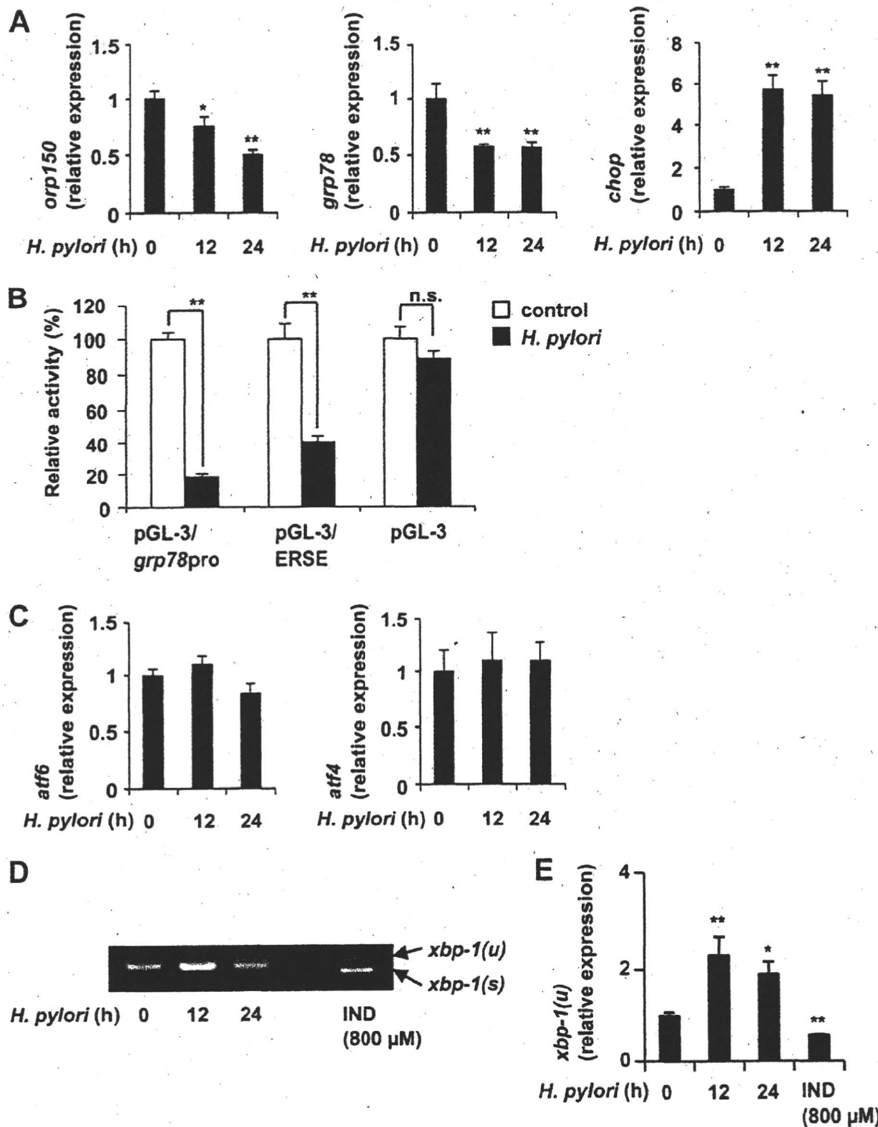

FIGURE 2. **Inhibitory effects of *H. pylori* on transcription of ER stress response-related genes.** AGS cells were co-cultured with *H. pylori* at a bacteria:cell ratio of 200:1 for the indicated periods (*A* and *C–E*) or treated with indomethacin (*IND*) for 24 h (*D* and *E*). *A*, *C*, and *E*, the relative expression of each gene was monitored by real-time RT-PCR using a specific primer for each gene. Values normalized to the *actin* gene are expressed relative to the control sample. *B*, AGS cells were co-transfected with pRL-SV40 (internal control plasmid carrying the *R. reniformis* luciferase gene) and pGL-3 or its derivatives (pGL-3/*grp78*pro and pGL-3/ERSE) and cultured for 24 h. Cells were then co-cultured with or without *H. pylori* at a bacteria: cell ratio of 200:1 for 24 h, and *P. pyralis* luciferase activity was measured and normalized for *R. reniformis* luciferase activity. The 100% value of the *P. pyralis* luciferase activity is 5.4×10^6, 7.4×10^5, or 2.4×10^4 units for pGL-3/*grp78*pro, pGL-3/ERSE, or pGL-3, respectively. *D*, RT-PCR was performed with total RNAs and primer sets for detecting the un-spliced (*xbp-1(u)*) and spliced (*xbp-1(s)*) forms of *xbp-1* mRNA, which were separated by agarose gel electrophoresis. Values are the mean ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

treatment of cells with *H. pylori* cell lysates, but the decrease occurred more slowly than that of ORP150 and GRP78.

As described above, *atf6* mRNA expression was not affected by *H. pylori* (Fig. 2*C*). Thus, either suppression of translation or post-translational modification of ATF6 (such as degradation by proteases) may be responsible for the observed reduction in the level of ATF6 after co-culture of cells with *H. pylori*. To address this issue, we first examined the effect of *H. pylori* on the level of ATF6 in cells pretreated with cycloheximide, an inhibitor of protein synthesis. As shown in Fig. 4*A*, the *H. pylori*-dependent decrease in the level of ATF6 was observed even in cells pretreated with cycloheximide. We also found that the *H. pylori*-dependent decrease in the level of ATF6 was observed for GFP-ATF6, whose expression is regulated by the strong cytomegalovirus promoter (Fig. 4*B*). We also examined the effect of *H. pylori* on the stability of p90-ATF6 by the pulse-chase experiment. As shown in Fig. 4, *C* and *D*, the labeled p90-ATF6 disappeared more rapidly in the presence of *H. pylori* treatment than its absence. These results suggest that post-translational modification of ATF6, such as protein degradation, is responsible for the lower level of ATF6 observed after treatment of cells with *H. pylori*.

In addition to cleavage by S1P and S2P, it is known that ATF6 is continuously degraded by the proteasome-ubiquitin pathway (50, 51). Thus, using specific inhibitors, we examined the contribution of these systems to the *H. pylori*-dependent decrease in the level of ATF6. As shown in Fig. 4*E*, an inhibitor of the proteasome-ubiquitin system, epoxomycin, weakly suppressed the *H. pylori*-dependent decrease in the level of ATF6. On the other hand, an inhibitor of S1P, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), did not affect the level of ATF6 in the presence of *H. pylori* (Fig. 4*F*). Furthermore, we found that inhibitors of lysosomal proteases (pepstatin A (an inhibitor of aspartate proteases) and E-64-d (an inhibitor of cysteine protease)) also weakly suppressed the *H. pylori*-dependent decrease in the level of ATF6 (Fig. 4*G*). Interestingly, combination of epoxomycin and inhibitors of lysosomal proteases resulted in clear suppression of the *H. pylori*-dependent decrease in the level of ATF6 (Fig. 4*H*). The results in Fig. 4 suggest that *H. pylori* decreases the level of ATF6 partly through modulation of its degradation by the proteasome-ubiquitin and lysosomal systems.

We also examined the effect of *H. pylori* on subcellular localization of ATF6 using GFP-ATF6. As shown in Fig. 4*I*, GFP-ATF6 co-localized with GRP94 (ER marker). Although the level of GFP-ATF6 was decreased, the localization of ATF6 was not clearly affected by treatment of cells with *H. pylori* (Fig. 4*I*).

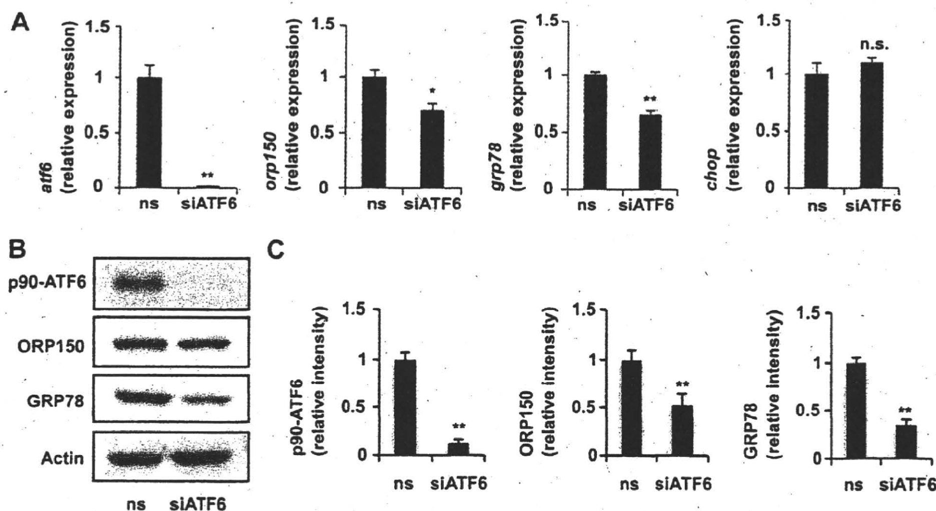

FIGURE 3. **Effect of siRNA for ATF6 on expression of ER chaperones.** AGS cells were transfected with siRNA for ATF6 (*siATF6*) or nonspecific siRNA (*ns*) and were incubated for 48 h (*A*) or 72 h (*B*). The mRNA (*A*) and protein (*B* and *C*) expression was monitored and expressed as described in the legends of Figs. 1 and 2. Values are the mean ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

Effect of H. pylori on the Gastric Ulcerogenic Response and Expression of ER Chaperones Induced by Indomethacin in Mice— To evaluate the *in vivo* relevance of our *in vitro* observation that *H. pylori* suppress the expression of ER chaperones, we first examined the effect of oral inoculation of *H. pylori* on the expression of ER chaperones at gastric mucosa. We monitored by real-time RT-PCR analysis the mRNA expression of ER chaperones and CHOP after administration of indomethacin and/or *H. pylori* in gastric tissues. Oral inoculation of *H. pylori* to mice suppressed the background (without indomethacin administration) expression of *orp150* and *grp78* mRNAs but not *chop* mRNA (Fig. 5*A*). Indomethacin administration up-regulated the expression of *orp150*, *grp78*, and *chop* mRNAs, whereas the expression of *orp150* and *grp78* mRNAs but not that of *chop* mRNA was significantly suppressed by prior administration of *H. pylori* (Fig. 5*A*). Immunohistochemical analyses also demonstrated that oral inoculation with *H. pylori* decreased the levels of ORP150 and GRP78 at gastric mucosa in both the presence and absence of indomethacin administration (Fig. 5*B*). We consider that the staining of ORP150 and GRP78 in Fig. 5*B* is specific, because no positive staining was observed without a primary antibody (supplemental Fig. S2). We also performed immunoblotting analysis, and suppression of the gastric expression of ORP150 and GRP78 by inoculation with *H. pylori* in both the presence and absence of indomethacin treatment was confirmed (Fig. 5, *C* and *D*). Indomethacin-induced expression of GRP78 and CHOP was also confirmed (Fig. 5, *C* and *D*). Furthermore, we found that the gastric level of p90-ATF6 was decreased by inoculation with *H. pylori* in both the presence and absence of indomethacin treatment (Fig. 5, *C* and *D*). We also found that there is a tendency that inoculation with *H. pylori* decreases the levels of ORP150 and GRP78 in the presence of indomethacin treatment in the small intestine but not other organs (supplemental Fig. S3*A*).

The observation that inoculation with *H. pylori* reduces the gastric expression of ER chaperones suggests that this inoculation stimulates protein aggregation in ER in gastric cells. To address this idea, we examined the effect of *H. pylori* inoculation on the gastric level of connexin 43, which is known to be degraded by ER-associated degradation when it is aggregated in the ER (52, 53). As shown in supplemental Fig. S3, *B* and *C*, the gastric level of connexin 43 was decreased by *H. pylori* inoculation, suggesting that *H. pylori* stimulate aggregation of the protein in ER.

The effect of pre-administration of *H. pylori* on the development of gastric lesions after oral administration of indomethacin was examined. Administration of indomethacin produced gastric lesions, and this lesion production was significantly enhanced by pre-administration of *H. pylori* (Fig. 6*A*). Administration of *H. pylori* alone did not significantly produce gastric lesions under the conditions used (Fig. 6*A*). Histological analysis with H&E staining also supported the notion that indomethacin-produced gastric lesions are exacerbated by pre-administration of *H. pylori* (Fig. 6*B*).

As mentioned above, gastric mucosal cell death plays an important role in the formation of NSAID-induced gastric lesions. We, therefore, examined the effect of pre-administration of *H. pylori* on this process. The level of gastric mucosal cell death was determined by TUNEL assay. An increase in the number of TUNEL-positive cells was observed after indomethacin administration, and this increase was more apparent in mice pre-administered with *H. pylori* than in control mice (Fig. 6*C*). We also examined the effect of *H. pylori* on indomethacin-induced expression of ORP150, GRP78, and p90-ATF6 or apoptosis *in vitro*. Treatment of cells with *H. pylori* decreased the levels of these proteins and increased apoptotic cells in both the presence and absence of indomethacin treatment (supplemental Fig. S4, *A* and *B*). These results suggest that *H. pylori* exacerbate indomethacin-induced gastric lesion formation by stimulating indomethacin-induced gastric mucosal cell death.

The results in Figs. 5 and 6 suggest that *H. pylori* exacerbates indomethacin-induced gastric lesion formation through down-regulation of expression of ER chaperones. To test this idea using a genetic approach, the development of gastric lesions after oral administration of indomethacin was compared between heterozygous ORP150-deficient mice (ORP150$^{-/+}$) and wild-type mice (ORP150$^{+/+}$). Indomethacin-induced gastric lesions were significantly worse in heterozygous ORP150-deficient mice than in wild-type controls (Fig. 7*A*). ORP150 deficiency did not affect the background level of gastric lesions (Fig. 7*A*). Immunohistochemical analyses confirmed that the level of ORP150 in gastric mucosa was lower in heterozygous ORP150-deficient mice than wild-type mice in both the presence and absence of indomethacin administration (Fig. 7*B*). These results show that ORP150 plays an important role

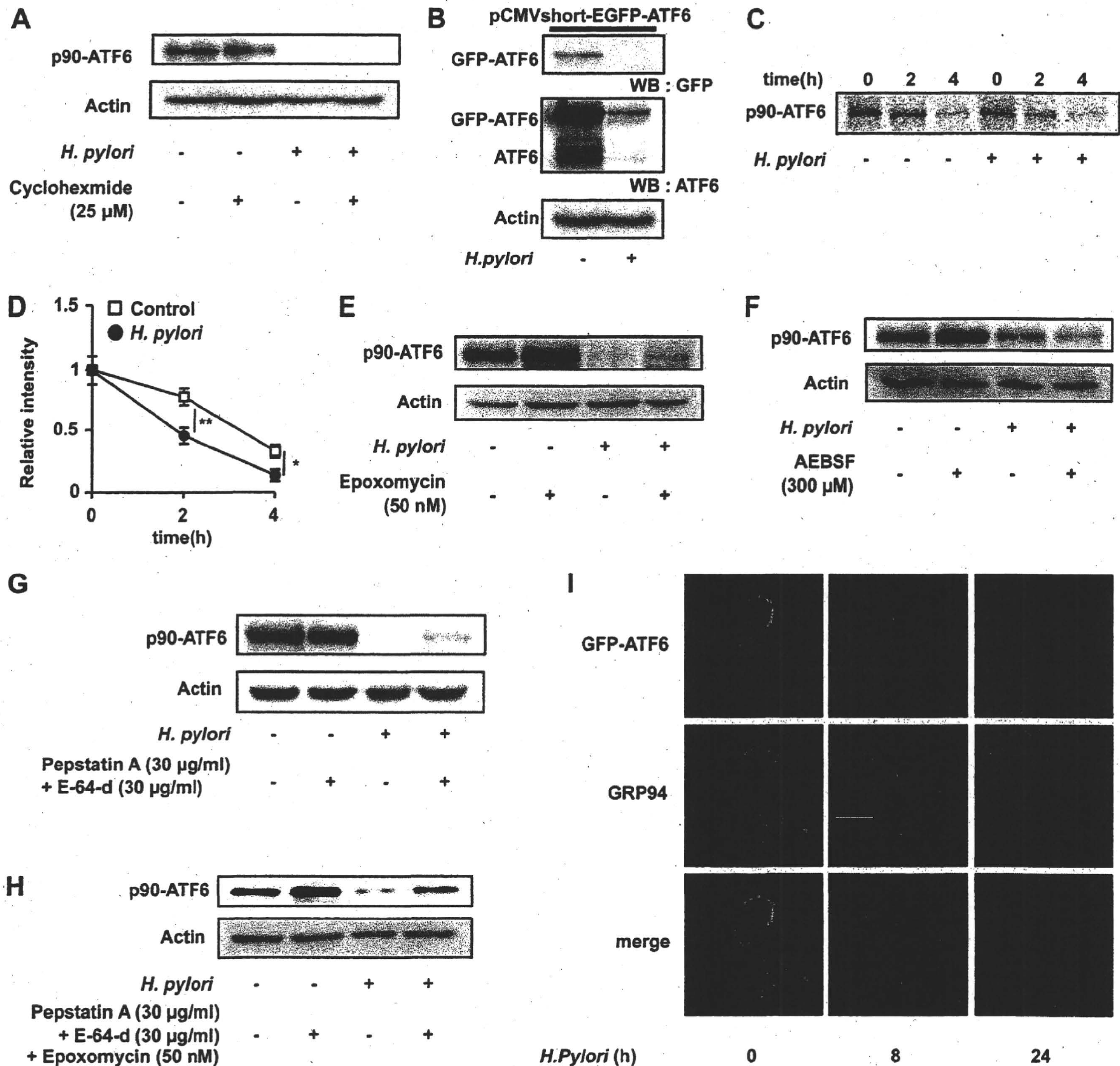

FIGURE 4. **Mechanism for the *H. pylori*-dependent decrease in the level of ATF6.** *A* and *E–H*, AGS cells were pre-incubated with or without each drug for 1 h and further incubated with or without *H. pylori* at a bacteria:cell ratio of 200:1 for 24 h in the presence (*E*, *G*, and *H*) or absence (*A* and *F*) of the same concentration of each drug as in the preincubation step. *B* and *I*, AGS cells were transfected with pCMVshort-EGFP-ATF6α (42) and co-cultured with or without *H. pylori* at a bacteria:cell ratio of 200:1 for 24 h (*B*) or indicated periods (*I*). *A*, *B*, and *E–H*, whole cell extracts were analyzed by immunoblotting (*WB*) with an antibody against GFP, ATF6, or actin. *C*, AGS cells were pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine and then chased with excess amounts of cold methionine and cysteine for the indicated periods in the absence or presence of *H. pylori* at a bacteria:cell ratio of 200:1. Labeled proteins were extracted, immunoprecipitated with antibody against ATF6, subjected to SDS-PAGE, and autoradiographed. *D*, the band intensity of p90-ATF6 was determined and expressed relative to the control. *I*, cells were fixed, stained with antibody against GRP94, and analyzed by confocal laser-scanning fluorescence microscope (magnification, 600 times). Values are the mean ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

in protecting gastric mucosa against indomethacin-induced lesions.

We also examined the indomethacin-induced gastric mucosal cell death in heterozygous ORP150-deficient mice. Indomethacin-induced gastric mucosal cell death was more apparent in heterozygous ORP150-deficient mice than in wild-type mice (Fig. 7*C*). These results suggest that ORP150 protects the gastric mucosa from indomethacin-induced cell death. Combining the results in Figs. 5–7, we consider that *H. pylori* exacerbates indomethacin-induced gastric lesion formation partly through down-regulation of ER chaperones and the resulting stimulation of indomethacin-induced gastric mucosal cell death.

DISCUSSION

There have been contradictory reports about whether infection with *H. pylori* increases the risk of developing NSAID-induced gastric lesions (in other words, whether eradication of *H. pylori* reduces the risk of developing NSAID-induced gastric lesions) (1–6, 54). This may be due to differences in diagnostic criteria (endpoints), standards for patient recruitment, and populations used for these studies. The most we can conclude is

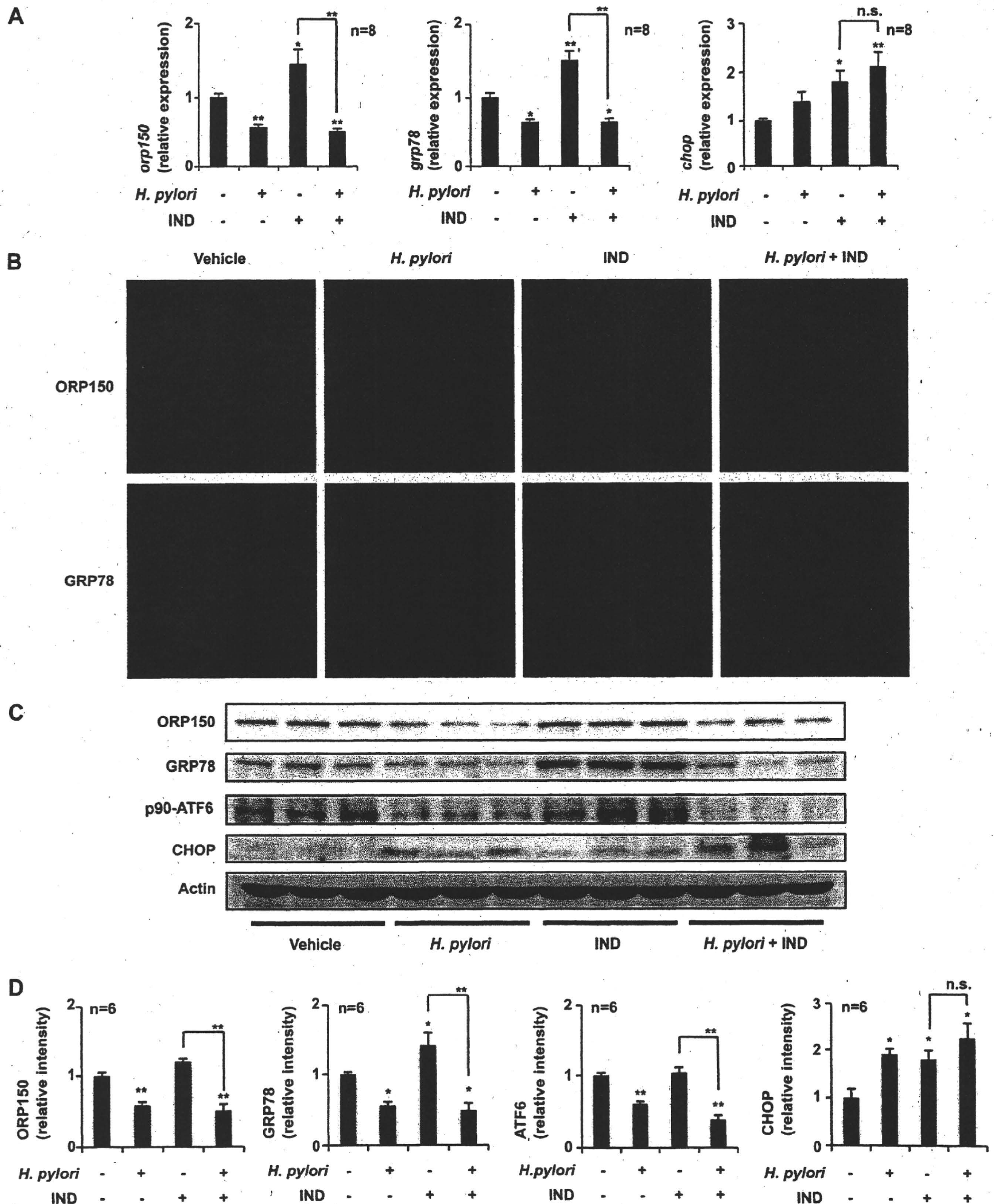

FIGURE 5. **Effect of *H. pylori* on expression of ER chaperones at gastric mucosa.** *H. pylori* were orally inoculated into mice (C57/BL6) at a dose of 2.0×10^8 *H. pylori*/animal every second day for 6 days (total 3 times). One day after the final inoculation, *H. pylori*-inoculated or control mice were orally administered 10 mg/kg of indomethacin (*IND*), and their stomachs were removed after 12 h. *A*, total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the *gapdh* gene are expressed relative to the control sample. *B*, sections of gastric tissues were subjected to immunohistochemical analysis with an antibody against ORP150 or GRP78 and DAPI staining (magnification, 200 times). *C* and *D*, protein expression was monitored and expressed as described in the legend of Fig. 1. Values are given as the mean ± S.E. **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

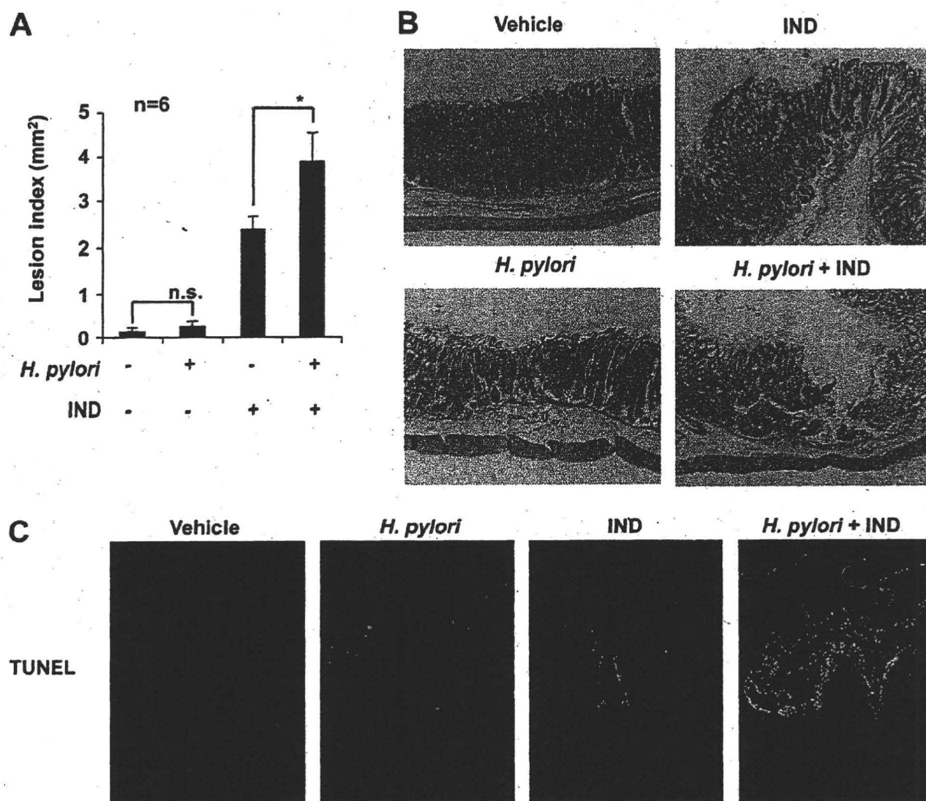

FIGURE 6. **Effect of *H. pylori* on indomethacin-induced gastric lesions and mucosal cell death.** Mice (C57/BL6) were administered *H. pylori* and indomethacin (*IND*), as described in the legend of Fig. 5. *A*, the stomach was scored for hemorrhagic damage. Values are the mean ± S.E. $*p < 0.05$; *n.s.*, not significant. *B*, sections of gastric tissues were subjected to H&E staining (magnification, 200 times). *C*, sections of gastric tissues were subjected to TUNEL assay and DAPI staining (magnification, 200 times). *n.s.*, not significant.

that under certain conditions infection with *H. pylori* increases the risk of developing NSAID-induced gastric lesions. Thus, it is important to examine the effect of *H. pylori* on factors that affect the formation of NSAID-induced gastric lesions. In this study we have focused on ER chaperones and found that co-culture of gastric cells with *H. pylori* decreases the level of ER chaperones. This is the first observation that *H. pylori* affect the expression of ER stress response-related proteins. However, although we used here the transient infection model of *H. pylori*, the infection in humans is chronic. The *H. pylori* strain used in this study does not colonize mice (data not shown), and thus, studies in the future need to be done with strains that do colonize mice.

By using real-time RT-PCR and luciferase reporter assays, we have shown that the *H. pylori*-dependent decrease in the level of ER chaperones *in vitro* is regulated at the level of transcription. Of three ER stress response-related transcription factors (ATF6, ATF4, and XBP-1), only the level of ATF6 (but not the *atf6* mRNA) was decreased by co-culture of cells with *H. pylori*, suggesting that ATF6 is involved in the *H. pylori*-dependent suppression of transcription of ER chaperone genes. Because the *H. pylori*-dependent decrease in the level of ER chaperones was observed in cells whose protein synthesis was inhibited and *H. pylori* decreased the stability of p90-ATF6, post-translational modification (protein degradation) of ATF6 would be involved in this process. Analysis with each inhibitor suggested that the proteasome-ubiquitin system rather than degradation by S1P is involved in this degradation of ATF6. The observation that the level of p50-ATF6 (the proteolytic product of S1P and S2P) did not increase after co-culture of cells with *H. pylori* further supports this notion. We also suggest that protein degradation in lysosomes is involved in this degradation of ATF6. It is known that VacA perturbs endocytic traffic at a late stage (55, 56), and as such it is possible that *H. pylori* affects the traffic of ATF6 to lysosomes and its degradation in lysosomes. Furthermore, because the suppression of *H. pylori*-dependent degradation of ATF6 by inhibitors of proteasomal and lysosomal proteases was weak, other proteases seem to be involved in this degradation.

We found that not only *H. pylori* themselves but also cell extracts of *H. pylori* suppress the expression of ER chaperones *in vitro*. However, the suppression of expression of ER chaperones by cell extracts of *H. pylori* was not as great as that induced by *H. pylori* themselves, and cell extracts of *H. pylori* did not affect the level of ATF6 so distinctly (Fig. 1, *D–G*). Furthermore, the decrease in level of ATF6 occurred more slowly that that of ORP150 and GRP78 in the presence of cell extracts of *H. pylori* (Fig. 1), suggesting that the decrease in levels of ORP150 and GRP78 observed with cell extracts of *H. pylori* is not due to the decrease in levels of ATF6. In other words, results suggest that the mechanism for the decrease in levels of ORP150 and GRP78 is different between *H. pylori* cells and cell extracts of *H. pylori*. We also compared the signal pathway for induction of apoptosis between *H. pylori* cells and cell extracts of *H. pylori*. As shown in supplemental Fig. S5, the decrease in Bax and increase in cytochrome *c* in cytosol fractions (an indicator for mitochondria-mediated apoptosis) and induction of expression of CHOP were not observed with apoptosis induced by cell extracts of *H. pylori* so apparently as that induced by *H. pylori* cells, suggesting that the signal pathway for induction of apoptosis is also different between *H. pylori* cells and cell extracts of *H. pylori*. Although siRNA for ATF6 suppressed the expression of ER chaperones, the extent of suppression was not as apparent as that seen with *H. pylori*. These results suggest that in addition to the mechanism described above (the ATF6-mediated mechanism), an ATF6-independent and as yet unknown mechanism that can be reproduced with cell extracts of *H. pylori* should also be mainly involved in the *H. pylori*-dependent suppression of expression of ER chaperones.

We have previously reported that suppression of expression of GRP78 and ORP150 by siRNA stimulated NSAID-induced

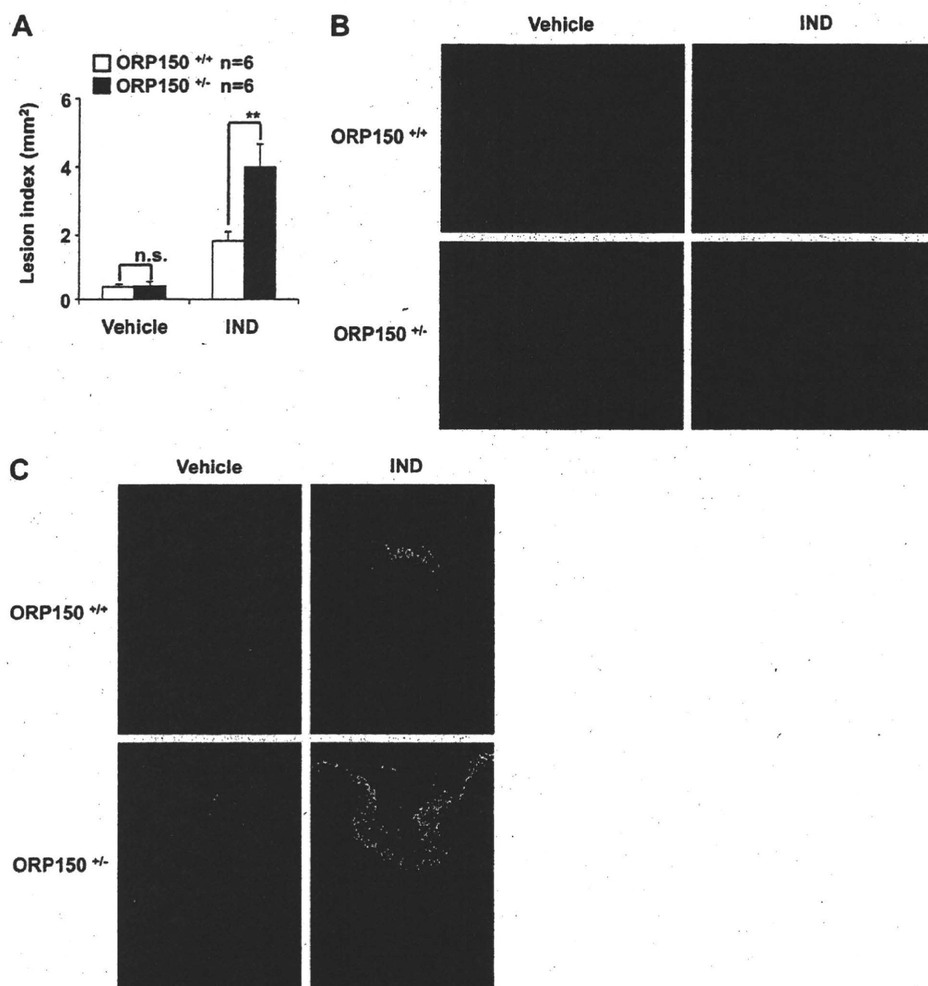

FIGURE 7. **Indomethacin-induced gastric lesions and mucosal cell death in heterozygous ORP150-deficient mice.** Heterozygous ORP150-deficient mice (ORP150$^{+/+}$) and wild-type mice (ORP150$^{+/+}$) were orally administered with 10 mg/kg of indomethacin (*IND*), and their stomachs were removed after 12 h. Gastric lesions (*A*), expression of ORP150 (*B*), and mucosal apoptosis (*C*) were assayed as described in the legends of Figs. 5 and 6. Values are mean ± S.E. **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant. *n.s.*, not significant.

apoptosis in cultured gastric cells (19, 20). We have also suggested that NSAID-induced apoptosis at gastric mucosa plays an important role in the formation of NSAID-induced gastric lesions (16–18). These results suggest that ER chaperones play a protective role against the development of NSAID-induced gastric lesions; however, there is no direct evidence supporting this notion. In this study we have shown that heterozygous ORP150-deficient mice display phenotypes sensitive to indomethacin-induced gastric mucosal cell death and gastric lesion formation. This is the first genetic evidence that an ER chaperone is protective against NSAID-induced gastric lesion formation. This result also suggests that inducers of ER chaperones may be therapeutically beneficial against NSAID-induced gastric lesions, as is the case for heat shock proteins inducers (18, 57).

The *in vitro* observation that expression of ER chaperones is suppressed by *H. pylori* suggests that *H. pylori* would suppress the expression of GRP78 and ORP150 at the gastric mucosa and stimulate NSAID-induced gastric mucosal cell death and lesion formation. In fact, we have shown that pre-inoculation of mice with *H. pylori* not only suppresses the expression of ER chaperones but also stimulates NSAID-induced cell death and gastric lesion formation.

We also showed *in vitro* that co-culture of gastric cells with *H. pylori* up-regulates the expression of CHOP, suggesting that this up-regulation is involved in the *H. pylori*-dependent stimulation of NSAID-induced cell death. There are two possible mechanisms that could explain this up-regulation of CHOP. One is that *H. pylori* directly affects the expression of CHOP. However, because siRNA for ATF6 did not up-regulate the expression of CHOP, *H. pylori*-dependent degradation of ATF6 must not be involved. The other possibility is that this up-regulation is a result of the suppression of expression of ER chaperones, as we previously reported that suppression of expression of GRP78 and ORP150 by siRNA induces the expression of CHOP in the presence of NSAIDs (19, 20).

Although we suggest that the *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesion formation is mediated by the suppression of expression of ER chaperones, various other mechanisms are likely to be involved in this exacerbation. For example, cytotoxic proteins produced by *H. pylori*, such as VacA and CagA, which are known to induce apoptosis in gastric cells (32–36), may stimulate indomethacin-induced cell death, resulting in exacerbation of indomethacin-induced gastric lesions. CagA disrupts the epithelial apical junction complex (58), which may also be involved in *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesions. We believe that this animal model for *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesion formation will be useful in future studies for examining the relationship between *H. pylori* and NSAIDs and their involvement in the production of gastric lesions.

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