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The focal complex of epithelial cells provides a signaling platform for interleukin-8 induction in response to bacterial pathogens

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Summary

Bacterial pathogens can induce an inflammatory response from epithelial tissues due to secretion of the pro-inflammatory chemokine interleukin-8 (IL-8). Many bacterial pathogens manipulate components of the focal complex (FC) to induce signaling events in host cells. We examined the interaction of several bacterial pathogens with host cells, including *Campylobacter jejuni*, to determine if the FC is required for induction of chemokine signaling in response to bacterial pathogens. Our data indicate that secretion of IL-8 is triggered by *C. jejuni*, *Helicobacter pylori*, and *Salmonella enterica* serovar Typhimurium in response to engagement of β_1 integrins. Additionally, we found that the secretion of IL-8 from *C. jejuni* infected epithelial cells requires FAK, Src, and paxillin, which in turn are necessary for Erk 1/2 recruitment and activation. Targeting the FC component paxillin with siRNA prevented IL-8 secretion from cells infected with several bacterial pathogens, including *C. jejuni*, *Helicobacter pylori*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Vibrio parahaemolyticus*. Our findings indicate that maximal IL-8 secretion from epithelial cells in response to bacterial infection is dependent on the FC. Based on the commonality of the host response to bacterial pathogens, we propose that the FC is a signaling platform for an epithelial cell response to pathogenic organisms.

Keywords

Bacteria-host cell interactions; cell signaling; integrin; FAK; Erk1/2; focal complex

Introduction

Interleukin-8 is a chemokine that can be secreted by epithelial cells in response to stress or injury, including contact with bacterial and viral pathogens (Hoffmann *et al.*, 2002). IL-8 diffuses locally upon secretion, acting as a chemo-attractant recruiting immune cells to the site of injury (Baggiolini *et al.*, 1997). Thus, IL-8 plays a key role in initiating the inflammatory response against bacterial pathogens. While expression of IL-8 is partially controlled through histone modification and mRNA stabilization, both NF- κ B and AP-1

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activation are required for IL-8 gene CXCL8 transcription. NF- κ B activation is often associated with activation of the Toll-like receptors that recognize bacterial components such as lipopolysaccharide (LPS) and flagellin (Hoffmann *et al.*, 2002). AP-1 is localized to the nucleus where it is activated by MAP kinases. Pathogenic bacteria produce unique proteins that activate the MAP kinases via a variety of mechanisms (Mitsuno *et al.*, 2001, Watson *et al.*, 2005, Matlawska-Wasowska *et al.*, 2010, Samuelson *et al.*, 2013).

Mitogen activated protein kinases (MAP kinases) are signaling proteins that are important in many cellular processes, including regulation of IL-8 expression (Schaeffer *et al.*, 1999, Hoffmann *et al.*, 2002). MAP kinase activation requires the activation of a MAP kinase-kinase [or MAP triple kinase (*e.g.*, Raf)]. The activated triple kinase then phosphorylates its target MAP kinase-kinase (*e.g.*, MEK 1/2), leading to phosphorylation of the MAP kinase (*e.g.*, Erk 1/2) (Schaeffer *et al.*, 1999). A scaffold protein is required for MAP kinase activation and the prototypical MAP kinase scaffold is KSR (kinase suppressor of Ras), but other proteins, including components of the focal complex (FC), may function as MAP kinase scaffolds (Morrison, 2001, Roy *et al.*, 2002, Ishibe *et al.*, 2003).

Integrins are surface exposed heterodimeric cell receptors, which are common to many types of metazoan (animal) life, consisting of one α and one β subunit (Giancotti *et al.*, 1999). The integrins function both as anchoring points that interact with specific extracellular matrix (ECM) components, as well as intracellular signaling proteins (Schwartz *et al.*, 2002). The association of bacteria with the extracellular matrix of host cells or an associated integrin receptor is a common theme shared among pathogenic bacteria. Cellular association with the ECM results in recruitment and activation of focal complex components. The proteins of the FC are host cell structural and signaling molecules that contribute to cell motility and spreading by coordinating broad signaling cascades (Wozniak *et al.*, 2004). Bacteria have evolved interactions with these highly conserved integrins and their associated ECM proteins, particularly fibronectin (Henderson *et al.*, 2011).

Three proteins recruited to integrins, as part of the FC, are focal adhesion kinase (FAK), paxillin, and epidermal growth factor receptor (EGFR) (Giancotti *et al.*, 1999, Zouq *et al.*, 2009). The cytoplasmic tails of activated β_1 integrins interact with FAK leading to its auto-phosphorylation and the association of the non-receptor tyrosine kinase Src (Chen *et al.*, 1996, Orr *et al.*, 2004, Parsons *et al.*, 2004, Hall *et al.*, 2011). Following association, Src phosphorylates multiple tyrosine residues on FAK, leading to an increase in FAK kinase activity (Orr *et al.*, 2004). The fully activated FAK-Src complex then phosphorylates other associated proteins, including paxillin (Schaller *et al.*, 1995, Orr *et al.*, 2004, Hall *et al.*, 2011). Of particular interest FAK-Src phosphorylation of paxillin can result in the recruitment of the MAP kinase Erk 1/2 (Schaller *et al.*, 1995, Ishibe *et al.*, 2003). Similarly, the EGFR can be activated through integrin activation utilizing the scaffold protein p130Cas as well as Src (Cabodi *et al.*, 2004). Active Erk 1/2 translocates to the nucleus where it contributes to the transcription of IL-8 through activation of AP-1 (Hoffmann *et al.*, 2002).

Pathogens often induce host inflammatory responses. For example, *Campylobacter jejuni*, which is a leading bacterial cause of gastroenteritis worldwide, causes an intense inflammatory response and diarrhea with blood and leukocytes in the stools. The latter is

characteristic of a pathogen that invades the intestinal epithelium as cell invasion results in epithelial barrier disruption. *C. jejuni* possesses two fibronectin binding proteins termed CadF and FlpA (Konkel *et al.*, 1997, Flanagan *et al.*, 2009). Invasion of the cells lining the intestinal track is requires the binding of the bacteria to the host cells via the adhesins followed by the delivery of effector proteins termed the *Campylobacter* invasion antigens (Cia proteins) to host cells (Konkel *et al.*, 2010). The Cia proteins are synthesized when *C. jejuni* are co-cultured with epithelial cells (Konkel *et al.*, 1993). We have recently demonstrated that the CiaD effector is, in part, responsible for the activation of Erk 1/2, though the cellular components of this activation remain unknown (Samuelson *et al.*, 2013). We hypothesize that the *C. jejuni* adhesins and secreted proteins act cooperatively to subvert components of the host cell focal adhesion complex to facilitate internalization, and that these virulence proteins also contribute to IL-8 secretion.

The recurring theme of bacterial interaction with components of the FC system, including the ECM components and the integrins, led us to hypothesize that these proteins are serving a critical role in bacterial pathogenesis. The goal of this work was to identify membrane associated and cytosolic signaling components required for Erk 1/2 activation as a prerequisite for IL-8 secretion in response to bacterial pathogens. We hypothesized that bacterial activation of the FC directly contributes to the activation of the MAP kinase signaling pathway in epithelial cells. We demonstrate that β_1 integrins, FAK, Src, and paxillin are required for Erk 1/2 activation and IL-8 secretion in response to *C. jejuni*. We further show that several bacterial pathogens require paxillin for the generation of an IL-8 response, including *Helicobacter pylori*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Staphylococcus aureus*. This work suggests an expanded role for the FC in the detection of pathogenic bacteria.

Results

β_1 integrin is required for IL-8 secretion from multiple pathogens

We hypothesized that host epithelial cells have evolved the capacity to detect pathogenic bacteria via their interactions with the extracellular matrix (ECM). Given the prevalence of fibronectin binding proteins among these bacteria, we hypothesized that pathogen detection requires β_1 integrin receptors. To test this hypothesis, we treated INT 407 human epithelial cells with siRNA specific to β_1 integrin or a scrambled siRNA control, infected the cells with various pathogenic bacteria, and used an ELISA to measure the level of IL-8 in the supernatants. The cells were infected with *Campylobacter jejuni*, *Helicobacter pylori*, and *Salmonella enterica* Serovar Typhimurium. Uninfected cells served as a negative control. Knockdown of the β_1 integrin with siRNA lead to a significant decrease in the level of IL-8 secreted following infection with all three organisms (Fig. 1A–C). The knockdown of β_1 integrin in the siRNA treated cells was confirmed by immunoblot analysis (Fig. 1D). Based on these data, we concluded that the β_1 integrin is required for a maximal IL-8 response.

C. jejuni must be metabolically active to promote IL-8 secretion from epithelial cells

We chose to use *C. jejuni* to dissect the role of the IL-8 response, as this pathogen activates a robust inflammatory response. To determine the role of the bacteria in inducing IL-8

secretion from epithelial cells, experiments were initially performed to determine if the bacteria must be metabolically active. IL-8 was not detected in the supernatants when *C. jejuni* was incubated with host cells for 24 hr in the presence of the bacterial protein synthesis inhibitor chloramphenicol (Fig. S1), indicating that the bacteria must be metabolically active to generate a host response. This result is consistent with previous findings (Samuelson *et al.*, 2013), and demonstrates that constitutively synthesized bacterial components are not sufficient to promote IL-8 secretion.

***C. jejuni* activates the Raf/MEK/Erk MAPK signaling pathway**

Erk 1/2 is highly activated in response to *C. jejuni* at every time point over a course of a 24 hr infection period (time points: 30 min, 3 hr, and 24 hr), as judged by experiments using a Map Kinase phospho-array (not shown). This finding is consistent with a previous report (Watson *et al.*, 2005). To determine if *C. jejuni* activates the entire Raf/MEK/Erk MAP kinase pathway, INT 407 cells were infected with *C. jejuni* and lysed following a 30 min incubation. The lysates were immunoblotted with phospho-specific antibodies for Raf (S-338), MEK (S-217/221), and Erk 1/2 (T-202/Y-204), with total Erk 1/2 serving as a loading control. Uninfected cells served as a negative control. Erk 1/2, MEK 1/2 and c-Raf were all activated in response to *C. jejuni* (Fig. 2A and 2B). To determine if the activation of the Raf/MEK/Erk MAP kinase pathway leads to IL-8 secretion, INT 407 cells were treated with Raf Inhibitor I and Erk 1/2-activation inhibitor PD98059. Vehicle treated INT 407 cells infected with *C. jejuni* served as a positive control and uninfected cells served as a negative control. A reduction in the level of IL-8 secreted from *C. jejuni* infected host cells was observed following treatment of the cells with Raf Inhibitor I (Fig. 2C) and Erk 1/2-activation inhibitor PD98059 (Fig. 2D). The effect of Raf inhibition was not as potent as that of the Erk 1/2 inhibition regardless of the Raf inhibitors tested (Fig. S2), suggesting that other MAP triple kinases may be acting on the pathway. Taken together, these results indicate that *C. jejuni* activates the Raf/MEK/Erk MAP kinase pathway and that the Raf-1, MEK 1/2, and Erk 1/2 components are required for maximal IL-8 secretion.

β_1 integrins are required for Erk 1/2 activation

We hypothesized that β_1 integrin activation is linked with Erk 1/2 activation and is required for IL-8 secretion. To test this hypothesis, we used siRNA to knockdown β_1 integrin expression, infected cells with *C. jejuni*, and examined the level of Erk 1/2 activation. Non-specific scrambled siRNA was used to control for the effect of transfection. Knockdown of the β_1 integrin in host cells resulted in a significant reduction in the amount of activated (phosphorylated) Erk 1/2 when compared to untreated cells (Fig. 3A). To determine if the decrease in β_1 integrin expression was effecting the level of IL-8 gene activation, transcripts were measured by RT-PCR analysis. Scrambled siRNA treated cells infected with *C. jejuni* served as a positive control and uninfected cells served as a negative control. INT 407 cells treated with β_1 integrin siRNA were significantly reduced in IL-8 mRNA levels as compared to controls treated with nonspecific siRNA (Fig. 3B). This finding is consistent with our hypothesis, indicating that the β_1 integrin is necessary for Erk 1/2 activation and IL-8 gene expression.

Src is required for IL-8 secretion

Several proteins are known to associate with the clustered β_1 integrins, including the EGFR, the scaffold protein p130Cas, and the tyrosine kinase Src (Cabodi *et al.*, 2004). Importantly, the EGFR and Src kinase are both known to be required for *C. jejuni* invasion of host cells (Eucker *et al.*, 2012). Therefore, we performed studies to determine if the secretion of IL-8 from *C. jejuni* infected epithelial cells involves EGFR, p130Cas, and Src (Fig. 4).

To test the contribution of the EGFR in IL-8 secretion, INT 407 cells were treated with the EGFR inhibitor erlotinib and infected with *C. jejuni*. Uninfected cells served as a negative control. We measured the amount of IL-8 secreted from infected cells and found that inhibition of the EGFR did not significantly reduce IL-8 secretion (Fig. 4A). Because activated EGFR can lead to Erk 1/2 activation (Roberts *et al.*, 2007), we also examined if Erk 1/2 was activated in response to *C. jejuni* when EGFR activation was prevented by erlotinib. Inhibition of EGFR activation with erlotinib did not prevent Erk 1/2 activation following *C. jejuni* infection (Fig. 4B). This finding indicates that Erk 1/2 is activated independent of EGFR. We also infected INT 407 cells with the *C. jejuni cadF flpA* double mutant, as this isolate does not activate the EGFR (Eucker *et al.*, 2012). No difference was observed in the level of IL-8 secretion from INT 407 cells infected with the *C. jejuni cadF flpA* double mutant when compared to the wild-type strain (Fig. S3). This supports the finding that the EGFR is not contributing to IL-8 secretion.

To determine the role of p130Cas in IL-8 secretion, INT 407 cells were treated with siRNA to knockdown p130Cas expression and infected with *C. jejuni*. Uninfected cells served as a negative control. The level of IL-8 in supernatants was measured by ELISA. The level of IL-8 secreted from *C. jejuni* infected INT 407 cells treated with p130Cas siRNA was not different from *C. jejuni* infected cells treated with scrambled siRNA (Fig. 4C). This result suggests that p130Cas is not involved in triggering IL-8 secretion.

To determine the role of Src in IL-8 secretion, INT 407 cells were treated with Src kinase inhibitor PP2 and infected with *C. jejuni*. Uninfected cells served as a negative control. In contrast to the EGFR and p130Cas, treatment of INT 407 cells with PP2 significantly reduced the amount of secreted IL-8 following *C. jejuni* infection (Fig. 4A). We then probed lysates of *C. jejuni* infected INT 407 cells treated with PP2 for Erk 1/2 activation. We found that Src inhibition reduced the level of Erk 1/2 activation following *C. jejuni* infection (Fig. 4C). Taken together, these data suggest that Erk 1/2 activation is independent of the EGFR and p130Cas, but indicate that Src is playing a role in Erk 1/2 activation and IL-8 secretion.

Rac1 activation and host cell invasion are not required for IL-8 secretion

We have shown previously that the *C. jejuni* CiaC effector protein is delivered to host cells and is required for cell invasion. We further reported that a *C. jejuni ciaC* mutant is deficient in the activation of the Rho-GTPase Rac1. In agreement with the latter finding, knockdown of the Rac1 guanine exchange factor Dock180 reduced the internalization of the *C. jejuni* wild-type strain to that of the *ciaC* mutant (Eucker *et al.*, 2012). Here we found that infection of INT 407 cells with the *C. jejuni ciaC* mutant, which is deficient in host cell invasion and Rac1 activation, triggered IL-8 secretion to the same extent as cells infected

with a *C. jejuni* wild-type strain (Fig. S4A and B). We also found that a knockdown of Dock180 expression using siRNA did not result in a reduction in IL-8 secretion (Fig. S4C). These data indicate that host cell invasion and Rac1 activation are not required for an IL-8 response to *C. jejuni*.

Focal adhesion kinase (FAK) is critical for IL-8 secretion in response to *C. jejuni*

FAK is a non-receptor tyrosine kinase recruited to activated β_1 integrin receptors (Mitra *et al.*, 2006). Following association with the β_1 integrin, FAK autophosphorylates Y-397, resulting in the recruitment and activation of Src (Mitra *et al.*, 2005). Src activation leads to the subsequent phosphorylation of FAK at Y-925. To reduce FAK activation and subsequent phosphorylation events, INT 407 cells were treated with TAE 226, an inhibitor of FAK Y-397 and Y-925 phosphorylation. The cells were then infected with *C. jejuni* and Erk 1/2 phosphorylation (activation) was assessed by immunoblot analysis. Vehicle treated cells infected with *C. jejuni* and untreated cells served as positive and negative controls, respectively. Treatment of cells with the FAK inhibitor significantly reduced Erk 1/2 activation following *C. jejuni* infection, as compared to vehicle treated cells (Fig. 5A). IL-8 mRNA accumulation was then measured by RT-PCR following TAE 226 treatment of host cells. Treatment with TAE 226 significantly reduced IL-8 gene expression as compared to vehicle treated cells (Fig. 5B). We also measured the level of secreted IL-8 and found that TAE 226 treatment resulted in a dose-dependent decrease in secreted IL-8 as compared to vehicle treated cells infected with *C. jejuni* (Fig. 5C). This result is in agreement with the observation that inhibition of Src with PP2 reduces IL-8 secretion from *C. jejuni* infected cells as Src is required for full activation of FAK. To ensure that the effects observed in INT 407 cells were not cell-type specific, human colonic carcinoma cells (Caco-2 cells) were treated with TAE 226 and infected with *C. jejuni*. While Caco-2 cells secreted less IL-8 than the INT 407 cells in response to *C. jejuni* infection, the result obtained with TAE 226 treatment was consistent with the two cell lines (Fig. 5D). Collectively, these data suggest that FAK is an essential component of the IL-8 signaling cascade.

Focal adhesion kinase (FAK) activates PI3 kinase

FAK signaling leads to the recruitment and activation of PI3 kinase, which leads to the recruitment of molecules linked to numerous signaling cascades that could trigger IL-8 secretion from cells. To explore the importance of FAK, we further examined the PI3 kinase response of *C. jejuni* infected cells when FAK was inhibited with TAE 226 treatment. Because the activation state of PI3 kinase is not governed by posttranslational modification and is not directly measurable, we measured activation of the downstream signaling protein Akt as an indicator of PI3 kinase activity. A significant reduction in the level of Akt activation was observed following TAE 226 treatment (Fig. S5A). This finding supports the proposal that *C. jejuni* activation of FAK leads to PI3 kinase activation.

Activation of PI3 kinase and Akt can also lead to activation of NF- κ B, another signaling protein required for transcriptional activation of IL-8 (Hoffmann *et al.*, 2002). Because Akt can activate NF- κ B, INT 407 cells were treated with TAE 226 or the PI3 kinase inhibitor LY294002, infected with *C. jejuni*, and NF- κ B activation was measured by immunoblot analysis. Treatment of cells with TAE 226 or LY294002 did not alter NF- κ B activation (Fig.

S5B), indicating that the NF- κ B pathway is not dependent on cross-talk with FAK or PI3 kinase. Noteworthy is that the inhibition of PI3 kinase by treatment of INT 407 cells with LY294002 resulted in a significant decrease in IL-8 secretion from *C. jejuni* infected INT 407 cells. This suggests that PI3 kinase is playing a role in modulating a host response (Fig S5C).

Paxillin is in a complex with the MAP kinase Erk 1/2 and is required for an IL-8 response

Paxillin, which is activated by the FAK-Src complex, leads to the recruitment of the inactive (non-phosphorylated) form of Erk 1/2 (Ishibe *et al.*, 2003, Ishibe *et al.*, 2004, Mitra *et al.*, 2005, Sen *et al.*, 2010). Because paxillin is activated in response to *C. jejuni*, we hypothesized that paxillin associates with Erk 1/2 and is necessary for secretion of IL-8 (Monteville *et al.*, 2003). To determine if *C. jejuni* infection results in an increase in Erk 1/2 association, immunoprecipitation experiments were performed (Figure 6). We found that Erk 1/2 co-precipitated with paxillin from cells infected with *C. jejuni*, while uninfected cells did not yield a paxillin-Erk 1/2 co-immunoprecipitate (Fig. 6A). To determine if paxillin is involved in Erk 1/2 activation and IL-8 secretion, knockdown of paxillin expression was performed using siRNA. Cells transfected with scrambled siRNA served as a control. Cells treated with paxillin siRNA secreted significantly less IL-8 than cells treated with control siRNA (Fig. 6B). In addition, paxillin knockdown resulted in a significant decrease in IL-8 gene expression when compared to infected cells treated with control siRNA (Fig. 6C). The level of activated Erk 1/2 was also reduced in cells treated with paxillin siRNA when compared to infected cells treated with control siRNA, as judged by immunoblot analysis (Fig. 6D). These findings indicate that paxillin is in a complex with Erk 1/2 and that paxillin is required for IL-8 secretion.

Paxillin is required for IL-8 secretion from host epithelial cells in response to multiple bacterial pathogens

In this study, we determined that the β_1 integrin, FAK, Src, and paxillin, which are all components of the focal complex, are required for maximal Erk 1/2 activation and IL-8 induction in response to infection with *C. jejuni*. Based on these findings, we hypothesized that *Helicobacter pylori* and *Salmonella enterica* Serovar Typhimurium also required paxillin for maximal activation of an IL-8 response in epithelial cells. To test this hypothesis, INT 407 cells were treated with siRNA to paxillin and the amount of IL-8 secreted from infected cells was measured (Figure 7A and B). Cells treated with scrambled siRNA served as a positive control and untreated cells served as a negative control. Knockdown of paxillin resulted in a significant reduction in the level of IL-8 secreted from cells infected with either *H. pylori* or *S. Typhimurium*. To determine if paxillin is a part of a conserved response of epithelial cells to other pathogenic bacteria, cells were also infected with *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. We chose these pathogens because they are known to induce inflammation and have been shown to interact with the FC. INT 407 cells were also incubated with *Citrobacter freundii*, which is a commensal organism, as a negative control as it should not induce an inflammatory response. Paxillin siRNA treatment of INT 407 cells was also found to significantly reduce the amount of IL-8 secreted in response to *V. parahaemolyticus*, *P. aeruginosa*, and *S.*

aureus (Fig. S6). Based on these findings, we concluded that the focal complex is a central player in epithelial cells detecting and responding to bacterial pathogens.

Discussion

The benefit of inflammation to invading bacterial pathogens is debatable. On the one side, inflammation and the resultant inundation by immune cells can be seen as detrimental to invasive pathogens. The recruited immune cells can kill the invading pathogenic bacteria and prevent dissemination into other tissues. However, some benefits to the pathogen can be derived from the effects of IL-8 induction. Macrophages recruited to infected tissues may disrupt the epithelium, allowing increased access to the basement membrane necessary for disease progression. Such a strategy of epithelial disruption has been reported for *Shigella* species (Sansonetti *et al.*, 1999). Another benefit for pathogens is the increase in nutrients in the exudate as well as the clearance of competing organisms through host secreted antibacterial agents and increased peristaltic flushing (Winter *et al.*, 2010). Bacteria may have evolved specific mechanisms for inducing inflammation due to the positive aspects of inflammation.

The purpose of this study was to identify the host epithelial cell signaling cascade that *C. jejuni* activate to cause IL-8 secretion. We utilized *C. jejuni*, which is known to trigger an intense inflammatory response in an infected host, to dissect the host cell components necessary for IL-8 secretion. We have shown that components of the focal complex, including β_1 integrins, FAK, and Src, play a key role in the activation of Erk 1/2. We have also shown that Erk 1/2 activation is Src dependent, but independent of the EGFR and p130Cas. Noteworthy is that activated Erk 1/2 translocates to the nucleus where it activates transcription factors such as AP-1, resulting in IL-8 secretion (Romashkova *et al.*, 1999, Tak *et al.*, 2001, Hoffmann *et al.*, 2002). We have demonstrated that Erk 1/2 is associated with paxillin in response to *C. jejuni* infection, as evidenced by paxillin immunoprecipitation experiments. Knockdown of paxillin resulted in a decrease in Erk 1/2 activation and IL-8 secretion. Based on these data, we generated a model illustrating the focal complex components involved in triggering IL-8 secretion (Fig. 8). To our knowledge, this is the first report that identifies the host cell proteins required for Erk 1/2 activation in response to *C. jejuni* infection.

Many pathogens, including *Salmonella* Typhimurium and *Helicobacter pylori*, are known to exploit the focal complex to their advantage (Shi *et al.*, 2006, Kwok *et al.*, 2007). In the case of *Salmonella*, manipulation of the focal complex results in host cell invasion. One of the focal complex components that *Salmonella* requires for host cell invasion is FAK, but it does not require the kinase function of the protein, suggesting that FAK may be required solely as a scaffold (Shi *et al.*, 2006). In contrast to *Salmonella* as well as *C. jejuni*, *H. pylori* does not invade host cells, but does interact with β_1 integrins for the delivery of the effector protein CagA. Following delivery to the cytosol of the host cell, CagA promotes the activation of FAK (Kwok *et al.*, 2007). In agreement with our findings, paxillin is known to be required for IL-8 secretion in response to *H. pylori* (Tabassam *et al.*, 2011). Our results show that disruption of the focal complex results in a decrease in IL-8 secretion from cells infected with both *S. Typhimurium* and *H. pylori*. Collectively these findings show that IL-8

regulation in this system is independent of host cell invasion. Our findings further indicate that the role of the FC is not limited to one or two pathogenic organisms, but is a conserved epithelial response to pathogens.

The bacterial component(s) required for the activation of the IL-8 signaling cascade through the focal complex are not yet known. The *C. jejuni* CadF and FlpA proteins bind to fibronectin and promote integrin activation (Monteville *et al.*, 2003). Thus, it was unexpected that the level of IL-8 in supernatants from INT 407 cells infected with the *C. jejuni cadF flpA* double mutant was not different (reduced) compared to cells infected with the *C. jejuni* wild-type strain (Fig. S3). While the result with the *C. jejuni cadF flpA* double mutant is confounding, especially given that the knockdown of the β_1 integrin with siRNA resulted in a significant decrease in secreted IL-8 following *C. jejuni* infection (Fig. 1A), several explanations are possible for why the integrin β_1 subunit is required for IL-8 production. The simplest explanation is that *C. jejuni* activates the β_1 subunit via a third Fn-binding protein or a secreted effector protein. It is also possible that the cytoplasmic tail of integrin β_1 subunit may be activated via inside-out signaling. Well documented is that the short cytoplasmic tail of the β subunit interacts with cytosolic scaffold and regulatory proteins, leading to the activation of signaling cascades (Hynes, 2002). Noteworthy is that *C. jejuni* delivers multiple effector proteins to the cytosol of epithelial cells that engage components of the focal complex that may promote inside-out signaling by engagement of the integrin β_1 subunit. Studies are currently being performed in our laboratory to dissect the cellular mechanism of β_1 subunit activation (outside-in versus inside-out signaling) in response to the *C. jejuni cadF flpA* double mutant.

We have shown that *C. jejuni*, *S. Typhimurium*, and *H. pylori* trigger IL-8 secretion from epithelial cells by activating the focal complex. Of additional interest is the fact that these pathogens bind to or have components that interact with fibronectin. *C. jejuni* has two fibronectin binding proteins, termed CadF and FlpA; *H. pylori* produces a fibronectin interacting toxin VacA; and *S. Typhimurium* interacts with fibronectin through ShdA and MisL (Konkel *et al.*, 1997, Cover *et al.*, 2005, Flanagan *et al.*, 2009, Henderson *et al.*, 2011). Although each of these pathogens interacts with fibronectin in a distinctive manner, we have shown that all of these pathogens require β_1 integrin and paxillin to induce a maximal IL-8 response. Consistent with our model shown in Figure 8, others have shown that *S. Typhimurium* and *H. pylori* activate Erk 1/2 and that Erk 1/2 activation is required for IL-8 secretion (Mitsuno *et al.*, 2001, Galdiero *et al.*, 2002, Vitiello *et al.*, 2004, Torok *et al.*, 2005). Based on these findings, it is tempting to speculate that certain bacterial pathogens have evolved to activate the focal complex, resulting in an inflammatory response. Alternatively, host cells may have evolved mechanisms that allow them to detect pathogen association with fibronectin and/or integrin receptors.

Our understanding of the MAP kinase activation pathways has significantly increased over the past decade. Cellular biologists have shown that paxillin acts as a scaffold for Erk 1/2 activation following its phosphorylation by FAK/Src (Ishibe *et al.*, 2003). Thus, paxillin has been found to respond to contact-dependent signaling, allowing for rapid activation of cell signaling cascades. We show that Erk 1/2 co-precipitates with paxillin in response to *C. jejuni* infection. Based on this finding, we propose that paxillin is acting as an Erk 1/2

scaffold upon *C. jejuni* infection. In the context of inflammation, the function of paxillin may not be limited to peripheral functions. Recent work has shown that paxillin translocates with Erk 1/2 to the nucleus where it interacts directly with DNA to regulate gene expression (Sen *et al.*, 2010). Therefore, the knockdown of paxillin may alter the regulation of various genes. To address this possibility, we transfected INT 407 cells with a paxillin-GFP construct and infected them with *C. jejuni*. Paxillin was not observed in the nucleus of the *C. jejuni*-infected cells after 6 hr period, as judged by confocal microscopy (not shown). Based on this finding, we favor the hypothesis that components of the focal complex, particularly paxillin, are modulating a peripheral host cell response to pathogenic bacteria.

In summary, we have shown that several bacterial pathogens utilize components of the focal complex to induce IL-8 secretion. This result is significant because it provides deeper insight into the cellular mechanism of pathogen detection by an epithelial cell. Future work will focus on identifying the bacterial proteins (*e.g.*, adhesins and effector proteins) that initiate host cell activation.

Experimental procedures

Cell culture and bacterial strains

Human INT 407 intestinal epithelial cells (ATCC CCL6; American Type Culture Collection, Manassas, VA) were grown in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). The *C. jejuni* F38011 clinical strain, *ciaC* mutant, and *cadF flpA* double mutant were grown on Mueller-Hinton blood (MHB) agar as described previously (Konkel *et al.*, 1992). *H. pylori* strain 43504 was grown on MHB agar in 100% humidity. *P. aeruginosa* strain MEK1109, *C. freundii* ATCC 8090, *S. aureus* strain MEK1111, and *V. parahaemolyticus* strain MEK1110 were passaged on LB agar in microaerobic conditions (85% N₂, 10% CO₂, 5% O₂, and 80% humidity). Prior to an assay, the bacteria were subcultured onto MHB plates for 18 to 24 hr.

Antibodies and Reagents

Paxillin antibody was purchased from BD Transduction Laboratories (San Diego, CA). Antibodies against β_1 integrin (4706), pErk (4370), p c-Raf (9427), and pMEK1/2 (9154) were acquired from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Erk 1/2 (sc-94), Dock 180 (sc-13163), and actin (sc-1616) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Antibody against p130Cas (610271) was purchased from BD Signal Transduction Laboratories (San Jose, CA, USA). Secondary goat anti-rabbit (A6154) and goat anti-mouse (A4416) HRP-conjugated antibodies were purchased from Sigma Aldrich (St Louis, MO, USA). All antibodies were used according to the manufacturer's instructions.

The inhibitors were used at the following concentrations: Bacterial protein synthesis inhibitor: chloramphenicol at 1 mg/mL (Sigma), MEK 1/2: PD98059 at 50 μ M (Selleck, Houston, TX, USA), FAK: TAE 226 at 5, 2.5, 1.25 and 0.625 μ M (Novartis, Basel, Switzerland), Src: PP2 at 10 μ g/mL (Sigma), Raf: Raf Inhibitor I at 9 nM (Merck, Darmstadt, Germany), PLX-4720 at 80 nM (Selleck), GDC-0879 at 25 nM (Selleck), and

EGFR: erlotinib at 20 μ M (LC Laboratories, Woburn, MA). Inhibitor experiments were performed with samples treated with each inhibitor or the appropriate vehicle. Using the trypan blue viability assay, we assessed host cell survival upon treatment with each inhibitor. All treatments reported herein resulted in no detectable change in host cell viability. The concentration of chloramphenicol has been previously demonstrated to completely prevent bacterial protein synthesis in this strain of *C. jejuni* (Konkel et al., 1992).

IL-8 secretion assay

INT 407 cells were seeded into 24-well tissue culture trays at a confluency of 1.5×10^5 cells per well and cultured overnight at 37°C. Inhibitors were added to cells 30 min prior to bacterial inoculation at the indicated concentrations. *C. jejuni* were re-suspended from MHB plates in MEM 1% FBS to an optical density of OD₅₄₀ 0.03 and then added to the INT 407 cells. The tissue culture plates were centrifuged for 1 min at $800 \times g$ to promote host cell contact. After a 30 min incubation period at 37°C, supernatant was removed from each well and replaced with MEM 1% FBS containing the appropriate inhibitor. The trays were incubated overnight at 37°C and the supernatant was collected and analyzed by ELISA using the BD optEIA human IL-8 detection set. Samples for each condition were generated in quadruplicate and assayed by ELISA in duplicate. The results were confirmed by repeating the assays on multiple days.

Immunoblot analysis

INT 407 cells were seeded into 6-well tissue culture trays at a confluency of 2.5×10^5 cells per well and incubated at 37°C overnight. The cells were rinsed and serum starved in MEM without FBS for 3 hr. Prior to adding *C. jejuni*, the INT 407 cells were pretreated with each inhibitor for 30 min. Bacteria were added in serum free MEM at an optical density of OD₅₄₀ 0.03 and incubated for 40 min. Following incubation the cells were rinsed once with ice-cold PBS supplemented with 50 mM NaF and lysed in lysis buffer (10 mM HEPES, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 10% glycerol, 0.1% Triton X-100, pH 7.4) supplemented with protease inhibitor cocktail P2714 (Sigma). The protein in the cell lysates were separated by SDS-polyacrylamide gel electrophoresis (12% SDS-PAGE), transferred to Immobilon P^{SQ} (PVDF) membranes (Millipore, Billerica, MA, USA), and probed with specific antibodies. Blots were developed with Perkin Elmer ECL Western Lightning and visualized with a GE ImageQuant LAS-4000 mini, (GE Healthcare Biosciences Pittsburgh, PA, USA).

siRNA treatment

The siRNA against β_1 integrin (ITGB1HSS105559), paxillin (PXNHSS108927), and a High GC duplex (46-2000) negative control (Scrm) were obtained from Invitrogen (Grand Island, NY). Target gene knockdown was performed by reverse transfection according to the manufacturer's instructions. Briefly, INT 407 cells were seeded into 24-well trays containing 1 pmol siRNA and Lipofectamine RNAi MAX (Invitrogen) at 1.5×10^6 cells per well. The cells were incubated 24 hr at 37°C prior to inoculation with *C. jejuni* according to the IL-8 secretion assay described above. After the supernatants were removed for IL-8

concentration determination, lysates of the INT 407 cells were prepared for immunoblot analysis to determine the effectiveness of protein knockdown.

Immunoprecipitation

INT 407 cells were grown in T75 flasks to 60% confluency, rinsed, and serum starved for 24 hr. Cells were infected with the *C. jejuni* wild-type strain and incubated for 40 min. The cells were rinsed with ice-cold PBS supplemented with 50 mM NaF prior to lysis. Lysates were generated using IP lysis buffer (25 mM Tris, 150 mM NaCl, 50 mM NaF, 1mM EDTA, 1 mM Na₃VO₄ 5% glycerol, 0.1% Triton X-100 pH 7.4) and centrifuged at 12,000 × *g* at 4°C for 10 min to remove insoluble proteins. Following centrifugation, the protein concentration of each supernatant was determined by Precision Red protein quantification assay (Cytoskeleton, Denver, CO, USA) and normalized. The cell lysates were then incubated with mouse monoclonal anti-paxillin antibody (BD Transduction Labs) and protein A/G conjugated agarose beads (Santa Cruz) while rocking for 24 hr at 4°C. The beads were pelleted by centrifugation at 5000 × *g* for 3 min. The pellets were washed with IP wash buffer (20 mM HEPES, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 10% glycerol, 0.1% Triton X-100 pH 7.4) in three subsequent wash-centrifugation cycles. The final pellet was suspended in Laemmli sample buffer.

RT-PCR analysis

INT 407 cells were cultured in a 24-well tissue culture tray. For the use of chemical inhibitors the INT 407 cells were pretreated with vehicle or TAE 226 for 30 min and then infected with *C. jejuni*. INT 407 cells treated with siRNA were infected with *C. jejuni* 18 hr after transfection. After a 6 hr infection, the RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue Kit, (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Following total RNA extraction cDNA was generated using SuperScript® III First-Strand Synthesis System (Life Technologies, Grand Island, NY, USA). The relative cDNA was quantified using Power Sybr Green (Life Technologies). Analysis of relative gene expression was performed using $2^{-\Delta\Delta CT}$, with GAPDH serving as the reference gene.

Statistical analysis

All results presented in this work represent at least three biological replicates from experiments repeated on multiple days. Averages and standard error mean (SEM) were generated from each sample set. *F* tests were performed to ensure normal deviation between sets. Those data that did not have significant differences in their deviations were tested with an unpaired, two-tailed Student's *t* test. Data with significant differences between standard deviations were tested with an unpaired, two-tailed Welch's *t* test. To make multiple comparisons within one data set the data was analyzed by one-way ANOVA and individual comparisons were then made by Tukey's posthoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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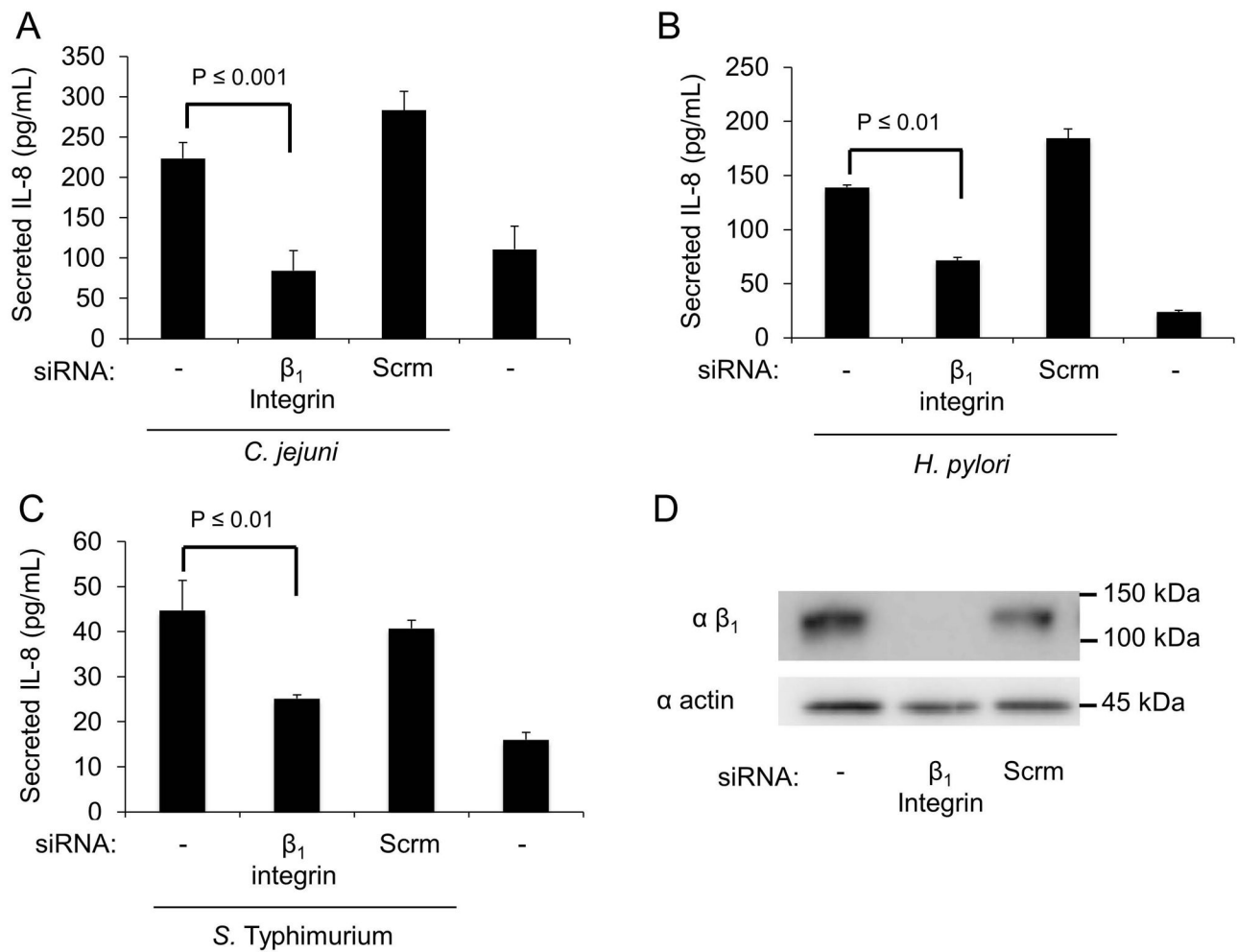


Fig. 1. The β_1 integrin in epithelial cells contributes to the IL-8 response to multiple bacterial pathogens

Panels: (A–C) INT 407 cells were transfected with either siRNA specific for the β_1 integrin or scrambled (Scrm) siRNA. The cells were then infected with *C. jejuni*, *H. pylori*, or *S. Typhimurium*. Secreted IL-8 levels were determined by ELISA following 24 hr (*C. jejuni*) or 5 hr (*H. pylori* and *S. Typhimurium*) incubations. Non-transfected cells infected with each bacteria served as a positive control and untreated uninfected cells served as a negative control. (D) The efficiency of the β_1 integrin knockdown was tested by immunoblot analysis of cell lysates, with actin serving as a loading control.

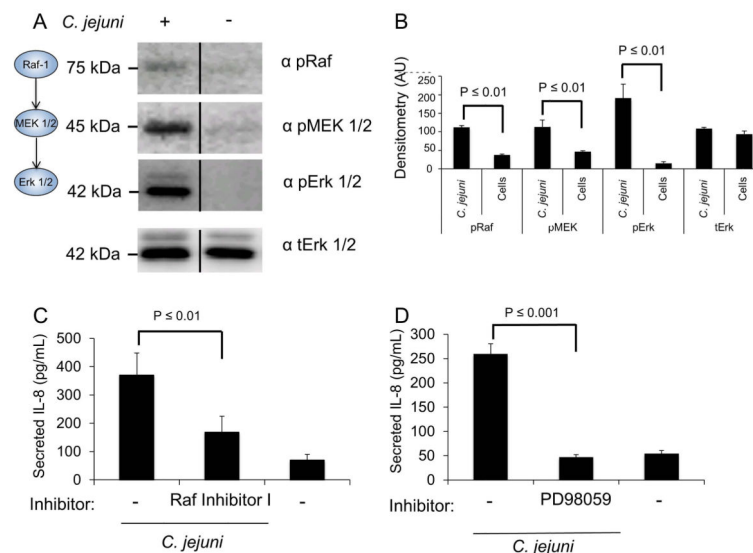


Fig. 2. *C. jejuni* activates Erk 1/2 by the canonical MAPK activation pathway

Panels: **(A)** Immunoblot analysis of INT 407 cells infected with *C. jejuni* following a 30 min incubation. The level of phosphorylated c-Raf, MEK 1/2 and Erk 1/2 were determined by immunoblot with phospho-specific monoclonal antibodies for each protein. Total Erk 1/2 (tErk) served as a loading control. The images shown are a composite of different sections of the same immunoblot. **(B)** Densitometric analysis of phosphorylated Raf/MEK/Erk from three samples. **(C)** INT 407 cells were treated with Raf Inhibitor I and IL-8 secretion was determined by ELISA 24 hr following infection with *C. jejuni*. Uninfected cells served as a negative control. **(D)** INT 407 cells were treated with the Erk 1/2 activation inhibitor PD98059, infected with *C. jejuni*, and the level of IL-8 in supernatants were measured by ELISA. Vehicle treated cells infected with *C. jejuni* served as a positive control and uninfected cells served as a negative control.

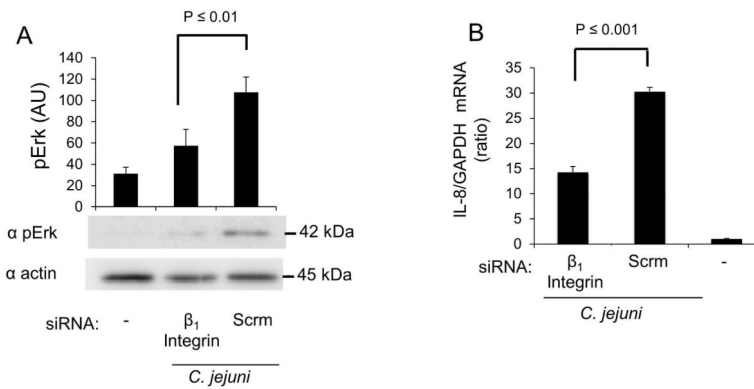


Fig. 3. The β_1 integrin is required for full activation of Erk 1/2 and IL-8 gene transcription in response to *C. jejuni*

Panels: **(A)** INT 407 cells were transfected with siRNA specific for the β_1 integrin or scramble (Scrm) siRNA. The cells were infected with *C. jejuni* and incubated for 30 min. Uninfected cells served as a negative control. The cell lysates were analyzed by immunoblot using antibody against pErk 1/2. The membranes were re-probed using antibody against actin as a loading control. Densitometric analysis was performed to quantify the level of Erk 1/2 activation. **(B)** INT 407 cells were treated with siRNA and infected with *C. jejuni*. The level of IL-8 gene expression following a 6 hr infection was measured by RT-PCR. IL-8 expression was normalized to GAPDH transcript levels with uninfected cells serving as negative control.

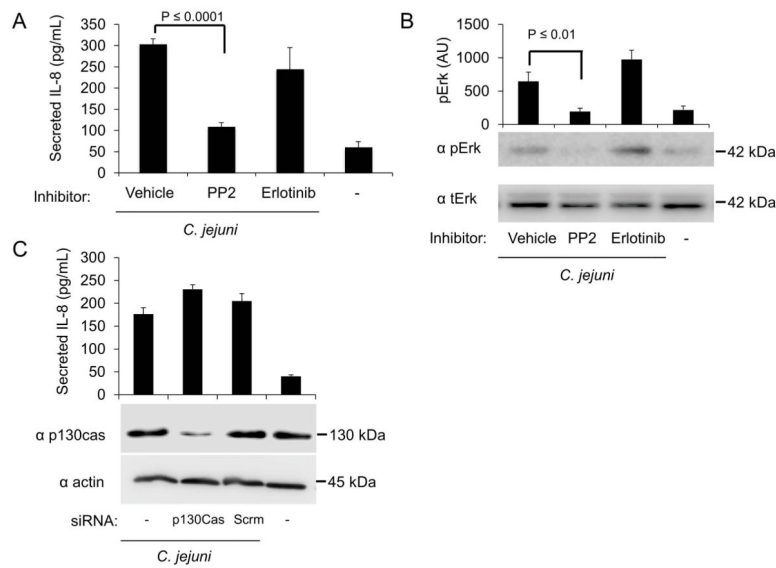


Fig. 4. Src is required for full Erk 1/2 activation and IL-8 secretion

Panels: **(A)** INT 407 cells were treated with PP2 and erlotinib to inhibit Src and the EGFR, respectively. Vehicle treated cells infected with *C. jejuni* served as a positive control and uninfected cells served as a negative control. Secreted IL-8 was quantified by ELISA. **(B)** INT 407 cells were treated with PP2 and erlotinib to inhibit Src and the EGFR, respectively. Erk 1/2 phosphorylation (activation) following a 30 min infection was measured by immunoblot using antibodies against phospho-Erk 1/2 (pErk). The membranes were re-probed using antibody against total Erk 1/2 (tErk) as a loading control. Densitometric analysis was used to quantify the level of pErk activation. **(C)** INT 407 cells were transfected with siRNA specific to p130Cas or scrambled siRNA (control). Non-transfected cells infected with *C. jejuni* served as a positive control and untreated cells served as a negative control. The efficiency of p130Cas knockdown was determined by immunoblot analysis. The membranes were re-probed using antibody against actin as a loading control.

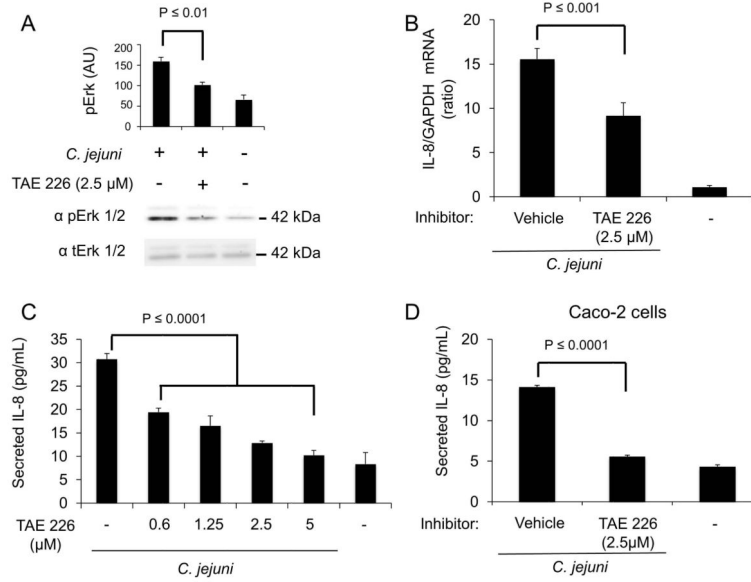


Fig. 5. FAK is critical to Erk 1/2 activation and IL-8 secretion in response to *C. jejuni*
 Panels: (A) INT 407 cells were treated with focal adhesion kinase (FAK) inhibitor TAE 226 and infected with *C. jejuni*. Immunoblots coupled with densitometric analysis were used to determine the level of Erk 1/2 activation following a 40 min incubation. (B) INT 407 cells were treated with TAE 226 and infected with *C. jejuni*. The level of IL-8 gene expression was measured by RT-PCR following a 6 hr infection. IL-8 expression was normalized to GAPDH transcript levels with uninfected cells serving as a negative control. (C) INT 407 cells were treated with TAE 226 and infected with *C. jejuni*. Supernatants were collected after 6 hr and IL-8 was quantified by ELISA. Multiple TAE 226 concentrations were tested to determine if there was a dose-dependent effect. Uninfected cells served as a negative control. (D) Caco-2 human intestinal epithelial cells were treated with TAE 226 and infected with *C. jejuni*. Supernatants were collected after 6 hr incubation, and IL-8 was quantified by ELISA. Uninfected cells served as negative control.

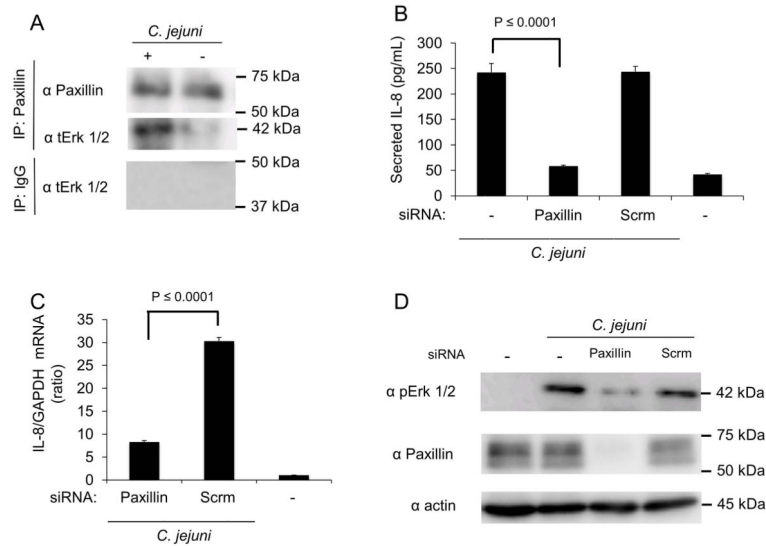


Fig. 6. Erk 1/2 is recruited to and activated at paxillin in response to *C. jejuni* infection
 Panels: **(A)** INT 407 cells were infected with *C. jejuni* and incubated for 40 min. Uninfected cells served as a negative control. Paxillin was immunoprecipitated from cell lysates with anti-paxillin antibody. The cell lysates were analyzed by immunoblot and probed with antibody against total Erk 1/2. Immunoprecipitations using antibodies against IgG were used to examine nonspecific interactions. **(B)** INT 407 cells were treated with siRNA specific to paxillin and infected with *C. jejuni*. Lysates were collected and immunoblots probed with antibodies directed against phospho Erk 1/2 (pErk), with total Erk 1/2 (tErk) serving as a loading control. The efficiency of paxillin knockdown using siRNA treatment was tested by immunoblot analysis. **(C)** INT 407 cells were transfected with siRNA specific to paxillin or a scrambled (Scrm) control and infected with *C. jejuni*. The level of IL-8 gene expression was measured by RT-PCR following a 6 hr infection. IL-8 expression was normalized to GAPDH transcript levels. Uninfected cells served as negative control. **(D)** ELISA quantification of IL-8 secretion by INT 407 cells treated with siRNA specific to paxillin or scrambled siRNA following incubation with *C. jejuni*. Uninfected cells served as a negative control.

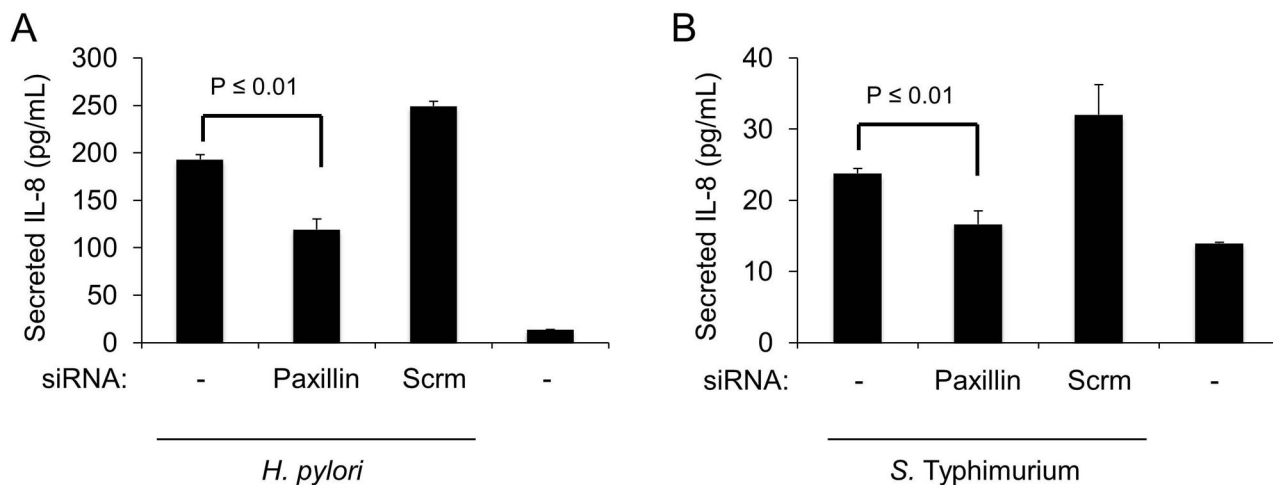


Fig. 7. Knockdown of paxillin results in a decrease in IL-8 secretion from epithelial cells in response to *H. pylori* and *S. Typhimurium*

Panels: (A, B) INT 407 cells were treated with siRNA specific to paxillin or scrambled siRNA. The cells were infected with *H. pylori* or *S. Typhimurium* and supernatants were collected after a 5 hr incubation and tested for IL-8 by ELISA. Uninfected cells served as a negative control.

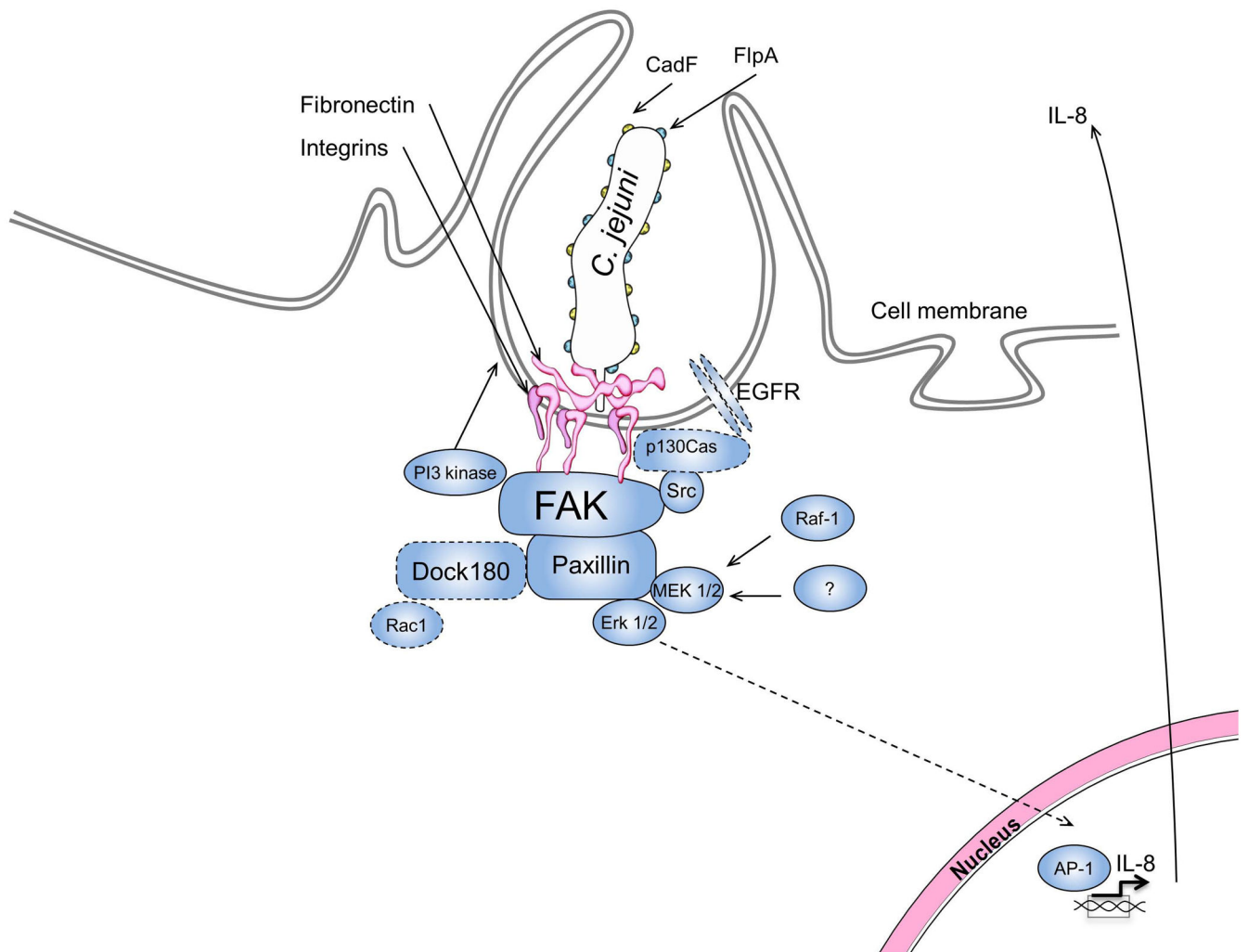


Fig. 8. The focal complex is required for IL-8 induction

Shown is a model illustrating the signaling cascade leading to IL-8 secretion from *C. jejuni* infected cells. We propose that *C. jejuni* associates with fibronectin leading to activation of β_1 integrins. The β_1 integrins serve as a scaffold for the association and activation of FAK. Src is then recruited to FAK and becomes activated. The FAK-Src complex phosphorylates paxillin, leading to the recruitment and activation of Erk 1/2, and IL-8 gene transcription. The bacterial proteins CadF and FlpA (yellow and blue dots on the bacteria respectively) and host proteins outlined with a dashed line (EGFR, p130Cas, Dock180, and Rac1) are required for *C. jejuni* host cell invasion, but do not play a significant role in IL-8 induction.