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Epithelial cell ADAM17 activation by *Helicobacter pylori*: role of ADAM17

C-terminus and Threonine-735 phosphorylation

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Abstract

Helicobacter pylori transactivates the epidermal growth factor receptor (EGFR) on gastric epithelial cells via a signalling cascade involving a disintegrin and metalloprotease 17 (ADAM17) cleavage of membrane bound heparin binding-epidermal growth factor (HB-EGF). The effects of *H. pylori* on ADAM17 C-terminus in epithelial cells have been examined. Total cellular ADAM17 and surface expression of ADAM17 were significantly increased by *H. pylori* in AGS gastric epithelial cells. These changes were associated with ADAM17 C-terminal phosphorylation at T375 and S791. AGS cells lacking the ADAM17 C-terminal domain induced significantly attenuated cleavage of HB-EGF and were also unable to upregulate *HB-EGF* and *EGFR* transcripts to the same extent as cells expressing full length ADAM17. In mitotic unstimulated AGS and ADAM17 over-expressing AGS cells, ADAM17 was highly T735 phosphorylated indicating ADAM17 T735 phosphorylation is modified during the cell cycle. In conclusion, *H. pylori* induced ADAM17 C-terminal T735 and/or S791 phosphorylation in gastric epithelial cells are likely to be an important trigger inducing ADAM17 activation and shedding of HB-EGF leading to EGFR transactivation. ADAM17 over-expression in gastric cancer represents a potential target for therapeutic intervention.

Keywords

Helicobacter pylori; ADAM 17; phosphorylation; EGFR; HB-EGF; TNF- α

1. Introduction

The predominant cause of gastric cancer is chronic infection with the gastric bacterium *Helicobacter pylori*. The incidence of gastric cancer is highest in those with atrophic gastritis and intestinal metaplasia resulting from long-term *H. pylori* infection [1]. Potential contributory factors linking *H. pylori* with development of gastric cancer include the chronic inflammatory response to infection, *H. pylori*-induced perturbations in gastric epithelial cell signalling [2-5] and disruption of gastric epithelial cell intercellular and cell-matrix junctions [6].

Gastric epithelial hyperproliferation is observed both clinically [7,8] and in animal models [9,10]. One of the potential mechanisms by which *H. pylori* induces hyperproliferative epithelial responses involves *H. pylori* stimulated transactivation of the Epidermal Growth Factor Receptor (EGFR). *H. pylori* strains both with, and without, the *cag* pathogenicity island (*cag* PAI) [11] transactivate the EGFR on gastric epithelial cells [12-16]. In line with the study of Prenzel *et al*, 1999, which identified that EGFR activation occurs via a Triple Membrane Passing Signal (TMPS) cascade [17], the EGF ligand heparin binding epidermal growth factor (HB-EGF) is essential for *H. pylori* stimulated EGFR transactivation and requires membrane metalloprotease and Mek1 activity for cleavage of membrane anchored pro-HB-EGF [12]. *H. pylori* stimulated cleavage of proHB-EGF and EGFR transactivation is mediated by ADAM17 [18].

ADAM17 was originally described as a tumour necrosis factor- α converting enzyme [19,20]. It has subsequently been shown to cleave numerous transmembrane proteins and is over expressed in tumours at many sites [21]. In gastric cancer both *ADAM17* transcripts [22] and ADAM17 protein are increased [23,24] compared to non-involved gastric tissue and HB-EGF is similarly upregulated in gastric cancer [25]. Our previous studies have shown that *H. pylori* infection upregulates *ADAM17* transcripts in the gastric mucosa *in vivo* both clinically

[22] and in the gerbil *H. pylori* infection model [26]. In cultured gastric epithelial cells *H. pylori* stimulates increase in transcripts of several *ADAM* genes [27] and upregulation of *ADAM17* transcripts is independent of a functional *cag* PAI [22].

ADAM17 activity is regulated by multiple post-translational modifications including phosphorylation [21]. *ADAM17* has three phosphorylation sites on its C-terminal cytoplasmic domain located at Threonine 735 (T735) and Serines 791 (S791) and 819 (S819). Phosphorylation at T735 regulates *ADAM17* maturation and trafficking to the cell surface and is important for docking of Erk1/2 [28]. S791 and S819 also have a role in *ADAM17* activity. Phosphorylation of *ADAM17* at S819 and dephosphorylation at S791 occurs following EGF stimulation of HeLa cells [29]. Phosphorylation of S819 is dependent on Erk1/2 while S791 dephosphorylation is not. There are varying results regarding the importance of the cytoplasmic tail for *ADAM17* enzymatic activity following exposure to different stimuli [28-33].

Numerous pathogens transactivate the EGFR in epithelial cells yet little is known regarding their effects on the activation of the key *ADAM* proteins involved in EGFR transactivation [34]. *H. pylori*-induced EGFR transactivation can be mediated directly, or following pathogen-induced upregulation of other proteins capable of EGFR transactivation such as IL-8 [35]. The aims of the present study were firstly to investigate the effects of *H. pylori* on upregulation of *ADAM17* in epithelial cells and on C-terminus *ADAM17* phosphorylation; and secondly to investigate *ADAM17* substrate cleavage and autocrine EGFR signaling pathways in gastric epithelial cells over expressing *ADAM17* and an *ADAM17* C-terminus deficient mutant.

2. Materials and Methods

2.1. Cell culture and transfections

AGS gastric epithelial cells, MKN28 and A431 human epidermoid carcinoma were cultured in RPMI 1640 medium (Gibco, Paisley, UK) containing 10% (v/v) heat inactivated fetal calf serum, 2 mM glutamine and 40 mg/ml gentamicin (Gibco, Paisley, UK) at 37°C, 95% air and 5%CO₂. Where indicated, transfected cells were cultured with the addition of appropriate antibiotics. For transfection of AGS cells Lipofectamin2000 (Invitrogen, Paisley, UK) was used following the manufacturer's instructions. The plasmid solution was added to AGS cells for 24h following replacement with media containing 500 µg/ml of appropriate selective antibiotic.

2.2. Bacterial culture

H. pylori *cag* pathogenicity island (*cag* PAI) positive strains NCTC 11637, G27 and a G27Δ *cagM* isogenic mutant (kindly provided by Dr. A. Covacci, Chiron Vaccines, Siena, Italy) were cultured on 8% (v/v) horse blood, 7.5% (w/v) agar plates (Oxoid base 2) with 50 µg/ml Amphotericin B at 37°C in a microaerophilic atmosphere using the Campygen system (Oxoid, Hampshire, UK).

2.3 Antibodies and plasmids

Antibodies used were mouse anti-ADAM17 monoclonal antibody (Abcam, Cambridge, UK), mouse anti-ADAM17 (M220) monoclonal antibody [36] kindly provided by Dr. R. Black, Amgen Inc., California, USA, rabbit anti-pADAM17 polyclonal antibodies targeting individually pThr735, pSer791 and pSer819 (GenScript, Piscataway, USA) and rabbit anti-ERK1 polyclonal antibody (Santa Cruz, Heidelberg, Germany). Secondary antibodies Alexa Fluor 568 anti-rabbit and Alexa Fluor 488 anti-mouse (Invitrogen) were used. Plasmids used

were part of the Tet-off system (Clontech, France) and included pTet-off Advanced repressor plasmid, pTre-Tight-ADAM17 and pTre-Tight- ΔC -terminus ADAM17. To deactivate the over expression cells were cultured in the presence of 20 $\mu\text{g/ml}$ of tetracycline.

2.4 Bacterial-epithelial co-culture

MKN28, A341, AGS cells were seeded into 96-well plates (Nunc, Rochester, NY, USA) at a density of $3\sim 5\times 10^4$ cells per well and cultured at 37°C in 95% air and 5% CO_2 until 80%~90% confluent. Prior to assay cells were cultured in serum free and antibiotic free medium for 24 hours. *H. pylori* G27 and G27 ΔcagM were harvested into serum and antibiotic-free RPMI 1640 media, centrifuged at 1000 rpm for 10 min, re-suspended at $5\times 10^7/\text{ml}$ and used immediately. Bacteria were co-cultured in duplicate with MKN28, A431 and AGS cells at a MOI of 100:1 for 1-6h. Untreated cells were used as negative controls at each time point. In some experiments epithelial cells were pre-incubated for 1h with 0.001-1 μM EKB-569 kindly provided by Dr. L. Greenberger (Wyeth Research Chemical Sciences, Pearl River, USA) before bacterial stimulation.

2.5 “In Cell Western” assay

“In Cell Western” (ICW) assay was undertaken as previously described [14]. Following aspiration of media, the cells were fixed in 96 well plates with 3.7% (v/v) formaldehyde in phosphate buffered saline (PBS) for 20 min at 20°C . After cell permeabilisation by washing four times with 0.1% (v/v) Triton X-100 in PBS, nonspecific protein binding sites were blocked with Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE, USA) for 1h before incubation for 2h with anti-ADAM17 monoclonal antibody (M220) and rabbit polyclonal anti-ERK1 diluted in blocking buffer. Following further washing cells were incubated for 1h in the dark with goat anti-mouse IgG Alexa Fluor 680 (Molecular Probes, Eugene, OR, USA) and goat anti-rabbit IRD800 antibody (Rockland, Gilbertsville, PA, USA)

before rewashing. Unstimulated control cells were included at each time point to determine endogenous ADAM17. A row of stimulated cells was included on every plate for incubation with secondary antibodies alone to determine any background levels of labelling. Plates were imaged on an Odyssey Reader (LICOR Biosciences) at 700nm and 800nm and data analysed using Odyssey Software 2.0. The 800nm value from ERK1 labelling was used to control for the intrawell variation in cell density and derive the 700nm Relative Value (Rel₇₀₀) of ADAM17. The Rel₇₀₀ value was used for comparisons between different cell lines with reference to unstimulated controls at each time point (100% standard).

To evaluate surface ADAM17 an “On Cell Western” was performed where incubation with the ADAM17 antibody M220 against the extracellular domain [36] and secondary antibody labeling was undertaken without cell permeabilisation. Cells were subsequently permeabilised, labelled for ERK1, imaged at 700nm and 800 nm in an Odyssey Reader and analysed as above. Total cellular ADAM17 was measured by ICW on the same plate as the OCW assay to measure surface ADAM17 under identical experimental conditions.

2.6 Immunofluorescent microscopy

AGS cells grown on sterile cover slips were stimulated either with *H. pylori* NCTC 11637 or G27 strains at MOI 100:1 for 90 min or with TNF- α 50 ng/ml (Sigma, Dorset, UK) for 15 min at 37°C. Unstimulated cells were used as controls. Cells were fixed with 4% (v/v) paraformaldehyde (Calbiochem, Darmstadt, Germany) and permeabilised with 0.1% (v/v) Triton X-100. Samples were labelled with primary antibodies followed by Alexa-fluor conjugated secondary antibodies (Invitrogen) and DAPI (Invitrogen) and examined using a Nikon D-Eclipse C1 confocal microscope. Cells stained with secondary antibodies only and stained with non-immunised rabbit serum were used as negative controls. To confirm the specificity of the phosphorylation epitope permeabilised cells were incubated prior to

immunolabelling with 10 U/ml phosphatase (Sigma) in 0.5M Tris-HCL buffer pH 8.5 containing 1mM EDTA at 37°C for 1h.

2.7 IRDye Near Infrared Fluorescence Resonance Energy Transfer assay (NIR-FRET)

To assay ADAM17 shedding activity in cell lines, a proTNF- α (SPLAQAVRSSSR) and proHB-EGF (LPVENRLY) synthetic NIR-FRET based peptide substrates were synthesised (kindly provided by Dr. Mike Olive, LI-COR Biosciences, Lincoln, NE, USA). The synthesised substrates were linked to IRDye 800 CW NHS Ester (LICOR Biosciences) and quenched with the non-fluorescent quencher IRDye QC-1 (LICOR Biosciences). Cleavage of the peptide substrate results in separation of the quencher and IRDye 800 CW and excitation at 780nm leads to the emission of an Infrared signal at 810-830nm.

AGS cells were seeded in a flat bottom 96-well plate (Corning, NY, USA) at cell density of 2.5×10^5 cells per ml, and incubated for 24h at 37°C, 95% air 5% CO₂ followed by incubation in antibiotic and serum-free media for 15h. FRET substrates were added at 40 nM for 3h and incubated in the dark at 37°C, 95% air 5% CO₂. Substrate diluted in media alone was used as a background control. Plates were centrifuged at 400g at 4°C and cell free medium transferred to 96-well flat bottomed plates (Nunc, Paisley, UK). The OD of each well was read on an Odyssey reader at 800nm and data analysed with Odyssey 2.0 Software after subtracting background substrate only readings from test wells. Following fixation and permeabilisation nuclear staining of AGS cells with DRAQ5 DNA stain and reading in the 700nm channel normalised for differences in cell density.

2.8 Polymerase chain reaction (PCR)

Cellular RNA was isolated using Bioline RNA isolation kit (Bioline, London, UK) as per the manufacturer's protocol. cDNA was prepared from 2.5 μ g RNA using MMLuV reverse

transcriptase (NEB, Ipswich, USA) and 1 µg random hexamer primers. For Quantitative Real-Time Polymerase chain reaction (qRT-PCR) RNA isolation and cDNA synthesis was as above. Quantitative RT-PCR and data analysis was performed as described previously using SYBR Green I [37]. Primers used are listed in Table 1.

2.9 Fluorescence activated cell sorting (FACS)

FACS analysis of cell cycle using propidium iodide was performed and labelling with rabbit polyclonal antibodies to pADAM17-T735, pADAM17-S791, pADAM17-S819. Cells were fixed with 1% paraformaldehyde, permeabilised with 0.5% saponin and blocked with 5% bovine serum albumin. Cells were incubated individually with the three rabbit polyclonal antibodies to pADAM17 followed by Alexa conjugated secondary antibody (Invitrogen, Paisley, UK). Following RNA degradation with RNase A, propidium iodide was used to stain cellular DNA and permit cell cycle determination. Following the selection of intact cells stained with PI and the exclusion of doublets a plot of PI intensity (y axis) against the number of events (x axis) was created.

2.10 Statistics

All data was initially analysed with the Kolmogorow-Smirnow test to determine if it was parametric. Parametric data was analysed by comparing each group to controls using t-test. Groups with p values below 0.05 were interpreted as significantly different from controls.

3. Results

3.1 *H. pylori* stimulates ADAM 17 in epithelial cells independent of a functional *cag* PAI

To extend results on *H. pylori* induced upregulation of *ADAM17* transcripts in gastric epithelial cells [22], ADAM17 protein was quantified using “In Cell Western” analysis in AGS and MKN28 gastric epithelial cells and A431 cells following stimulation with the *cag* PAI+ *H. pylori* strain G27 and G27 Δ *cagM* isogenic mutant. The 700nm Relative Value (Rel₇₀₀) of unstimulated controls for each cell line at both 1 and 3h time points (defined as 100% standard) was used for comparisons of changes in ADAM17 induced by *H. pylori*. In all three cell lines both G27 and the G27 Δ *cagM* isogenic strain significantly increased ADAM17 in a time dependant manner (Figure 1A). Previous studies have demonstrated that G27 Δ *cagM* fails to activate NF- κ B [38] and IL-8 secretion in gastric epithelial cells [11]. The greatest increase in ADAM17 following *H. pylori* stimulation was observed in the AGS gastric epithelial cell line.

As ADAM17 is produced as a pro-protein which undergoes maturation and cell trafficking to the cell membrane where ADAM17 acts as a sheddase [39], “On Cell Western” assays [40] were used to assess the effects of *H. pylori* infection on surface ADAM17 in AGS cells. ADAM17 immunolabelling of the extracellular domain of the protein was carried out in unpermeabilised AGS cells and following permeabilisation, total cellular ERK1 was measured to control for cell density variations. Parallel assays of total cellular ADAM17 were undertaken by ICW on the same assay plates under identical experimental conditions. In the OCW both the wild type strain G27 and the Δ *cagM* mutant significantly increased surface cell membrane ADAM17 (Figure 1B). At 1h post-stimulation there was a 90% increase in surface ADAM17 relative to unstimulated control cells following G27 stimulation and 100% increase following G27 Δ *cagM* (Figure 1B). Whilst there was a temporal increase in total

cellular ADAM17 after bacterial stimulation as determined by the ICW assay over the course of the 6h experiment, the major increase in surface ADAM17 in the OCW assay was detected 1h post-stimulation with G27 and the isogenic mutant G27 Δ cagM (Figure 1B).

3.2 ADAM17 over expression in AGS gastric epithelial cells

As *H. pylori* stimulated the highest ADAM17 levels in the AGS gastric epithelial cell line (Figure 1A), AGS cells were chosen for constructing ADAM17 over expression and C-terminus deletion mutants. ADAM 17 has three phosphorylation sites located within its C-terminus. To analyse the effects of *H. pylori* on ADAM17 activity and its C-terminal phosphorylation at T735, S791 and S819, AGS gastric epithelial cells over expressing both wild type and C-terminus deficient ADAM17 were constructed. The Δ C-terminus ADAM17 mutant had a translational stop signal introduced resulting in the absence of the cytoplasmic tail and T735, S791 and S819 phosphorylation sites. Protein expression from plasmid constructs was controlled via a Tet-off system, which allowed for inducible over expression of ADAM17 in the absence of tetracycline. ADAM17 over expressing monoclones were selected and over expression validated using reverse transcription followed by PCR and immunofluorescence microscopy. Over expressed ADAM17 had the same cellular localisation profile as the endogenous ADAM17 and no compartmentalisation was observed. Due to the very high efficiency of ADAM17 overexpression we hypothesise that in these cells overexpressed ADAM17 will be the dominant clone and the biological driver.

3.3 Phosphorylation of ADAM 17 at T735 is cell cycle dependant and induced by *H. pylori* and TNF- α

Examination of the localisation of the three phosphorylated forms of ADAM17 (p-T735, p-S719 and p-S819) in unstimulated AGS wild type cells and AGS cells over expressing ADAM17 was undertaken by immunofluorescent microscopy (Figure 2A and B) and FACS

analysis (Figure 2C and D). High levels of ADAM17 phosphorylated at T735 were present in cells that were undergoing mitosis in both wild type AGS gastric epithelial cells (Figure 2A) and ADAM17 over expressing cells (Figure 2B) but this was not observed with ADAM p-S719 or ADAM p-S819 immunofluorescence microscopy (data not shown). Confocal microscope analysis indicated that pT735ADAM17 constitutes approximately 7% of total ADAM17 in unstimulated cells and 32% and 39% of total ADAM17 respectively after TNF- α and *H. pylori* stimulation. pS791 constitutes 21% of total ADAM17 in unstimulated cells and 34% and 57% of total ADAM17 respectively following TNF- α and *H. pylori* stimulation. I would strongly advise to delete this section, reasons explained in the response to reviewers document.

To quantify further the phosphorylation of ADAM17 at these three sites in relation to cell cycle, FACS analysis of cell cycle using propidium iodide (PI) was performed along with staining for each of the phosphorylated forms of ADAM17 in wild type AGS cells (Figure 2C) and ADAM17 over expressing AGS cells (Figure 2D). In AGS gastric epithelial cells treated with PI and the Alexa 488 conjugated secondary antibody only, there was a signal from the Alexa 488 indicating that there was some non-specific binding of the secondary antibody but the intensity of this staining was much lower than that for any of the primary phospho-ADAM17 antibody treated cells (Figure 2C and D). The PI intensity of AGS cells plotted against the intensity of ADAM17 phosphorylated at S791 or S819 indicated that phosphorylation of ADAM17 occurs at these sites but the AGS cells fell within one cluster suggesting phosphorylation at S791 and S819 was not linked to specific phases of the cell cycle. In contrast, co-staining with PI and an antibody against phospho-T735 identified two populations of cells in both wild type AGS cells (Figure 2C) and AGS ADAM17 over expressing cells (Figure 2D). Phospho-T735 was detected in the majority of cells and these cells were in all phases of the cell cycle, however a population of cells with high PI staining

in G2/M phase of the cell cycle had much higher expression of p-T735 (Figure 2C and 2D). Unstimulated AGS wild type cells and AGS cells over expressing ADAM17 had respectively 0.95 ± 0.16 (mean $\pm$ SEM) % and 3.4 ± 0.77 % (n = 4 independent experiments) of total cells with high expression of p-T735 in G2/M phase of the cell cycle (p <0.02). These data confirm the findings of the immunofluorescence microscopy where high levels of p-T735 were detected in AGS cells undergoing mitosis. This suggests that either phosphorylation of ADAM17 at T735 may have a role in regulating the cell cycle or that ADAM17 phosphorylated at T735 plays some role in cell division.

To investigate whether *H. pylori* induced phosphorylation of ADAM17 in AGS gastric epithelial cells, AGS cells were cultured with *H. pylori* strain NCTC 11637 for 90 min and TNF- α (50ng/ml) for 15 min and cells were immunolabelled for ADAM17 and phospho-T735 and phospho-S719 ADAM17 (Figure 2E and F) and phospho-S819 ADAM17. Phosphorylation of ADAM17 T735 (Figure 2E) and ADAM17 S791 (Figure 2F) was stimulated by both *H. pylori* NCTC 11637 strain and TNF- α in AGS gastric epithelial cells. Similar results were observed with *H. pylori* G27 strain. Background levels of ADAM phospho-T735 positivity in unstimulated AGS cells were lower than that of phospho-S791 ADAM17. Confocal microscope analysis indicated that pT735ADAM17 constitutes approximately 7% of total ADAM17 in unstimulated cells and 32% and 39% of total ADAM17 respectively after TNF- α and *H. pylori* stimulation. pS791 constitutes 21% of total ADAM17 in unstimulated cells and 34% and 57% of total ADAM17 respectively following TNF- α and *H. pylori* stimulation. I would strongly advise to delete this section, reasons explained in the response to reviewers document, also this is a repetition. Pre-incubation of permeabilised cells with phosphatase prior to immunolabelling confirmed specificity of phospho-T735 and S791 ADAM17 antibodies for phosphorylation epitopes. No specific

upregulation of phospho-S819 ADAM17 was observed with *H. pylori* or TNF- α stimulation (data not shown).

3.4 C-terminus of ADAM 17 is required for substrate cleavage

To evaluate the importance of ADAM17 phosphorylation in the TMPS cascade leading to EGFR transactivation, the ability of unstimulated AGS gastric epithelial cells, AGS cells over expressing wild type ADAM17 or Δ C-terminus ADAM17 to cleave ADAM17 substrates was determined using an IRDye Near Infrared Fluorescence Resonance Energy Transfer assay (NIR-FRET). The cleavage of HB-EGF and TNF- α quenched probes by the AGS cell lines was examined. AGS Δ C-terminus ADAM17 induced a small but significant ($p < 0.05$) increase in HB-EGF cleavage compared to non-transfected AGS cells. In contrast, AGS cells over expressing ADAM17 significantly increased ($p < 0.05$) HB-EGF cleavage relative to both non-transfected AGS cells and AGS Δ C-terminus ADAM17 cells (Figure 3A). Analysis of cleavage of the ADAM17 substrate TNF- α using a TNF- α quenched probe showed that the AGS Δ C-terminus ADAM17 cell line was able to significantly ($p < 0.05$) increase TNF- α cleavage relative to that induced by non-transfected gastric epithelial AGS cells. However cleavage induced by the AGS Δ C-terminus ADAM17 cell line was significantly lower ($p < 0.05$) than that observed with AGS cells over expressing wild type ADAM17 (Figure 3B).

To investigate further the importance of the ADAM17 cytoplasmic tail and its phosphorylation sites in autocrine cell signalling, *HB-EGF* and *EGFR* transcripts in unstimulated AGS cells, AGS cells overexpressing ADAM17 and AGS cells with Δ C-terminus ADAM17 were examined. Transfected cells were examined after culture with, and without, tetracycline. Both ADAM17 over expressing wild type and Δ C-terminus ADAM17 AGS cells had significantly ($p < 0.01$) increased abundance of *HB-EGF* and *EGFR* transcripts in the absence of tetracycline compared to AGS epithelial cells (Figure 4A and B).

However both *HB-EGF* and *EGFR* transcript abundance was significantly ($p < 0.05$) higher in AGS cells over expressing ADAM17 than AGS cells with ΔC -terminus ADAM17 in cells cultured in the absence of tetracycline (Figure 4A and B). This demonstrates that ADAM17 over expression in gastric epithelial cells in the absence of *H. pylori* stimulation will upregulate key components of the TMPS pathway promoting autocrine EGFR signalling.

3.5 *H. pylori*-induced ADAM17 upregulation in epithelial cells is not blocked by EGFR inhibition

To examine whether *H. pylori*-induced upregulation of ADAM17 was dependent on EGFR transactivation, A431 cells were pre-incubated with the selective EGFR tyrosine kinase inhibitor EKB-569 for 1h at 37°C before co-culture with *H. pylori*. In contrast to results on *H. pylori* stimulated ERK1/2 phosphorylation [14], EKB-569 had no effect on *H. pylori* induced ADAM17 upregulation at 3 hours post-infection. There was no effect of EKB-569 on endogenous ADAM17 in uninfected A431 cells (Figure 5).

4. Discussion

The activity of ADAM17 can be post-translationally regulated via a number of different cellular processes. These include post-translational modifications such as phosphorylation, targeted degradation, enzymatic cleavage and inhibition by TIMPs [39]. It has been hypothesised that ADAM17 phosphorylation is required for its activation and hence would be required for *H. pylori* activation of ADAM17 during the TMPS cascade leading to EGFR transactivation.

Previous studies have shown ERK-mediated phosphorylation of T735 ADAM17 induces trafficking of ADAM17 to the surface of COS-7 cells [28]. Our previous studies using “In Cell Western” assays have shown maximal ERK1/2 phosphorylation in AGS gastric epithelial cells occurs 90 min following stimulation with *H. pylori* G27 strain and G27 Δ cagM [14]. In the present study using “On Cell Westerns” and ICW assays a significant increase in both cell surface and total ADAM17 in AGS epithelial cells was observed following culture with *H. pylori* G27 strain and G27 Δ cagM. The OCW cell-based assay is a recently developed valuable tool which allows rapid quantitative monitoring of changes in cell surface protein expression [40]. With G27 stimulation total cellular ADAM17 increased over 6h. With both G27 and G27 Δ cagM stimulation the majority of the increase in surface ADAM17, as determined by OCW assay, was evident 1h after bacterial stimulation indicating a rapid surface translocation of ADAM17. Additionally, *H. pylori*-upregulation of ADAM17 in the AGS gastric epithelial cells was independent of a functional *cag* PAI, concurring with previous reports on *H. pylori*-stimulated ERK1/2 activation [14].

ADAM17 is active only on the cell membrane where it works as a sheddase [21,39]. After *H. pylori* stimulation, both the ADAM17 T735 and S791 phosphorylation status increased. *H. pylori*-induced ADAM17 phosphorylation at T735 and S791 in gastric epithelial cells was

observed 90min post-infection, a time point concomitant with maximal *H. pylori*-induced ERK activation [14]. These results on *H. pylori*-induced ADAM17 phosphorylation of T735 and increased cell surface ADAM17 fit the model proposed by Soond *et al* (2005) that ERK-mediated ADAM17 T735 phosphorylation is important in ADAM17 trafficking and maturation [28]. However whilst the EGFR inhibitor EKB-569 reduces *H. pylori* stimulated ERK1/2 phosphorylation *in vitro* [14], the present study indicates that upregulation of ADAM17 by *H. pylori* was independent of EGFR transactivation occurring in the presence of the EGFR inhibitor EKB-569. Both pERK1/2 dependent and independent *H. pylori* activation of ADAM17 is likely to occur.

The present results indicate ADAM17 T735 phosphorylation **could be** involved in *H. pylori*-induced EGFR transactivation in gastric epithelial cells [12]. Additionally, the observation that AGS cells transfected with ADAM17 lacking the C-terminus stimulated reduced cleavage of HB-EGF and TNF- α quenched probes compared to AGS cells transfected with wild type ADAM17 also supports a role for ADAM17 phosphorylation sites in surface proHB-EGF cleavage leading to EGFR transactivation. In addition, reduction in *EGFR* and *HB-EGF* transcripts in unstimulated AGS cells transfected with Δ C-terminus ADAM17 compared to cells transfected with wild type ADAM17 suggests ADAM17 phosphorylation, and over expression in gastric cancer cells, will promote autocrine EGFR signalling. In addition to the current studies with *H. pylori*, ADAM17 activation has also been observed in airway epithelial cells following culture with pulmonary bacteria [41]. However, the current study with *H. pylori* is the first report on ADAM17 phosphorylation as a result of bacterial stimulation.

ADAM17 was previously found to co-localise to membrane ruffles [42]. During Golgi transport, ADAM17 is inserted into lipid rafts for removal of the prodomain by furin [43]. This results in active ADAM17 and increased levels of its substrates within lipid rafts [43].

When cholesterol is depleted, ADAM17 increases substrate shedding, highlighting the importance of cholesterol in ADAM17 regulation via substrate availability and spacial organisation [43].

Recently *H. pylori* has been shown to extract cholesterol rapidly from lipid rafts in epithelial plasma membranes in a *cag* PAI independent manner for subsequent glucosylation and immune evasion [44]. The resulting destruction of lipid rafts as a consequence of *H. pylori* cholesterol extraction could be a potential trigger for ADAM17 phosphorylation and increased sheddase activity. It would also explain why in the present study the EGFR inhibitor EKB-569 failed to modify *H. pylori*-induced ADAM17 upregulation.

However, both ADAM17 [42,45] and the *H. pylori* protein CagL protein [46] bind $\alpha 5\beta 1$ integrin. Additionally *H. pylori* protein CagL can directly interact with ADAM17 leading to ADAM17 dissociation from the $\alpha 5\beta 1$ integrin resulting in ADAM17 dependent pro-HB-EGF cleavage [47]. Both the disruption of lipid rafts in epithelial cells by *H. pylori* [44] and upregulation of ADAM17 transcripts [22] and ADAM17 protein in gastric epithelial cells, as observed in the present study, was independent of a functional *cag* PAI. Further studies are required to evaluate fully the effect of cholesterol depletion on *H. pylori* induced ADAM17 shedding and EGFR activation.

The present study has also identified that ADAM17 T735 phosphorylation is cell cycle dependent and ADAM17 T735 phosphorylation is markedly upregulated during mitosis in gastric epithelial cells. This, to our knowledge, is the first observation of phosphorylated ADAM17 T735 being associated with mitosis. Interestingly, earlier yeast two-hybrid studies showed ADAM17 binds to the protein MAD2 (mitotic arrest deficient 2), a component of the mitotic checkpoint mechanism [48]. The screen identified MAD2 as a binding partner to ADAM17 cytoplasmic domain containing residues 706-740 [48]. Further investigation is

419 necessary to determine if the MAD2 interaction is dependant on T735 phosphorylation of
420 ADAM17.

421 In summary, these studies have identified the importance of the ADAM17 C-terminal domain
422 in *H. pylori* stimulated TMPS which results in EGFR transactivation. ADAM17 C-terminal
423 ADAM17 T735 and S791 phosphorylation in gastric epithelial cells is induced by *H. pylori*.
424 ADAM17 T735 phosphorylation is modified in the cell cycle being markedly upregulated in
425 mitotic cells. ADAM17 over-expression in gastric cancer represents a potential target for
426 therapeutic intervention.

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572 **Table 1: Primer sequences.**

Target Gene	Direction	Sequence 5'-3'
EGFR	F	dAGTGACTGCTGCCACAACCAGTGTGCTGCAGGC
EGFR	R	dGCCTTGCCGCAAAGTGTGTAACGG
HB-EGF	F	dTGGTGCTGAAGCTCTTTCTGGCTGCAG
HB-EGF	R	dCCAAGCCACAAGCACTGGCCACAC
Actin	F	dTGCCCTGAGGCACTCTTCCAG
Actin	R	dCACACGGAGTACTTGCGCTC
ADAM 17	F	dGTGGATAAGAAATTGGATAAACAG
ADAM 17	R	dGCGTTAGCACTCTGTTTCTTTGCTGTCAACACG

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Figure legends

Figure 1. *H. pylori* strain G27 and G27 Δ cagM stimulate ADAM17 in human epithelial cells. A) MKN28, AGS gastric epithelial cells and A431 human epidermoid carcinoma cells were co-cultured with *H. pylori* cag PAI positive strain G27 and G27 Δ cagM at MOI 100:1 or left unstimulated for 1 to 3h. ADAM17 was quantified by “In Cell Western” (ICW) analysis using a mouse monoclonal antibody to ADAM17 and a rabbit-polyclonal ERK1 antibody. Fluorescence intensity was measured at 800 nm and 700nm using a Licor Odyssey imager. The 700nm Relative Value was obtained after normalisation with 800nm values to control for cell density variation. Individual cell line data are presented as mean $\pm$ SEM (n = 4 - 7) of 700/800 % Relative Response (Res₇₀₀) with reference to unstimulated controls at each time point (100% standard). Statistical significance was analysed using t-test * p < 0.05; ** p < 0.01. # p < 0.05 versus control; ## p < 0.01 versus control. B) “On Cell Western- In Cell Western” (OCW- ICW) and ICW assay of cell surface and total **cellular** ADAM17 respectively in AGS gastric epithelial cells stimulated with *H. pylori* cag PAI positive strain G27, and G27 Δ cagM at MOI 100:1 or left unstimulated for 1 to 6h. Cell surface ADAM17 was measured in unpermeabilised AGS cells by OCW using a mouse monoclonal antibody to **the extracellular domain of** ADAM17 as above. Following cell permeabilisation, total cell ERK-1 was measured using a rabbit polyclonal ERK1 antibody as above. ICW of ADAM17 was measured as in A). OCW/ICW and ICW data are presented as mean $\pm$ SEM (n = 4) of 700/800% Relative Response (Res₇₀₀) with reference to unstimulated controls (100% standard) at each time point. Statistical significance was analysed using t-test, * p < 0.05; ** p < 0.01.

Figure 2. Phosphorylation of ADAM17 at T735 is cell cycle dependant and induced by TNF- α and *H. pylori*. (A) Immunofluorescence microscopy of AGS cells. Bar = 20 μ m. (B)

599 Immunofluorescence microscopy of AGS cells over expressing full length ADAM17, Bar =
 600 20µm. In both (A) and (B) DAPI nuclear stain (blue); total ADAM17 (green); ADAM17
 601 phosphorylated at T735 (red); followed by the merged image. Cells undergoing mitosis with
 602 high levels of pT735 are indicated with white arrow heads. (C) Cell cycle analysis of AGS
 603 cells and (D) AGS cells over expressing full length ADAM17 was undertaken using
 604 fluorescence activated cell sorting. Cells were fixed with 1% PFA, permeabilised with 0.5%
 605 saponin and blocked with 5% BSA. ADAM17 phosphorylated at T735, S791 and S819 was
 606 detected with rabbit antibodies α -ADAM17-pT735, α -ADAM17-pS791 and α -ADAM17-
 607 pS819, respectively and an Alexa conjugated secondary antibody. Propidium iodide was used
 608 to stain the DNA and allow cell cycle phase determination. Contour plots of propidium iodide
 609 staining intensity (y axis) against the intensity of pADAM17 staining (x axis) are shown in C
 610 and D. (E) Immunofluorescence microscopy of AGS cells showing ADAM17
 611 phosphorylation at T735; total ADAM17 (green), pADAM17 T735 (red) and DAPI nuclear
 612 stain (blue), lower right hand image (merged). Bar= 20µm. Stimulation with both TNF- α and
 613 *H. pylori* increases ADAM17 T735 phosphorylation. (F) Immunofluorescence microscopy of
 614 AGS cells for ADAM17 phosphorylation at S791; total ADAM17 (green), pADAM17 S791
 615 (red) and DAPI nuclear stain (blue), lower right hand image (merged). Bar 20µm. In both (E)
 616 and (F) cells were either non-treated (Control) or stimulated with 50 ng/ml TNF- α for 15
 617 mins or *H. pylori* NCTC 11637 *cag* PAI+ at MOI 100:1, for 90 mins.

618 **Figure 3. C-terminus of ADAM17 is required for substrate cleavage.** Cleavage of
 619 quenched fluorescent substrates with cleavage sites corresponding to (A) HB-EGF and (B)
 620 TNF- α by AGS gastric epithelial cells and AGS over expressing ADAM17 or ADAM17 Δ C-
 621 terminus (Δ C-ter) was measured. Cells were incubated for 3h with quenched fluorescent
 622 substrates containing cleavage sites corresponding to HB-EGF or TNF- α . Fluorescence

intensity was measured at 800 nm using a Licor Odyssey imager. Error bars represent SEM of $n = 3$, statistical significance was analysed using t-test. * $p < 0.05$.

Figure 4. ADAM17 deficient in its C-terminus does not increase EGFR and HB-EGF transcripts to the same extent as WT ADAM17 in gastric epithelial cells. (A-B) AGS gastric epithelial cells were transfected with wild type ADAM17 and C-terminus deficient ADAM17 mutant (ΔC -ter). Cells were cultured in both the presence (inhibited over expression [off]) and absence (active over expression [on]) of Tetracycline (as described in materials and methods). Untransfected AGS cells were included for comparison. Cellular RNA was isolated using Bioline RNA isolation kit. cDNA was prepared from 2.5 mg of RNA per sample. Transcript levels were analysed using qRT-PCR. (A) EGFR transcript levels relative to actin (B) HB-EGF transcript level relative to actin. Error bars represent SEM of $n = 3$, statistical significance between the cell lines was analysed using unpaired t-test. A paired t test was used for comparison of removal of tetracycline in ADAM17 over expressing cells and ΔC -terminus ADAM17 expressing cells. * $p < 0.05$, ** $p < 0.01$.

Figure 5. *H. pylori* stimulation of ADAM17 in epithelial cells is not blocked by EGFR inhibition. A431 human epidermoid carcinoma cells were pre-incubated with 0-1000nM EKB-569 for 1 hour before co-culture with *H. pylori* *cag* PAI positive strain G27 and G27 $\Delta cagM$ at MOI 100:1 or left unstimulated for 1.5 to 3h. ADAM17 was quantified by “In Cell Western” (ICW) analysis using a mouse monoclonal antibody to ADAM17 and a rabbit-polyclonal ERK1 antibody. Fluorescence intensity was measured at 800 nm and 700nm using a Licor Odyssey imager. The 700nm Relative Value was obtained after normalisation with 800nm values to control for cell density variation. Individual cell line data are presented as mean $\pm$ SEM ($n = 3$) of 700/800 % Relative Response (Res700) with reference to unstimulated controls at each time point (100% standard). Statistical significance was

647 analysed using t-test * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$ versus control; ## $p < 0.01$ versus
648 control.