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H. pylori CagL Activates ADAM17 to Induce Repression of the Gastric H, K-ATPase α Subunit

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Abstract

Background & Aims—Infection with *H. pylori* represses expression of the gastric H, K-ATPase α subunit (HKα), which could contribute to transient hypochlorhydria. CagL, a pilus protein component of the *H. pylori* type IV secretion system, binds to the integrin α5β1 to mediate translocation of virulence factors into the host cell and initiate signaling. α5β1 binds ADAM17, a metalloenzyme that catalyzes ectodomain shedding of receptor tyrosine kinase ligands. We investigated whether *H.* pylori-induced repression of *HK*α is mediated by CagL activation of ADAM17 and release of heparin-binding epidermal growth factor (HB-EGF).

Methods—HKα promoter and ADAM17 activity were measured in AGS gastric epithelial cells transfected with HKα promoter-reporter constructs or ADAM17-specific small interfering (si)RNAs and infected with *H. pylori*. HB-EGF secretion was measured by ELISA analysis and ADAM17 interaction with integrins were investigated by co-immunoprecipitation analyses.

Results—Infection of AGS cells with wild-type *H. pylori* or an *H. pylori cagL*-deficient isogenic mutant that also contained a wild-type version of *cagL* (P12Δ*cagL*/*cagL*) repressed HKα promoter-*Luc* reporter activity and stimulated ADAM17 activity. Both responses were inhibited by point mutations in the NF-κB binding site of HKα or by infection with P12Δ*cagL*. siRNA-mediated silencing of ADAM17 in AGS cells inhibited the repression of wild-type *HK*α promoter and reduced

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ADAM17 activity and HB-EGF production, compared to controls. Coimmunoprecipitation studies of AGS lysates showed that wild-type *H.* pylori disrupted ADAM17–α5β1 complexes.

Conclusions—During acute *H. pylori* infection, CagL dissociates ADAM17 from the integrin α5β1 and activates ADAM17-dependent, NF-κB–mediated repression of *HK*α. This might contribute to transient hypochlorhydria in patients with *H. pylori* infection.

Keywords

H. pylori; CagL; ADAM17; H,K-ATPase

Introduction

Acute *Helicobacter pylori* infection of the human gastric corpus reduces acid secretion causing transient hypochlorhydria which may facilitate bacterial colonization[1, 2]. Hypochlorhydria provokes superficial gastritis, which may progress to atrophic gastritis, intestinal metaplasia, dysplasia, and eventually carcinoma[3–5]. The mechanisms underlying *H. pylori*-mediated transient hypochorhydria are poorly understood. *H. pylori* expresses a type IV secretion system (T4SS) which forms a pilus to inject cytotoxin-associated gene A (CagA) protein into host cells[6]. T4SS proteins are encoded by a 40 kb genetic locus termed the *cag* pathogenicity island (PAI). CagL is a pilus protein that interacts with host cellular $\alpha_5\beta_1$ integrins through its arginine-glycine-aspartate (RGD) motif, guiding proper positioning of the T4SS and translocation of CagA[7]. Consequent activation of host signaling pathways stimulates the transcription factor NF-κB and up-regulates inflammation-associated genes such as IL-8 and Cox2[8–12] Another important repercussion of NF-κB activation in gastric epithelial cells is transcriptional repression of H,K-ATPase, the enzyme responsible for acid secretion. We previously reported that *H. pylori* down-regulates H,K-ATPase α subunit (HKα) promoter-reporter constructs transfected into gastric epithelial AGS cells[13] and that NF-κB p50 subunit homodimer binding to the HKα promoter underlies this transcriptional repression[14].

ADAMs (a disintegrin and metalloprotease) are glycosylated transmembrane metalloproteases containing an N-terminal pro-domain, metalloenzyme domain, cysteine-rich disintegrin domain, and C-terminal cytoplasmic domain. ADAMs catalyze ectodomain cleavage of cell surface integral membrane proteins, generating soluble forms of TNFα and the EGF receptor (EGFR) ligands EGF and heparin-binding EGF (HB-EGF)[15, 16]. Disintegrin domains in many ADAMs possess RGD analogous acidic motifs that facilitate integrin binding, and numerous instances of ADAM family members binding to $\alpha_n\beta_1$ integrin heterodimers have been reported[17, 18]. ADAM17, also known as TACE (TNFα converting enzyme), was first identified by its generation of soluble TNFα from a membrane-bound precursor[19]. ADAM17 co-localizes with $\alpha_5\beta_1$ integrin heterodimer in HeLa cells during cell migration[20] although the significance of integrin binding in ADAM17 activation is not clearly understood.

Antral gastric biopsies from *H. pylori*-infected patients express more ADAM17 mRNA than normal controls[21], and *H. pylori*-infected gastritis patients showed increased levels of the EGFR ligands EGF, amphiregulin and HB-EGF[22, 23]. EGFR expression and signaling is perturbed in head and neck, lung, breast, and bladder cancers[24, 25], and *H. pylori* is known to up-regulate EGFR in gastric epithelial cells[26]. We hypothesized that *H. pylori* CagL displaces ADAM17 from $\alpha_5\beta_1$ integrins, activating a signaling pathway that culminates in suppression of HKα gene transcription and expression. Data presented in this paper indicate that *H. pylori* infection of AGS cells causes a CagL-dependent dissociation of ADAM17 from $\alpha_5\beta_1$ integrin, activation of the enzyme, cellular secretion of HB-EGF, and NF-κB-dependent repression of transfected HKα promoter activity. These results further elucidate *H. pylori* orchestration of host signal transduction mechanisms in favor of bacterial pathogenicity.

Materials and Methods

Cells, Bacteria and Reagents

Human gastric epithelial AGS cells (ATCC, Manassas, VA) were maintained in Ham's F12 medium containing 10% FBS as described[14]. *H. pylori* wild-type (WT) strains P12 and 7.13 were grown on *Brucella*-agar plates at 37°C using a micro-aerophilic gas pack system (BD Biosciences, Sparks, MD). WT *H. pylori* strains 7.13, P12, P12Δ*cagL*-deficient isogenic mutant strain and complemented P12Δ*cagL*/$_{cagL}$ were grown on *Brucella*-agar plates[7]. Bacterial multiplicities of infection (MOI) were calculated as described[14]. *E. coli* strain BL21 expressing pET28 His-tagged CagL (*H. pylori* TIGR strain 26695) expression plasmid was grown overnight in LB containing kanamycin (60 μg/ml) and induced with IPTG (1 mM, 1.5 hours)[7]. Overexpressed CagL in the inclusion bodies was purified as described[7]. RGD and control peptides were from Enzo Life Sciences (Plymouth Meeting, PA). All reagents were of molecular biology grade.

HKα Promoter-Luc Reporter Plasmid Transfection

HKα promoter-*Luc* reporter constructs (HKα2179 and HKα206) were formed by independently integrating two nesting segments (2179 bp and 206 bp) of proximal human gastric HKα 5′-flanking sequence into the luciferase reporter plasmid pGL2-Basic Vector as described[13]. Point mutations at −159 bp (АПС, forward strand; ТПG, reverse strand) and at −161 bp (GПA, forward strand; СПТ, reverse strand) were introduced into the NF-κB$_1$ site of both constructs using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene)[14], and mutagenesis was verified by dideoxy sequencing. AGS cells were co-transfected with wild-type or mutated HKα promoter-*Luc* reporter constructs and pMaxGFP for transfection efficiency control and normalization, and promoter-reporter activities were measured and normalized as described[27]. Transfected cells were incubated with recombinant (r)CagL protein or recombinant human (rh)ADAM17 (R&D Systems, Minneapolis, MN) in Ham's F12, 10% FBS at concentrations and times as specified.

ADAM17 Activity Measurement

AGS cells (3×10^4 cells/well) were plated overnight in 96-well plates, serum-deprived for 15–20 h and infected with WT *H. pylori* or corresponding mutant strains (MOI=50) for varying times. Cells were washed 3X with ice-cold phosphate-buffered saline (PBS) and overlaid with serum-free F12 medium (200 μl) containing quenched fluorogenic peptide substrate (10 μM; MCA-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2; Cat. No. 616402, Calbiochem, CA). ADAM17 activity was measured spectrofluorometrically as the rate of change of relative fluorescence units ($\Delta RFU.s^{-1}$) as described[16]. rhADAM17 served as a positive control.

Small Interfering RNA (siRNA) Transfection

AGS cells (1.25×10^5, 75%–85% confluent) were incubated for 24 h with Dharmafect transfection reagent 1 (T-2001-07) and 100 nM pooled siRNAs against human ADAM17 (J-003453-05) or non-targeting siRNA (D-001810-03) (Dharmacon RNA Technology, Lafayette, CO). Effects of ADAM17-specific siRNA on HKα promoter activity were studied in AGS cells transiently-transfected with HKα2179 for 24 h and then infected with WT *H. pylori* strain P12 or P12Δ*cagL* (MOI=50, 8 h). Transcriptional activities of HKα2179-*Luc* constructs were measured as described[27].

Real-time RT-PCR

Total RNA was isolated from AGS cells (80% confluent, serum-deprived for 15–20 h) using STAT-60 (Tel Test, Friendswood, TX) and reverse-transcribed using iScript™ cDNA synthesis

kit (Bio-Rad, Hercules, CA). AGS cell ADAM17 mRNA was quantitated by RT-PCR using an iCycler iQ with iQ™ SYBR ® Green Super mix (Bio-Rad, Hercules, CA) and ADAM17-specific primer mix (PPH00343A; SuperArray Bioscience, Frederick, MD). Cellular β2-microglobin mRNA was used as a normalization control.

Immunoprecipitation and Immunoblotting

AGS cells (80% confluent, serum-deprived for 15–20 h) were infected (MOI=50, 1 h) with WT *H. pylori* strain P12 or P12Δ*cagL*, washed and harvested in ice-cold PBS, and centrifuged (520g, 5 min). Cell pellets were dissolved in Triton Lysis Buffer (Boston BioProducts, Worcester, MA) containing protease inhibitor cocktail (P8340, Sigma, St. Louis, MI) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford IL) in a rotating shaker at 4°C for 30 min. Cell lysates were incubated overnight at 4°C with ADAM17-specific antibody (Santa Cruz Biotechnology, CA) or β_1 integrin-specific antibody (AIIB2, developed by CH Damsky and NICHD, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA 52242) (5 μg antibody/mg lysate protein). After incubation at 4°C for 1 h with protein G-agarose beads, immunoprecipitated protein complexes were eluted with SDS-PAGE sample buffer and analyzed by immunoblotting as described[16] using ADAM17- or β1 integrin-specific antibodies. Immunoprecipitations with non-immune IgG or protein G-agarose beads alone served as negative controls. ADAM17 protein levels following ADAM17-specific siRNA transfection and/or *H. pylori* infection were assessed by immunoblotting[16], using ADAM17-specific antibody (QED, San Diego, CA) and p44/42 MAP kinase (ERK)-specific antibody (Cell Signaling Technology, Danvers, MA) or β-actin as gel loading controls. Immunoblots shown are representative of 3 replicates.

HB-EGF Shedding

AGS cells (80% confluent, serum-deprived for 15–20 h) were infected (MOI=50, 1 h) with WT *H. pylori* strain P12 or P12Δ*cagL*. Cellular supernatants were concentrated using 9 kDa cut-off protein concentrators (Thermo Scientific, Rockford, IL) and HB-EGF concentration was measured using HB-EGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN).

Statistical Analysis

Statistical significance between treatment groups was determined by two-way ANOVA and Bonferroni post-test method using PRISM 4.0 (GraphPad, San Diego, CA). A P value <0.05 was defined as statistically significant.

Results

CagL Mediates H. pylori-induced Repression of HKα Promoter

H. pylori represses HKα promoter activity in gastric epithelial cells by an NF-κB-dependent mechanism[14]. To test the hypothesis that CagL mediates this down-regulation of HKα expression, AGS cells were transfected with HKα promoter-*Luc* reporter constructs encompassing 206 bp of human HKα 5′-flanking sequence containing either the wild-type (HKα206) or 2 bp-mutated NF-κB binding site (HKαΔ206). The cells were then infected (MOI=50, 8h) with WT *H. pylori* strain P12, a *cagL*-deficient mutant strain (P12Δ*cagL*), or P12Δ*cagL* genetically-complemented with WT *cagL* (P12Δ*cagL*/*cagL*). Figure 1A shows that WT *H. pylori* and P12Δ*cagL*/*cagL* infection repressed HKα promoter activity by ~50%, while P12Δ*cagL* infection caused no significant repression, unambiguously implicating the *cagL* gene in HKα repression, and confirming that polar or other unrecognized mutations in P12Δ*cagL* play no role in this repression. Absence or presence of *cagL* in P12Δ*cagL* and P12Δ*cagL*/*cagL* was confirmed by immunoblotting (Figure 1B), and bacterial-AGS cell adhesion assay showed WT *H. pylori* to be marginally more adherent than P12Δ*cagL* (3019

±118 vs 2610±116 cfu/10^3 cells, P=0.07). The mechanism through which CagL modulates HKα promoter activity depends on a functional NF-κB site in the promoter, because neither P12WT nor P12Δ*cagL*/*cagL* infections significantly affected HKαΔ206 activity (Figure 1A). Constitutive HKαΔ206 promoter activities in AGS cells were significantly higher than the wild-type promoter, suggesting that NF-κB exerts a tonic repression of HKα promoter activity in these cells. Subsequent experiments further investigated the mechanistic basis of CagL-mediated HKα repression.

H. pylori Activates ADAM17 in AGS Cells

To determine the effect of the bacteria on metalloprotease activity, AGS cells were infected with *H. pylori* (MOI=50, 1 h) and metalloprotease activity was measured fluorometrically in $\Delta RFU.s^{-1}$ after medium replacement and addition of quenched fluorogenic substrate. WT *H. pylori* strains P12 or 7.13 increased metalloprotease activity ~9-fold and ~8-fold respectively compared to control (Figure 2A). However, P12 and 7.13 *H. pylori* infection of AGS cells transfected with ADAM17-specific siRNA increased metalloproteinase activity only 4-fold and 3.5-fold respectively. *H. pylori*-induced metalloprotease activation was comparable in cells transfected with nt siRNA and in untransfected cells. AGS cells and *H. pylori* alone showed low levels of metalloprotease activity (0.14 and 0.19 $\Delta RFU.s^{-1}$ respectively; not shown). AGS cell ADAM17 mRNA and ADAM17 protein levels were measured by real-time PCR and immunoblotting respectively 72 hrs after cell transfection with ADAM17-specific pooled siRNA comprising four individual siRNAs. Pooled siRNAs significantly repressed ADAM17 mRNA in AGS cells and also in AGS cells previously transfected with an HKα2179-*Luc* reporter plasmid, while nt siRNA transfection had no effect on ADAM17 expression (Figure 2B). AGS cell ADAM10 mRNA was unaffected by ADAM17-specific siRNA transfections (not shown). siRNA transfection also markedly repressed ADAM17 protein (92% by densitometric quantitation) compared to mock transfection (insert in Figure 2B). Total cell ADAM17 expression was unchanged over an 8 h time-course of *H. pylori* P12 infection (Figure 2C). Taken together, these data indicate that *H. pylori* infection of AGS cells causes activation of ADAM17.

CagL Mediates H. pylori-induced ADAM17 Activation in AGS Cells

AGS cells were plated into untreated, rCagL-treated or fibronectin (FN)-treated 96-well plates and then infected with *H. pylori* (MOI=50, 8 h). Cellular ADAM17 activity was measured after medium replacement and addition of quenched fluorogenic substrate. WT *H. pylori* and P12Δ*cagL*/*cagL* infections induced a significantly greater increase in ADAM17 activity than P12Δ*cagL* mutant in AGS cells plated in untreated wells, and the presence of plate-adsorbed rCagL significantly restored ADAM17 activity in P12Δ*cagL*-infected cells (Figure 3). Plate-adsorbed FN (a natural ligand of integrins) or rCagL alone had no effect on ADAM17 activity, possibly due to adsorption-induced steric effects and/or low bound concentrations. These data indicate that *H. pylori* T4SS pilus CagL interaction with the host cell constitutes an extracellular signal to activate ADAM17. The finding that the P12Δ*cagL* mutant alone stimulated ADAM17 to a small extent implies that bacterial factors other than CagL are also involved in this activation.

ADAM17 Down-regulation Abrogates H. pylori-induced HKα Promoter Repression

The coordinated roles of *cagL* and ADAM17 in repressing HKα gene expression were further investigated by measuring *H. pylori*-mediated HKα promoter activity in AGS cells with attenuated expression of ADAM17. AGS cells were transfected for 48 h with ADAM17-specific pooled siRNA or non-targeting siRNA, and then transfected for 24 h with HKα2179 promoter-*Luc* construct. The doubly-transfected AGS cells were infected with WT P12 *H. pylori* or P12Δ*cagL* (MOI=50, 8 h). As shown in Figure 4, ADAM17-specific pooled siRNA

cell transfections had no significant effect on HKα2179 activity in mock-infected cells. HKα 2179 activity was repressed by 64% in control AGS cells infected with WT P12 *H. pylori*, and repressed by only 38% in ADAM17-silenced AGS cells. Inoculation of control AGS cells or ADAM17-silenced AGS cells with P12Δ*cagL* had no significant effect on HKα2179 activity. These data confirm that ADAM17 is a crucial component of the signaling pathway that links *H. pylori* infection of gastric epithelial cells to inhibition of HKα gene expression, and further emphasize the CagL-dependency of this repression.

CagL Mediates Dissociation of ADAM17 from β_1 Integrin

Because CagL has been reported to interact with host cell $\alpha_5\beta_1$ integrin in the early stages of gastric epithelial cell infection by *cag*PAI-positive *H. pylori*[7], we hypothesized that ADAM17 is bound to β_1 integrin in non-infected AGS cells and that CagL may induce the dissociation of ADAM17 from this complex during infection. To test this idea, AGS cells were infected with WT *H. pylori* strain P12 or P12Δ*cagL* (MOI=50, 1 h), and the cell lysates were incubated with anti-ADAM17 antibody or with anti-β_1 integrin antibody. The presence of bound β_1 integrin or ADAM17 in the immunoprecipitated protein complexes was assessed by immunoblotting. As shown in Figure 5A, β_1 integrin signal intensity was markedly reduced in ADAM17 immunoprecipitates of AGS cell lysates infected with WT P12 *H. pylori*, but not in lysates of cells infected with P12Δ*cagL*. Conversely, ADAM17 signal intensity was markedly reduced in β_1 integrin immunoprecipitates of AGS cell lysates infected with WT P12 *H. pylori*, but not in lysates of cells infected with P12Δ*cagL* (Figure 5B). ADAM17 and β_1 integrin immunoblots of corresponding immunoprecipitated lysates served as gel-loading controls (lower panels, Figure 5A and B). Figure 5C shows that total cell ADAM17 and β_1 integrin were unaffected by *H. pylori* infection, indicating that *H. pylori* increases the ratio of active to inactive ADAM17 in AGS cells although the total cellular ADAM17 level remains unchanged. These data are consistent with binding of ADAM17 to β_1 integrin in AGS cells, and with *H. pylori*-induced, CagL-dependent dissociation of ADAM17 from β_1 integrin following exposure of AGS cells to *H. pylori*. Because WT *H. pylori* infection of AGS cells increased ADAM17 specific activity, the results are also consistent with *H. pylori*-induced ADAM17 dissociation from β_1 integrin being accompanied by up-regulation of ADAM17 activity.

CagL Mediates H. pylori-induced Secretion of HB-EGF from AGS Cells

Given that ADAM17 releases membrane-bound precursors of EGFR ligands[28], evidence was sought for *H. pylori*-mediated cleavage of heparin-binding epidermal growth factor (HB-EGF) from AGS cells in culture. Figure 6 shows that infection of AGS cells with WT *H. pylori* P12 or P12Δ*cagL* (MOI=50, 1 h) increased medium [HB-EGF] by 7-fold and 2.7-fold respectively, compared to mock-infected controls. siRNA depletion of ADAM17 expression in AGS cells abrogated *H. pylori* P12 and P12Δ*cagL*-mediated secretion of HB-EGF. Taken together with the data in Figure 2A, which shows that ADAM17 siRNA significantly blunts *H. pylori*-mediated ADAM17 activation, these data are consistent with CagL-dependent activation of ADAM17 causing AGS cell ectodomain shedding of HB-EGF.

RGD-Peptide, rCagL and rhADAM17 Independently Repress HKα Promoter Activity

Given our finding that *H. pylori*-associated CagL activates host cell ADAM17 and represses HKα gene expression, and that CagL interaction with $\alpha_5\beta_1$ integrins is mediated by an CagL N-terminal RGD motif[7], we reasoned that treatment of AGS cells with synthetic RGD-containing peptide, rCagL, or rhADAM17 should repress transfected HKα promoter activity. To test these hypotheses, AGS cells were transfected with the HKα promoter-*Luc* constructs HKα206 or HKαΔ206. The cells were incubated for 8 h with increasing concentrations of RGD or RAD peptides, rCagL or rhADAM17, and HKα promoter activity was measured in cell lysates as RLU of luciferin luminescence. The RGD peptide, but not an RAD peptide used as

a specificity control, caused a dose-dependent repression of transfected wild-type HKα promoter (Figure 7A). Co-incubation of AGS cells with rCagL also caused dose-dependent repression of transfected wild-type HKα promoter (Figure 7B). In contrast, constitutive activity of the NF-κB site-mutated HKα promoter (HKαΔ206) was higher than the wild-type promoter, and was stimulated with increased [rCagL]. Figure 7C shows that co-incubation of the wild-type HKα-transfected cells with enzymatically-active rhADAM17 (0.6 $\Delta RFU.s^{-1}$) resulted in dose-responsive repression of promoter activity, compared to untreated vehicle controls, whereas activity of the mutated HKα promoter remained relatively constant regardless of rhADAM17 concentration. Taken together, the data indicate that both CagL and ADAM17 participate mechanistically in *H. pylori*-mediated regulation of HKα promoter activity, and that the mechanism is dependent on the presence of a functional NF-κB binding site on the HKα promoter.

Discussion

This study sought to identify the mechanistic basis of *H. pylori*-induced repression of proton pump gene expression[13, 14, 29]. Prolonged inhibition of acid secretion leads to intestinal metaplasia, a premalignant lesion, and to gastric cancer. In susceptible individuals, the *H. pylori*-induced mucosal progression to premalignancy occurs in this setting of reduced acid secretion, which in addition to facilitating initial colonization also activates pro-inflammatory pathways involved in development of disease[10, 30, 31]. Animal studies have shown that hypochlorhydria is a permissive factor in gastric cancer, rather than an epiphenomenon. Gastrin-deficient ($G^{-/-}$) mice became hypochlorhydric, their stomachs becoming chronically inflamed, progressing to fundic atrophy, intestinal metaplasia, dysplasia, and eventually antral carcinoma[3]. HKα knock-out mice are achlorhydric, have abnormal parietal cells, and have ciliated metaplasia associated with dysplasia[32]. This recapitulation of the phenotypic progression to gastric cancer, triggered not by *H. pylori* but by perturbation of gastric pH, provides an important rationale for clarifying mechanisms whereby *H. pylori* inhibits acid secretion.

The *cag*PAI and the secreted bacterial oncoprotein CagA[6, 33] facilitate *H. pylori* gastric corpus colonization, rather than the antrum[34], placing the bacteria close to parietal cells and potentially jeopardizing acid secretion. Another *cag*PAI gene product, CagL, is dispensable for gastric colonization, but through its interaction with $\alpha_5\beta_1$ integrin facilitates binding of the *H. pylori* T4SS-pilus to the cell surface enabling transfer of CagA into the cytoplasm[7]. To further clarify mechanisms of *H. pylori*-induced hypochlorhydria, we investigated the hypothesis that CagL initiates signaling that culminates in NF-κB activation and HKα repression by inducing dissociation of ADAM17 from $\alpha_5\beta_1$ integrin.

Experimental evidence presented here is consistent with this hypothesis. AGS cell infection with WT *H. pylori* or an *H. pylori cagL*-deficient isogenic mutant (P12Δ*cagL*) genetically-complemented with *cagL* (P12Δ*cagL*/*cagL*) repressed transfected HKα promoter-*Luc* reporter activity and stimulated AGS cell ADAM17 activity. Both responses were abrogated by P12Δ*cagL* infection or by point mutations in the HKα NF-κB binding site. siRNA silencing of ADAM17 in AGS cells abrogated WT *H. pylori*-mediated HKα promoter repression and significantly repressed ADAM17 enzymatic activity and HB-EGF secretion induced by WT *H. pylori* strains P12 and 7.13. Co-immunoprecipitation and immunoblotting of AGS lysates revealed WT *H. pylori*-dependent attenuation of ADAM17/β_1 complexes. Recombinant CagL or ADAM17 added to AGS cells transfected with HKα promoter-*Luc* reporter construct induced a dose-responsive repression of HKα promoter activity, as did addition of an RGD-containing synthetic peptide. CagL has been reported to interact with $\alpha_5\beta_1$ integrin through its RGD motif[7], and competitive displacement of ADAM17 from $\alpha_5\beta_1$ integrin at this motif, as suggested by our data, may constitute a novel mechanism of ADAM17 activation. $\alpha_5\beta_1$ integrin

complexes and associated ADAM17 are expressed on gastric epithelial cell basolateral membranes, separated from apical membrane-adherent *H. pylori* by tight junctions. However, *H. pylori* is known to disrupt cell-cell junctions, gaining access to intercellular basolateral spaces and consequent contact with integrins[35, 36]. Thus, T4SS CagL may play a dual role by positioning T4SS on the host cell basolateral membrane to facilitate CagA translocation, and concurrently dissociating ADAM17 from $\alpha_5\beta_1$ integrin with resulting activation of ADAM17. Chronic *H. pylori* infection of human gastric mucosa increases ADAM17 mRNA levels[21] which may also increase ADAM17 enzymatic activity, but we propose that in the acute phase of *H. pylori* infection, ADAM17 activation is regulated by CagL-mediated dissociation from $\alpha_5\beta_1$ integrin.

H. pylori infection of gastric epithelial cells transactivates EGFR by metalloproteinase-mediated shedding of HB-EGF[37, 38]. *H. pylori* is also known to induce cell proliferation through EGFR transactivation by ADAM activation[39]. ADAM17 catalyzes the release of membrane-bound precursors of the receptor ligands TNFα, EGF, HB-EGF and amphiregulin, among others[20, 28]. These ligands are inflammatory mediators and pro-proliferation factors, interacting with their receptors to activate receptor tyrosine kinase signaling leading to ERK1/2 activation. *H. pylori* is a known activator of ERK-1/2-mediated NF-κB signaling cascade[9, 26] and this study demonstrates that *H. pylori*-induced ADAM17 activation in AGS cells is accompanied by shedding of the potent EGFR ligand HB-EGF into the medium, a down-stream consequence of which would be ERK-1/2-mediated NF-κB activation. We have previously documented *H. pylori*-mediated, EGFR-dependent NF-κB mobilization using MEK1/2 inhibitors[14], and that NF-κB p50 homodimer interaction with a *cis*-response element on the HKα promoter represses HKα gene transcription[14]. Consistent with this finding, the present study demonstrates that mutation of the core NF-κB binding site of the HKα promoter abrogates *H. pylori cagL*-mediated, rCagL-mediated, RGD oligo peptide-mediated, and rhADAM17-mediated repression of promoter activity. Mutation of the NF-κB binding site significantly increased the constitutive activity of transfected HKα promoter-reporter constructs, suggesting that in transfected AGS cells, NF-κB p50 homodimer activity is constitutively elevated and exerts a tonic repression of HKα promoter by interaction with the NF-κB binding site.

Partial abrogation of *H. pylori*-induced HKα promoter repression in AGS cells expressing reduced levels of ADAM17 due to siRNA silencing (Figure 4), and the fact that the Δ*cagL* mutant strain also induces ADAM17 activation (although significantly less than WT *H. pylori*, Figure 3), indicate that while ADAM17 has a role in HKα gene repression, other CagL-dependent mechanisms are probably involved. The critical participation of CagL in binding of the *H. pylori* pilus to the cell surface, and the resultant capacitation of T4SS transfer functions, enable CagA, GM-tripeptide peptidoglycan and possibly other virulence factors to penetrate host cells. Interestingly, a yeast two-hybrid screen and GST-pulldown assays revealed that T4SS-associated CagY and CagA protein also bind to β_1 integrin, which was proposed to facilitate the injection of CagA in an RGD-independent manner[40]. However, it remained unclear if and how binding of CagY and CagA to extracellular β_1 integrin receptor could trigger intracellular signaling. Irrespective of the specific contribution of individual T4SS proteins (CagL, CagY and CagA) towards CagA injection, the present data from complementation of P12Δ*cagL* either genetically by WT *cagL* restoration or by extracellular addition of rCagL clearly demonstrate the crucial role of surface-exposed CagL in triggering signaling through β_1 integrin and ADAM17. Following these interactions, intracellular CagA and GM-tripeptide both cause mobilization and nuclear localization of NF-κB, the former through activation of receptor tyrosine kinases that in turn up-regulate ERK-1/2 MAPK pathways[9, 41], and the latter through interaction with the intracellular nuclear-oligomerization domain (Nod) 1 receptor, thereby inducing NF-κB activation in AGS cells[42]. The convergence of CagA, GM-tripeptide, and ADAM17 signaling at IκK complex degradation with emergence of NF-κB subunits allows

for the formation of NF-κB p50 subunit homodimers, which have been shown to be necessary and sufficient for repression of HKα gene transcription[14].

The extent to which the CagL-mediated repression of HKα promoter activity described here contributes to *H. pylori*-induced clinical hypochlorhydria is unclear. *H pylori* deploys at least two other mechanisms of acid inhibition during colonization. Vacuolating toxin secreted by *H. pylori* disrupts actin interaction with parietal cell apical membranes, preventing recruitment and fusion of H,K-ATPase-containing tubulovesicles and causing hypochlorhydria[43]. The inflammatory cytokine IL-1β, secreted by monocytes and neutrophils recruited by *H. pylori*-induced mucosal IL-8 production, also inhibits H,K-ATPase by impairing PI3K-mediated increases in $[Ca^{2+}]_i$[44, 45]. Indeed, host IL-1 gene polymorphisms causing increased IL-1β production in *H. pylori* infection magnify the risk of hypochlorhydria and gastric cancer[46]. Transcriptional repression of HKα contributing to *H. pylori*-induced acid inhibition would be consistent with the finding that *H. pylori* eradication results in increased H,K-ATPase expression[47]. We have also observed that HKα mRNA and H,K-ATPase levels in human gastric corpus biopsies are significantly depleted within 24 hours of WT *H. pylori* infection, but remained unaffected by infection with the Δ*cagL* mutant[48].

In summary, this study demonstrates that *H. pylori* represses HKα transcriptional activity in gastric epithelial cells by inducing CagL-dependent ADAM17/β_1 integrin dissociation, ADAM17 activation, HB-EGF secretion, and NF-κB binding to the HKα promoter. The marked perturbation of downstream signaling pathways caused by CagL interaction with the host cell constitutes a novel mechanism of *H. pylori* acid secretory pathogenesis, exemplifying how *H. pylori* orchestrates host cell signaling pathways to attain the goal of gastric colonization and persistence.

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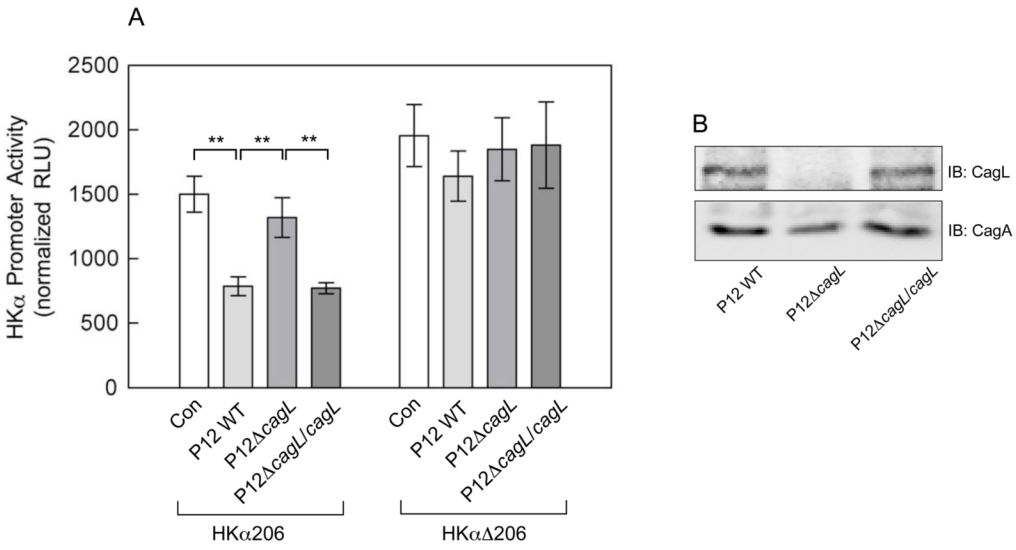

Figure 1.

(**A**) CagL mediates *H. pylori*-induced repression of HKα promoter. AGS cells transfected with HKα206 or HKαΔ206 promoter-*Luc* reporter constructs were infected (MOI=50, 8h) with *H. pylori* WT P12, P12Δ*cagL*, or P12Δ*cagL*/*cagL* strains. HKα promoter activities in cell lysates were then measured as RLU of luciferin luminescence normalized to co-transfected GFP fluorescence (means, SD, n=3; ***P*<0.01). (**B**) Immunoblot of *H. pylori* WT P12, P12Δ*cagL*, and P12Δ*cagL*/*cagL* strain lysates probed with CagL-specific or CagA-specific (loading control) antibodies.

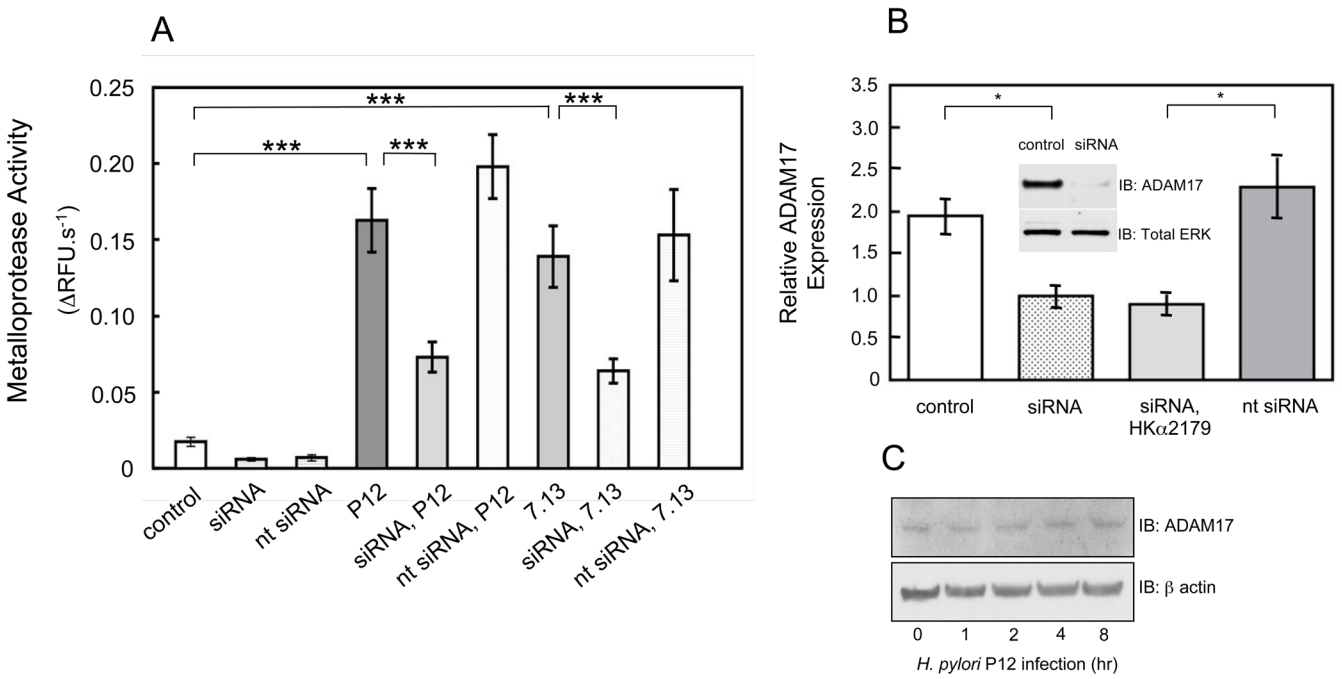

Figure 2.

H. pylori activates ADAM17 in AGS cells. (**A**) AGS cells transfected with ADAM17-specific siRNA or non-targeting siRNA for 72 h were infected (MOI=50, 1 h) with WT *H. pylori* strains P12 or 7.13. After medium replacement, metalloproteinase activity was measured using a quenched fluorogenic oligopeptide substrate. Mock siRNA-transfected AGS cells served as a control (mean, SD, n=8; ***P<0.001). (**B**) ADAM17 siRNA-tranfected cells were subsequently transfected with HKα promoter-*Luc* reporter construct (HKα2179) and were then processed to extract RNA or lysed for immunoblot analysis. ADAM17 mRNA was measured 72 h after siRNA transfection using RT-PCR and β microglobin as the internal control (mean, SD, n=3; *P <0.05). (**B** insert) Immunoblot of ADAM17 protein in cell lysates 72 h after siRNA transfection (total ERK is the gel loading control. (**C**) Immunoblot of total cell ADAM17 expression over an 8 h time-course of *H. pylori* P12 infection.

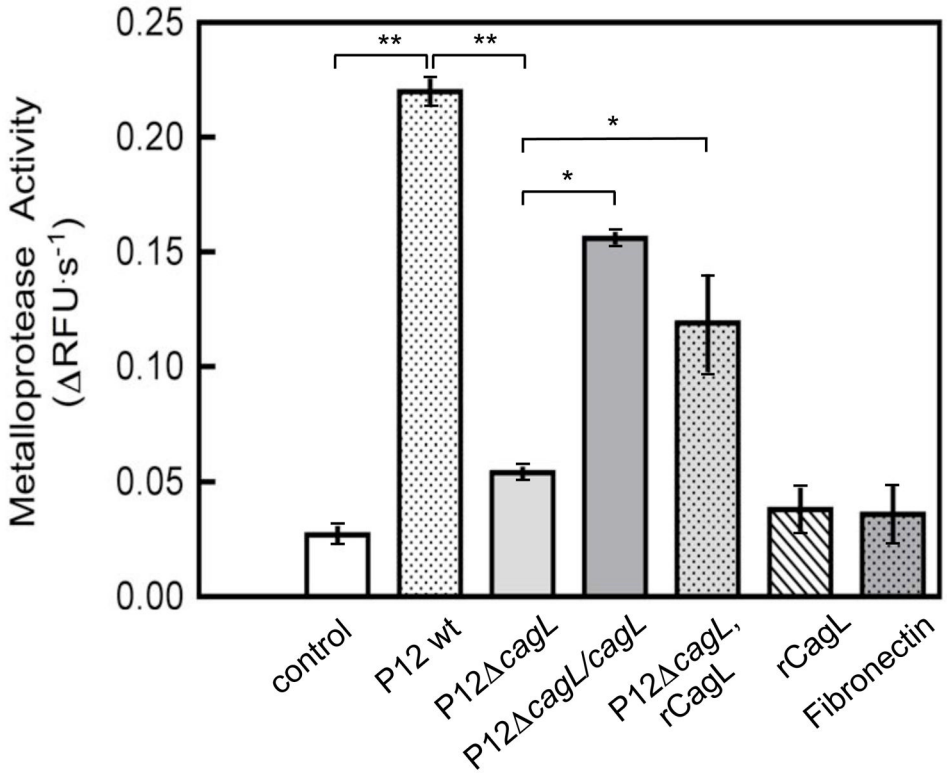

Figure 3.

CagL mediates *H. pylori*-induced ADAM17 activation in AGS cells. AGS cells (3×10^4 cells/well) were incubated overnight in untreated, human recombinant (r) CagL-treated (5 μg/ml), or fibronectin-treated (5 μg/ml) 96-well plates, infected (MOI=50, 8 h) with WT *H. pylori* strain P12, P12 Δ*cagL* or genetically-complemented P12 Δ*cagL*/*cagL*, and after medium replacement cellular ADAM17 activity was measured using a quenched fluorogenic oligopeptide substrate (means, SD, n=8; **P*<0.05, ***P*<0.01).

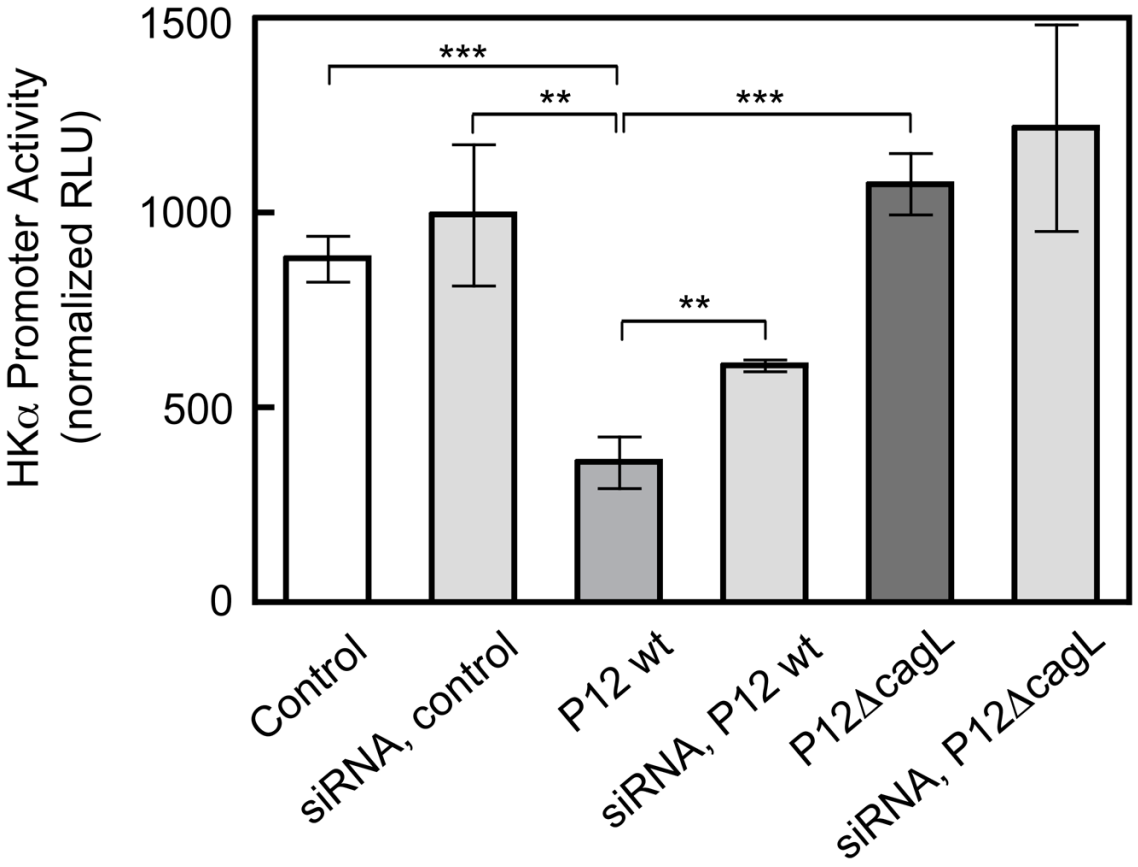

Figure 4.

ADAM17 down-regulation abrogates *H. pylori*-induced HKα promoter repression. AGS cells were first transfected with ADAM17-specific pooled siRNAs and then with HKα2197 promoter-*Luc* constructs. The cells were then infected (MOI=50, 8 h) with WT *H. pylori* strain P12 or P12 Δ*cagL*. HKα promoter activity was measured as RLU of luciferin luminescence normalized to co-transfected GFP fluorescence (means, SD, n=3; ***P*<0.01, ****P*<0.001).

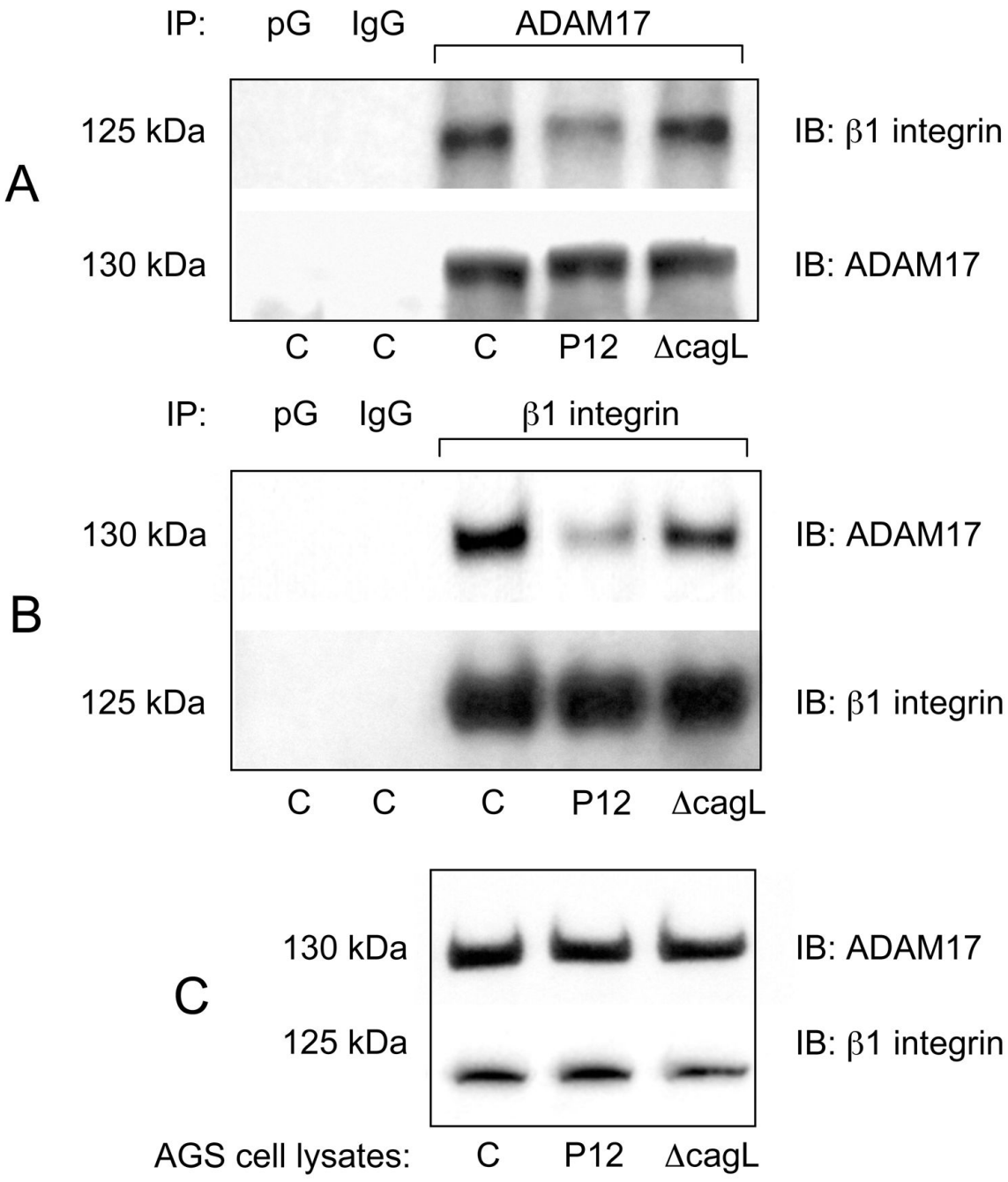

Figure 5.

CagL mediates dissociation of ADAM17 from β_1 integrin. AGS cells were infected (MOI=50, 1 h) with WT *H. pylori* strain P12 or P12 Δ*cagL*. Cell lysates were immunoprecipitated with (**A**) ADAM17-specific antibody followed by immunoblotting (IB) of IP protein with β_1 integrin-specific antibody; ADAM17 IB served as loading control. Reverse IP was performed by immunoprecipitating the lysates with (**B**) β_1 integrin-specific antibody followed by IB with ADAM17-specific antibody; β_1 integrin IB served as loading control.. (**C**) Direct ADAM17 and β_1 integrin IB of cell lysates served as a gel loading control and a positive control of antibody specificity respectively. Protein G beads (pG) and non-immune immunoglobulin Gs

(IgG)-precipitated lysates served as negative controls. Mock-infected AGS cells are shown in lanes “C”.

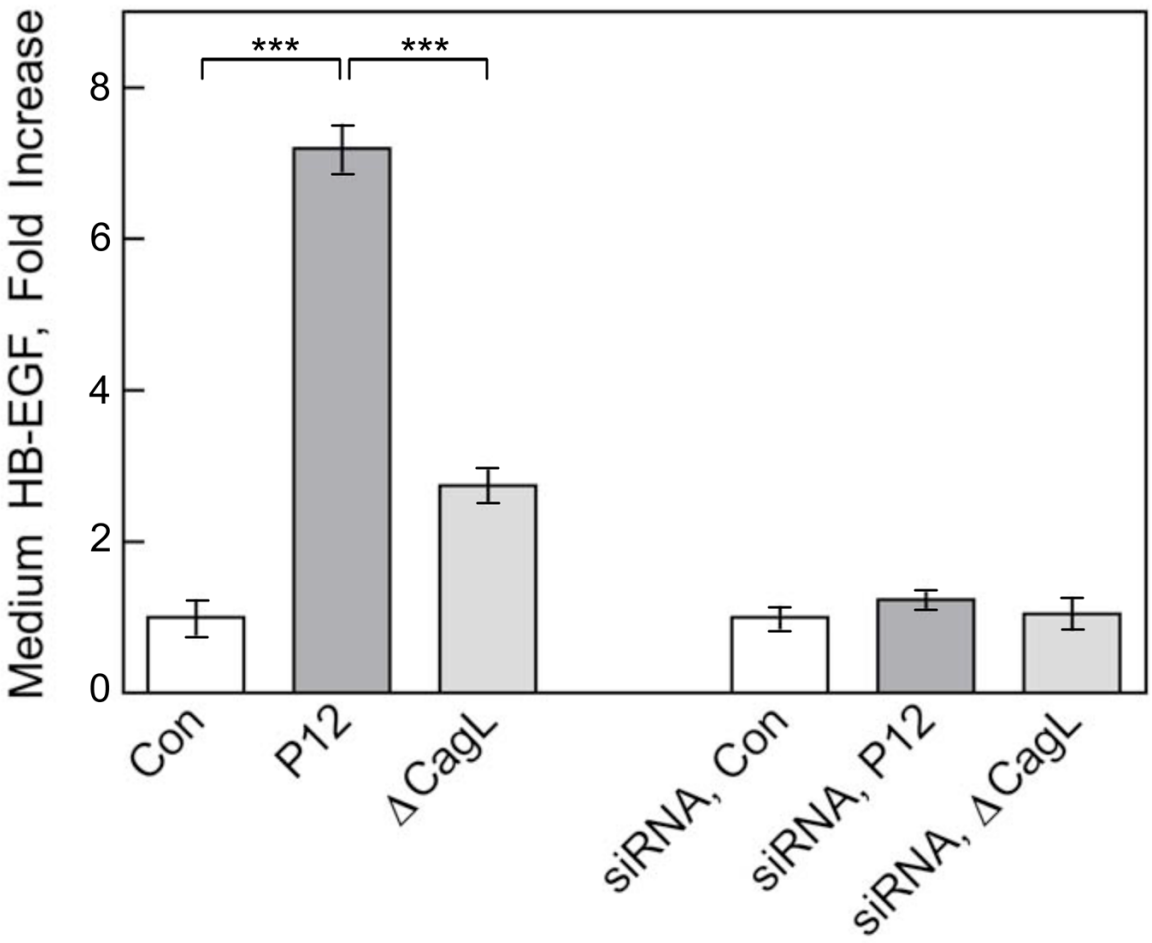

Figure 6.

CagL mediates *H. pylori*-induced shedding of HB-EGF from AGS cells. AGS cells were infected (MOI=50, 1 h) with WT *H. pylori* strain P12 or P12 Δ*cagL* with or without previously silencing ADAM17 expression by siRNA. Culture medium supernatants were concentrated by centrifugal filtration and HB-EGF in the retentates was measured by ELISA (mean, SD, n=2; ***P<0.001).

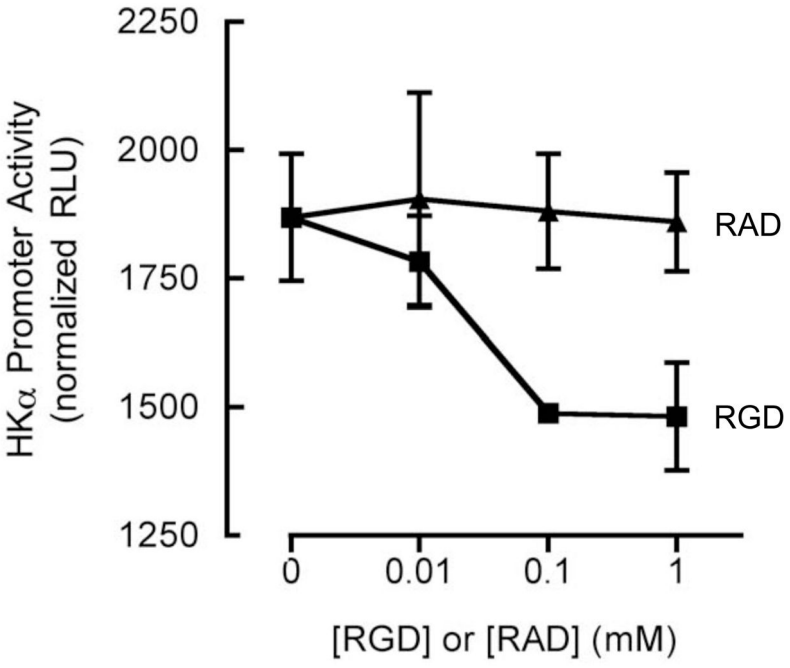

Figure 7A

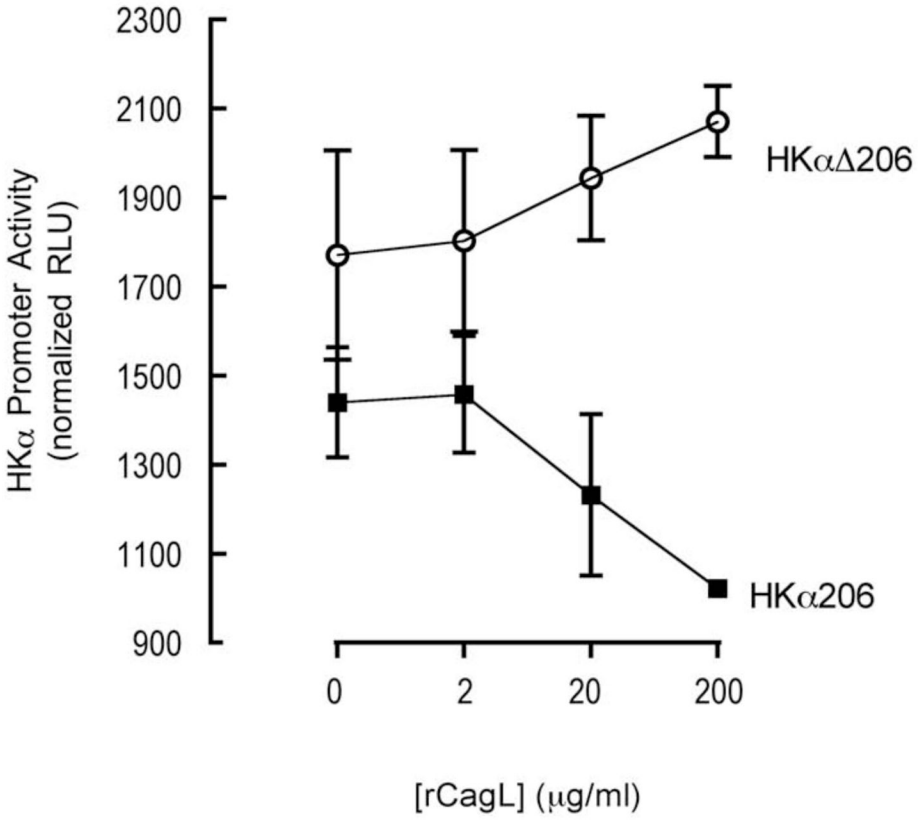

Figure 7B

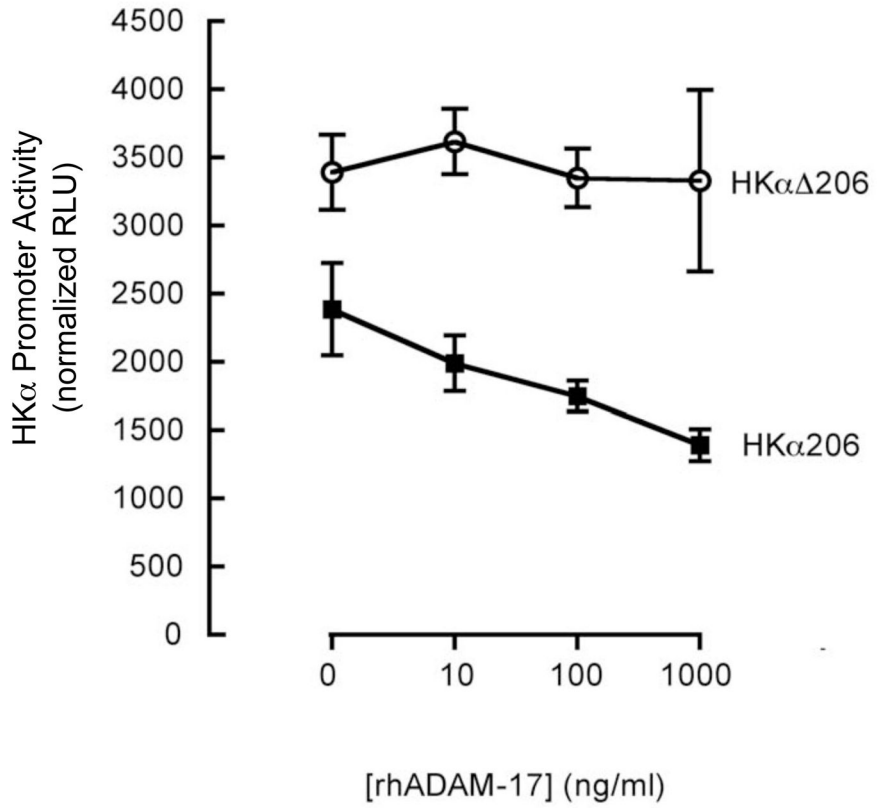

Figure 7C

Figure 7.

RGD peptide, rCagL and rhADAM17 independently repress HKα promoter activity. AGS cells were transfected with a WT HKα promoter-*Luc* reporter construct (HKα206) or an HKα206 construct with two point mutations in the NF-κB binding site (HKαΔ206). Transfected AGS cells were incubated for 8 h with (**A**) synthetic RGD or RAD-containing peptides; (**B**) recombinant CagL (rCagL); or (**C**) recombinant human ADAM17 (rhADAM17) and HKα promoter activity was measured as RLU of luciferin luminescence normalized to co-transfected GFP fluorescence (means, SD, n=3).