

Insertion of the Enteropathogenic *Escherichia coli* Tir Virulence Protein into Membranes *in Vitro**

Received for publication, December 20, 2005, and in revised form, January 18, 2006 Published, JBC Papers in Press, January 24, 2006, DOI 10.1074/jbc.M513532200

Paul R. Race, Jeremy H. Lakey¹, and Mark J. Banfield²

From the Institute for Cell and Molecular Biosciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom

Insertion of the enteropathogenic *Escherichia coli* Tir protein into the plasma membrane of intestinal epithelial cells is a crucial event in infection because it provides a receptor for intimate bacterial adherence. This interaction with the bacterial outer membrane protein intimin is also essential in generating a number of signaling activities associated with virulence. Tir can be modified at various sites by phosphorylation and functionally interacts with multiple host proteins. To investigate the mechanism of membrane insertion and to establish a model system in which the multiple interactions/functions of Tir can be uncoupled and independently characterized, we used intrinsic tryptophan fluorescence, surface plasmon resonance, and protease digestion assays to show that Tir can insert directly into phospholipid vesicles in a composition-dependent manner to generate the topology reported *in vivo*. This is the first time that Tir has been shown to insert into membranes in a simple model system in the absence of chemical modification or other factors. These data are consistent with the protein interacting with lipids through two sites. The major site is localized to the transmembrane/intimin-binding domain region and includes Trp²³⁵, which is shown to be an effective reporter of interaction. The minor site is located within the C-terminal domain. Together, these data support a model in which Tir is released into the cytoplasm by the type III translocon and then independently inserts into the plasma membrane from a cytoplasmic location. A thorough understanding of this mechanism will be crucial to understand the subtleties of enteropathogenic *E. coli* pathogenesis.

Enteropathogenic *Escherichia coli* (EPEC)³ is a major cause of infantile diarrheal disease in developing countries (1, 2) and is closely related to enterohemorrhagic *E. coli* (O157), which is an emerging health concern throughout the world (3). EPEC pathogenesis is dependent on (a) an active virulence-associated type III secretion system (TTSS) that directly injects “effector” proteins into host cells and (b) the interaction of Tir (translocated intimin receptor; one such effector protein), inserted into the host cell membrane, with the bacterial outer mem-

brane protein intimin (recently reviewed in Refs. 4 and 5). The intimate adhesion of bacteria to host cell membranes as mediated by the intimin-Tir interaction is essential for virulence (6–8) and results in subversion of host cell signaling processes promoting colonization of the intestinal tract (reviewed in Ref. 9).

Following delivery by the TTSS, Tir inserts into the host cell plasma membrane, adopting a distinct topology composed of a large extracellular loop (the intimin-binding domain (IBD), residues 255–364) and N-terminal (residues 1–233) and C-terminal (residues 385–550) domains, which remain on the cytosolic face of the membrane (7, 10). These domains are linked by two predicted transmembrane helices (residues 234–254 and 365–384). In this topology, the extracellular domain is available to bind intimin (see Ref. 11 for the x-ray crystal structure of this interaction), and the N- and C-terminal domains are exposed to the cytoplasm for interaction with host cell proteins.

The mechanism by which Tir inserts into the target cell membrane following delivery by the TTSS is poorly understood, and very little detail is available at the molecular level. Ectopic expression of Tir within host cells can produce a functional inserted protein that interacts with intimin (12), demonstrating that the protein can insert from the cytoplasm and does not require other bacterial factors. However, it remains possible that insertion can be enhanced by bacterial factors (acting as efficiency factors) and/or host factors such as kinases that modify Tir (e.g. phosphorylation). To uncouple the multiple factors involved in Tir localization and function and to investigate the properties described above requires an *in vitro* model system in which tight control can be maintained over contributing factors.

Following expression *in vivo*, Tir is bound by its TTSS chaperone CesT. This interaction stabilizes the protein in the bacteria and assists in trafficking to the bacterial side of the TTSS (13–15). Previous studies with purified soluble Tir have shown that it adopts a folded structure (16), and recent data show that the protein exists as a monomer (as determined by analytical ultracentrifugation), is monodisperse (as determined by dynamic light scattering), and displays cooperative unfolding kinetics (as measured by thermal denaturation circular dichroism).⁴ Therefore, current evidence shows that Tir can be produced in the absence of CesT and appears to adopt a stable state in solution.

To date, some of the best studied examples of proteins that undergo transitions from soluble to membrane-inserted forms are bacterial pore-forming toxins such as the colicins and perfringolysin O (reviewed in Ref. 17). There are also well characterized examples of proteins that transfer a catalytic domain across membranes upon insertion (e.g. cholera toxin) (reviewed in Ref. 17). Furthermore, SipB, a virulence factor from *Salmonella*, has been shown to insert into membranes from a soluble state *in vitro*, translocating a 50-residue hydrophilic domain across the bilayer (18). Generally, these proteins initially adhere to and

* This work was supported in part by the University of Newcastle upon Tyne. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by a research development fellowship from the Biotechnology and Biological Sciences Research Council (United Kingdom).

² Supported by a university research fellowship from The Royal Society (United Kingdom). To whom correspondence should be addressed. Tel.: 44-191-222-5810; Fax: 44-191-222-7424; E-mail: M.J.Banfield@ncl.ac.uk.

³ The abbreviations used are: EPEC, enteropathogenic *E. coli*; TTSS, type III secretion system; IBD, intimin-binding domain; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; FL-Tir, full-length Tir; cTir, C-terminal domain of Tir; nTir, N-terminal domain of Tir; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; SPR, surface plasmon resonance; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization.

⁴ P. R. Race, J. H. Lakey, and M. J. Banfield, unpublished data.

penetrate through the outer leaflet of membranes. In contrast, Tir has the unusual property of initial interaction and penetration through the inner leaflet. Given the different lipid compositions and membrane environments of these surfaces (fluidity, charge), studying Tir membrane insertion may provide important insights into the general mechanisms used by proteins to insert into membranes.

To investigate the mechanism of Tir insertion into the plasma membrane *in vivo*, we used biophysical and biochemical techniques to probe the protein's interaction with membranes, including a mimic of the human erythrocyte inner leaflet (15% phosphatidylcholine (PC), 10% sphingomyelin (SM), 45% phosphatidylethanolamine (PE), and 30% phosphatidylserine (PS)) (19) as an model of the Tir target membrane and 100% PC as a control model membrane *in vitro*. These membranes were prepared in the form of small and large unilamellar vesicles, as appropriate. Similar *in vitro* approaches have been extensively used in the study of how both non-integral membrane proteins and lipid-binding proteins interact with membranes (20–22), in addition to the bacterial toxin examples given above. The results are consistent with Tir interacting with membranes via two sites. The first encompasses the transmembrane/IBD region of the protein, which translocates across the membrane; the second is within the C-terminal domain. Data showing that membrane composition is a key determinant of Tir-membrane interaction are also presented. These data not only illustrate that Tir possesses sufficient structural information for insertion into specific membranes (mimicking target composition), but also provide a simple model with which to further study this novel insertion mechanism and the role of accessory factors and/or protein modification.

EXPERIMENTAL PROCEDURES

Materials—PC, PS, PE, and SM were purchased from Lipid Products (South Nutfield, UK). All other materials were from Sigma (Poole, UK) unless specified.

Generation of Wild-type Tir Constructs—Generation of the full-length (FL-Tir) and C-terminal domain (cTir) constructs of Tir has been described previously (16). The N-terminal domain (nTir) was generated by PCR using the vector encoding FL-Tir as a template and primers 5'-TAATACGACTCACTATA-3' (T7 promoter region) and 5'-GCTAGCAACGCTGGAATTTGTTGTTGAAGT-3' (coding region up to Val²²⁸, followed by an NheI site (underlined)). This fragment was cloned into pGEM-T. The pGEM-T/nTir vector was then cut with BamHI and NheI and cloned into pET27b, pre-cut with the same enzymes. For the transmembrane/IBD region deletion mutant (Δ TM-Tir), the N-terminal region of the protein was cloned into pGEM-T as described above using primer 5'-AAGCTTAACGCTGAATTTGTTGTTGAAGT-3', which encodes a HindIII site (underlined) rather than an NheI site. The pGEM-T/nTir-HindIII vector was then cut with BamHI and HindIII and cloned into the pET27b/cTir vector, pre-cut with BamHI and HindIII, as described (16). This leads to a vector encoding residues 1–228 and 387–550 with a non-native Lys-Leu linker between the domains. Constructs were checked for any PCR-induced errors by DNA sequencing. All plasmids were transformed in *E. coli* BL21(DE3) cells for protein expression. A schematic representation of the constructs is shown in Fig. 4B.

Generation of Tryptophan Mutants—Trp-to-Phe mutants of FL-Tir were generated using the QuikChange mutagenesis kit (Stratagene) following the manufacturer's protocol. Mutants (underlined) were generated using the following primers: W145F, 5'-CATTAAGTCCTCAAGAATTTAGCAGCTTGC-3' (forward) and 5'-GCAAGCTGCTAAATTCCTTGAGGACTTAATG-3' (reverse); W235F, 5'-GATCC-TAAATTCCTTTGTTTCTGTGCGCGCA-3' (forward) and 5'-TGCG-

CCGACAGAAACAAAGAATTTAGGATC-3' (reverse); and W443F, 5'-ACACACTTTTCAGATTCTCTAGCGAAGTG-3' (forward) and 5'-CACTTCGCTAGAGGAATCTGAAAAGTGTGT-3' (reverse). For all mutagenesis reactions, the template used was pET27b/FL-Tir (wild-type) (16), except for generation of the double mutant W145F/W443F, where W443F was generated using pET27b/FL-Tir(W145F) as a template. Mutagenized PCR products were transformed directly into BL21(DE3) cells for protein expression. Trp-to-Phe mutations were confirmed by DNA sequencing.

Expression and Purification of Wild-type and Mutant Tir Proteins—FL-Tir (wild-type and tryptophan mutants), nTir, cTir, and Δ TM-Tir were expressed and purified essentially as described previously (16), except a final gel filtration step was included to ensure high purity, and the final buffer contained 20 mM Tris and 150 mM NaCl (pH 7.5) (vesicle resuspension buffer). The proteins used in this study contained a vector-encoded C-terminal herpes simplex virus and His tag (pET27b) and yielded a single band when examined by 15% SDS-PAGE, confirming sample purity. The previously documented (16) unusual SDS-PAGE resolving properties of FL-Tir and cTir were reproduced here and were also observed for nTir (predicted molecular mass of 24 kDa and resolved molecular mass on SDS-polyacrylamide gel of 31 kDa) and Δ TM-Tir (predicted molecular mass of 40 kDa and resolved molecular mass on SDS-polyacrylamide gel of 55 kDa). Protein concentrations were calculated from absorbance at 280 nm using extinction coefficients of 24,750 M⁻¹ cm⁻¹ for FL-Tir; 19,060 M⁻¹ cm⁻¹ for the W145F, W235F, and W445F mutants; 5690 M⁻¹ cm⁻¹ for nTir; 10,810 M⁻¹ cm⁻¹ for cTir; and 16,500 M⁻¹ cm⁻¹ for Δ TM-Tir.

Preparation of Lipid Vesicles—Lipids in chloroform/methanol were dried under a stream of nitrogen gas. Dried samples were placed under vacuum for 3 h to remove any remaining organic solvent. The subsequent lipid cake was resuspended in vesicle resuspension buffer by vigorous mixing. Small unilamellar vesicles (SUVs) were prepared from the lipid suspension (2 mg/ml) by sonication (with a probe) on ice for 8 × 3 min with a 2-min cooling period between each burst. SUV suspensions were then centrifuged at 21,000 × *g* for 45 min at 20 °C to remove lipid aggregates. For large unilamellar vesicles (LUVs), the lipid suspension (10 mg/ml) was extruded 21 times through two 100- μ m polycarbonate filters using a mini extruder (Avanti Polar Lipids). LUV suspensions were centrifuged at 21,000 × *g* for 45 min at 20 °C to remove lipid aggregates. The formation of a single population of both SUVs and LUVs of suitable size (35–45- and 100–110-nm diameters, respectively) was confirmed by dynamic light scattering. The final SUV and LUV suspensions were stored at 4 °C for 24 h prior to use. The lipid vesicles used in this study were composed of 15% PC, 10% SM, 45% PE, and 30% PS (based on the composition of the human erythrocyte inner leaflet (19), which serves as a mimic of the Tir target membrane *in vivo*); 100% PC; 75% PC and 25% PE; 75% PC and 25% PS; or 75% PC and 25% SM.

Fluorescence Measurements—Fluorescence measurements were performed using a Cary Eclipse spectrofluorometer (Varian Inc.). Measurements were performed at 25 °C in vesicle resuspension buffer. SUVs (2 mg/ml) were incrementally added in 20- μ l aliquots to 300 μ l of wild-type or mutant FL-Tir (0.2 mg/ml), and tryptophan emission spectra were recorded from 300 to 450 nm at 124 nm/min, with excitation and emission slit widths set at 5 nm. The excitation wavelength used was 295 nm. SUVs were used to minimize the effects of light scattering. The intensities were corrected for the dilution factor, and the background was subtracted using appropriate blanks with SUVs only. Data were plotted without smoothing using ExcelTM and analyzed by calculation of the barycentric mean of the peaks (23) in the range of 320–370 nm.

Interaction of Tir with Lipids

Binding Experiments Using Surface Plasmon Resonance (SPR)—SPR measurements were performed using a Biacore 2000 system (Biacore AB, Bedfordshire, UK) at 25 °C. SUVs (50 μ l, 2 mg/ml) were immobilized on the surface of a Pioneer L1 sensor chip (Biacore AB) in vesicle resuspension buffer at 2 μ l/min. Loosely bound lipids were removed by two 10- μ l injections of water and equilibrated in buffer at a flow rate of 2 μ l/min. The surface of the chip was “blocked” using a 50- μ l injection of bovine serum albumin (1 mg/ml) at 2 μ l/min to prevent nonspecific interactions between the analyte and the chip surface. The analytes, FL-Tir (wild-type and tryptophan mutants), nTir, cTir, and Δ TM-Tir (20 μ l) were injected at a flow rate of 2 μ l/min over the immobilized vesicles. Finally, the chip surface was washed using two injections of buffer containing 1 M NaCl to remove nonspecifically bound proteins from the immobilized vesicles. Following each measurement, the chip was regenerated by washing with 20 mM CHAPS, which removed the membranes along with the bound protein. The chip was considered regenerated (free from lipid) when the base line of the sensorgram returned to the previous level.

Trypsin Encapsulation and Protection Assays—For vesicle-encapsulated trypsin assays, LUVs composed of the erythrocyte inner leaflet mixture (10 mg/ml) and containing trypsin were prepared by extrusion. Trypsin (5 mg/ml) in vesicle resuspension buffer was used to hydrate and extrude the lipid mixture. Non-encapsulated trypsin was removed by passage through two 1-ml benzamidine-Sepharose columns (Amersham Biosciences) joined sequentially and equilibrated in vesicle resuspension buffer. Trypsin-containing vesicles were initially incubated in the presence of 20 mM 4-(2-aminoethyl)benzenesulfonyl fluoride at 25 °C for 5 min. Following the addition of Tir (200 μ g), samples were incubated at 37 °C for 40 min. Proteins from the digests were precipitated with trichloroacetic acid and resolved by 15% SDS-PAGE. Encapsulated trypsin digestion products were characterized by whole protein MALDI mass spectrometry and either N-terminal sequencing or peptide mass fingerprinting (where protein concentrations were insufficient to permit sequencing). For the protease protection assays, FL-Tir, nTir, cTir, and Δ TM-Tir were mixed with an excess of erythrocyte inner leaflet lipid vesicles (200 μ g) and incubated at 20 °C for 1 h to allow protein binding. Trypsin was added to the proteoliposomes, and the mixture was incubated at either 20 or 37 °C for 1–16 h. After the addition of 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, samples were analyzed by 15% SDS-PAGE. Protein bands were visualized by Coomassie Blue staining. For control experiments, Tir proteins were mixed with trypsin in the absence of liposomes. Protected protein fragments were resolved by SDS-PAGE and identified by MALDI mass spectrometry and N-terminal sequencing (as described for trypsin encapsulation experiments).

RESULTS

The Interaction of Tir with Membranes Is Dependent on Lipid Composition—Bioinformatic analysis of the predicted transmembrane regions of Tir revealed the presence of a single tryptophan residue (Trp²³⁵) that could act as a fluorescent reporter of membrane interaction. As the lipid composition of membranes can have a major influence on the propensity of proteins to interact with or to insert into lipid bilayers (24, 25), the binding of wild-type FL-Tir to vesicles of varying composition was monitored after the addition of SUVs. The interaction of the protein with vesicles was shown to be composition-dependent. Initially, binding to vesicles composed of either the human erythrocyte inner leaflet mixture (as a mimic of the Tir host cell target membrane) or PC only (as a generic neutral membrane) was investigated. The binding of FL-Tir to the inner leaflet mixture vesicles resulted in a blue shift in

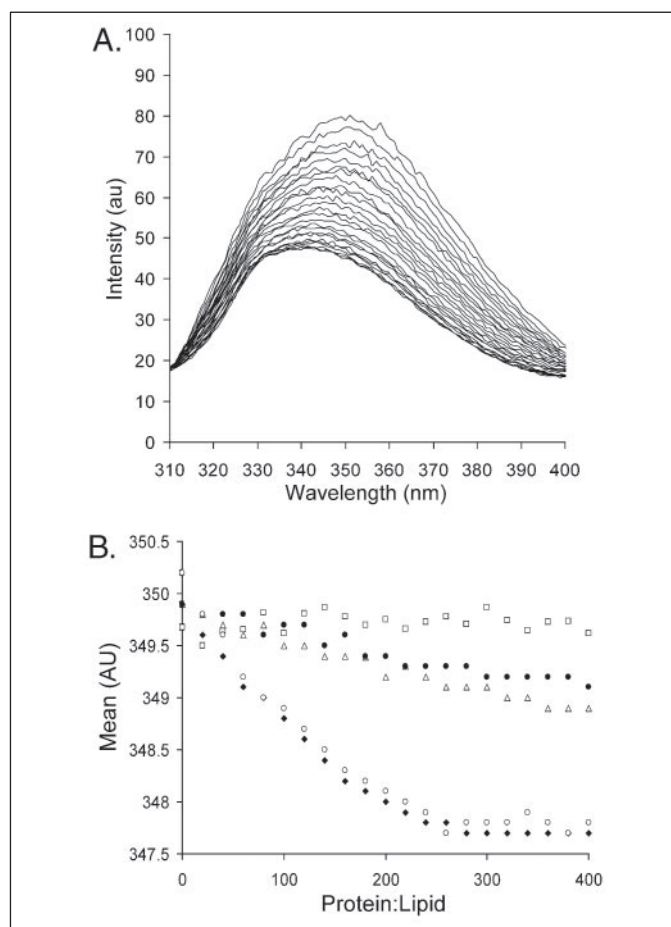


FIGURE 1. Interaction of FL-Tir with membranes monitored by intrinsic tryptophan fluorescence. A, raw data showing the change in intrinsic tryptophan fluorescence upon titration of SUVs into wild-type FL-Tir; B, plot of the shift in the barycentric mean of fluorescence versus protein/lipid molar ratio for membranes composed of 100% PC (\square), 75% PC and 25% PE (\triangle), 75% PC and 25% PS (\bullet), 75% PC and 25% SM (\circ), and the inner leaflet mixture (\blacklozenge). au, absorbance units.

intrinsic tryptophan fluorescence (Fig. 1A), consistent with the transfer of solvent-exposed tryptophan residues to a more hydrophobic phase (21, 26). The blue shift was accompanied by a quenching of the fluorescence signal (Fig. 1A). When presented as a function of SUV concentration, the shift in the barycentric mean of the data yields a curve describing Tir-SUV interaction (Fig. 1B). The degree of blue shift was proportional to the amount of SUV added up to a molar ratio of 260:1 (lipid/protein), with subsequent additions consistent with saturation of the effect and therefore representing a clear end state for the interaction. Where a blue shift (\sim 2 nm) was observed for the inner leaflet mixture, the experiment using pure PC vesicles displayed a shift of only \sim 0.2 nm (Fig. 1B). Also, where a quenching of fluorescence was observed with the inner leaflet mixture, negligible quenching was observed with pure PC (data not shown). To investigate which of the individual components of the complex inner leaflet mixture may be important for FL-Tir binding, interactions with 7.5:2.5 PC/PS, 7.5:2.5 PC/PE, and 7.5:2.5 PC/SM were investigated. In turn, each of these probed whether negative charge (PS), PE, or SM is important for the interaction. The plots of the barycentric means describing the interactions are shown in Fig. 1B. It is clear that, whereas a small change in fluorescence signal was observed with the 7.5:2.5 PC/PS and 7.5:2.5 PC/PE vesicles, only the interaction with the 7.5:2.5 PC/SM vesicles reproduced the specific saturable effect observed for the interaction with the inner leaflet mixture. Given these results, further experiments focused on vesicles composed of the inner leaflet

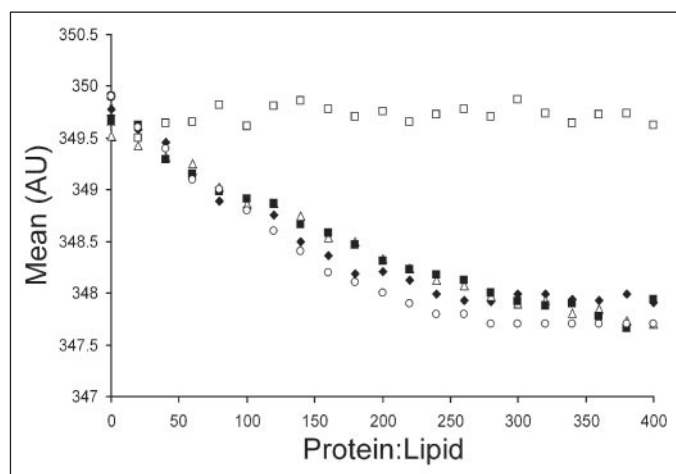


FIGURE 2. Intrinsic tryptophan fluorescence measurements following interaction of FL-Tir tryptophan mutants with membranes. Shown is a plot of the shift in the barycentric mean of fluorescence versus protein/lipid molar ratio for FL-Tir (○), FL-Tir(W145F) (■), FL-Tir(W235F) (□), FL-Tir(W443F) (◆), and FL-Tir(W145F/W443F) (△).

mixture (as a mimic of the Tir target membrane) and pure PC (as a negative control). These were chosen in an effort to ensure the *in vitro* studies may be of relevance to *in vivo* mechanisms.

Tir-Membrane Interaction Involves the Region Including Trp²³⁵—The tryptophan fluorescence experiments described above demonstrated that Tir interacted best with membranes composed of the inner leaflet mixture, and not pure PC. However, predicting the region of the protein that contributes to this signal (and therefore partitions to the membrane) is complicated by the presence of three tryptophans in the protein's sequence. As mentioned above, one of these is within a predicted transmembrane region (Trp²³⁵); there is also one in the N-terminal domain (Trp¹⁴⁵) and one in the C-terminal domain (Trp⁴⁴³) (see Fig. 4B). To determine the relative contributions of the three tryptophans to the observed fluorescence change, each residue was independently mutated to phenylalanine (W145F, W235F, and W445F). These mutants were expressed and purified using the same protocol described for the wild-type protein. The far-UV CD spectrum of each purified protein overlays on that of the wild-type protein, suggesting that the overall fold of the protein is maintained (data not shown). Also, the binding of the mutants to lipid vesicles was comparable with that of the wild-type protein as measured by SPR (data not shown), demonstrating that tryptophans at these positions are not essential for lipid binding. The W145F and W443F mutants exhibited blue shifts identical to those observed for the wild-type protein (~2 nm) (Fig. 2), indicating that regions surrounding these residues do not interact directly with the vesicle bilayer. In contrast, the W235F mutant showed no shift or quenching of the fluorescence signal following the addition of SUVs (Fig. 2). To confirm that the fluorescence blue shift can be completely accounted for by Trp²³⁵, the double mutant W145F/W443F was constructed. This protein displayed essentially the same blue shift/fluorescence quench change as observed for the wild-type protein (Fig. 2). In fact, the individual blue shift was slightly larger, as would be expected following the removal of Trp¹⁴⁵ and Trp⁴⁴³, which do not contribute to the shift.

The Interaction of Tir with Lipids Is Mediated by Two Binding Sites—The interaction of FL-Tir and individual Tir domains with vesicles was further investigated by SPR. SPR can be used to investigate the interaction of proteins with lipid vesicles immobilized on a hydrophobic surface (26, 27). The system employed utilized SUVs (composed of the inner leaflet mixture and pure PC) immobilized on a Pioneer L1 gold chip (28) as the ligand and FL-Tir (or individual domains) over a range of concentrations as the analyte. Changes in response as a result of protein binding were measured (Fig.

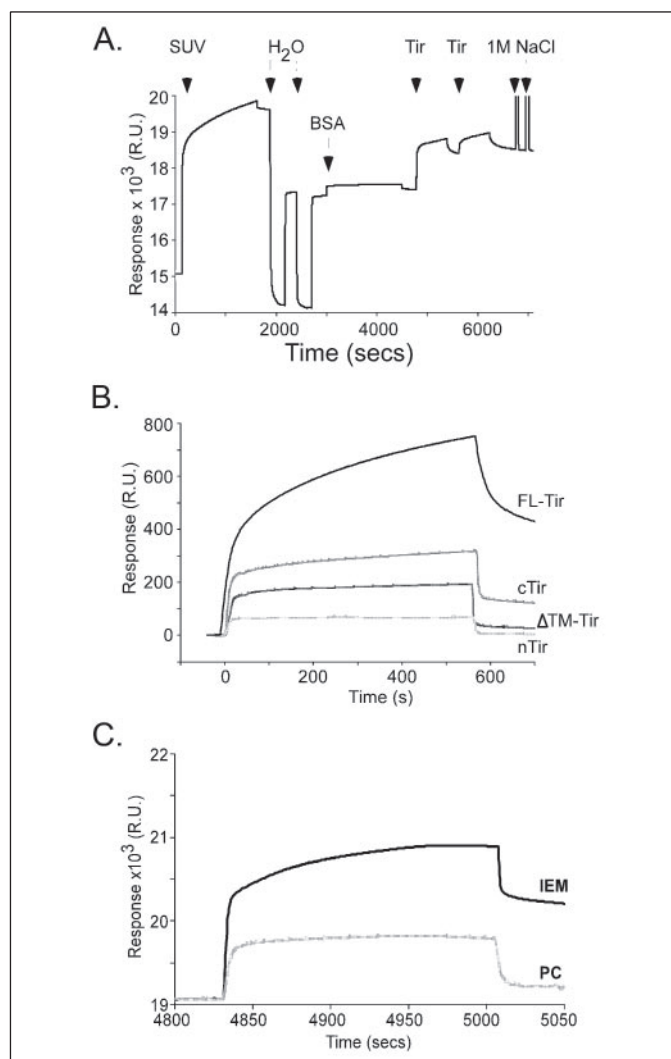


FIGURE 3. SPR experiments. A, sensorgram showing the interaction of FL-Tir with immobilized lipid vesicles composed of the inner leaflet mixture. Arrows indicate the time points at which each of the compounds was injected over the chip surface. B, comparative sensorgram showing the binding of FL-Tir, nTir, cTir, and Δ TM-Tir to immobilized vesicles (inner leaflet mixture). All proteins are at 150 nM. C, comparative sensorgram showing the interaction of FL-Tir with immobilized vesicles composed of the inner leaflet mixture (IEM, inner leaflet erythrocyte membrane; black trace) and PC only (gray trace). Protein is at 350 nM. BSA, bovine serum albumin; r.u., response units.

3A), and the interaction of each of the individual Tir constructs was compared (Fig. 3B). FL-Tir, cTir, Δ TM-Tir, and nTir all showed some interaction with lipid using this technique. FL-Tir, cTir, and Δ TM-Tir all bound to immobilized vesicles in a dose-dependent fashion (data not shown). There was a strong interaction between FL-Tir and vesicles, indicative of insertion, as a significant proportion of the protein was not desorbed from the lipid surface by buffer alone, and a 1 M NaCl wash removed only a very small proportion of the bound protein (Fig. 3A). The interaction of cTir and Δ TM-Tir with immobilized vesicles was significantly weaker than that of the full-length protein (Fig. 3B). nTir showed only minimal binding and may be nonspecific. Whereas nTir and, to a lesser extent, Δ TM-Tir were effectively removed from the surface when washed with buffer (and therefore were not inserted into the membrane), somewhat surprisingly, a proportion of cTir was retained on the chip (Fig. 3B). This response returned to the base line following a 1 M NaCl wash, suggesting that cTir binding also forms only a peripheral interaction with membranes. It was common to observe a further increase in response during a subsequent injection of protein (full-length and domain constructs) (e.g. Fig. 3A), suggesting that

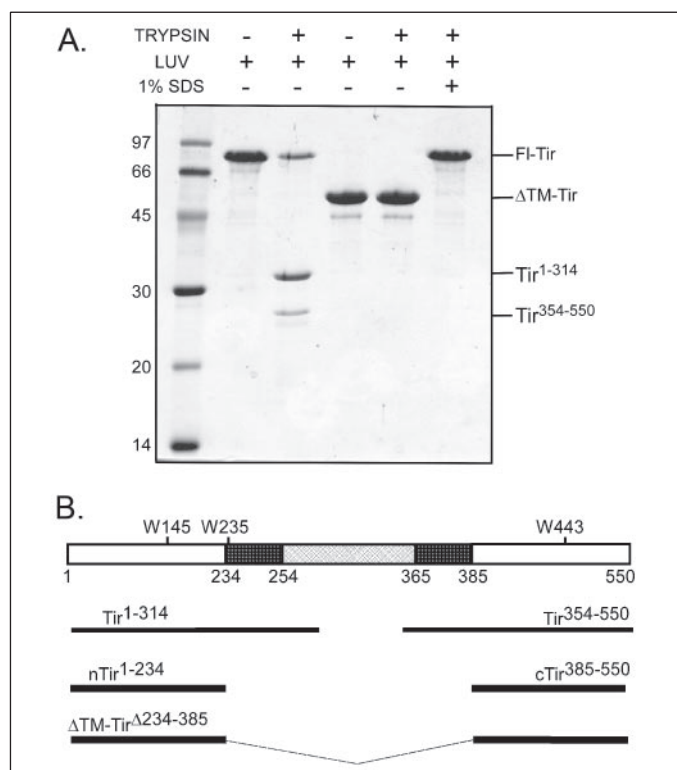


FIGURE 4. *A*, vesicle-encapsulated trypsin assays. The proteolytic fragments of FL-Tir following incubation with vesicle-encapsulated trypsin are shown on a 15% SDS-polyacrylamide gel. *First lane*, markers; *second lane*, FL-Tir + LUVs not containing trypsin; *third lane*, FL-Tir + LUVs containing trypsin; *fourth lane*, ΔTM-Tir + non-trypsin-containing LUVs; *fifth lane*, ΔTM-Tir + trypsin-containing LUVs; *sixth lane*, FL-Tir + trypsin-containing LUVs and 1% SDS. *B*, schematic representation of FL-Tir showing the domain structure, positions of tryptophan residues, fragments generated following vesicle-encapsulated trypsin assays, and Tir domain constructs generated for this study.

the system was not saturated following the first injection. Global fitting of FL-Tir, cTir, and ΔTM-Tir data at different analyte concentrations fitted poorly to any of the binding models described in the BIAevaluation software supplied with the machine. However, binding data generated at low concentrations of FL-Tir (45, 70, and 110 nM) globally fitted best to a two-state reaction model. Low concentrations of cTir (55, 110, and 160 nM) and ΔTM-Tir (60, 95, and 125 nM) globally fitted best to a 1:1 Langmuir binding model. The inability to model the binding data at high concentrations (>250 nM) is suggestive of saturation resulting in artifactual binding events.

SPR was also used to investigate the effects of lipid composition on insertion of Tir using the inner leaflet mixture and pure PC vesicle systems. SPR analysis revealed the binding of FL-Tir to be significantly weaker in PC-only vesicles, and proportionally less was retained following the buffer wash compared with the inner leaflet mixture (Fig. 3C), supporting the previously described fluorescence data.

Determination of the Membrane-inserted Domains of Tir—To adopt a native *in vivo*-like topology, Tir not only has to insert into membranes, but also has to translocate the IBD region across the membrane. To determine whether the IBD or any region of purified Tir crosses the bilayer *in vitro*, FL-Tir and domain truncations were incubated with vesicle-encapsulated trypsin. Incubation of FL-Tir for 40 min resulted in the formation of two significant digestion products (Fig. 4A). Each of these was analyzed by whole protein MALDI mass spectrometry following excision of the bands from SDS-polyacrylamide gels. The fragments were identified as having molecular masses of 32,144 and 22,520 Da, respectively. N-terminal sequencing of the ~32-kDa band resulted in the unambiguous assignment of only three amino acid residues (IGN)

due to low protein concentrations obtained from the proteolysis experiments. However, this tripeptide sequence appears only once in FL-Tir as residues 3–5. Analysis of the Tir sequence identified a trypsin cleavage site after Lys³¹⁴ that would generate a fragment 32,161 Da in size, 17 Da different from that determined experimentally. The ~32-kDa fragment was therefore identified as spanning Tir residues 1–314 (Fig. 4B). To date, it has not been possible to generate a sufficient amount of the ~23-kDa species to permit N-terminal sequencing. However, tryptic peptide mass fingerprinting revealed the presence of fragments containing Arg³⁸⁹–Arg⁴²³, Asp⁴³³–Arg⁴⁶¹, and Leu⁴⁹⁹–Arg⁵⁴⁸, clearly identifying this fragment as the C-terminal domain of the protein. Analysis of the Tir sequence suggests that cleavage after Arg³⁵⁴ would liberate a fragment 22,492 Da in size (including the herpes simplex virus and His₆ tag), 28 Da different from that determined experimentally. The ~23-kDa fragment was therefore identified as spanning Tir residues 354–550 (Fig. 4B). When Tir was mixed in identical experiments but with LUVs prepared in the absence of trypsin or when trypsin-containing vesicles were lysed with 1% SDS immediately prior to the incubation, only the intact full-length protein was recovered (Fig. 4A). The latter demonstrates that there was sufficient 4-(2-aminoethyl)benzenesulfonyl fluoride in the surrounding buffer to completely inhibit trypsin activity if there was any leakage from the vesicles. Also, mixing ΔTM-Tir with trypsin-containing vesicles failed to generate any new species (Fig. 4A), suggesting that transfer across the membrane does not occur. Furthermore, no digestion was detected when either nTir or cTir was mixed and incubated with vesicle-encapsulated trypsin (data not shown). Vesicle-encapsulated trypsin experiments with FL-Tir were also performed using vesicles composed of pure PC. Following incubation, only FL-Tir could be detected by SDS-PAGE and Coomassie Blue staining (data not shown).

In addition to vesicle-encapsulated protease experiments, protease protection assays were also undertaken. No protected fragments corresponding to the transmembrane/IBD region of Tir were observed following the addition of exogenous trypsin to LUVs preincubated with Tir, despite numerous experiments varying the Tir and protease concentrations and incubation times and temperatures (1 h to overnight, 20 and 37 °C) (data not shown). The only fragment of the protein that remained resistant to trypsin (identified by mass spectrometry and N-terminal sequencing) was a 10,734-Da region spanning residues 76–178, within the N-terminal domain (theoretical molecular mass of 10,721 Da). This fragment could also be recovered by trypsin digestion of the protein in the absence of vesicles and therefore does not represent a lipid-protected region.

DISCUSSION

In this study, we used biochemical and biophysical methods to investigate the interaction of the EPEC TTSS effector protein Tir with biological membranes in the absence of other bacterial or host cell factors. A combination of data from intrinsic tryptophan fluorescence spectroscopy (Figs. 1 (A and B) and 2), SPR (Fig. 3, A–C), and protease digestion (Fig. 4) experiments are consistent with purified Tir inserting into lipid bilayers *in vitro* and adopting a transmembrane topology as found *in vivo*. This is the first time that biophysical analysis has revealed Tir partitioning to a membrane *in vitro*. The mechanisms used by non-co-translationally inserted soluble proteins to penetrate and/or span/cross biological membranes are poorly understood. The development of this model system provides a tool to investigate the fine details of the Tir insertion mechanism and to dissect the multiple functions of the protein upon infection of host cells by EPEC.

Role of Lipid Composition in Tir Insertion—Membrane composition can play a significant role in the insertion propensity of proteins (24, 25, 29). This can be due to effects such as the specific binding activity of a protein for an individual lipid (or other membrane component) (30–32), charge on the membrane, membrane fluidity, or a combination of these. This study has focused on the interaction of Tir with vesicles composed of two lipid systems: the first based on the composition of the erythrocyte inner leaflet as a mimic of the Tir target membrane (15% PC, 10% SM, 45% PE, and 30% PS) and the second based on a neutral model membrane (PC alone). These systems were chosen in an effort to ensure that the *in vitro* studies may be of relevance to *in vivo* mechanisms. The results show that the interaction and insertion of Tir are highly dependent on lipid composition. Intrinsic tryptophan fluorescence and SPR showed that Tir interacted strongly with the inner leaflet mixture and, to a much lesser extent, with vesicles composed of pure PC and that this interaction was largely mediated by the transmembrane/IBD region (see below). Also, using vesicle-encapsulated trypsin, it was possible to detect transfer of the IBD region of Tir into vesicles composed of only the inner leaflet mixture, and not pure PC vesicles (see below). The inner leaflet mixture is a complex system that introduces charge (30% PS), a second neutral phospholipid (45% PE), and a sphingolipid (10% SM) compared with pure zwitterionic PC. Initial intrinsic tryptophan fluorescence experiments suggested that that most influential lipid in the inner leaflet mixture is SM, as Tir inserted into 75% PC and 25% SM with the same binding curve as obtained with the more complex mixture (Fig. 1B). It was possible to follow an interaction of Tir with vesicles in which a portion of the PC had been substituted with another neutral lipid (75% PC and 25% PE) or a charged lipid (75% PC and 25% PS) (Fig. 1B). However, these interactions are difficult to interpret and are most likely nonspecific, as the binding did not become saturated, and no end point was achieved (Fig. 1B).

The increased and saturable binding of Tir to vesicles containing SM compared with those containing pure PC suggests that SM is important for Tir-membrane interaction. This could demonstrate either a specific requirement for protein-SM interaction in the insertion mechanism or the more general effect the presence of SM has on the dynamics of the membrane. Interestingly, the vesicles composed of 75% PC and 25% SM reflect to some extent the composition of the outer leaflet of the erythrocyte membrane (19). However, as Tir is translocated across the host cell membrane by the TTSS and does not come into contact with the outer leaflet prior to insertion, it is likely that the interaction with these vesicles reflects only the presence of SM. Although further study is required to correlate the effects of lipid composition on Tir insertion, it is interesting to note that recent studies have demonstrated the importance of lipid raft structures, which are rich in SM, in the binding and activation of the TTSS machinery upon interaction with the host cell (33, 34). It seems plausible that the insertion mechanism of Tir has evolved to target membranes that would be localized to the point of TTSS machinery attachment, as the ultimate Tir target (post-membrane localization) is the bacterial surface protein intimin. Although it is known that intimin can rapidly sequester Tir dispersed in cell membranes to the point of bacterial attachment (12),⁵ insertion at the point of TTSS contact *in vivo* will increase the local concentration of Tir on the surface of the host cell in the vicinity of the bacteria, maximizing the chance for intimin-Tir interaction and efficient bacterial adherence.

Tryptophan Fluorescence and SPR Reveal the Regions of the Protein Important for Membrane Interaction—Having demonstrated that vesicles composed of the inner leaflet mixture are an appropriate model for

investigating Tir insertion into membranes (and pure PC as a negative control), the regions of the protein important for the interaction were investigated by intrinsic tryptophan fluorescence and SPR. If a conformational change associated with a protein partitioning into membranes is accompanied by a change in the environment of tryptophan residues, this interaction can be effectively followed by fluorescence, as tryptophan residues are acutely sensitive to the hydrophilicity/hydrophobicity of their immediate environment. They can act as “sensors” to demonstrate whether the residues are in a solvent-exposed or solvent-protected environment, such as buried in a protein fold or membrane. The addition of SUVs composed of the inner leaflet mixture to a solution of FL-Tir resulted in a fluorescence blue shift indicative of tryptophan residues moving from a hydrophilic to more hydrophobic environment (Fig. 1, A and B). Tir has three tryptophan residues, well separated in sequence, and this fluorescence shift may represent movement of one or more tryptophans into the membrane during insertion. Using site-directed mutagenesis to analyze the relative contributions of each tryptophan, the fluorescence shift of FL-Tir was mapped to Trp²³⁵, which has been shown to be both necessary and sufficient to account for the wild-type protein shift. Sequence analysis showed that Trp²³⁵ is positioned within the first of the two predicted transmembrane helices of Tir and demonstrated that partitioning of the transmembrane regions of Tir into bilayers can be directly followed using biophysical techniques.

Furthermore, SPR analysis also revealed that the region comprising the transmembrane domains (and the extracellular IBD loop) forms the major site for the protein's interaction with the membrane. It would appear that this region is multifunctional in that it not only encodes the intimin-binding site and the ability to maintain a transmembrane topology, but also may act as a membrane binding/insertion signal. The two-state model that best describes the interaction of the full-length protein with membranes is consistent with an initial binding event followed by a conformational change, a model that seems entirely appropriate for Tir interaction with membranes and that has been previously observed for other membrane-inserted proteins (20). However, this interpretation is complicated by the SPR data from the cTir and, to some extent, Δ TM-Tir constructs, which retained the ability to interact with membranes, but not to the same extent as FL-Tir. Both the Δ TM-Tir and cTir interactions were also susceptible to a 1 M NaCl wash, unlike FL-Tir, indicating that the binding of these proteins to membranes is not associated with stable insertion (supported by the protease digestion assays, in which no translocation was observed). As the nTir protein demonstrated only minimal interaction with membranes as reported by SPR (Fig. 3B), it is likely that a common site in both Δ TM-Tir and cTir is responsible for this peripheral membrane interaction. It is possible that the presence of the N-terminal domain partly occludes the cTir binding site, which would explain why the response of Δ TM-Tir is weaker than that of cTir. The binding of cTir to membranes was not detected by intrinsic tryptophan fluorescence, indicating that the interaction does not involve a change in the environment of Trp⁴⁴³ (the tryptophan present in the C-terminal domain of Tir). It has been reported previously that cTir in isolation can adopt a partially unfolded but soluble state in solution (16). It is therefore possible that the cTir-lipid interaction and indeed the Δ TM-Tir-lipid interaction may be non-specific, or as these proteins are non-native constructs, they may be prone to aggregation in the presence of vesicles.

As the region of Tir previously demonstrated to span the membrane *in vivo*, it is not surprising to observe the importance of the transmembrane/IBD region as the major component of membrane interaction. However, Tir localization to the membrane *in vivo* does not require the IBD region (6, 10), and combined with the data in this study, it seems

⁵ B. Kenny, personal communication.

Interaction of Tir with Lipids

likely that the transmembrane helices only are sufficient to promote insertion, with the intervening sequence playing no role but to undergo translocation across the membrane. This mechanism of membrane insertion and translocation has parallels with colicins, in which the native translocated region can be replaced with peptide epitopes (35) or even functional proteins (36), which become translocated upon insertion. Whether any sequence within the IBD is important in the insertion mechanism of Tir is currently being addressed.

Protease Digestion Shows That Tir Spans the Membrane in an *In Vivo*-like Topology—To be consistent with the *in vivo* topology of Tir, an *in vitro* model system must support the translocation of the ~130-amino acid hydrophilic IBD across the membrane as part of the insertion mechanism. The addition of FL-Tir to the inner leaflet mixture vesicles has been shown to result in translocation of the IBD using protease digestion assays with vesicle-encapsulated trypsin. Whole protein mass spectrometry, N-terminal sequencing, and tryptic peptide mass fingerprinting identified cleavage of Tir after Arg³¹⁴ and Arg³⁵⁴. The resulting fragments encompass (a) the N-terminal domain, the first transmembrane helix, and ~60 residues of the IBD domain and (b) ~10 residues of the IBD domain, the second transmembrane helix, and the C-terminal domain. Within the IBD, there are nine potential trypsin cleavage sites (lysine or arginine residues), and the identified N- and C-terminal fragments result in cleavage at the fourth and ninth of these. Therefore, whereas the IBD is cleaved at the last available position before transverse the membrane to generate the C-terminal fragment, the protease cannot access any of the first three potential cleavage sites in the IBD to generate a shorter N-terminal fragment. Presumably, these sites are protected from proteolytic cleavage in the translocated form, possibly through interaction with the membrane of the vesicles.

Protease protection assays following the addition of exogenous trypsin to vesicles preincubated with Tir failed to generate any protected fragments corresponding to the transmembrane/IBD region, which might be expected if the protein has been inserted into the membrane. One model that accounts for this observation is that, following proteolytic removal of the N- and C-terminal domains from Tir inserted into the membrane, the remaining transmembrane/IBD region causes destabilization of the vesicle's structure, allowing access of the protease to the rest of the protein.

Model for Tir Insertion into the Membrane—Taken together, the data generated in this study show that Tir insertion into membranes does not require any accessory factors (be they bacterial or host cell-derived) or a switch such as receptor binding or a pH shift. As Tir can be obtained in a soluble state, the hydrophobic regions that are destined to span the bilayer are presumably protected in the protein structure prior to membrane interaction. Two alternative hypotheses for how Tir can insert into membranes can be derived (Fig. 5). In the first model (*Model A*), the region either encompassing or close to the transmembrane/IBD region that is exposed in solution binds peripherally to the membrane. This leads to a conformational change in which the hydrophobic regions adopt their transmembrane orientation, and the IBD is translocated across the membrane. This model is supported by the SPR data, which showed that the most appropriate model for FL-Tir binding to vesicles is a two-state conformational change, indicating initial binding followed by a structural rearrangement. This model of interaction has been observed previously for other membrane-inserted proteins (20, 35, 36). Following this insertion, the C-terminal domain of Tir could then bind to the membrane adjacent to the transmembrane regions (via the second cTir-located binding site) in an orientation such that an appropriate surface is presented to the cytoplasm for interaction with host cell proteins, which include specific kinases and the actin nucleating machin-

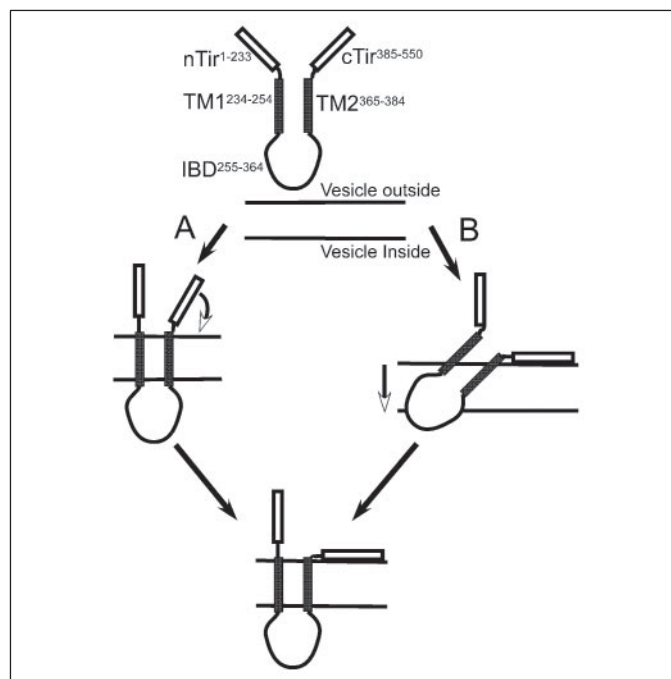


FIGURE 5. Schematic representation of the two alternative models for Tir insertion into membranes as discussed under "Discussion." In *Model A*, direct interaction of the transmembrane/IBD region mediates membrane binding and insertion, followed by binding of the C-terminal domain to the membrane surface. In *Model B*, the initial interaction of the C-terminal domain and the membrane induces structural rearrangements permitting penetration and translocation of the transmembrane/IBD region.

ery. The positioning of this domain could also be important for transducing the intimin-binding signal through the protein into the host cell. Alternatively, a second model (*Model B*) that is also consistent with the data in this study is that cTir initially binds membranes, followed by a conformational change that may drive binding and insertion at the second site, the transmembrane/IBD. Following insertion, the binding site in the C-terminal domain could still act to maintain a suitable orientation promoting interactions in signaling as described above. A third model in which the two binding sites act in tandem to bring about insertion and translocation also remains possible. The complex interplay between the binding sites is difficult to dissect given currently available data. These models do not take into account any role for oligomerization of Tir in the insertion mechanism, which requires further study. Although the protein used in these studies is monomeric in solution (as determined by analytical ultracentrifugation), a dimeric state was observed for a peptide derived from the IBD in the intimin-IBD crystal structure (11) and for both FL-Tir and cTir on SDS-polyacrylamide gels under certain conditions (16). The existence of oligomeric states in solution or in membranes has not yet been definitively shown. A role for the clustering of Tir in the membrane resulting in host cell signaling has also been suggested, but again, it is not known if this results in Tir oligomerization (37).

In conclusion, the mechanism of membrane insertion by the EPEC TTSS effector protein Tir has been investigated using biochemical and biophysical techniques. This study has shown that Tir has an inherent affinity for membranes and can insert into model bilayers *in vitro*, generating an *in vivo*-like topology, and that this interaction is dependent on the composition of the bilayer. The protein has two sites that appear to mediate interaction with membranes. The major site encompasses the transmembrane/IBD region and is essential for insertion into membranes. Future investigations will focus on using this *in vitro* model system to further dissect the mechanism of Tir insertion, addressing

both protein-based (role for oligomerization, protein modification/accessory factors, how the hydrophilic IBD is translocated across the hydrophobic membrane) and lipid-based (lipid composition, role for cholesterol) effects. This system may also prove useful for following the downstream signaling effects of Tir after membrane localization and activation.

Acknowledgments—We thank Duncan McNulty for technical assistance, Patrick Allen for help constructing the tryptophan mutants, and Prof. Brendan Kenny for discussion and critical review of the manuscript.

REFERENCES

- Nataro, J. P., and Kaper, J. B. (1998) *Clin. Microbiol. Rev.* **11**, 142–201
- Vallance, B. A., and Finlay, B. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8799–8806
- Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B., and Knutton, S. (1998) *Mol. Microbiol.* **30**, 911–921
- Nougayrede, J. P., Fernandes, P. J., and Donnenberg, M. S. (2003) *Cell. Microbiol.* **5**, 359–372
- Chen, H. D., and Frankel, G. (2005) *FEMS Microbiol. Rev.* **29**, 83–98
- de Grado, M., Abe, A., Gauthier, A., Steele-Mortimer, O., DeVinney, R., and Finlay, B. B. (1999) *Cell. Microbiol.* **1**, 7–17
- Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., and Finlay, B. B. (1997) *Cell* **91**, 511–520
- Hartland, E. L., Batchelor, M., Delahay, R. M., Hale, C., Matthews, S., Dougan, G., Knutton, S., Connerton, I., and Frankel, G. (1999) *Mol. Microbiol.* **32**, 151–158
- Dean, P., Maresca, M., and Kenny, B. (2005) *Curr. Opin. Microbiol.* **8**, 28–34
- Kenny, B. (1999) *Mol. Microbiol.* **31**, 1229–1241
- Luo, Y., Frey, E. A., Pfuetzner, R. A., Creagh, A. L., Knoechel, D. G., Haynes, C. A., Finlay, B. B., and Strynadka, N. C. (2000) *Nature* **405**, 1073–1077
- Campellone, K. G., Rankin, S., Pawson, T., Kirschner, M. W., Tipper, D. J., and Leong, J. M. (2004) *J. Cell Biol.* **164**, 407–416
- Thomas, N. A., Deng, W., Puente, J. L., Frey, E. A., Yip, C. K., Strynadka, N. C., and Finlay, B. B. (2005) *Mol. Microbiol.* **57**, 1762–1779
- Abe, A., de Grado, M., Pfuetzner, R. A., Sanchez-Sanmartin, C., DeVinney, R., Puente, J. L., Strynadka, N. C., and Finlay, B. B. (1999) *Mol. Microbiol.* **33**, 1162–1175
- Page, A. L., and Parsot, C. (2002) *Mol. Microbiol.* **46**, 1–11
- Hawrani, A., Dempsey, C. E., Banfield, M. J., Scott, D. J., Clarke, A. R., and Kenny, B. (2003) *J. Biol. Chem.* **278**, 25839–25846
- Parker, M. W., and Feil, S. C. (2005) *Prog. Biophys. Mol. Biol.* **88**, 91–142
- McGhie, E. J., Hume, P. J., Hayward, R. D., Torres, J., and Koronakis, V. (2002) *Mol. Microbiol.* **44**, 1309–1321
- Virtanen, J. A., Cheng, K. H., and Somerharju, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4964–4969
- Hong, Q., Gutierrez-Aguirre, I., Barlic, A., Malovrh, P., Kristan, K., Podlessek, Z., Macek, P., Turk, D., Gonzalez-Manas, J. M., Lakey, J. H., and Anderluh, G. (2002) *J. Biol. Chem.* **277**, 41916–41924
- Saito, H., Dhanasekaran, P., Nguyen, D., Deridder, E., Holvoet, P., Lund-Katz, S., and Phillips, M. C. (2004) *J. Biol. Chem.* **279**, 20974–20981
- Colomer-Pallas, A., Petit-Glatron, M. F., and Chambert, R. (2004) *Biochim. Biophys. Acta* **1660**, 16–23
- Lakey, J. H., Massotte, D., Heitz, F., Dasseux, J. L., Faucon, J. F., Parker, M. W., and Pattus, F. (1991) *Eur. J. Biochem.* **196**, 599–607
- Barlic, A., Gutierrez-Aguirre, I., Caaveiro, J. M., Cruz, A., Ruiz-Arguello, M. B., Perez-Gil, J., and Gonzalez-Manas, J. M. (2004) *J. Biol. Chem.* **279**, 34209–34216
- Ray, S., and Chakrabarti, A. (2004) *Mol. Membr. Biol.* **21**, 93–100
- Macek, P., Zecchini, M., Pederzoli, C., Dalla Serra, M., and Menestrina, G. (1995) *Eur. J. Biochem.* **234**, 329–335
- Malovrh, P., Barlic, A., Podlessek, Z., Macek, P., Menestrina, G., and Anderluh, G. (2000) *Biochem. J.* **346**, 223–232
- Cooper, M. A., Hansson, A., Lofas, S., and Williams, D. H. (2000) *Anal. Biochem.* **277**, 196–205
- Daghastanli, K. R., Ferreira, R. B., Thedei, G., Jr., Maggio, B., and Ciancaglini, P. (2004) *Colloids Surf. B: Biointerfaces* **36**, 127–137
- Berne, S., Sepcic, K., Anderluh, G., Turk, T., Macek, P., and Poklar Ulrih, N. (2005) *Biochemistry* **44**, 11137–11147
- Mahfoud, R., Garmy, N., Maresca, M., Yah, N., Puigserver, A., and Fantini, J. (2002) *J. Biol. Chem.* **277**, 11292–11296
- Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996) *J. Biol. Chem.* **271**, 8472–8480
- van der Goot, F. G., Tran van Nhieu, G., Allaoui, A., Sansonetti, P., and Lafont, F. (2004) *J. Biol. Chem.* **279**, 47792–47798
- Hayward, R. D., Cain, R. J., McGhie, E. J., Phillips, N., Garner, M. J., and Koronakis, V. (2005) *Mol. Microbiol.* **56**, 590–603
- Jakes, K. S., Kienker, P. K., Slatin, S. L., and Finkelstein, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4321–4326
- Slatin, S. L., Nardi, A., Jakes, K. S., Baty, D., and Duche, D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 1286–1291
- Touze, T., Hayward, R. D., Eswaran, J., Leong, J. M., and Koronakis, V. (2004) *Mol. Microbiol.* **51**, 73–87