Stimulation of MMP-7 (matrilysin) by *Helicobacter pylori* in human gastric epithelial cells: role in epithelial cell migration

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Summary

Epithelial cell responses to bacterial infection include induction of matrix metalloproteinase 7 (MMP-7). Here, we identify increased MMP-7 expression in the gastric epithelium in response to the oncogenic bacterium *Helicobacter pylori*, and report on the mechanisms and consequences for gastric epithelial cell migration. In patients infected with *H. pylori*, there was increased MMP-7 in gastric biopsies detected by western blot. MMP-7 was localized to the advancing edge of migrating gastric epithelial cell colonies, including lamellipodia. Rates of spreading of gastric gland cells were higher in *H. pylori*-infected cultures compared with control, and this was inhibited by antisense oligonucleotides to MMP-7. Complementary data were obtained in a gastric cancer cell line (AGS cells). In the latter, *H. pylori* induced expression of an MMP-7-luciferase promoter/reporter vector through mechanisms that involved activation of Rho and Rac. RhoA acted through activation of both NF-κB and AP-1, whereas Rac activated NF-κB but not AP-1. MMP-7 is commonly upregulated in gastric cancer; since *H. pylori* is a recognized gastric carcinogen, the data suggest a new mechanism by which the bacterium might predispose towards gastric neoplasia.

Key words: MMP-7, Migration, *Helicobacter pylori*, Gastric epithelium, Matrix metalloproteinase

Introduction

Epithelial cells in the gastrointestinal, urogenital and respiratory tracts are frequently exposed to bacteria. The maintenance of normal epithelial integrity and function therefore depends on the execution of appropriate adaptive responses, which may include interactions with immune cells (e.g. by release of cytokines, chemokines or antigen presentation) and control of epithelial cell proliferation, migration or apoptosis. Recent studies suggest that matrix metalloproteinase 7 (MMP-7), or matrilysin, plays a role in mediating responses to bacteria in several different systems, although the relevant cellular mechanisms are uncertain (Lopez-Boado et al., 2000; Lopez-Boado et al., 2001). Induction of MMP-7 also occurs in tumours in many organs including colon, stomach and pancreas (Crawford et al., 2002; McDonnell et al., 1991). In the stomach, it is well established that infection with *Helicobacter pylori* predisposes to gastric cancer in some subjects (Uemura et al., 2001); this appears to be one of the clearest examples of a growing number of instances in which bacteria facilitate tumourigenesis (Lax and Thomas, 2002; Peek and Blaser, 2002). The responses of epithelial cells to *H. pylori* infection that might influence the subsequent progression to cancer are still largely unknown, and the possible role of MMP-7 in this system is uncertain.

The MMPs comprise a family of over 25 members implicated in the proteolysis of extracellular matrix, growth factors, cytokines, adhesion molecules and protease inhibitors such as the serpins, resulting in both gain and loss of function (Nagase and Woessner, 1999; Seiki, 2002). It is now widely appreciated that induction of distinct profiles of MMPs, or of their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), is a feature of the tissue remodelling that occurs in many normal and disease processes (e.g. in development, wound healing, cancer and a wide variety of inflammatory conditions) (Murphy and Gavrilovic, 1999). In the gastrointestinal tract, epithelial cells are often not the source of the increased MMP production that occurs in response to disease. Instead, it is the sub-epithelial (i.e. stromal or mesenchymal) cells that produce the MMPs that play a role in mucosal responses, at least in part by influencing the environment at the epithelial basolateral membrane. However, an exception is MMP-7, which is characteristically produced by epithelial cells and plays a role in a variety of epithelial responses. Thus, for example, in murine small intestinal Paneth cells, MMP-7 may act as a processing enzyme to convert prodefensins to their active form, thereby playing a role in the innate defence system of the gut (Wilson et al., 1999).

The epithelial cells of the gastrointestinal tract are protected from ingested bacteria in part by the barrier presented by gastric acid secretion that generates an initial environment unfavourable to the survival of microoganisms. However, unusually, the gastric pathogen *H. pylori* (Tomb et al., 1997) colonizes the microenvironment generated between the surface of gastric epithelial cells and the overlying mucus glycoprotein

gel. In many subjects, infection with *H. pylori* is clinically silent; but in some patients it is associated with duodenal ulcer, and in others it is associated with a progression through chronic atrophic gastritis to gastric adenocarcinoma (Fox and Wang, 2001; Uemura et al., 2001). The cellular and molecular mechanisms that determine the precise constellation of epithelial cell responses to infection are far from clear. However, this is a relatively attractive system for studies of the way that bacterial infection might control MMP-7 expression because: (1) it is relatively straightforward to obtain primary gastric epithelial cells from *H. pylori* positive and control subjects; (2) there is a clear link between *H. pylori* and gastric cancer; and (3) MMP-7 expression is known to be increased in gastric cancer (Honda et al., 1996). We report here increased expression of MMP-7 in response to *H. pylori*, the consequences for epithelial cell migration, and the relevant signalling pathways.

Materials and Methods

Cells, plasmids and drugs

AGS cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), routinely cultured in HAMS/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and subcultured with 1% trypsin/versene (Life Technologies, Paisley, UK) as described (Wroblewski et al., 2002). Expression vectors for constitutively active (CA)-RhoA (L63RhoA), Rac (L61Rac) and cdc42 (L61cdc42), and for dominant-negative (DN)-RhoA (N19RhoA) and DN-Rac (N17Rac) were gifts from Alan Hall (University College, London, UK). A DN-c-jun plasmid was provided by Tim Wang (UMAS, Worcester, MA) and the c-fos-overexpressing vector was generated as described previously (Deavall et al., 2000). Lynn Matrisian (Vanderbilt University, Nashville, TN) generously donated a vector consisting of 2.3 kb of human MMP-7 promoter coupled to firefly luciferase (i.e. MMP-7-luc). BAY11-0782, PD98059, epidermal growth factor (EGF), human recombinant MMP-7, MMP-7 antisense (AS) oligonucleotides (both unlabelled and fluorescein labelled), and control oligonucleotide were obtained from Calbiochem (Nottingham, UK). Interleukin 8 (IL-8), IL-6 and tumour necrosis factor α (TNF-α) were purchased from R&D Systems (Minneapolis, MN). *Clostridum botulinum* C3 toxin was obtained from Sigma (Poole, Dorset, UK).

Bacterial infection of AGS cells and gastric glands in vitro

We routinely used *H. pylori* strain 60190 (ATCC). In a few experiments, we also used two gastric isolates, strain E5 (*cag*$^+$, *vacA*$^-$) and E6 (*cag*$^+$, *vacA*$^+$). Bacteria were grown in a microaerophilic atmosphere at 37°C on fresh chocolatized Columbia blood agar for 3-9 days (Oxoid, Basingstoke, UK). Unless otherwise stated, *H. pylori* was added to human gastric glands or AGS cells at a multiplicity of infection (MOI) of 100. In some experiments, bacteria were added to 0.2 μm Anapore filter inserts (Life Technologies) that were suspended above AGS cells. Two such systems were studied: either bacteria was added directly to the filters, or to AGS cells cultured on filters that were then inserted over AGS cells transfected with MMP-7-luc (see below). In other experiments, *H. pylori* were sonicated for 20 minutes and added directly to AGS cells.

Patients

Endoscopic pinch biopsies of the gastric corpus and antrum were obtained from patients attending routine gastroscopy for investigation of dyspepsia. Most patients had no macroscopic gastroduodenal pathology; none of them had neoplastic disease, and a total of four *H. pylori*-positive patients had peptic ulcer (3 duodenal, 1 antral). *H. pylori* status was initially assessed by a rapid urease test (Prontodry, Medical Instruments Corporation, Solothurn, Switzerland), and subsequently confirmed by histology. On routine histology, *H. pylori*-positive biopsies typically exhibited active chronic gastritis as expected. The present report is based on studies of 41 patients (25 female, mean age 53.5±1.6 years) who were *H. pylori* positive, and 45 who were *H. pylori* negative (29 female, mean age 50.9±1.8 years) and were used as controls. Samples were assigned randomly within the two groups either to extraction for western blotting or to gastric gland culture. The study was approved by the Ethics Committee of Royal Liverpool and Broadgreen University Hospitals NHS Trust. All patients gave informed consent.

Gastrin radioimmunoassay (RIA)

The concentration of amidated gastrins in plasma was determined by RIA using antibody L2 specific for the C-terminal amide sequence of gastrin, as described previously (Varro et al., 1997).

Western blotting

Protein extracts were prepared in RIPA or lysis buffer and western blotting was performed as previously described (Varro et al., 2002) using a rabbit anti-MMP-7 antibody (Chemicon International, Temecula, CA) or anti-NF-κB (p65, p50) antibodies (Santa Cruz Biotechnology, Santa Cruz, MA). Samples were reprobed with a goat anti-β-actin antibody (Santa Cruz Biotechnology).

Immunohistochemistry

For immunohistochemistry (IHC), a goat anti-MMP-7 antibody (Santa Cruz Biotechnology) was used with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (Jackson IR, West Grove, PA) as previously described (Wroblewski et al., 2002). In colocalization studies, the following antibodies were used: mouse anti-gastric mucin (Sigma), rabbit anti-pepsinogen (gift from Mike Samloff, Center for Ulcer Research, Los Angeles, CA), rabbit anti-H^+/K^+ATP-ase (Calbiochem), rabbit anti-somatostatin (Santa Cruz Biotechnology), rabbit anti-chromogranin A and guinea-pig anti-gastrin (Hussain et al., 1999), mouse anti-vimentin and α-smooth muscle actin (Research Diagnostics, Flanders, NJ). Texas-Red-labelled donkey anti-mouse, anti-rabbit or anti-guinea-pig IgG (Jackson IR) were used as appropriate. For studies of the localization of MMP-7 in gastric biopsies, formalin-fixed paraffin sections were treated with 0.9% hydrogen peroxide in methanol to block endogenous peroxide, and antigen was retrieved by heating in 10 mM EDTA, pH 7.0 for 3 minutes at full pressure in a pressure cooker. Primary rabbit anti-MMP-7 (Chemicon) was employed with detection by the ChemMate EnVision HRP/DAB system (DakoCytomation, Ely, UK).

Human primary gland culture

Human biopsies were sliced into 2 mm^2 segments using a razor blade and washed in three changes of Hank's Balanced Salt Solution (HBSS). Tissue was incubated in 5 ml 1 mM dithiothreitol for 15 minutes, with continuous gassing with 5% CO_2/95% O_2 at 37°C, and shaking at 100 cycles per minute. Tissue was then washed in HBSS (three times), incubated in 0.5 mg ml^{-1} collagenase for 30 minutes (Roche, Lewes, UK), washed again in HBSS (three times) and incubated for a further 30 minutes in collagenase (0.5 mg ml^{-1}). The tissue was then triturated using a wide mouthed pipette and larger fragments allowed to settle under gravity for 45 seconds. The supernatant containing isolated glands was removed and transferred to a clean tube, and shaken vigorously to release additional glands that were allowed to sediment for 45 minutes. The supernatant (which contained individual cells and debris) was carefully removed and

discarded. The isolated glands were routinely cultured up to 72 hours in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Sigma), at 37°C in 5% CO_2/95% O_2, and the medium was changed every 24 hours.

Spreading of human gastric glands

Human gastric glands were cultured in 6-well dishes. In experiments where AS oligonucleotides were used, either MMP-7 AS oligonucleotide (2 μM) or negative control oligonucleotides (2 μM) were added at the start of culture. Medium and oligonucleotides were replaced daily. Intracellular accumulation of oligonucleotides was confirmed using FITC-labelled MMP-7 AS oligonucleotide (2 μM) viewed live under a Leica DMIRE2 microscope 17 hours after addition. Routinely, gastric colonies were cultured for 17 hours and then mounted on a motorized stage fitted on a Leica DMIRE2 microscope in a heated, humidified chamber (Solent Scientific, Portsmouth, UK) and images captured with a Hamamatsu Orca ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) controlled by Kinetic Imaging AQM-2001 software (Kinetic Imaging, Liverpool, UK). Serial images were analysed using Lucida 4.0:Analyse software (Kinetic Imaging), and gland cell spreading was calculated as the mean area of a gland normalized for cell number determined by Hoechst 33342 (Molecular Probes, Eugene, OR) nuclear staining.

AGS cell migration and invasion assays

Transwell migration and invasion assays were performed using AGS cells cultured in 24-well plates containing either 8 μm pore Biocoat® control inserts (migration assays) or Matrigel-coated inserts (invasion assays), according to the manufacturer's instructions (Becton Dickinson, Bedford, MA). Briefly, 2.5×10^4 AGS cells were diluted in 0.5 ml serum free (SF) medium and placed in the insert immersed in 0.75 ml SF medium with or without *H. pylori* and a mouse monoclonal MMP-7 neutralizing antibody (Chemicon) for 15-22 hours. After incubation, non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were stained with Diff-Quik reagent (Dade Behring, Dudingen, Switzerland). Membranes were then removed, mounted in immersion oil and invading cells counted using a Zeiss 25 Axiovert microscope (Carl Zeiss, Welwyn Garden City, UK) and Intellicam software (Matrox, Stoke Poges, UK).

Transient transfection and luciferase assay

Cells (2×10^5) were plated in 6-well plates in full medium (FM). The following day, medium was removed and cells were transfected using TransFast (Promega, Madison, WI) in SF medium for 1 hour. Routinely, MMP-7-luc was used at 0.5-1.0 μg $well^{-1}$. After transfection, 2 ml FM was added and cells incubated for 20-24 hours. Media was then replaced with 2 ml SF medium and cells were incubated with *H. pylori* and other compounds as indicated for 6 hours. Luciferase activity was measured with Bright-Glo™ or Dual Glo™ (Promega) using a LumiCount Platereader (Packard BioScience, Pangbourne, UK) according the manufacturer's protocol. Results are presented as fold increase over unstimulated control, so 1.0 signifies no change in luciferase activity. Protein concentration was determined when appropriate using Lowry protein assay kit (Sigma) to monitor plating efficiency and cell death.

Transcription factor activation

To identify the transcription factors that might bind cis-acting DNA elements in nuclear extracts in response to *H. pylori*, we used Mercury™ Transfactor Profiling Kit-Inflammation 1 (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol. Nuclear extracts were prepared from control cells, and cells were incubated with *H. pylori* using NE-PER™ (Pierce Biotechnology, Rockford, IL).

Statistics

Results are presented as means±s.e.m.; comparisons were made using a *t* test, and were considered significant at $P<0.05$.

Results

Increased MMP-7 abundance in gastric mucosa from *H. pylori*-positive subjects

In initial studies, we examined the abundance of MMP-7 in western blots of gastric biopsies from control and *H. pylori*-infected subjects. In all subjects, a band corresponding to 28 kDa pro-MMP-7 was detected. However, the abundance was greater in both the corpus

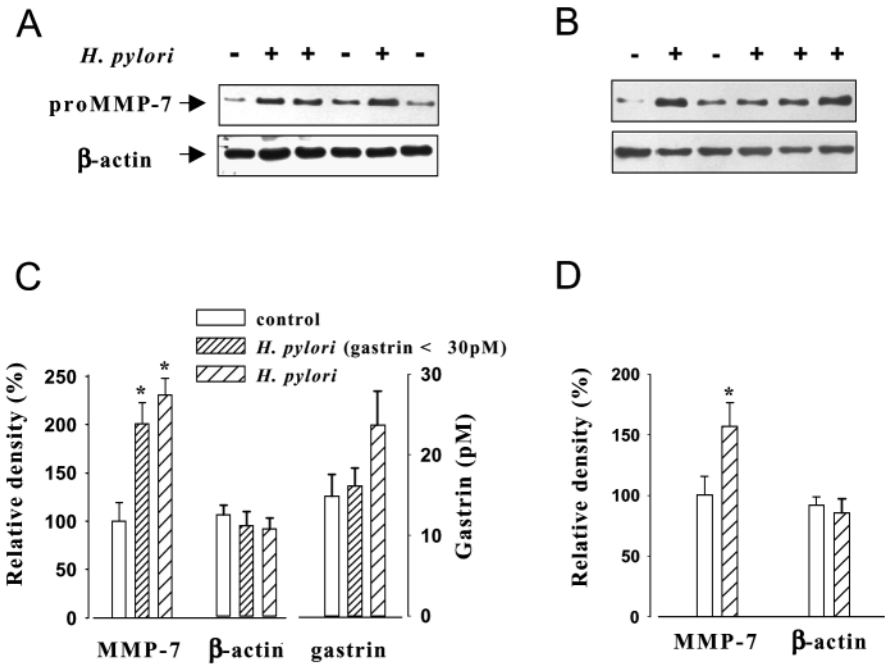

Fig. 1. Western blots of MMP-7 and β-actin in gastric biopsies from control and *H. pylori*-infected subjects. Top panels, representative blots of MMP-7 and β-actin from control subjects (–) and subjects infected with *H. pylori* (+) in biopsies from the gastric corpus (A) and antrum (B). Bottom panels, the relative abundance in corpus (C) and antrum (D) of MMP-7 and β-actin. (C) Plasma gastrin concentrations are indicated in panel C, where MMP-7 and β-actin data are also shown for the subset of *H. pylori*-positive patients in which plasma gastrin concentrations were in the normal range (i.e. <30 pM). Data for corpus based on $n=11$ control subjects (open bars) and 16 *H. pylori*-positive subjects; data for antrum based on $n=10$ control subjects and $n=6$ *H. pylori*-positive subjects. Means±s.e.m. *$P<0.05$.

and antral region of the stomach from *H. pylori*-positive subjects compared with controls (Fig. 1). There is a tendency in *H. pylori* infection towards elevated plasma concentrations of the gastric hormone gastrin (Calam et al., 1997), and so we considered the possibility that gastrin might mediate the effect of *H. pylori* on MMP-7. However, when *H. pylori*-positive subjects with plasma gastrin concentrations in the normal range for this assay (<30 pM) were compared with control subjects, there remained a significant difference in the relative abundance of MMP-7 in western blots in the two groups (Fig. 1).

Cellular localization of MMP-7 in *H. pylori*-positive subjects

The localization of MMP-7 was determined in both paraffin sections of gastric biopsies and cultured gastric glands. In paraffin sections, MMP-7 immunoreactivity was identified in surface epithelial cells where there was cytoplasmic localization that tended to be subnuclear (i.e. on the basolateral side); there was also staining deep in the gastric gland compatible with localization to chief cells (Fig. 2). The staining in samples from *H. pylori*-positive subjects was more intense than in those from *H. pylori*-negative subjects, but the cellular distribution was similar. Cultured gastric glands from the corpus and antrum of normal and *H. pylori*-positive subjects contained the major cell types found in vivo. Thus, in corpus cultures, antibody to H^+/K^+ATPase identified approximately 10% of the total cells as parietal cells, and antibodies to pepsinogen and mucus glycoprotein identified chief and mucus cells (approximately 25 and 35%) respectively; enterochromaffin-like (ECL) cells, revealed by chromogranin A immunoreactivity, were typically <5% total. In antral cultures, parietal cells were absent, and there were abundant mucus (>50% total) and pepsinogen (approximately 40% total) cells, but G- and D-cells revealed by gastrin and somatostatin immunoreactivity, respectively, were typically <5% of total. MMP-7 immunoreactivity was found in many cells that were also stained with antibodies to mucus glycoprotein (Fig. 2), or to pepsinogen, compatible with localization to mucus and chief cells, respectively. In both cases, only a subset of the two cell types expressed MMP-7. By contrast, in the corpus, neither parietal cells nor ECL cells exhibited MMP-7 immunoreactivity

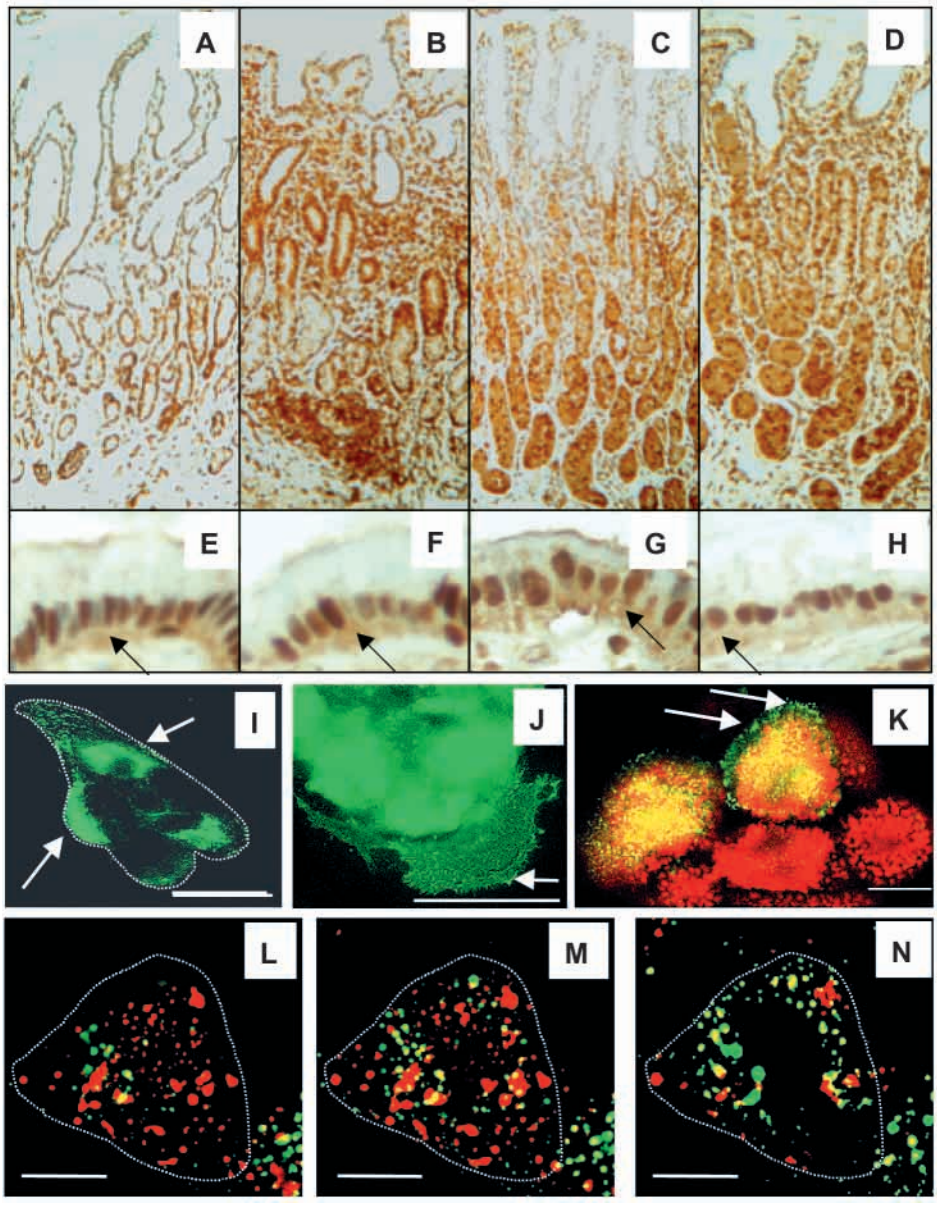

Fig. 2. Immunohistochemical localization of MMP-7 in human gastric epithelial cells. Immunoperoxidase labelling of MMP-7 and counter-staining with haematoxylin in antral biopsies from (A) *H. pylori*-negative and (B) *H. pylori*-positive subjects, and (C) in corpus biopsies from *H. pylori*-negative and (D) *H. pylori*-positive subjects (all ×10). Higher magnification (×40) of the same samples (E-H, respectively) reveals cytosolic localization to a subnuclear region of surface mucus cells (arrow). (I) Immunofluorescence localization of MMP-7 to cells in a cultured antral gland from a *H. pylori*-positive subject (bar, 25 μm). The outline of the gland is indicated with a white broken line. (J) MMP-7 immunoreactivity in a cell at the periphery of a cultured antral gland from a control subject after *H. pylori* addition, demonstrating a lamellipodium with punctate MMP-7 immunoreactivity (bar, 50 μm). (K) Co-expression of MMP-7 (green) and mucin (red) in antral cells, demonstrating MMP-7 is not exclusively localized with mucin and showing the presence close to the plasma membrane of cells at the periphery of the cultured gland (bar, 10 μm). (L-N) Deconvolved images from a z-series through a single antral cell (outlined by a white broken line) showing that MMP-7 and mucin exhibit punctate staining in distinct subcellular compartments (bar, 10 μm).

(not shown). Moreover, in the gastric antrum, MMP-7 immunoreactivity was not identified in G- or D-cells (not shown). The pattern of localization in different cell types did not differ between *H. pylori*-positive and -negative subjects.

The majority of cultured glands did not contain cells staining with antibodies to α-smooth muscle actin, or to vimentin (not shown). In those glands in which positive staining was encountered with these antibodies, the stained cells never accounted for more than 3% of the total. Thus, the cultured gastric glands were largely epithelial with little or no contamination by myofibroblasts or fibroblasts. Moreover, the localization of MMP-7 in cultured epithelial cells was compatible with that seen in tissue fixed immediately after sampling.

In cultured glands, MMP-7 was typically sequestered in vesicles that were distinct from those reacting with either mucin or pepsinogen antibodies (Fig. 2). Interestingly, there was a clear localization of MMP-7 to the plasma membrane of cells at the periphery (i.e. the migrating edge) of cultured gastric glands. This pattern was seen in both *H. pylori*-positive and -negative subjects. When *H. pylori* was added to control gastric gland cultures, the peripheral cells exhibited a tendency to extend lamellipodia, and MMP-7 immunoreactivity was identified in these structures (Fig. 2).

Role of MMP-7 in gastric gland spreading

We then examined the spreading of cultured gastric glands of both *H. pylori*-positive and -negative subjects. Over periods of up to 48 hours in culture, cells migrated from isolated gastric glands to form monolayer islands, or colonies, of cells. During this process, cell-cell contacts were maintained and, although time-lapse video-microscopy revealed cellular movements indicative of remodelling of these contacts, cells characteristically did not migrate away from the colony. Careful tracking of individual cells provided no evidence for mitosis of gland cells, and in separate studies we also found no evidence of proliferation indicated by nuclear PCNA in cultured glands (S. Kennedy and A. Varro, unpublished). The progressive increase in area covered by individual glands therefore represents both migration and spreading, but not proliferation. Importantly, the expansion of these islands of gastric gland cells from *H. pylori*-positive subjects was significantly greater than in controls (Fig. 3). In order to determine whether there was a

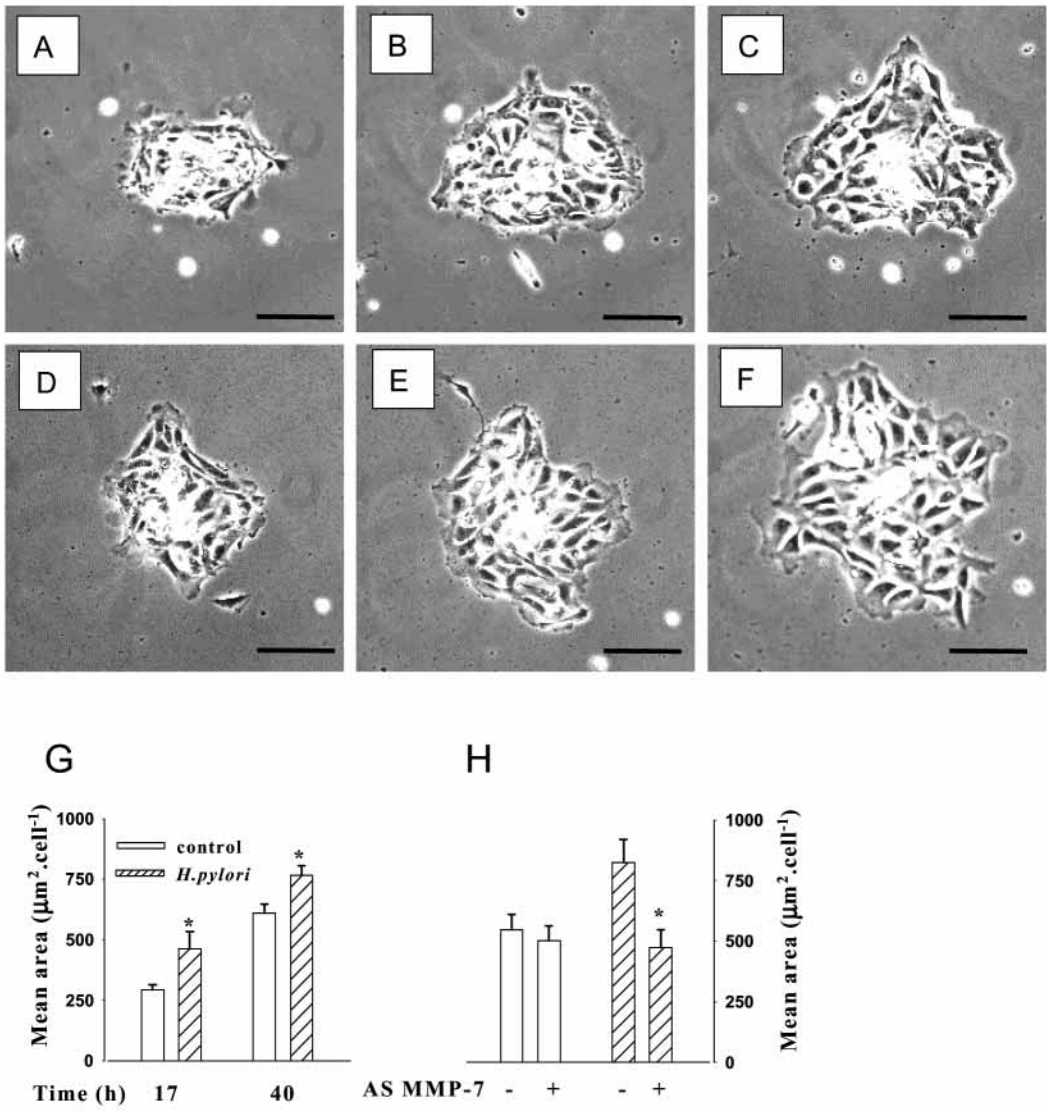

Fig. 3. Spreading of human gastric epithelial cells is stimulated by *H. pylori* by induction of MMP-7. (A-C) Frames taken at 17, 32 and 52 hours from the same human gastric gland (control subject) during time-lapse video-microscopy. (D-F) Similar frames taken from a gland from a *H. pylori*-positive subject. Subsequent staining of cell nuclei with Hoechst 33342 revealed 86 cells in A-C and 88 cells in D-F. (G) The area of gastric colonies expressed relative to cell numbers at 17 and 40 hours indicates increased migration in *H. pylori*-positive (n=8; mean data from 4-15 glands per subject) compared with control subjects (n=12; mean data of 4-15 glands per subject). (H) Pretreatment with antisense (AS) oligonucleotides to MMP-7 (2 μM) inhibited the spreading of gastric glands at 40 hours from *H. pylori*-positive subjects (n=7), but had only a slight effect on control glands (n=7). Data expressed as means±s.e.m. *P<0.05. In A-F: bar, 200 μm.

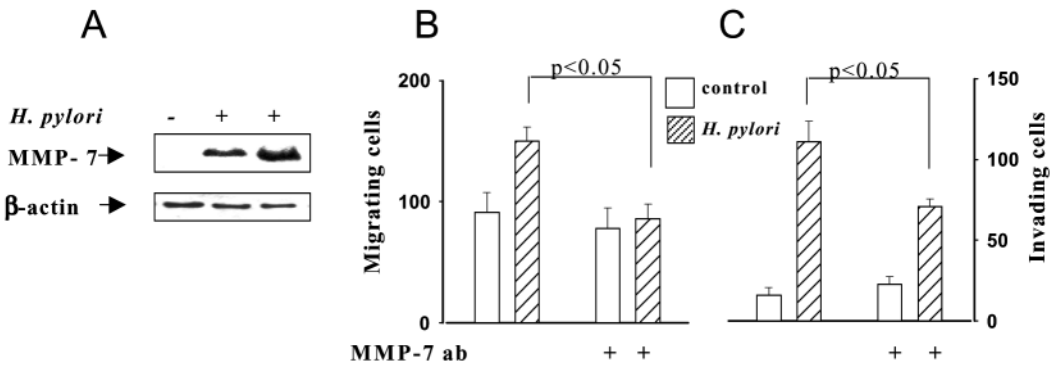

Fig. 4. *H. pylori* induction of MMP-7 in AGS cells is associated with stimulation of migration and invasion. (A) Western blots indicate that addition of *H. pylori* to cultured AGS cells with a multiplicity of infection [MOI] of 100 (centre lane) or 200 (right lane) produced a graded increase in the abundance of MMP-7; the latter co-migrated with the 19 kDa human recombinant MMP-7 (arrow). (B) *H. pylori* (hatched bars), increased migration of AGS cells through Transwell filters. The number of migrating cells per field are presented; mean±s.e.m., n=3. (C) *H. pylori* (hatched bars) increased invasion of AGS cells through Matrigel-coated Transwell filters. The sum of the invading cells in five fields is presented; mean±s.e.m., n=3. Neutralization of MMP-7 using an MMP-7 monoclonal antibody (4 μg ml^{-1}) inhibited both migration and invasion.

role for MMP-7 in this response, we examined the effect of application of MMP-7 AS oligonucleotides. In validation experiments, the latter were shown to suppress MMP-7 induction by *H. pylori* in AGS cells (data not shown). We then showed that MMP-7 AS oligonucleotides inhibited migration of cultured glands from *H. pylori*-positive subjects (Fig. 3). By contrast, application of MMP-7 AS oligonucleotides had no effect on the spreading of gland cells from control subjects. In addition, scrambled oligonucleotides used as a control had no effect on the gland cell spreading.

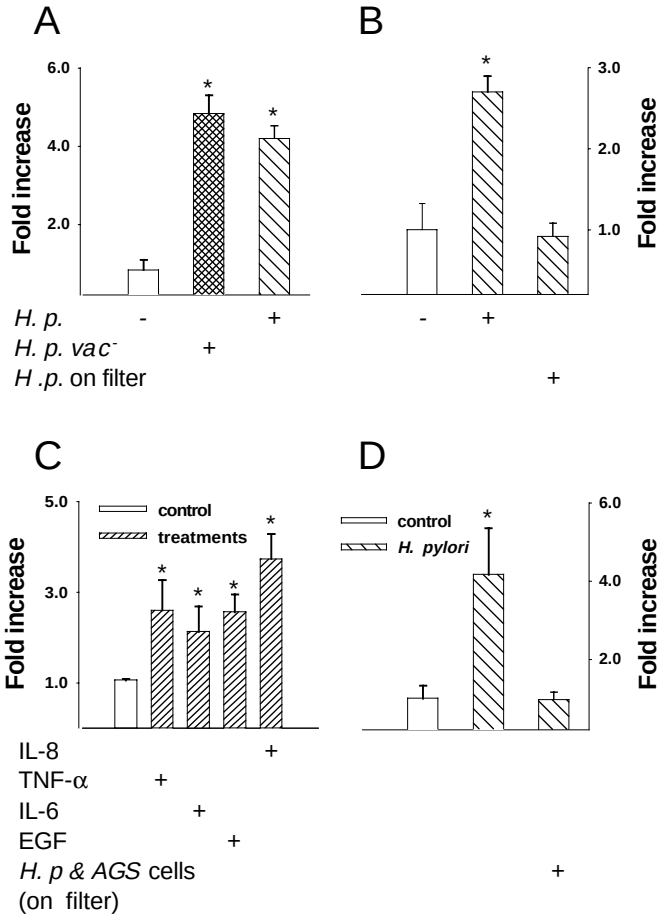

Fig. 5. Addition of *H. pylori* stimulates the expression of the 2.3 kb human MMP-7 promoter coupled to a luciferase reporter. (A) Stimulation of MMP-7-luc (1.0 μg well^{-1}) by two different isolates of *H. pylori*, one *vacA*$^+$ and *cagA*$^+$ (E6), the other *vacA*$^-$ and *cagA*$^+$ (E5). (B) Failure of *H. pylori* (60190) to stimulate MMP-7-luc when added to a filter over transfected AGS cells. (C) Stimulation of MMP-7-luc by the chemokine IL-8 (250 ng ml^{-1}), proinflammatory cytokines TNF-α (2.5 ng ml^{-1}) and IL-6 (5 ng ml^{-1}), and the growth factor EGF (100 ng ml^{-1}). (D) However, the effect of *H. pylori* (60190) on MMP-7-luc was not mediated by soluble AGS cell factors, because application of *H. pylori* to AGS cells on filters cultured above MMP-7-luc transfected AGS cells had no effect on MMP-7-luc compared with direct application to AGS cells. Means±s.e.m., n=3.

H. pylori stimulation of MMP-7: invasion and migration of a gastric cancer cell line

We explored the induction of MMP-7 by *H. pylori* in a gastric epithelial cell line (AGS cells). Application of *H. pylori* to AGS cells for 16 hours increased the abundance of the active 19 kDa form of MMP-7 determined by western blot (Fig. 4). The functional significance of this induction was then examined using migration and invasion assays. In this model, *H. pylori* increased migration of AGS cells through 8 μm Transwell filters, and stimulated invasion through Matrigel-coated filters compared with control cells (Fig. 4). A role for MMP-7 in both migration and invasion was shown by the inhibition of *H. pylori*-stimulated migration and invasion by addition of neutralizing MMP-7 antibody (Fig. 4). Application of sonicates of *H. pylori* had no effect on AGS cell migration or invasion, suggesting that a soluble mediator was not involved.

Putative bacterial and host mediators of increased MMP-7 in response to *H. pylori*

In order to study the mechanism of *H. pylori*-induced expression of MMP-7, we first showed that addition of *H. pylori* to cultured AGS cells increased the expression of a construct consisting of 2.3 kb of the human MMP-7 promoter coupled to a luciferase (i.e. MMP-7-luc) reporter. The induction of MMP-7-luc in AGS cells was similar for two strains that were *cag*$^+$ and *vacA*$^+$ (E6 and 60190). A third strain that was *vacA*$^-$ (E5) also produced similar responses. Thus,

although the secreted cytotoxin VacA has been suggested to activate the small GTPase Rac (Hotchin et al., 2000), and studies described below implicate Rac in the induction of MMP-7, the data suggest this toxin was not required for the AGS cell responses studied here (Fig. 5). In addition, sonicates of *H. pylori* had no stimulatory effect on MMP-7-luc (not shown). Moreover, when *H. pylori* was applied to 0.2 μm filters cultured over AGS cells transfected with MMP-7-luc, there was again no increase in MMP-7-luc expression (Fig. 5). Together, these lines of evidence suggest that release of a soluble mediator by the bacterium is unlikely to be the main mechanism involved in MMP-7 induction.

It is known that *H. pylori* increases shedding of HB-EGF and stimulates production of proinflammatory cytokines (IL-6, TNF-α) and chemokines (IL-8) (Crabtree et al., 1994; Moss et al., 1994; Romano et al., 1998; Wallasch et al., 2002). In AGS cells, EGF-family members, IL-8, TNF-α and IL-6 were all found to increase MMP-7-luc expression (Fig. 5). We therefore examined the possibility that *H. pylori* increased MMP-7 expression in AGS cells via a paracrine mediator. AGS cells were transfected with MMP-7-luc, and then cocultured with untransfected AGS cells grown on 0.2 μm pore filters suspended above the transfected cells. Addition of *H. pylori* to AGS cells grown on 0.2 μm Transwell filters had no effect on MMP-7-luc expression in the cocultured cells. Putative paracrine signals may of course be diluted in this system, but in the same experimental system using luciferase expression driven by a promoter sequence of plasminogen activator inhibitor-2 (Varro et al., 2002) there was induction of luciferase by *H. pylori* up to 3-5 fold. Thus, the data support the view that a direct effect due to adherence of the bacteria was responsible for stimulating MMP-7-luc expression (Fig. 5).

Putative transcription factors mediating the effect of *H. pylori* on MMP-7

Previous studies have implicated NF-κB and AP-1 in *H. pylori*-stimulated transcriptional responses (Meyer-ter-Vehn et al., 2000; Munzenmaier et al., 1997). We first confirmed that addition of *H. pylori* to AGS cells increased p50 and p65 NF-κB translocation to the nucleus by western blotting, and that binding of p50 and p65 NF-κB to a consensus NF-κB cis-element was increased in *H. pylori*-stimulated AGS cell nuclear extracts (Fig. 6). The functional significance of these findings for MMP-7 expression was then demonstrated by showing that an inhibitor of IκB degradation (BAY11-7082) reduced the MMP-7-luc response to *H. pylori* to control levels. In addition, an increase in c-fos binding to a consensus AP-1 cis-element was demonstrated in nuclear extracts, and cotransfection of AGS cells with MMP-7-luc and a vector encoding c-fos increased expression 2-3 fold (Fig. 6). It is known that *H. pylori* increases AP-1 activity via the Erk1/2 pathway (Meyer-ter-Vehn et al., 2000) and, consistent with the involvement of this pathway in the present studies, we found that PD98059 (which inhibits the Erk1/2 kinase, MEK) inhibited the response to *H. pylori* (Fig. 6).

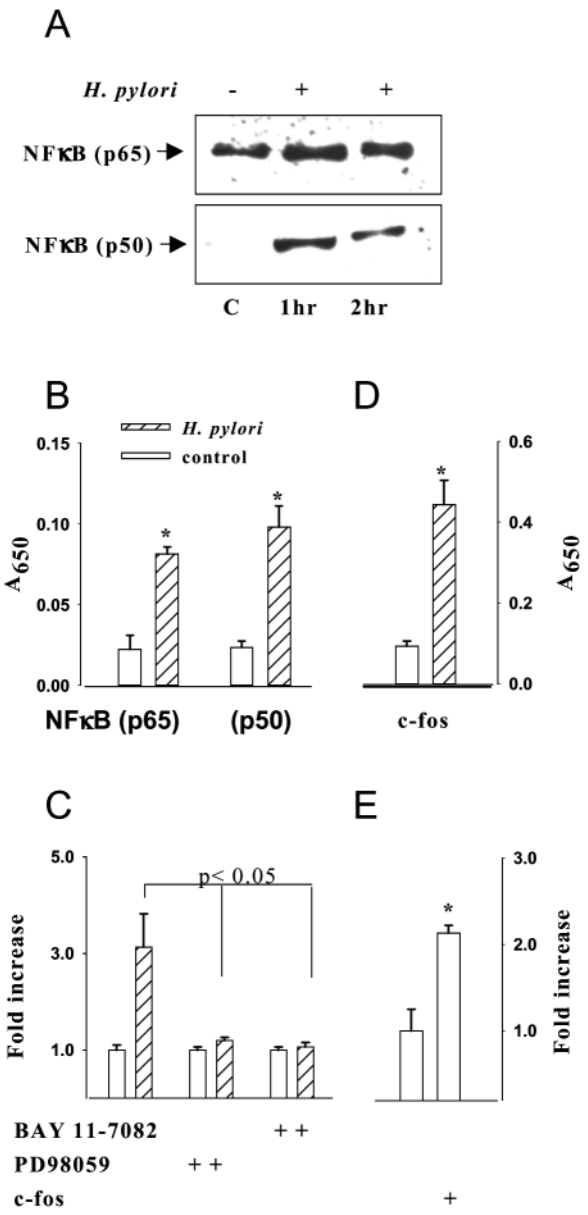

Fig. 6. *H. pylori*-increased p50, p65 and c-fos activity in AGS cells mediates MMP-7-luc expression. (A) In western blots of nuclear extracts, *H. pylori* increased NF-κB p50 and p65 compatible with nuclear translocation. (B) In nuclear extracts of *H. pylori*-treated cells there was increased p65 and p50 binding to the NF-κB consensus cis-element. (C) The inhibitor of IκB degradation, BAY11-7082 (10 μM), depressed MMP-7-luc responses to *H. pylori*, as did the MEK inhibitor PD98059 (20 μM). (D) In nuclear extracts, there was also activation of c-fos, as indicated by binding to oligonucleotides with relevant consensus sequences. (E) Cotransfection with c-fos (0.6 μg well^{-1}) increased expression of MMP-7-luc. Means±s.e.m., n=3.

Role of RhoA and Rac in mediating the effect of *H. pylori* on MMP-7-luc expression

H. pylori is reported to activate small GTPases of the Rho family (Palovuori et al., 2000). We therefore screened three representatives of this GTPase family (RhoA, Rac1, cdc42) for the capacity to stimulate MMP-7-luc expression. Cotransfection of AGS cells with a vector encoding a constitutively active form of RhoA (L63RhoA) strongly stimulated MMP-7-luc; there was a moderate response to a

constitutively active form of Rac (L61Rac), whereas a constitutively active form of cdc42 (L61cdc42) had little effect (Fig. 7). We then showed that induction of MMP-7-luc by *H. pylori* was mediated by RhoA and Rac because cotransfection of MMP-7-luc and a DN-RhoA vector (N19RhoA), or DN-Rac (N17Rac), reduced the *H. pylori*-increased expression of MMP-7-luc (Fig. 7). In addition, application of the RhoA inhibitory toxin, C3-transferase, reduced MMP-7-luc responses to *H. pylori* by 76.9±4.4%. The MMP-7-luc responses to L63RhoA and L61Rac were inhibited by BAY11-7082, indicating that NF-κB was downstream of both RhoA and Rac (Fig. 7). Interestingly, cotransfection of MMP-7-luc and L63RhoA with a DN-jun vector resulted in significant inhibition of MMP-7-luc expression, compatible with activation of AP-1 downstream of RhoA. By contrast, the DN-jun vector had no effect on Rac activation of MMP-7-luc expression (Fig. 7).

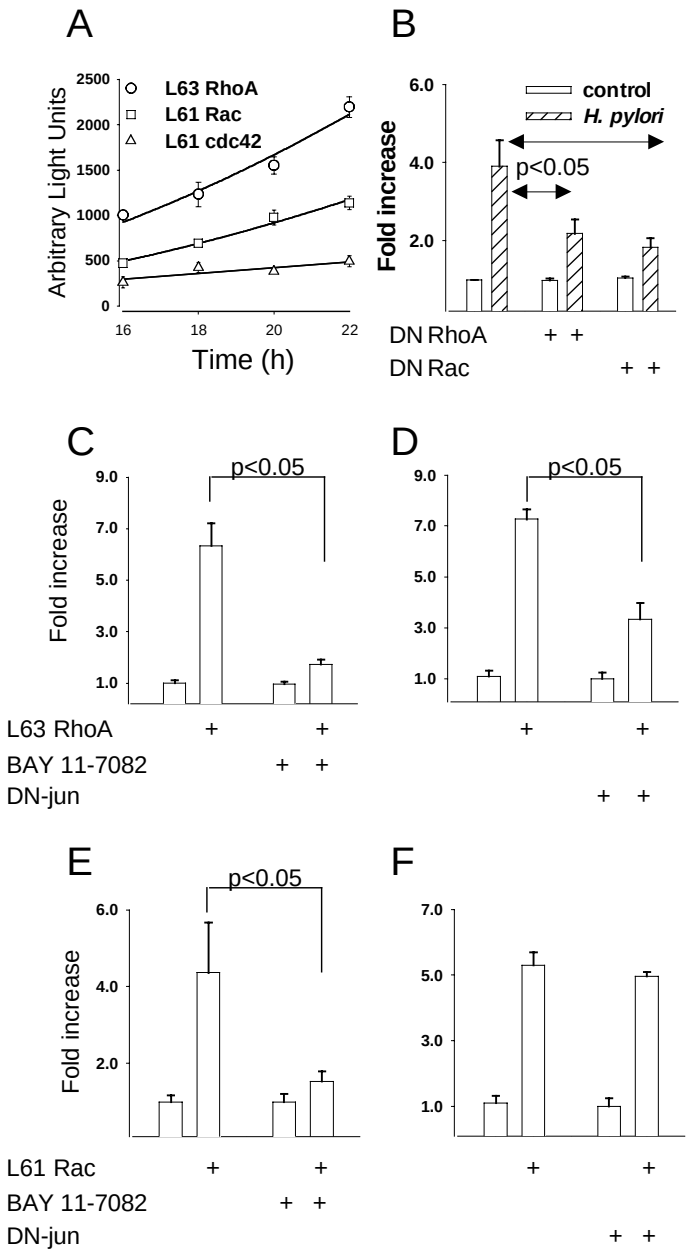

Fig. 7. Induction of MMP-7 by *H. pylori* is mediated by RhoA. (A) Expression of the MMP-7-luc plasmid (0.5 μg well^{-1}) was increased in a time-dependent manner (hours after transfection) by cotransfection with plasmids encoding constitutively active forms of Rho (L63RhoA, 0.25 μg well^{-1}) and, to lesser extent, Rac (L61Rac, 0.25 μg well^{-1}); constitutively active cdc42 (L61cdc42, 0.25 μg well^{-1}) had only a slight effect. (B) *H. pylori*-stimulated MMP-7-luc expression was inhibited by cotransfection with dominant-negative (DN) RhoA (N19RhoA, 0.5 μg well^{-1}) and DN-Rac (N17Rac, 0.5 μg well^{-1}). (C,D) L63RhoA-stimulated MMP-7-luc expression was inhibited by BAY11-7082 (10 μM) and by cotransfection with DN-jun vector (0.5 μg well^{-1}). (E,F) L63Rac-stimulated MMP-7-luc expression was inhibited by BAY11-7082, but not by cotransfection with a DN-jun vector. Means±s.e.m., *n*=3.

Discussion

The present study shows that the gastric oncogenic pathogen *H. pylori* is associated with increased expression of MMP-7 in a subset of gastric epithelial cells. Moreover, we found that induction of MMP-7 by *H. pylori* plays a role in stimulating gastric epithelial cell migration. The induction of MMP-7 by *H. pylori* appears to require adhesion of bacteria to epithelial cells and to be mediated by small GTPases of the Rho family through activation of NF-κB and AP-1 transcription factors.

Recent reports have noted that MMP-7 expression is induced by bacteria in several different epithelia, including colon, bladder and airways, but the cellular mechanisms responsible for this response are unclear (Lopez-Boado et al., 2000; Lopez-Boado et al., 2001; Parks et al., 2001). In mouse small intestine, MMP-7 is expressed in Paneth cells, where it is proposed to function as a processing enzyme for mediators of innate immunity, the α-defensins (Wilson et al., 1999). However, this function seems to be species specific since, in human small intestine, trypsin has been reported to function as a prodefensin-processing enzyme (Ghosh et al., 2002). Moreover, in normal human small and large intestine, MMP-7 was reported to be absent (Ghosh et al., 2002), although expression may increase in ulcerative colitis in association with dysplasia (Newell et al., 2002). By contrast, we found detectable expression of MMP-7 in epithelial cells in both antral and corpus regions of normal human stomach, and in both cases expression was increased in the presence of the gastric pathogen *H. pylori*.

In mucus and pepsinogen-secreting cells, which were the main MMP-7-containing cells, MMP-7 was found in vesicles distinct from those containing the primary exocrine secretory product. Interestingly, MMP-7 was found at the leading edge of migrating cells, including lamellipodia. By using time-lapse video-microscopy of isolated gastric gland fragments, we showed that cells progressively migrated to form islands, or monolayer colonies of cells, over 2-3 days through a combination of cell migration and cell spreading. This process was accelerated in *H. pylori*-positive biopsies, and the response was reversed in the presence of MMP-7 AS oligonucleotides. Moreover, in a cancer cell line (AGS cells) *H. pylori* stimulated both migration and invasion through artificial basement membrane, and in both cases this was inhibited by neutralization of MMP-7. Together, these data support the idea that *H. pylori* induction of MMP-7 is a functionally important host cell response mediating cell migration in the stomach.

The stimulation of gastric epithelial cell migration in response to *H. pylori* occurs in otherwise normal gastric gland

cells. Related processes have also been described in airways (Dunsmore et al., 1998; Parks et al., 2001). These phenomena may well be protective and serve to maintain epithelial integrity in the face of epithelial damage and infection. However, in stomach, the induction of MMP-7 may also play a role in initiating events that predispose towards malignancy. Thus, there is increased expression of MMP-7 in gastric cancer, particularly at the migrating front of tumours (Adachi et al., 1998; Ajisaka et al., 2001; Honda et al., 1996), and AS inhibition of MMP-7 expression suppressed tumour invasion but not proliferation (Yonemura et al., 2001). In addition, MMP-7 degrades pro-apoptotic factors and proinflammatory cytokines (e.g. TNF-α and Fas ligand). Decreased availability of the latter would tend to suppress apoptosis and may therefore serve to preserve cells after DNA damage and so contribute to tumorigenesis (Mitsiades et al., 2001; Vargo-Gogola et al., 2002).

Previous work has shown that MMP-7 expression is regulated by the PEA3 Ets transcription factor, which appears to enhance transcriptional responses to both β-catenin and AP-1 transcription factors (Crawford et al., 1999; Crawford et al., 2001). Our data suggest that activation of AP-1 by *H. pylori* (Naumann et al., 1999) is one component of the MMP-7 transcriptional response. But, in addition, we found that *H. pylori* stimulation of NF-κB activity contributed to the induction of MMP-7. The activation of NF-κB by *H. pylori* has been described by several groups and has been linked to induction of IL-8 (Gupta et al., 2001; Keates et al., 1997; Munzenmaier et al., 1997; Wada et al., 2001). The present data linking NF-κB activation to induction of MMP-7 suggest a wider range of responsive genes than previously supposed. Moreover, although IL-8 increased MMP-7 expression, the present data suggest that this is not an important component of the response to *H. pylori.* Similarly, the data suggest that other possible endocrine, paracrine, cytokine or bacterial mediators are unlikely to mediate the MMP-7 response to *H. pylori.* Instead, we suggest that cell adhesion of the bacterium and activation of small GTPases of the Rho family (Churin et al., 2001; Hotchin et al., 2000; Palovuori et al., 2000) are responsible for induction of MMP-7. In particular, our data suggest that induction of MMP-7 via NF-κB occurs as a consequence of activation of Rho and Rac (Montaner et al., 1999). Interestingly, there was differential activation of AP-1 and NF-κB, since both Rho and Rac activated the latter, but only Rho activated AP-1.

Infection with *H. pylori* affects approximately 50% of the western population; some patients exhibit a progression through chronic gastric atrophy to cancer, others develop peptic ulcer, but most do not exhibit either disease (Blaser and Berg, 2001; Peek and Blaser, 2002; Uemura et al., 2001). The different outcomes from infection are thought to reflect host, pathogen and environmental variables (Blaser and Berg, 2001; Fox and Wang, 2001). The ability of epithelial cells to migrate after damage is usually considered to be an important component of the host defence mechanism. In this context, we suggest that *H. pylori* induction of MMP-7 can be considered part of a protective host response. In addition, though, we also suggest that, over longer periods, the prolonged induction of MMP-7 accelerates an oncogenic progression via disruption of epithelial organization and increased invasion. The identification of increased MMP-7 expression as a consequence of *H. pylori* infection should provide a basis for detailed study of the links between bacterial infection and cancer.

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