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Helicobacter pylori Induces IκB Kinase α Nuclear Translocation and Chemokine Production in Gastric Epithelial Cells

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NF-κB is an important transcriptional factor that is involved in multiple cellular responses, such as inflammation and antiapoptosis. IκB kinase α (IKKα) and IKKβ, which are critical regulators of NF-κB activity, possess various mechanisms for NF-κB activation. This variability in NF-κB signaling may be associated with distinct inflammatory responses in specific cell types. The gastric pathogen *Helicobacter pylori* is known to activate NF-κB. However, the role of IKK in *H. pylori* infection remains unclear. In this report, we show that *H. pylori* activates both IKKα and IKKβ in gastric cancer cells and enhances NF-κB signaling in distinct manners. We found that IKKβ acted as an IκBα kinase during *H. pylori* infection, whereas IKKα did not. *H. pylori* induced IKKα nuclear translocation in time-, multiplicity of infection-, and *cag* pathogenicity island-dependent manners. In contrast, p100 processing, which is a known IKKα activity induced by several cytokines, was not induced by *H. pylori*. Both IKKs were responsible for chemokine secretion by infected cells. However, the antiapoptotic effect of *H. pylori* was merely transduced by IKKβ. Microarray analysis and real-time PCR indicated that both IKKs were involved in the transcriptional activation of genes associated with inflammation, antiapoptosis, and signal transduction. Our results indicate that *H. pylori* activates NF-κB via both IKKα and IKKβ using distinct mechanisms. IKKα nuclear translocation induced by *H. pylori* is indispensable for appropriate inflammatory responses but not for antiapoptosis, which suggests a critical role for IKKα in gastritis development.

Helicobacter pylori is a pathogen that causes human gastric disease. About half of the world population is infected with this bacterium, although only a relatively small proportion of infected patients develop symptomatic disease, such as gastroduodenal ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. Bacterial, environmental, and host genetic factors may affect the progress and outcome of gastric disease. One such factor that is responsible for severe disease is the bacterial virulence factor *cag* pathogenicity island (PAI) (reviewed in references 6, 32, and 36). *H. pylori* strains that carry *cag* PAI genes, called type I strains, are highly prevalent in patients with gastroduodenal ulcer and gastric cancer (2, 4, 8). Previous studies have revealed that type I *H. pylori* strains are capable of activating multiple intracellular signaling pathways in infected epithelial cells (22, 26). The inflammatory, proliferative, and antiapoptotic responses observed in *H. pylori*-infected cells in culture and in gastric tissues are possibly mediated by the activation of intracellular signaling pathways, such as those for NF-κB and mitogen-activated protein kinase.

NF-κB is an important transcriptional factor that controls various biological processes, such as inflammation, cell survival or death, and cell cycle (reviewed in references 5, 14, 18, 20, and 23). The mechanism of NF-κB activation by a variety of extracellular stimuli is unique in that it is induced rapidly and does not require de novo protein synthesis, thereby allowing the cells to respond quickly to emergent situations, such as bacterial infection (49). Most forms of NF-κB, especially the most common form of the p50-RelA dimer, are rendered inactive through binding of the inhibitory protein IκBs. Phosphorylation-induced ubiquitination of IκBs promotes its degradation, which in turn liberates NF-κB dimers as their active forms (reviewed in references 5, 14, 18, and 23). The IκB kinase (IKK) complex is a protein complex that phosphorylates IκBs in response to upstream stimuli, and it is considered to be a critical regulator of NF-κB activity (14, 20, 49).

The IKK complex contains three subunits: the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ (10, 30, 37, 38, 45, 50). When overexpressed exogenously or synthesized in an in vitro system, both IKKα and IKKβ phosphorylate IκB proteins and activate NF-κB (10, 30, 37, 45, 50). Earlier studies on IKKβ knockout cells have indicated that IKKβ is indispensable for IκB phosphorylation, NF-κB activation, and subsequent gene expression in response to proinflammatory stimuli (24, 25). In contrast, IKKα knockout cells show normal IκBα phosphorylation and RelA nuclear translocation in response to lipopolysaccharide or cytokines (16, 43). These results have raised the question of whether IKKα is involved physiologically in NF-κB activation. Interestingly, IKKα-deficient mice show morphological abnormalities, indicating the specific role of IKKα that cannot be compensated for by IKKβ (16, 43). One of the specific activities of IKKα is the induction of p100 processing to p52. The phosphorylation of p100 by IKKα results in p100 degradation and the generation of p52, which in turn dimerizes with RelB to form the NF-κB subunit (9, 39). This pathway, which is called the alternative pathway, is considered to play an essential role in secondary lymphoid organ development and adaptive immunity (9, 39). Another

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specific function of IKKα is to control gene expression by direct translocation into the nucleus, which appears to be important in epidermal differentiation and craniofacial morphogenesis (1, 41, 46). These functional diversities of IKKα and IKKβ, as well as the variations in extra- and intracellular signaling that lead to the activation of each IKK, may provide information on the numerous biological roles of these molecules with respect to NF-κB.

As NF-κB is especially important for the immune system, the constitutive activation of NF-κB is associated with inflammatory diseases (20, 23). Furthermore, aberrant NF-κB activation leads to tumorigenesis via antiapoptotic gene expression (19, 20). Thus, the detailed analysis of this signaling in disease states will be useful for therapy development. Although *H. pylori* persistently infects the human stomach and activates NF-κB in gastric tissues, the way in which it activates NF-κB is not well understood. Furthermore, the subunit of IKK complex that is involved in *H. pylori*-induced NF-κB activation and the effects of these molecules on gastric disease remain to be resolved. To achieve a better understanding of NF-κB signaling in *H. pylori*-related gastric disease, we examined the role of IKK in *H. pylori*-infected gastric cancer cells.

MATERIALS AND METHODS

Cell line and *H. pylori* strains. Cells of the AGS human gastric cancer cell line (ATCC CRL-1739) were maintained at 37°C in 5% CO_2 in Ham's F12 medium that was supplemented with 10% fetal bovine serum. TN2, a type I *H. pylori* isolate, and its isogenic mutants, TN2-Δ*cagA*, TN2-Δ*cagE*, TN2-ΔPAI, and TN2-Δ*vacA*, were maintained under microaerophilic conditions in Brucella broth that was supplemented with 5% horse serum (15, 26). The bacterial strains were centrifuged at 3,500 × *g* for 5 min at 4°C and washed with phosphate-buffered saline (PBS), and the concentrations were estimated, using an optical density at 560 nm (OD_{560}) of 0.1, as 4×10^7 CFU/ml *H. pylori*.

The AGS cells were washed once with PBS, incubated in fresh medium, and then infected with *H. pylori* at a multiplicity of infection (MOI) of 100, except in indicated instances.

Plasmids and small interfering RNA (siRNA). Dominant-negative IKKα, IKKβ, and its empty vector pRK5 were kindly donated by D. Goeddel (26). The reporter plasmids, pNF-κB-Luc and pRL-TK, have also been described previously (26). RNA oligonucleotides for silencing IKKα (5′-GCAGGCUCUUUC AGGGACA-3′), IKKβ (5′-GGUGAAGAGGUGGUGGUGAGC-3′), TAK1 (5′-UGGCUUAUCUUACACUGGA-3′) (42), and the nonsilencing control (5′-UUCUCCGAACGUGUCACGU-3′) with two thymidine residues (dTdT) at the 3′ end were synthesized together with their corresponding antisense RNAs and were annealed (QIAGEN, Hilden, Germany).

Antibodies and reagents. Human lymphotoxin (LT) α1/β2 and human tumor necrosis factor alpha (TNF-α) were purchased from R&D Systems (Minneapolis, MN). As a positive control, 50 ng/ml LT α1/β2 or 10 ng/ml TNF-α was added to the culture medium. Polyclonal anti-phospho-IκB-α (Ser32), anti-phospho-JNK (Thr183/Tyr185), and phospho-NFκB2 p100 (Ser864) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The polyclonal anti-IKKα, anti-IKKβ, anti-p50, and anti-TF-IID antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and the monoclonal anti-IKKα and polyclonal anti-p100/p52 antibodies were from Upstate Biotechnology (Lake Placid, NY). The monoclonal anti-TRAF2 antibody was purchased from BD Biosciences (San Jose, CA), the polyclonal anti-TAK1 antibody was from StressGen Biotechnologies Corp. (Victoria, Canada), and the monoclonal anti-actin antibody was from Sigma (St. Louis, MO).

Transfection and reporter assays. In the RNA interference experiments, AGS cells were seeded in tissue culture plates 24 h before transfection and grown to 30 to 50% confluence. The siRNA oligonucleotides were introduced at a concentration of 100 nM into the cells using Lipofectamine (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Forty-eight hours after siRNA transfection, the cells were washed and infected with *H. pylori* for the indicated time.

In the reporter analysis, AGS cells were seeded in 12-well tissue culture plates and transfected with 50 ng pNF-κB-Luc, 10 ng pRL-TK, and 400 ng pRK or the dominant-negative IKK vector for 24 h. Where indicated, siRNA oligonucleotides were transfected as described above 24 h before transfection of the reporter plasmids. The cells were supplemented with fresh culture medium and infected with *H. pylori* for 8 h. Luciferase activity was measured and calculated from cell lysates as described previously, and the results are represented as fold induction compared to the control in three independent experiments.

Immunoblot analysis. For the preparation of total cell lysates, AGS cells that were treated with the indicated siRNAs and infected with *H. pylori* for different time periods were washed once with cold PBS and lysed in ice-cold Triton X-100 buffer (50 mM Tris/HCl [pH 7.6], 1% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatants were stored as total cell lysates. For the preparation of nuclear and cytosolic extracts, AGS cells were seeded in a 6-cm dish, transfected with the indicated siRNAs, and infected with *H. pylori*. The cells were washed with Tris-buffered saline (TBS), suspended in 200 μl of Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75% Nonidet P-40, 1 mM dithiothreitol, protease inhibitor cocktail [Roche Molecular Biochemicals]), incubated on ice for 3 min, and centrifuged at 1,500 × *g* for 4 min at 4°C. The supernatant was removed and used as the cytosolic extract. The pellet was washed once with Buffer A without Nonidet P-40 and centrifuged as described above, followed by resuspension in 50 μl Buffer C (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol, protease inhibitor cocktail) and incubation on ice for 10 min with frequent mixing. Finally, the suspension was centrifuged at 14,000 × *g* for 10 min at 4°C and the supernatant was used as the nuclear extract. Equal amounts of cell lysates and extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was probed with the indicated primary antibody followed by the horseradish peroxidase-conjugated secondary antibody and developed using the ECL plus kit (Amersham, Buckinghamshire, United Kingdom). The protein levels of phospho-IκB-α, phospho-JNK, IKKα, and p100/52 were determined by densitometry using KODAK 1D Image Analyzer software and normalized with the level of actin, TF-IID, or TRAF2.

Immunofluorescence. AGS cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) and infected with *H. pylori* for the indicated time. The cells were washed twice with PBS, fixed in 2% paraformaldehyde for 30 min, washed with PBS, and permeabilized with 0.2% Triton X-100 for 1 h. After blocking with 10% normal goat serum, the cells were incubated overnight at 4°C with the polyclonal anti-IKKα antibody diluted in PBS. The cells were then washed three times with PBS and incubated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 h. The nuclei were visualized by staining with propidium iodide. Images were obtained using the LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkohen, Germany).

TUNEL assay. To investigate the effect of *H. pylori* infection on cell apoptosis, we used the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay. AGS cells, which were treated with control or IKK siRNAs in Lab-Tek chamber slides, were maintained in serum-free medium for 24 h and infected with *H. pylori* at an MOI of 100 for a further 8 h. The cells were washed three times with PBS, and apoptotic cells were stained with Apoptag (Serologicals Inc., Norcross, GA) in accordance with the manufacturer's instructions. Apoptotic cells were visualized by fluorescein isothiocyanate, and the nuclei were stained with propidium iodide, followed by microscopic examination with the LSM510 confocal laser scanning microscope. The number of apoptotic cells, in a total of 1,500 to 2,000 cells in each well, was counted in three independent experiments, and the percentage of apoptotic cells was calculated.

Quantification of chemokines by ELISA. The interleukin-8 (IL-8) and GROα concentrations in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) as specified by the manufacturer (Techne, Minneapolis, MN). AGS cells were plated in 24-well plates, transfected with siRNAs for 48 h, and infected with *H. pylori* for a further 8 h. The culture supernatants were then aspirated and stored at −70°C until they were subjected to the ELISA. The concentrations of IL-8 and GROα were determined using standard curves obtained with the respective recombinant proteins. The values are represented as the averages ± standard deviations (SD) of three independent experiments.

Microarray procedures. For RNA preparation, AGS cells were transfected with control or IKK-specific siRNAs for 48 h. The cultures were supplemented with fresh medium and subsequently infected with *H. pylori* for 3 h. The RNA was extracted using Isogen (Wako, Osaka, Japan), the samples were treated with DNase for 1 h, and then the samples were purified using an RNA purification kit (QIAGEN). The cDNA microarray analysis was performed according to the manufacturer's instructions using the Human Chip Oligo DNA Microarray

(DNA Chip Consortium, Hokkaido, Japan), which contains approximately 29,000 open reading frame oligo probes. Briefly, 5 μg of total RNA was amplified using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX). Antisense RNA (5 μg) from control or experimental samples, e.g., unstimulated versus infected or control siRNA-transfected versus IKK siRNA-transfected, were labeled with Cy5 or Cy3, respectively. The two fluorescently labeled probes were mixed and applied to a microarray, followed by incubation under humidified conditions at 60°C overnight. Fluorescent images of the hybridized microarrays were scanned with a fluorescence laser confocal slide scanner (Affymetrix 428 Array Scanner; Santa Clara, CA). The images were analyzed using the ImaGene 4.2 software (Bio-Discovery, Marina Del Rey, CA) according to the manufacturer's instructions. To control for labeling differences and to reduce hybridization errors, all of the reactions were carried out in duplicate, whereby the fluorescent dyes were switched.

Quantitative real-time PCR. RNA was prepared as described above from AGS cells that were infected with *H. pylori* for the indicated time. For quantitative PCR, cDNA was prepared using a combination of oligo(dT), random primers, and the Impron II Reverse Transcription System (Promega). Each PCR was carried out in triplicate in a 25-μl volume that contained the SYBR Green Master mix (Applied Biosystems, Foster City, CA) and using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The following PCR conditions were used: 15 min at 95°C for the initial denaturation, followed by 45 cycles of 95°C for 30 s and 60°C for 30 s. Relative quantification of gene expression was performed using GAPDH mRNA as the internal standard. Two independent experiments were performed with similar results, and a representative was shown. The oligonucleotide primers for IL-8, A20, c-IAP2, MCL1, and survivin have been described previously (12, 35, 42, 48). The primer sequences for other genes, which were designed using the Primer Express software (Applied Biosystems), were as follows: XIAP sense, 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′, and antisense, 5′-CCGCACGGTATCTCCTTCA-3′; GADD45β sense, 5′-CAC GCTCATCCAGTCCTTCTG-3′, and antisense, 5′-CCGACACCCGCACGAT-3′; BCL10 sense, 5′-TTTTTTGAGACAGTCTTGCTCTATCG-3′, and antisense, 5′-AGCATGGGAGGCAGAAGTTG-3′.

Statistical methods. Statistical analysis was performed using the Student's *t* test, two sided, and Dunnett's post hoc tests for multiple comparisons. Differences were considered statistically significant with $P < 0.05$.

RESULTS

Role of IKKs on NF-κB signaling in *H. pylori*-infected AGS cells. To investigate the roles of IKKα and IKKβ in *H. pylori*-infected AGS cells, we initially performed a reporter assay for NF-κB-dependent transcription using kinase mutant forms of IKKα and IKKβ. As reported previously (11, 26), cotransfection of dominant-negative IKKs decreased *H. pylori*-mediated NF-κB reporter activity (Fig. 1A), which indicates that the overexpression of dominant-negative IKKα or dominant-negative IKKβ inhibits NF-κB activation. We also assessed the effect of IKK gene silencing on this signaling pathway. The siRNAs for IKKα, IKKβ, or nonsilencing control RNA were transfected in AGS cells, and NF-κB reporter activity was analyzed with or without *H. pylori* infection. Similar to the effect of the dominant-negative molecules, IKKα and IKKβ gene silencing decreased by 50% the *H. pylori*-induced NF-κB reporter activation (Fig. 1B).

We then performed immunoblots for phosphorylated IκBα to reveal the upstream event that leads to NF-κB activation. As shown in Fig. 1C, IKKβ silencing dramatically reduced *H. pylori*-mediated IκBα phosphorylation. In contrast, IKKα silencing had a very limited effect on IκBα phosphorylation, although the siRNA for IKKα apparently decreased the IKKα protein level. These siRNAs for IKKs had only slight effects on *H. pylori*-induced JNK phosphorylation. Thus, we believe that IKKα is involved in the NF-κB signaling activation induced by *H. pylori* through a mechanism that is distinct from IκB phosphorylation, which is transduced via IKKβ activation.

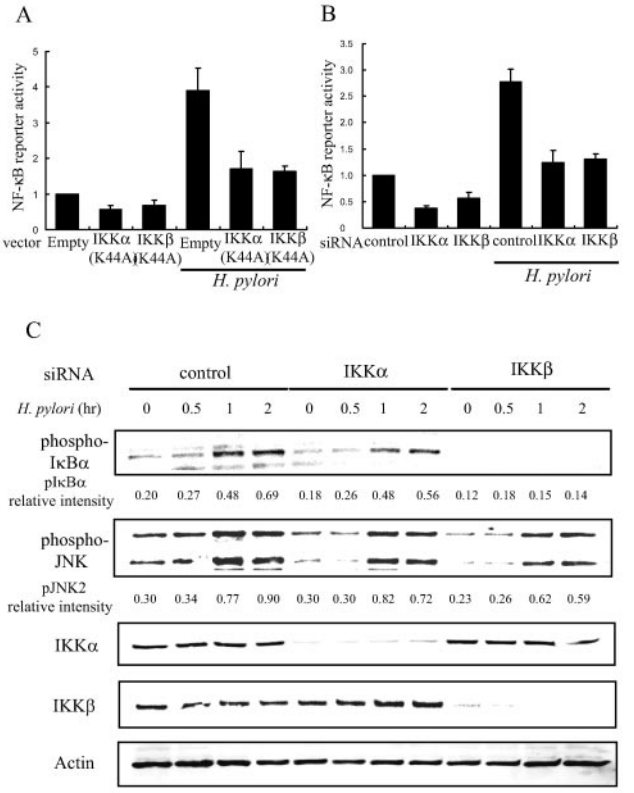

FIG. 1. IKKα and IKKβ are involved in *H. pylori*-induced NF-κB activation. (A) The kinase mutant expression vector for IKKα or IKKβ, or the empty vector, was transfected into AGS cells together with the NF-κB reporter and internal control plasmids. After 24 h, the cells were infected with *H. pylori* or left unstimulated for 8 h. NF-κB reporter activation was measured. The values shown are the means ± SD; $n = 3$. (B) AGS cells were transfected with siRNA oligonucleotides for IKKα, IKKβ, or the nonsilencing control for 24 h, followed by transfection of the reporter and control plasmids. The cells were then infected with *H. pylori* or left unstimulated for 8 h. NF-κB reporter activation is shown, as described for panel A. (C) AGS cells were treated with siRNA for the control or IKKs. The cells were serum starved for 24 h and then infected with *H. pylori* for the indicated time. Cell lysates were analyzed by immunoblotting with the indicated antibodies. The protein levels of phospho-IκBα or phospho-JNK were measured and normalized with that of actin. A representative of three independent experiments with similar results is shown.

Nuclear translocation of IKKα, but not p100 processing, is induced by *H. pylori* infection of AGS cells. Recent studies on IKKα or IKKβ knockout cells have revealed the specific functions of IKKs in cytokine signaling. To elucidate the role of IKKα in *H. pylori* infection, we examined the IKKα-specific signaling pathway, namely the processing of p100, and the nuclear translocation of IKKα. As reported for other cell lines (9), LT induced both IκBα and p100 phosphorylation in AGS cells (Fig. 2A). In LT-treated cells, the p100 level gradually decreased and that of p52 increased, which indicates that LT activates the NF-κB alternative pathway in this cell line. In contrast, *H. pylori* infection induced only IκBα phosphorylation. Neither p100 phosphorylation nor processing to p52 was observed in *H. pylori*-treated cells (Fig. 2A). This suggests that the alternative pathway of

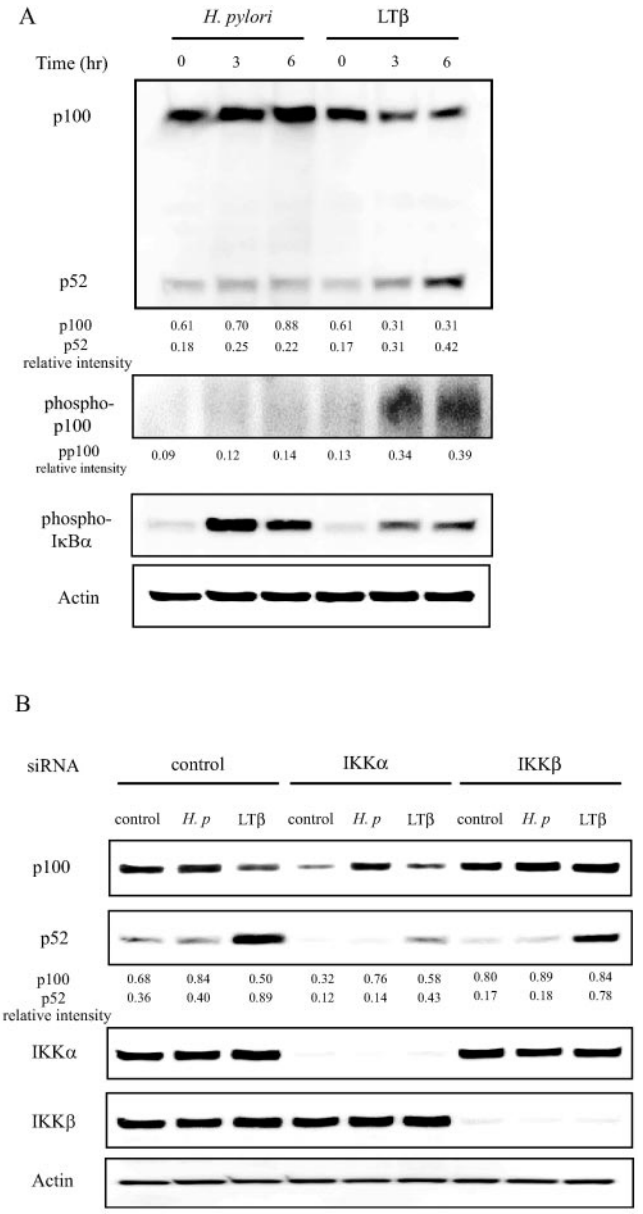

FIG. 2. *H. pylori* does not induce p100 processing to p52 in AGS cells. (A) AGS cells were treated with *H. pylori* or LT (50 ng/ml) for the indicated time. The cell lysates were analyzed by immunoblotting with the indicated antibodies. The protein levels of p100 and p52 relative to that of actin were determined. (B) AGS cells were transfected with siRNA oligonucleotides for the control, IKKα, or IKKβ and then treated with *H. pylori* or LT for 6 h. The cell lysates were analyzed by immunoblotting as described for panel A. *H. p*, *H. pylori*.

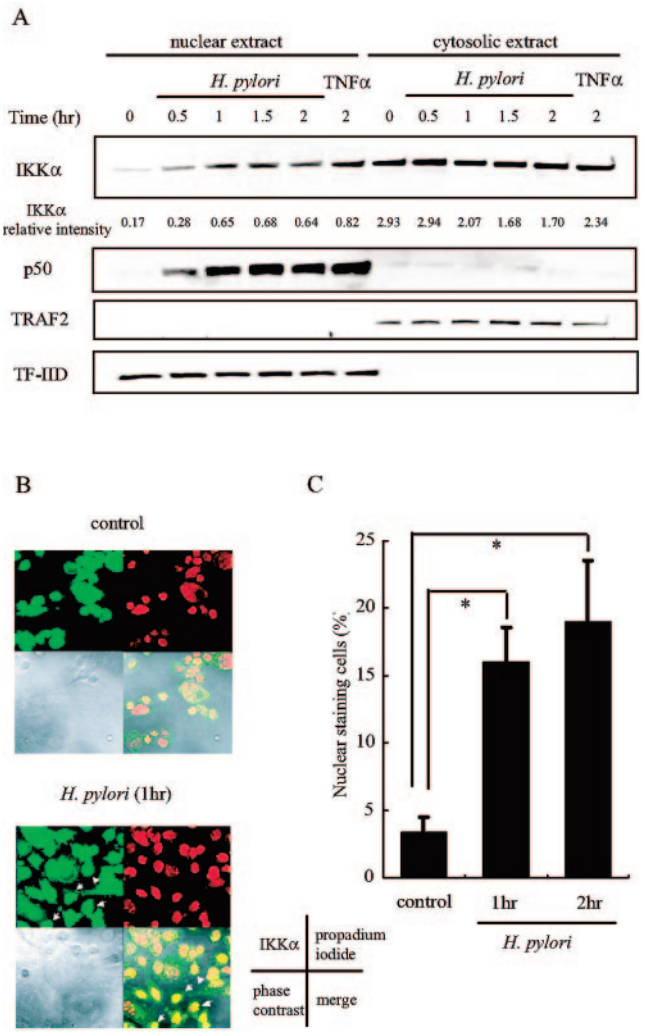

FIG. 3. *H. pylori* infection induces IKKα nuclear translocation in AGS cells. (A) AGS cells were infected with *H. pylori* for the indicated time or treated with TNF-α for 2 h. The cells were fractioned into nuclear and cytosolic extracts. Aliquots of these extracts were analyzed by immunoblotting for IKKα and p50. Antibodies directed against TRAF2 and TF-IID were used to verify the integrity of the fractionation procedure and to ensure equal loading. A representative of three independent experiments with similar results is shown. (B) AGS cells seeded in 4-well chamber slides were left untreated or were infected with *H. pylori* for 1 h. The cells were immunostained with the anti-IKKα antibody and visualized by staining with Alexa Fluor 488. The nucleus was stained with propidium iodide. Phase-contrast images and merged images are shown. Arrows indicate the cells with IKKα nuclear staining. (C) AGS cells were infected with *H. pylori* for the indicated time, and IKKα nuclear translocation was assessed by immunofluorescence. The percentages of cells with IKKα nuclear staining cells are calculated from the observation of 300 cells in three independent experiments and are indicated as the means ± SD. *, $P < 0.05$.

NF-κB activation, which includes p100 processing to p52, is not induced by *H. pylori* in AGS cells. To confirm these findings, we also investigated the effect of *H. pylori* infection or LT treatment on IKK-silenced cells. As shown in Fig. 2B, p52 protein induced by LT was severely reduced by IKKα siRNA but not by IKKβ siRNA, which demonstrates the essential role of IKKα in the alternative pathway. In contrast, *H. pylori* infection did not increase the p52 protein level in any cell type, although the basal p52 protein level was slightly reduced in IKK-silenced cells. We also found that the p100 protein level was increased significantly in *H. pylori*-infected IKKα-silenced cells but not in IKKβ-silenced cells. This result also indicates that p100 is a target gene of NF-κB in *H. pylori*-infected cells, especially via the IKKβ-dependent classical pathway. Collectively, these results clearly demonstrate that *H. pylori* does not

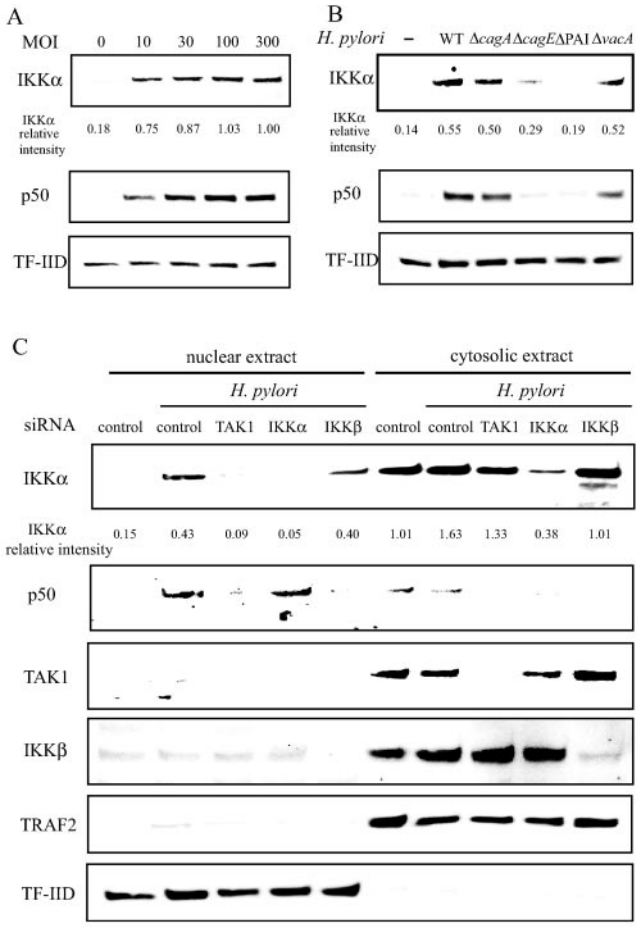

FIG. 4. Factors associated with *H. pylori*-induced IKKα nuclear translocation. (A) AGS cells were infected with *H. pylori* at the indicated MOI for 2 h. The nuclear extracts were immunoblotted for IKKα, p50, and TF-IID. (B) AGS cells were infected with various isogenic mutants of *H. pylori* strain TN2 at an MOI of 100 for 2 h. Immunoblotting of nuclear extracts was performed as described for panel A. (C) AGS cells were transfected with siRNA for the nonsilencing control, TAK1, IKKα, or IKKβ for 48 h and subsequently infected with *H. pylori* for 2 h. The nuclear and cytosolic fractions were extracted and analyzed by immunoblotting. WT, wild type.

induce IKKα-dependent p100 phosphorylation or its processing to p52 in AGS cells, despite p100 induction via the IKKβ-dependent classical pathway.

We also investigated whether *H. pylori* induces IKKα nuclear translocation. Nuclear and cytosolic fractions of *H. pylori*-infected cells were analyzed by immunoblotting for IKKα. As shown in Fig. 3A, *H. pylori* induced IKKα nuclear accumulation in a time-dependent manner. Nuclear accumulation of IKKα was observed 30 min after infection and increased for 1.5 h, after which the level remained the same. The time course of IKKα nuclear translocation was similar to that of p50 nuclear translocation.

We also performed immunofluorescence staining to confirm IKKα nuclear translocation. In uninfected AGS cells, IKKα was localized, mainly in the cytosol. However, upon infection, nuclear staining of IKKα was observed in about 15 to 20% of the cells (Fig. 3B and C). These results indicate that *H. pylori* activates IKKα and induces its nuclear translocation but does not induce p100 processing in AGS cells.

Factors associated with *H. pylori*-induced IKKα nuclear translocation. *H. pylori* activates the intracellular signaling pathways of epithelial cell lines in MOI-dependent and *cag* PAI-dependent manners (21, 22, 26). Thus, we investigated whether these bacterial factors also affect IKKα nuclear translocation. AGS cells were infected with *H. pylori* at the indicated MOI for 2 h, and nuclear extracts were subjected to immunoblotting for IKKα. As shown in Fig. 4A, IKKα nuclear accumulation was observed in cells that were infected with *H. pylori* at an MOI of 10. The levels of IKKα and p50 in the nucleus increased in relation to increases in the infection ratio, up to an MOI of 100.

We also investigated the roles of bacterial virulence factors in IKKα nuclear translocation using *cagA*, *cagE*, *cag* PAI, and *vacA* mutant strains. Immunoblot analysis revealed that gene disruption of *cagE* or *cag* PAI reduced IKKα nuclear accumulation (Fig. 4B). In contrast, in *cagA* and *vacA* mutant-infected cells we observed almost the same level of nuclear IKKα and p50 as in wild-type-infected cells. These results indicate that the *cag* PAI molecular transportation system is required for

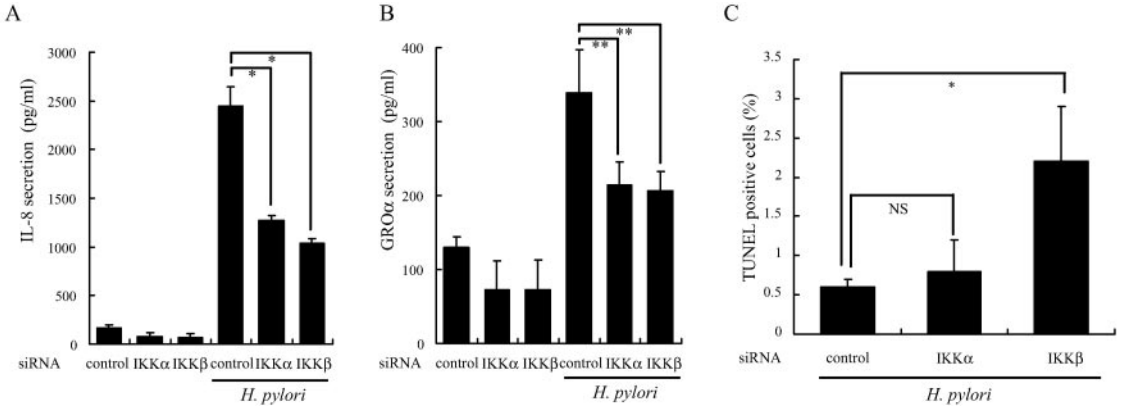

FIG. 5. The roles of IKKα and IKKβ in *H. pylori*-induced inflammatory and antiapoptotic responses. (A and B) AGS cells were transfected with the indicated siRNAs for 48 h and were cultured with or without *H. pylori* for 8 h. The culture supernatants were assayed for IL-8 (A) or GROα (B) by ELISA. The values are the means ± SD from three independent experiments. *, $P < 0.01$; **, $P < 0.05$. (C) AGS cells were transfected with the indicated siRNAs, serum starved for 24 h, and subsequently infected with *H. pylori* for 8 h. The numbers of TUNEL-positive cells were calculated from more than 1,500 cells per slide in three independent experiments. The values are the means ± SD. *, $P < 0.05$; NS, not significant.

IKKα activation. Although the CagA and VacA proteins are bacterial cytotoxins that enter epithelial cells (32, 36), these bacterial toxins themselves do not induce either NF-κB activation or IKKα nuclear translocation.

We then assessed the upstream signaling event for IKK complex activation. Several studies have revealed that TAK1 transduces cytokine signaling to the IKK complex. Therefore, we used siRNAs for TAK1 and IKKs to examine the importance of these molecules for *H. pylori*-induced IKKα nuclear translocation. As shown in Fig. 4C, TAK1 silencing reduced the nuclear translocation of IKKα as well as that of p50. In contrast, IKKβ silencing had no effect on IKKα localization while p50 nuclear translocation was severely inhibited. These results indicate that TAK1 is an important signaling intermediate for *H. pylori*-induced NF-κB activation, which bifurcates upstream of the stimulus to both IKKα and IKKβ.

The role of IKKs in *H. pylori*-induced epithelial cell responses. It has been reported that NF-κB activation induced by *H. pylori* mediates cytokine production and antiapoptosis in gastric epithelial cells. Therefore, we assessed whether IKKα activation in gastric cells affects these cellular responses. IL-8 production by *H. pylori*-infected AGS cells was measured by ELISA. In the control cells, approximately 2,400 pg/ml IL-8 was produced after 8 h of *H. pylori* infection. However, cells treated with the IKKα or IKKβ siRNA showed severely decreased levels of IL-8 production. In the IKKα-silenced cells, *H. pylori* induced about 1,200 pg/ml IL-8, which was approximately half the level induced in the control cells (Fig. 5A). Another chemokine observed in *H. pylori*-infected gastric mucosa, GROα, has also been reported to have chemotactic activities for neutrophils (47). The production of GROα by *H. pylori*-infected cells was also reduced by IKKα or IKKβ silencing (Fig. 5B). These results indicate that both IKKα and IKKβ are necessary for chemokine production.

We also assessed the role of each IKK on cellular apoptosis. Using TUNEL staining, we evaluated the effect of *H. pylori* infection on serum starvation-induced apoptosis. In control siRNA-transfected cells, about 0.6% of the cells were apoptotic (Fig. 5C). The percentage of apoptotic cells was similar after IKKα silencing. However, in the case of IKKβ silencing, *H. pylori* infection enhanced the apoptosis of AGS cells (2.2% ± 0.7%; $P < 0.05$ compared to control transfected cells). These results indicate that the antiapoptotic effect of *H. pylori* is transduced mainly through IKKβ activation.

The role of IKKs in *H. pylori*-induced gene transcription. We next investigated the IKKα target genes in *H. pylori*-infected AGS cells. Cells that were treated with control or IKK-specific siRNAs were infected with *H. pylori* for 3 h, and the transcriptional profiles were determined by microarray analysis. In the control oligonucleotide-transfected AGS cells, *H. pylori* infection up-regulated 181 out of 21,000 genes. The 181 genes included those for immune responses, antiapoptosis, and signal transduction; representative genes are shown in Table 1. Using siRNA, we found 15 of the genes with enhanced expression were down-regulated more than 20% by IKKα silencing, and 25 of the genes were down-regulated by IKKβ silencing (Table 2). Interestingly, 12 out of 15 of the IKKα-regulated genes were identical to IKKβ-regulated genes. These results, based on microarray experiments, indicate that most of the IKKα target genes in *H. pylori*-infected AGS cells are similar to IKKβ target genes, which are activated via the NF-κB classical pathway. In addition, it appears that the induction of several genes, such as NF-κB1 and CXCL2, requires signaling via

TABLE 1. Identification of genes that are enhanced by *H. pylori* infection[a]

Gene type and accession no.	Gene name	Fold change
Apoptosis-related genes		
NM_001165	cIAP2(BIRC3)	5.3
NM_014350	GG2-1	3.7
NM_003806	HRK(harakiri)	2.9
NM_021960	MCL1	2.1
Signal transduction-related genes		
NM_020529	IκBα (NFKBIA)	2.7
NM_021913	AXL	2.2
NM_002401	MAP3K3	1.7
NM_005043	MAP2K7	1.7
Immune response-related genes		
NM_000584	IL-8	7.8
NM_001511	CXCL1	6.3
NM_002089	CXCL2	2.3
NM_002090	CXCL3	3.5
NM_003811	TNFSF9	1.7
Regulation of transcription		
NM_002729	HHEX	3.1
NM_003998	NF-κB 1 (p105)	2.6
NM_006509	RelB	2.4
NM_138714	NFAT5	2.4
NM_001300	COPEB	2.2
NM_001730	KLF5	1.8
NM_002228	JUN	1.8
NM_005902	MADH3	1.7
NM_006147	IRF6	1.5
Cell surface receptor		
NM_000640	IL13RA2	5.1
NM_005534	IFNGR2	1.9
NM_000201	ICAM1	1.8
NM_012211	ITGA11	1.6
G-protein-coupled receptor protein		
NM_002082	GPRK6	4.3
X64980	HTPCRX02	3.4
NM_005274	GNG5	1.9
NM_005308	GPRK5	1.8
Proteolysis and peptidolysis		
NM_002421	MMP1	3.7
NM_002543	OLR1	1.8
NM_002425	MMP10	1.8
Cytoskeleton-related genes		
NM_012334	MYO10	2.7
X13100	Myosin heavy chain	2.7
NM_000526	KRT14	1.6
NM_005557	KRT16	1.6
Others		
NM_000041	APOE	14.6
NM_030952	SNARK	9.6
NM_014172	PHP14	4.3
NM_000499	CYP1A1	3.2
NM_002526	NT5E	3.2
NM_004583	RAB5C	2.4
NM_000527	LDLR	2.4
NM_001731	BTG1	2.4
NM_002999	SDC4	2.2
NM_001142	AMELX	2.1

[a] Microarray analysis was performed in duplicate, as described in Materials and Methods. The genes shown in this table are representative genes that were up-regulated more than 1.5-fold in both arrays. For each functional category of genes, the GenBank accession number, the common name, and the mean signal ratio are indicated.

TABLE 2. Genes regulated by IKKs in *H. pylori*-infected AGS cells[a]

Gene type and accession no.	Gene name	Ontology	Fold induction in control cells	% Down-regulation by: IKKα siRNA	% Down-regulation by: IKKβ siRNA
Genes suppressed in both IKK silencing cells					
NM_002421	MMP1	Proteolysis and peptidolysis	3.7	43	57
NM_000584	IL-8	Immune response	7.8	55	67
NM_001165	cIAP2 (BIRC3)	Antiapoptosis	5.3	33	49
NM_001511	CXCL1	Immune response	6.3	37	34
NM_002090	CXCL3	Immune response	3.5	41	30
NM_002575	SERPINB2	Antiapoptosis	3.3	42	54
NM_000201	ICAM1	Cell surface receptor	1.8	29	35
NM_002597	PDC	Electron transport	1.9	34	37
BC039669	RIKEN cDNA 1810046K07	Unknown	1.5	30	30
NM_002999	SDC4	Unknown	2.2	27	38
NM_014350	GG2-1	Antiapoptosis	3.7	26	47
NM_003900	SQSTM1	Tyrosine kinase ligand binding	2.1	21	24
Genes suppressed in IKKα silencing cells					
NM_000526	KRT14	Cell organization	1.6	39	1
NM_002659	PLAUR	Cell surface receptor	1.6	27	0
L77608	clone SEL289 17q YAC	Unknown	2.2	25	0
Genes suppressed in IKKβ silencing cells					
NM_002638	SKALP	Unknown	2.1	ND	61
NM_002089	CXCL2	Immune response	2.3	10	51
AK001903	cDNA FLJ11041 fis	Unknown	13.9	−36	39
AK090485	FLJ00409 protein	Unknown	2.1	ND	52
NM_004864	PLAB	Cell-cell signaling	1.9	ND	56
NM_030952	SNARK	Protein phosphorylation	9.6	ND	43
NM_002543	OLR1	Proteolysis and peptidolysis	1.8	15	43
NM_005534	IFNGR2	Cell surface receptor	1.9	ND	30
NM_020529	IkBa (NFKBIA)	Signal transduction	2.7	8	33
NM_003998	NF-kB1 (NFKB1)	Regulation of transcription	2.6	13	27
NM_021913	AXL	Signal transduction	2.2	19	37
NM_001730	KLF5	Regulation of transcription	1.8	−6	32
NM_002729	HHEX	Regulation of transcription	3.1	ND	23

[a] Microarray analysis was performed on RNA samples from *H. pylori*-infected AGS cells, which were treated with the control or IKK-specific siRNA. Among the genes induced by *H. pylori* infection (Table 1), those genes that were suppressed >20% by IKK siRNA treatment in duplicate were considered to be IKK-regulated genes. The GenBank accession number, the common name, the average fold change in control siRNA transfected cells and the mean inhibition rate by IKKs are shown. The 30% inhibition level represents 0.7-fold expression in IKK-silenced *H. pylori*-infected cells. ND represents data that were not available, owing to the absence of results from at least one microarray.

IKKβ, but not via IKKα, for stimulus-dependent transcriptional activation.

To confirm the microarray data and to evaluate sequential changes in gene induction, we performed real-time PCR for several genes. As shown in Fig. 6, the expression of IL-8 (A), cIAP2 (B), and MCL1 (C) was up-regulated by *H. pylori* infection but was effectively inhibited by the siRNAs for either IKK. These results are in accordance with the microarray data. A20 (D), which negatively regulates NF-κB activity and is involved in antiapoptosis, was also up-regulated by *H. pylori* infection in a time-dependent manner and down-regulated by IKK silencing. A20 induction was not observed in the current microarray experiments, although it was observed in a previous study (28). In contrast to these *H. pylori*-inducible genes, other antiapoptotic genes were not affected (E to H). Thus, IKKα and IKKβ appear to be equally involved in *H. pylori*-induced antiapoptotic gene expression, although the antiapoptotic phenotypes observed in cells silenced for individual IKK subunits were different (Fig. 5C).

DISCUSSION

In this report, we have examined the roles of IKKα and IKKβ in *H. pylori*-infected gastric cancer cells. Both of these kinases are involved in NF-κB activation and inflammatory cytokine production. IKKβ is considered to act as a physiological IκB kinase during *H. pylori* infection, while IKKα does not have this activity. Our results reveal that *H. pylori* induces the nuclear translocation of IKKα, which may be one of the important roles of IKKα in gastric cancer cells. Chemokine expression induced by *H. pylori* infection was repressed by both IKK siRNAs, although the antiapoptotic effects were abrogated only in IKKβ-silenced cells. Thus, IKKα and IKKβ seem to regulate independent cell responses through different mechanisms of NF-κB activation in *H. pylori*-infected gastric cancer cells.

Although both IKKα and IKKβ were discovered as stimulus-dependent kinases of IκB that are structurally related to each other, their roles in cell biology may be different. IKKβ is

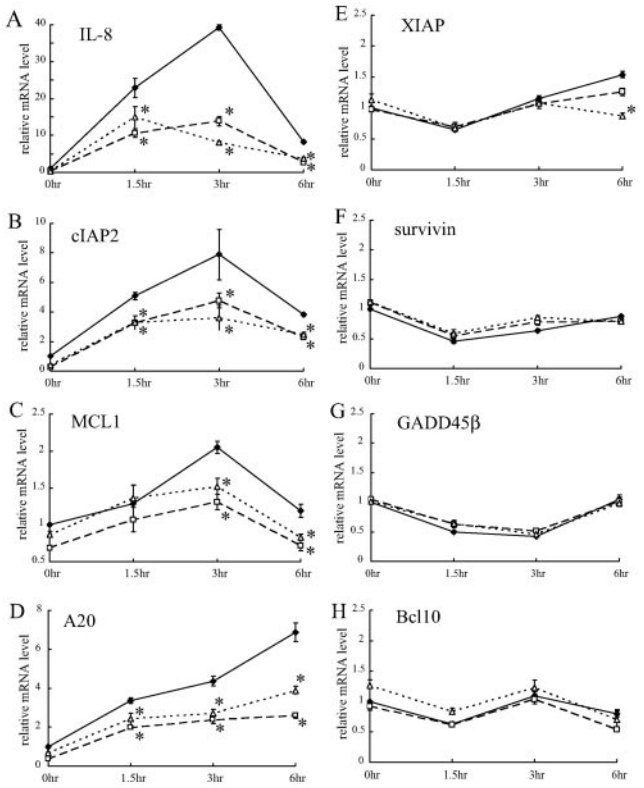

FIG. 6. Sequential analysis of *H. pylori*-induced gene expression in IKK-silenced cells. (A to H) AGS cells were transfected with the indicated siRNAs for 48 h and were then infected with *H. pylori* for 0, 1.5, 3, and 6 h. The cDNA prepared from these cells was investigated for the indicated gene expression relative to GAPDH by real-time PCR. The reaction was performed in triplicate, and the data are shown as the means ± SD. Reverse transcription-PCR was performed from two independent infection experiments with similar results. The black diamond (◆) represents the control siRNA-transfected cells, and the open square (□) and open triangle (△) represent IKKα and IKKβ siRNA-transfected cells, respectively. ∗, $P < 0.05$ versus control siRNA-transfected cells.

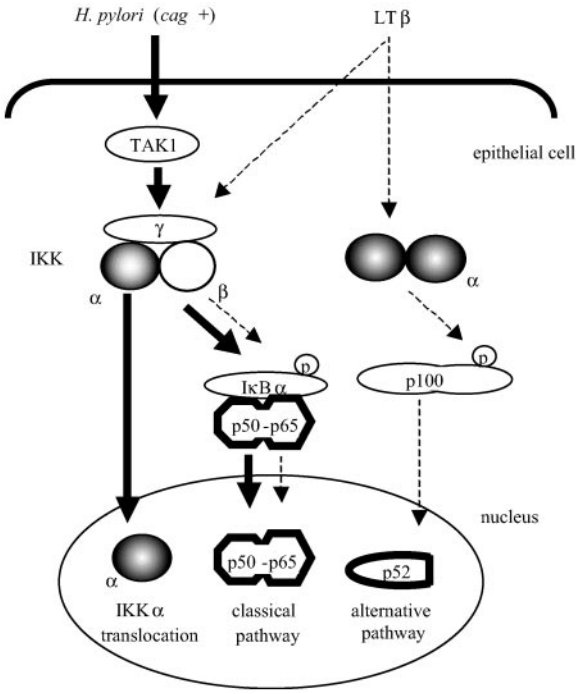

FIG. 7. Schematic representation of the role of IKK in human gastric cancer cells. The signalings activated by *H. pylori* are indicated with a boldface solid line, and those induced by LT are indicated with a broken line. *cag*-positive *H. pylori* activated TAK1 and IKK complex, followed by a classical pathway activation via IKKβ and by IKKα nuclear translocation in AGS cells. In contrast, LT induced classical pathway activation as well as alternative pathway activation through p100 processing.

considered to be an essential signal transducer in cytokine-mediated NF-κB activation, thereby promoting cell survival and preventing apoptosis (24, 25, 49). However, in our analysis of *H. pylori*-infected cells, not only the siRNA for IKKβ but also the siRNA for IKKα reduced NF-κB reporter activity. Therefore, we investigated the role of IKKα in the NF-κB pathway in AGS cells, especially with respect to IKKα-specific signaling. We found that *H. pylori* induces the nuclear translocation of IKKα, which was first reported in TNF-α-treated cells (1, 46). Similar to the classical NF-κB activation mode (21, 26), IKKα nuclear translocation is induced by *H. pylori* in *cag* PAI- and MOI-dependent manners. Many bacterial components, such as peptidoglycan, lipopolysaccharide, and flagellin, are known to target cellular receptors, called Toll-like receptors, and to induce IκBα phosphorylation and NF-κB activation (reviewed in references 17 and 29). However, it has not been established whether these bacterial components induce IKKα nuclear translocation and inflammatory gene expression. Since the nontoxic *H. pylori* *cag* PAI mutant did not induce this type of signaling, the IKKα activation observed for *cag*-positive strains in our experiments is possibly associated with severe gastric disease.

Interestingly, *H. pylori* did not induce the activation of the alternative NF-κB pathway in AGS cells. Recent studies have shown that certain stimuli, such as LT, BAFF, and CD40, induce p100 processing to p52, which then translocates into the nucleus together with RelB (3, 9, 39). Furthermore, lipopolysaccharide activates the alternative pathway in pre-B cells or primary dendritic cells (31). In our experiment, LT stimulation induced p100 phosphorylation and increased p52 in the AGS cells. Thus, this cell line has a normal response with respect to alternative pathway signal transduction but is defective for activation of the *H. pylori*-mediated alternative pathway. *H. pylori* also failed to phosphorylate p100 in AGS cells. Collectively, these results indicate that *H. pylori* does not activate IKKα kinase activity in this cell line, in spite of the ability of IKKα to undergo nuclear translocation (Fig. 7). These results also suggest that epithelial cell lines, such as AGS and MKN45, are not stimulated by *H. pylori* lipopolysaccharide to activate either the classical or alternative NF-κB pathway (27). In contrast to the unresponsiveness of epithelial cells, we have found that *H. pylori* induces activation of the alternative pathway in lymphocytes and fibroblasts (34). Thus, it appears that the ability of *H. pylori* to activate the alternative NF-κB pathway is cell type dependent.

Nuclear translocation of IKKα is reported to regulate gene expression by modifying histone function in TNF-stimulated

cells. The kinase activity of IKKα is considered to be essential for this process (1, 46). In contrast, for keratinocyte differentiation and normal morphological development, which are also reported to be dependent on IKKα, the kinase activity is not required, although its nuclear translocation is indispensable (41). In this process, IKKα is associated with the suppression of the fibroblast growth factor family of genes, possibly via an indirect mechanism (41). Since it is difficult to determine the essential role of IKKα in vivo, which may depend on the type and strength of the stimulus, the cell type, and cell environment, we have investigated IKKα function in *H. pylori*-infected gastric cells. In our experiments, IKKα appeared to act as a positive regulator of gene expression, thereby resembling IKKβ, since in microarray experiments *H. pylori*-induced expression of chemokines and antiapoptotic genes was repressed to a similar extent by IKKα or IKKβ silencing.

In this study, IKKα nuclear translocation was observed within 30 min of *H. pylori* infection, which is similar to the time required for IκB phosphorylation by IKKβ and which is clearly different from the kinetics of alternative pathway activation by other ligands, which usually takes several hours (9, 31). Furthermore, we have clarified that TAK1 is an important upstream molecule for both IKKα nuclear translocation and IKKβ-dependent p50 nuclear translocation. As TAK1 is reported to be the critical activator of IKK in cytokine stimulation (33, 42), it is possible that TAK1 is the common upstream molecule for the IKKβ-dependent classical pathway and IKKα nuclear translocation in *H. pylori*-infected cells. Furthermore, we found that both IKKs were involved in NF-κB activation and chemokine production in *H. pylori*-infected cells. Thus, we speculate that both IKKα nuclear translocation and IKKβ-dependent IκB phosphorylation are required for sufficient gene expression by *H. pylori* (Fig. 7).

The antiapoptotic responses induced by *H. pylori* seemed to be transduced via IKKβ. To elucidate these IKK phenotypic differences, we carried out a comprehensive and sequential analysis of the antiapoptotic genes in IKK silencing cells. Similar to previous reports on cDNA array experiments of *H. pylori*-induced gene expression, we found that genes associated with immune responses and signal transductions, such as IL-8, CXCL1, CXCL2, IkBa, p105, and ICAM-1, were up-regulated in AGS cells by *H. pylori* infection (7, 13, 28). Most of these genes were shown to be induced by *cag*-positive *H. pylori* infection. This is consistent with our results demonstrating that these genes were suppressed by IKK silencing, as IKK activation by *H. pylori* was dependent on *cag* PAI. Furthermore, it has been reported that *H. pylori* upregulates antiapoptotic genes like MCL1, cIAP2, A20, and GG2.1 (13, 28, 40, 48). We also found a critical role of IKKs in antiapoptotic gene regulation (Table 2). However, in spite of the differences in antiapoptotic effects (Fig. 5C), we could not find the differences in antiapoptotic gene regulation between IKKα-silencing cells and IKKβ-silencing cells. Thus, it is possible that IKKβ affects antiapoptosis not through gene regulation but through other biological processes, such as posttranscriptional modification via its kinase activity. Previous reports on IKKβ knockout cells have shown that the inactivation of NF-κB signaling enhances JNK activity and affects proapoptosis (44). In our experiments using IKK-silenced cells, the enhancement of JNK activity was not apparent. Furthermore, *H. pylori* infection did not enhance the expression of the XIAP gene (Fig. 6E), which is reported to activate JNK. Since our experiments failed to clarify the IKKβ-dependent antiapoptotic mechanism, further investigations of IKKβ will facilitate the understanding of gastric diseases associated with dysregulated apoptosis.

In conclusion, we have investigated the function of IKKα in the *H. pylori* infection model using AGS cells. IKKα is translocated into the nucleus upon infection, and chemokine expression is induced via IKKα. These results suggest that IKKα activation in the gastric mucosa is associated with severe inflammation and inflammation-induced carcinogenesis in vivo, as is IKKβ.

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