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Induction of Cyclooxygenase-2 Overexpression in Human Gastric Epithelial Cells by *Helicobacter pylori* Involves TLR2/TLR9 and c-Src-Dependent Nuclear Factor-κB Activation

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ABSTRACT

Gastric epithelial cells were incubated with a panel of clinical isolates of *Helicobacter pylori*, including nonulcer dyspepsia with gastritis (HS, $n = 20$), gastric ulcer (HU, $n = 20$), duodenal ulcer (HD, $n = 21$), and gastric cancer (HC, $n = 20$). HC strains induced a higher cyclooxygenase-2 (COX-2) expression than those from HS, HD, and HU. The bacterial virulence factors and the host cellular pathways were investigated. Virulence genes of *iceA, vacA, babA2, cagA 3′* repeat region, and *hrgA* failed to show any association with the disease status and COX-2 expression. Methylation-specific polymerase chain reaction revealed HC strains not affecting the methylation status of COX-2 promoter. Nuclear factor (NF)-κB, NF-interleukin 6, and cAMP response element were found to be involved in COX-2 induction. We explored a novel NF-κB activation pathway. The mutants of TLR2 and TLR9, but not TLR4, inhibited *H. pylori*-induced COX-2 promoter activity, and neutralizing antibodies for TLR2 and TLR9 abolished *H. pylori*-induced COX-2 expression. Phosphatidylinositol-specific phospholipase C (PI-PLC), protein kinase C (PKC), and Src inhibitors inhibited COX-2 induction. The dominant-negative mutants of NIK and various IκB kinase complexes, including IKKβ (Y188F), IKKβ (Y199F), and IKKβ (FF), inhibited the COX-2 promoter activity. Phosphorylation of GST-IKKβ (132–206) at Tyr^{188} and Tyr^{199} by c-Src was found after *H. pylori* infection. In summary, *H. pylori* induces COX-2 expression via activations of NF-κB, NF-interleukin 6, the cAMP response element. In NF-κB activation, *H. pylori* acts through TLR2/TLR9 to activate both the cascade of PI-PLCγ/PKCα/c-Src/IKKα/β and the cascade of NIK/IKKα/β, resulting in the IκBα degradation and the expression of COX-2 gene. The COX-2 overexpression may contribute to the carcinogenesis in patients colonized with these strains.

Helicobacter pylori has been identified as a major pathogen leading to the development of a wide range of gastroduodenal diseases (Passaro et al., 2002). However, only a small portion of infected patients suffered from the more severe gastric pathological conditions, such as gastric malignancy (Peek and Blaser, 2002). Evidence has emerged that the inappropriate inflammation of gastric mucosa would dictate the clinical outcomes after exposure to *H. pylori* (Bodger and Crabtree, 1998). Clinical outcomes associated with *H. pylori* infection include gastritis, duodenal ulcer, gastric ulcer, gas-

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ABBREVIATIONS: GC, gastric cancer; COX, cyclooxygenase; NF, nuclear factor; IL, interleukin; CRE, cAMP response element; TLR, Toll-like receptor; LPS, lipopolysaccharide; ICAM, intercellular adhesion molecule; FCS, fetal calf serum; U73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5- pyrrolidine-dione; Ro 31-8220, 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide (Bisindolylmaleimide IX), methanesulfonate; HC, *H. pylori* isolate from gastric cancer; HU, *H. pylori* isolate from gastric ulcer; HD, *H. pylori* isolate from duodenal ulcer; HS, *H. pylori* isolate from gastritis; PBS, phosphate-buffered saline; m.o.i., multiplicity of infection; Ab, antibody; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; wt, wild type; PI-PLC phosphoinositide-specific phospholipase C; PKC, protein kinase C; TNF, tumor necrosis factor; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP response element-binding protein; KBM, κB site mutation; ILM, NF-IL6 site mutation; CRM, CRE site mutation; P/H, proline→histidine; ICD, Intracellular domain deletion; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; K/R, lysine→arginine; SH2(N), arginine564→alanine; GST, glutathione *S*-transferase; bp, base pair(s).

tric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (Parsonnet et al., 1991). Both host factors and the characteristics of infecting strains have been postulated to contribute to the variable outcome and have been the focus of intensive investigations (Blaser, 2002). The reported strain-specific virulence factors for gastric cancer (GC) include CagA, VacA, IceA, BabA2, and HrgA (Yamaoka et al., 1998; Kidd et al., 2001; Nogueira et al., 2001; Prinz et al., 2001; Ando et al., 2002; Bravo et al., 2002).

Cyclooxygenase (COX)-2 is the key enzyme responsible for the prostaglandin production during gastric inflammation and ulcer healing (Jackson et al., 2000). The overexpression of COX-2 has been implicated in the development and progression of GC (van Rees et al., 2002). *H. pylori* infection is the most important factor for the induction of COX-2 in the stomach. Both animal studies and human samples have confirmed that *H. pylori*-induced inflammation is linked to COX-2 expression (Takahashi et al., 2000). The degree of COX-2 expression is strongly correlated with the extent of inflammation and the severity of gastric disease (Sung et al., 2000). In addition, levels of the COX-2 protein were significantly higher in patients suffering from gastric cancer who were infected with *H. pylori* than in those with nonulcer dyspepsia (Wambura et al., 2002), and the up-regulation of COX-2 in *H. pylori*-associated GC is related to vascular invasion (Chen et al., 2001). Therefore, COX-2 expression was assumed to play a crucial role in *H. pylori*-associated GC in addition to gastric inflammation. Recent in vitro studies demonstrated the modulation of COX-2 expression by *H. pylori* (Romano et al., 1998). It is of interest to investigate whether *H. pylori* isolates from patients with different disease status vary in their capabilities to induce COX-2 expression, because such data are limited. Furthermore, the roles of *hrgA, iceA, vacA, babA2*, and *cagA* genotypes in relation to COX-2 expression and disease status are also determined.

The induction of COX-2 expression requires the de novo mRNA and protein synthesis (Kosaka et al., 1994), indicating the regulation at transcriptional level. The promoter region of human COX-2 gene has been cloned and sequenced and has been shown to contain the putative recognition sequences for several transcriptional factors, including two NF-κB sites, a nuclear factor for IL-6 expression (NF-IL6)/C/EBP, an activator protein 1, and a cAMP response element (CRE) (Kosaka et al., 1994). Although NF-κB activation by *H. pylori* has been reported previously (Keates et al., 1997), whether it is involved in the *H. pylori*-induced COX-2 expression and whether other transcriptional factor is also activated was not explored. These issues are addressed in the present study.

It has been reported that epithelial cells and macrophages recognize microbial infections via Toll-like receptors (TLRs) (Rock et al., 1998). These receptors are oligospecific and recognize conserved motifs on pathogens. So far, 10 human TLRs have been identified (Rock et al., 1998), and their ligands are beginning to be unraveled. TLR4 recognizes lipopolysaccharide (LPS), which is the major outer membrane component of Gram-negative bacteria (Poltorak et al., 1998). TLR2 is involved in the recognition of Gram-positive bacteria (Takeuchi et al., 1999). TLR9 recognizes bacterial DNA (Hemmi et al., 2000). In this study, the types of TLRs and their role in *H. pylori*-regulated COX-2 expression are evaluated.

The phosphorylations of Ser^{177} and Ser^{181} on IKKβ by the upstream mitogen-activated protein kinase kinase kinase leading to NF-κB activation are well recognized (Malinin et al., 1997). However, our recent studies found the additional phosphorylations of Tyr^{188} and Tyr^{199} by c-Src through PKC activation resulting in COX-2 and ICAM-1 expressions (Huang et al., 2003a,b). The upstream signaling molecule involving in *H. pylori*-induced NF-κB activation is investigated in the present study. Whether the PKC/c-Src/IKKβ pathway is also involved in the *H. pylori*-induced NF-κB activation leading to COX-2 expression is examined to determine whether this pathway exists in different types of cells despite different stimuli.

Materials and Methods

Materials. The rabbit polyclonal antibodies specific to TLR2, TLR4, IκBα, IKKβ, and c-Src and the goat polyclonal antibodies specific to COX-2, COX-1, TLR9, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN). RPMI, fetal calf serum (FCS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Staurosporine was obtained from Sigma (St. Louis, MO). U73122, U73343, and PP2 were obtained from Calbiochem (San Diego, CA). Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Hercules, CA). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA), poly(dI-dC) was from Amersham Biosciences, [γ-^{32}P]ATP (3000 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA), the SuperFect reagent was from QIAGEN (Valencia, CA), and the luciferase assay kit was from Promega (Madison, WI).

Bacterial Strains and Growth Conditions. We studied *H. pylori* strains from patients undergoing gastroscopy for the evaluation of upper gastrointestinal symptoms. At the time of gastroscopy, two biopsy specimens were taken from antrum for bacterial culture. A total of 81 clinical isolates from patients with nonulcer dyspepsia with gastritis (HS, $n = 20$), gastric ulcer (HU, $n = 20$), duodenal ulcer (HD, $n = 21$), and gastric cancer (HC; $n = 20$, 11 from intestinal type and nine from diffuse type) were collected. Columbia agar with 5% sheep blood (Invitrogen) was used for *H. pylori* culture. The bacterial cells were cultured at 37°C in a microaerophilic chamber (Don Whitley, West Yorkshire, England) containing 10% CO_2, 5% O_2, and 85% N_2. Bacterial cells were grown to 48 h on Columbia agar plates, collected, washed with PBS, pH 7.4, and pelleted. Cell pellets were then resuspended in PBS, pH 7.4, and used for infection experiment (Wang et al., 1998).

Cell Culture and *H. pylori* Infection Experiments. The human gastric cancer epithelial cell lines AGS and MKN45 were obtained from the American Type Culture Collection (Manassas, VA) and RIKEN (Saitama, Japan), respectively. Both were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. AGS cells and *H. pylori* were cocultured in antibiotic-free RPMI 1640 supplemented with 10% FCS. Bacteria were resuspended in PBS, pH 7.4, and diluted corresponding to the multiplicity of infection (m.o.i.) at 150:1. Cells were incubated in the absence (controls) or in the presence of bacteria for 16 h.

Preparation of Cell Extracts and Western Blot Analysis. After 16 h incubation with *H. pylori*, AGS or MKN45 cells were rapidly washed with PBS to remove bacteria and then lysed with the ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM NaF, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and 1% Triton X-100). The cell lysate was subjected to SDS-polyacrylamide gel electrophoresis using 10% running gels. The proteins were transferred to the nitrocellulose paper, and the Western blot was performed as described

previously (Huang et al., 2003b). The quantitative data were obtained using a computing densitometer with ImageQuant software and normalized by the actin expression (Amersham Biosciences).

Immunofluorescence Staining. AGS or MKN45 cells grown on coverslips were cocultured for 16 h with *H. pylori* in antibiotic-free growth medium, rapidly washed with PBS, then fixed at room temperature for 30 min with 3.7% paraformaldehyde. After washing with PBS, the cells were blocked for 30 min with 3% bovine serum albumin in Tris-buffered saline-Tween 20 containing 0.1% Triton X-100, then incubated with anti-COX-2 Ab (1:100) for 1 h, washed extensively, and stained for 30 min with anti-goat IgG-fluorescein (1:2000). After further washes, the coverslips were mounted on glass slides using mounting medium (2% *n*-propyl gallate in 60% glycerol and 0.1 M PBS, pH 8). Optical sections of the immunostained cells were observed and photographed using a Zeiss Axiovert inverted microscope equipped with a photoMicroGraph Digitized Integration System (Zeiss, Oberkochen, Germany).

RT-PCR. Total RNA was isolated from AGS cells using TRIzol reagent (Invitrogen). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse-transcribed into cDNA using the oligo-dT primer, then the cDNA was amplified for 30 cycles using two oligonucleotide primers derived from a published COX-2 sequence (5′-CAGCACTTCACGCATCAGTT-3′ and 5′-TCTGGTCAATGGAAGCCTGT-3′) and two oligonucleotide primers from a β-actin sequence (5′-TGAC GGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGGGACGATGGAGGG-3′). For COX-2, a polymerase chain reaction (PCR) cycle consisted of a denaturation step (94°C, 1 min), an annealing step (60°C, 1 min), and an elongation step (72°C, 1.5 min). There were 35 cycles, followed by an additional extension step (72°C, 7 min). For β-actin, PCR cycle was carried out for 30 s at 94°C, 30 s at 65°C, and 1 min at 70°C. The PCR products were subjected to electrophoresis on a 1.5% agarose gel. Quantitative data were obtained using a computing densitometer and ImageQuant software (Amersham Biosciences).

Detection of iceA, cagA 3′ Repeat Region, hrgA, babA2, and vacA Genotypes. PCR with specific primers was used to detect genotypes of *H. pylori* as described previously (Yamaoka et al., 1998; Kidd et al., 2001; Ando et al., 2002; Wang et al., 1998; Sheu et al., 2003). The primers used are shown in Table 1. The amplification condition was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min (for *cagA, hrgA*, and *iceA*). The annealing temperature was changed to 55°C for *vacA* and 45°C for *babA2*. All samples with negative results were tested at least twice. The strains from randomly selected samples with positive PCR results were subjected for sequencing using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

TABLE 1
Primers

ice A (Kidd et al., 2001)	
Forward	5′-GTTGGGTATATCACAATTAT-3′
Reverse	5′-TTACCCTATTTTCTAGTAGGT-3′
CagA (Yamaoka et al., 1998)	
Forward	5′-ACCCTAGTCGGTAATGGGTTA-3′
Reverse	5′-GTAATTGTCTAGTTTCGC-3′
hrgA	
Forward	5′-TCTCGTGAAAGAGAATTTCC-3′
Reverse	5′-TAAGTGTGGGTATATCAATC-3′
hpyIIIR (Ando et al., 2002)	
Forward	5′-CTCATTGCTGTGAGGGAT-3′
Reverse	5′-TCTTGATAGGATCTTGCG-3′
babA2 (Sheu et al., 2003)	
Forward	5′-CCAAACGAAACAAAAAGCGT-3′
Reverse	5′-GCTTGTGTAAAAGCCGTCGT-3′
vacA s1a	
Forward	5′-GTCAGCATCACACCGCAAC-3′
Reverse	5′-CTGCTTGAATGCGCCAAACTTTATC-3′
vacA s2	
Forward	5′-GCTAACACGCCAAATGATCC-3′
Reverse	5′-CTGCTTGAATGCGCCAAACTTTATC-3′
vacA m1	
Forward	5′-GGCCACAATGCAGTCATGG-3′
Reverse	5′-CTCTTAGTGCCTAAAGAAACA-3′
vacA m2 (Wang et al., 1998)	
Forward	5′-GGAGCCCCAGGAAACATTG-3′
Reverse	5′-CATAACTAGCGCCTTGCAC-3′

Methylation Status of the COX-2 Promoter. The methylation status of the COX-2 promoter was determined by methylation-specific PCR as detailed previously (Akhtar et al., 2001). In brief, 1 μg of genomic DNA isolated from AGS cells was bisulfite-modified. PCR was performed with unmethylated primers [5′-ATAGATTATATGGTGGTGGTGGT-3′/5′-CACAATCTTTACCCAAACACTTCC-3′ (171-bp product)] and methylated primers [5′-TTAGATACGGCGGCGGCGGC- 3′/5′-TCTTTACCCGAACGCTTCCG3′ (161-bp product)]. The PCR condition was 30 s at 95°C, 45 s at 65°C, 45 s at 72°C for 35 cycles followed by a final 5-min extension at 72°C.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay. AGS cells were cocultured with *H. pylori* isolates from patients with GC for 30, 60, or 120 min, and then nuclear extracts were prepared as described previously (Huang et al., 2003a). Oligonucleotides corresponding to the downstream κB (5′-GAGTG**GGGACTACCC**CCTC-3′), NF-IL6 (5′-CGGC**TTACGCAAT**TTTT-3′), and CRE (5′-TCAT**TTCGTCA**CATG-3′) consensus sequences in the human COX-2 promoter were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and EMSA was performed as described previously (Huang et al., 2003b). Bold letters represent the binding sequences for transcription factor.

Plasmids. The COX-2 promoter constructs pGS-459/+9, −327/+59, CRM, ILM, or KBM (Luc) were generous gifts from Dr. L. H. Wang (University of Texas, Houston, TX). NF-κB luciferase reporter (κB-Luc) was from Stratagene (La Jolla, CA). The PLC-γ2 wild type and the mutant SH2(N), in which the Arg at position 564 is replaced by Ala, and the PKC-α constitutively active mutant (PKC-α AE) and the PKC-α dominant-negative mutant (PKCα/KR) were gifts from Drs. T. Kurosaki (Kansai Medical University, Japan) and A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA) respectively. The dominant-negative mutants of NIK (KKAA), IKKα (KM), and IKKβ (KM) were gifts from Signal Pharmaceuticals (San Diego, CA). The dominant-negative mutant of IKKβ (AA) was from Dr. Karin (University of California San Diego, San Diego, CA). pGEX-IκB$\alpha\alpha$ (1–100) was a gift from Dr. Nakano (University of Juntendo, Tokyo, Japan). pGEX-IKKβ (132–206) was a gift from Dr. Nakanishi (University of Nagoya, Nagoya, Japan). TLR2 and TLR4 plasmids, including wt TLR2, wt TLR4, mutants of TLR2 (P/H) and TLR4 (P/H) were as described previously (Muta and Takeshige, 2001). TLR9 plasmids including wt TLR9 and mutant of TLR9 (ICD) from Ken J. Ishii (United States Food and Drug Administration, Bethesda, MD) were as described previously (Takeshita et al., 2001). The dominant-negative mutants of c-Src (K295M), IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) were prepared as described previously (Huang et al., 2003b).

Transient Transfection and Luciferase Activity Assay. AGS cells grown to 60% confluence in 12-well plates were transfected with either the human COX-2 promoter construct or kB-Luc using SuperFect (Qiagen) according to the manufacturer's recommendations. In brief, reporter DNA (0.3 μg) and β-galactosidase DNA (0.15 μg; pRK plasmid containing the β-galactosidase gene driven by the constitutively active simian virus 40 promoter was used to normalize the transfection efficiency) were mixed with 0.45 μl of SuperFect in 0.4 ml of serum-free RPMI 1640. After 10 to 15 min of incubation at room temperature, the mixture was applied to the cells. Six hours later, 0.4 ml of RPMI 1640 with 20% FCS was added. Twenty-four hours after transfection and change to an antibiotic-free medium, the cells were incubated with *H. pylori* isolate from GC patients for 6 h. Cell extracts were then prepared, and the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized to

the β-galactosidase activity. In experiments using dominant-negative mutants, cells were cotransfected with reporter (0.3 μg) and β-galactosidase (0.15 μg) and either the dominant-negative TLRs, PLCγ2, PKCα, NIK, IKKα, IKKβ, and c-Src mutants or the empty vector (0.6 μg). In experiments using wt plasmids, cells were cotransfected with 0.3 μg of reporter plasmid, 0.15 μg of β-galactosidase plasmid, 0.45 μg of the wt PLCγ2, constitutively active PKCα (A/E) plasmid, wt c-Src plasmid, or empty vector, and 0.6 μg of the dominant-negative PLCγ2, PKCα, NIK, IKKα, IKKβ, or c-Src mutant or empty vector.

Immunoprecipitation and Kinase Activity Assay. After incubation with *H. pylori* isolates from patients with GC, with or without 30-min pretreatments with PI-PLC, PKC, and Src kinase inhibitors, AGS cells were rapidly washed with PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM NaF, and 1 mM Na_3VO_4), then either IKK or c-Src was immunoprecipitated. For the in vitro kinase assay, 100 μg of total cell extract was incubated for 1 h at 4°C with either 0.5 μg of rabbit anti-IKKβ or anti-c-Src Ab. The protein A-Sepharose CL-4B beads were then added to the mixture, and incubation continued for 4 h at 4°C. The immunoprecipitates were collected by centrifugation, washed three times with lysis buffer without Triton X-100, and then incubated for 30 min at 30°C in 20 μl of kinase reaction mixture (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, and 1 mM dithiothreitol) containing 10 μM [γ-^{32}P]ATP and either 1 μg of bacterially expressed GST-IκBα (1–100) as IKK substrate, 1 μg of acidic denatured enolase, or 6 μg of bacterially expressed GST-IKKβ (132–206) as c-Src substrate. The reaction was stopped by the addition of an equal volume of Laemmli buffer, the proteins were separated by electrophoresis on 10% SDS polyacrylamide gels, and the phosphorylated-GST-IκBα (1–100), phosphorylated enolase, and phosphorylated GST-IKKβ (132–206) were visualized by autoradiography. Quantitative data were obtained using a densitometer with ImageQuant software and normalized by the protein expression.

Statistical Analysis. To establish the significance of the results, the Student's t test was used for numerical data. Fisher's exact test or χ^2 test was used for categorical data as appropriate. A p value less than 0.05 was considered statistically significant.

Results

COX-2 Protein Expression Induced by *H. pylori* Isolates from Patients with Different Disease Status. Compared with basal levels, overexpression of COX-2 protein was seen in the AGS cells treated with either TNF-α or HC at a bacterium/cell ratio of 50:1, 150:1, or 350:1. In contrast, HS and HD showed no obvious COX-2 overexpression [Fig. 1A, (a)]. At a bacterium/cell ratio of 150:1, the capabilities of representative HS, HU, HD, and HC to induce COX-2 expression were shown in Fig. 1A, (b). The clinical isolate from GC had a greater capability to induce COX-2 protein expression, whereas relatively lower induction of COX-2 protein among isolates of nonulcer dyspepsia, gastric ulcer, and duodenal ulcer was seen. To determine whether *H. pylori*-induced COX-2 expression occurred at the transcriptional level, the induction of COX-2 mRNA expression stimulated by HC in AGS cells was examined by RT-PCR and a time-dependent increase was found [Fig. 1A, (c)]. A box plot of COX-2 protein expression in AGS cells induced by different clinical isolates is shown in Fig. 1B. The increase induced by these 20 isolates from gastric cancer (6.19 $\pm$ 0.86-fold, mean $\pm$ S.E.) was significantly higher than that from nonulcer dyspepsia (1.48 $\pm$ 0.16-fold; $p < 0.001$), gastric ulcer (1.92 $\pm$ 0.38-fold; $p < 0.001$), and duodenal ulcer (1.97 $\pm$ 0.46-fold; $p < 0.001$) (Fig. 1B). Compared with HS, HU, and HD, HC also exhibited a stronger ability to induce the COX-2 expression in MKN45 cells, another gastric adenocarcinoma cell line (Fig. 1C).

The induction of COX-2 by HC in AGS and MKN45 cells was further demonstrated by immunofluorescence staining. As shown in Fig. 1D, no COX-2 expression was seen in the basal state but was apparent in the nuclear envelope of AGS cells and in the cytosol of MKN45 cells after coculture with HC.

Genotypes of *H. pylori* Isolates from Patients with Different Disease Status. Table 2 shows the genotypes of *hrg, iceA, babA2, cagA*, and *vacA* from 20 patients of HS, HU, and HC, and 21 patients of HD. There was no difference in the genotypes among the *H. pylori* isolates from patients with different disease status despite the higher COX-2 induction capability of HC. Furthermore, no difference in COX-2 induction capability was seen among HC isolates from 11 intestinal and nine diffused subtypes of GC. Regardless of the difference in induction capacity, all *H. pylori* strains can induce COX-2 expression in MKN45 cells (Fig. 1C), suggesting that *H. pylori*-induced COX-2 expression is a common phenomenon. To further study the mechanism of this effect, the *H. pylori* isolates that exhibited stronger ability of inducing COX-2 overexpression were chosen for the following studies using AGS cells.

***H. pylori*-Induced COX-2 Promoter Activity via NF-κB, C/EBP, and CREB Activation without Change in the Methylation Status.** To analyze the transcriptional regulation, the methylation status of COX-2 promoter in AGS cells before and after incubation with *H. pylori* was examined. By methylation-specific PCR, both the unmethylated and methylated products were shown before the incubation with *H. pylori* (Fig. 2A, lanes 1–2). The incubation with *H. pylori* did not affect the methylation status (Fig. 2A, lanes 3–4).

To identify which *cis*-acting element was involved, the COX-2 promoter-Luc constructs, including $-327/+59$, KBM with κB site ($-223/-214$) mutation, ILM with NF-IL6 site ($-132/-124$) mutation, and CRM with CRE site ($-59/-53$) mutation, were used (Huang et al., 2003b). Our results showed a decrease in the induction of COX-2 promoter activity by *H. pylori* using KBM, ILM, and CRM (Fig. 2B), demonstrating that NF-κB, NF-IL6, and CRE elements contribute to the *H. pylori*-induced COX-2 transcription.

Because NF-κB, NF-IL6, and CRE elements were involved in the COX-2 gene transcription after *H. pylori* infection, the DNA-protein complex formation was examined with the use of EMSA. An increase in NF-κB DNA-protein binding was seen after coculture with *H. pylori* for 30 min and reached a maximum at 60 min (Fig. 2C). Similar induction of C/EBP and CREB DNA-protein complex formation was also seen after 60 and 120 min of coculture (Fig. 2C).

Involvement of Toll-Like Receptors in *H. pylori*-Mediated COX-2 Expression. Several studies have shown the involvement of TLRs in bacterial infections (Rock et al.,1998), whether TLR is involved in the *H. pylori*-induced NF-κB activation leading to COX-2 expression is elucidated. Expression levels of TLR2, TLR4, and TLR9 were found in AGS and MKN45 cells (Fig. 3A), and we examined their role in the *H. pylori*-induced COX-2 promoter activity. As shown in Fig. 3B, the induction of COX-2 promoter activity by *H.*

pylori was inhibited in a dose-dependent manner by the mutant of TLR2 (P/H) and TLR9 (ICD), but not TLR4 (P/H), indicating the involvements of TLR2 and TLR9 but not TLR4. The neutralizing antibody for TLR2 and TLR9 also attenuated the *H. pylori*-induced COX-2 expression (Fig. 3C). To further identify the TLRs-mediated COX-2 promoter activity and its downstream signaling, we overexpressed the wild-type TLRs in AGS cells. The wt TLR2, but not the wt TLR4, increased the COX-2 promoter activity (Fig. 3D). Although TLR9 mutant blocked the induction of *H. pylori*-mediated COX-2 promoter activity, the wt TLR9 was unable to induce the COX-2 promoter activity (Fig. 3D). Combination of TLR2 or TLR9 with *H. pylori* by transfected cells with TLR2 or TLR9 followed by *H. pylori* coculture showed a synergistic effect on the COX-2 promoter activity (Fig. 3D), confirming their involvement in *H. pylori*-mediated COX-2 induction. Although HS alone had no effect on COX-2 induction, the increases of COX-2 promoter activity were seen in the presence of overexpression of TLR2 and TLR9 but not TLR4 (Fig. 3D).

Because TLR2 and TLR9 were demonstrated to be involved in *H. pylori*-induced COX-2 promoter activity (Fig. 3E), their role in *H. pylori*-induced NF-κB activity was examined. As shown in Fig. 3E, HC, but not HS, HU, or HD, induced NF-κB activation, and the dominant-negative mutants of TLR2 and TLR9 inhibited this effect.

Involvements of PLCγ, PKC, and c-Src in *H. pylori*-Mediated COX-2 Expression. The COX-2 expression induced by *H. pylori* was inhibited by either 10 μM U73122 (PI-PLC inhibitor) or 1 μM Ro 31-8220 (PKC inhibitor), whereas 10 μM U73343 (an inactive analog of U73122) had no effect (Fig. 4A, lanes 3–5), indicating the involvement of PI-PLC/PKC pathway. The Src inhibitor PP2 also abolished the *H. pylori*-induced COX-2 expression (Fig. 4A, lane 6), suggesting the role of Src kinase in this regulation. To further confirm the involvement of the PI-PLC/PKC pathway in

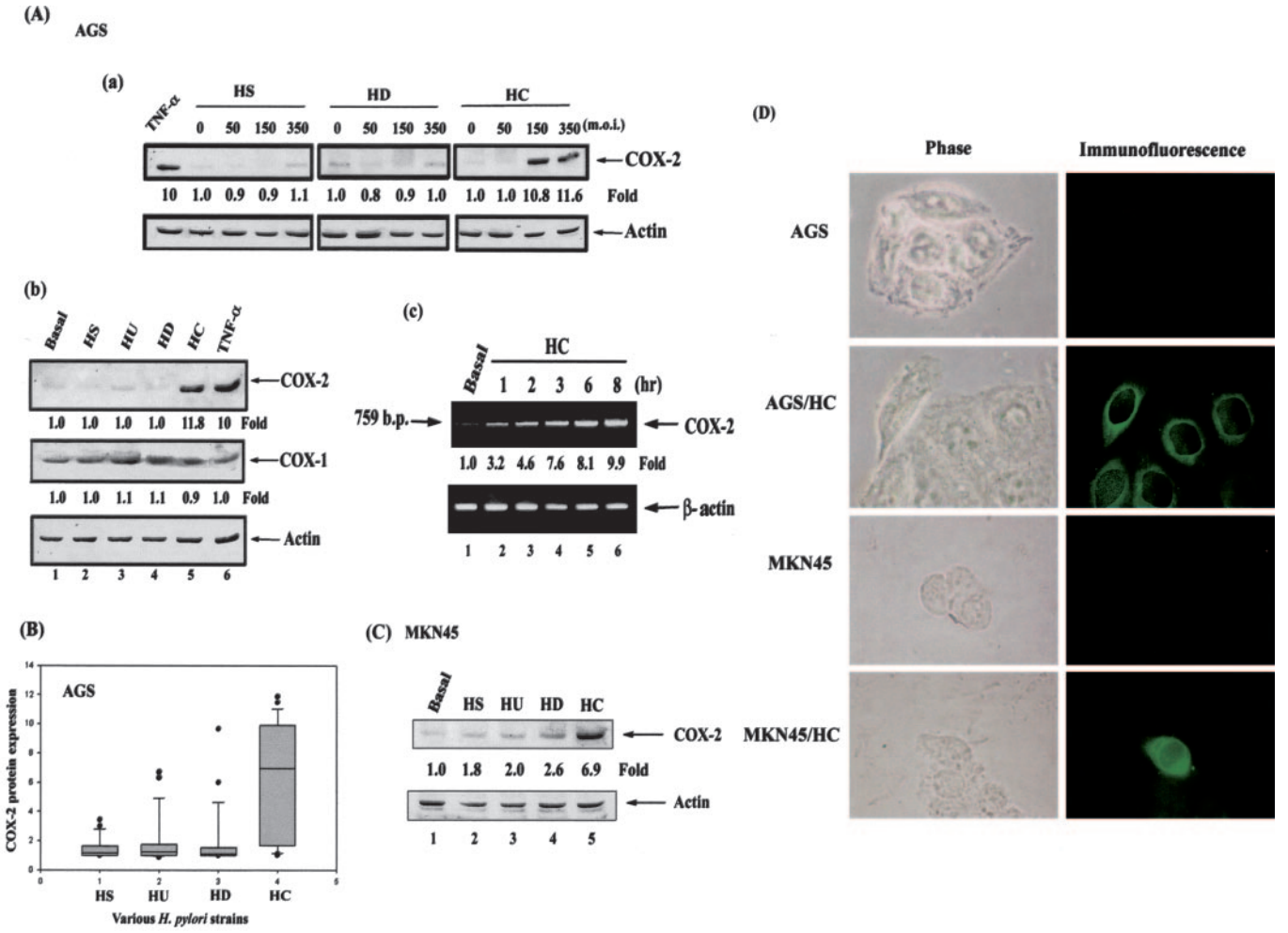

Fig. 1. COX-2 expression in AGS and MKN45 cells induced by *Helicobacter pylori* isolates from patients with gastritis (HS), gastric ulcer (HU), duodenal ulcer (HD), gastric cancer (HC) and by TNF-α. Cells were treated with *H. pylori* isolates from different patients at indicating bacterium/cell ratio in (a) or at a bacterium/cell ratio of 150:1 in (b) and (c) or 10 ng/ml of TNF-α for 16 h. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2, COX-1, or Actin. In (c), AGS cells were cocultured with HC for the indicated time. Total RNA (2 μg) was used for RT-PCR as described under *Materials and Methods*. B, box plot of COX-2 expression in AGS cells summarized from different clinical isolates of *H. pylori*. Cells were cocultured with *H. pylori* strains from patients with gastritis (HS) (n = 20), gastric ulcer (HU) (n = 20), duodenal ulcer (HD) (n = 21), and gastric cancer (HC) (n = 20). The quantitative data were normalized by the actin level. In D, COX-2 is located around the nuclear envelope or cytosol. Immunofluorescence staining of AGS or MKN45 cells with affinity-purified anti-COX-2 Ab (1:100) were performed as described under *Materials and Methods*. Control AGS and MKN45 cells or those cocultured with HC for 16 h are shown.

H. pylori-induced COX-2 expression (Fig. 4A), cotransfections of the PLCγ mutant with reporter, the dominant-negative mutants of PKCα (K/R) with reporter, and the c-Src (KM) with reporter were performed. The induction of COX-2 promoter activity by *H. pylori* was attenuated by PLCγ2 (SH2(N)), PKCα (K/R), and c-Src (KM) in a dose-dependent manner, confirming the involvement of these signaling molecules in the COX-2 expression.

TABLE 2
The relationship between the extent of cyclooxygenase-2 expression in AGS cells and genotypes in different clinical isolates of *H. pylori*

	HS (n = 20)	HU (n = 20)	HD (n = 21)	HC (n = 20)
COX-2 expression				
Mean ± S.E.	1.48 ± 0.16	1.92 ± 0.38	1.97 ± 0.46	6.19 ± 0.89*
Restriction endonuclease-replacing gene				
hrgA	7	8	7	7
hpyIIIR	13	12	14	13
IceA				
A1A1	17	18	18	17
A1A2	2	1	2	2
A2A2	1	1	1	1
babA2				
positive	20	20	21	20
negative	0	0	0	0
CagA 3′ repeat region				
A	19	18	21	19
B, C, D	1	2	0	1
vacA				
s1a/m1	3	6	8	6
s1a/m2	17	14	13	14

* $P < 0.001$ versus HS, HU, or HD.

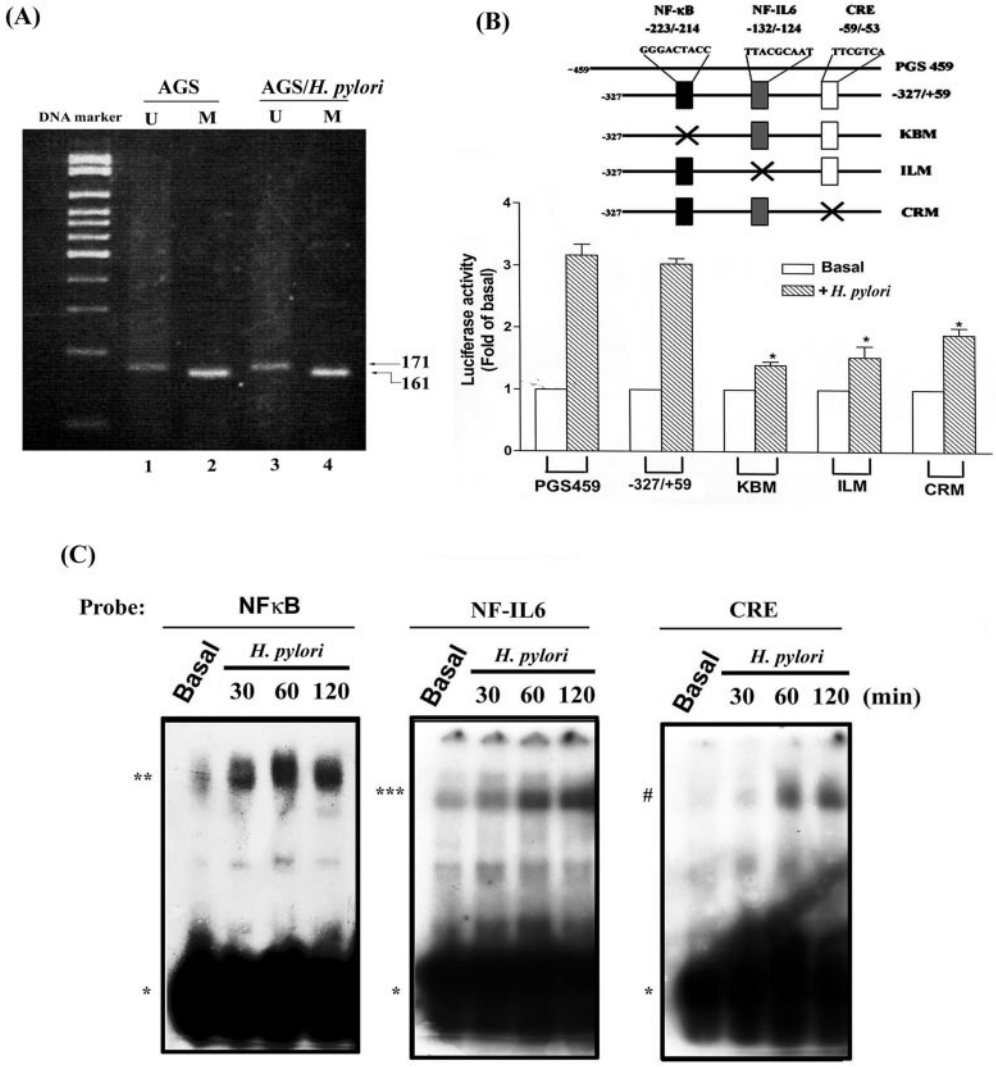

Fig. 2. Methylation status and activation of the COX-2 promoter, and kinetics of DNA-protein complex formation induced by *Helicobacter pylori*. A, methylation-specific PCR was performed after bisulfite modification of DNA as described under *Materials and Methods*. *M* indicates methylated COX-2 and *U* indicates unmethylated COX-2. B, top, schematic diagram of the 5′ regulatory region of the human COX-2 gene. Rectangles indicate the location of the NF-κB, NF-IL6, and CRE sites. Cells were transfected with the pGS459/+9, −327/+59, KBM, ILM, or CRM luciferase expression vector, and then infected with *H. pylori*. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity. The luciferase activity was normalized using the β-galactosidase activity and expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $p < 0.05$ compared with −327/+59. C, cells were cocultured with *H. pylori* (m.o.i., 150) for 30 min, 1 h, or 2 h, then nuclear extracts were prepared. NF-κB, NF-IL6, or CRE oligonucleotide probe was used to measure the DNA-protein complex formation by EMSA as described under *Materials and Methods*. *, free probe; **, NF-κB DNA-protein complex; ***, NF-IL6 DNA-protein complex; #, CRE DNA-protein complex.

Demonstration of TLR2/PLCγ/PKC/c-Src/IKK Pathway in *H. pylori*-Mediated COX-2 Promoter Activity. Involvements of NIK and IKKα/β in the *H. pylori*-induced COX-2 promoter activity were demonstrated using the dominant-negative mutants of NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (AA), IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) [Fig. 5A, (a)]. wt TLR2-induced COX-2 promoter activity was inhibited by either the PLCγ2 mutant or the dominant-negative mutants of PKCα (K/R), c-Src (KM), NIK(KKAA), IKKα (KM) and IKKβ (KM), IKKβ(Y188F), IKKβ(Y199F) and IKKβ(YYFF) [Fig. 5A, (b)], suggesting that *H. pylori* induced COX-2 expression through the activation of PLCγ2, PKCα, c-Src, NIK, IKKα, and IKKβ.

To characterize the relationship between PLCγ2, PKCα, c-Src, and IKKβ, overexpression of the constitutively active PKCα(A/E), wt PLCγ2, and wt c-Src were performed. wt PLCγ2, PKCα (A/E), and wt c-Src increased the COX-2 promoter activity by 7.7-, 2.5-, and 42-fold, respectively (Fig. 5B). The COX-2 promoter activity induced by either wt PLCγ2 or PKCα (A/E) was inhibited by the dominant-negative PKCα (K/R), c-Src (KM), IKKα (KM), and IKKβ (KM) mutants [Fig. 5B, (a) and (b)], whereas that induced by wt c-Src was inhibited by the dominant-negative IKKα (KM) and IKKβ (KM), IKKβ (YF) and IKKβ (YYFF), but not IKKβ (AA) and PLCγ2 (SH2(N)) mutants [Fig. 5B, (c)]. These results indicated the involvement of PI-PLCγ/PKC/c-Src/IKKα/β pathway in *H. pylori*-induced COX-2 expression.

Our recent studies have revealed that phosphorylation of IKKβ at Tyr^{188} and Tyr^{199} is required for the TNF-α-induced ICAM-1 and COX-2 expressions in the lung epithelial cells and also demonstrated that these two tyrosine residues are the targets of c-Src (Huang et al., 2003a,b). Overexpressions of the dominant-negative tyrosine mutants IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) attenuated the *H. pylori*-induced, the wt TLR2-induced, and the wt c-Src-induced COX-2 promoter activity. The dominant-negative IKKβ (KM) mutant with Lys^{44} mutated to methionine had a similar inhibitory effect (Fig. 5, A and B). On the other hand, IKKβ (AA) with Ser^{177} and Ser^{181} mutated to alanine had no effect on the wt c-Src-induced COX-2 promoter activity ([Fig. 5B, (c)]), but was as effective as IKKβ (Y188F) and IKKβ (Y199F) in inhibiting the *H. pylori*-induced COX-2 promoter activity [Fig. 5B, (a)].

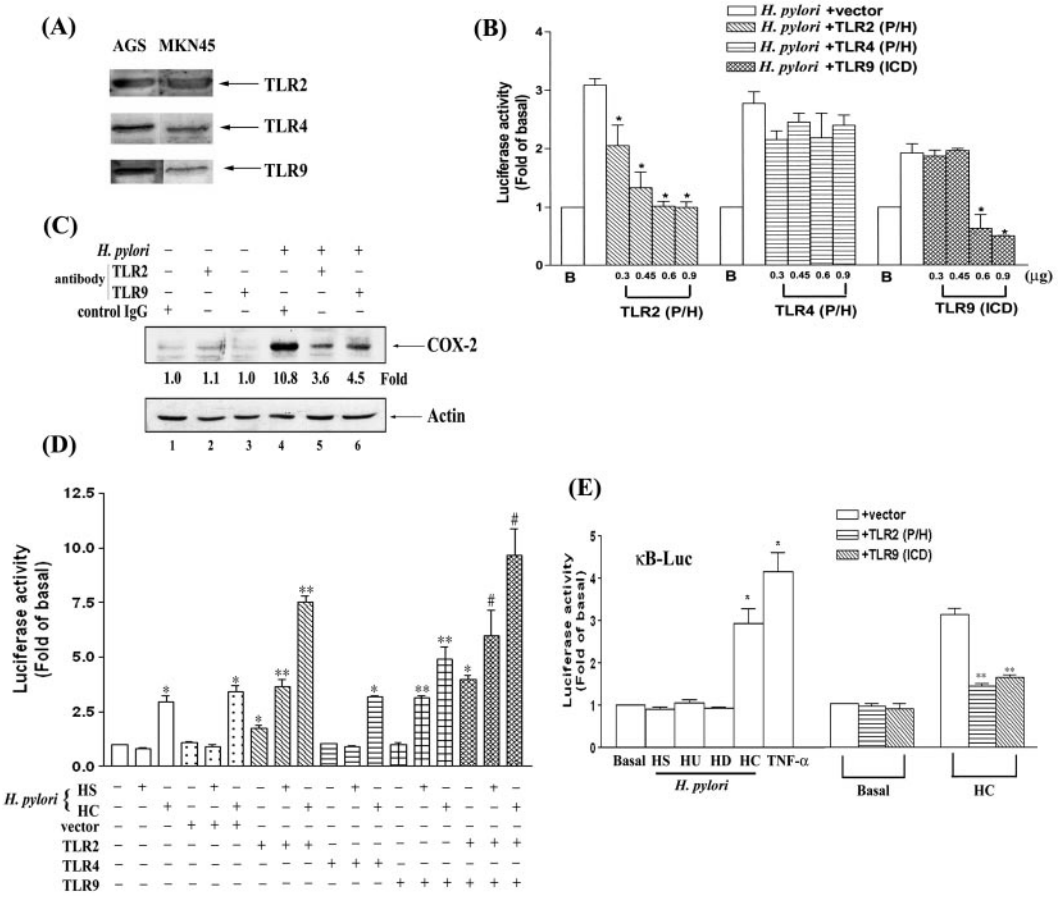

Fig. 3. Effects of TLR mutant or neutralizing antibody on COX-2 promoter activity or COX-2 expression induced by *H. pylori*. A, cell lysates were prepared and 100 μg of total proteins were subjected to Western blot analyses using anti-TLR2, TLR4, or TLR9 Ab. B and D, AGS cells were cotransfected with pGS459 and the mutant of TLR2 (P/H), TLR4 (P/H), or TLR9 (ICD) (B), or cotransfected with pGS459 and the wild-type TLR2, TLR4, TLR9, or the respective empty vector after coculture with *H. pylori* (D). E, cells were transfected with the κB-Luc plasmid, then infected with HS, HU, HD, or HC, treated with 10 ng/ml TNF-α for 6 h, or cotransfected with κB-Luc plasmid and TLR2 (P/H) or TLR9 (ICD) mutant, or the respective empty vector, then cocultured with *H. pylori* for 6 h. Luciferase activity was measured as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean $\pm$ S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone in B and E or with empty vector (D). **, $p < 0.05$ compared with the respective wt TLR alone. #, $p < 0.05$ compared with wt TLR2+wt TLR9 (D). C, AGS cells were incubated with anti-TLR2, TLR9, or control Ab for 3 h, then cocultured with *H. pylori* (m.o.i., 150) for 16 h. Whole-cell lysates were prepared and subjected to Western blotting using anti-COX-2 or anti-Actin Abs.

Induction of c-Src Activation by *H. pylori* Infection and the Inhibitory Effect of PI-PLC, PKC, or Src Kinase Inhibitor. To further demonstrate that the *H. pylori*-activated PKCα/c-Src/IKKβ pathway induced tyrosine phosphorylation of IKKβ, in vitro c-Src activity was measured. c-Src was isolated by immunoprecipitation using anti-c-Src Ab, and activity was measured using enolase as the substrate. As shown in Fig. 6A, the maximal c-Src activity (enolase phosphorylation) was seen after 30-min coculture with *H. pylori*, and this effect declined after 60 min (Fig. 6A, lanes 3–5). Marked autophosphorylation of c-Src was also seen (Fig. 6A). The *H. pylori*-induced c-Src activation was inhibited by 10 μM U73122, 1 μM Ro 31-8220, and PP2 at 1 and 10 μM (Fig. 6B, lanes 3–6).

Induction of IKK Activation and IκBα Degradation by *H. pylori* Infection, and the Inhibitory Effect of PI-PLC, PKC, or Src Kinase Inhibitor. Because the dominant-negative IKKα/β mutant inhibited *H. pylori*-induced COX-2 promoter activity, the endogenous IKK was immunoprecipitated with anti-IKKβ antibody, and its activity was measured. When cells were cocultured with *H. pylori* for periods of 10, 30, 60, or 120 min, a significant IKK activity was measured after 30 min [Fig. 7A, (a), lane 3] that paralleled with the degradation of IκBα [Fig. 7A, (b), lane 3]. IκBα was restored to the resting level after coculture with *H. pylori* for 24 h (data not shown). *H. pylori*-induced IKK activation was inhibited by the PI-PLC, PKC, and Src kinase inhibitors in a dose-dependent manner [Fig. 7B, (a), lanes 3–8], which was paralleled with the recovery of IκBα degradation [Fig. 7B, (b), lanes 3–8].

Involvement of c-Src-Dependent Tyrosine Phosphorylation of IKKβ by *H. pylori* Infection. Because Tyr^{188} and Tyr^{199} of IKKβ were found to be critical in the PKCα/c-Src/IKKβ pathway eliciting NF-κB activation and inducing COX-2 promoter activity (Fig. 5, A and B), the tyrosine phosphorylation of IKKβ by c-Src was examined further. c-Src was immunoprecipitated using anti-c-Src antibody, and its ability to phosphorylate IKKβ was measured using GST-IKKβ-(132–206) as an in vitro substrate. When cells were cocultured with *H. pylori*, IKKβ was phosphorylated by c-Src in a time-dependent manner; the maximal effect was seen at 30 min (Fig. 8A, lane 3), and this effect was inhibited by 1 and 10 μM PP2 (Fig. 8B, lanes 3 and 4).

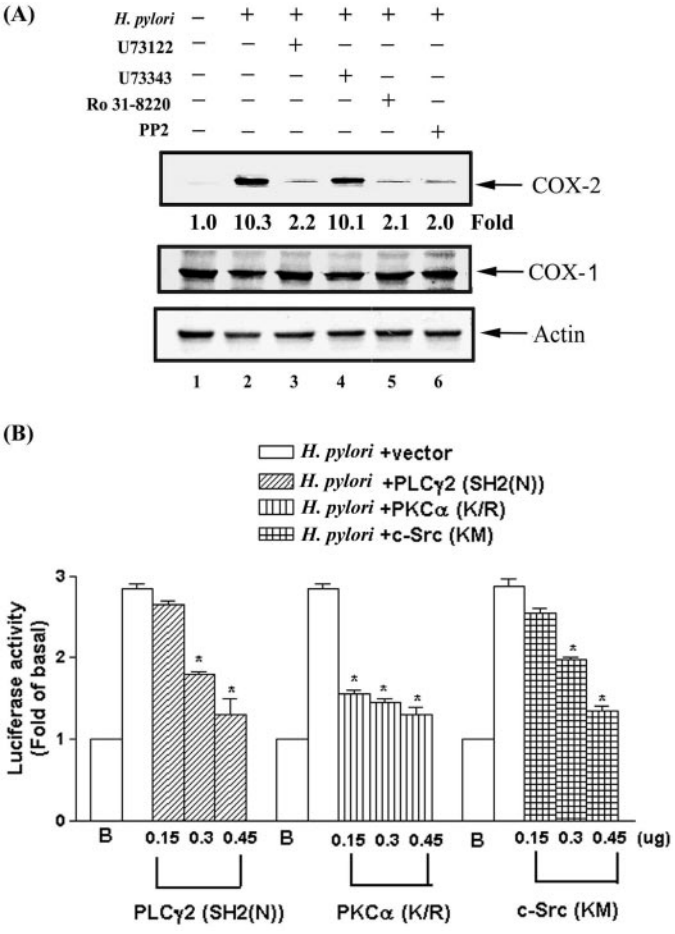

Fig. 4. Effects of various inhibitors or PLCγ2 mutant or dominant-negative mutants on *H. pylori*-induced COX-2 expression or promoter activity in AGS cells. A, cells were pretreated with U73122 (10 μM), U73343 (10 μM), Ro 31-8220 (1 μM), or PP2 (10 μM) for 30 min before coculture with *H. pylori* for 16 h. Whole-cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2, COX-1, or actin. B, cells were cotransfected with pGS459 and the PLCγ2 (SH2(N)) mutant or the dominant-negative mutants of PKCα (K/R) or c-Src (KM), or the respective empty vector, then cocultured with *H. pylori* (m.o.i.,150) for 6 h. Luciferase activity was measured as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean $\pm$ S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone.

Discussion

In the present study, we have demonstrated different capabilities among clinical isolates of *H. pylori* to stimulate COX-2 expression in vitro. Our results demonstrated that *H. pylori* strains isolated from GC patients induced higher expression of COX-2 protein in vitro. It is likely that overexpression of COX-2 by *H. pylori* isolates from GC patients may contribute to the carcinogenesis in host induced by these strains. This phenomenon is also in agreement with the in vivo observation that COX-2 overexpression is found in 50~80% of gastric cancer patients (Sung et al., 2000; Chen et al., 2001; Wambura et al., 2002). The bacterial virulence factors and host cellular pathways of *H. pylori*-mediated COX-2 expression were further investigated.

The clinical outcome of *H. pylori* infection is determined by a complex interaction of host, environmental influences, and microbial virulence factors. The relevance of several specific *H. pylori* genes has been studied in the past. Although *cagA, vacA, iceA, babA2*, and *hrgA* genotypes have been reported to associate with GC (Yamaoka et al., 1998; Kidd et al., 2001; Nogueira et al., 2001; Prinz et al., 2001; Ando et al., 2002; Bravo et al., 2002), our data show no difference in these virulence genes among *H. pylori* isolates from patients with different disease statuses. The significances of VacA and CagA in *H. pylori*-induced COX-2 expression have been reported (Caputo et al., 2003; Juttner et al., 2003). Although we failed to show correlation of *cagA* and *vacA* genotypes with COX-2 expression in various clinical isolates, our data could not exclude their crucial roles in *H. pylori*-induced COX-2 expression. It is also probable that *H. pylori* might have CagA- and VacA-independent pathways to induce COX-2 expression, because all experimental strains are live bacteria and contain such toxins. The high induction of COX-2 expression by the HC strains might be exerted through other novel factors, such as γ-glutamyl transpeptidase (Busiello et al.,

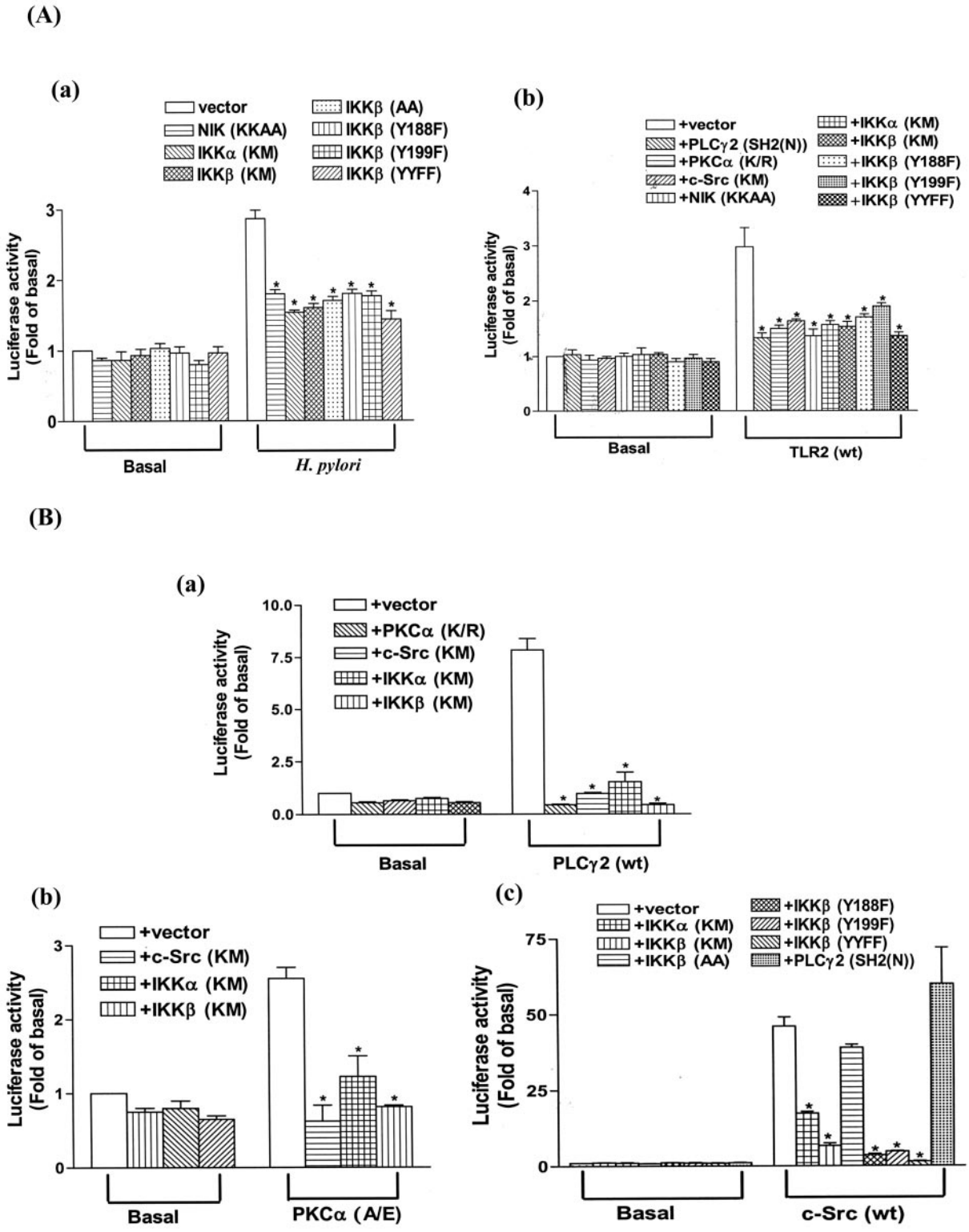

Fig. 5. Effect of PLCγ2 mutant or various dominant-negative mutants on wild-type or constitutive active plasmid-induced COX-2 promoter activity. A, (a), AGS cells were cotransfected with the dominant-negative NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (Y188F), IKKβ (Y199F), or IKKβ (FF) mutant or the respective empty vector, then cocultured with *H. pylori* (m.o.i., 150) for 6 h. A, (b), and B, cells were cotransfected with wild-type TLR2 and PLCγ2 (SH2(N)) mutant or the dominant-negative mutants of PKCα (K/R), c-Src (KM), NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (Y188F), IKKβ (Y199F), or IKKβ (FF) or the respective empty vector [A, (b)], or cotransfected with PLCγ2 (wt) and the dominant-negative PKCα (K/R), c-Src (KM), IKKα (KM), or IKKβ (KM) mutant [A, (a)], or cotransfected with PKCα (A/E) and the dominant-negative c-Src (KM), IKKα (KM) or IKKβ (KM) mutant [B, (b)], or cotransfected with c-Src (wt) and the PLCγ2 (SH2(N)) mutant or the dominant negative mutants of IKKα (KM), IKKβ (KM), IKKβ (AA), IKKβ (Y188F), IKKβ (Y199F), or IKKβ (FF), or the respective empty vector[B, (c)]. Luciferase activity was measured as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean $\pm$ S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone [A, (a)], wt TLR2 alone [A, (b)], wt PLCγ2 alone [B, (a)], PKCα (A/E) alone [B, (b)] or c-Src (wt) alone [B, (c)].

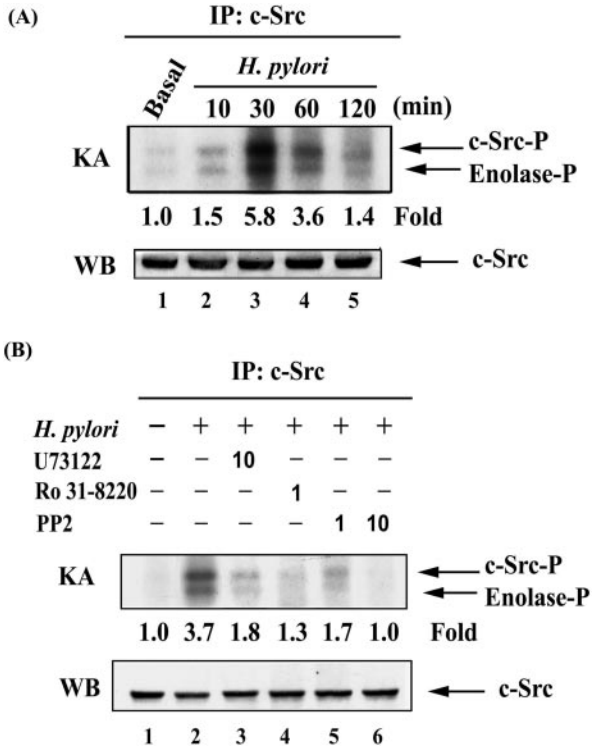

Fig. 6. Time-dependent activation of c-Src by *H. pylori* and effects of various inhibitors. AGS cells were cocultured with *H. pylori* for 10, 30, 60, or 120 min (A) or pretreated with 10 μM U73122, 1 μM Ro 31-8220, or 1 or 10 μM PP2 for 30 min before coculture with *H. pylori* for 60 min (B). Whole-cell lysates were prepared and immunoprecipitated with anti-c-Src antibody. The kinase assay (KA) and autoradiography for phosphorylated enolase were performed on the precipitates as described under *Materials and Methods*. Levels of immunoprecipitated c-Src were estimated by Western blotting (WB) using anti-c-Src antibody.

2004). It is also possible that some strains express virulence factors only at the gene level but not at the protein level. Further exploration of the molecular mechanisms involved in the enhancing effects of *H. pylori* on COX-2 expression in vitro and in vivo is warranted to provide deep insights into the role of *H. pylori* and COX-2 in the gastric carcinogenesis.

Methylation of the gene promoter DNA at the areas of CpG islands has been linked to the silencing of gene expression (Song et al., 2001). The induction of COX-2 expression in AGS cells by *H. pylori* was not through its demethylation of gene promoter, because the methylated and unmethylated status of COX-2 promoter in AGS cells was not changed after coculture with HC. Akhtar et al. (2001) also found that *H. pylori* did not change the methylation status of COX-2 promoter in AGS cells, and an increase in COX-2 protein expression was also seen after cells were cocultured with *H. pylori* (Akhtar et al., 2001) (Fig. 3C, compare lanes 1 and 2).

In addition to the methylation status, transcription factor-binding sites on the COX-2 promoter and their individual role as *cis*-acting elements regulating the transcription are of particular interest. Rodents have only one NF-κB site (-401/-393 bp in mouse), which has been shown to be involved in the TNF-α-induced COX-2 induction in a mouse osteoblast cell line (Yamamoto et al., 1995). The NF-κB-3′ site (-223/-214 bp) on the human COX-2 promoter, in concert with the NF-IL6 and CRE sites, may play a role in facilitating the induction of COX-2 by LPS and phorbol ester (Inoue et al., 1995). Our results clearly showed the indispensable role of the downstream NF-κB site (-223/-214) in TNF-α-induced COX-2 expression in human alveolar epithelial cells (Huang et al., 2003b). Our study presents the first evidence that NF-κB, NF-IL6, and CRE are involved in *H. pylori*-induced COX-2 expression. Activation of the CRE site has also been reported to be involved in the *H. pylori*-stimulated COX-2

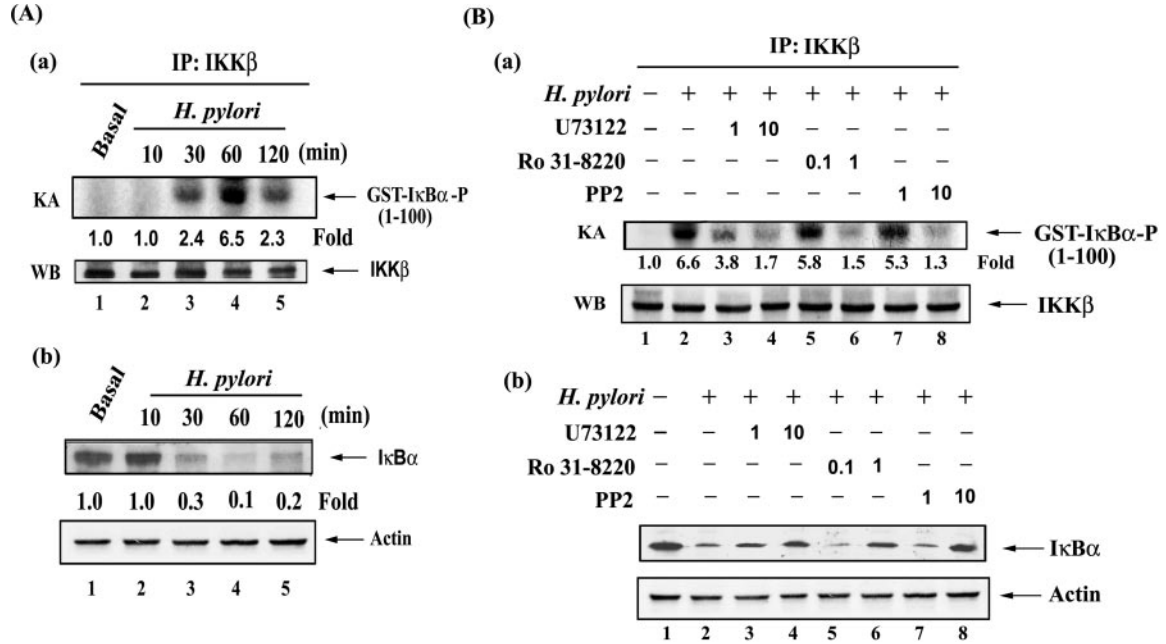

Fig. 7. Kinetics of *H. pylori*-induced IKK activation and IκB-α degradation and effects of various inhibitors. AGS cells were cocultured with *H. pylori* for 10, 30, 60, or 120 min (A) or pretreated with 1 or 10 μM U73122, 0.1 or 1 μM Ro 31-8220, or 1 or 10 μM PP2 for 30 min before coculture with *H. pylori* for 60 min (B), and then whole-cell lysates were prepared. Cell lysates were immunoprecipitated with anti-IKKβ antibody, then kinase assay (KA) and autoradiography for phosphorylated GST-IκBα (1–100) were performed on the precipitates as described under *Materials and Methods*. Levels of immunoprecipitated IKKβ protein were estimated by Western blotting (WB) using anti-IKKβ antibody [A (a) and B (a)]. Cytosolic levels of IκB-α and actin were measured using anti-IκB-α and actin antibody, respectively [A (b) and B (b)].

gene transcription (Juttner et al., 2003). However, Juttner et al. (2003) used mouse but not human COX-2 promoter construct to transfect the human AGS cells (Fig. 2). In addition to the differences in number of the NF-κB site, there are still some differences in the *cis*-acting elements on the COX-2 promoter between these two species (Inoue et al., 1995). For instance, the relative position and sequences of CRE site (5′-CGTCACGTG-3′ at −56 to −48 bp) on the mouse promoter are different from those on the human (5′-TTCGTCA-3′ at −59 to −53). Using consensus sequences containing human CRE site, we found the bindings of CREB-1, ATF-2, and c-jun to this site. TLR2 and TLR9 also initiated CRE activation (Y.-J. Chang and C.-C. Chen, unpublished data). C/EBPβ and C/EBPδ were found to bind *H. pylori*-activated NF-IL6 site (Y.-J. Chang and C.-C. Chen, unpublished data), and the involvement of TLRs in this signaling is under investigation.

All TLRs activate a common signaling pathway that culminates in the activation of NF-κB as well as the mitogen-activated protein kinases (Barton and Medzhitov, 2003). Expression of three TLRs (TLR2, TLR4, and TLR9) in both AGS and MKN45 cells was found in the present study. Although Smith et al. (2003) demonstrated the involvements of TLR2 and TLR5 in *H. pylori*-induced chemokine releases, we first revealed the roles of TLR2/9 in COX-2 induction and further explored their role in the *H. pylori*-mediated signaling pathway. Our data showed the involvements of TLR2 and TLR9 but not TLR4. When wild-type TLR was overexpressed, TLR2 and TLR9, but not TLR4, synergistically increased the *H. pylori*-induced COX-2 promoter activity. The neutralizing antibody for TLR2 or TLR9 also inhibited the *H. pylori*-induced COX-2 expression. The mutant and neutralizing antibody of TLR9 blocked the induction of *H. pylori*-mediated COX-2 promoter and expression, respectively, suggesting that *H. pylori* might mediate signaling via its CpG DNA (Hemmi et al., 2001). The finding that Gram-negative bacterial LPS stimulated TLR2 leading to NF-κB activation had been reported (Yang et al., 1998). Our data demonstrated that TLR2/9 are the major receptors mediating *H. pylori*-induced NF-κB activation leading to the COX-2 expression. This is the first report that links TLR2/9, NF-κB, and COX-2. In addition, the expression level of TLR2/9 might be a sensitive factor. HS alone did not induce COX-2 promoter activity but was enhanced in the presence of overexpression of these receptors (Fig. 3D). TLR2, but not TLR4, was also found to mediate *H. pylori*-induced NF-κB activation in MKN45 cells (Smith et al., 2003), and *Candida albicans* also acted through TLRs to activate NF-κB and induce COX-2 expression in Hela cells (Deva et al., 2003).

Because *H. pylori*-induced NF-κB activation through TLR2/TLR9 in AGS cells was demonstrated, the existence of the PKC/c-Src/IKKβ pathway downstream of TLR2/9 was examined. Several lines of evidence showed that gastric epithelial cells also exist in this pathway. First, both *H. pylori*- and wt TLR2-induced COX-2 promoter activities were inhibited by the dominant-negative tyrosine mutants IKKβ (Y188F), IKKβ (Y199F), or IKKβ (FF). Second, wt c-Src-

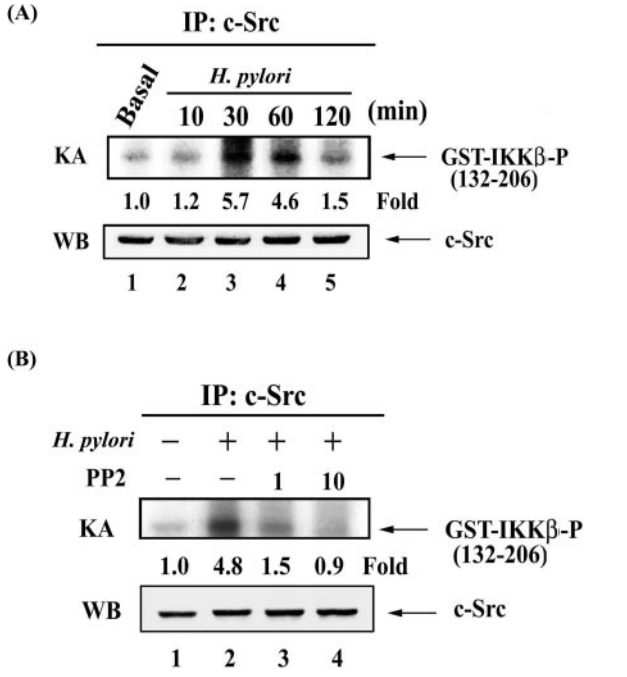

Fig. 8. c-Src-dependent phosphorylation of IKKβ induced by *H. pylori* and the inhibition by PP2. AGS cells were cocultured with *H. pylori* for 10, 30, 60, or 120 min (A) or pretreated with 1 or 10 μM PP2 for 30 min before coculture with *H. pylori* for 60 min (*B*). Whole-cell lysates were prepared and immunoprecipitated with anti-c-Src antibody, then kinase assay (KA) and autoradiography were performed for phosphorylated GST-IKKβ (132–206). The amount of immunoprecipitated c-Src was detected by Western blotting (WB) using anti-c-Src antibody.

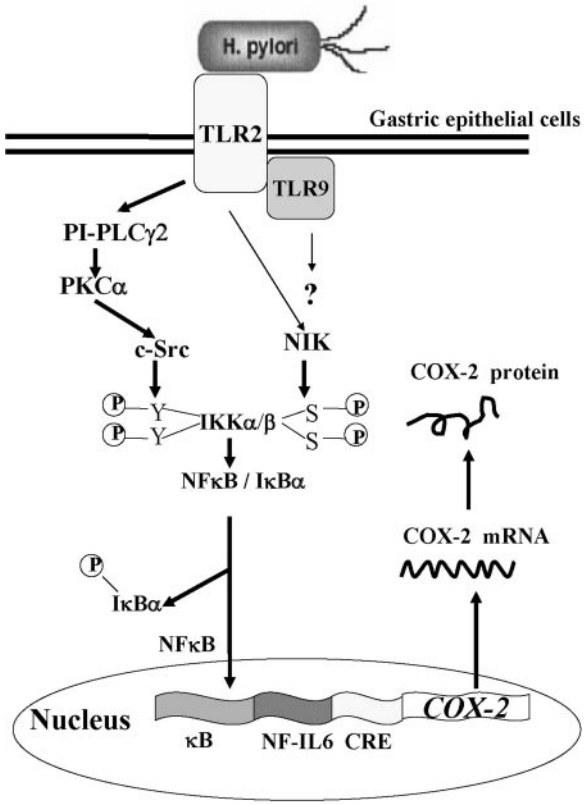

Fig. 9. Schematic representation of the signaling pathways involved in *H. pylori*-induced COX-2 expression in the AGS epithelial cells. The κB, NF-IL6, and CRE elements are involved in *H. pylori*-induced COX-2 expression. *H. pylori* acts through TLR2 and TLR9, then activates PI-PLCγ to induce PKCα and c-Src activation, leading to tyrosine phosphorylation of IKKα/β. *H. pylori* also activates NIK, leading to serine phosphorylation of IKKα/β. These two pathways converge at IKKα/β, resulting in the phosphorylation and degradation of IκBα, stimulation of NF-κB in the COX-2 promoter, and finally initiation of COX-2 expression.

induced COX-2 promoter activity was inhibited by the dominant-negative tyrosine mutants but not by the IKKβ (AA) mutant, in which Ser^{177} and Ser^{181} are mutated. Third, *H. pylori* induced c-Src activation as well as IKK activation and IκBα degradation, and the PI-PLC, PKC, and Src inhibitors inhibited these effects. Fourth, an in vitro kinase assay demonstrated that *H. pylori*-stimulated c-Src phosphorylates IKKβ at Tyr^{188} and Tyr^{199}, and this effect was inhibited by PP2. It is already known that these two tyrosine residues in IKKβ are conserved with other Ser/Thr kinases such as Akt1 and PKCδ (Huang et al., 2003a,b). Therefore, two signal pathways are involved in the *H. pylori*-induced NF-κB activation leading to COX-2 expression. One is the activation of NIK/IKK pathway, which was already recognized (Malinin et al., 1997), and the other is the activation of PKC-dependent c-Src pathway demonstrated here. These two pathways converge at IKKα/β. The PKC/c-Src/IKK pathway, shown to be involved in the induction of COX-2 expression here, might be a common pathway for the inducible gene expression, because the TNF-α-, IL-1β-, and IFN-γ-induced COX-2 or ICAM-1 expression in lung epithelial cells also involved the PKC-dependent activation of c-Src (Chang et al., 2002, 2004; Huang et al., 2003a,b). Although the *H. pylori*-up-regulated COX-2 mRNA expression and PGE2 synthesis were already found (Romano et al., 1998), we are the first to identify the involvement of NF-κB, NF-IL6, and CRE sites in *H. pylori*-induced COX-2 expression and to further explore the existence of a novel TLR2/9/c-Src/NF-κB/COX-2 pathway.

In summary, the identification of clinical isolates of *H. pylori* from GC patients induced high levels of COX-2 expression in vitro. Although the genetic bases for this phenomenon are not known at present, these clinical isolates may represent a group of *H. pylori* strains harboring novel virulence factors that could be pursued in the future. The involvement of NF-κB, NF-IL6, and CRE sites in the regulation of *H. pylori*-induced COX-2 expression are demonstrated. The mechanism of TLR2/TLR9 mediated *H. pylori*-induced NF-κB activation is further examined. *H. pylori* acts through the TLR2/TLR9 to activate both the PI-PLCγ/PKCα/c-Src/IKKα/β and NIK/IKKα/β pathways, resulting in the phosphorylation and degradation of IκBα, which in turn leads to the stimulation of NF-κB and COX-2 gene expression. A schematic presentation of the involvements of these pathways in the AGS cells is shown in Fig. 9. This COX-2 expression may contribute to the carcinogenesis in patients colonized with these cancer strains.

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