Helicobacter pylori

ORIGINAL ARTICLE

Helicobacter pylori CagL dependent induction of gastrin expression via a novel αvβ5-integrin—integrin linked kinase signalling complex

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ABSTRACT

Objective One of the most important hormones in the human stomach is the peptide gastrin. It is mainly required for the regulation of gastric pH but is also involved in growth and differentiation of gastric epithelial cells. In Helicobacter pylori infected patients, gastrin secretion can be upregulated by the pathogen, resulting in hypergastrinaemia. H pylori induced hypergastrinaemia is described as being a major risk factor for the development of gastric adenocarcinoma.

Design In this study, the upstream receptor complex and bacterial factors involved in H pylori induced gastrin gene expression were investigated, utilising gastric epithelial cells which were stably transfected with a human gastrin promoter luciferase reporter construct.

Results Integrin linked kinase (ILK) and integrin β5, but not integrin β1, played an important role in gastrin promoter activation. Interestingly, a novel Cag/IL/K integrin β5/ILK signalling complex was characterised as being important for H pylori induced gastrin expression. On interaction of H pylori with αvβ5-integrin and ILK, the epidermal growth factor receptor (EGFR)→Raf→mitogen activated protein kinase kinase (MEK)→extracellular signal regulated kinase (Erk) downstream signalling cascade was identified which plays a central role in H pylori gastrin induction.

Conclusion The newly discovered recognition receptor complex could be a useful target in treating precancerous conditions triggered by H pylori induced hypergastrinaemia.

Significance of this study

To investigate the H pylori induced gastrin promoter activation, the bacterial ligand and its host receptor as well as the signalling pathway upstream of the promoter.

What is already known about this subject?

► H pylori induces the gastrin promoter.
► The induction proceeds in a CagA and VacA independent manner.
► The actual H pylori factor inducing gastrin is unknown.

What are the new findings?

► H pylori CagL is a novel ligand activating the gastrin promoter.
► CagL is interacting with αvβ5-integrin to activate gastrin expression via integrin linked kinase (ILK) signalling complex.
► The upstream signalling pathway of the gastrin promoter involves the epidermal growth factor receptor (EGFR)→Raf cascade leading to mitogen activated protein (MAP) kinase activation.

How might it impact on clinical practice in the foreseeable future?

► This newly discovered interaction partner CagL/β5-integrin could be a useful target in treating gastric precancerous conditions triggered by H pylori induced hypergastrinaemia.

INTRODUCTION

Helicobacter pylori is a gram negative, spiral shaped bacterium that colonises the gastric mucosa of approximately 50% of the world’s population. This pathogen causes gastritis, peptic ulcer and, in some cases, gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma.1 2 Gastric cancer is the fourth leading cancer causing death worldwide. In 1994, the WHO declared H pylori as a class I carcinogen based on epidemiological studies. H pylori strains harbouring the cytotoxic associated gene pathogenicity island (cagPAI) and certain alleles of the vacuolating cytotoxin (VacA) are more virulent than strains deficient in both virulence factors.3–5 The cagPAI encodes a functional type IV secretion system (T4SS) to inject the effector protein CagA into host target cells6 where it is phosphorylated by Src and Abl kinases.7 8 Both phosphorylation dependent and independent signalling mechanisms of injected CagA have been described, such as activation of proinflammatory cytokines, cell proliferation, motility and elongation.9 A recent study indicated that CagL, a VirB5 orthologue, is located at the tip of the T4SS pilus and interacts with the integrin αvβ5 receptor of the gastric epithelial cells via an Arg-Gly-Asp (RGD) motif.6 CagL has been also shown to activate epidermal growth factor receptor (EGFR) and is essential for injection of CagA into gastric epithelial cells.10

Gastrin is one of the most important peptide hormones in the human stomach. It was first described in 1906 as a tissue factor which stimulates acid secretion in the corpus mucosa.11 Gastrin is released into the gastric vasculature from...
endocrine G cells in the antrum in response to food intake. Gastrin binds to cholecystokinin 2 receptors on enterochromaffin-like cells to stimulate histamine release and also binds to cholecystokinin 2 receptors on parietal cells to activate acid secretion in corpus tissue.\textsuperscript{12, 13} Histamine stimulates adjacent parietal cells through histamine 2 receptors and thus activates acid secretion also. It is well established that epidermal growth factor (EGF) binds to its receptor EGFR activating the gastrin promoter via the mitogen activated protein (MAP) kinase members, mitogen activated protein kinase kinase (MEK) and extracellular signal regulated kinase (ERK) 1/2.\textsuperscript{14, 15} 

Studies in diverse animal models, usage of various human gastric cancer cell lines and the exploration of human biopsies established gastrin as a type of growth factor which affects proliferation, apoptosis, migration, invasion, tissue remodelling and angiogenesis both in vitro and in vivo.\textsuperscript{16–25} INS-GAS and GAS-KO mice models are two animal models frequently used to investigate the physiological and pathological role of gastrin. INS-GAS mice which overexpress human amidated gastrin (GAS) under an insulin (INS) promoter exhibit initial gastric mucosal hypertrophy and excess gastric acid secretion. Gastrin deficient (GAS-KO) mice are achlorhydric due to a severe impairment of acid secretion. After 5 months, the INS-GAS mice display progressive changes in histology and physiology and after 20 months they are essentially achlorhydric and all animals develop gastric dysplasia. When these hypergastrinaemic mice were infected with \textit{H pylori} expressing both CagA and VacA (\textit{cagA}\textsuperscript{+}/\textit{vacA}\textsuperscript{+}) they developed gastric adenocarcinoma after 7 months of infection.\textsuperscript{24, 25} However, in contrast with the INS-GAS mice, GAS-KO mice are characterised by reduced parietal cell numbers and are predisposed to bacterial colonisation and, therefore, are often overgrown by multiple bacterial species.\textsuperscript{26–29} When these mice are infected with \textit{cagA}\textsuperscript{+}/\textit{vacA}\textsuperscript{+} \textit{H pylori}, there is an alteration in acid secretion, thought to be stimulated by a vagal response mechanism, but no increased risk of tumour development is observed after 6 months.\textsuperscript{30} The absence of capacity for increased tumour development observed could be attributed to the lack of gastrin and its mitogenic function.

Mongolian gerbils are another useful model system to study \textit{H pylori} induced gastric pathology. In our recently published study, gerbils were infected with the \textit{H pylori} wild-type (WT) strain B8 which expresses a functional T4SS for injection and phosphorylation of CagA,\textsuperscript{31} and an isogenic T4SS mutant strain B8\textit{ΔcagY} (also called Δ\textit{wv}B1\textit{Δc}) which is not able to translocate the CagA into the host cells. The results revealed early (4–8 weeks) T4SS dependent inflammation only in WT infected animals that was followed by severe active and chronic gastritis, mucous gland metaplasia and dysplasia. Interestingly, WT infected animals exhibited hyperchlorhydria after 16 weeks and hypergastrinaemia after 32 weeks post infection but none of the mutant infected mice did. To summarise these observations, we assert that the early proinflammatory responses (increase in interleukin (IL)-1β, interferon γ, tumour necrosis factor α, IL-6 and keratinocyte-derived cytokine) are major drivers for physiological changes such as hyperchlorhydria and hypergastrinaemia.\textsuperscript{32, 33} 

To date, the role of \textit{H pylori} in the pathology of developing gastritis, disturbed acid and gastric homeostasis was not well understood. Recently, it was shown that live \textit{H pylori} are able to target the gastrin promoter through GC-rich DNA elements.\textsuperscript{34} However, it is not clear which \textit{H pylori} factor is responsible for activating the gastrin promoter and which downstream signalling cascades are involved. Hence in the present study, we investigated in detail the molecular mechanisms of \textit{H pylori} induced gastrin expression. To explore the regulation of the human gastrin gene by \textit{H pylori}, we used an in vitro approach with AGS gastric epithelial cells stably transfected with a human gastrin promoter luciferase reporter construct (GAS-Luc). Our main interests were to systematically identify the \textit{H pylori} ligand, its binding partner on the cell surface as well as the signalling cascade involved in inducing the human gastrin promoter. 

**MATERIAL AND METHODS**

**Bacterial strains and culture conditions**

\textit{H pylori} P12, B8, 7.15, P1, 26695, J99, Tx50a, Ka12\textsuperscript{35} and 1061\textsuperscript{36} were grown on GC agar plates (Oxoid, Vienna, Austria) supplemented with horse serum (5%), vancomycin (10 μg/ml), trimethoprim (5 μg/ml) and nystatin (1 μg/ml) (serum plates) and incubated for 2–3 days in a microaerobic atmosphere (85% N\textsubscript{2}, 10% CO\textsubscript{2}, 5% O\textsubscript{2}) at 37°C. \textit{E coli} strain DH5α (BRL) was grown on Luria–Bertani (LB) agar plates under oxoid conditions at 37°C. \textit{Campylobacter jejuni} C64\textsuperscript{37} was grown at 37°C on Columbia blood agar plates with sheep blood plus (Oxoid) under microaerobic conditions. Re-isolates out of gastric mucosa of Mongolian gerbils were verified with a positive urea urease broth (Oxoid), oxidase (DrySlide, BBL), and catalase (3% peroxiredoxin solution) activity and streptomycin resistance.

**Materials, reagents and antibodies**

Anti-β3-integrin (blocking, MAB1388Z), anti-αvβ3-integrin (blocking, MAB2025Z), anti-β4-integrin (blocking, MAB2058Z), anti-αvβ5-integrin (blocking, MAB1961Z), anti-αv-integrin antibodies and cell culture inserts were purchased from Millipore (Bedford, Massachusetts). Anti-IL1β (neutralisation, I6421), anti-IL15 linked kinase (ILK) and IL-8 were purchased from Sigma–Aldrich (Taufkirchen, Germany). Inhibitors ZM336372, PD98059, SB203580, Gefitinib and AG1478 were purchased from Merck (Vienna, Austria). The phospho-tyrosine specific antibody PY99, anti-Erk, anti-p38, anti-focal adhesion kinase (FAK) and anti-GAPDH were purchased from Santa Cruz (Heidelberg, Germany). For the experiments in figure 6C, the phospho specific antibodies anti-EGFR-PY-845, anti-Raf-PS-259, anti-MEK-PS-217/221 and anti-ERK-PT-202/PY-204 were purchased from NEB Cell Signalling (Frankfurt, Germany). Pro- and anti-inflammatory cytokines IL-1β, interferon γ, tumour necrosis factor α, IL-4 and IL-10 were purchased from ImmunoTools (Friesoythe, Germany). Antibodies against phospho-p38 and phospho-Erk were purchased from Cell Signalling. The antibody against CagA\textsuperscript{38} anti-β3-integrin (blocking, ALIB2), and the purified VacA\textsuperscript{39} were kindly provided by the laboratory of Rainer Haas (Max-von-Pettenkofer Institute, Munich, Germany). Anti-β3-integrin antibody (blocking, 121004) was purchased from BioLegend (Fell, Germany). As western blot loading control, an anti-α-tubulin antibody (Upstate, Frankfurt, Germany) was used.

**Construction of ΔcagL and ΔcagY mutants**

Standard techniques were used for routine DNA manipulation, subcloning and plasmid construction, as previously described by Sambrook and Russell.\textsuperscript{40} The ΔcagY mutant was constructed as previously described.\textsuperscript{33} To produce a \textit{H pylori} B8 ΔcagL mutant, the plasmid pSH6 was constructed. pSH6 carries a human gastrin cDNA cassette in a XbaI site between two adjacent nucleotide regions of about 1000 bp up and down stream of the \textit{cagL} gene. The PCR products were generated by using genomic DNA from \textit{H pylori} B8 strain applying the primers for 5'-cagL fragment: FP 5’-CGG AGG TCA TGA GGT TCA GAC ATC TTG CTT GG-3’ and RP 5’-GCT CTA GAG CAT CTG CCT CTT CAC CCA TTT C-3’ and 5’-cagL fragment: FP 5’-CCT CTA GAG CCA ATT TTG AAG CGA ATG AGC-3’ and RP 5’-CCG ATT TCG ACA ACA CCT
GAG TGG TTT AAA AC-3'. The amplified products were cloned into the restriction sites SacI and EcoRI of pBluescript SK+ (Stratagene, La Jolla, California, USA).

The resulting plasmid was transformed into E. coli DH5α and the re-isolated plasmid (QiAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) subjected to DNA sequencing to verify sequence integrity.

The deletion mutant was obtained by transforming the constructed plasmid pSH6 into the natural competent H. pylori B8 cells kept in liquid culture medium (Brucella broth plus 10% fetal calf serum). The transformed bacteria were selected on serum plates supplemented with kanamycin. The P12-cagL-RGD mutants were constructed as previously described.41

In vitro gastrin promoter stimulation assay
AM0 cells are AGS cells stably transfected with a plasmid containing 240 base pairs of the human gastrin promoter expressing the luciferase reporter (240 GasLuc).42 AM4 cells express the 240 GasLuc construct with a 4 base pair mutation within the GC-rich element preventing EGF responsiveness (gERE) to the gastrin promoter.43 AM4 cells equally released served as negative controls. Both cell lines were cultured in RPMI 1640 (Gibco BRL, Darmstadt, Germany) supplemented with horse serum (10%) under standard conditions. Cells at 70% confluence and starved for 24 h in Nutrient Mixture F12 (HAM; Gibco) without supplements were stimulated with bacterial suspensions of WT and mutant strains at a multiplicity of infection of 100:1 (bacteria per cell) or purified VacA and CagL for 5 h. Cells were harvested in lysis buffer (14 g/l K2HPO4, 2.67 g/l KH2PO4, 0.74 g/l EDTA, 1 g/l Triton X-100, 1 mM DTT), and luciferase activity was measured (MicroLumat Plus LB 96 V; Berthold Technologies, Bad Wildbad, Germany) and normalised to total cell protein. Blocking antibodies and inhibitors were preincubated 45 min before bacterial stimulation. To generate metabolic inactive bacteria, pretreatment with 0.15% NaN3 was carried out for 20 min.

Purification of recombinant CagL
To clone WT CagL for protein expression in E. coli, a PCR product corresponding to amino acid residues 21–237 of the protein (minus the predicted signal sequence) was cloned into the pET-28a vector (Novagen, Darmstadt, Germany) and sequenced. The CagL-RGA mutant was constructed using the QuickChange mutagenesis kit (Novagen, Vienna, Austria). WT and RGA mutant CagL were overexpressed and purified by means of 6×His tag applying a standard protocol, as described previously.5 In brief, E. coli strain BL21 (DE3) harbouring the CagL vectors was grown in 5 ml of LB medium at 37°C overnight. Then 500 ml of fresh LB medium were added and shaken for about 3 h until OD600 = 1. IPTG (1 mM) was added and the bacteria were grown for another 2 h to induce CagL protein expression. Bacterial pellets were harvested, pelleted by centrifugation and then resuspended in ice cold CW buffer (50 mM KH2PO4-KHPO4, pH 7.5, 200 mM NaCl) supplemented with protease inhibitor cocktail (Roche, Dublin, Ireland). After sonication, CagL protein present in the inclusion bodies was solubilised in buffer LW (50 mM KH2PO4-KHPO4, pH 7.5, 200 mM NaCl, 6 mM guanidine hydrochloride) and refolded in ice cold refolding buffer (52 mM Tris HCl, pH 8.2, 20 mM NaCl, 854 mM KCl, 1.1 mM EDTA, 2.1 mM reduced glutathione, 210 µM oxidised glutathione). CagL proteins were then further purified through Talon resin affinity chromatography (BD Biosciences, Heidelberg, Germany) and gel filtration in buffer CW through Sephacryl S-200 (16/60) according to the suppliers' recommendation (Pharmacia Biosciences, Munich, Germany). CagL protein concentrations were measured by BCA protein assay (Pierce, Dublin, Ireland), typically yielding a total amount of about 1.5 mg CagL in 10 ml of buffer. Purification of CagL was judged to be of >95% homogeneity by SDS-PAGE/Coomassie Blue staining. The folded conformation of the purified CagL proteins was confirmed by circular dichroism.6 No indication of post-translational modifications of purified CagL such as disulphide formation or methylation was detected.10

Binding of CagL to integrin αβ3 determined by Biacore surface plasmon resonance
Purified integrin αβ3 (Chemicon, Darmstadt, Germany) was immobilised by amino coupling to the surface of a CM5 carboxymethyl dextran sensorchip. The analysis was performed as described previously.6 Data were processed using the Biaevaluation software (V4.1).

In vitro assays of AGS cells with recombinant CagL
Assays for the interaction of AGS cells with purified CagL were performed according to procedures described previously.10 Briefly, six well plates were coated overnight at 4°C with 100 µl of 50 µg/ml purified CagL or BSA (Sigma, Vienna, Austria) in buffer CW as control. Non-specific binding sites were then blocked with 5% bovine serum albumin (BSA) in buffer CW for 2 h at 37°C. The AGS cells were grown as described above, trypsinised and then treated with soybean trypsin inhibitor according to the protocol of the manufacturer (Sigma). After washing with phosphate buffered saline, 4×105 AGS cells in RPMI were added to the wells and incubated for 3 h. In this time period, AGS cells attached to immobilised CagL (but not BSA) and the cells were harvested with a cell scraper, pelleted by centrifugation and resolved in 1× SDS-PAGE buffer for western blot analyses.

Plasmid transfection
Low passage AGS cells with 40–50% confluency were transfected with either pSP65SR+ or pSP65SR+CagA and 240GasLuc for 24 h using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) and Genejuice Transfection Reagent (Novagen), respectively, according to the manufacturer’s instructions. The CagA overexpression plasmid pSP65SR+CagA contains the cagA gene of H. pylori strain NCTC11637 which was cloned into basic vector pSP65SRα.44

RNA interference
Low passage cells with 20–30% confluence were transfected with 50 nM small interfering RNA (siRNA) using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions. siRNA against FAK, ILK and control siRNA (with an equal GC content) were purchased from Invitrogen and (for figure 5D) from Santa Cruz. Three days after transfection, cells were harvested and subjected to immunoblotting and luciferase measurement.

RNA isolation and real time RT-PCR
Gastric epithelial cells were harvested 24 h post transfection with FAK or ILK siRNA in RTL buffer (Qiagen RNeasy Mini Kit) with 1% β-mercaptoethanol. RNA isolation was performed as described in the Qiagen RNeasy Mini Kit protocol. Using the TaqMan reverse transcription reagents (Roche) with random primers according to the kit protocol, 1 µg mRNA was transcribed into cDNA. Oligonucleotide primers specific for FAK, ILK and the housekeeping gene 18S rRNA were applied for real time RT-PCR (ABI PRISM 7000, Applied Biosystems, Darmstadt, Germany). For amplification, the FastStart Universal SYBR
Green Master (ROX) kit (Roche) was used according to the manufacturer’s instructions. All data were normalised with the corresponding 18S rRNA transcription level using a comparative delta Ct method.

**Immunoblotting**

Cells were harvested in lysis buffer (PhosSTOP (Roche) phosphatase inhibitor cocktail tablets resolved in DPBS (Gibco) by scraping). These cell lysates were boiled in Laemmli sample buffer for 10 min. Boiled samples were subjected to SDS-PAGE using a minigel apparatus (Bio-Rad, Munich, Germany) and blotted onto a PVDF membrane using a semi-dry blot system (Biotec Fischer, Reiskirchen, Germany). The membranes were blocked with either 5% milk powder or 5% BSA in TBS buffer (50 mM Tris HCl; pH 7.5; 150 mM NaCl) and incubated overnight with the respective primary antibody at 4°C followed by secondary antirabbit IgG or antimouse IgG conjugated HRP for 1 h at room temperature. Immunoreactive bands were detected by ECL and radiography.

**Animals and infection experiments**

Specific pathogen free Mongolian gerbils (n=20 females) were infected as previously described. Animals were challenged orogastrically with approximately $10^9$ *H pylori* suspended in Brucella broth. All experiments and procedures carried out were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Regierung von Oberbayern (AZ 55.2-1-54-2531-78/05). The animals were sacrificed after 8 weeks of infection, the stomach opened along the greater curvature and the gastric tissue conserved in antrum and corpus separately.

**Statistics**

Data are presented as mean±SEM. The results (raw data) were statistically analysed using the Student t test with SigmaStat statistical software (**p<0.001; *p<0.01; #p<0.05 were considered significant; p>0.05 was considered not significant). **RESULTS**

**H pylori strains with functional T4SS specifically induce the gastrin promoter**

Earlier reports showed that *H pylori* infected patients exhibited elevated serum gastrin concentrations relative to control populations. In line with these observations, *H pylori* eradication in humans was accompanied by reduction of gastrin concentrations. To better understand how gastrin expression is influenced by *H pylori*, an in vitro gastrin promoter study was performed. We used AGS gastric epithelial cells that were stably transfected with a luciferase reporter construct regulated by the human gastrin WT gene promoter (AM0) to examine human gastrin expression. In the first set of experiments, the cells were stimulated with sodium azide (NaN3) resulting in structural changes to the cells and subsequently gastrin promoter transactivation was determined. As a positive control, EGF, a natural agonist for stimulation of gastrin gene expression, showed elevated gastrin activation as expected (figure 1). Interestingly, clinical *H pylori* strains lacking the entire *cag*PAI (Tx50a, Ka125 and 1061) showed a significantly reduced gastrin promoter activation in contrast with all other applied WT strains expressing the *cag*PAI, such as B8, P12, 7.13 and P1. Two other *cag*PAI positive *H pylori* WT strains, 26695 and J99, also revealed gastrin promoter activation but at moderate levels. As negative controls, *C jejuni* and *E coli*, two bacterial species usually resident in the gastrointestinal tract, were unable to activate the gastrin promoter. Thus transcriptional activation of the gastrin promoter in vitro appears to be a specific feature of *H pylori* WT strains that express a functional T4SS in the *cag*PAI (figure 1).

**Binding of *H pylori* strains is essential for gastrin promoter activation**

*H pylori* causes persistent infection in the gastric mucosa, which is the major colonisation site for the bacterium. To explore whether bacterial contact with gastric epithelial cells is necessary to induce the gastrin promoter, filter inserts were used to prevent contact between the bacteria and the cells. In this experimental setting, WT *H pylori* were unable to induce promoter activation, suggesting that direct bacterial contact is necessary (figure 2A).

In a second step, we focused on the investigation of the *H pylori* ligand that binds to the epithelial cells inducing activation of the gastrin promoter. As binding of bacteria to epithelial cells is mainly mediated through its major adhesins BabA, SabA and AlpA/B, we underlined the above observation by applying the corresponding adhesin defective mutants. Therefore, *H pylori* J99 WT and isogenic single and double adhesin mutant strains were used to stimulate AM0 cells. No differences in gastrin promoter induction between the J99 WT and its isogenic mutant strains were observed (figure 2B). These results confirmed the above data that binding of *H pylori* via the major adhesins BabA, SabA and AlpA/B is not required to activate the gastrin signalling pathway. In agreement with a previous study, a series of infection and transfection experiments showed that CagA or VacA are also not required for gastrin activation (see supplementary figure S1, available online only).

Next, we investigated if vital and metabolically active *H pylori* are required for elevated gastrin promoter activity during infection. To answer this question, we stimulated AM0 cells with either heat killed or sonicated *H pylori* lysates. The results of these experiments showed that neither heat killed nor sonicated cells were able to induce gastrin activity (figure 2C). In the following experiment, we prepared metabolically inactive *H pylori* cells by preincubation with sodium azide (NaN3) resulting in structurally intact but metabolically inactive bacteria. NaN3 treated
**Helicobacter pylori**

**Figure 2** Contact of viable *H. pylori* wild-type (WT) is essential for gastrin induction. (A) Filter inserts (FI) were placed in each well containing AM0 cells. *H. pylori* B8 WT or B8ΔcagY mutant strain were added. (B) AM0 cells were stimulated with *H. pylori* J99 and isogenic adhesine mutant strains. (C) Heat killed (hk) and sonicated (so) B8 WT or (B) J99 (represents 100%; interpolated broken line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student’s t test) was applied using SigmaStat software. ***p ≤ 0.001; **p ≤ 0.01; *p ≤ 0.05.

*H. pylori* were no longer able to actively translocate CagA, as indicated by the absence of a phospho-CagA signal (figure 2D), but these metabolically inactive bacteria induced gastrin promoter activation similar to untreated *H. pylori* WT (figure 2C). Taken together, these data indicate that a heat labile surface component of *H. pylori* other than the well known adhesins BabA, SabA and AlpA/B or factors injected by the T4SS is sufficient to induce profound gastrin promoter activity. This indicates that a structural member of the *cag*PAI might be involved in promoter activation.

**H. pylori** CagL induces the gastrin promoter

To identify the surface component responsible for gastrin promoter induction, we tested a series of *H. pylori* mutants with defects in structural components of the T4SS, such as ΔcagY or ΔcagL. Interestingly, neither ΔcagY nor ΔcagL mutant was able to trigger gastrin promoter activation (figure 3A). The ΔcagY mutant was chosen because it can still produce CagL protein but does not produce T4SS pili for protein exposure. This suggested that T4SS itself or a pilus exposed protein could trigger this signalling. A potential candidate was the CagL protein that decorates the T4SS pilus surface and makes contact with the integrin receptor. Importantly, complementation of the ΔcagL mutant with the WT cagL gene restored promoter activity to WT levels, indicating that CagL plays a crucial role in gastrin promoter induction (figure 3A). In agreement with this conclusion, we also found that addition of recombinant CagL protein alone stimulated gastrin promoter profoundly (figure 3B).

**H. pylori** CagL induces gastrin promoter activity via binding to αvβ3-integrin

The above results suggest that CagL via interaction with the known integrin family member integrin αvβ3 may trigger...
downstream signalling leading to gastrin promoter activation. Interestingly, we found in infection experiments using AM0 cells preincubated with function blocking α-integrin antibodies (figure 4A) that binding to integrin α5β1 is not required for gastrin activation. We therefore tested a number of other function blocking antibodies for major integrins expressed on AGS cells, including β2, β3, β4, β5 and β7 (figure 4A). Most strikingly, preincubation of cells with the α-αvβ5-integrin blocking antibody significantly decreased gastrin promoter stimulation by more than 50% while the other antibodies had almost no effect (figure 4A). To make sure that CagL really interacted with integrin α5β1 during infection, we infected cells with WT H pylori, a ΔcagY (ΔvirB10) and ΔcagL mutant for 3 h followed by immunoprecipitation (IP) with an α-CagL antibody.
CagL interacts with both α5β1-integrin and αvβ3-integrin

We have recently shown in Biacore in vitro binding studies that recombinant WT CagL bound to α5β1 integrin with high affinity (K_D of 0.09 μM) whereas the CagL_{L5C5} mutant, in which the aspartate residue in the RGD motif was substituted by alanine, bound to α5β1 integrin with fourfold less affinity. To test if CagL can bind in the same manner to αvβ3-integrin, we performed Biacore binding studies using the same settings. The results showed that WT CagL bound to purified αvβ3-integrin with about the same affinity (K_D of 0.20 μM) compared with α5β1 integrin (figure 4C). Interestingly, the CagL_{L5C5} mutant bound αvβ3-integrin with a similarly high affinity at a K_D of 0.35 μM (figure 4C), suggesting that the latter interaction proceeds in an RGD independent manner. In agreement with these data and previously reported findings, we also observed that infection with H pylori expressing a CagL_{L5C5} mutant bound less to integrin β1. In contrast, in the same experiment, CagL_{L5C5} bound about equally to integrin β3 compared with WT CagL. To verify an RGD independent binding of CagL with integrin β3, we infected cells with WT H pylori and a ΔcagL mutant complemented with a cagL_{L5C5} mutated construct followed by IP with α-CagL antibodies (figure 4D). In each experiment, equal amounts of CagL were precipitated. The western blot analyses with α-integrin β1 and β3 antibodies clearly showed a significantly weaker and an equally intense band compared with the WT infected cells, respectively (figure 4D). Furthermore, reprobing of the IP with an α-αv integrin antibody revealed that the αv subunit is also present in the complex, as proposed (figure 4D).

CagL induces the gastrin promoter activity in an RGD independent manner

As the RGD motif seems to be dispensable for interaction of CagL with αvβ3-integrin, we next aimed to investigate if this is also the case for signalling leading to gastrin promoter activation. For this purpose, we infected AM0 cells with WT H pylori and a series of additional RGD mutants (CagL_{R5C5}, CagL_{D5C5} or CagL_{D5R5}). In agreement with the results above, we found that each of these mutants substantially induced gastrin promoter activation similar to that of WT bacteria (figure 4E). This further underlines the fact that CagL plays a crucial role in gastrin promoter activation in an RGD independent manner.

H pylori triggered host communication is mediated through integrin linked kinase

Integrins are specialised receptors in focal adhesions which link signalling from the extracellular matrix to intracellular factors. ILK and FAK are the most important focal adhesion components in mediating signals on β-integrin binding. To analyse whether the binding of H pylori WT to αvβ3-integrin triggers integrin signalling via FAK or ILK and eventually induces gastrin promoter stimulation, an RNA interference approach was established. siRNA was applied to achieve knockdown of endogenous ILK and FAK levels in AM0 cells. Real time PCR analyses revealed a knockdown efficiency of 80–90% on the mRNA level for FAK and ILK siRNA (figure 5A). A subsequent step was to confirm the knockdown of FAK and ILK on the protein level by western blotting. Following optimisation, after 72 h of siRNA transfection, neither ILK nor FAK proteins could be detected which verified an effective knockdown (figure 5B,D). AM0 cells were transfected with FAK or ILK siRNA for 72 h and then stimulated with H pylori WT. Our results established that downregulation of FAK did not abolish gastrin promoter activity but slightly enhanced it. However, all samples transfected with ILK siRNA showed a significant decrease in gastrin promoter induction of more than 50% (figure 5C). Therefore, we assume that induction of gastrin expression also involves ILK as a downstream signalling component.

EGFR/Raf/Map/Erk signalling cascade involved in gastrin gene expression

Observations in various cell systems revealed that different intracellular MAP kinase signalling pathways (ERK, JNK and p38) downstream of membrane receptors may be involved in the transmission of gastrin inducing cellular effects. In contrast with the signalling pathways activated by gastrin, the signal transduction leading to gastrin gene induction is not well characterised. In a previous study it was suggested that H pylori involves MAP kinase using inhibitors targeting MEK1, JNK and p38. To obtain a deeper insight into the regulation of the gastrin promoter by H pylori, we performed a detailed study of the upstream signalling.

Therefore, in this part of the study, we stimulated AM0 cells with H pylori WT, preincubated with kinase inhibitors against Raf-1 (ZM336372), MAP kinase MEK1 (PD98059) and p38 (SB203580). The data clearly revealed the involvement of the MAP kinase cascade (figure 6A). Western blot analysis showed that, compared with the H pyloriΔcagY mutant strain, H pylori WT activates the p38 signalling cascade to a higher extent. Erk signalling in AM0 cells was induced comparably in H pylori WT and H pyloriΔB8cagY mutant strains. The phosphorylation of Erk was no longer detectable when the cells were preincubated with the MEK1 kinase inhibitor PD98059 (figure 6B). Further western blot analysis of AGS cells stimulated with recombinant CagL protein clearly demonstrated activation of the signal cascade Raf→MEK→Erk (figure 6C) transactivating the gastrin promoter.

For several years it has been shown that EGF is a crucial signalling molecule in the stimulation of gastrin promoter activity via EGF-R. Whether H pylori induced transactivation of the gastrin promoter involves EGF-R activation was investigated in this part of the study. Western blot analysis of CagL stimulated cells with α-phospho EGF-R antibodies revealed EGF-R as a candidate upstream receptor for the signal cascade involved in gastrin promoter activation (figure 6C). To verify the involvement of the EGF-R in H pylori WT induced gastrin induction, we preincubated AM0 cells with Gefitinib and AG1478, tyrosine kinase inhibitors of the EGF-R family resulting in significantly reduced luciferase activity (figure 6D). In parallel, we utilised a neutralising α-EGF antibody to bind free EGF which would also stimulate the gastrin promoter via EGF-R. However, H pylori WT strain was still able to stimulate the gastrin promoter, independently of EGF (figure 6D). These data suggest that the H pylori induced gastrin promoter activation involves the phosphorylation of the EGF-R receptor independent of its classical EGF stimuli.

Furthermore, we analysed the impact of ILK siRNA treatment on EGF-R/MAP kinase signalling in infected cells.
Therefore, AGS cells were pretreated with ILK siRNA and then stimulated with \( H\) pylori WT. Activated EGFR and Erk were significantly reduced after ILK silencing. These data indicate that EGFR activation as well as MAP kinase signalling are linked to CagL binding to αβ3-integrin and ILK activation (figure 5D).

**WT H pylori but not \( \Delta cagL \) mutant induces a severe antral gastritis in Mongolian gerbils**

To investigate the newly discovered functional role of CagL in vivo, we used the well established Mongolian gerbil model for infection studies. For this purpose, Mongolian gerbils were orogastrically challenged with a suspension of \( H\) pylori WT strain B8 or an isogenic \( \Delta cagL \) mutant. Eight weeks after infection the animals were sacrificed and longitudinal sections of antral and corpus mucosa were prepared for haematoxylin–eosin staining. The stained gastric sections of non-infected, WT and \( \Delta cagL \) mutant infected animals were used for grading the inflammatory response in the antrum but not in the corpus mucosa (figure 7C). These data are consistent with our previous observations of animals infected with a \( H\) pylori B8\( \Delta cagY \) mutant that only reveal a proinflammatory response in the antral mucosa, after 8 weeks of infection, whereas animals infected with \( H\) pylori WT, carrying a intact T4SS, were able to induce a corpus dominant gastritis after only 8 weeks of infection, resulting in a significant hypochlorhydria and hypergastrinaemia after 16 and 32 weeks of infection, respectively. This indicates a role for CagL in early proinflammatory responses as major drivers for such physiological changes, as reported previously.32-35

**DISCUSSION**

Secreted gastrin is a crucial hormone in mammals and regulates not only the pH of the stomach but also the growth and differentiation of gastric epithelial cells.36 \( H\) pylori induced hypergastrinaemia was postulated to be a major risk factor promoting the development of gastric cancer.34,32,33,35 To date the mechanism by which \( H\) pylori induces the gastrin signalling pathway is poorly understood. To elucidate how \( H\) pylori induces gastrin gene expression, an in vitro promoter study was performed. We report here the identification of a bacterial factor, CagL, which can activate a novel integrin linked signalling cascade to induce gastrin secretion as recombinant protein in vitro and during infection in vivo.
Our data demonstrate for the first time that *H pylori* uses a αvβ5-integrin–ILK signalling complex to induce gastrin expression. We have shown that αvβ5-integrin is a novel human receptor exploited by a bacterial pathogen to induce precancerous conditions in gastric epithelial cells. Kwok et al found that β3-integrin functioned as an important receptor for binding of the *H pylori* T4SS to host cells. The authors demonstrated that this interaction triggers CagA translocation into the host cells and activates the downstream cascade via FAK and Src. In the present study, we established that T4SS, but not β3-integrin receptor or the FAK/Src cascade, plays a major role in gastrin promoter induction. In contrast, by means of silencing, blocking and immunoprecipitation experiments, a newly discovered interaction could be identified between *H pylori* WT and αvβ5-integrin that was followed by an ILK dependent downstream signalling cascade. A series of experiments, including function blocking antibodies, siRNA and inhibitors conclusively revealed that *H pylori* induced gastrin promoter activation involves EGFR followed by an MEK/Erk signalling cascade. However, the mechanism by which CagL binds with very high affinity to αvβ5 integrin is still unexplored.

Using a variety of mutant *H pylori* strains during infection, our data revealed that the major bacterial virulence factors CagA, VacA, BabA, SabA and AlpA/B are not required for induction of gastrin gene expression. However, binding of *H pylori* WT strains carrying an intact T4SS of the cagPAI was necessary to induce the gastrin promoter. This is in agreement with several patient studies demonstrating that the *H pylori* CagA status, linked to the presence of the entire cagPAI, is correlated with an increased plasma gastrin level in gastritis and gastric cancer patients. Those observations raised the question which bacterial T4SS factor is essential for gastrin gene induction on binding to αvβ5-integrin. Several lines of evidence presented here demonstrate that this factor is CagL. Firstly, deletion of *cagL* gene in *H pylori* causes loss of gastrin promoter activation during infection of AM0 cells. Secondly, immunoprecipitation experiments during infection demonstrate that CagL forms a complex with αvβ5-integrin. Thirdly, purified recombinant CagL alone is sufficient to induce gastrin promoter activation on contact with AM0 cells. Fourthly, our Biacore protein–protein interaction studies in vitro demonstrate that CagL binds with very high affinity to αvβ5 integrin (K₈ = 0.20 μM). Fifthly, recombinant CagL, on binding to αvβ5-integrin, triggers profound activation of the EGFR→Raf→MEK→Erk signalling cascade upstream of the gastrin promoter.

Apart from the fact that CagL triggers αvβ5-integrin mediated gastrin promoter activation, our study suggests that this signalling proceeds by an intriguing RGD independent mechanism. The first report demonstrating the importance of CagL, which is located at the surface of the T4SS injection pilus, inducing the translocation of the oncprotein CagA in a RGD independent manner, let us originally propose that the CagL dependent activation of the gastrin promoter might be RGD dependent also. To our surprise, applying several different isogenic *H pylori* mutant strains that carry a mutated or total deleted RGD motif of the *cagL* gene, exhibited strong gastrin induction compared with WT levels. In line with these observations, both WT CagL and mutated CagL₉₉₄ bound with similarly strong affinity to αvβ5-integrin, both during infection in vivo (as supported by IP experiments) and as...
purified proteins in vitro (as supported by Biacore binding studies).

The development of gastric cancer is a highly complex process and proinflammatory cytokines play an important role in the progression of precancerous conditions. In our recently published animal study it was shown that \textit{H pylori} B8 WT strain can induce an early and severe inflammation in antral and corpus mucosa that is associated with increased expression of proinflammatory cytokines.\textsuperscript{32} In contrast, isogenic \textit{H pylori} mutant strains with a defective T4SS revealed an early moderate to severe inflammation only in the antral but not in the corpus mucosa. This indicates that induction of early inflammation in the corpus mucosa by \textit{H pylori} is part of a pathomechanism which causes later precancerous physiological changes, such as hypochlorhydria and hypergastrinaemia.\textsuperscript{32} These observations demonstrate that an infection of 8 weeks is sufficient to distinguish between bacterial factors harbouring a risk factor for developing cancerous conditions or not. As shown in the in vivo part of this study, \textit{\textDelta cac}L mutants revealed strongly attenuated responses compared with WT bacteria. Thus CagL, a functional protein of the T4SS, is an essential bacterial ligand interacting with the host receptor family \(\alpha_v\beta_5\)-integrin to induce precancerous conditions.

The question arises how our newly discovered in vitro data can be extrapolated to understand the interactions of \textit{H pylori} with G cells and gastrin production in vivo. It is known that \textit{H pylori} is able to release proteases and other soluble factors that open the tight and adherence junctions of epithelial cells.\textsuperscript{62} This enables the bacteria to reach the basolateral side of the cells where integrin is expressed. We assume a comparable mechanism regarding G cells and their stimulation by \textit{H pylori}. This is not an early side effect of infection but requires a solid colonisation of \textit{H pylori} with a severe inflammation associated with histopathological changes of the gastric mucosa. The occurrence of hypergastrinaemia in our in vivo experiments was based on a late physiological change due to an infection with \textit{H pylori} type I strain.

Taken together, in this report we characterised a novel pathway leading to \textit{H pylori} induced gastrin promoter activation and presented evidence for a new role of the CagL protein to induce the gastrin promoter in a \(\alpha_v\beta_5\)-integrin dependent manner. These findings represent an important \textit{H pylori} associated signalling cascade in gastrin gene induction. Using a combination of in vitro and in vivo experiments, we elucidated a \textit{H pylori} induced upstream signalling pathway of the gastrin promoter via the EGFR \textrightarrow Raf \textrightarrow MEK \textrightarrow Erk cascade. In a time of increasing \textit{H pylori} antibiotic resistance, the newly discovered interaction of \textit{H pylori} CagL with \(\alpha_v\beta_5\)-integrin could be a useful target in treating gastric precancerous conditions triggered by \textit{H pylori} induced hypergastrinaemia.

**Figure 7** CagL deficiency prevents inflammation in corpus mucosa of infected Mongolian gerbils. Haematoxylin–eosin stained paraffin embedded antral and corpus tissue of (A) non-infected, (B) \textit{H pylori} B8 and (C) isogenic B8\textit{\textDelta cac}L mutant strain infected Mongolian gerbils for 8 weeks (original magnification \(\times\)10).
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Helicobacter pylori CagL dependent induction of gastrin expression via a novel $\alpha_v\beta_5$-integrin–integrin linked kinase signalling complex

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