in survival (Fig. 1) and apoptosis (Fig. 3) experiments and included strains that were *cagA*$^+$ *vacA* s1a (60190), *cagA*$^+$ *vacA* s1b (291, 166), and *cagA*$^-$ *vacA* s2 (68, 9366). As shown in Fig. 4, expression of p53, p21, and MDM2 was not increased 6–72 h after incubation with either the *cagA*$^+$ *vacA* s1b strain 291 or the *cagA*$^-$ *vacA* s2 strain 9366, and similar effects were seen in AGS cells after incubation with strains 60190, 166, and 68 (data not shown). In fact, a slight time-dependent decrease in levels of protein expression were seen after incubation with all of the strains. Because these findings are opposite to the corresponding alterations in cell cycle induced by these strains (apoptosis from *cag*$^+$ strains and G_1 arrest from *cag*$^-$ strains), these data indicate that the eukaryotic signaling pathways responsible for *H. pylori*-induced effects on AGS cell cycle events are not mediated by elevations in p53.

Correlation of *H. pylori vacA* Genotypes with Differential Cell Cycle Effects. Heterogeneity among *cag*$^+$ *H. pylori* isolates in their ability to alter cell cycle events suggests that strain-specific traits in addition to the presence of the *cag* island may be important in inducing these responses. We therefore examined AGS cell cycle distribution after coincubation with 11 *cag*$^+$ wild-type *H. pylori* strains that differed in *vacA* allelic types and toxigenicity (Table 1). All six *cag*$^+$ strains that significantly induced both G_2-M progression and apoptosis possessed a type s1/m1 *vacA* allele, whereas three of the remaining five *cag*$^+$ strains possessed a type s1/m2 *vacA* allele (Table 1; $P = 0.03$). The six *cag*$^+$ strains that increased both G_2-M progression and apoptosis also were more likely to be toxigenic for HeLa cells (83%), compared with *cag*$^+$ strains that lacked these effects (40%; $P = 0.1$; Table 1). These findings raised the hypothesis that particular *vacA* genotypes, as well as genes within the *cag* island (*i.e.*, *picB*), may contribute to strain-specific effects on cell cycle events *in vitro*.

Effects of *vacA* and *picB* Inactivation on Gastric Epithelial Cell Cycle Events. To examine the effects of two known *H. pylori* virulence-related genes on cell cycle progression in gastric epithelial cells, we next cocultured AGS cells with the *cag*$^+$ toxigenic *H. pylori* reference strain 60190 or its isogenic *vacA*$^-$ null mutant or *picB*$^-$ null mutant derivatives. Analyzing cells by flow cytometry, as expected, incubation with strain 60190 induced significant AGS cell accumulation at G_2-M at 6 h ($P = 0.04$ *versus* controls), followed by a significant increase in the sub-G_1 population at 24–72 h ($P \leq 0.04$ for each time point *versus* controls; Fig. 5*A*). AGS cells incubated with *aphA* (km)-derived and *cat*-derived *H. pylori* mutants demonstrated nearly identical flow patterns (data not shown); therefore, results for isogenic *H. pylori vacA*$^-$ and *picB*$^-$ derivatives at each time point were combined. The parental 60190 strain and *vacA*$^-$ null mutant strains did not differ in effects on cell cycle progression up to 48 h (Fig. 5*A*). However, the parental strain induced enhanced progression of AGS cells into sub-G_1 at 48 and 72 h compared with the *vacA*$^-$ null mutant ($P = 0.04$; Fig. 5*A*). Filtered supernatant containing VacA (10 μg/ml) from wild-type strain 60190 did not significantly increase apoptosis in the AGS cells (data not shown). Because mutation in *vacA* decreased the sub-G_1 population of AGS cells at 48–72 h (Fig. 5*A*), we incubated AGS cells with strain 60190 *vacA*$^-$ in the presence

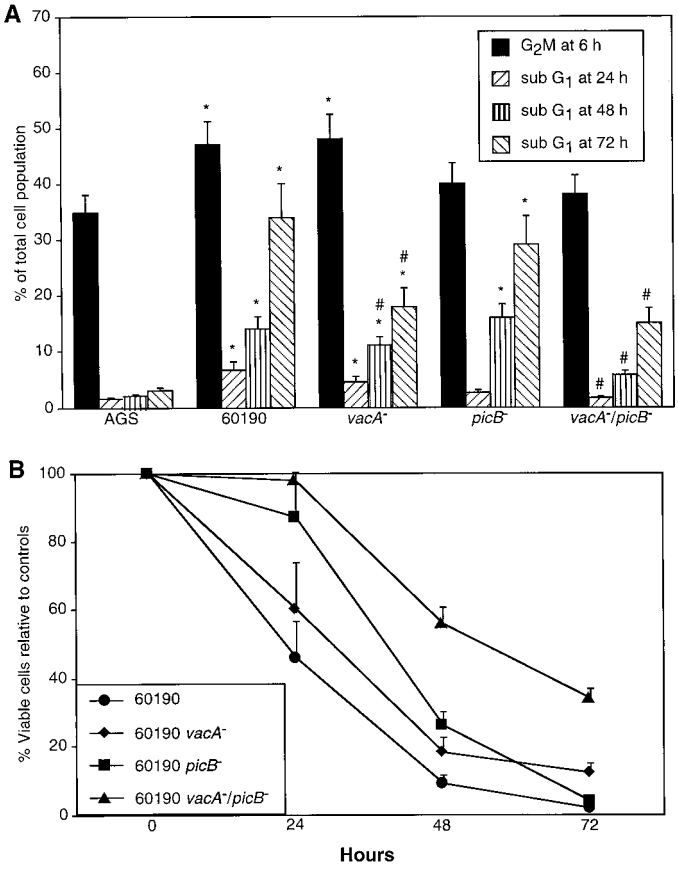

Fig. 5. Cell cycle distribution (*A*) and viability (*B*) of AGS cells grown alone or in the presence of *H. pylori* reference strain 60190 or its *vacA*$^-$, *picB*$^-$, or *vacA*$^-$/*picB*$^-$ derivatives. *A*, DNA content of adherent cells was examined by flow cytometry using propidium iodide staining. Data were plotted using Cell Quest software (Becton-Dickinson), and 15,000 events were analyzed for each sample. Mean values are shown and represent at least three independent experiments; bars, SE. *, $P \leq 0.05$ compared with AGS cells alone; #, $P \leq 0.05$ compared with wild-type strain 60190. The Mann Whitney *U* test was used for statistical comparisons. Data shown represent the proportion of AGS cells in G_2-M at 6 h, and sub-G_1 at 24, 48, and 72 h, respectively, after incubation with medium alone or parental wild-type strain 60190 or corresponding *H. pylori* mutants. *B*, AGS cells (5×10^4/well) were grown alone or in the presence of cells of various strains of *H. pylori* (5×10^6/well) in RPMI 1640 supplemented with 10% FBS. Cell viability was assessed by trypan blue exclusion 24–72 h after inoculation. Data represent means of at least three independent experiments and are expressed as percentages of viable cells relative to controls; *bars*, SE.

of 60190 supernatant containing VacA to determine whether adding the toxin reconstituted the increased sub-G_1 AGS cell population found after coincubation with the wild-type 60190 strain. No additional alterations in cell cycle were found when supernatants were incubated with the *vacA*$^-$ isogenic derivative (data not shown), suggesting that VacA must be presented by *H. pylori* cells to increase apoptosis.

Coculture of AGS cells with the *cagA*$^-$ derivative and the wild-type strain did not result in significant differences in cell cycle events (data not shown), consistent with previous reports (29). In contrast to the parental 60190 strain, deletion of *picB* did not induce any significant alterations in G_1 to G_2-M transition at 6 h (Fig. 5*A*) and decreased the sub-G_1 AGS cell population at 24 h (Fig. 5*A*). Nucleosomal release in AGS cell lysates at 24 h was significantly lower in samples incubated with the *picB*$^-$ strains (absorbance, 0.40 $\pm$ 0.04) compared with the wild-type strain (0.67 $\pm$ 0.08; $P = 0.005$), confirming the decrease in apoptosis. At the 48- and 72-h time points, the parental strain and its *picB*$^-$ mutant induced similar effects (Fig. 5*A*). In total, these findings indicate that *vacA* and *picB* products contribute to cell cycle progression and apoptosis in AGS cells.

Effect of Inactivating Both *vacA* and *picB* in the Same Strain on AGS Cell Cycle Events. The results described above indicate that loss of *vacA* and *picB* may have independent effects on cell cycle progression or apoptosis. To analyze the effect of simultaneous loss of both genes, reciprocal double *H. pylori* mutants (*vacA*::km/*picB*::*cat* and *vacA*::*cat*/*picB*::km) were created in the strain 60190 background, and their ability to modulate AGS cell cycle distribution was determined. In preliminary studies, we cocultured each *vacA*$^-$/*picB*$^-$ strain with AGS cells and measured toxigenicity of supernatants for HeLa cells to confirm that *vacA* expression had been inactivated. The *vacA*$^-$/*picB*$^-$ derivatives failed to induce cytoplasmic vacuolation, in contrast to the wild-type strain and the isogenic *picB*$^-$ mutants (data not shown), indicating that toxin production was successfully interrupted. Data from flow cytometry and DNA fragmentation experiments were similar for experiments involving both double mutants; therefore, results for both mutants were combined. The *vacA*$^-$/*picB*$^-$ derivatives did not induce a significant transition into G_2-M at 6 h and significantly ($P = 0.01$ at 24–72 h) decreased the sub-G_1 population of AGS cells compared with the wild-type strain (Fig. 5*A*). To confirm that the reduced population of sub-G_1 cells reflected cells undergoing apoptosis, DNA fragmentation was quantitated in AGS cell lysates after coincubation with strain 60190 or the double mutant strains. Compared with wild-type (absorbance, 0.67 $\pm$ 0.08 and 1.5 $\pm$ 0.1 at 24 and 48 h, respectively), the *vacA*$^-$/*picB*$^-$ strains significantly attenuated nucleosomal release (absorbance, 0.15 $\pm$ 0.01 and 1.1 $\pm$ 0.1 at 24 and 48 h, respectively; $P \leq 0.02$ for each time point *versus* 60190), findings consistent with results from the flow cytometry experiments. However, the *vacA*$^-$/*picB*$^-$ derivatives still caused detectable apoptosis, suggesting that gene products in addition to *vacA* or *picB* may affect gastric epithelial cell cycle events.

Effect of *vacA* and *picB* Inactivation on AGS Gastric Epithelial Cell Viability. Because *vacA* and *picB* products had independent effects on cell cycle progression and/or apoptosis, we next examined the effect of strains harboring either or both of these mutations on the viability of AGS cells (Fig. 5*B*). As expected, coincubation with strain 60190 significantly reduced AGS cell survival at all time points compared with AGS cells in medium alone ($P \leq 0.002$ for each time point), a pattern consistent with our earlier results (Fig. 1). At 24 and 48 h, the parental strain caused significantly greater loss of viability than the *picB*$^-$ null mutant ($P \leq 0.05$), but these differences disappeared by 72 h. The parental strain caused significantly greater loss of viability at 48 and 72 h compared with the *vacA*$^-$ null mutant ($P \leq 0.02$ *versus* 60190). The *vacA*$^-$/*picB*$^-$ double mutant caused significantly less cell death than the parental strain at each time point ($P \leq 0.0001$). In total, these data indicate that expression of *vacA* and *picB* together are necessary for the maximal *H. pylori*-induced modulation of AGS cell cycle progression, consistent with the flow cytometry and DNA fragmentation results.

DISCUSSION

Apoptosis is a normal component of epithelial cell turnover in the gastrointestinal tract, but the effect of *H. pylori* on gastric cell cycle events is not uniform. Carriage of *H. pylori* is associated with either increased or reduced rates of gastric mucosal proliferation and apoptosis, depending on the study (20–27). *H. pylori* strain-specific characteristics may contribute to this heterogeneity, as we have reported that strains that possess the *cag* island and produce a functional cytotoxin are associated with increased epithelial cell proliferation but reduced apoptosis (22). However, other studies have found no differences in levels of epithelial cell growth and loss among *H. pylori*-colonized persons harboring *cag*$^+$ or *cag*$^-$ strains (24). One explanation for these discrepant findings is that host differences may contribute to cell turnover variation in persons carrying *H. pylori*. Binding of class II MHC molecules by *H. pylori* induces gastric epithelial cell apoptosis (36). Signaling pathways differ after engagement of bacterial superantigens to class II MHC molecules, depending on host haplotype (36), and thus variations in the apoptotic response to *H. pylori* may reflect heterogeneity of MHC genotypes. Inflammatory mediators present in *H. pylori*-colonized mucosa also may influence rates of epithelial cell growth and loss. For example, tumor necrosis factor α, a proinflammatory cytokine present in *H. pylori*-infected mucosa, can inhibit apoptosis in certain cell types (44–46). Another possible mechanism is that certain *H. pylori* strains express proteins that can directly affect cell cycle events. By using an *in vitro* model system, we have identified specific components that may vary the ability of *H. pylori* strains to modulate gastric epithelial cell cycle events, which may explain in part the heterogeneity in cell growth and death rates reported previously in *H. pylori*-colonized persons (20–27).

Because *cag*$^+$ and *cag*$^-$ *H. pylori* strains are associated with differing risks for development of distal gastric cancer (14–16), we first compared the ability of clinical *cag*$^+$ and *cag*$^-$ isolates to alter gastric epithelial cell cycle events *in vitro*. Our results demonstrate that a majority of *cag*$^+$, but not *cag*$^-$, strains reduce AGS cell viability, induce a transient accumulation of cells in G_2-M, and increase apoptosis by 72 h. These effects were not seen when cells were incubated with *E. coli*, *S. flexneri*, or *C. jejuni*, which is consistent with the previous demonstration that *C. jejuni* does not alter gastric epithelial cell viability or apoptosis *in vitro* (28, 47), indicating that the response of AGS cells to *H. pylori* strains was specific. In contrast, Wagner *et al.* (28) reported no difference in the ability of *H. pylori* to alter proliferation or apoptosis *in vitro* when strains were analyzed by *cagA* status and toxin production. These discordant findings may be attributable to differences in study design. Wagner *et al.* (28) used different gastric epithelial cell lines to assess apoptosis and proliferation, and the number of *in vitro* passages for the *H. pylori* strains examined was not reported. We cocultured minimally passaged *H. pylori* strains with asynchronously grown AGS cells so as to recapitulate events occurring within actively replicating gastric mucosa. We examined a larger number ($n = 21$) of clinical isolates and analyzed the effect on cell cycle, not only by segregating strains on the basis of *cagA* genotype and toxigenicity, but also by *vacA* genotype. This characterization of strain-specific modulation of cell cycle events and apoptosis facilitated the identification of specific bacterial components that regulated these events. We also found that *H. pylori*-

induced apoptosis occurred relatively late after coculture (by 72 h), in contrast to previous studies examining the effects of *H. pylori* on epithelial cell cycle events *in vitro* (29, 33). However, we studied *in vivo*-adapted *H. pylori* clinical strains that may differ from *in vitro*-passaged reference strains used by other investigators (29, 33). Consistent with this hypothesis, apoptosis was more rapidly induced in our system when reference strain 60190 was used, indicating that *in vivo* selection may attenuate or delay the apoptotic response to *H. pylori in vitro*.

Analysis of a *picB*$^-$ null mutant *H. pylori* strain indicated that expression of the *picB* product contributes to AGS cell cycle progression and to an apoptotic response. The predicted protein product of *picB* shares homology with the *Bordetella pertussis* toxin secretion protein (PtlC) and the VirB4 protein from *Agrobacterium tumefaciens,* both of which are involved in bacterial type IV secretion pathways (8). The current finding that *picB* is a necessary component in the *H. pylori*-induced apoptotic response is consistent with earlier data that demonstrated that *picB,* as well as other genes within the *cag* island, were necessary for induction of interleukin 8 from gastric epithelial cells *in vitro* (11). Analysis of a *vacA*$^-$ null mutant strain indicated that *vacA* expression also contributes to the apoptotic response, but at a later time point. *H. pylori* VacA induces vacuolation in various types of eukaryotic cells, and although the mechanism by which VacA exerts its cytotoxic effect is not completely elucidated, it is believed to involve disruption of intracellular membrane trafficking at the level of the late endosome as well as pore formation in cellular membranes (48–50). In contrast to vacuolation, induction of apoptosis in this study could not be reconstituted when the 60190 *vacA*$^-$ derivative was incubated with supernatant containing VacA derived from the wild-type 60190 strain. Similarly, purified supernatant from wild-type 60190 alone only slightly increased apoptosis in AGS cells. It is possible that higher concentrations of VacA-containing supernatant than those used in our study (10 μg/ml) might have significantly increased apoptosis, similar to results reported by Rudi *et al.* (35). However, the absence of apoptosis in the setting of vacuolation also suggests that cell vacuolation may not be related to enhanced apoptosis. A potential explanation for these findings is that VacA may need to be specifically presented by the *H. pylori* cell to interact with a signal transduction pathway that stimulates apoptosis. Another possibility is that induction of apoptosis is dependent on the amount of VacA present intracellularly. For example, *Staphylococcus aureus* α-toxin only induces apoptosis at low concentrations ($<$300 nM; Ref. 51). Finally, the ability of VacA to stimulate apoptosis may depend on environmental conditions, because pH variations can alter VacA structure (52).

The results of the experiments focusing on *picB* and *vacA* raise the hypothesis that their gene products may have complementary effects on AGS cell cycle events, because inactivation of both genes together reconstituted the phenotype observed when AGS cells were cocultured with clinical *cag*$^-$ strains. In addition, inactivation of *picB* predominantly modulated cell cycle events early after coincubation with *H. pylori*, whereas loss of *vacA* had effects later during the time course. These complementary effects are consistent with the linkage dysequilibrium reported previously for *vacA* alleles and presence of the *cag* island (18) and may indicate natural selection based on related interactions with host epithelial cells. However, coincubation of AGS cells with the *vacA*$^-$/*picB*$^-$ double mutant strains still resulted in apoptosis, at levels similar to those produced by *cag*$^-$ wild-type isolates, suggesting that *H. pylori* determinants independent of the *cag* island or of *vacA* may affect epithelial cell cycle events. The recent observation that urease, a highly conserved *H. pylori* constituent, can induce gastric epithelial cell apoptosis *in vitro* by binding to MHC class II molecules (53) is consistent with this hypothesis. Thus, *H. pylori* appears to possess a repertoire of both conserved and nonconserved activation determinants that are capable of affecting epithelial cell growth and death *in vitro*.

Expression of p53 can inhibit cell growth and/or induce apoptosis, depending on the cell type and growth conditions, and p53 overexpression has been demonstrated in gastric tissue from *H. pylori*-colonized children compared with children without the organism (23). Because the majority of *cag*$^+$ strains in the current study increased apoptosis, whereas *cag*$^-$ strains induced G_1 arrest, we investigated whether these alterations were mediated by p53. However, levels of p53, p21, and MDM2 were essentially unchanged, and even slightly reduced, after coculture with either *cag*$^+$ or *cag*$^-$ strains, findings that are consistent with previous reports (33). The uniformity of responses among all strains tested, combined with the fact that these findings are opposite to the corresponding alterations in cell cycle induced by the same strains (apoptosis from *cag*$^+$ strains and G_1 arrest from *cag*$^-$ strains), indicates that modulation of the epithelial cell cycle by *H. pylori* is not dependent upon induction of p53. Furthermore, these results suggest that *in vivo* elevations of p53 previously demonstrated (23) may result from mediators present in concomitantly inflamed gastric tissue, and not from a direct *H. pylori* effect. Consistent with the current data, other investigators have reported that signaling pathways independent of p53 may be involved in *H. pylori*-induced apoptosis (28, 29, 35, 47). For example, *H. pylori*-induced apoptosis in certain gastric cells appears to be mediated by activation of the CD95 (APO-1/Fas) receptor and ligand system (28, 35, 47). In addition, induction of apoptosis in AGS cells after incubation with *H. pylori* is accompanied by increased expression of the proapoptotic protein Bak (29). In total, the literature and our current data suggest that *H. pylori*-induced apoptosis is mediated by multiple eukaryotic signaling cascades that are not dependent upon increased p53 levels.

In conclusion, we have demonstrated that the diminished AGS cell viability, progression to G_2-M, and apoptosis associated with coincubation with particular *cag*$^+$ *H. pylori* strains are mediated in part by expression of *vacA* and by genes in the *cag* island. These results suggest that differential expression of *H. pylori* genes *in vivo* may in part explain heterogeneity in levels of gastric mucosal cell proliferation and apoptosis.

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