CMLS, Cell. Mol. Life Sci. 55 (1999) 961–976
1420-682X/99/070961-16 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Enteropathogenic *Escherichia coli*: a pathogen that inserts its own receptor into host cells

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Received 23 November 1998; received after revision 21 December 1998; accepted 21 December 1998

Abstract. Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infant diarrhea, killing hundreds of thousands of children per year worldwide. Intimate attachment to the host cell leading to the formation of actin-rich pedestals beneath the adhering bacteria is an essential feature of EPEC pathogenesis. EPEC attaches to host cells via the outer membrane adhesin, intimin. It was recently shown that EPEC inserts its own receptor for intimate adherence, Tir (translocated intimin receptor) into the host cell membrane. The focus of this review is on the discovery and characterization of this novel receptor, and our current understanding of its role in pedestal formation. Gram-negative bacterial secretion systems, including type III secretion systems, are reviewed and discussed in the context of Tir delivery into the host cell membrane. The relationship and relevance of in vitro models compared to the actual in vivo situation is essential to understanding disease. We have critically reviewed the use of animal models in studying EPEC infection. Elucidating the function of Tir will contribute to our understanding of how EPEC mediates disease.

Key words. Enteropathogenic *Escherichia coli* (EPEC); diarrhea; Tir (translocated intimin receptor); cytoskeleton; signal transduction; type III secretion system; in vivo models; rabbits.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea in the developing world. EPEC belongs to a family of Gram-negative pathogens that cause attaching and effacing (A/E) lesions. Upon infection, EPEC colonizes the small intestine [1] and forms small microcolonies on the epithelial cell surface. Intimate adherence between EPEC and the host epithelium leads to the formation of A/E lesions, which are characterized by the degeneration of epithelial brush border microvilli, and the formation of actin-rich pedestals beneath the adherent bacteria [2, 3] (fig. 1). Other related A/E pathogens include enterohemorrhagic *E. coli* (EHEC), the causative agent of the 'hamburger' disease, *Citrobacter rodentium*, *Hafnia alvei* and the rabbit pathogen rabbit diarrheagenic *E. coli* (RDEC-1). In EPEC and EHEC O157:H7, all the genes needed for A/E formation are encoded in a chromosomal pathogenicity island called the LEE (locus of enterocyte effacement) [4]. This region contains genes encoding a type III secretion apparatus, the secreted Esp (*E. coli* secreted protein) proteins and factors necessary for intimate attachment to the host cell (Tir and intimin). EPEC also has plasmids of 50–70 MDa in size which encode an adhesin (bundle forming pilus—BFP) and a transcriptional activator (Per).

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Much progress has been made in determining the factors involved in A/E lesion formation, but many questions remain regarding what role these factors play in disease. Recently, the EPEC receptor for intimate adherence was identified, and surprisingly found to be a bacterial protein inserted into the epithelial cell membrane [5]. This is the first report of a bacteria inserting its own receptor, Tir, into host cells. This review will focus on the characterization of Tir, its various functions and our understanding of how Tir is inserted into the host cell membrane. This review will discuss the role of Tir and other factors involved in A/E lesion formation in terms of the current animal models for studying EPEC-mediated disease and relate this to in vitro models.

From Hp90 to Tir

Initial work described several tyrosine phosphorylation events induced by EPEC, including the appearance of a 90-kDa band in the membranes of infected cultured epithelial cells [6]. This protein, called Hp90, was later shown to be the receptor for the bacterial outer membrane protein intimin, and is found at the tip of the EPEC pedestal. Hp90 was believed to be a host membrane protein that is tyrosine-phosphorylated in response to EPEC infection [7]. Surprisingly, that was not the case. Hp90 is actually a bacterial protein, renamed to Tir (translocated intimin receptor), which is inserted into the host cell membrane where it is tyrosine-phosphorylated and binds intimin on the bacterial surface [5]. Several lines of evidence support this finding. First, antibodies to a previously uncharacterized 78-kDa EPEC secreted protein recognized the 90-kDa tyrosine-phosphorylated protein in membranes from infected, but not uninfected, host cells. Phosphatase treatment converted the 90-kDa protein to 78 kDa, which was no longer recognized by anti-phosphotyrosine antisera. Immunofluorescence microscopy demonstrated that Tir has the same cellular localization as Hp90, which is found at the tip of the EPEC pedestal. EPEC *tir* deletion mutants were transformed with epitope-tagged Tir to demonstrate delivery of the 78-kDa bacterial protein to the host cell membrane, and its subsequent tyrosine phosphorylation. Intimin bound to both the secreted unphosphorylated 78-kDa, and the 90-kDa tyrosine-phosphorylated host membrane form of Tir, using in vitro binding techniques. This suggests that Tir tyrosine phosphorylation may not be required for intimin binding. Tir delivery to the host cell is facilitated by the type III secretion system, and the EPEC secreted proteins EspA and B.

Tir is probably a common feature of many A/E pathogens. Tir sequences have been identified from a variety of these pathogens, including several strains of Shiga toxin-producing *E. coli* (STEC), Hafnia alvei and the rabbit pathogen RDEC-1 ([8, 9] and GenBank accession number AF045568). Tir translocation to the host cell membrane has been described for a second A/E pathogen, the O26:H$^-$ STEC strain 413/89-1 [8]. Tir is encoded within the LEE pathogenicity island, and is contained in an operon with its ligand, intimin and a putative chaperone, orfU [4]. Sequence analysis of Tir proteins suggests they are integral membrane proteins that contain two transmembrane-spanning domains. In EPEC, Tir has six tyrosine residues that are potential substrates for phosphorylation, the majority of which are located in the carboxy terminus [5]. These tyrosine residues are predicted to reside in an intracellular domain due to their inaccessibility to labelling with antiphosphotyrosine in unpermeabilized cells using immunofluorescense microscopy experiments [7]. Based on this we propose a model for Tir topology, with the amino and carboxy termini inside the host cell, and the intimin binding domain as an extracellular loop (fig. 2). Tir has at least three major functions: it is the receptor for the bacterial outer membrane protein intimin; it serves as a cornerstone for EPEC-induced cytoskeletal rearrangements; and it may be involved in other signal transduction events.

Intimin binding

One major functional role for Tir is to serve as a receptor for the bacterial outer membrane protein in-

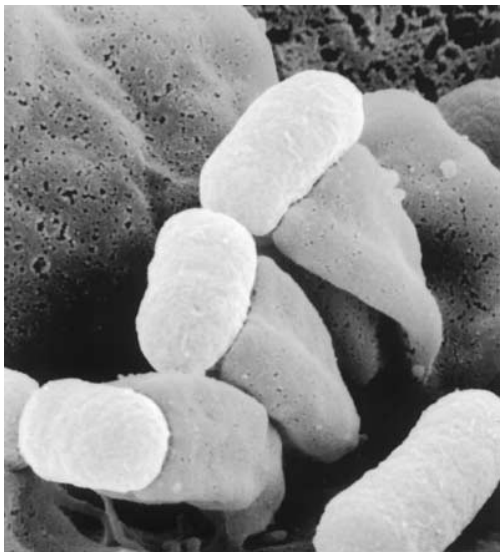

Figure 1. EPEC infection leads to the formation of pedestals beneath the adherent bacteria. Scanning electron micrograph of an EPEC-infected HeLa cell. Micrograph courtesy of Dr. Ilan Rosenshine.

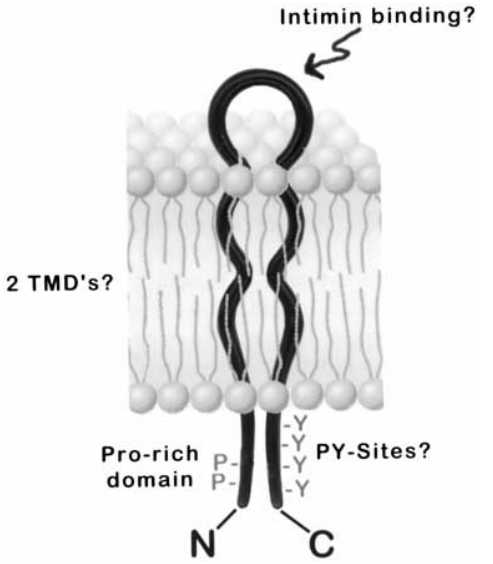

Figure 2. Hairpin model of Tir structure. Tir is predicted to be an integral membrane with two transmembrane domains. Both the amino and carboxy termini are predicted to be intracellular domains, and intimin is thought to bind to an extracellular loop. Tyrosine residues that are potential substrates for phosphorylation are located within the carboxy-terminal tail.

timin. Intimin is a 94-kDa protein conserved throughout the family of A/E pathogens [10, 11]. Intimin expression is essential for A/E lesion formation both in vitro and in vivo, and is required for full virulence in both human and animal disease models [12–16]. Intimins show a high degree of amino acid sequence identity at their amino termini, but diverge significantly at their carboxy terminal cell binding domain [11]. Despite these differences in primary sequence, intimins from different A/E pathogens are functionally interchangeable [10, 13, 15]. For example, expression of EPEC intimin in an EHEC intimin deletion mutant resulted in A/E lesion formation, but altered the pattern in which the bacteria localized within the intestine [15]. Unlike wild-type EHEC, which colonizes both the small and large intestine, EHEC expressing EPEC intimin colonized only the small intestine, in a pattern indistinguishable from wild-type EPEC. This has led to speculation that EPEC and EHEC intimins bind to different receptors on the host cell, and that this difference drives the tissue specificity observed. This is hard to reconcile given the finding that these pathogens can insert their own intimin receptor (Tir), and suggests that intimin may have multiple binding activities. Frankel et al. report that the carboxy terminal domain can bind to cultured epithelial cells in the absence of bacterial secretion or signal transduction, and describe binding to $\beta 1$ integrins [10]. The role of this binding is still not known.

Cytoskeletal rearrangements

Intimin binding to Tir elicits dramatic cytoskeletal rearrangements within the host cell, leading to the formation of actin-rich pedestals beneath the adherent EPEC. These EPEC-induced pedestals can extend as much as 10 μm above the epithelial cell surface [7]. In addition to polymerized actin, EPEC pedestals contain α-actinin, ezrin, talin [17], myosin light chain [18] and villin [19]. Surprisingly, pedestal formation does not involve the small guanosine triphosphate (GTP)-binding proteins Rac, Rho and Cdc42 factors known to be involved in cytoskeletal rearrangements within eukaryotic cells [20, 21]. Inhibition of Rac, Rho and Cdc42 by *Clostridium difficile* Tox B and compactin, along with expression of dominant negative alleles of these GTP-binding proteins, had no effect on pedestal formation, suggesting that EPEC may use a novel mechanism to reorganize actin [20]. The role Tir tyrosine-phosphorylation plays in pedestal formation is unknown. EHEC, a closely related A/E pathogen, can induce pedestal formation without the accumulation of tyrosine-phosphorylated proteins beneath adhering bacteria [22], suggesting that tyrosine-phosphorylation is not essential for pedestal formation.

Disruption of the host cytoskeleton is not unique to A/E pathogens. Other bacteria, including *Salmonella*, *Shigella* and *Listeria* have profound effects on host cytoskeletal organization [23]. *Shigella flexneri* and *Listeria monocytogenes* pirate the host cytoskeleton to form actin 'comets' behind intracellular bacteria, facilitating mobility and cell-to-cell spreading. These pathogens use two analogous but unrelated systems to achieve this [24]. *Shigella* expresses IcsA, which is localized to one pole of the bacterial outer membrane, and has adenosine triphosphate (ATP) hydrolytic activity [25]. IcsA binds to the host cytoskeletal proteins vinculin and neural-Wiscott-Aldrich syndrome protein (N-WASP) [26], leading to the recruitment of vasodilator-stimulated phosphoprotein (VASP) [27], α-actinin [28], filamin, fimbrin [29] and profilin [30] to the actin tails. In *Listeria*, VASP binds to the bacterial protein ActA, which is then localized to one pole of the bacterium [27, 31], leading to the association of the actin-related protein complex (Arp2/3) [32], cofilin [33], α-actinin [34], profilin, vinculin, villin, talin, ezrin and fimbrin [35], with the comet tail. Although many of the cytoskeletal components identified so far in the EPEC pedestal are also found in *Listeria* and *Shigella* actin comets, the organization of these proteins within the

pedestal and the protein(s) directly binding to Tir are not known.

Signal transduction

Tir may play a role in transducing signals within the host cell. EPEC infection leads to the modulation of a variety of signalling events, although a role for Tir has yet to be defined. One effect is to stimulate the release of calcium from intracellular stores [36, 37]. This requires active bacterial signalling via the type III secretory system, and is dependent upon intimate attachment to the host cell. Blocking the increase in internal calcium with an intracellular calcium buffer abolishes pedestal formation [36]. EPEC also stimulates inositol phosphate (IP) fluxes, in a manner dependent on an intact type III secretory system and the EPEC secreted protein EspB [37–39]. IP fluxes are enhanced, but not dependent on intimate adherence to the host cell. EPEC activation of protein kinase C (PKC) has also been reported [18, 40]. EPEC enhances membrane-associated PKC activity [40]. This enhancement is dependent on intimate adherence to the host cell, and is not observed in EPEC intimin mutant strains.

EPEC induced IP and calcium fluxes, and PKC activation suggest the involvement of a phospholipase. Recently, a second wave of tyrosine phosphorylation events was described in EPEC-infected HeLa cells [41]. One of these proteins was identified as PLCγ, which requires tyrosine phosphorylation for activation. EPEC-induced phospholipase Cγ (PLCγ) activation is dependent on intimin binding, but occurs at a later time point than Tir phosphorylation. It is unknown whether Tir phosphorylation is required for PLCγ activation. PLCγ is most likely not responsible for the IP fluxes observed, as these can occur in the absence of intimate adherence [39]. Whether PLCγ activation provides the secondary messengers needed for the intimin-dependent PKC activation and intracellular calcium release has yet to be determined.

A compelling question is what relevance these EPEC-induced signalling events have in A/E lesion formation and disease. The temporal sequence of these events, and the bacterial factors that are required, are not yet known. It is uncertain whether these signalling events occur in vivo, and whether they are required for pathogenicity. It is also not known what role Tir plays in signal transduction, and whether Tir tyrosine phosphorylation is required. Many of the EPEC-activated signalling events require intimate adherence and presumably Tir, but whether this is a direct effect of Tir, or a consequence of the extensive cytoskeletal rearrangements within the cell have yet to be determined.

EPEC and diarrhea

How EPEC causes diarrhea is still unknown, although recent studies have suggested several possible mechanisms. The loss of absorptive surface area due the effacement of epithelial cell microvilli may contribute to EPEC-induced diarrhea [42]. Increasing chloride secretion is a common method used by other bacteria to cause diarrhea [23]. Work from two laboratories suggests that EPEC may alter cellular electrolyte transport. Stein and co-workers demonstrated that EPEC infection led to the depolarization of epithelial cell membranes, suggesting an efflux of chloride ions or the influx of sodium or potassium [43]. This was investigated further by Collington et al. who report that EPEC increases short-circuit current (Isc) in cultured epithelial cells [44]. This increase coincided with pedestal formation and requires EPEC secreted Esp proteins. The initial rise in Isc was due in part to an increase in chloride secretion from the epithelial cells, with the balance due to either influx of sodium or amino acids. As EPEC also activates PKC, this effect could be due in part to PKC-stimulated chloride secretion via the cystic fibrosis transmembrane conductance regulator (CFTR) [42].

Another potential mechanism is disruption of epithelial cell tight junctions, which could contribute to diarrhea by the perturbation of electrochemical gradients, and increasing intestinal permeability. EPEC has been reported to modulate two different pathways that could lead to this result. First, EPEC alters the transepithelial electrical resistance (TEER) in polarized epithelial monolayers [45, 46]. This effect is due to myosin light chain kinase phosphorylation, which leads to the disruption of epithelial cell tight junctions and a decrease in TEER [46]. Pretreatment with myosin light chain kinase (MLCK) inhibitors, but not inhibitors of PKC or tyrosine kinases, protected cells from tight junction disruption and decrease in TEER, suggesting an involvement for MLCK. EPEC-induced disruption of barrier function is also dependent upon release of intracellular calcium, which is necessary for MLCK activation [47]. Tight junction disruption has also been reported in response to EHEC infection [48]. In EHEC, both MLCK and PKC play a role, suggesting different mechanisms are used by these two related pathogens.

EPEC infection also leads to the transmigration of polymorphonuclear leukocytes (PMN) across the epithelial cell monolayers, which may contribute to the disruption of epithelial barrier function [49]. EPEC-induced PMN transmigration requires EspB but not intimate adherence, and is not due to n-formyl peptides, which are potent bacterial-derived chemoattractants [49]. PMN transmigration is due to increased IL-8 secretion, in response to EPEC-induced activation of the transcription factor nuclear factor-κB (NF-κB) [50].

Interestingly, NF-κB activation is stimulated only by pathogenic *E. coli* and not by nonpathogenic species, and requires bacterial signalling but not intimate adherence. PMN transmigration can also affect chloride secretion. Once the PMNs are on the luminal side of the epithelial border, they release 5′-adenosine monophosphate. This is converted to adenosine, which binds to host adenosine receptors, stimulating chloride secretion [42].

Delivery of Tir into host cell membrane

One of the most basic and yet most complex questions is how EPEC delivers Tir into host cell membranes. While several clues have been found, the mechanism of insertion is not known. Only the 90-kDa phosphorylated form of Tir has been detected with both anti-phosphotyrosine and anti-Tir antisera in host cell membranes infected with EPEC [5–7, 41], whereas both the unphosphorylated and phosphorylated forms were found in cells infected with STEC [8]. As of yet, Tir has not been detected in the host cell cytoplasm. This is suggestive that Tir is inserted directly into the host cell membrane. A puzzle concerning Tir insertion into the host membrane is that this protein possesses both a soluble bacterial form and a membrane-associated form. Most membrane proteins are not stable without lipid bilayers, yet Tir can be secreted by the bacteria and is stable, as demonstrated by the fact that the secreted form of Tir can bind intimin [5]. Type III secretion systems, which are found in many pathogenic bacteria, transfer proteins directly from the bacterial cytosol into host cell membrane and cytoplasm, and Tir delivery probably uses a similar mechanism.

Bacterial secretion systems

Most bacteria, pathogenic or not, can secrete proteins. Four secretion mechanisms for proteins and a fifth system for macromolecular secretion have been identified in Gram-negative bacteria (reviewed in [51]). This fifth system is involved in conjugal transfer of plasmids, transfer DNA (T-DNA) transfer by *Agrobacterium tumefaciens*, and secretion of *Bordetella pertussis* toxin [52]. The type I secretion system involves the direct translocation of a protein from the cytoplasm of the bacteria to the exterior, and the prototype for this system is the *E. coli* protein hemolysin [53]. Although type I secretion is *sec*-independent and therefore does not involve amino-terminal processing, proteins secreted via this pathway have a secretion signal within the 60 carboxy-terminal amino acids which is not proteolytically cleaved. In contrast with other secretion systems, type I secretion requires only three secretory proteins: an inner membrane transport ATPase; an outer membrane pore; and a membrane fusion protein that spans the periplasmic space.

Both the type II and type IV secretion pathways involve a *sec*-dependent transport of their effectors into the periplasm. An identifying feature of a *sec*-dependent protein is a 30-amino acid hydrophobic amino-terminal signal sequence that aids in export and is cleaved off by periplasmic signal peptidases. The type II secretion system, or general secretory pathway, exports a large variety of proteins and is by no means restricted to virulence factors. The best-studied protein that is secreted by a type II system is pullulanase from *Klebsiella oxytoca* which, in addition to the multiple proteins involved in the *sec* pathway, needs 14 additional factors for secretion which are found in both the inner and outer membrane [54]. Interestingly, the outer membrane component of the pullulanase secretion system, PulD, is conserved in many Gram-negative protein transport systems.

The type IV secretion system is quite interesting in that the proteins themselves mediate their own transport from the periplasm to the extracellular space and thus are referred to as the autotransporters (reviewed in [23]). *Neissereria gonorrhoeae* immunoglobulin A (IgA) protease is the archetypal example of this family of proteins that apparently form a pore in the outer membrane through which they pass, and autoproteolysis releases proteins into the extracellular space. In EPEC, the secreted protein EspC is believed to be transported out of the cell via this pathway [51, 55].

The type III secretion systems, which are activated in response to host factors, have been found in many human, animal and plant pathogens, including EPEC (reviewed in [51]). This apparatus delivers its effectors directly from the cytoplasm of the bacteria, through both inner and outer membranes, to the surface with no distinct periplasmic intermediates. Like the type I secretion system, type III secretion is independent of the *sec* pathway. Unlike the type I pathway, the type III secretion system is complex and composed of approximately 20 proteins. Most of these proteins are found in the inner membrane and are homologous to components of the flagellar biosynthesis apparatus of bacteria. Some of the outer membrane proteins of the type III secretion system are homologous to PulD, the outer membrane secretin of the type II general secretion pathway. This suggests that while different secretion systems do exist in Gram-negative bacteria, they all have a common origin.

The most striking feature of the type III secretion system is that bacterial virulence proteins can be delivered directly into the cytosol of eukaryotic cells. The term ‘translocation’ is used to describe the transport of a protein from the bacterial cell through the eukaryotic

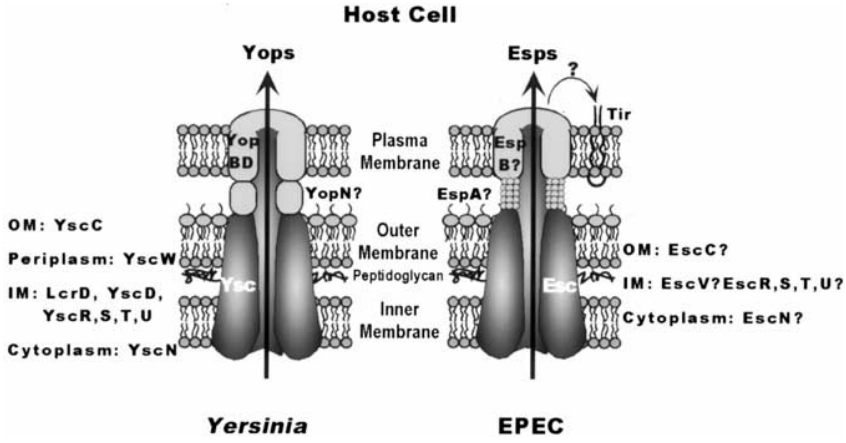

Figure 3. Comparison of the type III secretion systems of *Yersinia* and EPEC. A model for EPEC's type III secretion system was constructed based on its similarity to the Ysc proteins, and the localization of the Ysc components in *Yersinia*. In EPEC, EspA is thought to act as a channel to deliver proteins inside the host cell [77], and EspB might be involved in forming the translocation channel in the host cell membrane [82]. The mechanism for Tir insertion into the host cell membrane has not been elucidated.

cytoplasmic membrane into the cytosol of the target host cell, while 'secretion' refers to the transport of proteins from the bacterial cytoplasm to the extracellular space [51]. It has been proposed that protein secretion in vitro may be an experimental artifact which only occurs under conditions that mimic those encountered by the bacteria, and that in reality the type III system is used for translocation [51].

The Yop system in yersiniae represents the archetype of the type III secretion family (fig. 3) [56]. The Yop virulon, located on a 70-kb plasmid (pYV), encodes a secretion apparatus, chaperone proteins and 14 Yop proteins. The yersiniae secretion complex (Ysc) is comprised of 22 proteins of which only a few have been characterized [57]. YscN is the putative energizer of the secretion machinery, sharing homology with the F_0F_1 ATPase and having an ATP-binding site [58]. LcrD, YscU and YscD and many others have been localized to the inner membrane [59–61], whereas YscC was found in the outer membrane [60]. YscC belongs to a family of proteins which are involved in transporting large molecules across the outer membrane likely by forming a channel [51]. Two domains in the carboxy terminus are conserved, and the amino terminus is similar within the subfamilies which are (i) type III secretion pathway, (ii) type II secretion pathway (PulD), (iii) extrusion and assembly of filamentous phage, and (iv) export of pilus subunits in the assembly of type IV pili.

YopN acts as a gatekeeper controlling the deployment of the translocation apparatus and effector proteins. YopN itself is not delivered into the host cell, but is surface-located and is involved in signalling and regulation of secretion of the effectors [62, 63]. Recently, YopN has been found in the eukaryotic cytosol as well as associated with the bacterium using selective solubilization [64], although a role for this is not known. YopB and D are directly involved in the delivery or translocation of the effector Yops into the host cell. Both proteins are required for the internalization of the Yops and seem to form a pore in the membrane of the eukaryotic cell [62, 65–69].

The sequence required for secretion of a type III effector is located in the amino-terminal region of the protein. YopE, YopH and YopN secretion sequences have been mapped by deletion analysis and reporter gene fusion to the first 15 to 17 amino acid residues or codons [69–71]. No common features are shared by the amino acid sequences at the beginning of type III secreted proteins. Recently it has been suggested that the signal that leads to the type III secretion of Yop proteins is encoded by the 5′ messenger RNA (mRNA) [70]. Individually replacing all codons in the secretion signal with alanine or glutamate had no effect on secretion; however, translation was affected in some mutants. Strikingly, secretion was not affected by frameshift mutations that caused very few changes in the DNA and mRNA but completely altered the amino acid sequence. The authors not only suggest that the signal recognized by the secretion apparatus is mRNA, but also propose that secretion and translation are coupled. The 5' ends of the YopE and YopN mRNAs are predicted to form a stem-loop structure where the AUG translational start codon is buried in a duplex,

and it is postulated that the mRNA assumes a translatable conformation by interacting with the secretion apparatus [70].

Type III secreted proteins appear to have a modular structure composed of a secretion domain, a translocation domain and an effector domain. At least 50 amino-terminal amino acids of YopE and approximately 70 amino acids of YopH are required for these proteins to be translocated into eukaryotic cells [69, 71].

EPEC's type III secretion system

EPEC's type III secretion apparatus contains many genes that are homologous to the *Yersinia* system [4, 72], although the function of each protein remains to be determined (fig. 3). Striking similarities are seen between components of the type III system across species [73]. In fact, functional conservation of the type III secretion and translocation machinery has been shown by cross-complementation in yersiniae, salmonellae and shigellae [74], and it was recently demonstrated that *Erwinia chrysanthemi* type III secretion system functions in *E. coli* to deliver and secrete avirulence proteins [75]. With any system that has many components and thus many laboratories investigating them, nomenclature difficulties arise. Those EPEC genes that are homologous to *Yersinia* type III secretion (*ysc*) genes are referred to as *esc* (*E. coli secretion*) genes with the same suffix as the *Yersinia* homologue [4]. Those genes that are not *ysc* homologues but are involved in type III secretion are named *sep* (secretion of *E. coli* proteins). Four genes (*escU*, *escC*, *sepZ* and *escN*) have been shown experimentally to be involved in the system; 19 genes are putatively involved on the basis of homology or position in the LEE [4]. EscV, which is similar to YscV/LcrD, is predicted to be an inner membrane protein [72]. EscN is hypothesized to supply ATP for protein secretion and is similar to YscN [72]. SepZ is predicted to be an integral membrane protein and is not homologous to any of the known components of the type III secretion apparatus [76]. EscC is similar to YscC, the outer membrane secretin of the *Yersinia* type III secretion system [72].

EPEC appendages form bridges to the host cell

Recently, using an antibody to one of the EPEC-secreted proteins, EspA, filamentous structures were seen on the bacterial surface by fluorescence microscopy [77]. The anti-EspA-labelled structures were 50 nm in diameter and up to 2 μm long. These structures were produced on wild-type EPEC but were not detected on strains with mutations in the type III secretion apparatus or on cells of the *espA*$^-$ strain. These anti-EspA-labelled filaments formed a bridge between the bacteria and epithelial cell as visualized by both immunofluorescence microscopy and transmission electron microscopy [77]. Combined with the evidence that EspA is required for translocation of EspB into host cells [77], it is appealing to postulate that the EspA filaments act as a channel to deliver proteins inside the host, although the mechanism for translocation is far from proven. When A/E lesions are formed, EspA filaments are excluded from the region of intimate contact, and fully developed A/E lesions are devoid of EspA filaments [77]. Similar filamentous structures were observed in STEC [78]. Contrary to EPEC, the STEC *espA* deletion mutant abolished binding of the bacteria to HeLa cells [78]. The reason for this discrepancy could be that while EPEC has the plasmid-encoded bundle-forming pilus adhesin, whereas no similar adhesins have been identified in STEC. Hence, the EspA filaments may act as adhesins as well as portals for delivery of proteins into eukaryotic cells.

Electron microscopy of *Salmonella typhimurium* revealed supramolecular structures spanning the inner and outer membrane [79]. A type III-dependent surface appendage on the plant bacterial pathogen *Pseudomonas syringae* has been recently discovered [80]. It has been postulated that the surface projections and associated fimbrial extensions found on the obligate intracellular pathogen *Chlamydia* are the type III secretion apparatus, and associated virulence effectors, respectively [81].

EspB might form translocation channel

It was recently shown that EspB is translocated directly into the host cell, where it is distributed between the membrane and cytoplasm [82]. Previously it was shown that EspB is resistant to protease digestion upon association with epithelial cells [83]. EspB-CyaA fusion protein was secreted but not translocated by an *espB* mutant, and soluble EspB cannot transcomplement this mutant, indicating that EspB is both translocated and required for protein translocation [82]. In addition to these lines of evidence, EspB has weak structural similarity to YopB, suggesting that EspB might be involved in forming the translocation channel in the host cell membrane [82].

Role of the type III in Tir delivery

The transfer of Tir into host cell membranes is facilitated by the type III secretion apparatus and secreted proteins, EspA and EspB [5]. No Tir was found in the membrane of host cells infected with mutants in the type III secretion apparatus, *espA*, or *espB*. It is important to note that other than in one instance [5], Tir

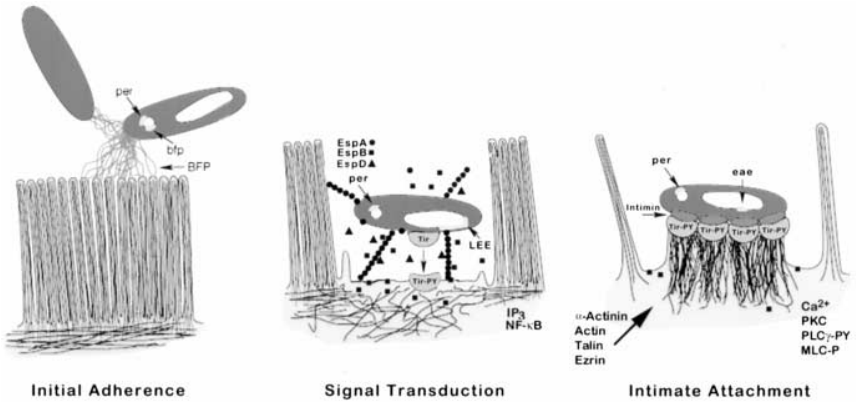

Figure 4. Three-step model of EPEC interactions with cultured epithelial cells. The first step, initial adherence, is mediated by the plasmid-encoded BFP, and brings the bacteria into close proximity with the host cell. In the second step, signal transduction, EPEC transmits a variety of signals to the host cell, including the formation of EspA filaments, and the translocation of Tir and EspB into the host cell. Signalling pathways activated in the host cell in response to EPEC include tyrosine kinases, IP fluxes and activation of NF-κB. This leads to the third step, intimate adherence, in which the bacterial outer membrane protein intimin binds to its receptor, Tir, leading to cytoskeletal rearrangements and the formation of actin-rich pedestals beneath the adhering EPE. Adapted from [91].

delivery has been monitored by probing with anti-phosphotyrosine antisera [77, 82]. Studying Tir delivery by monitoring its modified form precludes the understanding of what form of Tir is delivered and its dependence on the type III secretion system and secreted proteins.

Tir does not retain its N-terminal methionine

Type III secreted proteins, including Tir, are characteristic in that they lack an amino-terminal signal sequence that is present in sec-dependent secreted proteins. Unlike many proteins secreted by type III systems, purified Tir does not retain its amino-terminal methionine [5]. SopE of *S. dublin* also has this modification [84]. While this was thought to indicate that Tir may not directly use a type III secretion system, the lack of the amino-terminal methionine could be due to the activity of amino-terminal methionine specific peptidases that cleave when the second amino acid is glycine, alanine, serine, valine, threonine (as in the case of SopE) or proline (as in the case of Tir) [85].

Cytosolic chaperones

Secretion of the Yops involves cytosolic chaperones called Syc (specific yop chaperone). These chaperones are small and usually acidic proteins that bind specifically to secreted proteins for secretion and presecretory stabilization. YopE secretion requires SycE/YerA, and interestingly YopE does not accumulate in *sycE*/*yerA* mutants [86, 87]. The chaperone for YopH, SycH, affects secretion, and YopH accumulates in the cytoplasm of the *sycH* mutants [53]. These cytosolic chaperones recognize an amino-terminal motif in the proteins to be secreted that overlaps the translocation domain [88]. There is only one Syc-binding domain in YopE (residues 15–20) and YopH (residues 20–70) [88]. Deleting this domain does not affect secretion of YopE and YopH and even suppressed the need for the chaperone in the secretion process [88]. Thus the chaperones seem to prevent premature interactions with other proteins during secretion and translocation, and therefore are referred to as 'bodyguards' to reflect their preventative and protective functions [88]. Recently it was shown that EspB and EspD require a specific chaperone, CesD, for proper secretion from EPEC [89]. OrfU is a hypothetical EPEC protein that is weakly similar to SycH and is predicted to be small (17 kDa) with an acidic isoelectric point [90]. The similarity of OrfU to SycH and its location suggest that it may act a chaperone for Tir [4].

Tir insertion needs to be elucidated

The mechanism of Tir delivery into host cell membranes is unknown, although it seems clear that a type III secretion system is involved. This leads to several questions of how the type III secretion/translocation system works with Tir. What does Tir interact with inside the bacteria during its translocation? What is the role of Tir's putative chaperone? When is Tir delivered? Does Tir get inserted directly into the host cell membrane or is there a cytoplasmic intermediate? When does Tir become

phosphorylated, and is this involved in insertion? Elucidating the mechanism by which Tir is injected into the host cell membrane by the type III secretion system will impact on several areas since many pathogens, both animal and plant, inject virulence factors into host cells.

Three-stage model of EPEC pathogenesis

Using the information collected from these in vitro experiments, a three-step model for EPEC interacting with epithelial cells to form an A/E lesion has been postulated (fig. 4) [7, 91]. The initial nonintimate interaction with the host cell is mediated through the plasmid-encoded BFP in a pattern known as the localized adherence phenotype [92]. Adherence of EPEC to epithelial cells triggers a variety of signal transduction pathways via EspA, EspB and EspD and possibly other secreted proteins. Recent evidence indicates that the EspA filaments might act as a channel, and EspB might form a pore in the host cell membrane to act in combination with the type III secretion apparatus to deliver proteins inside the host cell [77, 82]. The insertion of Tir into the host cell membrane and its tyrosine phosphorylation are facilitated by these structures [5]. Intimate adherence is mediated through the binding of intimin to the tyrosine phosphorylated form of Tir, and this probably causes cytoskeletal rearrangements to form pedestals and pseudopods upon which EPEC resides. While understanding these in vitro systems is important and can shed light on EPEC-mediated disease, in reality the disease occurs inside an organism, thus in order to obtain a complete picture, EPEC infection must be studied in in vivo systems.

In vivo models for EPEC disease

The ability of EPEC to form A/E lesions is a major characteristic of EPEC pathogenesis. Despite increasing information about factors involved in A/E lesion formation in vitro, little is known about their role in virulence. One approach has been to infect human volunteers. One study examined the role of the BFP, which is believed to mediate initial, nonintimate adherence to the host epithelium [93]. The results from this study implicated several BFP genes that are necessary for full EPEC virulence. Intimin has been shown in vitro to be essential for A/E lesion formation, and intimin's role in disease was exam- ined by infecting adult human volunteers with either EPEC wild-type or intimin mutant strains [13]. Although the importance of intimin and intimate adherence in EPEC pathogenesis was demonstrated in this study, diarrhea still developed in 4 of 11 volunteers receiving the intimin mutant strain, indicating that other virulence factors are involved in EPEC pathogenesis.

Although much valuable data has been obtained using human studies, there are several major limitations to using adult humans as model for EPEC infection. Infants, rather than adults, are the normal host for EPEC [1]. Additionally, high doses of EPEC (2×10^{10}) are needed to elicit diarrhea in adults, which does not reflect the natural infection state [13]. An alternate strategy has been to develop an animal model of EPEC infections. This has proven difficult due to the human specificity of EPEC infections. Several animal pathogens related to human EPEC have been identified. These include REPEC (rabbits) [94, 95], *E. coli* O45 (pigs) [96], DEPEC strain 4221 (dogs) [97] and *Citrobacter rodentium* (mice) [98]. Interestingly, adaptation of these strains to host body temperature may contribute to the species specificity observed. For example, maximal expression of EPEC Esp proteins was observed at 37 °C (human body temperature), whereas expression was reduced at 39 °C (rabbit body temperature) [99]. In contrast, maximal expression of REPEC Esp proteins occurred at 39 °C. The most well characterized animal EPEC strain is REPEC, which has been useful in revealing some of the mechanisms of EPEC virulence.

History and characteristics of REPEC pathogenesis

In 1977, Cantey and Blake first isolated the RDEC-1 (rabbit diarrheagic *E. coli* serotype O15) strain from rabbits suffering from diarrhea [94]. Takeuchi et al. [100] showed that RDEC-1 triggered the formation of A/E lesions within the rabbit intestine, although the terminology of 'attaching and effacing' was defined later [2]. Since RDEC-1 was first isolated from rabbits in the United States [94], strains with the same serotype have been isolated in Europe [95, 101]. Furthermore, many variants of REPEC with different serotypes have been isolated, and serotype O103 was the most predominant, and is pathogenic in weaned rabbits by oral inoculation [95, 102]. Since RDEC-1 and REPEC O103 form A/E lesions, both strains have been used to develop a relevant EPEC animal model.

REPEC pathogenicity

Interestingly, REPEC also appears to have host specificity (rabbit) like EPEC (human). As described above, maximal expression of REPEC Esp proteins was observed at normal rabbit body temperature [99]. Furthermore, adherence of the REPEC strain RDEC-1 was characterized by incubating RDEC-1 with intestinal brush borders prepared from rats, guinea pigs, rabbits

and humans [103]. RDEC-1 adherence to rabbit brush borders was at least 13-fold greater than that to rat, guinea pig and human tissue, with RDEC-1 eliciting diarrhea in rabbits, but not in rats or guinea pigs. RDEC-1 virulence appears to depend on age and/or body weight [94]. When 0.7–1.1-kg rabbits were inoculated with RDEC-1 via an oral route, all rabbits showed diarrhea. In contrast, when 1.4–1.8-kg rabbits were infected with the same dose, two of five rabbits showed no diarrheal symptoms. Although as few as 100 RDEC-1 bacteria can cause diarrhea in 0.7–1.1-kg rabbits, infection of older or heavier rabbits with this same dose does not necessarily result in diarrhea. The age or body weight dependence of the infection mechanism may be a common feature of many attaching and effacing pathogens.

The Peyer's patch as a REPEC colonization site

Vibrio cholerae and enterotoxic and enteroinvasive *E. coli* colonize the gut within a few hours and produce diarrhea rapidly. In contrast to these pathogens, REPEC-mediated diarrhea is observed 6 to 7 days after infection. Although RDEC-1 adheres to epithelial surfaces of the ileum, cecum and colon, this pathogen does not colonize the rabbit intestine extensively until 3 to 4 days after infection [94]. The delay in RDEC-1 colonization may be a reason for the later onset of diarrhea. Although the adherence of human EPEC to the Peyer's patch is still unclear, REPEC preferentially adheres to and colonizes the Peyer's patch [104–107]. RDEC-1 adheres to the Peyer's patches to a significantly greater extent than REPEC O103 at early time points after infection [107]. However, REPEC O103 produced severe inflammation despite the decreased adherence. It can be postulated that REPEC colonizes the Peyer's patches, is shed and migrates into the ileum, cecum and colon.

Analysis of EPEC virulence factors using rabbit infection models

Many characteristics of REPEC pathogenesis are quite similar to that observed with EPEC, in that multiple steps are involved with A/E lesion formation, suggesting that the EPEC three-stage infection model [91] can be extended to REPEC pathogenesis. A major difference is that EPEC and REPEC show different patterns of adherence to epithelial cell surfaces. As the pattern of initial adherence does not affect A/E lesion formation [99, 108], REPEC can still serve as a relevant EPEC animal model. In EPEC, the genes involved in A/E lesion formation are encoded in the LEE pathogenicity island, and recently the LEE was characterized in REPEC serotypes O15 and O103 [109, 110]. The profile of LEE-encoded secreted proteins in REPEC O103 and RDEC-1 is similar to that in EPEC. Both rabbit strains secrete EspA, EspB and EspD into the culture media [99, 110, 111]. Cultured epithelial cells infected with either EPEC, REPEC O103 or RDEC-1 accumulated actin and tyrosine-phosphorylated proteins beneath adherent bacteria [6, 99, 111], and deletion of either the EspA or EspB genes from all three strains abolished this effect [99, 111–113]. These results suggest that EspA and EspB, in both EPEC and the REPEC strains, are necessary for triggering cytoskeletal rearrangements, and indicate that the function of LEE in REPEC, including the induction of host signalling, is similar to that of EPEC.

Initial adherence factor involved in virulence

Unlike EPEC, REPEC strains including RDEC-1 and serotype O103 show a 'diffuse adherence (DA)', with bacteria adhering evenly over the monolayer of cultured cells [114]. Different adhesive factors associated with the DA phenotype have been identified in REPEC strains. In RDEC-1, adherence is mediated by a fimbria termed AF/R1 (adherence factor/rabbit 1) [115, 116], encoded on an 86-MDa plasmid. The major subunit of AF/R1 is a 17-kDa protein termed AfrA [116] that shares 43% homology to the FimA subunit of type I pilus from *E. coli* [117] and 42% homology to the PapA pilus from human uropathogenic *E. coli* [118]. Strains lacking AF/R1 still elicit disease in infant rabbits and form A/E lesions, but are less virulent than the wild-type strain [108]. This is analogous to the BFP in EPEC, where the factor involved in initial adherence is not involved in formation of A/E lesions. Although AF/R1 was first characterized as the REPEC adhesin, the genes encoding AF/R1 have not been detected in other REPEC strains. Two different types of fimbriae were isolated from REPEC strains: Ral from serotype O15 [119], and AF/R2 [120] from serotype O103. Both adhesins showed homology to K88 [121] and CS31A [122], fimbrial adhesins from swine and bovine enterotoxigenic *E. coli*, and may represent new members of the *E. coli* K88 adhesion family.

Type III secretion apparatus as a virulence factor

The type III secretion apparatus was demonstrated to be a virulence factor using a rabbit infection model [110]. All rabbits infected with the wild-type strain suffered weight loss, whereas 17 of 18 had diarrhea, and 16 of 18 rabbits died. These results indicate that REPEC O103 is highly virulent in weaned rabbits, and has a greater than 80% mortality rate. In contrast, rabbits inoculated with the same dose of the type III mutant strain did not show any diarrheal symptoms or

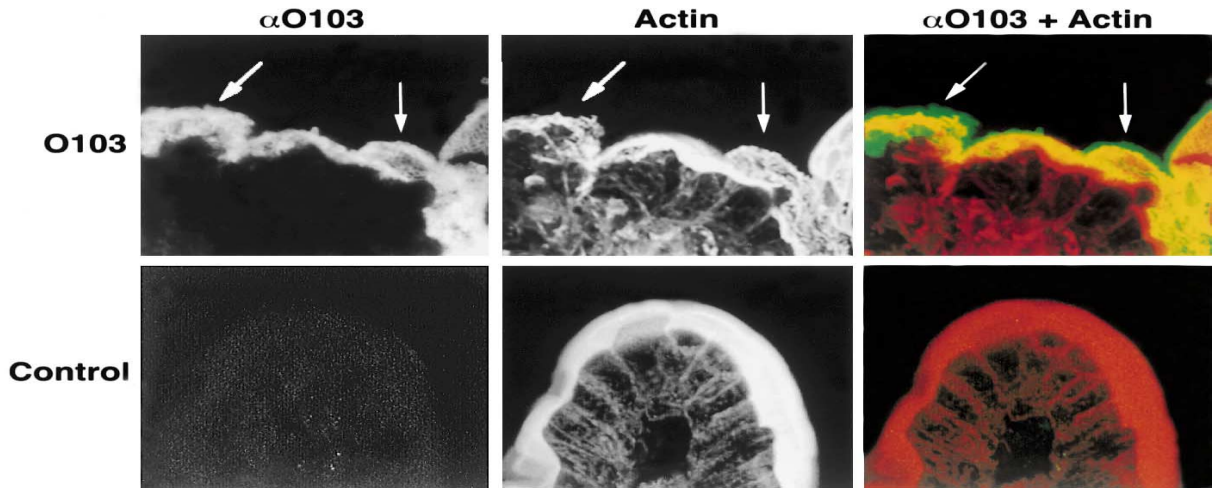

Figure 5. Confocal laser scanning micrographs of ileal surfaces from rabbits infected with REPEC O103 or control (10 μm thickness) were stained with phalloidin-Texas red (red for overlay) and anti-O103 antibody (green for overlay). REPEC O103 adhered to and colonized the ileal surface, cytoskeletal actin beneath the attached bacteria was rearranged and cuplike structures were also observed as indicated by arrows. In contrast, no actin rearrangements occurred in the PBS control,

weight loss. However, bacterial colonization of the intestinal tract was unaffected by this mutation, suggesting that other factors, such as the adhesin AF/R2, may be involved in the persistent colonization observed with the mutant strain.

EspA and EspB are virulence factors

The type III secretion apparatus mutants do not secrete EspA, EspB or EspD [110]. Accordingly, the loss of virulence by the mutation in type III apparatus might be due to a lack of these secreted proteins. To determine if EspA and EspB are involved in the disease process, we have carried out in vivo infection studies using REPEC O103 EspA and EspB mutant strains [111]. Rabbits were infected orally with either REPEC O103 or the mutant strains, and the virulence was compared to the wild-type strain. Rabbits infected with REPEC O103 suffered weight loss, and one rabbit died with watery diarrhea 7 days after infection. Rabbits that survived the wild-type infection showed significant weight loss compared with rabbits given phosphate buffered saline (PBS). In contrast, rabbits infected with the EspA and EspB mutant strains did not show any symptoms of diarrhea or weight loss. These results clearly show that EspA and EspB are critical for the disease process.

The difference between wild-type and both mutant strains was also observed in the histological analysis of intestinal tissues. Only rabbits infected with wild-type showed evidence of blunting of intestinal villi and necrosis of mucosal epithelial cells. In contrast, rabbits infected with either mutant strain did not show these effects, indicating that EspA and EspB are involved in inflammation and disruption of the mucosal epithelial surface in vivo. Furthermore, the disruption of the epithelial surface does not correlate with adherence ability. Although the adherence of EspA and EspB mutant strains was somewhat delayed when compared with that of wild-type, both strains adhered to the ileum, Peyer's patches and proximal colon. These results indicate that EspA and EspB are not involved in initial adherence.

EspA and EspB are critical for A/E lesion formation. Rabbits infected with the wild-type strain showed typical A/E lesions associated with the ileum, Peyer's patches and proximal colon. In contrast, when rabbits were infected with either mutant strain, no A/E lesions were detected in intestinal tissue, suggesting that EspA and EspB are needed to trigger A/E lesions. Actin rearrangements beneath adhering bacteria were visualized using confocal scanning laser microscopy. Wild-type REPEC O103 dramatically reorganized the epithelial cell cytoskeleton, with actin accumulation beneath the adherent bacteria (fig. 5). In contrast, neither mutant strain triggered cytoskeletal rearrangements. These results indicate that EspA and EspB are involved in triggering cytoskeletal actin rearrangements, and the formation of cuplike structures in vivo, and that these processes are necessary for disease.

As described earlier in this text, a three-step model for in vitro EPEC interactions with epithelial cells has been

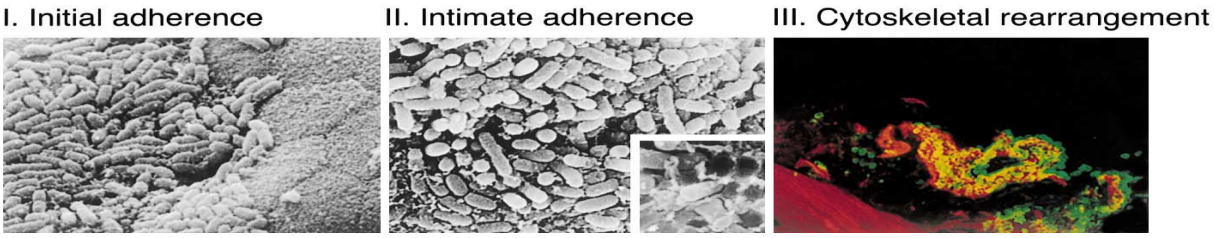

Figure 6. Three-step model for in vivo REPEC interactions with epithelial cells. In step one, REPEC adheres to intestinal tracts with diffuse adherence. This nonintimate adherence is mediated by the AF/R2 and/or other adhesins, and EspA and EspB are not involved. Signal transduction involving EspA and EspB results in intimate adherence of REPEC to the epithelial cell surface, and the formation of cuplike structures (see inset, center panel). Intimate adherence leads to extensive rearrangement of host actin beneath adhering bacteria, and disruption of ileal villi. Scanning electron micrograph courtesy of Dr. Ursula Heczko.

described (fig. 4). Based on findings using the rabbit EPEC model, we can exend this model to the situation in vivo (fig. 6). The first step, nonintimate adherence, was demonstrated using the REPEC EspA and EspB mutant strains, which adhere to the rabbit intestinal epithelium without leading to A/E lesion formation. In vivo, as in vitro, signal transduction events lead to intimate adherence and A/E lesion formation. The A/E lesions formed are similar to those observed with in vitro: REPEC infection causes rearrangement of the host actin cytoskeleton, and the formation of pedestals beneath the adherent bacteria.

The rabbit model of EPEC infection has provided much useful information about how factors identified in in vitro studies contribute to disease. These findings include a role for EspA and EspB in both A/E lesion formation and disease. However, the role of Tir in virulence is still unclear. Tir may be involved in intimate adherence, or in transducing signals within the host cell, but little is known concerning how induction of host signal transduction pathways contributes to EPEC virulence. The rabbit model for EPEC infection provides a useful tool for examining the role of virulence factors in a system that is more akin to the situation of human disease.

Conclusions

The ability to attach intimately to the host cell and form A/E lesions is essential to EPEC pathogenesis. Recent work has led to the discovery of bacterial factors and host responses involved in this function. These include the identification of the type III secretion of the EPEC virulence factors EspA and EspB, along with the unexpected finding that EPEC translocates its own receptor for intimate adherence to the host cell membrane. The finding that Tir is of bacterial origin has raised several intriguing questions. One compelling issue is where Tir came from and how it evolved. Additionally, the role played by Tir and Tir tyrosine phosphorylation in signal transduction and cytoskeletal reorganization is unknown. How intimin binding focuses the cytoskeletal rearrangements around Tir, and the role of Tir and other EPEC virulence factors in inducing these rearrangements remains uncharacterized.

Another area of interest is elucidating how Tir is inserted into the host cell membrane. It is clear that the type III secretion apparatus and secreted proteins facilitate the delivery of Tir into host cell membranes, but what is not immediately evident is how this apparatus functions to do this. If the delivery of bacterial receptors into host cells is far-reaching, then it is crucial to understand how EPEC goes about this.

A critical question is how EPEC causes diarrhea. Studies have implicated several mechanisms in vitro, but whether these occur in vivo is not known. The development of in vivo models for EPEC infection has greatly increased our understanding of the roles factors identified in vitro play in disease. Based on these studies, intimin, the type III secretory apparatus, EspA and EspB make up a growing list of proven EPEC virulence factors. We have only begun to understand how this enteric pathogen affects and effects host cells. Ultimately, this information will contribute to increasing our understanding of how EPEC mediates disease, and to our efforts to design treatments, including antibiotics and vaccines.

Acknowledgements. Work in B.B.F.'s laboratory is supported by operating grants from the Medical Research Council of Canada (MRC) and ID Vaccine Corp. A.G. is supported by a post-graduate scholarship from the Natural Sciences and Engineering Research Council of Canada, and B.B.F. is a Howard Hughes International Research Scholar Award and an MRC scientist.

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