Mutagenesis of conserved tryptophan residues within the receptor-binding domain of intimin: influence on binding activity and virulence

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Intimate bacterial adhesion to intestinal epithelium is a pathogenic mechanism shared by several human and animal enteric pathogens, including enteropathogenic and enterohaemorrhagic Escherichia coli and Citrobacter rodentium. The proteins directly involved in this process are the outer-membrane adhesion molecule intimin and the translocated intimin receptor, Tir. The receptor-binding activity of intimin resides within the carboxy terminus 280 aa (Int280) of the polypeptide. Four tryptophan residues, W117/776, W136/795, W222/881 and W240/899, are conserved within different Int280 molecules that otherwise show considerable sequence variation. In this study the influence of site-directed mutagenesis of each of the four tryptophan residues on intimin-Tir interactions and on intimin-mediated intimate attachment was determined. The mutant intimins were also studied using a variety of in vitro and in vivo infection models. The results show that all the substitutions modulated intimin activity, although some mutations had more profound effects than others.

Keywords: Citrobacter rodentium, EPEC, EHEC, Tir

INTRODUCTION

Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) Escherichia coli are important causes of severe infantile diarrhoeal disease (Nataro & Kaper, 1998). EPEC and EHEC colonize the gastrointestinal mucosa and, by subverting intestinal epithelial cell function, produce a characteristic histopathological feature known as the ‘attaching and effacing’ (A/E) lesion (Frankel et al., 1998a; Kaper et al., 1998). The A/E lesion is characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to the host-cell plasma membrane and the formation of an underlying pedestal-like structure in the host cell consisting of polymerized actin, ezrin, talin and myosin (Frankel et al., 1998a; Kaper et al., 1998), as well as WASP (Wiskott–Aldrich syndrome family of proteins) and the Arp2/3 complex (Kalman et al., 1999). E. coli capable of forming A/E lesions have also been recovered from diseased cattle, dogs, cats, rabbits and pigs (Nataro & Kaper, 1998). In mice, Citrobacter rodentium colonizes gut enterocytes via A/E lesion formation and, like EHEC in humans, causes disease in the large bowel (Barthold et al., 1976; Schauer & Falkow, 1993a).

The genes encoding the A/E phenotype are encoded on a pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). The majority of the LEE genes are organized in five polycistronic operons (LEE1, LEE2, LEE3, tir and LEE4) (Mellies et al., 1999). LEE1, LEE2 and LEE3 encode components of a type III secretion system (TTSS), LEE4 encodes proteins secreted by the TTSS, termed E. coli secreted proteins (EspS) (Elliott et al., 1998; Frankel et al., 1998a), and the tir operon encodes the outer-membrane adhesion molecule, intimin (Jerse & Kaper, 1991; Jerse et al., 1990), the translocated intimin receptor, Tir (Deibl et al., 1998; Kenny et al., 1997), and CesT (the Tir chaperone) (Abe et al., 1999; Elliott et al., 1999).
Studies on intimin in EPEC, EHEC and C. rodentium have demonstrated its importance in bacterial colonization and virulence (Dean-Nystrom et al., 1998; Donnenberg et al., 1993b; McKee et al., 1995; Marches et al., 2000; Schauer & Falkow, 1993b). The receptor-binding domain of intimin molecules is localized to the C-terminal 280 aa (Int280) (Frankel et al., 1994, 1995). Furthermore, based on sequence variation within Int280, five distinct intimin subtypes (α, β, γ, δ and ε) have been described (Adu-Bobie et al., 1998; Oswald et al., 2000). Recently, the structure of Int280α complexed with Tir was determined by NMR and X-ray crystallography (Batchelor et al., 2000; Luo et al., 2000). The global fold of Int280α shows three globular domains. The first two domains (residues 1–91 and 93–181) each comprise β-sheet sandwiches that resemble the immunoglobulin super family (IgSF). Despite no significant sequence homology, the topology of the C-terminal domain (residues 183–280) is reminiscent of the C-type lectin domains (CTLD), a family of proteins responsible for cell-surface carbohydrate recognition (Weis & Drickamer, 1996).

The overall organization and structure of the receptor-binding domain of other intimin types is predicted to be similar to Int280α, despite the fact that sequence analysis of the Int280 domains reveals high levels of sequence diversity. Nevertheless, four Trp residues (W117/776, W136/795, W222/881 and W240/899) (positions numbered according to Int280α/whole intimin) α are conserved in all intimins sequenced to date. Two of the four Trp residues (W222/881 and W240/899) are part of short conserved islands in the CTLD modules that have been hypothesized to play a role in receptor-binding activity. In this report we describe results of site-directed mutagenesis of the Trp residues. Using *in vitro* (HEp-2 cells) and *in vivo* (mice) infection models, as well as biochemical approaches, we report diverse modulation of intimin activity.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains used in this study include wild-type EPEC strain E2348/69 (O127:H6) (Levine et al., 1985), *eae* deletion mutants of E2348/69, strain CVD206 (Donnenberg & Kaper, 1991), *C. rodentium* strain DS525 (Schauer & Falkow, 1993b), and *E. coli* strains XL-1 Blue and BL21. Bacterial strains were grown in L-broth. Media were supplemented with 30 μg kanamycin ml⁻¹, 30 μg chloramphenicol ml⁻¹ or 100 μg ampicillin ml⁻¹. Plasmids are listed in Table 1.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene), following the manufacturer’s instructions, employing plasmid pCVD438 that encodes intimin α (Donnenberg & Kaper, 1991) as a template. Complimentary mutagenesis oligonucleotide pairs, incorporating single amino acid substitutions, were as follows. Sense oligonucleotides: W776/117A, 5′-GGTACCACCTGATGCTTGCATATGGG-3′; W797/136A, 5′-GGAAAATATACAGCTGCGTCA-GCAATCCC-3′; W881/222A, 5′-CTTTAAACGCTGGGGCTGCAATAATAGG-3′; W899/240A, 5′-GACTATATTTCAGCTGACATCAACACAGC-3′. Antisense oligonucleotides: W776/117A, 5′-CCATATTGAAGCTACGTGGTACAGG-3′; W797/136A, 5′-GGATTGCTGACGGAGTGTATATTTTCC-3′; W881/222A, 5′-CATATTATTGGCAGCCCCAGCTGTTTTAAAG-3′; W899/240A, 5′-GCTGGTTGTGTGTCAGTGAATTTAAGTGC-3′.

Mutated plasmids containing staggered nicks were generated by extension of primers annealed to opposite strands of the denatured plasmid by temperature cycling (1 cycle of 95 °C for 30 s, then 16 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 18 min) in the presence of the high fidelity *Pfu* DNA polymerase. Synthesized DNA containing the desired mutation was selected from the original DNA template by incubation with *DpnI* at 37 °C for 1 h, on the basis that dam-methylated parental DNA template would be susceptible to digestion, whereas the newly synthesized unmethylated mutated plasmid would not. Nicks in the plasmid were repaired following transformation of 1 μl of the synthesized products into competent *E. coli* XL-1 Blue cells. Chloramphenicol-resistant transformants were randomly selected and inoculated into overnight L-broth cultures for use in plasmid minipreps (Qiagen). Correct incorporation of each mutation was monitored by DNA sequencing using a labeled DNA sequencer. The mutated plasmids were then transformed into an *eae* deletion mutant of EPEC strain CVD206 (Donnenberg & Kaper, 1991).

**Construction of maltose-binding protein (MBP)–Int280 fusions.** The mutated pCVD438-derivative plasmids were then used as templates to amplify the DNA fragments encoding the mutated Int280 regions by PCR using one set of primers (Forward primer, 5′-GGAAATTCATTACTGAGATTAAGGCT-3′; Reverse primer, 5′-GGGATCCCTTTATTGTTACAACAGTGGC-3′). Following digestion with EcoRI and BamHI the DNA fragments were subcloned into pMAL-c2 for expression as MBP fusions (Table 1).

**Gel overlays.** Purified His-Tir-M, expressed from pICCl26 in *E. coli* BL21, was purified as described by Hartland et al. (1999). MBP–Int280 derivatives were expressed from recombinants pMal-c2 and purified from *E. coli* XL-1 Blue as described by Frankel et al. (1994). His-Tir-M was separated by SDS-PAGE and blotted onto a nitrocellulose membrane which was blocked with 10% skim-milk in PBS/0.1% Tween-20 overnight. The nitrocellulose membranes were reacted with 5 μl of the purified MBP–Int280 fusions ml⁻¹ or MBP in PBS/0.1% Tween-20 for 2 h and washed twice for 5 min in PBS/0.1% Tween-20. MBP–Int280 fusion proteins binding to Tir were detected with anti-MBP antiserum (1:2000 for 1 h) and then anti-rabbit antibodies conjugated to alkaline phosphatase (1:2000 for 1 h) as described by Hartland et al. (1999).

**Fluorescent actin stain (FAS) assays, detection of surface intimin expression and Western blots.** Expression of the intimin derivatives on the surface of CVD206 and their ability to mediate A/E lesion formation was assessed using rabbit Int280α antisera (Frankel et al., 1998b) and the FAS test developed by Knutton et al. (1989), respectively. Briefly, HEp2 cells were grown to approximately 80% confluency on coverslips in 24-well plates. Cells were infected with 10 μl static overnight L-broth cultures in medium lacking antibiotics for 3 h. The monolayers were then washed with PBS, fixed by the addition of 300 μl 10% formalin for 20 min and washed again. For detection of FPEC-associated intimin, coverslips were incubated with the anti-Int280α polyclonal antisera (1:50 dilution) for 45 min in Dulbecco’s modified Eagle medium (DMEM) and then with a secondary tetramethylrhodamine isothiocyanate (TRITC)-labelled anti-rabbit antibody (1:250 dilution) for 30 min. Following washes, the
Table 1. List of plasmids used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pCVD438</td>
<td>pACYC184 encoding intimin α</td>
<td>Donnenberg &amp; Kaper (1991)</td>
</tr>
<tr>
<td>pICC201</td>
<td>pCVD438 encoding intimin W776A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC202</td>
<td>pCVD438 encoding intimin W797A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC203</td>
<td>pCVD438 encoding intimin W881A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC62</td>
<td>pCVD438 encoding intimin W8896A</td>
<td>Batchelor et al. (2000)</td>
</tr>
<tr>
<td>pMal-C2</td>
<td>Cloning vector generating translational fusions with MBP</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pICC22</td>
<td>pMal encoding MBP–Int280α</td>
<td>Frankel et al. (1994)</td>
</tr>
<tr>
<td>pICC209</td>
<td>pMal encoding MBP–Int280w117A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC210</td>
<td>pMal encoding MBP–Int280w136A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC211</td>
<td>pMal encoding MBP–Int280w222A</td>
<td>This study</td>
</tr>
<tr>
<td>pICC63</td>
<td>pMal encoding MBP–Int280w240A</td>
<td>Batchelor et al. (2000)</td>
</tr>
<tr>
<td>pICC18</td>
<td>pET28a encoding Tir-M</td>
<td>Hartland et al. (1999)</td>
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Monolayers were permeabilized with 0.1% Triton X-100 for 4 min. Filamentous actin was subsequently stained with 5 µl fluorescein-isothiocyanate-labelled phalloidin in PBS (0.1 µg ml⁻¹) for 20 min (FAS test). Following extensive washes with PBS, the infected cultures were visualized by fluorescence microscopy.

Expression of the intimin derivatives was also determined by Western blotting. Briefly, stationary-phase L-broth cultures were diluted 1:100 in DMEM and incubated for 3 h at 37 °C. An equivalent of an OD₆₀₀ of 0.5 was loaded onto a 7.5% SDS-polyacrylamide gel as described (Adu-Bobie et al., 1998). The electrophoresed polypeptides were transferred to a nitrocellulose membrane and immunodetection of intimin was performed using anti-Int280, diluted 1:500 as described previously (Adu-Bobie et al., 1998; Frankel et al., 1998b).

Fluorescence-activated cell sorter (FACS) analysis of surface-expressed intimin by indirect immunofluorescence. CVD206(pCVD438) derivatives were grown overnight in 5 ml L-broth. Cultures were diluted 1/100 in DMEM and grown to an OD₆₀₀ of 0.6–0.8. Bacteria were washed three times in PBS and fixed for 20 min in 3% formaldehyde in PBS. Bacteria were washed with PBS and incubated with anti-Int280 polyclonal antisera (Adu-Bobie et al., 1998) for 30 min at room temperature. Bacteria were then washed with PBS and incubated with FITC-conjugated anti-rabbit IgG (Sigma) at room temperature for 30 min. Bacteria were washed with PBS and 50,000 events were acquired from a Beckman Dickinson FACS Analyser using the FL1 channel.

Challenge of mice with C. rodentium. Female, specific-pathogen-free, C3H/Hje mice (6–8 weeks old) were purchased from Harlan Olac. All mice were housed in individual ventilated cages with free access to food and water. Bacterial inoculums were prepared by culturing bacteria overnight at 37 °C in 10 ml L-broth containing 100 µg nalidixic acid ml⁻¹ plus 50 µg chloramphenicol ml⁻¹. After incubation, bacteria were harvested by centrifugation and resuspended in a 1 ml volume of PBS. Unanaesthetized mice were then orally inoculated with 200 µl of the bacterial suspension using a gavage needle. The viable count of the inoculum was determined by retrospective plating on L-agar containing appropriate antibiotics.

Measurement of pathogen burden. Mice were killed 14 days post-infection by cervical dislocation. The distal 6 cm of colon was removed and weighed after removal of faecal pellets. Colons were then homogenized mechanically using a Seward 80 Stomacher and the number of viable bacteria in colonic homogenates was determined by viable count on L-agar containing appropriate antibiotics.

RESULTS

The effect of mutagenesis on intimin expression and A/E lesion formation in vitro

Four tryptophans, namely W117/776, W136/795, W222/881 and W240/899, lie in the Tir-binding domain of intimin (D2 and D3 of Int280) (Batchelor et al., 2000). These are distributed throughout the structure (Fig. 1), and based on sequence alignment all four are conserved.

Fig. 1. Schematic representation of the structure of D2 and D3 of Int280 comprising the Int190 super domain (Batchelor et al., 2000). The location within the super domain of the four Trp residues selected for mutagenesis is indicated. Numbers by the amino acid residues represent positions in the full-length intimin α.
within all intimin types (Fig. 2). Although W240/889 is buried proximal to the residues involved in Tir-binding, experimental evidence supports a role as a structural scaffold for the Tir binding site within D3 (Batchelor et al., 2000). W222/881 is also particularly interesting as it lies in one of the most highly conserved regions (WAGGANKY) and is present at the interface between D2 and D3 (Figs 1 and 2). W117/776 is buried within D2 and lies proximal to the D2/D3 interface. W136/795 lies centrally within D2 and appears to fulfill largely structural requirements for the folding of this domain (Fig. 1).

To determine the importance of the W117/776, W136/795 and W222/881 residues for intimin-mediated A/E lesion formation on HEp-2 cells, the mutations were introduced, individually, into pCVD438 harbouring the eae gene encoding intimin α (Donnenberg & Kaper, 1991), generating plasmids pICC201 (intimin W117/776), pICC202 (intimin W136/776) and pICC203 (intimin W222/881) (Table 1). The mutant CVD206 derivatives were introduced into CVD206 (Donnenberg & Kaper, 1991), which harbours a null deletion in eae, and the CVD206 derivatives were subjected to a number of biological assays designed to determine the influence of the mutations on intimin function in vitro. Western blot analysis of whole-cell lysates prepared from the different CVD206(pCVD438) derivatives reacted similarly with anti-Int280α antiserum (Fig. 3). No reactivity was seen with CVD206, suggesting that the multiple bands are intimin degradation products. Similar patterns of intimin blots have been reported previously (Adu-Bobie et al., 1998; Frankel et al., 1998b; Reece et al., 2001). Indirect immunofluorescence staining showed that after a 3 h incubation with HEp-2 cells, all the adhering CVD206(pCVD438)-derivatives, but not CVD206, expressed intimin (Fig. 4). Surface intimin expression was quantified using FACS analysis. Although CVD206(pICC201) presented somewhat higher fluorescent intensity, an overall similar level of surface intimin expression was observed with all the CVD206(pCVD438) derivatives, while no signal was observed using CVD206 (Fig. 5).

Since expression of intimin was not markedly affected by the introduction of site-directed mutations, the ability of the different CVD206(pCVD438) derivatives to induce actin polymerization in infected HEp-2 cells (FAS test; Knutton et al., 1989), a marker for A/E lesion formation, was determined. This assay showed that like CVD206 and CVD206(pICC62) (W240/899A) (Fig. 4; Batchelor et al., 2000), CVD206(pICC202) (W136/795A) was unable to induce actin polymerization while CVD206(pICC201) (W117/776A) and CVD206(pICC203) (W222/881A) were FAS-positive (Fig. 4).

**Binding of the modified intimins to Tir**

To determine if the site-directed mutagenesis data and the phenotypes described above were due an impaired binding to Tir, Int280 domains harbouring the mutation...
were amplified by PCR and cloned into pMALc-2. This allowed us to express the Int280 domains as translational fusions with MBP. The MBP–Int280 fusion proteins were purified by affinity chromatography (Frankel et al., 1994) and equal amounts (Fig. 6) were used together with purified Tir-M (consisting of the intimin-binding region of Tir; Hartland et al., 1999) in a gel-overlay binding assay. This revealed that MBP–Int280, MBP–Int280 W117/776, MBP–Int280 W222/881 A and MBP–Int280 W136/795 A bound to the immobilized Tir-M, al-
though the latter two seemed to bind somewhat less efficiently. No binding was detected with MBP–Int280_W240A or MBP only (Fig. 7).

Colonic colonization of mice challenged with DBS255(pCVD438) derivatives

*C. rodentium* causes transmissible colonic hyperplasia in mice (Barthold *et al.*, 1976), an infection associated with the formation of A/E lesions similar to those described for human EPEC infection (Schauer & Falkow, 1993a). Accordingly, this model provided an opportunity to evaluate the *in vivo* biological properties of EPEC intimin mutants in mice. To evaluate the effect of site-directed mutagenesis on the biological activity of intimin *in vivo*, the different pCVD438 derivatives were transformed into DBS255, which harbours a null deletion in *eae*, and used to infect mice orally. Fourteen days post-challenge, measurement of pathogen burden in the colons of these mice revealed marked differences in the ability of each strain to colonize the colonic epithelium. Mice infected with DBS255(pCVD438) had high numbers of challenge bacteria in their colons (Fig. 8a) and induced colonic hyperplasia as measured by colonic weight (Fig. 8b).

**DISCUSSION**

In the present investigation we targeted the four Trp residues within the receptor-binding superdomain of intimin (Batchelor *et al.*, 2000) for site-directed mutagenesis. These residues were selected because: (i) they are conserved among all the different intimin types reported thus far; (ii) Trp residues are large hydrophobic moieties that potentially have key structural roles and can be involved in protein interactions; and (iii) two of the residues are part of conserved motifs (Fig. 2) and for this reason were hypothesized to have a role in the binding activity of intimin (Adu-Bobie *et al.*, 1998).

Previous work has shown that W240/899, located on a conserved loop on the D3 domain, is important for intimin–Tir interactions and A/E lesion formation, despite the fact that it does not directly contact Tir (Batchelor *et al.*, 2000). The other residue that forms part of a conserved island within D3 is W222/881. This residue lies at the D2 interface and, together with the adjacent Tyr residues and W117/776, stabilizes a surface pocket, which may be involved in intermolecular interactions, perhaps with a receptor other than Tir. Indeed, mutating either W117/776 or W222/881 did not abolish...
the ability of intimin to bind Tir. In addition, EPEC expressing intimin W117/776A or W222/881A induced A/E lesions on HEP-2 cells. In contrast, a mutation at position W136/795, which is located at the core of D2, resulted in an intimin molecule that when presented on the surface of EPEC could not mediate A/E lesion formation on HEP-2 cells. This was despite the fact that on gel overlays Int280 W136/795A bound to immobi-
lized Tir-M. Importantly, we demonstrated using Western blots, immunofluorescence and FACS that neither mutation affected the level or surface localization of intimin.

The mouse pathogen C. rodentium was used to assess the effect of mutagenesis on the function of intimin in vivo. The expression of intimin α in C. rodentium DBS255 restores the ability of the strain to colonize the mouse colon and to induce colonic hyperplasia (Higgins et al., 1999b). In addition, intimin α can bind Tir from C. rodentium (Hartland et al., 2000). Evaluating intimin mutants in the C. rodentium infection model, where the bacteria have to compete with the normal intestinal flora and establish a long-term association with the mucosal surface, demonstrated that mutation at each of the Trp residues resulted in an attenuated phenotype. No detectable colonic hyperplasia and a reduced pathogen burden in comparison with DBS255(pCVD438) were observed. Nevertheless, in a few individual mice the level of colonization by each of the mutants was higher than that observed for DBS255. No C. rodentium bacteria were recovered at any time from the colons of DBS255-infected mice. We have reported similar results for other intimin mutations (Reece et al., 2001). The absence of detectable colonic hyperplasia in animals infected with C. rodentium derivatives expressing intimin mutants may suggest that a sustained and substantial level of pathogen colonization is required to trigger colonic pathology. Alternatively, these intimin mutations may affect the in vivo stability of the molecule, or potentially, its ability to interact with other receptors in the mouse colon.

Substitution W240/899A produced the most extreme phenotype. This intimin did not bind Tir, nor induce A/E lesions on HEP-2 cells or colonic hyperplasia in vivo. As reported, this residue might have an important structural role within the Tir-binding site (Batchelor et al., 2000). The phenotype associated with W136/795 differed from W240/899 only by the fact that the strain still bound Tir. This indicates that binding to Tir, although necessary, may not be sufficient for production of A/E lesions or that reduced intimin/Tir affinity could not mediate A/E lesion formation. W136/795 is believed to have a structural role in maintaining the integrity of the D2/D3 super domain within intimin. In contrast, residues W222/881 and W117/776, which exhibited similar phenotypes (including binding to Tir, A/E lesion formation on HEP-2 cells with reduced colonization of the mouse colon and no hyperplasia), seem to play a part in a further function of intimin. These conserved residues perhaps stabilize a binding pocket that is implicated in a host–receptor interaction, possibly β1 integrins (Frankel et al., 1996). Indeed, oral infection of mice with live wild-type C. rodentium or intracolonic inoculation of dead bacteria [wild-type and DBS255(pCVD438)] induces a pronounced colitis (Higgins et al., 1999a). This response was not observed in mice inoculated with any of the intimin mutants of C. rodentium.

Intimin is an abundant outer-membrane adhesion molecule that is essential for full virulence both in human volunteers (Donnenberg et al., 1993a) and animal models (Dean-Nystrom et al., 1998; Donnenberg et al., 1993b; McKee et al., 1995; Schauer & Falkow, 1993b). As such it has potential as a vaccine component against A/E-lesion-forming bacterial pathogens. Indeed, we have recently shown that immunization with Int280 can protect mice from oral infection with C. rodentium (Ghaem-Maghami et al., 2001). However, the fact that intimin can mediate colonic hyperplasia on its own (Higgins et al., 1999a) means that to reduce the risk of side effects, this activity of intimin would have to be eliminated. In this study we have produced a number of attenuated (detoxified) intimins. Their use as potential vaccines is currently being evaluated.

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