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Helicobacter pylori stimulates host cyclooxygenase-2 gene transcription: critical importance of MEK/ERK-dependent activation of USF1/-2 and CREB transcription factors

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Summary

Cyclooxygenase-2 (COX-2) represents the inducible key enzyme of arachidonic acid metabolism and contributes to the pathogenesis of gastroduodenal ulcers and gastric cancer. *Helicobacter pylori* infection is associated with elevated gastric COX-2 levels, but the mechanisms underlying *H. pylori*-dependent *cox-2* gene expression are unclear. *H. pylori* stimulated *cox-2* mRNA and protein abundance in gastric epithelial cells *in vitro* and *in vivo*, and functional analysis of the *cox-2* gene promoter mapped its *H. pylori*-responsive region to a proximal CRE/Ebox element at –56 to –48. Moreover, USF1/-2 and CREB transcription factors binding to this site were identified to transmit *H. pylori*-dependent *cox-2* transcription. Activation of MEK/ERK1/-2 signalling by bacterial virulence factors located outside the *H. pylori cag* pathogenicity island (*cagPAI*) was found to mediate bacterial effects on the *cox-2* promoter. Our study provides a detailed description of the molecular pathways underlying *H. pylori*-dependent *cox-2* gene expression in gastric epithelial cells, and may thus contribute to a better understanding of mechanisms underlying *H. pylori* pathogenicity.

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Introduction

Gastric *Helicobacter pylori* infection is associated with a variety of disorders including chronic gastritis, peptic ulcer disease, mucosa-associated lymphatic tissue (MALT) lymphoma and gastric adenocarcinoma (Ernst, 1999; Scheiman and Cutler, 1999; Megraud, 2001; Peek and Blaser, 2002). The main feature of gastric *H. pylori* pathogenicity is a chronic inflammatory infiltrate, which is characterized by enhanced release of proinflammatory cytokines, growth factors and reactive oxygen metabolites (Crabtree, 1998; Ernst, 1999; Ernst *et al*., 2001; Peek and Blaser, 2002). Moreover, recent studies have suggested that increased expression of the *cox-2* gene represents an important step in *H. pylori* pathogenicity (Fu *et al*., 1999; McCarthy *et al*., 1999; Jackson *et al*., 2000), although the molecular pathways through which the bacterium influences *cox-2* gene expression have not yet been defined.

COX-2 is the inducible key enzyme of arachidonic acid metabolism (Williams *et al*., 1999), and the end-products of its enzymatic activity have been identified as critical regulators of fundamental biological processes including parturition, T-cell development, inflammation, wound healing and proliferation (Williams *et al*., 1999; Smith *et al*., 2000). COX-2 enzymatic activity is largely regulated through *de novo* protein synthesis, which is controlled to a major extent through transcriptional activation of the *cox-2* gene (Williams *et al*., 1999; Smith *et al*., 2000; Howe *et al*., 2001). In the stomach, elevated COX-2 levels were found in *H. pylori*-associated gastritis, mucosal stress lesions, gastroduodenal ulcers and after ischaemia/reperfusion damage (McCarthy *et al*., 1999). In addition, studies in rodents using COX-2-selective inhibitors demonstrated that COX-2-derived prostanoids are critical for the healing of ulcerative mucosal lesions (McCarthy *et al*., 1999; Jackson *et al*., 2000; Halter *et al*., 2001). In *H. pylori* gastritis, increased COX-2 expression has been postulated to be part of the mucosal protective

response against microbial infection, a mechanism that has also been observed in *Salmonella* infection of intestinal cells (Eckmann *et al*., 1997; DuBois *et al*., 1998). Moreover, COX-2-derived prostanoids have been suspected to shift T-cell-mediated responses of the intestinal mucosa after lipopolysaccharide challenge towards immunosuppression (Newberry *et al*., 1999), supporting the concept that COX-2 is part of regulatory circuits controlling mucosal immune responses triggered by external factors (Morteau, 1999). In addition to its role in benign gastric diseases, COX-2 also appears to contribute to the pathogenesis of gastric cancer and its metastasis. Gastric adenocarcinomas and premalignant mucosal lesions frequently overexpress the *cox-2* gene (Ristimaki *et al*., 1997; Lim *et al*., 2000; Sung *et al*., 2000), and elevated COX-2 levels were found to be associated with deeper tumour invasion (Ohno *et al*., 2001) and increased frequency of lymphatic metastasis (Murata *et al*., 1999). In addition, it has been shown that application of COX-2 inhibitors can potently suppress proliferation of gastric cancer cells *in vitro* and *in vivo* (Sawaoka *et al*., 1998a,b). The clinical significance of these observations is further supported by the finding that individuals taking COX inhibitors display a reduced risk of development of gastric carcinoma (Farrow *et al*., 1998). Overall, expression of the *cox-2* gene is regarded as a critical aspect in the pathobiology of inflammatory and malignant gastric diseases and, therefore, clarification of its regulation by *H. pylori* appears to be of special pathobiological relevance.

Interaction of *H. pylori* with mucosal epithelial cells leading to changes in expression and release of inflammatory mediators and growth factors represents a core feature of gastric *H. pylori* infection (Crabtree, 1998; Megraud, 2001; Naumann, 2001). Several studies have demonstrated that the bacterium exerts its effects through activation of distinct epithelial signalling pathways, and that this activation requires the presence of particular bacterial virulence factors (Naumann, 2001). In this context, the *H. pylori cag* pathogenicity island (*cagPAI*) region has been linked to JNK- and NFκB-dependent signalling pathways (Glocker *et al*., 1998; Keates *et al*., 1999; Naumann *et al*., 1999; Foryst-Ludwig and Naumann, 2000), whereas the MEK/ERK cascade has been found to be primarily activated through *cagPAI*-independent mechanisms (Keates *et al*., 1999; Wessler *et al*., 2000). However, the molecular identity of bacterial virulence factors and epithelial signalling pathways mediating the effects of *H. pylori* on gastric *cox-2* gene expression has not yet been elucidated.

Here, we demonstrate that *H. pylori* potently upregulates *cox-2* gene expression in gastric epithelial cells *in vivo* and *in vitro*, and provide a detailed delineation of the underlying molecular mechanisms including epithelial signalling pathways as well as participating *cis*- and *trans*-regulatory factors. Our study identifies for the first time the pathways linking *H. pylori* to *cox-2* gene expression in gastric epithelial cells, and thus helps to uncover further the mechanisms through which the bacterium exerts its pathogenic effects in the human stomach.

Results

Helicobacter pylori *infection stimulates* cox-2 *gene expression in gastric epithelial cells* in vivo *and* in vitro

Oral infection of mice with *H. pylori* resulted in rapid upregulation of mucosal *cox-2* mRNA levels (Fig. 1A, top). Immunohistochemical analysis of COX-2 expression in response to *H. pylori* infection showed that COX-2-positive cells were found in the superficial epithelial layer and epithelial cells located in deeper areas of gastric pits (Fig. 1A, bottom). Within the glandular stomach, there was no detectable difference in the epithelial COX-2 response to *H. pylori* infection, whereas the epithelium of the forestomach did not show upregulation of COX-2 (Fig. 1A, bottom). Although no COX-2-expressing inflammatory cells were found in the murine gastric mucosa at these early stages of infection, at later time points (e.g. 13 weeks after infection), COX-2 expression was detected in epithelial as well as myeloid cells of the submucosa (data not shown). In contrast to these findings after *H. pylori* exposure, gastric infection with *Salmonella typhimurium*, which cannot colonize the stomach permanently, did not alter gastric *cox-2* mRNA expression (data not shown). Similar to the *in vivo* findings, *in vitro* infection of gastric epithelial cell lines (MKN-28, AGS) with *H. pylori* resulted in an immediate and pronounced elevation of *cox-2* mRNA levels that were comparable to the effects of phorbol ester PMA (Fig. 1B, data for AGS cells shown in Fig. 5B), which has been described as a potent stimulus of *cox-2* gene expression (Subbaramaiah *et al*., 2001). These data show that *H. pylori* infection is associated with an immediate upregulation of *cox-2* gene mRNA and protein expression *in vivo* and *in vitro* and strongly suggest that transcriptional activation of the *cox-2* gene represents a major underlying mechanism.

The proximal CRE/Ebox element represents the H. pylori*-responsive region of the* cox-2 *promoter*

To explore whether *H. pylori* is capable of activating the *cox-2* gene promoter in gastric epithelial cells, we performed reporter gene assays using *cox-2* promoter luciferase constructs. We found that *H. pylori* infection potently stimulated *cox-2* promoter activity (three- to fivefold) in AGS (Fig. 2A) and MKN-28 cells (data not shown). Maximal *cox-2* transactivation was obtained at a multiplicity of infection (MOI) of 100 bacteria per cell (Fig. 2A). To identify *cox-2 cis*-regulatory elements mediating the

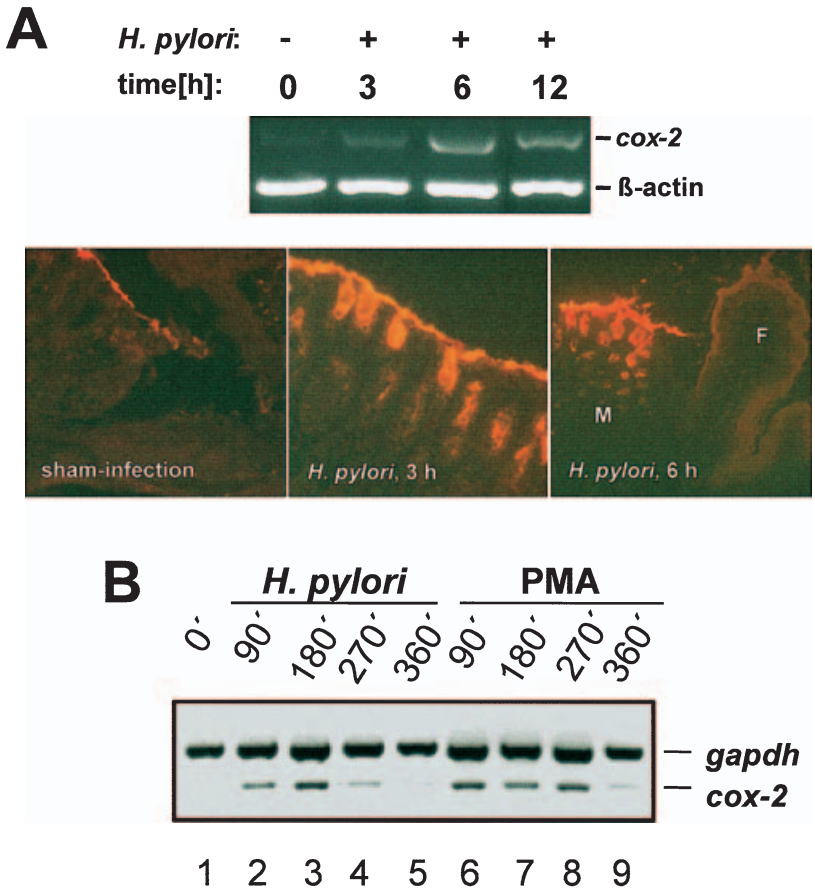

Fig. 1. *H. pylori* infection stimulates *cox-2* gene expression in gastric epithelial cells.
A. Mice were infected with mouse-adapted *H. pylori* strain *Hp*76 or received PBS (control), and *cox-2* mRNA abundance in gastric mucosal scrapings was analysed at the indicated time points (top) using semi-quantitative duplex RT-PCR. To visualize the cell types expressing COX-2 in response to *H. pylori* infection, immunohistochemistry was performed on frozen sections obtained from mouse stomachs using a COX-2-specific antibody. Results shown represent typical findings obtained from three independent experiments. Note the clear discrepancy in *H. pylori*-induced COX-2 abundance between the gastric mucosa (M) and the squamous epithelium of the forestomach (F).
B MKN-28 cells were infected with *H. pylori* or received 10 nM PMA. Cells were lysed at the indicated time points (given in minutes after infection), and *cox-2* and *gapdh* mRNA levels were determined by RT-PCR. The figure shows the results of one typical out of a series of three independent experiments.

effects of *H. pylori*, we performed 5′-deletion analysis of *cox-2* 5′-flanking DNA. Although 5′-deletion down to –69 had virtually no influence on the *H. pylori* responsiveness of the *cox-2* promoter, loss of an additional 18 bp, which contain an overlapping CRE/Ebox element, abrogated the transactivating effect of the bacterium and also strongly reduced basal promoter activity (Fig. 2B). To confirm these results, internal deletion mutants lacking *cox-2*–68/–51 (mutant Δ–68/–51) or *cox-2*–371/–69 (mutant Δ–371/–69) were applied in transfection assays. In agreement with the results obtained in 5′-deletion assays, loss of *cox-2*–68/–51 reduced *H. pylori* responsiveness to background levels (mutant Δ–68/–51), whereas deletion of more 5′ located sequences (mutant Δ–371/–69) had no significant effect (Fig. 2C). After obtaining strong evidence that the proximal *cox-2* promoter region containing the CRE/Ebox is of key importance for *H. pylori*-dependent *cox-2* gene regulation, a fragment containing this element (*cox-2*–66/–38) was subcloned into the enhancerless construct pT81-luc (Nordeen, 1988). The presence of *cox-2*–66/–38 elevated basal activity of the TK minimal promoter present in pT81-luc fivefold (data not shown), and also conferred *H. pylori* and PMA responsiveness (Fig. 2D), demonstrating that this promoter fragment possesses typical features of a 'true' enhancer element. In addition, *H. pylori* and PMA responsiveness of *cox-2* (–66/–38)-luc was strongly diminished by mutating the core sequence of the overlapping *cox-2* CRE/Ebox element, further confirming that this element is essential for transmission of *H. pylori*-dependent effects on the *cox-2* promoter.

USF1, USF2 and CREB transcription factors bind to the proximal cox-2 *CRE/Ebox element*

In order to identify protein(s) binding to the *cox-2*–66/–38 sequence, electrophoretic mobility shift assay (EMSA) analysis was performed. Applying the –66/–38 sequence as a radiolabelled probe, a single nuclear complex (complex 1) was formed (Fig. 3A, lane 1), and time course studies revealed that *H. pylori* infection did not alter the shape or intensity of this complex (data not shown). In competition studies, mutation of the core sequence of the CRE/Ebox abrogated its ability to interact with binding protein(s) (Fig. 3A, lane 3). As this mutant also abrogated basal and *H. pylori*-dependent *cox-2* transactivation in transfection studies (Fig. 2D), the presence of nuclear factors binding to the *cox-2*–66/–38 element is indispensable for the functional integrity of this element.

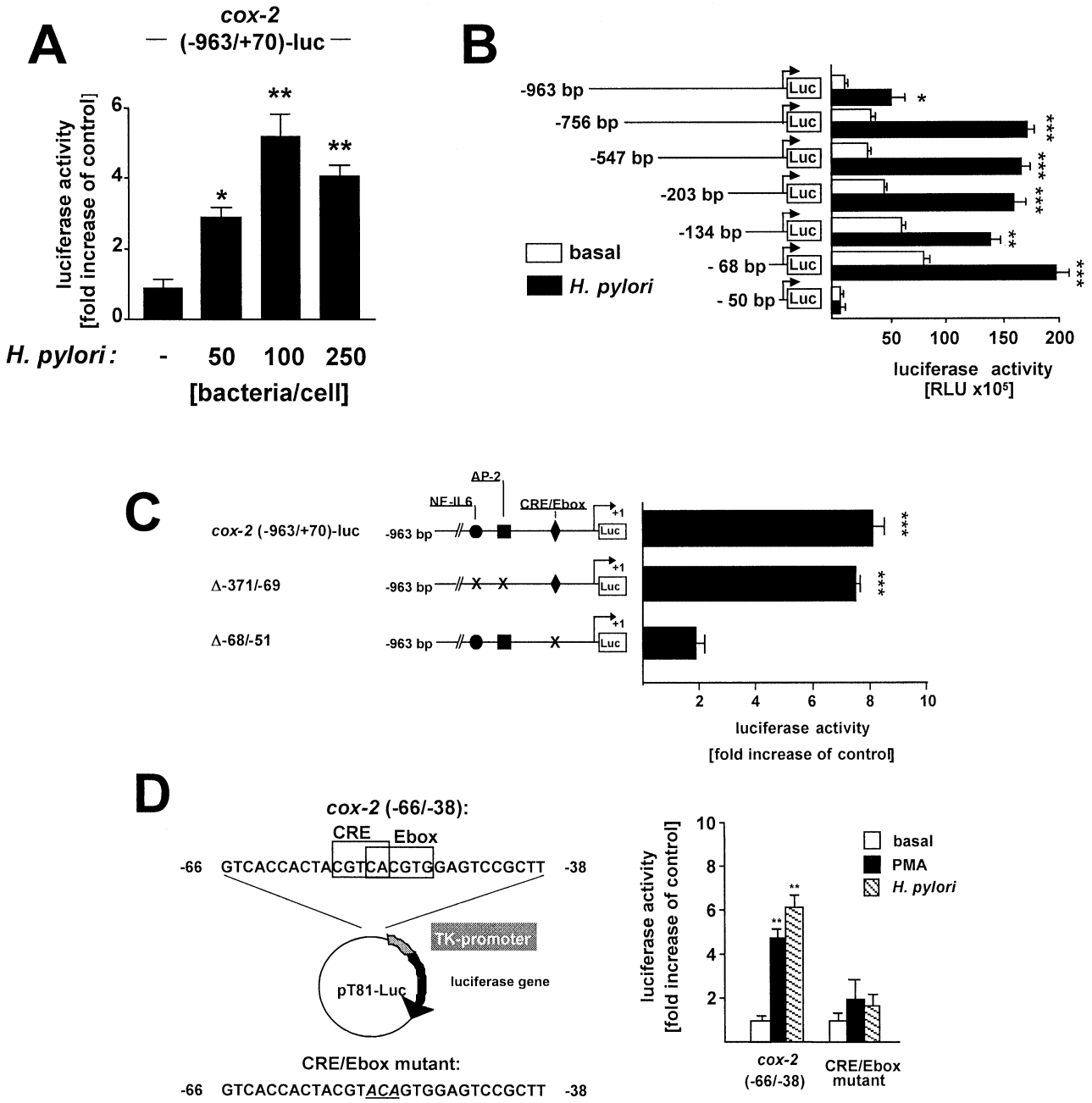

Fig. 2. The proximal CRE/Ebox element represents the *H. pylori*-responsive region of the *cox-2* promoter.
A. AGS cells were transiently transfected with *cox-2* (–963/+70)-luc construct and infected with *H. pylori* at the indicated MOIs. Four hours after inoculation of bacteria, cells were harvested, and lysates were analysed for luciferase activities. Data shown represent the mean ± SEM obtained from three independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).
B. AGS cells were transfected with *cox-2* 5′-deletion constructs and infected with *H. pylori*. Control infections received the diluent PBS. Four hours after inoculation of bacteria, cells were harvested, and lysates were analysed for luciferase activities. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
C. AGS cells were transfected with *cox-2* (–963/+70)-luc construct or the indicated mutant constructs. After transfection, cells were infected with *H. pylori*, whereas control infections received PBS. Four hours after inoculation of bacteria, cells were harvested, and lysates were analysed for luciferase activities. Asterisks indicate statistically significant differences compared with the PBS-treated controls (***$P < 0.001$).
D. AGS cells were transfected with the sequence *cox-2*–66/–38 subcloned into the enhancer-free reporter gene vector pT81-luc yielding construct *cox-2* (–66/–38) or a mutant construct in which nucleotides *cox-2*–53/–51 had been exchanged (CRE/Ebox mutant). After infection with *H. pylori* or treatment with PBS or PMA (10 nM), cells were harvested, and lysates were analysed for luciferase activities (**$P < 0.01$). In all panels, data shown represent a typical result obtained from a series of three independent experiments.

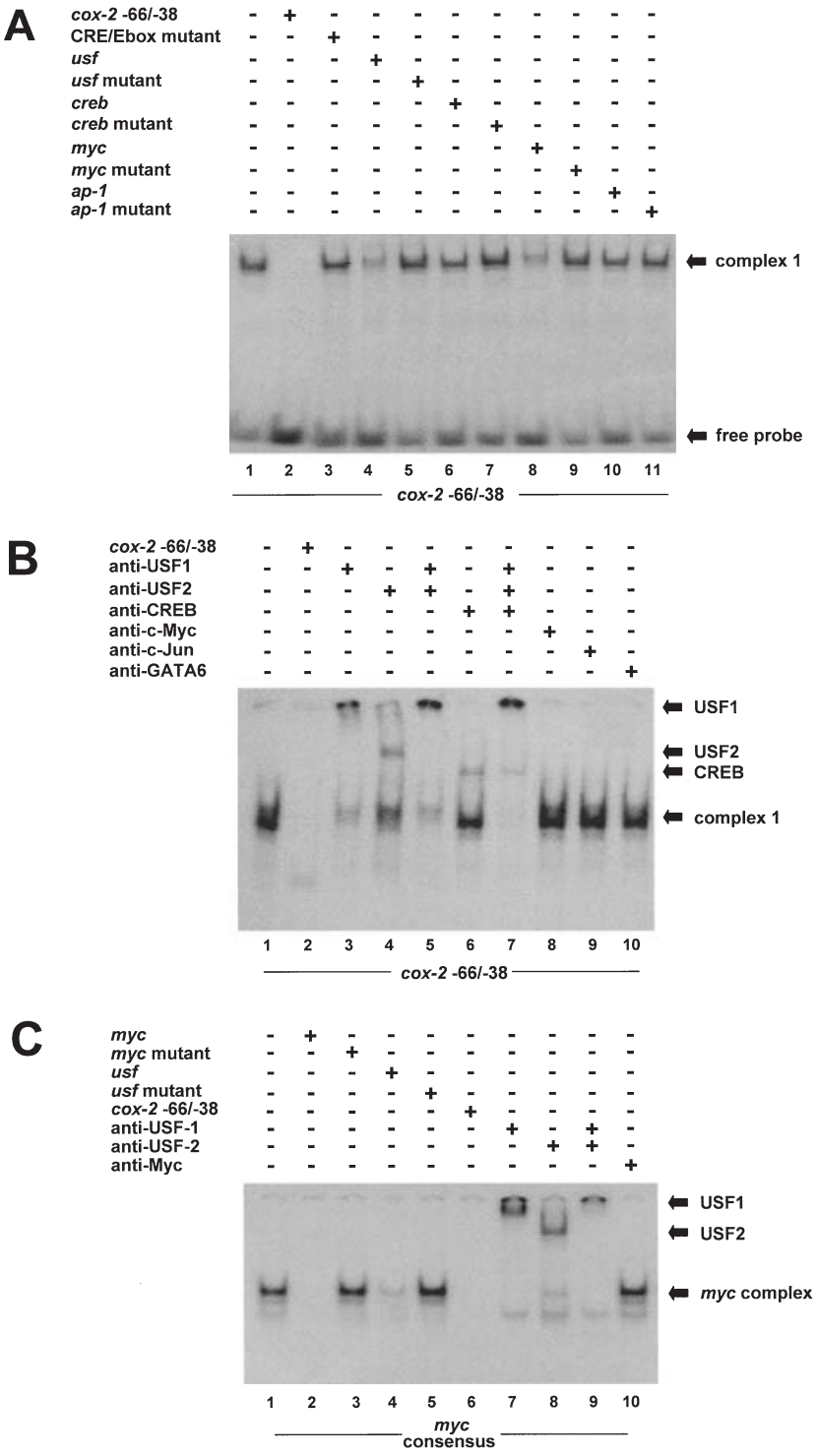

Fig. 3. USF1, USF2 and CREB transcription factors bind to the proximal *cox-2* CRE/Ebox element.
A. Competition studies were performed incubating nuclear extracts with the radiolabelled *cox-2*–66/–38 probe and an excess of unlabelled oligonucleotides as indicated. Mutant oligos served as negative controls. Arrows indicate the major complex obtained with the *cox-2*–66/–38 probe (complex 1) or the localization of unbound radioactive probe.
B. To identify nuclear proteins contained in the complex formed at the *cox-2*–66/–38 probe, antibodies specifically recognizing USF1, USF2, CREB, Myc, c-Jun or GATA6 were used.
C. To investigate the binding of nuclear factors to the Myc consensus binding site, a *myc* oligonucleotide was used as radiolabelled EMSA probe. Competition experiments were performed using unlabelled oligonucleotides as indicated. For supershift analyses, antibodies recognizing USF1, USF2 or c-Myc were applied. Arrows indicate supershifted complexes containing USF1 or USF2 transcription factors or the major complex obtained with the *myc* probe.

To clarify the molecular nature of nuclear proteins binding to the CRE/Ebox element, competition experiments using oligonucleotides encoding consensus binding motifs present within this sequence were performed (Fig. 3A). Application of *usf*, *myc* and *creb* consensus sequences yielded marked reduction of complex 1 (Fig. 3A, lanes 4, 8 and 6). To confirm the results obtained in competition studies, EMSA supershift experiments were performed (Fig. 3B). Application of anti-USF1, anti-USF2 and anti-CREB antibodies supershifted parts of complex 1 (Fig. 3A, lanes 3–6), and application of anti-USF1-, anti-USF2- and anti-CREB antibodies together

completely supershifted complex 1 (Fig. 3A, lane 7). In contrast, antibodies recognizing c-Myc, c-Jun or GATA6 had no effect (Fig. 3A, lanes 8–10), demonstrating that the presence of nuclear proteins other than USF1/-2 and CREB can be largely excluded. Excess of unlabelled *creb* consensus sequence as well as preincubation with anti-CREB antibodies reduced the intensity of the upper part of complex 1 (Fig. 3A and B, lanes 6). In contrast, excess of *usf* and *myc* consensus sequences, which both show high homology to the Ebox sequence, competed out the lower part of complex 1 (Fig. 3A, lanes 4 and 8). Similarly, application of anti-USF1 and/or -USF2 antibodies supershifted the lower part of this complex (Fig. 3B, lanes 3–5). These observations suggest that CREB binds to the CRE site, whereas USF1/-2 interact with the Ebox sequence of the CRE/Ebox element.

To address the discrepancy between competition and supershift studies, showing effectiveness of *myc* consensus oligonucleotide in competition studies, whereas a c-Myc-specific antibody was without effect in supershift experiments, *myc* consensus oligonucleotide served as radiolabelled probe (Fig. 3C). Binding of nuclear proteins to the *myc* probe was effectively inhibited by *myc* or *usf* oligonucleotides. Supershift experiments identified USF1 and USF2 as nuclear proteins binding to the *myc* sequence (Fig. 3C, lanes 7–9), whereas application of a c-Myc-specific antibody had no effect (Fig. 3C, lane 10). These results strongly suggest that the nuclear proteins competed out by cold *myc* oligonucleotides from the *cox-2* promoter represent USF1 and USF2 proteins, but not Myc.

USF and CREB transcription factors are indispensable for H. pylori*-triggered* cox-2 *transactivation*

Co-transfection of a construct encoding a dominant-negative USF mutant (A-USF), which has been shown to interrupt USF1- and USF2-dependent effects (Qyang *et al*., 1999), dramatically impaired *H. pylori*- or PMA-stimulated *cox-2* promoter activation (Fig. 4A). Similarly, application of A-CREB strongly reduced the *cox-2*-transactivating effect of *H. pylori* and PMA (Fig. 4B). These results clearly confirm that, in gastric epithelial cells, USF1/2 and CREB transcription factors are required for *cox-2* transactivation in response to *H. pylori.* As EMSA studies demonstrated that *H. pylori* infection of AGS cells did not influence the configuration of the nuclear protein complex binding to the *cox-2*–66/–38 probe (data not shown), we aimed to analyse whether modification of the transcription-activating potency of USF and/or CREB may represent a mechanism mediating *H. pylori*-dependent activation of the *cox-2* gene. For this purpose, appropriate Gal4/Gal4-luc co-transfection systems were applied (see detailed description in *Experimental procedures*). As interaction of Gal4/USF or Gal4/CREB fusion proteins with their binding sites in the Gal4-luc reporter gene construct is determined by post-translational modification(s) of their USF or CREB residues, these systems allow the determination of the transactivating capacity of the transcription factors under investigation. These studies revealed that *H. pylori* infection significantly stimulated the transactivating capacity of USF1, USF2 (Fig. 4C) and CREB transcription factors in gastric epithelial cells (Fig. 4D), providing a potential molecular mechanism mediating *H. pylori*-dependent transactivation of the *cox-2* gene.

*The vacuolating toxin A (*vacA*) gene and* cagPAI*-encoded virulence factors are not involved in* H. pylori*-dependent* cox-2 *gene expression*

To elucidate the importance of *cagPAI*- and *vacA*-encoded virulence factors for regulation of the *cox-2* gene by *H. pylori*, AGS cells were infected with isogenic *H. pylori* mutants lacking these virulence factors (mutants *ΔcagPAI* and *ΔvacA*). Both mutants transactivated the *cox-2* gene promoter (Fig. 5A) as effectively as the wild-type strain. In addition, the *ΔcagPAI* mutant displayed very similar capacity to stimulate *cox-2* mRNA expression in AGS cells (Fig. 5B) and MKN-28 cells (data not shown) when compared with its corresponding wild-type strain. In contrast, as described previously (Naumann *et al*., 1999), *cagPAI*-deficient bacteria were unable to transactivate an AP-1-regulated reporter gene construct (Fig. 5C), confirming the functionality of this *ΔcagPAI* mutant. Taken together, these data strongly suggest that *cagPAI*- and *vacA*-encoded virulence factors are not involved in *H. pylori*-dependent *cox-2* gene expression. Preliminary results from transwell filter assays and transfer experiments using *H. pylori* culture supernatants indicate that the *cox-2*-transactivating effects of *H. pylori* do not require physical interaction of bacteria with gastric epithelial cells and are probably mediated by (a) secreted bacterial factor(s) (data not shown).

Helicobacter pylori*-dependent* cox-2 *transactivation is mediated via the MEK/ERK1/-2 kinase cascade*

To investigate the role of MEK/ERK and JNK cascades in *H. pylori*-dependent *cox-2* transactivation, phosphorylation of MEK1/-2, ERK1/-2 and JNK was assessed. Bacteria lacking *cagPAI* stimulated MEK1/-2 and ERK1/-2 phosphorylation to the same extent and with similar kinetics to the wild-type strain, whereas the capability of the mutant bacteria to induce JNK phosphorylation was dramatically impaired (Fig. 6A). To determine a functional contribution of the MEK/ERK or the JNK pathway to *H. pylori*-dependent *cox-2* gene regulation, vectors

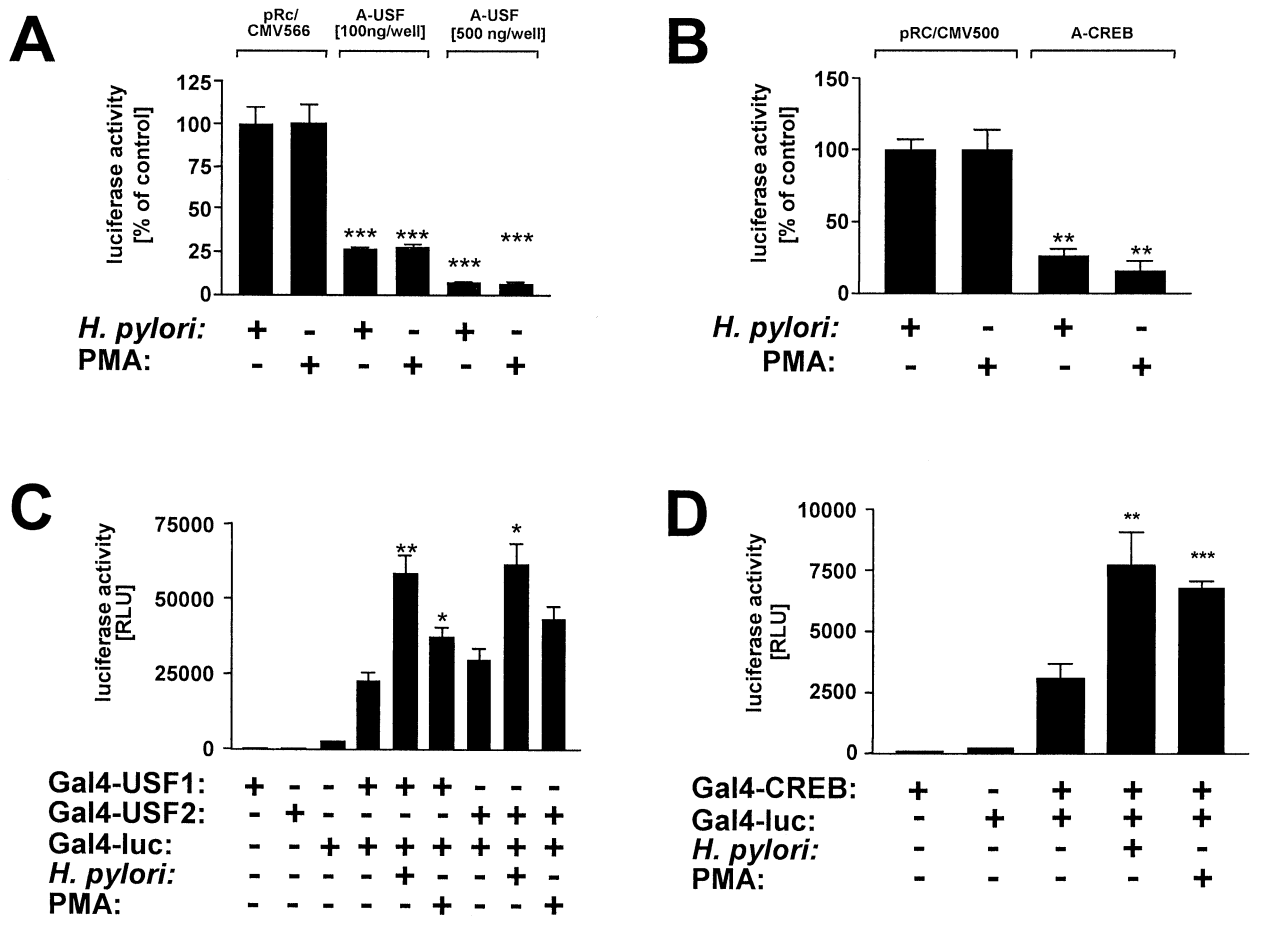

Fig. 4. USF and CREB transcription factors are indispensable for *H. pylori*-triggered *cox-2* transactivation. AGS gastric epithelial cells were transiently transfected with *cox-2* (–963/+70)-luc reporter gene construct along with expression constructs encoding dominant-negative mutants of USF (A-USF) (A) or CREB (A-CREB) (B). Control transfectants received corresponding amounts of the appropriate empty expression construct (pRc/CMV500; pRc/CMV566). Four hours after inoculation of bacteria, cells were harvested, and lysates were analysed for luciferase activities. Data shown represent mean values (± SEM) obtained from three independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). AGS cells were transiently transfected with a Gal4-luc reporter plasmid along with Gal4–USF1, Gal4–USF2 (C) or a Gal4–CREB transactivator plasmid (D). After transfection, cells were infected with *H. pylori*, whereas control infections received PBS or PMA (10 nM). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data shown represent typical results obtained from a series of three independent experiments.

encoding dominant-negative kinase mutants were applied. Co-transfection of dominant-negative ERK1 and ERK2 mutants strongly suppressed *H. pylori*-dependent *cox-2* transactivation, whereas interruption of the MKK4/JNK pathway by application of a dominant-negative MKK4 mutant had no effect (Fig. 6B). Proper function of this mutant was confirmed by applying an AP-1-dependent reporter gene construct (Fig. 6C). Overexpression of MEK1, ERK1 and ERK2 potently transactivated the *cox-2* gene promoter, and co-expression of ERK1 and ERK2 resulted in additive effects (Fig. 6B). These data strongly suggest that *H. pylori* stimulates *cox-2* transcription via the MEK/ERK1/-2 pathway, whereas the MKK4/JNK cascade does not contribute significantly to this process.

Discussion

In the present study, we demonstrate that *in vitro H. pylori* infection potently stimulates *cox-2* mRNA levels in gastric epithelial cell lines as well as in an *in vivo* mouse infection model, confirming that increased expression of the *cox-2* gene is part of the gastric epithelial response triggered by the bacterium. Moreover, these results suggest that enhanced transcription of the *cox-2* gene represents an important mechanism underlying *H. pylori*-dependent COX-2 upregulation. This is further supported by the observation that *H. pylori* infection *in vitro* potently transactivated *cox-2* promoter reporter gene constructs transiently transfected into gastric epithelial cells.

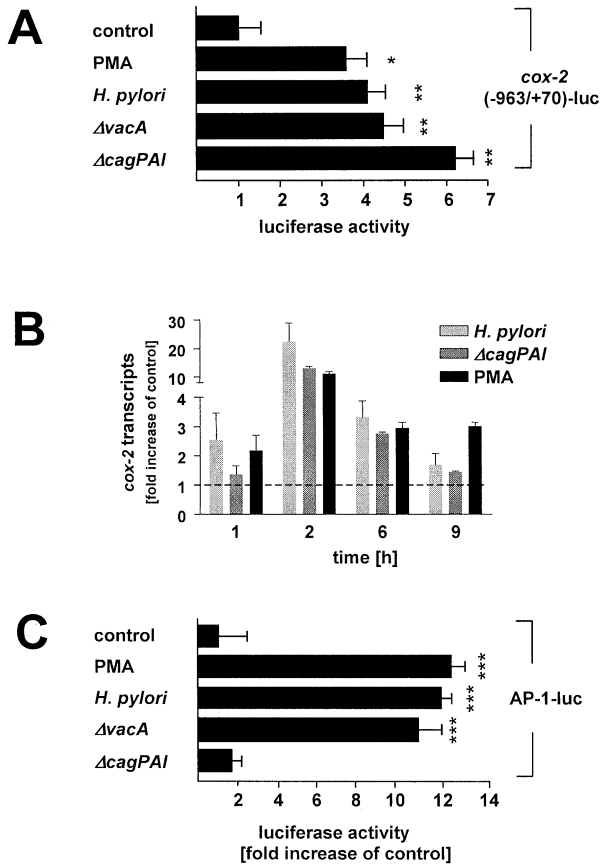

Fig. 5. *vacA*- and *cagPAI*-encoded virulence factors are not involved in *H. pylori*-dependent *cox-2* promoter regulation.
A. AGS cells were transiently transfected with the *cox-2* (–963/+70)-luc construct and infected with wild-type *H. pylori* strain *P12* or its isogenic mutants *ΔvacA* and *ΔcagPAI*, whereas control infections received PBS. In addition, a subset of cells was treated with PMA (10 nM). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
B. AGS gastric carcinoma cells were exposed to *H. pylori* strain *P12*, its isogenic mutant Δ*cagPAI* or PMA (50 nM) for the indicated times, and *cox-2* mRNA levels were determined by real-time RT-PCR. Determinations were performed in duplicate, and results are expressed as the increase in normalized *cox-2* levels (± SEM) compared with sham-infected controls.
C. As a control, the effects of wild-type *H. pylori* and its isogenic mutants on AP-1-dependent transcription were explored using a luciferase reporter construct, in which the firefly luciferase reporter gene is controlled by a multimer of the consensus AP-1 binding sequence (AP-1-luc). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data shown represent typical results obtained in a series of three independent experiments.

To explore *cis*-regulatory element(s) mediating the effects of *H. pylori* on the *cox-2* gene, we performed an extensive functional promoter analysis using deletion and mutational strategies. These studies revealed that an overlapping CRE/Ebox element located in the proximal *cox-2* promoter functions as an *H. pylori*-responsive element (Fig. 2). This proximal CRE/Ebox element, which is highly conserved among mammalian *cox-2* gene promoters, consists of a 'classical' CRE site, which overlaps an adjacent consensus Ebox motif by two nucleotides (Kim and Fischer, 1998; Smith *et al*., 2000). In several previous studies, this CRE/Ebox sequence was identified as an important regulatory element involved in the control of basal and regulated *cox-2* gene expression (Smith *et al*., 2000; Howe *et al*., 2001). In most of these investigations, however, the CRE sequence was identified as the critical *cis*-regulatory sequence, whereas the Ebox half-site had little or no functional importance (Subbaramaiah *et al*., 1998; Shao *et al*., 2000; Smith *et al*., 2000; Howe *et al*., 2001; Tang *et al*., 2001). The CRE/Ebox element has also been shown frequently to act in concert with other, more 5′-located regulatory elements including NF-κB and/or NF-IL6 consensus sites (Subbaramaiah *et al*., 1998; Kim *et al*., 1998; Shao *et al*., 2000; Smith *et al*., 2000; Howe *et al*., 2001; Tang *et al*., 2001). In contrast to these findings, our study demonstrates that *H. pylori*-triggered *cox-2* transcription in gastric epithelial cells depends on the proximal CRE/Ebox sequence, and does not require additional *cis*-regulatory elements.

Several transcription factors, including CREB, ATF1, AP-1, USF1/-2 and NF-κB, have been shown to bind and transactivate the *cox-2* gene promoter (Smith *et al*., 2000; Howe *et al*., 2001). Our results revealed that USF1, USF2 and CREB represent the nuclear proteins responsible for basal and *H. pylori*-dependent activation of the *cox-2* gene in gastric epithelial cells. Moreover, EMSAs showed binding of CREB to the CRE region, and USF1/-2 to the Ebox element of the CRE/Ebox sequence (Fig. 3A and B). Therefore, both half-sites of the CRE/Ebox element appear to participate in *H. pylori*-triggered *cox-2* transcription in gastric epithelial cells. Similar findings have been reported in two previous studies (Mestre *et al*., 2001; Schroer *et al*., 2002). In contrast to our results, USF factors binding to the *cox-2* CRE/Ebox motif in human endothelial cells had no functional importance (Schroer *et al*., 2002), whereas in macrophages, CREB and USF1 were found to interact with c-Jun at the CRE/Ebox element (Mestre *et al*., 2001). Furthermore, interplay of CREB and USF has been described for an overlapping CRE/Ebox site of the rat *brain-derived neurotrophic factor* gene promoter (Tabuchi *et al*., 2002). However, an uncharacterized nuclear factor has been demonstrated to bind to the CRE/Ebox site of this promoter, in addition to USF and CREB (Tabuchi *et al*., 2002). Therefore, *H. pylori*-dependent activation of USF1/-2 and CREB at the *cox-2* promoter in gastric epithelial cells appears to represent an as yet undescribed example for the regulatory interplay of these transcription factors at an CRE/Ebox element.

Helicobacter pylori has been shown to exert its transcriptional effects in gastric epithelial cells via activation of different transcription factors including AP-1 (Naumann *et al*., 1999), NF-κB (Foryst-Ludwig *et al*., 2000) and the as yet uncharacterized GASREBP transcription

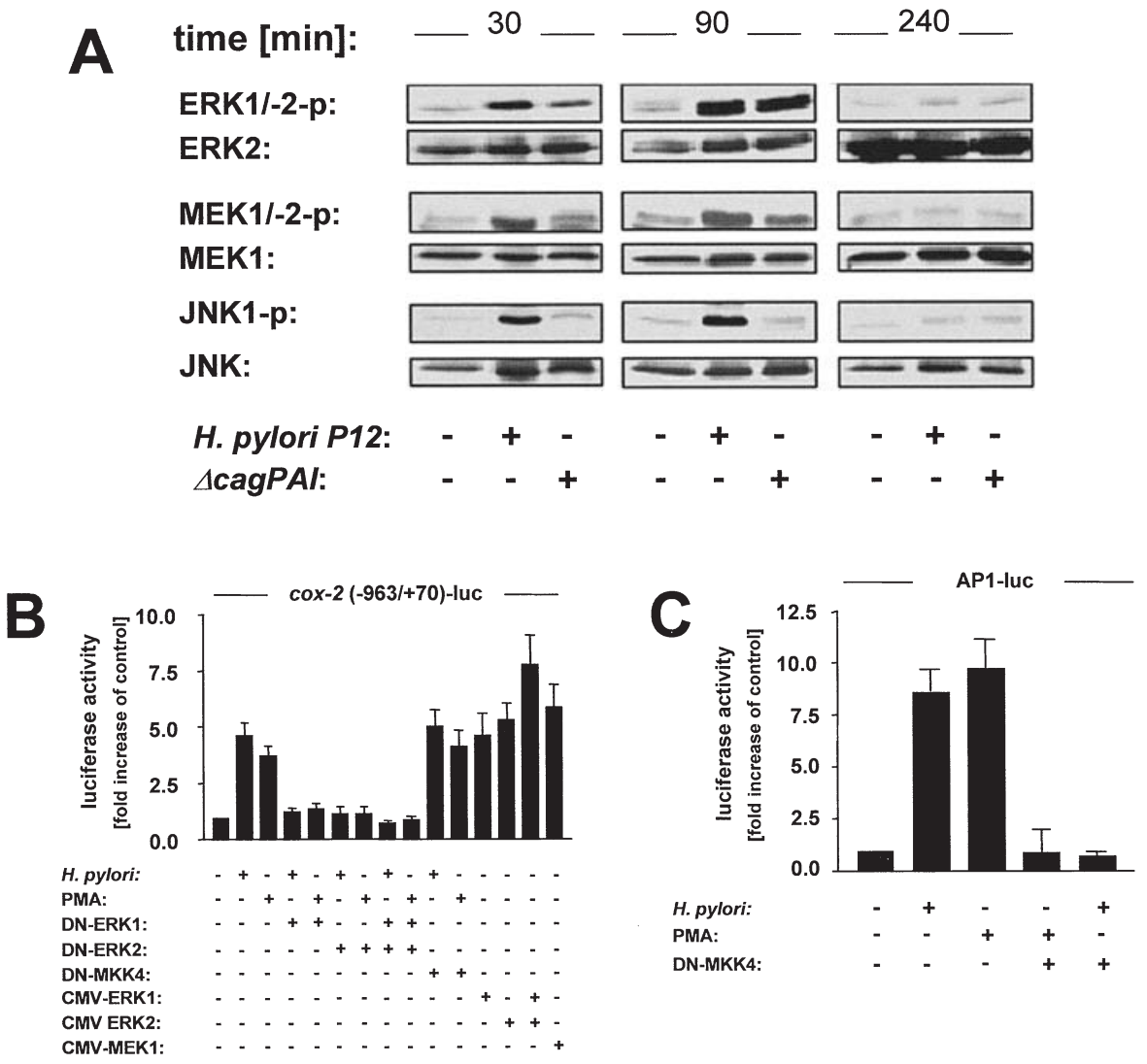

Fig. 6. *H. pylori*-dependent *cox-2* transactivation is mediated via the MEK/ERK1/-2 kinase cascade.
A. AGS cells were infected with wild-type or mutated *H. pylori*. After 30, 90 or 240 min, cells were lysed, and phosphorylation of ERK1/-2, MEK1/-2 and JNK1 was detected by immunoblotting using phosphospecific antibodies (top). To visualize protein amounts loaded, blots were stripped and reprobed with corresponding non-phosphospecific antibodies (bottom).
B and C. AGS cells were co-transfected with *cox-2* (–963/+70)-luc and the indicated dominant-negative (DN) mutants of ERK1-, ERK2- or JNK-activating kinase MKK4 (B). Effectiveness of the DN-MKK4 construct was confirmed using the AP-1-driven luciferase reporter construct AP-1-luc (C). The stimulatory effect of MEK1 and ERK1/-2 kinases on the *cox-2* gene promoter was investigated by co-transfection of constructs encoding human ERK1 (CMV-ERK1), ERK2 (CMV-ERK2) or MEK1 (CMV-MEK1) along with a *cox-2* reporter gene construct (B, lanes 12–15). Co-transfection of appropriate amounts of empty vector pCMV5 served as a control. After transfection, cells were infected with *H. pylori* or received PMA (10 nM) or PBS. Data shown represent mean values ± SEM obtained from three independent experiments.

factors (Wessler *et al*., 2000). CREB and/or USF1/-2 proteins, however, have not been linked to the bacterium yet. The '*leucine zipper*' transcription factor CREB belongs to the ATF/CREB family of nuclear proteins, which bind the palindromic CRE consensus sequence 5′-TGACGTCA-3′. CREB-dependent transcription is often regulated through phosphorylation of a serine residue at position 133 of the transcription factor (for a review, see Hai and Hartman, 2001). CREB plays an important role in a variety of biological processes including long-term memory and T-cell development (reviewed by Mayr and Montminy, 2001). Various growth factors such as nerve growth factor, epidermal growth factor and platelet-derived growth factor as well as hypoxia or ultraviolet B irradiation have been described to activate CREB (Mayr *et al*., 2001). In addition, viral pathogens including *human herpesvirus 6* (Janelle *et al*., 2002) and *human immunodeficiency virus type* 1 (Ross *et al*., 2001)

were identified to target CREB. Activation of the transcription factor by bacterial species, however, has not been reported yet. Therefore, *H. pylori*-dependent activation of CREB can serve as a novel molecular model for the activation of this transcription factor by extracellular pathogens and may allow novel insights into molecular mechanisms of CREB-dependent gene regulation associated with bacterial infections.

In contrast to CREB, USF transcription factors belong to the family of 'basic helix–loop–helix' proteins, and represent ubiquitously expressed mammalian nuclear proteins (Sirito *et al*., 1994). USFs were initially identified as regulators of genes regulating hepatic iron metabolism (Fleming and Sly, 2001) and glucose homeostasis (Vallet *et al*., 1998), but have not yet been linked to gastric gene regulation. They exist in two molecular forms with molecular weights of 43 (USF1) and 44 kDa (USF2), respectively, and these USF isoforms are encoded by two distinct genes (Sirito *et al*., 1994). USF proteins typically interact with the canonical Ebox sequence 5′-CACGTG-3′ as complexes consisting of USF1:USF1 or USF2:USF2 homodimers as well as USF1:USF2 heterodimers, the latter representing the major species of nuclear USF complexes (Sirito *et al*., 1994). Similar to CREB, USF transcription factors are mainly activated through reversible phosphorylation (Galibert *et al*., 1997). However, extracellular factors and related intracellular pathways regulating USF activity are not well understood. Moreover, regulation of USF transcription factors through a bacterial pathogen, as found in our study, has not been described so far. Our findings represent the first example of simultaneous activation of CREB and USF1/-2 transcription factors by a bacterial species and therefore contribute to a better understanding of how these transcription factors can act as nuclear effectors of bacterial pathogens. In addition, identification of CREB and USF transcription factors as targets of *H. pylori* can provide novel insights into the complex process of gene regulation associated with pathological situations such as gastric inflammation, ulcer disease and possibly carcinogenesis. A current report suggested a role for NF-κB as mediator of *H. pylori* effects on the *cox-2* gene in gastric epithelial cells (Kim *et al*., 2001). In contrast, our study clearly demonstrates that the upstream NF-κB site is not involved in *H. pylori*-dependent *cox-2* gene regulation in these cells. In agreement with this view, we found that an isogenic *H. pylori* mutant defective in bacterial *cagPAI* genes has full capacity to activate the *cox-2* promoter (Fig. 5). As *cagPAI*-encoded virulence factors have been identified as a structural prerequisite for NF-κB activation (Glocker *et al*., 1998), the ability of isogenic *H. pylori* mutants lacking intact *cagPAI* genes potently to activate the *cox-2* gene in AGS cells clearly argues against participation of NF-κB in *H. pylori*-dependent *cox-2* gene regulation in this cell type.

By applying isogenic bacterial mutants, we found that *H. pylori*-dependent activation of the *cox-2* promoter *in vitro* does not require the *vacA* gene or *cagPAI*-encoded bacterial virulence genes (Fig. 5). These results are in agreement with previous reports showing that virulence factors outside *cagPAI* sequences can act as critical determinants of *H. pylori* gastric epithelial gene expression (Wessler *et al*., 2000; Cox *et al*., 2001). Given the increasing evidence that *cagA*-negative *H. pylori* strains can cause severe gastric pathology including gastric carcinoma (Graham and Yamaoka, 2000; Ekstrom *et al*., 2001), analysis of the mechanisms underlying control of *cox-2* gene expression through non-*cagPAI*-encoded *H. pylori* virulence factors may provide important insights into the pathogenesis of such diseases.

Similar to our observations, a recent report showed that mutation of the bacterial *vacA* gene had no influence on *H. pylori's* ability to stimulate *cox-2* mRNA abundance *in vitro* (Romano *et al*., 1998). In contrast to our results, deletion of *cagPAI*-encoded bacterial genes (*picA* and *picB*) in this study reduced the ability of *H. pylori* to stimulate *cox-2* mRNA expression. These discrepancies can be explained by substantial differences in the experimental protocols and bacteria used. Romano *et al*. (1998) used the ATCC 49503 *H. pylori* strain and performed incubations with bacteria or culture broth supernatants thereof for up to 48 h. In contrast, we have chosen a short-term infection with living bacteria (strain *P12*), which allowed us to avoid interference of bacterial decay products frequently developing after prolonged infection *in vitro* (Stassi *et al*., 2002).

To elucidate further the molecular pathways underlying *H. pylori*-dependent *cox-2* gene transcription in gastric epithelial cells, we analysed the signal transduction cascades mediating the effects of the bacterium on the *cox-2* promoter. Using a combination of kinase phosphorylation analysis and functional transfection studies with dominant-negative kinase mutants, we found that the transactivating effect of *H. pylori* on the *cox-2* gene promoter is preferentially transmitted via a MEK/ERK1/-2 signalling cascade. In contrast, the MKK4/JNK pathway does not contribute to this effect (Fig. 6B). The selectivity with which *H. pylori* triggers host cell signalling responses is largely determined by the presence of particular bacterial virulence factors (Glocker *et al*., 1998; Keates *et al*., 1999; Naumann *et al*., 1999; Wessler *et al*., 2000; Naumann, 2001). Although virulence factors encoded by *cagPAI* genes are of central importance for linking the bacterium to NF-κB-dependent signalling (Glocker *et al*., 1998; Naumann *et al*., 1999; Foryst-Ludwig *et al*., 2000) and the MKK-4/JNK cascade (Keates *et al*., 1999; Naumann *et al*., 1999), activation of the MEK/ERK1/-2 pathway occurs primarily through *cagPAI*-independent mechanisms (Keates *et al*., 1999; Wessler *et al*., 2000; 2002;

Naumann, 2001). Therefore, identification of MEK/ERK pathways as the primary signalling route through which *H. pylori* activates the *cox-2* gene in gastric epithelial cells is in full accordance with the finding that this effect depends on the presence of *cagPAI*-independent virulence factors.

Taken together, we demonstrate that *H. pylori* is capable of directly stimulating the transcription of the *cox-2* gene in gastric epithelial cells and provide a detailed characterization of the underlying molecular mechanism (Fig. 7). By identifying the transcription factors USF-1, USF-2 and CREB for the first time as molecular targets of *H. pylori*, these studies add to our understanding of mechanisms mediating the effects of *H. pylori* on host prostanoid formation as well as gastric gene expression in general, and may thus help to develop novel therapeutic and/or diagnostic approaches.

Experimental procedures

Bacterial strains, culture and infection

Mouse-adapted strain Hp76 (Lucas *et al*., 2001), *H. pylori* strain P12 (wild type) and related isogenic mutants deficient for the *vacA* gene (Δ*vacA*) and Δ*cagPAI* (lacking *cagPAI*) (Wessler *et al*., 2000) were grown on GC agar plates containing 10% horse serum as well as antibiotics (vancomycin, trimethoprim, nystatin and, in the case of Hp76 cultures, streptomycin) in a microaerophilic atmosphere (generated by Campy-Gen) at 37°C for 48–72 h. Harvesting of bacteria, infection of epithelial cells *in vitro* and of mice has been described in detail previously (Wessler *et al*., 2000; Lucas *et al*., 2001). Unless otherwise indicated, permanent cell lines were incubated with *H. pylori* strain P12 at an MOI of 100. Specific pathogen-free female BALB/c mice (6–8 weeks old), obtained from the Federal Institute for Health Protection of Consumers and Veterinary Medicine, were infected with 1.0×10^8–1.0×10^9 cfu of Hp76 per mouse. Sham-infected control mice received the diluent (PBS) only. As an additional control, another group of mice was infected with *Salmonella typhimurium aroA* (SL3261; 1.0×10^8 cfu per mouse) as described in detail elsewhere (Lucas *et al*., 2001). One-third of the tissue obtained from the stomach of bacteria- or sham-infected mice was frozen in liquid nitrogen, one-third was retained for histological analysis and the remainder was homogenized in brain–heart infusion broth and plated out to confirm colonization with *H. pylori*.

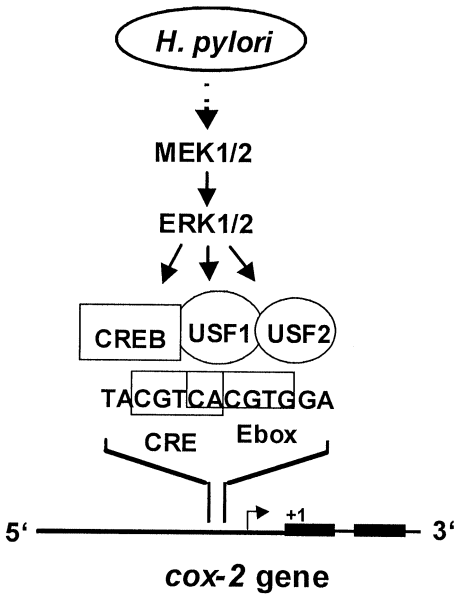

Fig. 7. Model of molecular pathways mediating the effects of *H. pylori* on the *cox-2* gene in gastric epithelial cells. *H. pylori* infection stimulates *cox-2* gene expression through *cagPAI*-independent activation of MEK1/-2 and ERK1/-2 kinases. At the promoter level, a proximal overlapping CRE/Ebox element functions as *H. pylori* response element of the *cox-2* gene. This element is bound and regulated by USF1/-2 and CREB transcription factors, the transactivating capacity of which is stimulated by *H. pylori*.

Immunohistochemistry

After fixation in 2.5% buffered paraformaldehyde for 6 h at 4°C and washes in PBS containing 30% sucrose, tissues were embedded in OCT medium (Sakura Finetech) and frozen immediately over liquid nitrogen. For staining procedures, frozen cryosections were briefly post-fixed in 2% paraformaldehyde (diluted in PBS) for 10 min at room temperature. OCT medium was removed by further washing for 10 min in Tris-buffered saline (TBS; 0.1 M Tris, pH 7.2). After blocking of Fc receptor (30 min, 10% normal goat serum) and endogenous biotin (ABC blocking kit; Vector Laboratories), slides were incubated with rabbit anti-COX-2 antibody (Cayman Chemical) diluted at 1:500 for 2 h at room temperature; control sections received normal rabbit sera. Between each step, slides were washed for 10 min with TBS containing 0.05% Tween 20. Detection of COX-2 antibody was performed using a Cy3-conjugated goat anti-rabbit antibody (Sigma-Aldrich).

RNA preparation, reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR

RNA isolation and DNase digestion were performed using the 'RNase-free DNase set' (Qiagen) according to the manufacturer's instructions. Conventional two-step RT-PCR was performed using 1 μg of total RNA and the 'Superscript II kit' (Gibco Life Sciences) as recommended by the manufacturer. Primer sequences for mouse duplex PCR were: mouse *cox-2* sense, 5′-AGATTGCTG GCCGGGTTGCT-3′; antisense, 5′-GGACACCCCTTCACATTAT TGCAG-3′, and mouse *cytoskeletal γ-actin* sense, 5′-GAAGAT GACGCAGATAATGTTTGAA-3′; antisense, 5′-CCAGGTCCAG ACGCAAGAT-3′. After 30 cycles of amplification, 5 μl of each product was electrophoresed in 1.2% agarose gels containing GelStar® (BioWhittaker). Mouse c*ox-2* mRNA expression was quantified by normalization to *γ-actin* expression using the TINA image analysis software (Division of Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). Histomorphological assessment of analysed mucosal scrapings obtained from the mouse stomach confirmed that mostly epithelial cells were obtained by this technique, whereas non-epithelial cells were only found occasionally (data not shown).

For analysis of human *cox-2* mRNA levels, *gapdh*-controlled duplex PCR was performed using the following primer sequences: *gapdh* sense, 5′-ACCACAGTCCATGCCATCAC-3′; *gapdh* antisense, 5′-TCCACCACCCTGTTGCTGTA-3′; *cox-2* sense, 5′-TTCAAATGAGATTGTGGGAAAAT-3′; *cox-2* antisense, 5′-AGATCATCTCTGCCTGAGTATCTT-3′. After 30 cycles (20 s at 94°C, 30 s at 60°C, 45 s at 72°C per cycle), 15 μl of PCR product was eletrophoresed in 2% agarose gels and stained with ethidium bromide. For real-time PCR analysis of human *cox-2* mRNA levels, sequences of primers, sensor and anchor oligonucleotides were as follows: *cox-2* sense, 5′-TTCAAATGAGATTGTG GAAAAATTGCT-3′; c*ox-2* antisense, 5′-AGATCATCTCTGCCT GAGTATCTT-3′; *cox-2* sensor, 5′-TGGGCCATGGGGTGGACT TAAATCA-FL-3′; *cox-2* anchor, 5′-LightCycler Red640-TTTACG GTGAAACTTGCTAGACACGTAAAC-p-3′. PCR was run for 45 cycles of 10 s at 95°C, 10 s at 54°C and 15 s at 72°C. For quantification of *cox-2* transcript levels, *cox-2* and *porphobilinogen deaminase* (*pbgd*) transcripts were determined in each sample, and *cox*-2-values were divided by the amount of *pbgd* transcripts using the LIGHTCYCLER relative quantification software, version 1.0® (Roche).

DNA constructs and reporter plasmids

Firefly luciferase reporter constructs and internal *cox-2* deletion constructs (Δ–371/–69 and Δ–68/–51) have been described in detail elsewhere (Kim and Fischer, 1998). Plasmids encoding Gal4–USF1, Gal4–USF2 and Gal4–CREB hybrid proteins, the Gal4-luciferase reporter gene vector (Gal4-luc) and the reporter gene plasmid AP-1-luc have been used before (Höcker *et al*., 1998; Naumann *et al*., 1999; Qyang *et al*., 1999). Plasmids encoding dominant-negative mutants of USF, CREB, MKK4, ERK1/-2 or wild-type MEK1, ERK1/-2 have also been described previously (Höcker *et al*., 1997; 1998; Naumann *et al*., 1999; Qyang *et al*., 1999). To examine the characteristics of potential *cox-2 cis*-regulatory elements in a heterologous promoter system, wild-type or mutated *cox-2*–66/–38 oligonucleotides were subcloned at *Hin*dIII (5′) and *Xho*I (3′) restriction sites into vector pT81-luc, which contains the enhancerless Herpes simplex thymidine kinase (TK) viral promoter (Nordeen, 1988). Before use, constructs were confirmed by dideoxy sequencing. To study the *H. pylori*-triggered transactivation potency of USF1, USF2 and CREB transcription factors, Gal4/Gal4-luciferase co-transfection systems were applied. In these systems, Gal4–USF1, Gal4–USF2 and Gal4–CREB transactivator plasmids encode fusion proteins consisting of USF1, USF2 or CREB transactivation domains linked to the Gal4 yeast transcription factor DNA-binding domain (Qyang *et al*., 1999). In the Gal4-luc reporter plasmid, expression of the firefly luciferase reporter gene is controlled by a multimer of the Gal4 binding element. Interaction of Gal4 fusion proteins with their binding sites in the Gal4-luc reporter gene construct is determined by post-translational modification(s) of their USF or CREB residues and, therefore, allows the determination of the transactivating capacity of these transcription factors. After co-transfection of AGS cells with 300 ng of Gal4-luc per well, Gal4–USF1, Gal4–USF2 or Gal4–CREB transactivator constructs (300 ng per well), cells were maintained under serum-free conditions overnight, incubated in the presence of *H. pylori* at an MOI of 100 or PMA (10 nM), harvested and assayed for luciferase activities as outlined below.

Cell culture and transfection studies

AGS and MKN-28 human gastric adenocarcinoma cell lines were grown in RPMI 1640 (Gibco Life Sciences) supplemented with 4 mM glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal calf serum (FCS; Gibco Life Sciences) in a humidified 5% CO_2 atmosphere, and transfections were carried out as described previously (Wessler *et al*., 2000) using 200 ng of reporter gene plasmid DNA per well and 200 ng of co-transfected expression constructs per well. Transfected cells were harvested at the indicated time points, and luciferase activities were assayed using the Dual Luciferase kit® (Promega) in a dual-channel luminometer. Statistical significances were calculated using Student's *t*-test for unpaired samples with the GraphPad PRISM 2.01® software. Unless otherwise indicated, experiments were carried out in triplicate.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously (Höcker *et al*., 1998). DNA-binding reactions were conducted in a buffer containing 20 mM Hepes (pH 8.4), 1 μg of poly-(dI–dC), 5 μg ml^{-1} bovine serum albumin, 60 mM KCl, 1 mM dithiothreitol (DTT), 1 mM $ZnCl_2$ and 10% glycerol for 30 min at 30°C. For competition experiments, nuclear extracts were incubated with a 100-fold molar excess of double-stranded competitor oligonucleotides at room temperature for 30 min before the addition of radiolabelled probes. For supershift experiments, nuclear extracts were incubated with 1 μl of the indicated antibodies (Santa Cruz Biotechnology).

Western blot analysis

To detect activated MEK1/2, ERK1/2 and JNK, total AGS cell extracts were prepared in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 10% glycerol, 1 mM Na_3VO_4, 10 mM NaF and 2 μg ml^{-1} aprotinin and pepstatin as described previously (Wessler *et al*., 2000). Equal amounts of protein extracts were separated by SDS-PAGE and blotted on membranes. Western blot analysis was performed using phosphospecific antibodies (Cell Signaling Technology) to detect pMEK1/2, pERK1/2 and pJNK. Each sample was reprobed with anti-MEK1, anti-ERK2 or anti-JNK antibodies to indicate equivalent protein amounts in all lanes.

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