Helicobacter pylori Activates the Cyclin D1 Gene through Mitogen-Activated Protein Kinase Pathway in Gastric Cancer Cells

YOSHIHIRO HIRATA,* SHIN MAEDA, YUZO MITSUNO, MASAO AKANUMA, YUTAKA YAMAJI, KEIJI OGURA, HARUHIKO YOSHIDA, YASUSHI SHIRATORI, AND MASAO OMATA

Department of Gastroenterology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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Helicobacter pylori induces cellular proliferation in host cells, but the mechanism remains unclear. Thus, we examined the effect of H. pylori on cyclin D1, an important regulator of the cell cycle, especially in relation to intracellular signaling pathways. In a Northern blot analysis, cyclin D1 transcription in gastric cancer (AGS) cells was enhanced by coculture with H. pylori strain TN2 in a time-dependent and multiplicity-of-infection-dependent manner. An isogenic mutant form of vacA also increased cyclin D1 transcription, but mutant forms of cagE or the entire cag pathogenicity island did not enhance cyclin D1 transcription. These effects were confirmed with a luciferase assay of the cyclin D1 promoter (pD1luc). Cyclin D1 promoter activation by H. pylori was inhibited by MEK inhibitors (U0126 and PD98059), indicating that the mitogen-activated protein kinase pathway may be involved in intracellular signal transduction. In contrast, transfection of a reporter plasmid having any point mutations of the NF-kB binding sites in the promoter (pD1-kB1M, pD1-kB2M, or pD1-kB1/2M) or cotransfection of dominant negative IκBα did not affect cyclin D1 activation by H. pylori. In conclusion, H. pylori activates cyclin D1 through the mitogen-activated protein kinase pathway and not through NF-kB activation in AGS cells. This activation of cyclin D1 is partly dependent on the cag pathogenicity island but not on vacA.

Colonization of the human gastric mucosa by Helicobacter pylori induces various diseases, such as atrophic gastritis, peptic ulcer diseases, and gastric adenocarcinoma (12, 21, 23, 28, 30, 34). It has been recently demonstrated that H. pylori affects intracellular signal conduction in host cells, leading to the activation of transcriptional factors (18, 19, 22, 24, 25, 42) and the induction of proinflammatory cytokines (8, 29, 32, 41). The cag pathogenicity island (PAI) genes of H. pylori and their products are responsible for the bacterium-host interactions, including activation of the NF-κB and mitogen-activated protein (MAP) kinase pathways (19, 22, 24, 25, 42). The cag PAI genes have been suggested as the cause of gastric diseases in vivo (3, 6, 21), and we confirmed this in the Mongolian gerbil model (33).

H. pylori infection is also associated with enhanced cellular proliferation of host cells (9, 14, 16, 36, 37); however, the mechanism of cellular proliferation induced by H. pylori infection remains unclear. In mammalian cells, cellular proliferation is regulated in a cell cycle governed by the sequential formation and degradation of cyclins and cyclin-dependent kinases. Among various cyclins, cyclin D1 regulates passage through the restriction point and entry into the S phase (43). Furthermore, cyclin D1 overexpression shortens the G1 phase and increases the rate of cellular proliferation (15, 38–40). Various factors, such as the MAP kinase cascade (20), NF-κB (13), and the Wnt signal (44, 47), are known regulators of cyclin D1 expression. In addition, some of these factors are already known to be activated by H. pylori infection; however, little is known about the effect of H. pylori infection on cyclin D1 expression. Thus, in this study, we tried to elucidate the mechanism of host cell proliferation caused by H. pylori in relation to cyclin D1 transcription.

MATERIALS AND METHODS

Cell culture. Human gastric adenocarcinoma cell line AGS cells were maintained in Ham’s F12 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, N.Y.) in an incubator with 5% CO2.

Bacterial strains and growth conditions. H. pylori strain TN2 possessing both the cag PAI and vacA was generously donated by M. Nakao (Takeda Chemical Industries, Ltd., Osaka, Japan). Infection with this strain induced gastric cancer in Mongolian gerbils (49). The isogenic mutants cagE-disrupted TN2-DcagE and vacA-disrupted TN2-DvacA were prepared as described previously (22, 32). TN2-DPAI, a strain in which all of the PAI genes are disrupted, was prepared as follows. A partial fragment of the cagE gene (700 bp) was amplified by PCR and cloned into the plasmid vector pCRII (Invitrogen, San Diego, Calif.). A 700-bp fragment of the cagE gene, which is localized in cagPAI, was also amplified and cloned into pCRII containing a cagE fragment at the KpnI site oriented in the same direction. A kanamycin resistance gene cassette was then inserted into the vector at the BamHI site between cagA and the cag5 fragment. The resulting construct was transferred into parental H. pylori cells (strain TN2) by electroporation. After selection by kanamycin resistance and Southern blot hybridization to confirm the disruption of the genes, a clone was selected for use as TN2-DPAI.

These strains were grown in brucella broth with 5% (vol/vol) fetal bovine serum under microaerobic conditions (Campy-Pak Systems; BBL, Cockeysville, Md.), diluted to the desired multiplicity of infection (MOI), and then used for experiments. The number of bacterial cells in the suspension was quantified by optical density measurements.

Heat-killed H. pylori was prepared by boiling the bacteria for 30 min. H. pylori filtrate was prepared by suspending the bacteria in antibiotic-free medium for 30 min, pelleting the bacteria by centrifugation, and then filtering the medium through a 0.22-μm-pore-size filter (Nihon Millipore Ltd., Tokyo, Japan).

Plasmids. Cyclin D1 promoter-containing construct pD1luc and vectors pD1-kB1M, pD1-kB2M, and pD1-kB1/2M, possessing mutations in their NF-kB binding sites, were kindly donated by M. Strauss ( Humboldt Universität, Berlin, Germany). Plasmid pD1luc is consistent with an EcoRI/PstI fragment (1,226 bp) of the cyclin D1 promoter. pD1-kB1M possesses mutations in the NF-kB binding site between nucleotides -840 and -831, pD1-kB2M has mutations in...
the NF-κB binding site between nucleotides −33 and −24, and pD1-luc/2M possesses both of these mutations (13, 27). The dominant-negative IκBα expression vector mutant (SS32/36AA) was generously donated by H. Suzuki (Yamanouchi Pharmaceutical Co., Ltd., Ibaraki, Japan) (46).

Northern blot analysis. AGS cells were serum starved for 72 h and subsequently cultured with H. pylori at an MOI of 10 to 300. Cells were harvested at the times indicated, and total RNA was isolated by using Isogen (Wako, Osaka, Japan) in accordance with the manufacturer’s instructions. Fifteen micrograms of total RNA was loaded onto a 1% agarose-formaldehyde gel, separated by electrophoresis, and then transferred onto a Hybond N membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Partial cDNA (200 bp) for cyclin D1 was produced by PCR using primers 5′-ATGGAACACAGCTCTCTGTG-3′ (forward) and 5′-ACCTCCAGCATCC AGTGGCC-3′ (reverse). The DNA sequence of the product was confirmed by using a cycle DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, Calif.). This cyclin D1 probe was labeled with an AlkPhos Direct Labelling Module (Amersham Pharmacia Biotech) used in accordance with the manufacturer’s instructions and then hybridized with the membrane for 12 h. After several washes of the membrane, cyclin D1 mRNA was detected by CDP-Star Detection Reagent (Amersham Pharmacia Biotech), followed by analysis using the LASi1000 imaging system (Fuji Photo Film Co., Ltd., Tokyo, Japan).

After the hybridization, the membrane was reprobed with the human gene for glyceraldehyde 3-phosphate dehydrogenase to control for equal loading of the RNA.

Transfections and luciferase assays. AGS cells (1.5 × 10⁵) were seeded into six-well plates and transfected 24 h later with 200 ng of pD1luc or the mutant vector and the indicated expression vectors using FuGENE 6 Transfection Reagent (Roche Diagnostic Corporation, Indianapolis, Ind.). Ten nanograms of Renilla luciferase vector (Promega, Madison, Wis.) was included in each sample for standardization of transfection efficiency. The total DNA amount was kept constant by supplementation with empty vectors. After 24 h, H. pylori (strain TN2 or an isogenic mutant) was added at an MOI of 10 to 100 and incubated for the indicated times. 12-O-Tetradecanoylphorbol-13-acetate (TPA) (5 to 50 ng/ml) was used as a positive control. When evaluating the effect of the MAP kinase signaling pathway on activation of the cyclin D1 promoter, the specific MEK inhibitor PD98059 (Wako) or U0126 (Promega) (10) or vehicle (dimethyl sulfoxide) alone was added 2 h before coculture with H. pylori. The cells were harvested and washed with phosphate-buffered saline and lysed in a luciferase lysis buffer (Piccagene; Toyo Ink, Tokyo, Japan). The lysates were assayed for luciferase and seapansy luciferase activity with a luminometer. After standardization buffer (Piccagene; Toyo Ink, Tokyo, Japan). The lysates were assayed for luciferase and seapansy luciferase activity with a luminometer. After standardization buffer (Piccagene; Toyo Ink, Tokyo, Japan). The lysates were assayed for luciferase and seapansy luciferase activity with a luminometer. After standardization buffer (Piccagene; Toyo Ink, Tokyo, Japan).

RESULTS

H. pylori increases transcription of the cyclin D1 gene in gastric cancer cells in a time- and MOI-dependent manner. When serum-starved AGS cells were cocultured with H. pylori strain TN2 at an MOI of 100, the amount of cyclin D1 mRNA was markedly increased from 60 to 120 min, as observed by Northern blot analysis (Fig. 1A). A similar increase in the cyclin D1 gene was observed when AGS cells were incubated with H. pylori at different MOIs. H. pylori increased transcription of the cyclin D1 gene in an MOI-dependent manner up to an MOI of 100 (Fig. 1B).

In the reporter assay using pD1luc, cyclin D1 promoter activity was enhanced in cells cocultured with H. pylori (Fig. 1C) in a time-dependent manner, in the same way as in cells...
treated with TPA at 50 ng/ml (Fig. 1D). Cyclin D1 promoter activity was increased 1.4-fold by 8 h, 3.0-fold by 16 h, and 6.4-fold by 24 h of incubation with \textit{H. pylori}, compared with that of an uninfected control. MOI-dependent transactivation was also observed in cells cocultured with \textit{H. pylori} for 24 h (Fig. 1E), as was observed in cells treated with TPA (Fig. 1F). This activation was observed only when live bacteria were added to the cells. Neither heat-killed bacteria nor bacterial filtrate activated the cyclin D1 promoter (Fig. 1G). Subsequent reporter assays were performed with cells cocultured with live \textit{H. pylori} at an MOI of 100 for 24 h.

Effects of virulence factors on cyclin D1 transcription in AGS cells. To assess the relationship between the virulence genes of \textit{H. pylori} and cyclin D1 activation, AGS cells were cultured with isogenic mutant strain TN2-\textit{ΔcagE}, TN2-\textit{ΔvacA}, or TN2-\textit{ΔPAI} at an MOI of 100. Northern blot analysis showed that the induction of cyclin D1 mRNA by TN2-\textit{ΔcagE} and TN2-\textit{ΔPAI} was significantly less than that by wild-type TN2; however, disruption of the \textit{vacA} gene did not affect cyclin D1 induction (Fig. 2A).

A luciferase reporter assay showed that TN2-\textit{ΔcagE} and TN2-\textit{ΔPAI} induced 4.5- and 3.7-fold increases in cyclin D1 promoter activity, respectively. The induction by TN2-\textit{ΔPAI} was significantly less than that by wild-type TN2 \((P < 0.05)\). In contrast, TN2-\textit{ΔvacA} induced a 6.7-fold increase in cyclin D1 promoter activity, a value similar to that of wild-type TN2 (Fig. 2B).

MAP kinase activation is required for induction of cyclin D1 by \textit{H. pylori}. Since cyclin D1 expression is regulated by MAP kinase cascades, we examined whether the MAP kinase cascade is involved in \textit{H. pylori}-induced cyclin D1 transcription by using the specific MEK inhibitors U0126 and PD98059. U0126 (5 to 20 \textmu M) and PD98059 (50 to 200 \textmu M) were added to the culture medium of AGS cells 2 h before coculture with \textit{H. pylori}. Activation of the cyclin D1 promoter by \textit{H. pylori} was markedly inhibited by each compound in a dose-dependent manner (Fig. 3A and B), as was observed in the cells treated with TPA (data not shown). U0126, at a dose of 20 \textmu M, reduced cyclin D1 promoter activation to 34\% \((P < 0.05)\), and PD98059, at a dose of 200 \textmu M, reduced promoter activation to 21\% \((P < 0.05)\). With the Northern blot analysis, preincubation of the cells with U0126 or PD98059 reduced cyclin D1 mRNA induction by coculture with \textit{H. pylori} (Fig. 3C).

Effect of NF-κB activation on \textit{H. pylori}-induced cyclin D1 promoter transactivation. We examined the role of NF-κB activation on \textit{H. pylori}-induced cyclin D1 transcription since FIG. 3. Role of MAP kinase activation in the induction of cyclin D1 promoter by \textit{H. pylori}. AGS cells transfected with pD1luc were infected with \textit{H. pylori} at an MOI of 100 or left uninfected in the presence of the MEK inhibitor U0126 (A) or PD98059 (B) or dimethyl sulfoxide (DMSO) (control). Luciferase activity is presented as a relative ratio against the basal level measured in untreated control cells. The values shown are the means \pm the standard deviations of more than three independent experiments. *, \(P < 0.05\) versus \textit{H. pylori} with DMSO preincubation. (C) Serum-starved AGS cells were infected with \textit{H. pylori} at an MOI of 100 for 90 min in the presence of 20 \textmu M U0126, 200 \textmu M PD98059, or DMSO alone. Fifteen micrograms of total RNA was tested for cyclin D1 mRNA by Northern blot analysis. GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
the type 1 strain of *H. pylori* can activate NF-κB and cyclin D1 transcription is also reportedly regulated by NF-κB. We examined the effect of *H. pylori* on the promoter constructs containing point mutations in the κB sites (pD1-κB1M, pD1-κB2M, and pD1-κB1/2M). These mutant reporters were transfected into AGS cells instead of pD1luc, and luciferase expression induced by *H. pylori* was measured. Coculture with *H. pylori* caused about a sixfold increase in the activation of luciferase expression on each promoter construct (Fig. 4A).

In addition, we cotransfected the dominant negative form of IκBα (SS32/36AA) with pD1luc to inhibit NF-κB activation by *H. pylori*. Although activation of NF-κB was completely inhibited by this dominant-negative form of IκBα (22), induction of the cyclin D1 promoter was not reduced by the mutant of IκBα (Fig. 4B). These data indicate that *H. pylori* transactivates the cyclin D1 promoter independently of NF-κB activation in AGS cells.

**DISCUSSION**

In this study, we demonstrated that live *H. pylori* transactivates cyclin D1, one of the critical regulators of the cell cycle, in AGS cells in a time- and dose-dependent manner. Although many studies have revealed that *H. pylori* induces cellular proliferation (9, 14, 16, 36, 37), the underlying mechanism has not yet been clarified. Our result indicating that *H. pylori* directly affects the cell cycle regulator molecule represents one possible reason for cellular proliferation. Furthermore, overexpression of cyclin D1 has been demonstrated to contribute to the oncogenic transformation of cells in vitro and in vivo (1, 4, 11, 26, 48, 50). Although little is known about the relationship between cyclin D1 and gastric cancer, transactivation of cyclin D1 and an accelerated cell cycle caused by *H. pylori* infection can be among the factors contributing to malignant transformation.

We also demonstrated the effects of virulence genes of *H. pylori* on cyclin D1 activation. In a Northern blot analysis, TN2-ΔcagE and TN2-ΔPAI exerted weaker effects on *H. pylori*-induced cyclin D1 activation compared with wild-type TN2, while TN2-ΔvacA induced cyclin D1 transcription at a level similar to that of the wild type. In the reporter assay, strains containing all of the cag PAI genes (TN2 and TN2-ΔvacA) activated the promoter more strongly than did strains partially or completely lacking cag PAI genes (TN2-ΔcagE and TN2-ΔPAI). Although we did not evaluate the cell cycle directly, these data may be compatible with a previous report by Peek et al. which demonstrated that *H. pylori* possessing cag PAI accelerated the progression of the cell cycle from G1 into G2-M in AGS cells at 6 h (37). We have recently demonstrated in the Mongolian gerbil model that TN2-ΔcagE did not induce any severe gastric diseases, in contrast to wild-type TN2 and TN2-ΔvacA, in spite of the similar colonization abilities (33). Together with other host responses such as transcriptional factor activation (18, 19, 22, 24, 25, 42) and apoptosis (17, 37), which can be enhanced by cag PAI-positive *H. pylori*, accelerated cell proliferation may provide one of the reasons for the high prevalence of gastric cancer in patients suffering from cag PAI-positive *H. pylori* (3, 21, 35).

It is not known, however, how cag PAI genes contribute to the differences in host response. Possibly only cag PAI-positive strains transport certain molecules of *H. pylori*, which directly activate intracellular signal transduction. Some of the cag PAI genes and their products have been thought to form a type IV secretion system (5, 7). Very recently, CagA protein was reported to be transported into AGS cells by this secretion system (5, 7). Very recently, CagA protein was reported to be transported into AGS cells by this secretion system (5, 7).

In the Northern blot analysis, an increased level of cyclin D1 mRNA was observed 60 to 120 min after coculture with *H. pylori*. The time-dependent increase in cyclin D1 mRNA levels was also observed when MKN-28 cells were cocultured with *H. pylori*.

**FIG. 4.** Role of NF-κB activation on *H. pylori*-induced cyclin D1 promoter transactivation. (A) AGS cells transiently transfected with 200 ng of pD1luc or a mutant reporter (pD1-κB1M, pD1-κB2M, or pD1-κB1/2M) were infected with *H. pylori* at an MOI of 100 for 24 h or left uninfected. The luciferase activity of each reporter is presented as a relative ratio against the basal level measured in uninfected cells. The values shown are the means ± the standard deviations of more than five independent experiments. (B) AGS cells were transfected with various amounts of the dominant-negative IκBα expression vector together with pD1luc for 24 h. The total DNA amount was kept constant by supplementation with an empty vector (pcDNA3). The cells were infected with *H. pylori* at an MOI of 100 for 24 h or left uninfected. Luciferase activity is presented as a relative ratio against the basal level measured in uninfected control cells. The values shown are the means ± the standard deviations of more than five independent experiments.
pneumonia (data not shown). This time lag suggests an involvement of intracellular signal transduction. Cyclin D1 expression is regulated by many signaling cascades, such as mitogen-activated protein kinase cascade, inflammation and NF-κB activation, and Wnt signaling (13, 20, 44, 47). Some of these signaling cascades are involved in H. pylori-mediated host responses. Thus, we determined the intracellular signaling pathway for H. pylori-induced cyclin D1 activation.

The present results showing that MEK inhibitors reduced H. pylori-induced cyclin D1 activation strongly suggest the involvement of MAP kinase cascades. Since the cag PAI mutant strains are known to be less potent in activating the MAP kinase cascades (19, 24, 25), less activation of cyclin D1, as shown by the mutant strains used in this study, is reasonable. We also performed in vitro kinase assays to measure MAP kinase activity, and we confirmed that H. pylori had the ability to activate MAP kinase, and this ability was correlated with the status of cag PAI (data not shown). On the other hand, the use of a mutant expression vector in the present study showed that NF-κB activation is not required for cyclin D1 transactivation in AGS cells.

In conclusion, we have demonstrated that H. pylori activates cyclin D1 expression and that this activation is partly dependent on the cag PAI genes. Furthermore, cyclin D1 expression was activated through the MAP kinase signaling pathway but not by activation of NF-κB. It is possible that other signaling pathways which can be induced by H. pylori exist, as well as another mechanism for cyclin D1 transactivation. These possibilities should be investigated in future studies.

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