

Helicobacter pylori γ -glutamyltranspeptidase upregulates COX-2 and EGF-related peptide expression in human gastric cells

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Summary

Gastric mucosa responds to *Helicobacter pylori*-induced cell damage by increasing the expression of COX-2 and EGF-related peptides. We sought to investigate the bacterial virulence factor/s and the host cellular pathways involved in the upregulation of COX-2, HB-EGF and amphiregulin in MKN 28 and AGS gastric mucosal cells. *H. pylori* strain CCUG 17874 was grown in *Brucella* broth supplemented with 0.2% (2,6-dimethyl)- β -cyclodextrins. The soluble proteins released in the culture medium by the bacterium were fractionated by exclusion size and anion exchange chromatography. A single peak retaining the ability to upregulate COX-2 and HB-EGF mRNA and protein expression was obtained. SDS-PAGE analysis of the peak showed two peptides with an apparent molecular weight of 38 and 22 kDa, which were identified by automated Edman degradation analysis as the N-terminal and C-terminal peptides of *H. pylori* γ -glutamyltranspeptidase respectively. Acivicin, a selective γ -glutamyltranspeptidase inhibitor, counteracted *H. pylori*-induced upregulation of COX-2 and EGF-related peptide mRNA expression. An *H. pylori*

isogenic mutant γ -glutamyltranspeptidase-deficient strain did not exert any effect on COX-2, HB-EGF and amphiregulin mRNA expression. Blockade of phosphatidylinositol-3 kinase and p38 kinase, but not MAP kinase kinase, inhibited *H. pylori* γ -glutamyltranspeptidase-induced upregulation of COX-2 and EGF-related peptide mRNA expression.

Introduction

Helicobacter pylori is the major causative agent of chronic superficial gastritis and peptic ulcer disease and plays an important role in the development of adenocarcinoma of the distal stomach in humans (Zarrilli *et al.*, 1999a; Peek and Blaser, 2002; Ricci *et al.*, 2002).

Helicobacter pylori-induced gastroduodenal disease depends on the inflammatory response of the host and on the production of a number of virulence factors, such as urease, responsible for ammonia generation (Ricci *et al.*, 1997), a vacuolating cytotoxin (VacA) (Cover and Blaser, 1992) and a cytotoxin-associated protein (CagA), one of the products of a 40 kb *cag* pathogenicity island (*cag* PAI) (Censini *et al.*, 1996). Colonization with *cag*+ strains induces a more intense tissue response, as reflected by enhanced mucosal inflammation, cell proliferation and cell death (Peek *et al.*, 1999). The *cag* PAI encodes a type IV secretion system, by which *H. pylori* delivers CagA into gastric epithelial cells (Censini *et al.*, 1996). The intracellular CagA activates the Ras/MAPKK/MAPK1 pathway and a growth factor-like cell response that results in abnormal proliferation and movement of gastric epithelial cells (Higashi *et al.*, 2002; Mimuro *et al.*, 2002). However, additional effector factors different from CagA seem to be involved in the epithelial cytokine/chemokine response to *H. pylori* infection (Fischer *et al.*, 2001).

The mechanism whereby *H. pylori* contributes to gastric carcinogenesis is still unknown. Infection in humans is associated with increased proliferation of gastric epithelial cells (Jones *et al.*, 1997; Peek *et al.*, 1997) that may be sustained by hypergastrinaemia (Wang *et al.*, 2000), increased expression of epidermal growth factor (EGF)-related peptides (Romano *et al.*, 1998a; Keates *et al.*, 2001; Tuccillo *et al.*, 2002; Wallasch *et al.*, 2002) and activation of the EGF receptor (EGFr) signal trans-

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duction pathway in gastric epithelial cells (Keates *et al.*, 2001; Wallasch *et al.*, 2002). In addition, it has been demonstrated that *H. pylori* upregulates the expression of cyclooxygenase-2 (COX-2), the inducible isoform of the enzyme responsible for prostaglandin production, in human gastric epithelial cells *in vitro* (Romano *et al.*, 1998b) as well as in human gastric mucosa *in vivo* (Fu *et al.*, 1999; Zarrilli *et al.*, 1999b). The upregulation of growth factors and COX-2 might increase the mitogenic activity of *H. pylori*-infected gastric mucosa in view of the absence of a corresponding increase in apoptosis (Jones *et al.*, 1997; Peek *et al.*, 1997), which may therefore be regarded as early events in gastric carcinogenesis associated with *H. pylori* infection (Zarrilli *et al.*, 1999a; Peek and Blaser, 2002). Upregulation of COX-2 and inducible nitric oxide synthase (iNOS) expression (Fu *et al.*, 1999; Zarrilli *et al.*, 1999b) may also contribute to the high oxidative DNA damage observed during *H. pylori* infection (Baik *et al.*, 1996), and this may increase the mutation rate in infected hyperproliferative gastric mucosa.

We have shown previously that either *H. pylori* whole bacteria or broth culture filtrates (BCFs) upregulated the expression of EGF-related growth factors and COX-2 in MKN 28 human gastric epithelial cells *in vitro* (Romano *et al.*, 1998a,b), and that these phenomena were independent of the expression of known bacterial virulence factors, such as VacA, CagA or urease (Romano *et al.*, 1998a,b). This study was designed to identify the bacterial virulence factor/s and the host cellular pathways involved in the upregulation of COX-2 and EGF-related peptides in gastric mucosal cells. We show that *H. pylori*-induced upregulation of COX-2 and EGF-related peptide expression in MKN 28 and AGS human gastric epithelial cells depends on the bacterial production of γ -glutamyl-transpeptidase (GGT). A common signal transduction pathway that relies on the activation of a phosphatidylinositol-3 kinase (PI-3 kinase) and p38 kinase, but not MAP kinase kinase, is responsible for *H. pylori* GGT-induced upregulation of COX-2 and EGF-related peptide expression in MKN 28 gastric mucosal cells.

Results

Biochemical fractionation of COX-2 and HB-EGF upregulating activities in MKN 28 gastric cells from H. pylori BCFs

Our previous data demonstrated that bacterial suspensions or BCFs from *H. pylori* were able to upregulate the expression of HB-EGF and amphiregulin (AR) growth factors (members of the EGF receptor family of ligands) and COX-2 in MKN 28 gastric mucosal cells. These effects were specifically related to *H. pylori* because they were

not observed with *Escherichia coli* and were independent of known *H. pylori* virulence factors such as VacA, CagA or urease (Romano *et al.*, 1998a,b). Also, increased expression of both EGF-related growth factors and COX-2 was mediated by a soluble factor (of apparent molecular mass >12 kDa) released by *H. pylori* in the culture medium (Romano *et al.*, 1998a; M. Romano and R. Zarrilli, unpublished data).

To identify the virulence factor(s) responsible for these phenomena, we performed a biochemical fractionation of soluble bacterial products released in BCFs and analysed these fractions for their COX-2 and EGF-related growth factor upregulating activities in MKN 28 gastric mucosal cells. *H. pylori* strain CCUG 17874 was grown in *Brucella* broth supplemented with 0.2% (2,6-dimethyl)- β -cyclodextrins (CD) instead of 5% fetal calf serum (FCS) to obtain the BCFs. Then, *H. pylori* soluble bacterial products were fractionated through a four-step biochemical procedure.

First, bacterial BCFs were fractionated through a G-50 gel filtration chromatography to exclude CD and low-molecular-mass components of *Brucella* broth medium. The fractions in either the exclusion or the inclusion volume of the column were pooled and analysed for their protein composition and COX-2 and EGF-related growth factor upregulating activities. As shown in Fig. 1A, pools of fractions from the exclusion volume of the column loaded with *H. pylori* broth filtrates, but not with control uBCFs, showed a complex protein pattern and retained COX-2 and HB-EGF upregulating activities. No biological activities were present in pools of fractions from the inclusion volume of columns loaded with either *H. pylori* BCFs or uninoculated broth culture filtrates (uBCFs; data not shown).

In the second fractionation step, the proteins in the exclusion volume of the G-50 column were loaded onto a cation exchange chromatography column and eluted with a 0.1–1 M NaCl linear gradient at pH 7.4. Two peaks (A and B) were eluted from *H. pylori* filtrates, showing patterns of 10 and five major proteins in the SDS-PAGE analysis respectively (Fig. 1A). COX-2 and HB-EGF upregulating activities were retained in the *H. pylori* proteins eluted in peak A, but not in peak B or in those not bound to the affinity column (Fig. 1A). Also, no COX-2 and HB-EGF upregulating activities were found in the not bound flowthrough (NB) or in the fractions eluted at the same ionic strength of peak A (A) from control uBCF (Fig. 1A). The affinity chromatography purification step enriched COX-2 and HB-EGF upregulating activities by approximately 15-fold compared with *H. pylori* BCFs (Table 1).

Next, *H. pylori* proteins in the peak of activity were separated through fast performance liquid chromatography (FPLC) exclusion size chromatography (Superdex 200 FPLC column), and the fractions were analysed by

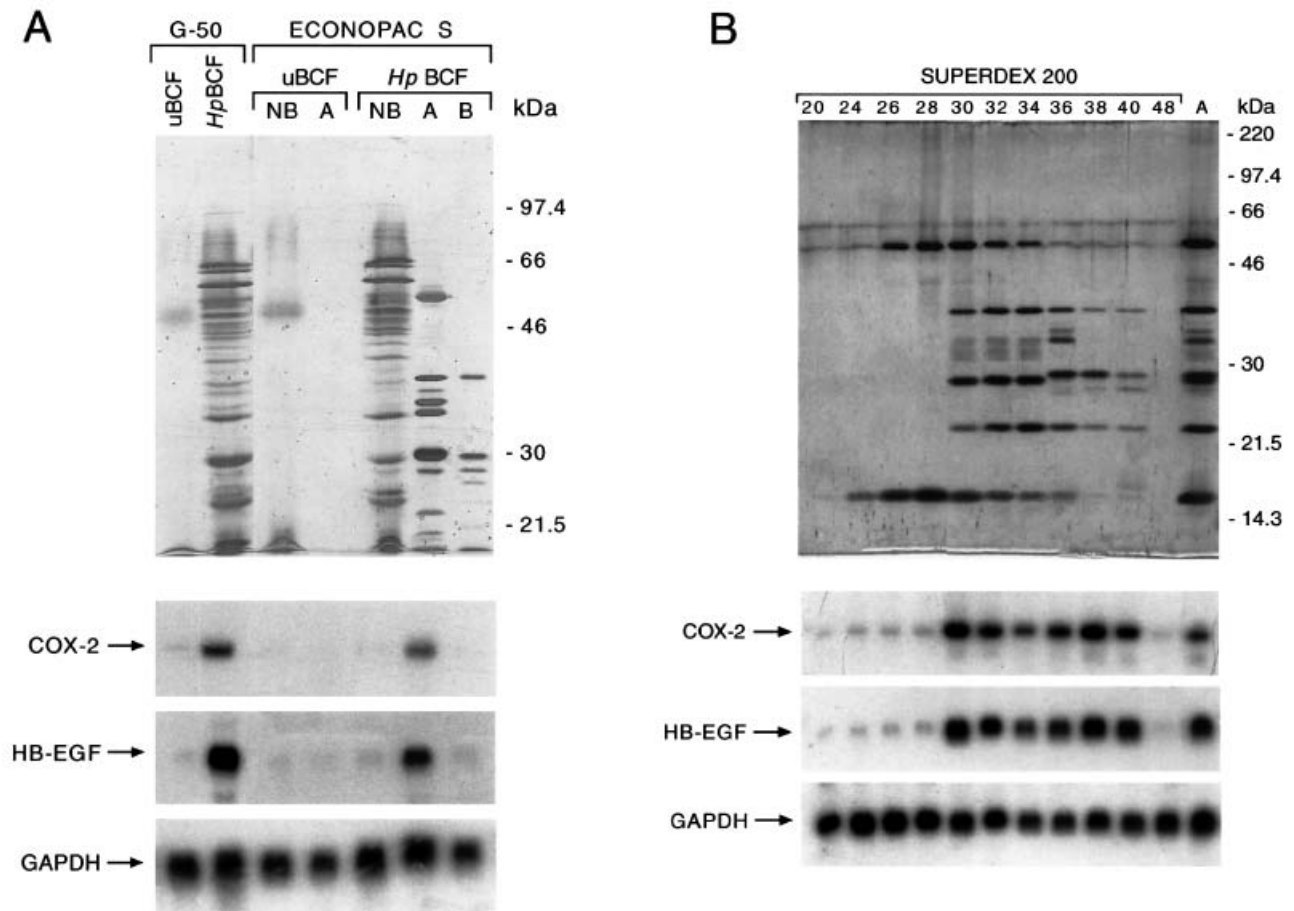


Fig. 1. Biochemical fractionation of COX-2 and HB-EGF upregulating activities from *H. pylori* broth culture filtrates. Top. A. Silver nitrate staining of 10% SDS-PAGE of uninoculated broth culture filtrate (uBCF) and *H. pylori* broth culture filtrate (*Hp* BCF) sequentially fractionated onto a Sephadex G-50 and an Econopac S FPLC. Pools of the exclusion volume fractions from Sephadex G-50 (G-50) and aliquots of either not bound samples (NB) or samples eluted in a first (A) or a second (B) peak from Econopac S FPLC are shown. B. Silver nitrate staining of 12% SDS-PAGE of *H. pylori* proteins eluted from peak A of the Econopac S FPLC column before (lane A) and after Superdex 200 FPLC fractionation. Fraction numbers of the Superdex 200 FPLC are indicated. Migration of molecular weight markers is indicated on the right. Bottom. Northern blot analysis of COX-2, HB-EGF and GAPDH mRNA expression in MKN 28 cells incubated for 18 h with *H. pylori* fractionated proteins shown in the top photos.

SDS-PAGE. As shown in Fig. 1B, fractions 24–28 contained two proteins with an apparent molecular mass of 60 kDa and 16 kDa, fractions 30–34 contained the same two proteins and three additional proteins with an apparent molecular mass of 38 kDa, 29 kDa and 22 kDa, fractions 36–40 contained three different proteins with an apparent molecular mass of 35 kDa, 34 kDa and 29.5 kDa and low amounts of the proteins eluted in fractions 30–34. Northern blot analysis of MKN 28 cells incubated with the eluted fractions from the column showed that COX-2 and HB-EGF upregulating activities were retained in fractions 30–40, but not in fractions 24–28 of the column (Fig. 1B). The calculated molecular mass of proteins eluted in fractions 30–40 from the FPLC exclusion size chromatography were estimated as being between 70 kDa and 50 kDa (data not shown).

Table 1. COX-2 and HB-EGF upregulating activities of *H. pylori* soluble virulence factors at the different purification steps.

Purification step	COX-2/GAPDH (mRNA levels μg^{-1} protein ^a)	HB-EGF/GAPDH (mRNA levels μg^{-1} protein ^a)
None	1	1
Sephadex G-50	2.5 ± 0.3	3.3 ± 0.4
Econo-Pac S FPLC	18.7 ± 1.2	15.8 ± 1.4
Superdex 200 FPLC	22.6 ± 1.8	19.6 ± 1.6
Econo-Pac S FPLC pH 8.3	44.4 ± 2.5	38.7 ± 3.1

a. COX-2/GAPDH and HB-EGF/GAPDH mRNA ratios from MKN 28 cells incubated with *H. pylori* soluble virulence factors at the different purification steps are shown. Values are expressed as arbitrary densitometric units normalized on the amount of *H. pylori* protein samples and are the mean ± SD of at least three independent experiments. None, *H. pylori* BCF. mRNA levels of MKN 28 cells incubated with crude *H. pylori* BCF are arbitrarily taken as 1. BCF, broth culture filtrate; FPLC, fast performance liquid chromatography.

Purification and identification of H. pylori COX-2 and HB-EGF upregulating activities in MKN 28 gastric mucosal cells

Based on the different protein composition of the fractions showing biological activity after exclusion size chromatography (Fig. 1B), the active eluted fractions were divided into two independent pools (pool I and pool II, from fractions 30–34 and fractions 36–40 respectively), loaded onto a cation exchange column and eluted with a 0.1–1 M NaCl linear gradient at pH 8.3. In fact, it has been shown that the proteins in *H. pylori* contained twice as many of the basic amino acids arginine and lysine as proteins in other organisms and that several surface and soluble proteins from *H. pylori* possessed basic isoelectric points (Tomb *et al.*, 1997; Alm *et al.*, 1999). At the end of the purification procedure, a single peak (A*) retaining both COX-2 and HB-EGF upregulating activities was obtained from both pools of fractions, whereas no activity was observed in the proteins that did not bind to the column (Fig. 2A). Moreover, COX-2 and HB-EGF upregulating activities at this purification step increased by ≈ 40 -fold compared with those in *H. pylori* BCFs (Table 1). SDS-PAGE analysis of the peak A* obtained from pool I showed two

peptides with an apparent molecular mass of 38 kDa and 22 kDa (Fig. 2A), whereas the same two peptides of 38 kDa and 22 kDa plus an additional one with an apparent molecular mass of 30 kDa were observed in the peak obtained from pool II (data not shown).

To determine whether COX-2 and HB-EGF protein synthesis correlated with mRNA expression, cellular extracts from peak A* soluble protein-treated or untreated (control) MKN 28 cells were analysed by Western blot analysis using antisera specific for COX-2 and HB-EGF respectively (Fig. 2B). A COX-2 immunoreactive peptide of ≈ 70 kDa was detected in untreated (control) cells that increased by eightfold in peak A*-treated cells (Fig. 2B, top). On the other hand, an HB-EGF immunoreactive peptide of ≈ 22 kDa was detected in peak A*-treated, but not in untreated (control), MKN 28 cells (Fig. 2B, bottom). In contrast, *H. pylori* soluble proteins eluted in peak A* did not affect the expression of COX-1 mRNA levels in MKN 28 cells, as assessed by reverse transcription polymerase chain reaction (RT-PCR) analysis (data not shown). We also studied whether induction of COX-2 mRNA expression was associated with an increased release of PGE₂ in the conditioned medium by MKN 28 cells. Incubation for 18 h with *H. pylori* soluble proteins eluted in peak A* caused an approximately fourfold increase in PGE₂

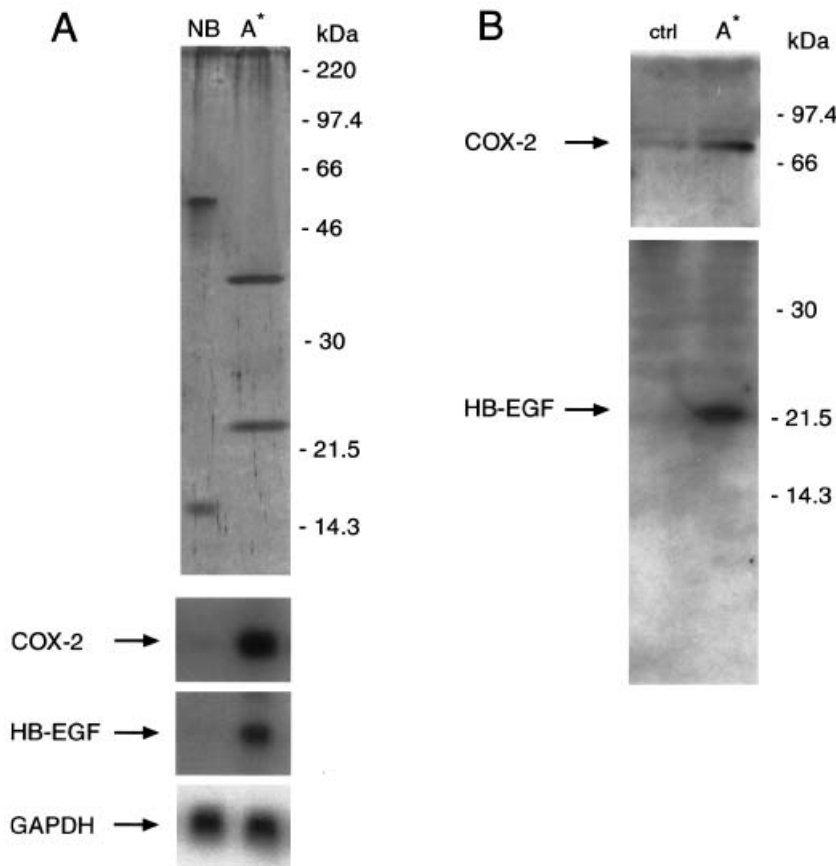


Fig. 2. pH 8.3 Econopac S FPLC fractionation of COX-2 and HB-EGF upregulating activities from *H. pylori* soluble proteins.

A. Top. Silver nitrate staining of 12% SDS-PAGE of *H. pylori* protein pool from fraction 30–34 of the Superdex 200 column after elution with Econopac S FPLC column run at pH 8.3. Aliquots of either not bound samples (NB) or samples eluted in the single A* peak from Econopac S FPLC are shown. Migration of molecular weight markers is indicated on the right. Bottom. Northern blot analysis of COX-2, HB-EGF and GAPDH mRNA expression in MKN 28 cells incubated for 18 h with *H. pylori* fractionated proteins shown at the top.

B. Western blot analysis of COX-2 and HB-EGF protein expression in MKN 28 cells after treatment with *H. pylori* soluble proteins eluted in the single A* peak from Econopac S FPLC column run at pH 8.3. COX-2 and HB-EGF immunoreactive bands are indicated on the left. Migration of molecular weight markers is shown on the right.

A

>>*H. pylori* 26695|HP1118|HP1118 gamma-glutamyltranspeptidase (567 aa)
 initn: 54 initl: 54 opt: 54 Z-score: 147.6 bits: 31.2 E(): 0.0057
 Smith-Waterman score: 54; 88.889% identity in 9 aa overlap (1-9:27-35)

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Query                               ASYPPPIXNT
                                     :::::
H.  MRRSFLKTIGLGVIALFLGLLNPLSAASYPPPIKNTKVLGLALSSHPLASEIGQKVLVEEGGN
      10          20          30          40          50          60
    
```

B

>>*H. pylori* 26695|HP1118|HP1118 gamma-glutamyltranspeptidase (567 aa)
 initn: 51 initl: 51 opt: 51 Z-score: 156.8 bits: 32.7 E(): 0.0018
 Smith-Waterman score: 51; 100.000% identity in 8 aa overlap (1-8:380-387)

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Query                               TTHYSVAD
                                     :::::
H.  KKIFDTIQPDTVTPSSQIKPGMGQLHEGSNTTHYSVADRWGNVSVTYTINASYGSAASI
      350          360          370          380          390          400
    
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Fig. 3. Amino acid alignment of N-terminal sequences from 38 kDa (A) and 22 kDa (B) *H. pylori* soluble proteins eluted in the single A* peak from Econopac S FPLC column run at pH 8.3 with *H. pylori* 26695 GGT. Amino acid residues of *H. pylori* 26695 GGT are indicated. Multiple sequence alignment has been performed by searching the PyloriGene™ database at Institut Pasteur (<http://pim.hybrigenics.com>) with the program FASTA (Pearson and Lipman, 1988).

release by MKN 28 cells compared with control cells (493 ± 134 pg mg⁻¹ protein versus 118 ± 37 pg mg⁻¹ protein respectively; $P < 0.05$).

Automated Edman degradation analysis was performed on the two proteins eluted in the peak A* of activity, and obtained peptide sequences were aligned with those deduced from the two complete sequences of *H. pylori* available (Tomb *et al.*, 1997; Alm *et al.*, 1999). As shown in Fig. 3, N-terminal sequences from 38 kDa and 22 kDa peptides aligned with residues 27–35 and 380–387 of *H. pylori* GGT, respectively, which corresponded to the N-terminal sequences of large and small subunits generated by *H. pylori* pro-GGT through cleavage of the N-terminal signal sequence and processing of the protein by autocatalytic activity at the amino acidic residue 379 (Chevalier *et al.*, 1999; Bumann *et al.*, 2002). Also, the calculated molecular mass of the large and small GGT subunit of 38 226 Da and 20 378 Da, respectively, as deduced from the published sequence of *H. pylori* 26695 (Tomb *et al.*, 1997), was in agreement with the apparent molecular mass of 38 kDa and 22 kDa of the purified proteins on which sequence analysis had been performed.

Effect of γ -glutamyltranspeptidase inhibitor acivicin on COX-2, HB-EGF and AR upregulating activities of purified γ -glutamyltranspeptidase from H. pylori filtrates in MKN 28 gastric cells

To demonstrate further that the induction COX-2 and HB-EGF expression was dependent on the bacterial production of GGT, we studied the effect of acivicin, a specific non-competitive inhibitor of GGT enzyme, on the ability of purified GGT from *H. pylori* BCFs to upregulate COX-2, HB-EGF and AR expression. As shown in Fig. 4, treatment of MKN 28 cells with acivicin at doses of 100 μ M and 200 μ M inhibited by more than 90% the upregulation

of COX-2, HB-EGF and AR expression induced by either the *H. pylori* proteins eluted in the exclusion volume of the G-50 column during the first purification step or the purified *H. pylori* GGT in the peak A* of activity from the fourth purification step. To support further a role for GGT in *H. pylori*-induced upregulation of COX-2 and EGF-related peptide expression, acivicin also counteracted COX-2, HB-EGF and AR overexpression induced by crude BCF obtained from wild-type *H. pylori* strain CCUG 17874 (data not shown). Acivicin treatment at the doses used did not affect COX-2 and EGF-related growth factor expression in

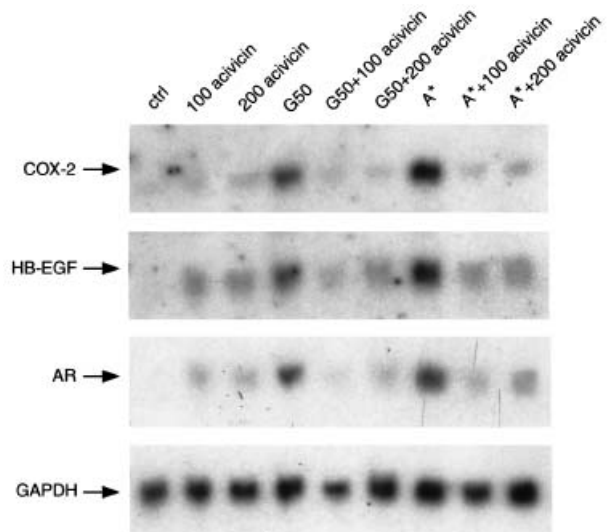


Fig. 4. Effect of acivicin, a selective GGT inhibitor, on COX-2, HB-EGF and AR upregulating activities of purified GGT from *H. pylori* filtrates in MKN 28 gastric mucosal cells. Northern blot analysis of COX-2, HB-EGF, AR and GAPDH mRNA expression in MKN 28 cells incubated for 18 h with *H. pylori* proteins eluted in the exclusion volume of the G-50 column or in the single A* peak from Econopac S FPLC column run at pH 8.3 in the presence of acivicin is shown. 100 acivicin and 200 acivicin, acivicin at a concentration of 100 μ M and 200 μ M respectively.

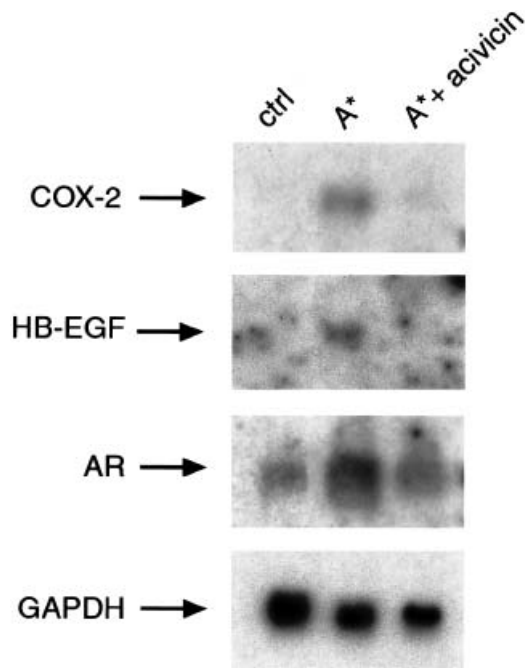


Fig. 5. Effect of acivicin, a selective GGT inhibitor, on COX-2, HB-EGF and AR upregulating activities of purified GGT from *H. pylori* filtrates in AGS gastric mucosal cells. Northern blot analysis of COX-2, HB-EGF, AR and GAPDH mRNA expression in AGS cells incubated for 18 h with *H. pylori* proteins eluted in the single A* peak from Econopac S FPLC column run at pH 8.3 in the absence and presence of acivicin GGT inhibitor at 100 μ M is shown.

MKN 28 cells (Fig. 4), nor did it alter cell viability (data not shown).

Effect of purified γ -glutamyltranspeptidase from H. pylori filtrates on COX-2, HB-EGF and AR mRNA expression in AGS gastric cells

In order to rule out the possibility that GGT-dependent upregulation of COX-2 and EGF-related peptides was a cell line-specific phenomenon, we investigated the effect of the purified *H. pylori* GGT in the peak A* of activity from the fourth purification step in the AGS gastric cell line. As shown in Fig. 5, *H. pylori* GGT induced the expression of COX-2, HB-EGF and AR mRNA, and this effect was inhibited by acivicin treatment. In contrast, purified *H. pylori* GGT did not affect the expression of COX-1 mRNA levels in AGS cells, as assessed by RT-PCR analysis (data not shown).

COX-2, HB-EGF and AR upregulating activities of an H. pylori isogenic mutant γ -glutamyltranspeptidase-defective strain in MKN 28 gastric mucosal cells

To elucidate further the role of GGT in the upregulation of COX-2 and EGF-related peptides, we evaluated whether

an *H. pylori* GGT null mutant strain retained these activities compared with its parental strain. As shown in Fig. 6, bacterial suspensions from the *H. pylori* wild-type strain M5 increased COX-2, HB-EGF and AR mRNA expression by 10-fold, whereas those from its isogenic GGT null mutant M5 *ggt::aph* did not. Accordingly, BCFs from wild-type strain M5, but not from its isogenic mutant lacking *ggt*, increased COX-2, HB-EGF and AR mRNA expression by threefold. On the other hand, bacterial suspension and BCFs from the *H. pylori* strain CCUG increased COX-2 and EGF-related peptide mRNA expression by 10- and five fold, respectively (Fig. 6).

Effect of PI-3 kinase, MAP kinase kinase or p38 kinase inhibitors on COX-2, HB-EGF and AR upregulating activities of purified γ -glutamyltranspeptidase from H. pylori filtrates

All the above data indicate that a single bacterial virulence factor was able to upregulate the expression of at least two different functions, COX-2 and EGF-related growth factors in MKN 28 cells. Because EGF-related growth factors are known to upregulate COX-2 expression through the activation of EGFR in colon cancer cells (Coffee *et al.*, 1997), we asked whether *H. pylori* GGT-dependent upregulation of COX-2 expression in gastric mucosal cells might be consequent on the induction of EGF-related growth factors. To address this issue, we performed a time

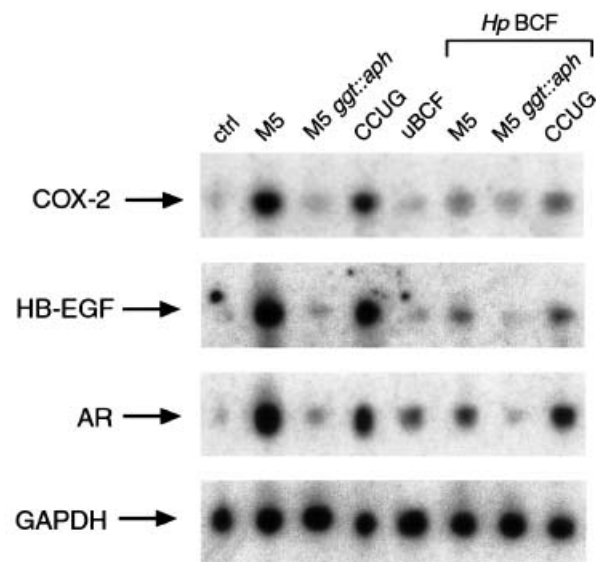


Fig. 6. COX-2, HB-EGF and AR upregulating activities of HpM5 parental strain and its γ -glutamyltranspeptidase-defective isogenic mutant strain. Northern blot analysis of COX-2, HB-EGF, AR and GAPDH mRNA expression in MKN 28 cells incubated for 18 h with DMEM (control), uninoculated broth culture filtrate (uBCF), a bacterial suspension (5×10^7 cfu ml⁻¹), broth culture filtrate (*Hp* BCF) from *H. pylori* M5 strain or its isogenic *ggt* mutant (M5 *ggt::aph*) or CCUG 17874 strain is shown.

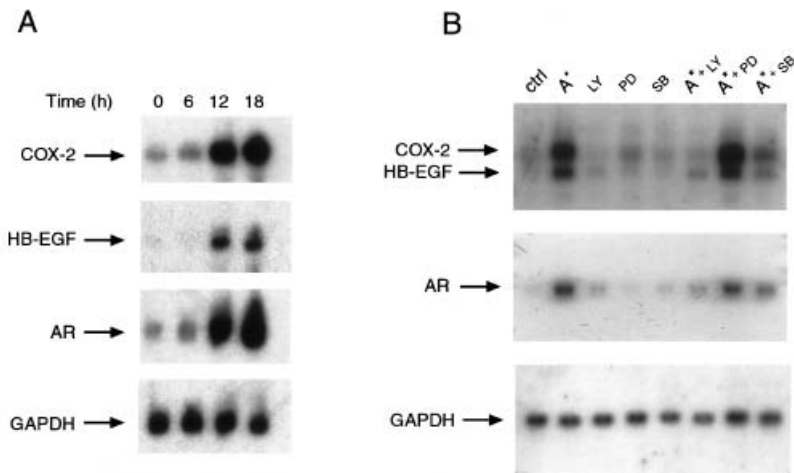


Fig. 7. Signal transduction pathways involved in *H. pylori* γ -glutamyltranspeptidase-dependent upregulation of COX-2 and EGF-related peptides.

A. Time course of COX-2, HB-EGF and AR expression in MKN 28 cells incubated with purified GGT from *H. pylori* filtrates. Northern blot analysis of COX-2, HB-EGF, AR and GAPDH mRNA expression in MKN 28 cells incubated with *H. pylori* proteins eluted in the single A* peak from Econopac S FPLC column run at pH 8.3 for up to 18 h is shown.

B. Effect of PI-3 kinase, MAP kinase or p38 kinase inhibitors on COX-2, HB-EGF and AR upregulating activities of purified GGT from *H. pylori* filtrates in MKN 28 cells. Northern blot analysis of COX-2, HB-EGF, AR and GAPDH mRNA expression in MKN 28 cells incubated for 18 h with *H. pylori* proteins eluted in the single A* peak from Econopac S FPLC column run at pH 8.3 and inhibitors is shown. LY, PI-3 kinase inhibitor LY294002 at a concentration of 50 μ M; PD, MAP kinase kinase inhibitor PD98059 at a concentration of 40 μ M; SB, p38 kinase inhibitor SB203580 at a concentration of 5 μ M.

course analysis of *H. pylori* GGT effect on COX-2, HB-EGF and AR expression. As shown in Fig. 7A, purified *H. pylori* GGT induced COX-2, HB-EGF and AR mRNA expression at similar time points (i.e. 12 and 18 h), thus making it unlikely that COX-2 upregulation is contributed to by EGF-related growth factors. It has been shown previously that different signal transduction pathways, either MAP kinase or PI-3 kinase, or stress kinase regulated the expression of COX-2 and EGF-related growth factors in inflammatory and epithelial eukaryotic cells (Barnard *et al.*, 1995; Barry *et al.*, 1999; Di Popolo *et al.*, 2000; Sheng *et al.*, 2001). Also, the above signal transduction pathways have been demonstrated to be activated in gastric mucosal cells during *H. pylori* infection (Naumann *et al.*, 1999; Higashi *et al.*, 2002; Mimuro *et al.*, 2002; Tuccillo *et al.*, 2002). To identify the signal transduction pathway/s responsible for GGT-dependent upregulation of COX-2 and EGF-related growth factor expression in MKN 28 gastric mucosal cells, we analysed whether this was affected by PI-3 kinase, MAP kinase kinase or p38 kinase inhibition. Treatment of MKN 28 cells with the PI-3 kinase inhibitor LY294002 or the p38 kinase inhibitor SB203580 reduced GGT-dependent upregulation of COX-2 and EGF-related growth factor expression by 80% and 70% respectively (Fig. 7B). On the other hand, treatment with the MAP kinase kinase inhibitor PD98059 did not affect GGT-dependent upregulation of COX-2 and EGF-related growth factor expression (Fig. 7B). All the pharmacological inhibitors at the doses used did not affect COX-2 and EGF-related peptide expression (Fig. 7B) or viability in MKN 28 cells (data not shown). Altogether, these data suggested that PI-3 kinase- and p38 kinase-dependent

common signal transduction pathways mediated the effect of *H. pylori* GGT on COX-2 and EGF-related growth factor expression in MKN 28 gastric mucosal cells.

Discussion

The present study was designed to identify the bacterial virulence factor(s) and the host cellular pathway(s) responsible for *H. pylori*-dependent upregulation of the expression of HB-EGF and AR growth factors (members of the EGF family of ligands) and of COX-2 expression in MKN 28 human gastric mucosal cells. Our previous data demonstrated that these effects were specifically related to *H. pylori* because they were not observed with *E. coli* and were independent of known *H. pylori* virulence factors, such as VacA, CagA or urease (Romano *et al.*, 1998a,b). Also, increased expression of both EGF-related growth factors and COX-2 was mediated by a soluble *H. pylori* factor >12 kDa in size (Romano *et al.*, 1998a; M. Romano and R. Zarrilli, unpublished data).

Here, we demonstrate that GGT is the bacterial product responsible for *H. pylori*-dependent upregulation of COX-2 and EGF-related peptide expression in MKN 28 and AGS human gastric mucosal cells. Moreover, we demonstrate that *H. pylori* GGT stimulates the release of PGE₂ in MKN 28 cells. A biochemical fractionation procedure of the soluble proteins released in the culture medium by the bacterium lead us to isolate two peptides with an apparent molecular mass of 38 kDa and 22 kDa, which retain the ability to upregulate EGF-related growth factors and COX-2 in gastric mucosal cells. The above two peptides were identified by automated Edman degradation analysis fol-

lowed by alignment with amino acid sequences deduced from the two complete sequences of *H. pylori* available (Tomb *et al.*, 1997; Alm *et al.*, 1999) as the N-terminal and C-terminal peptides of *H. pylori* GGT respectively. Also, the apparent molecular mass of 38 kDa and 22 kDa of the purified proteins is in agreement with the calculated molecular mass of the large and small GGT subunit of 38 226 Da and 20 378 Da, respectively, as deduced from the published sequence of *H. pylori* 26695 (Tomb *et al.*, 1997). In keeping with our data, it has been shown that two peptides of 38 kDa and 23 kDa are generated by *H. pylori* pro-GGT through cleavage of the N-terminal signal sequence and processing of the protein by autocatalytic activity at the amino acid residue 379 respectively (Chevalier *et al.*, 1999). Our data are also in agreement with a recent report showing that two GGT fragments of 38 kDa and 21 kDa are two major components of the proteins secreted by *H. pylori* (Bumann *et al.*, 2002). This finding, together with the presence of a signal peptide sequences for *sec*-dependent transport across the bacterial plasma membrane (Tomb *et al.*, 1997; Alm *et al.*, 1999; Chevalier *et al.*, 1999), is consistent with our previous studies showing that increased expression of both EGF-related growth factors and COX-2 was mediated by a soluble *H. pylori* factor >12 kDa in size (Romano *et al.*, 1998a; M. Romano and R. Zarrilli, unpublished data). We have shown previously that broth culture filtrates and suspensions from *E. coli* did not have any effect on EGF-related peptide and COX-2 mRNA expression in MKN 28 gastric mucosal cells (Romano *et al.*, 1998a,b). The enzyme GGT is expressed by several bacterial species, including *E. coli* (Suzuki *et al.*, 1986; reviewed and discussed in Chevalier *et al.*, 1999). One possible explanation for this discrepancy might be the different biochemical properties between *E. coli* GGT and *H. pylori* GGT, *E. coli* GGT activity being greatly affected by growth temperature and absent at 37°C (Suzuki *et al.*, 1986), whereas *H. pylori* GGT is not (Chevalier *et al.*, 1999). Also, the possibility exists that *H. pylori* secretes higher levels of GGT compared with other organisms. However, no quantitative data are available on GGT production from *H. pylori* in infected gastric mucosa. We also find that the ability of either purified *H. pylori* GGT or crude *H. pylori* BCF to upregulate EGF-related peptides and COX-2 expression in MKN 28 and AGS gastric epithelial cells is counteracted by acivicin, a selective non-competitive GGT inhibitor. Moreover, we demonstrate that an *H. pylori* isogenic mutant GGT-defective strain does not exert any effect on COX-2, HB-EGF and amphiregulin mRNA expression, whereas both M5 and CCUG wild-type strains are able to upregulate COX-2 and EGF-related peptide expression. The fact that BCFs from CCUG are more effective in the induction of COX-2 and EGF-related peptide mRNA than those from M5 suggests that differences in secretion/shed of GGT in these two strains may

exist. However, all the above data reinforce the concept that COX-2 and EGF-related peptide upregulation by *H. pylori* depends on the bacterial production of GGT.

GGT is an enzyme that plays a major role in glutathione metabolism (Tate and Meister, 1981). In mammals, GGT is a heterodimeric plasma membrane ectoenzyme, composed of two glycosylated subunits (heavy subunit of 55–60 kDa and light subunit of 21–30 kDa), which catalyses the transfer of a γ -glutamyl residue from glutathione (GSH) to amino acids or peptides, thus forming glutamate and cysteinylglycine. These components are then transported into cells for resynthesis of GSH (Tate and Meister, 1981). Because the maintenance of intracellular levels of glutathione is critical in response to oxidative stress injury, GGT has been interpreted as being an antioxidant enzyme. However, recent studies indicate that the extracellular cleavage of GSH by GGT leads to reactive oxygen species (ROS) production (del Bello *et al.*, 1999) and that purified GGT generates ROS in the presence of GSH and transferrin, as a source of iron (Drozd *et al.*, 1998), thus suggesting a pro-oxidant role for GGT. The fact that bacterial GGT may have a pro-oxidant effect during *H. pylori* infection is further supported by the finding that antral glutathione concentration and glutathione *S*-transferase activity are reduced during *H. pylori* infection (Verhulst *et al.*, 2000; Shirin *et al.*, 2001) and that incubation of cultured gastric mucosal cells with *H. pylori* depletes intracellular GSH stores (Shirin *et al.*, 2001). In keeping with this, it has been shown recently that the extracellular cleavage of GSH by GGT mediates nuclear factor- κ B activation in normal and transformed eukaryotic cells through ROS production (Accaoui *et al.*, 2000) or lipid peroxidation (Djavaheri *et al.*, 2002). In the above two experimental systems, the GGT pro-oxidant effect is based on the generation of cysteinylglycine (Accaoui *et al.*, 2000; Djavaheri *et al.*, 2002). We postulate that *H. pylori* GGT induces an oxidative response in gastric mucosal cells that results in upregulation of COX-2 and EGF-related growth factor expression. In partial support of this hypothesis, treatment of MKN 28 gastric mucosal cells (50 μ M) with the iron-chelator desferrioxamine, which inhibits the formation of ROS generated by cysteinylglycine in the presence of transition metals (Djavaheri *et al.*, 2002), inhibited by 40% purified *H. pylori* GGT-dependent upregulation of COX-2 and EGF-related peptide mRNA expression (data not shown).

Some experimental evidence indicates that GGT is an important virulence factor for *H. pylori*. A whole-genome microarray identified the presence of the gene in all *H. pylori* clinical isolates analysed (Salama *et al.*, 2000). Also, the synthesis of a catalytically active GGT has been demonstrated in *H. pylori* (Chevalier *et al.*, 1999). GGT seems to play an important role during the establishment of *H. pylori* infection, *H. pylori* GGT-defective isogenic strains

being unable to colonize (Chevalier *et al.*, 1999) or impaired (McGovern *et al.*, 2001) in the colonization of the gastric mucosa of mice or piglets. Dissecting out the response of the host to *H. pylori* infection and identifying the responsible bacterial virulence factors may be relevant to the understanding of the mechanism(s) whereby *H. pylori* alters gastric homeostasis and renders gastric mucosa more susceptible to disease. In particular, we identified a novel physiological role for *H. pylori* GGT, i.e. the activation of specific host signalling pathways that lead to the induction of cell cycle mediators such as EGF-related growth factors and COX-2. This contributes to unravelling the role of specific bacterial products in the pathogenesis of *H. pylori*-related gastric carcinogenesis and might provide a clue for targeted therapeutic intervention to overcome *H. pylori*-related gastric cancer. However, our data must be interpreted with caution, because the cells used in this study are from an adenocarcinoma, and the effects observed could potentially reflect the biology of a tumour cell as opposed to that of normal, non-transformed cells. On the other hand, MKN 28 and AGS cell lines have been proved to be appropriate models for the study of the response of gastric epithelial cells to *H. pylori* (Ricci *et al.*, 1997; Romano *et al.*, 1998a,b; Naumann *et al.*, 1999; Peek *et al.*, 1999; Pomorski *et al.*, 2001). Moreover, we have previously compared MKN 28 cells with human gastric epithelial cells in primary culture and found comparable results with regard to their response to damaging agents *in vitro* (Romano *et al.*, 1988).

Upregulation of COX-2 expression and activity has been considered as early events in *H. pylori*-associated gastric carcinogenesis, and its effect has been explained at least in part by the inhibition of cellular apoptosis (Zarrilli *et al.*, 1999a; Peek and Blaser, 2002). The fact that *H. pylori* GGT upregulates the expression of COX-2 therefore supports a role for GGT in *H. pylori*-related gastric carcinogenesis. However, this finding is apparently in contrast to a recent report demonstrating that GGT plays a significant role in *H. pylori*-mediated apoptosis of AGS gastric epithelial cells (Shibayama *et al.*, 2003). This discrepancy may depend on different experimental systems and/or different experimental conditions. In partial support of this hypothesis, our data show that *H. pylori* GGT-dependent induction of COX-2 mRNA levels is higher in MKN 28 cells compared with AGS cells. In further support of this hypothesis, we found that *H. pylori* is able to induce apoptosis in AGS cells but not in MKN 28 cells (R. Zarrilli, unpublished observation).

Several signal transduction pathways are activated during the inflammatory and proliferative response of gastric mucosal cells to *H. pylori* infection (Zarrilli *et al.*, 1999a; Peek and Blaser, 2002; Ricci *et al.*, 2002). The expression of genes within the *cag* island activates nuclear factor- κ B, mitogen-activated protein kinase- and stress kinase-

dependent signal transduction pathways, thus ultimately leading to chemokine secretion in gastric mucosal cells (Naumann *et al.*, 1999; Peek *et al.*, 1999). Also, translocation of CagA in gastric epithelial cells induces a growth factor like-response through Grb2 and the Ras/MAPKK/MAPK pathway (Higashi *et al.*, 2002; Mimuro *et al.*, 2002). On the other hand, *H. pylori* infection activates EGFr signalling and expression of EGF-related growth factors in gastric mucosal cells, and these effects are not dependent on genes in the *H. pylori cag* island (Romano *et al.*, 1998a; Tuccillo *et al.*, 2002).

Our data demonstrate that the blockade of the PI-3 kinase or the p38 kinase, but not the MAP kinase, pathway inhibits *H. pylori* GGT-dependent upregulation of COX-2 and EGF-related growth factor expression in MKN 28 gastric mucosal cells. We also show that purified *H. pylori* GGT induced COX-2, HB-EGF and AR mRNA expression at similar time points, thus making it unlikely that COX-2 upregulation is contributed to by EGF-related growth factors. In keeping with our finding, it has been demonstrated that *H. pylori* induces the release of prostaglandin E₂ from gastric epithelial cells by cytosolic phospholipase A₂ activation via the p38 kinase pathway (Pomorski *et al.*, 2001). Our data are also in agreement with those obtained in different experimental systems showing that COX-2 upregulation in platelets is mediated through PI-3 kinase, MAP kinase and p38 kinase (Barry *et al.*, 1999) and that COX-2 overexpression in colon cancer cells is regulated through a PI-3 kinase-dependent pathway (Di Popolo *et al.*, 2000; Sheng *et al.*, 2001).

In conclusion, we demonstrate that *H. pylori*-related upregulation of COX-2 and EGF-related peptide expression depends on the production by the bacterium of GGT and the subsequent activation of PI-3 kinase- and p38 kinase-dependent signal transduction pathways in human gastric mucosal cells. Because EGFr activation and COX-2 increased expression play an important role in the host response to *H. pylori* infection and during gastrointestinal carcinogenesis (Zarrilli *et al.*, 1999a; Peek and Blaser, 2002), based on our findings, we postulate that development of gastric adenocarcinoma associated with *H. pylori* infection may also depend on the activation of GGT-related events.

Experimental procedures

Bacterial strains and growth conditions

We used the wild-type (VacA+/cag+/urease+) CCUG 17874 strain (from the culture collection of the University of Goteborg, Goteborg, Sweden). We also used the mouse-adapted *H. pylori* strain M5 and its GGT-defective isogenic mutant M5 *ggt::aph*, in which the *ggt* gene was disrupted by insertional mutagenesis (McGovern *et al.*, 2001). Bacteria were grown routinely on Columbia agar supplemented with 1% Vitox and 10% defibrinated sheep blood (Oxoid) at 37°C under microaerobic

conditions. Bacteria were grown in *Brucella* broth (Difco) supplemented with 1% Vitox (Oxoid) and 5% fetal calf serum (FCS) to prepare standard crude BCFs (Romano *et al.*, 1998a,b) or 0.2% (2,6-dimethyl)- β -cyclodextrins (CD; from Sigma-Aldrich) (Marchini *et al.*, 1995) to purify soluble bacterial factors for 36–48 h at 37°C in a thermostatic shaker (150 oscillations min^{-1}) under microaerobic conditions. When bacterial suspensions reached 1.2 optical density units at 450 nm (corresponding to a bacterial concentration of 5×10^8 cfu ml^{-1}), bacteria were removed by centrifugation (12 000 *g* for 15 min), and the supernatants were sterilized by passage through a 0.22- μm pore size cellulose acetate filter (Nalgene) to obtain the BCFs. Proteins in the BCFs were processed further for biochemical analysis. In the experiments with crude *H. pylori* BCFs, BCFs were used at dilution of 1:3, and uninoculated BCF (uBCF) served as a control.

Biochemical fractionation of soluble bacterial proteins

Step 1. BCFs were 20-fold concentrated by a Heidolph Roto-vapor apparatus at 37°C and loaded onto a Sephadex G-50 column (Amersham Pharmacia Biotech, size 2.5 \times 60 cm), which was equilibrated and eluted with 25 mM Hepes, 100 mM NaCl, pH 7.4, at a flow rate of 1.5 ml min^{-1} and 4°C.

Step 2. The fractions eluted in the exclusion volume were pooled and loaded onto a cation exchanger column (Bio-Rad, Econo-Pac S-Cartridge, 5 ml) connected to an FPLC system (Amersham Pharmacia Biotech), equilibrated with 25 mM Hepes, 100 mM NaCl, pH 7.4, and eluted with a linear gradient of 0.1–1 M NaCl at a flow rate of 1 ml min^{-1} and room temperature.

Step 3. The eluted fractions retaining the biological activity were pooled, concentrated by Roto-vapor and loaded onto a Superdex 200 High Load column (Amersham Pharmacia Biotech, 1 \times 30 cm) connected to an FPLC system (Amersham Pharmacia Biotech) and eluted with 25 mM Hepes, 100 mM NaCl, pH 7.4, at a flow rate of 0.3 ml min^{-1} and room temperature. Gel filtration low- and high-molecular-weight calibration kits (Amersham Pharmacia Biotech) were used to calibrate the column and to estimate the molecular weight of the eluted samples.

Step 4. The eluted fractions retaining the biological activity were pooled, dialysed against 25 mM phosphate buffer, 100 mM NaCl, pH 8.3, and loaded onto a cation exchanger column (Bio-Rad Laboratories, Econo-Pac S-Cartridge, 5 ml) connected to an FPLC system (Amersham Pharmacia Biotech), equilibrated with the same buffer and eluted with a linear gradient of 0.1–1 M NaCl at a flow rate of 1 ml min^{-1} and room temperature.

SDS-PAGE analysis

Protein concentration of samples was determined by the dye-binding assay of Bradford (using protein assay reagent from Bio-Rad Laboratories). SDS-PAGE analysis was carried out under reducing conditions according to the method of Laemmli (1970); after the run, the proteins were stained by Coomassie brilliant blue R250 or silver nitrate as described previously (Rabilloud *et al.*, 1994).

Automated Edman degradation analysis

For amino acid sequence analysis, protein samples were trans-

ferred from 12% SDS-PAGE gels onto polyvinylidene difluoride (PVDF) membranes (ImmobilonP; Millipore) by electroblotting in 100 mM CAPS (3-cyclohexylamino-1-propanesulphonic acid), 20% methanol, pH 11.0. After Coomassie blue staining, the bands of interest were excised, and N-terminal sequence analysis was performed with a Perkin-Elmer–Applied Biosystems 477A pulsed-liquid protein sequencer equipped with a Perkin-Elmer–Applied Biosystems 120A high-performance liquid chromatography (HPLC) apparatus for phenylthiohydantoin (PTH)-amino acid identification as reported previously (Zappacosta *et al.*, 1997). The obtained peptide sequences were used to identify proteins by searching the PyloriGene™ database of Institut Pasteur (<http://pim.hybrigenics.com>) with the program FASTA (Pearson and Lipman, 1988).

Human gastric epithelial cells and culture conditions

We used two different human gastric cell lines, the MKN 28 cell line, which derives from a well-differentiated human gastric tubular adenocarcinoma and shows gastric-type differentiation (Romano *et al.*, 1988), and the human gastric adenocarcinoma cell line AGS (ATCC, CRL-1739). Both cell lines were grown as monolayers in DMEM supplemented with 10% FCS (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 . To evaluate COX-2 and EGF-related growth factor upregulating activities, MKN 28 cells were incubated for 18 h with *H. pylori* soluble bacterial factors in standard culture medium (DMEM supplemented with 10% FCS). In the experiments with bacterial suspensions, cells were incubated with bacterial preparations at a concentration of 5×10^7 cfu ml^{-1} in DMEM supplemented with 10% FCS. In the experiments with the pharmacological inhibitors, MKN 28 cells were treated with one or other of (i) acivicin, a specific non-competitive inhibitor, at a concentration of 100 μM and 200 μM (Maellaro *et al.*, 2000); (ii) PD98059, an inhibitor of the activation of MAP kinase kinase, at a concentration of 40 μM (Alessi *et al.*, 1995); (iii) SB203580, a specific inhibitor of p38 kinase, at a concentration of 5 μM (Cuenda *et al.*, 1995); (iii) LY294002, a specific inhibitor of PI-3 kinase, at a concentration of 50 μM (Vlahos *et al.*, 1994).

RNA isolation, Northern analysis and RT-PCR analysis

Total RNA was isolated from MKN 28 cells by the guanidinium thiocyanate acid–phenol procedure and subjected to Northern analysis as described previously (Romano *et al.*, 1998a,b). In brief, 10 μg of total RNA per lane was separated by electrophoresis in 1% agarose–formaldehyde gels. RNA was transferred to Hybond-N⁺ (Amersham Italia), cross-linked (UV Stratalinker-1800, Stratagene) and hybridized to ³²P-labelled cDNA probes. ³²P-labelled isotopes were from Amersham Italia. The human COX-2 probe was a 0.276 kb *EcoRI* cDNA, the human heparin-binding EGF-like growth factor (HB-EGF) probe was a 1.102 kb *EcoRI* cDNA, the human amphiregulin (AR) probe was a 0.87 kb *EcoRI*–*HindIII* cDNA, and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.5 kb *BamHI* cDNA. COX-2, HB-EGF and AR mRNA levels were normalized to mRNA levels of the constitutively expressed GAPDH gene (Romano *et al.*, 1998a,b). mRNA levels were quantified by densitometric scanning of the autoradiograph using a Howtek

Scanmaster-3 densitometer with RFL PRINT software (Amersham Pharmacia Biotech). RT-PCR analysis of COX-1 mRNA expression was performed as described previously (Zarrilli *et al.*, 1999b).

Preparation of cell extracts and Western blot analysis

At the end of the incubation period, cells were washed twice with PBS, scraped in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical), 1% NP40 in the presence of a protease inhibitor cocktail (Complete™ tablets; Roche Diagnostics) and then incubated for 30 min at 4°C with shaking. Total lysates were cleared by microfuging at 14 000 r.p.m. for 30 min at 4°C; 80 µg of proteins was separated by 12% SDS-PAGE and blotted to BA 85 0.45 µm protran nitrocellulose membrane (Schleicher and Schuell). After blocking with 10% BSA in TTBS (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20), filters were incubated with specific antibodies (sc-7951 rabbit polyclonal anti-human COX-2 from Santa Cruz Biotechnology; 2998 rabbit polyclonal anti-human HB-EGF, kindly provided by Dr M. Klagsbrun, Boston, MA, USA), and proteins were visualized with anti-rabbit IgG peroxidase-linked antibody (Amersham Pharmacia Biotech) using the ECLplus system (Amersham Pharmacia Biotech).

PGE₂ assay

PGE₂ concentration in the conditioned media of MKN 28 cells was measured with a ¹²⁵I radioimmunoassay as described previously (Romano *et al.*, 1998b).

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