Phosphorylation of the epidermal growth factor receptor (EGFR) is essential for interleukin-8 release from intestinal epithelial cells in response to challenge with *Escherichia coli* O157:H7 flagellin

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Enterohaemorrhagic *Escherichia coli* O157:H7 is a major foodborne and environmental pathogen responsible for both sporadic cases and outbreaks of food poisoning, which can lead to serious sequelae, such as haemolytic uraemic syndrome. The structural subunit of *E. coli* O157:H7 flagella is flagellin, which is both the antigenic determinant of the H7 serotype, an important factor in colonization, and an immunomodulatory protein that has been determined to be a major pro-inflammatory component through the instigation of host cell signalling pathways. Flagellin has highly conserved N- and C-terminal regions that are recognized by the host cell pattern recognition receptor Toll-like receptor (TLR) 5. Activation of this receptor triggers cell signalling cascades, which are known to activate host cell kinases and transcription factors that respond with the production of inflammatory mediators such as the chemokine interleukin-8 (IL-8), although the exact components of this pathway are not yet fully characterized. We demonstrate that *E. coli* O157:H7-derived flagellin induces rapid phosphorylation of the epidermal growth factor receptor (EGFR), as an early event in intestinal epithelial cell signalling, and that this is required for the release of the pro-inflammatory cytokine IL-8.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* is an important cause of foodborne and environmentally acquired intestinal infections. Infection in susceptible individuals can lead to serious sequelae, such as haemolytic uraemic syndrome, due to the production of Shiga-like toxins (Stxs), encoded by lysogenic phages, and the ingress of these toxins into the bloodstream. Infection occurs as a result of ingestion of contaminated foodstuffs, or environmental contact and accidental ingestion of contaminated material (Grif *et al.*, 2005), and cattle are the major animal reservoir of this

organism. Despite the potentially fatal outcomes of infection in humans, carriage in cattle appears to be asymptomatic. Serotype O157:H7 is the most common isolate in the USA, Canada, UK and Japan (Nataro & Kaper 1998).

Flagella are a prominent surface feature of many bacteria and are responsible for motility, with the major structural protein, known as flagellin, often being highly antigenic. The H7 flagellin protein can be isolated from *E. coli* O157:H7, and has been demonstrated to be immunogenic in experimentally infected rabbits and in humans previously infected with the organism (Sherman *et al.*, 1988). The N- and C-terminal regions are highly conserved and form the basis of an interaction with the mammalian pattern recognition receptor (PRR) Toll-like receptor (TLR) 5 (Hayashi *et al.*, 2001). A second, intracellular PRR, Ipaf, has also been found, and recognizes and initiates a response to cytosolic flagellin injected by, or present on, invasive bacteria such as *Salmonella* (Franchi *et al.*, 2006; Miao *et al.*, 2006). In the attaching–effacing *E. coli*

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Abbreviations: BCA, bicinchoninic acid; EGFR, epidermal growth factor receptor; EPEC, enteropathogenic *E. coli*; ERK, extracellular signal-regulated kinase; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; p-EGFR, phosphorylated EGFR; p-ERK, phosphorylated ERK; PKD, phosphokinase D; p-p38, phosphorylated p38; PRR, pattern recognition receptor; Stx, Shiga-like toxin; TLR, Toll-like receptor; WT, wild-type.

O157:H7, flagellin has been found to be more important than the carriage of the Stx-producing genes for the elicitation of pro-inflammatory responses in human intestinal epithelial cells (Berin *et al.*, 2002; Miyamoto *et al.*, 2006).

The flagella of enteropathogenic and enterohaemorrhagic strains of *E. coli* are also thought to be important in the colonization of the intestine (Best *et al.*, 2005), have been demonstrated to bind mucins and bovine mucus (Erdem *et al.*, 2007), and have also been shown to be an important factor for the initial binding to intestinal epithelial cells (Girón *et al.*, 2002; Mahajan *et al.*, 2009). The ability of *E. coli* O157:H7 to temporally coordinate the expression of flagellin with that of the type III secretion system is also thought to be of key importance (Iyoda *et al.*, 2006; Dobbin *et al.*, 2006; Mahajan *et al.*, 2009). Therefore, flagellin plays significant roles in pathogenesis, colonization and disease, and is important to both the pathogen response and the host immune response to infection. Investigation of the innate epithelial cell signalling responses to H7 flagellin further improves our comprehension of the means by which these surface structures modulate host responses and contribute to disease caused by *E. coli* O157:H7 and other flagellate pathogens.

Given the importance of flagella as a bacterial moiety in terms of their contribution to cell signalling events, there is now a wealth of information available on the consequences of *in vitro* or *in vivo* challenge with flagellate bacteria or purified flagellin protein.

Experimental evidence is accumulating to suggest that the activation of TLRs of epithelial cells can result in both pro-inflammatory and protective anti-apoptotic cell responses. This protective function has been described following the activation of TLR2 and TLR5, resulting in alterations to tight junctions and NF- κ B (Cario *et al.*, 2004; Vijay-Kumar *et al.*, 2006). Recent experimental data have also demonstrated that TLR2 and TLR5 stimulation leads to activation of the epidermal growth factor receptor (EGFR) in lung epithelial cells (Shaykhiev *et al.*, 2008), which is an important response in epithelial repair after wounding (Egan *et al.*, 2003), together with activation of transcription factor NF- κ B.

Our observations using the broad-spectrum tyrosine kinase inhibitor genistein suggested that tyrosine kinase signalling events play an important role in the epithelial cell interleukin-8 (IL-8) response to *E. coli* O157:H7 and other flagellate pathogens. As a major epithelial tyrosine kinase receptor involved, we examined the contribution of EGFR to the intestinal epithelial cell signalling response to *E. coli* O157:H7 flagellin.

METHODS

Culture of Caco-2 cells. Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) and were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented

with 10% fetal calf serum (Cambrex), 10 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ in a humid 5% CO₂ atmosphere at 37 °C. Three-day post-confluent cell monolayers were washed and serum-starved 12 h prior to experiments in order to reduce the background-level activation of cell signalling pathways. Inhibitor AG1478 (Sigma-Aldrich) was used at a concentration of 1 µM and added 1 h prior to challenge with bacteria or bacterial-derived flagellin (H7).

Bacterial challenge in pulse-chase experiments. *E. coli* O157:H7 strains NCTC12900 (wild-type; WT) and DM4 (Δ *fliC* mutant) were gifted by Martin Woodward (Veterinary Laboratories Agency, Weybridge, UK). NCTC12900 is a naturally occurring Stx-negative strain of *E. coli* O157:H7, and the DM4 strain was generated using insertional inactivation of the *fliC* gene (Allen-Vercos *et al.*, 1997; La Ragione *et al.*, 2000). Bacterial stocks were stored at -80 °C on Cryobeads (Protect) long-term. Prior to experimental challenge of epithelial cells, a bead of each stock culture was defrosted and streaked onto Luria-Bertani (LB) agar plates. The maintenance of the DM4 mutant requires the addition of streptomycin at 25 µg ml⁻¹. Individual colonies were used to inoculate overnight cultures in 10 ml serum-free cell culture medium containing the appropriate antibiotic at 37 °C. These were subcultured 1:20 the following day into fresh pre-warmed cell culture medium and incubated to mid-exponential phase (OD₆₀₀ 0.6), equating to a bacterial cell density of approximately 1 × 10⁸ c.f.u. ml⁻¹. Cultures were then diluted 1:10 in 1 ml pre-warmed cell culture medium in confluent 12-well plates (Cambrex) to give a bacterial cell density of 1 × 10⁷ c.f.u. ml⁻¹, equivalent to an m.o.i. of about 10 bacteria per epithelial cell. Cells were then challenged with the appropriate bacterial strain for 1 h at 37 °C in a humid 5% CO₂ atmosphere before the medium and any bacteria unattached to cell monolayers were aspirated and replaced with fresh pre-warmed serum-free cell culture medium containing gentamicin at 50 µg ml⁻¹. Cells were then incubated for a further 18 h and supernatants harvested for analysis.

Isolation and purification of H7 flagellin from *E. coli* O157:H7. H7 flagellin protein was derived from *E. coli* O157:H7 MCI24, originally isolated from a human patient in Washington State, USA (Ostroff *et al.*, 1990), a strain which does not possess the Stx2 bacteriophage, encoding verotoxin. Flagella were isolated using acidic dissociation and neutral pH reassociation, followed by ammonium sulphate precipitation as described by Ibrahim *et al.* (1985). Purity was assessed by SDS-PAGE and verification was obtained through Western blotting using rabbit polyclonal anti-H7 antibody (MAST ASSURE), as described previously (McNeilly *et al.*, 2008). Flagellin preparations used in these experiments were also assessed by MALDI-TOF MS, and were from the same batch as that used by McNeilly *et al.* (2010).

Bicinchoninic acid (BCA) protein estimation assay. The colorimetric method used to determine protein concentrations of flagellin preparations and cell lysates for Western blots was developed by Smith *et al.* (1985). A non-coated flat-bottomed 96-well plate (Fisher) was prepared by adding standards in triplicate using a dilution series of a stock solution of BSA (1.0 mg ml⁻¹ in sterile distilled water) to provide standard concentrations of 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1 mg ml⁻¹, and a blank control. Ten microlitre volumes of neat and diluted flagellin preparations or cell lysates were also loaded onto the plate in triplicate. Colour was developed using a combination of buffered BCA reagents and CuSO₄ solution (Smith *et al.*, 1985) freshly prepared prior to analysis. Plates were incubated at 60 °C for 1 h to develop, and absorbance was read at 570 nm on an ELx808IU Ultra plate reader (Endosave).

Antibodies and Western blot analysis. Goat polyclonal anti-human EGFR antibody and rabbit polyclonal anti-phosphorylated

EGFR (p-EGFR) (pTyr1173) were purchased from R&D Systems. Rabbit polyclonal anti-phosphorylated extracellular signal-regulated kinase (p-ERK) antibody was purchased from Sigma, whilst rabbit polyclonal anti-phosphokinase D (PKD) (pSer916) was purchased from Cell Signaling Technology and diluted 1:500. Finally, rabbit polyclonal anti-ERK antibody was purchased from Santa Cruz Biotechnology. Western blots were visualized using Pierce chemiluminescence reagents (Thermo Scientific) and developed against photographic film (Kodak). Densitometry was calculated using Scion Image software (Scion Corp.).

ELISA. Human IL-8 was detected and quantified using a DuoSet sandwich ELISA kit purchased from R&D Systems.

Statistical analysis. All experiments were performed at least three times and a Student's two-tailed *t* test was carried out on all data, with a confidence interval of 95 % or above being considered significant.

RESULTS

Inhibition of flagellin-dependent IL-8 release in Caco-2 cells by the EGFR-specific inhibitor AG1478

Three-day post-confluent Caco-2 cells were incubated with *E. coli* O157:H7 (*stx*[−]) parent strain NCTC12900 (WT) and its DM4 Δ *fliC* mutant, with and without the addition of the EGFR-specific inhibitor AG1478 (Fig. 1). This compound was developed from natural isoflavone inhibitors of tyrosine phosphorylation and is one of a group of compounds termed tyrphostins (Levitzki, 1992). It is a competitive and selective inhibitor of the EGFR ATP-binding

Fig. 1. Effect of AG1478 on WT *E. coli* O157:H7- and H7-deficient mutant-induced IL-8 release in Caco-2 cells. Caco-2 cells were incubated with cell culture medium alone (Control), 1.0 μ M AG1478 (AG), WT *E. coli* O157:H7, Δ *fliC* mutant of *E. coli* O157:H7, WT *E. coli* in the presence of 1.0 μ M AG1478, and the Δ *fliC* mutant of *E. coli* O157:H7 in the presence of 1.0 μ M AG1478 for 1 h prior to replacement of inocula with medium containing gentamicin at 50.0 μ g ml^{−1} and further incubation to 18 h. IL-8 release was measured by ELISA and values represent mean $\pm$ SEM of three independent experiments. ****P* < 0.0001 as compared with the control.

site and prevents phosphorylation of the receptor (Levitzki & Gazit, 1995). Live bacteria were incubated for a period of 1 h before replacement with fresh cell culture medium containing gentamicin. The antibiotics were introduced to inactivate the remaining bacteria and prevent the loss of epithelial cell viability due to exposure to live bacteria over the duration of the experiment. The supernatants were then collected after 18 h incubation. This limited experimental exposure to the viable bacteria also maximized IL-8 induction, as we observed that viable NCTC12900 cells were poor inducers of IL-8 at 6 h. IL-8 secretion by challenged Caco-2 cells was shown to be dependent upon the presence of flagella (Fig. 1), as the Δ *fliC* mutant did not elicit significant levels of IL-8 compared with controls, whereas WT challenges did. We also observed a significant reduction in the IL-8 response to WT challenge when Caco-2 cells were incubated with the tyrphostin AG1478 at a concentration known to be specific for the inhibition of EGFR (Lipson *et al.*, 1998).

The absence of TLR4 in the transformed Caco-2 epithelial cell line reflects *in vivo* findings for TLR4 expression in human intestinal epithelium, which is hyporesponsive to LPS (Naik *et al.*, 2001) and therefore rules out the contribution of bacterial LPS to IL-8 signalling. The lack of a functioning TLR4 and IL-8 response to LPS has made this cell line a popular choice for examining cell signalling responses to bacteria (Kim *et al.*, 2004). Earlier studies examining the role of flagella in enteropathogenic *E. coli* (EPEC) strain E2348/69 have examined WT and isogenic aflagellate mutants as well as purified flagellin, and found flagellin to be responsible for IL-8 induction in epithelial cells (Zhou *et al.*, 2003); however, the involvement of EGFR in this response has so far not been reported. Despite this, EGFR phosphorylation in intestinal epithelial cells has been demonstrated in response to EPEC (Roxas *et al.*, 2007), and we observed EGFR phosphorylation in response to WT *E. coli* O157:H7 only. Since the presence of flagella appeared to be the major determinant of the epithelial IL-8 response to *E. coli* O157:H7, we then examined epithelial responses to purified H7 flagellin protein.

Inhibition of the IL-8 response to purified H7 flagellin protein by the EGFR-specific inhibitor AG1478

H7 flagellin protein was obtained from *E. coli* strain ZAP198 and purified by pH-based dissociation–reassociation of flagella and ammonium sulphate precipitation (McNeilly *et al.*, 2008; Ibrahim *et al.*, 1985). Purified flagellin was shown to be free of major contamination with other proteins by SDS-PAGE, and the identity of the protein was confirmed by Western blotting with rabbit polyclonal anti-H7 antibody as well as by MS (data not shown); this protein was used in previously published work (McNeilly *et al.*, 2010).

Fig. 2 demonstrates the IL-8 response to a range of concentrations of H7 flagellin protein. For detection of

Fig. 2. Effect of AG1478 on H7-induced IL-8 release from Caco-2 cells. (a) IL-8 release after 18 h by Caco-2 cells in response to challenge with various concentrations of H7 flagella. (b) Caco-2 cells were stimulated with vehicle control (Control), 200 ng H7 ml⁻¹, 1 µM AG1478 (AG) and 200 ng H7 ml⁻¹ with 1 µM AG1478 for 18 h, and IL-8 release was quantified by ELISA, as detailed in Methods. Values represent the mean ± SEM of three independent experiments. ****P*=0.001 compared with the control.

IL-8 by ELISA, Caco-2 intestinal epithelial cells were pre-incubated for 1 h with or without 1.0 µM of the EGFR-specific inhibitor AG1478 prior to challenge with 200 ng H7 flagellin ml⁻¹. The cells were then incubated for a further 18 h at 37 °C in a 5% CO₂ atmosphere before supernatants were collected for analysis. We found that incubation with 1.0 µM AG1478 significantly reduced the level of IL-8 secreted by Caco-2 cells in response to 200 ng flagellin ml⁻¹. The results shown in Figs 1 and 2 confirm that flagellin is the most important moiety for eliciting an IL-8 response from Caco-2 epithelial cells, and therefore the response to flagellin as a ligand became the focus of our investigation. Fig. 3 demonstrates a rapid phosphorylation of EGFR in response to flagellin. Caco-2 cells were challenged with flagellin or EGF with or without the inhibitor AG1478 for 5 min, and were then lysed in cell lysis buffer containing protease and phosphatase inhibitors.

Total protein was estimated by BCA assay, and SDS-PAGE gels were equally loaded with 10 µg total protein per treatment per well for immunoblot examination of EGFR phosphorylation. The detection of total EGFR protein also acted as loading control. Immunoblots indicated a specific inhibition of EGFR by AG1478, demonstrated by a reduced phosphorylation of the receptor from 2.8 ± 0.9 - and 1.1 ± 0.6 -fold to 0.5 ± 0.3 - and 0.3 ± 0.2 -fold stimulation in response to treatment with flagella and EGF, respectively.

H7 flagellin protein rapidly transactivates EGFR in Caco-2 cells

To confirm the involvement of EGFR in H7 flagellin-dependent IL-8 release in Caco-2 cells we examined the phosphorylation of the receptor in response to challenge with purified flagellin over a period of time. Fig. 4 demonstrates EGFR phosphorylation in response to challenge with flagella, with a peak at 5 min (10.61 ± 4.6 -fold stimulation), which had returned to basal by 18 h, the longest time point studied. Examination of mitogen-activated protein kinase (MAPK) signalling events, which have been associated with EGFR activation (Tjabringa *et al.*, 2003), demonstrated that ERK phosphorylation was slower in onset than EGFR phosphorylation, reaching a maximum at 3 h of 321.7 ± 58 -fold stimulation, and returning to basal levels by 18 h. However, the kinetics of p38 phosphorylation were more rapid and more transient, reaching a maximum at 30 min, at 28.6 ± 1.1 -fold stimulation, and returning to basal levels by 3 h. Both ERK1/2 and p38 MAPK activation have been described following challenge with EHEC-derived H7 flagella (Berin *et al.*, 2002), and specific inhibition of these and of the transcription factor NF-κB has been shown to abrogate IL-8 responses.

Fig. 5 also shows that flagella- and EGF-induced ERK1/2 phosphorylation was inhibited by treatment with AG1478, decreasing from 6.2 ± 2.7 - and 6.0 ± 2.5 -fold stimulation to 2.6 ± 0.4 - and 2.4 ± 1.4 -fold, respectively. Immunoblot detection of total ERK protein was used as an internal loading control. Earlier data indicate that ERK is important for the activation of NF-κB and AP-1 transcription factors in response to flagellin (Kogut *et al.*, 2008). The effect of AG1478 on the phosphorylation of PKD was not inhibitory (data not shown), although this has been demonstrated to be a flagellin-dependent signalling event (Ivison *et al.*, 2007). Therefore, this suggests that the activation of EGFR is upstream of ERK1/2 signalling in Caco-2 cell responses to flagellin.

DISCUSSION

Epithelial cell signalling in response to challenge with bacterial pathogens is complex. The role of receptor tyrosine kinases (RTKs) in determining epithelial TLR responses is only just beginning to be understood, but our evidence suggests that EGFR may be an important RTK in

Fig. 3. Effect of AG1478 on H7- and EGF-induced EGFR phosphorylation in Caco-2 cells. (a) Immunoblots for the detection of EGFR and p-EGFR (pY1173) in cell lysates of 3 day post-confluent Caco-2 cells at 5 min in response to: (1) culture medium alone, (2) 200 ng purified H7 flagellin ml^{-1} , (3) 100 ng EGF ml^{-1} , with or without 1.0 μM AG1478 (AG), resolved by 10% SDS-PAGE and blotted onto nitrocellulose. Lysates from each treatment were pooled from three monolayers of 5×10^5 cells and the image is representative of three separate experiments. (b) Levels of stimulation were quantified by scanning densitometry, and each value represents the mean $\pm$ SEM of three experiments. * $P < 0.05$, significant increase, compared with the medium control; ** $P < 0.05$, significant decrease, compared with stimulated cells.

determining intestinal epithelial cell responses to *E. coli* O157:H7 flagella.

Current understanding of epithelial cell signalling responses to flagellin focuses on the cell signalling pathways triggered by cell membrane-bound TLR5 and the intracellular PRR Ipaf. Since our studies examined the cell response to the non-invasive, attaching-effacing pathogen *E. coli* O157:H7, and the introduction of *E. coli*-derived flagellin protein to cell supernatants, we did not consider the effect of EGFR inhibition on the function of Ipaf (Franchi *et al.*, 2006).

The TLR5 pathway initiates cell signalling through the induction of a conformational change induced by direct association with bacterial flagellin. The resulting signal transduction cascade leads to the nuclear translocation of the transcription factor NF- κ B, which in turn alters the transcriptional profile of the cell and has been well-described (Ballard *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990).

The involvement of protein tyrosine kinase adaptors and receptors downstream of TLR signals is a recent finding, and these proteins may be important to intestinal epithelial TLR5 signalling responses to bacterial flagellin. Indeed, TLR5 itself has been shown to be phosphorylated at tyrosine residue 798 in the Toll-interleukin-1 (TIR) domain, which is required for inflammatory responses in HEK293T cells (Iverson *et al.*, 2007). It has also been known for some time that signalling via TLR4 requires the phosphorylation of BTK/Bruton's tyrosine kinase (Jefferies *et al.*, 2003). In the present investigation, we examined the importance of protein tyrosine kinase involvement in

Caco-2 intestinal epithelial cells, after stimulation with *E. coli* O157:H7 and purified H7 flagellin protein, and report that phosphorylation of the receptor tyrosine kinase EGFR in response to *E. coli* O157:H7 flagellin is rapid, occurring within 5 min. Since H7 flagellin has been demonstrated to upregulate the release of the pro-inflammatory chemokine IL-8 (Berin *et al.*, 2002), we also investigated the importance of the activation of EGFR in flagellin/TLR5 cell signalling by the release of IL-8 from Caco-2 cells.

EGFR is a major tyrosine kinase receptor, and one of a family of proteins often designated ErbB1 from their discovery as human homologues of the viral oncogenes that cause avian erythroblastosis (Jansson *et al.*, 1983). It is central to many aspects of cell biology, including cell proliferation, differentiation and inflammation. In the colon, the expression of EGFR has primarily been demonstrated in the crypt cells of normal tissue, and also in epithelial-derived cancer cell lines (Rajagopal *et al.*, 1995), including Caco-2 cells (Tong *et al.*, 1998).

Fig. 1 confirms that flagella are a major bacterial determinant for IL-8 secretion in *E. coli* O157:H7-challenged cells, but it also demonstrates that a specific inhibitor of EGFR, the tyrphostin AG1478 (Gazit *et al.*, 1996), significantly reduces the level of IL-8 released in response to flagellate *E. coli* O157:H7. When we examined the response to H7 flagella alone we found that treatment with the inhibitor prevented both IL-8 secretion and EGFR phosphorylation at residue Tyr1173 (Fig. 3), which is responsible for binding Shc (Src homology 2 and collagen domain protein), one of a number of adaptor proteins that mediate signalling from EGFR and which have been found

Fig. 4. Dose-response, H7-induced activation of EGFR, ERK and p38 in Caco-2 cells. (a) Caco-2 cells were exposed to H7 for the times indicated. Samples were assayed for EGFR, p-EGFR, p-ERK and phosphorylated p38 (p-p38), as outlined in Methods. Each Western blot represents at least three experiments. The levels of stimulation were quantified by scanning densitometry (b), and each value represents the mean $\pm$ SEM of three experiments.

Fig. 5. Effect of AG1478 on H7- and EGF-induced ERK phosphorylation in Caco-2 cells. (a) Caco-2 cells were stimulated with either H7 or EGF for 30 min in the presence or absence of 1.0 μ M AG1478. Samples were assayed for ERK2 and p-ERK as outlined in Methods. Each Western blot represents at least three separate experiments. (b) The levels of stimulation were quantified by scanning densitometry, and each value represents the mean $\pm$ SEM of three experiments. * $P < 0.05$, significant increase, compared with medium control; ** $P < 0.05$, significant decrease, compared with stimulated cells. (c) Immunoblot of p-p38 of cell lysates treated as above with 200 ng H7 ml⁻¹, 1.0 μ M AG1478 (AG) and a combination of the two.

to be dominant but not essential for activation of the MAPKs ERK1/2 and p38 (Gong & Zhao, 2003). The kinetics of EGFR phosphorylation events have been examined in Caco-2 cells in response to other non-microbial stimuli, such as bile acids (Raimondi *et al.*, 2008), and the early spike in EGFR tyrosine phosphorylation in response to EGF has been described elsewhere (Zhang *et al.*, 2005). The peak in phosphorylation of EGFR demonstrated in response to *E. coli* O157:H7 flagellin waned by 120 min and returned to basal levels by 18 h. This mirrors the phosphorylation of ERK1/2 and p38 shown in Fig. 4, which also returned to basal levels after

18 h, probably reflecting the inter-relation of these signalling events.

EGFR phosphorylation in response to *E. coli* O157:H7-derived flagellin may be a conserved response to all bacterial flagellin proteins, and may explain earlier observations that have demonstrated EGFR phosphorylation in epithelial cell lines in response to challenge with a number of flagellate micro-organisms. The sterile-filtered culture supernatant of flagellate EPEC strain E2348/69 has been shown to result in phosphorylation of EGFR in Caco-2 cells (Roxas *et al.*, 2007). Those authors suggested that a soluble secreted component was responsible, and considered TLR ligands and flagellin as possible candidates; however, they state that their early indications did not suggest that flagellin was responsible. EGFR phosphorylation was also demonstrated in Henle-407 cells in response to the flagellate intestinal pathogen *Salmonella typhimurium* (Galán *et al.*, 1992), although the phenomenon of EGFR in cell signalling and *S. typhimurium*-induced chemokine release was not explored at the time. Recent studies have supported a role for metalloprotease EGFR ligand-shedding and receptor activation in response to many bacterial stimuli, including *Salmonella*-derived flagellin (Boots *et al.*, 2009).

Our results indicated an important role for tyrosine kinases in Caco-2 cell inflammatory responses to flagellate *E. coli* O157:H7 and purified flagellin. EGFR has also been implicated in other TLR signalling pathways, specifically that involving TLR4. LPS-induced activation of TLR4 enhances epithelial wound healing (Koff *et al.*, 2006) in a process requiring activation of EGFR. Similarly, transactivation of EGFR has also been demonstrated in *Helicobacter pylori*-infected gastric epithelial cells induced by the interaction of the secreted bacterial protein HP0175 with TLR4 (Basu *et al.*, 2008). Recent studies have also shown that LPS-induced phosphorylation of EGFR in biliary epithelial cells appears to follow similar kinetics to our results in Caco-2 cells using flagellin (Finzi *et al.*, 2009). Since we can rule out TLR4-mediated signalling events, it is tempting to speculate that transactivation of EGFR through flagellin engagement with TLR5 may have a role in flagellin/TLR5-dependent enhanced epithelial integrity (Gao *et al.*, 2010; Vijay-Kumar *et al.*, 2006, 2007a, b, 2008).

Multiple bacterial ligands and TLR receptors have now been demonstrated to activate EGFR; hence, it may be the case that epithelial cell systems for threat detection, cell repair, mucin production and mitogenesis are linked, and that this is a logical connection that provides a mechanism for minimizing damage to epithelial layers by microbial pathogens. We demonstrated EGFR-dependent activation of ERK1/2 in response to flagellin (Fig. 5). This provides some clues as to the downstream signalling events that are reliant on EGFR phosphorylation. It may be that the EGFR-ERK1/2 response is part of a necessary parallel pathway for the transcriptional activity of downstream transcription factors such as NF- κ B. There are likely to be a

number of other factors that affect the transcription of IL-8 via NF- κ B and AP-1, and these may also involve EGFR phosphorylation. The MAPK p38 has also been implicated in the regulation of transcriptional activity through the acetylation of the p65 subunit of NF- κ B (Saha *et al.*, 2007). Compared with that of ERK1/2, p38 activity has been found to be flagellin-specific in cell responses to flagellate and aflagellate EPEC (Khan *et al.*, 2008), and its importance to IL-8 production through studies using specific inhibitors has been known for some time, including the post-transcriptional control of IL-8 release in response to flagellin (Yu *et al.*, 2003). Therefore, further work delineating the downstream signalling events dependent upon EGFR phosphorylation, including the activation of MAPK, will be of great importance in determining the specific contribution of this major tyrosine kinase.

In conclusion, we have shown that EGFR phosphorylation is an essential requirement for the release of IL-8 in Caco-2 cells in response to challenge with *E. coli* O157:H7 flagellin. Hence, we propose that parallel signalling pathways lead to the production of IL-8 in response to *E. coli* O157:H7 flagellin in intestinal epithelial cells. The classical flagellin/TLR5 pathway leads to the nuclear localization of NF- κ B following conformational change of TLR5, recruitment of adaptor molecules, and activation of the IKK complex and degradation of I κ B. The proposed parallel pathway involves activation of EGFR, although whether this is TLR5-dependent or -independent, or whether other intermediaries (for example as proposed by Koff *et al.*, 2008; Kawahara *et al.*, 2004 using a lung epithelial cell challenge system) are involved remains to be resolved in further studies. We have demonstrated that ERK1/2 phosphorylation occurs subsequently to the EGFR signalling event, and propose that this and other unknown signalling intermediates are necessary for the release of IL-8 in response to flagellin, possibly through allowing or enhancing the transcriptional activity of NF- κ B or other transcription factors.

We have shown that flagellin activates intestinal epithelial cells not only via TLR5 but also via EGFR, another cell surface receptor. Activation of parallel NF- κ B and MAPK cascades is seemingly required for IL-8 induction, and it must be presumed that other consequences also arise. The interplay between pathogens and host epithelial cells is complex, even for 'simple' ligands, and the specific events leading to dependency on parallel signalling events and the consequences of those events require further elucidation to clarify the roles of bacterial determinants such as flagella in the outcome of infection.

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