Effect of Protein Kinase A-mediated Phosphorylation on the Structure and Association Properties of the Enteropathogenic Escherichia coli Tir Virulence Protein*

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Enteropathogenic Escherichia coli virulence is dependent on delivery of the translocated intimin receptor protein (Tir) into host cells. Tir phosphorylation on a single tyrosine (Tyr-474) is essential in mediating cytoskeletal rearrangement correlated with disease, Tir is also phosphorylated on other residues, with cAMP-dependent kinase (PKA) modification shown to play a role in Tir function. However, the mechanism by which non-tyrosine phosphorylation affects Tir function remains unclear. In this study, analytical ultracentrifugation, SDS and native gel electrophoresis revealed that both Tir and its C-terminal domain (residues 385–550 of Tir that include the PKA substrate sites) exist in an equilibrium of monomers, dimers, and in the case of Tir, higher oligomers. PKA phosphorylation (1:300, PKA/C-Tir, mol/mol) shifted the equilibrium of C-Tir, but not Tir, predominantly to the dimeric state, whereas, at 100-fold higher concentrations of PKA the phosphorylated C-Tir was largely monomeric. This monomeric state was also produced at the lower PKA concentration and physiological ionic strength. Phosphorylation-mediated effects were achieved without significant changes in secondary structure as determined by circular dichroism spectroscopy. The data presented indicate that PKA-mediated phosphorylation induces changes in the association properties of the C-terminal domain of Tir that may facilitate Tir-Tir interactions and subsequently C-Tir-host protein interactions in vivo.

The native EPEC Tir molecule resolves at a higher molecular mass (~78 kDa) on SDS-PAGE than is predicted from its sequence (~57 kDa), suggesting that the protein retains some form of residual structure in SDS (7). Anomalous SDS gel shifts may therefore act as a probe for Tir conformation. Upon translocation into host cells EPEC Tir undergoes a significant increase in its apparent molecular mass (78–85 kDa). This has been shown to be due to phosphorylation, and proceeds through sequential 5-kDa (designated T+) and 2-kDa (designated T−) shifts (6, 7, 11). More recently, Tir has been shown to be an in vitro and in vivo substrate for the cAMP-dependent protein kinase (PKA) with the sequential in vitro phosphorylation of Ser-434 and Ser-463 (within the C-terminal domain) mimicking those shifts displayed by Tir isolated from infected mammalian cells (11). Tir is also phosphorylated on a single tyrosine residue (Tyr-474), enabling the direct recruitment of the Nck adaptor molecule and the actin nucleation machinery (12, 13). However, tyrosine phosphorylation does not contribute to shifts in apparent molecular mass on SDS gels (6). Although the mechanism by which non-tyrosine phosphorylation affects Tir function has not been elucidated, it has been postulated that it may induce changes in Tir structure either to aid additional kinase modification and/or promote Tir insertion into the host cell membrane where it acts as a receptor for the bacterial surface protein, intimin, to mediate intimate EPEC adherence (6, 7). Topology analysis indicate that EPEC Tir spans the cell plasma membrane, presumably through the two predicted transmembrane domains (residues 234–259 and 353–382), exposing a large extracellular loop (residues 260–352) that contains the intimin-binding domain (IBD) (8, 9). The IBD is flanked by both N-terminal (residues 1–233) and C-terminal (residues 383–550) domains that are exposed to the cytoplasm for interaction with host proteins, such as α-actinin and host tyrosine kinases, respectively (9, 10). The x-ray crystal structure of the IBD of Tir bound to the C-terminal domain of intimin has been determined (9), providing direct information on how these molecules interact. Interaction of intimin with the extracellular central domain of Tir results not only in intimate adherence but also triggers cytoskeletal rearrangements resulting in the formation of pedestal-like extensions beneath the adherent non-invasive bacteria that are also evident in infected tissue (2, 3).

The Gram-negative organism enteropathogenic Escherichia coli (EPEC) induces a diarrheal disease and remains a major cause of infant death in developing countries (1–3). EPEC virulence is mediated by a number of effector molecules that cause of infant death in developing countries (1–3). EPEC

1 The abbreviations used are: EPEC, enteropathogenic E. coli; Tir, Translocated intimin receptor; C-Tir, C-terminal domain (residues 385–550) of Tir; PKA, cAMP-dependent kinase, GdnHCl, guanidine hydrochloride; CD, circular dichroism; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonic acid.
plasma membrane from a cytoplasmic location (2, 9). In this study biophysical methods have been used to characterize the effects of PKA-mediated phosphorylation on the properties of Tir in order to address the physiological relevance of such modifications. The results presented indicate a mechanism by which PKA modification of Tir may play a role in its function within host cells.

EXPERIMENTAL PROCEDURES

DNA Manipulation—A construct expressing a TirHSVHis fusion protein has been described previously (14). The C-terminal TirHSVHis gene (Tir residues 385–550) was amplified using a primer encoding a HindIII (0.85–1.35) site: CCAGACTTCAATAGACGACGCGAAGCTTCATACATATATCCTTTTAG that encodes a HindIII restriction site followed by an initiation ATG codon. The respective fragments (BamHI/HindIII and HindIII/XhoI) were cloned into pET27b (Novagen) and sequence analysis was used to confirm an identical sequence with that of the template.

Protein Purification—BL21-DE3 (15) E. coli were transformed with pET27b encoding either TirHSVHis or C-TirHSVHis. Protein expression was induced by addition of isopropyl-thiogalactopyranoside (1 mM) to cell cultures grown at 37 °C to an OD<sub>600</sub> of 0.6 and incubated for an additional 3 h. Cells pellets were resuspended in sonication/binding buffer (20 mM imidazole, 50 mM HEPES, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5) and lysed using a Vibra-Cell probe sonicator, 60% duty cycle (Sonics & Materials, Inc.). Soluble proteins were separated from cell debris and insoluble proteins by centrifugation (18,000 rpm, 30 min) and fast protein liquid chromatography (FPLC) was used to pass the supernatant through a nickel affinity chromatography column (5-ml Hi Trap™ nickel-chelating column; Amersham Biosciences) equilibrated in binding buffer. Bound TirHSVHis or C-TirHSVHis proteins were eluted with an imidazole solution. Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis (2D-GE) was performed using 6 M guanidine hydrochloride (GdnHCl) stock solutions prepared in 20 mM Tris, 50 mM NaCl, pH 7.5. A series of protein samples (30 μM) were denatured in 0–5 M GdnHCl in 0.2 M steps and left to equilibrate for 3 h at 25 °C before fluorescence intensity measurements were taken. GdnHCl unfolding was also carried out by addition of aliquots of GdnHCl to a single protein sample, and the data re-scaled to take into account differences in volume of denaturant at each concentration. Unfolding of unfolded proteins in this manner resulted in similar unfolding curves to those obtained by incubating the protein at each denaturant concentration in the range of 0–5 M.

Tryptophan Fluorescence—GdnHCl dependent intrinsic tryptophan fluorescence was carried out using a Perkin Elmer Luminescence (LS50B) spectrophotometer equipped with a thermostatted cell holder. Spectra were recorded using the following parameters: excitation wavelength 290 nm, slit width 10, emission 300–450 nm, and scan speed 60. The Raman spectral contribution was removed by subtraction of a buffer blank. All spectra were plotted using GraFit Data analysis and GraphPad Software, version 4.0 (GraphPad Software Ltd).

MALDI-TOF Mass Spectrometry Analysis of C-Tir—A mass spectrum of the C-Tir protein was determined using a PE Biosystems Voyager-DE STR MALDI-TOF mass spectrometer with a Nitrogen laser operating at 337 nm. The matrix solution was freshly prepared Sinapinic Acid (Fluka), at a concentration of 1 mg/100 μl in a 50:50 mixture of acetonitrile (Rathburn) and 0.1% trifluoroacetic acid (Aldrich). Sample and matrix, 0.5 μl of each, were spotted onto the sample plate. The sample was calibrated against Aldolase (Sigma) run as an external standard. The spectrum was acquired under linear conditions with an accelerating voltage of 25,000 V and an extraction time of 750 ns.

RESULTS

PKA Phosphorylation Does Not Induce Significant Changes in the Secondary Structure of Tir or C-Tir—To probe for phosphorylation-mediated structural changes in Tir, the protein was first purified as a C-terminally tagged HSVHis fusion protein and analyzed in both its native and PKA-phosphorylated forms by CD. The addition of HSVHis tags does not inhibit Tir delivery, modification or function within host cells (11). As the identified PKA phosphorylation sites (Ser-434, Ser-463) occur within the C terminus of Tir (11), this domain (C-Tir) was also purified for similar analysis. The CD spectra shown in Fig. 1 reveal very similar profiles for both the phosphorylated and native forms of Tir and C-Tir and indicate that phosphorylation (1:100, PKA/Tir, or 1:300, PKA/C-Tir, mol/mol, respectively) having protein concentrations in the range of 0.05–0.2 mg/ml were measured in 2-mm pathlength quartz cuvettes. Spectra at high protein concentration (0.5–1 mg/ml) were measured in 0.1-mm quartz cuvettes. All spectra are averages of between 5 and 11 scans with relevant protein-free buffer spectra subtracted and were plotted without smoothing using SigmaPlot (SPSS Inc.). Decoyn indolization of CD spectra into component secondary structural contributions was done using the CDPro suite of programs (16), which enable calculation of secondary structure using three independent methods (CONTIN, SELCON3, and CDSSSTR), allowing an internal check on the consistency of the analyses.
PKA-mediated Phosphorylation of EPEC Tir

Fig. 1. Circular dichroism spectra of Tir (A) and C-Tir (B). In each panel the solid line is the spectrum for the native (unphosphorylated) protein, and the dotted line is the spectrum for the protein phosphorylated by protein kinase A (1:100 PKA/Tir, or 1:300, PKA/C-Tir, mol/mol, respectively).

Table I
Deconvolution of circular dichroism spectra into secondary structural components

|     | α-Helix | β-Strand | Turn | "Unordered" |
|-----|---------|----------|------|-------------|
| Tir | 25.8    | 23.3     | 21.5 | 17          |
| C-Tir | 9.3 | 7.3      | 10   | 73          |

a Calculated values for regular and distorted helix are summed.

b Total number of residues in secondary structural element based on 550 residues in native Tir, and 165 residues in the C-terminal domain of Tir (C-Tir).

c Calculated values for regular and distorted β-strand are summed.

d Data are average values of separate calculations using CONTIN, CDSSTR, and SELCON3. Only data with r.m.s.d. (exp-calc) ≤ 0.3 was used.

has negligible effect on the secondary structure of these proteins.

Deconvolution of the data into secondary structural components for both Tir and C-Tir is shown in Table I. Since phosphorylation has little effect on the CD spectra of either protein the secondary structural components listed in Table I apply equally to both the phosphorylated and non-phosphorylated forms. Tir has secondary structure typical of a soluble protein with a high level of α-helical and β-sheet structure. The high helical content is consistent with the expectation that two hydrophobic (probably helical) transmembrane domains flank the extracellular intimin-binding domain. In contrast, the C-terminal domain contains a large amount of unordered or undefined structure. The accuracy of spectral deconvolution varies among the calculated secondary structural elements; in general, α-helix can be calculated with greatest accuracy, and the "unordered" component is not necessarily equivalent to polypeptide in "random coil" structure (17). These data suggest that the C terminus of Tir has a significant amount of unordered structure with phosphorylation in this region having no significant effect on secondary structure.

Attempts were made to obtain near UV data using the Tir protein in order to determine the effects of phosphorylation on the tertiary structure. Near UV data require large amounts of protein to obtain the concentrations (100 μM) necessary for measuring these weak signals and at these concentrations we found that Tir is prone to aggregation. However, the near UV data collected revealed no significant difference between the spectra of native and phosphorylated full-length Tir (not shown).

The C-terminal Domain of Tir Exists in a Monomer/Dimer Equilibrium—Given that PKA-phosphorylation induces shifts in the apparent molecular mass of Tir as determined by SDS-PAGE (11), both the native and phosphorylated Tir and C-Tir proteins were subjected to analytical ultracentrifugation to determine their molecular masses under non-denaturing conditions. Sedimentation velocity studies of native and phosphorylated Tir revealed the presence of multiple species of undeterminable mass, possibly indicative of aggregation (not shown). In contrast, the results with C-Tir show that phosphorylation using low PKA concentration (1:300, PKA/C-Tir, mol/mol) induces a shift in the sedimentation coefficient distribution to higher values (Fig. 2A). This is consistent with either a phosphorylation-induced conformational change and/or oligomerization. As each of these may be occurring, we carried out a sedimentation equilibrium experiment in order to assess the degree of oligomerization upon phosphorylation. The results are presented in Fig. 2B, which shows representative sedimentation profiles for unphosphorylated and phosphorylated forms of C-Tir. Each profile represents a global non-linear least-squares analysis of nine data sets collected at 3 protein concentrations and 3 speeds using the program NONLIN (19). The molecular mass determined from a single species fit were 27,562 (24,546, 30,553) and 46,601 (45,137, 48,058) for the unphosphorylated and phosphorylated forms, respectively. The figures in parentheses are confidence limits from the non-linear global fit quoted at the 67% level. Both of these are a non-integral value of the monomer molecular weight. A dissociation constant of 37 μM was calculated for the unphosphorylated C-Tir. The molecular mass of 27,562 kDa for unphosphorylated C-Tir is greater than that expected for a monomer (19,615 kDa), but less than that expected for a dimer (39,227 kDa), indicative of a system in equilibrium (Table II). The best model to fit our data set was that of a homogenous reversibly associating monomer/dimer equilibrium. The data can also be fitted to other models such as that describing a monomer/trimer. However, chromatography analysis of C-Tir at concentrations (50 μM) above that of the calculated dissociation constant (37 μM) on a Superdex 75 HR 10/30 gel-filtration column revealed a single peak with an elution volume corresponding to that of a dimer. In addition, observation of a small amount of C-Tir dimer by MALDI-TOF mass spectroscopy as well as the presence of dimers observed on SDS-PAGE gels (Fig. 3), both indicate that the model used (monomer/dimer) is the most
appropriate. As the molecular mass of phosphorylated C-Tir (46,601 kDa) as determined by analytical ultracentrifugation is higher than expected for a dimer (39,227) it appears that higher order forms are also present at the protein concentrations (5–20 µM) used.

**Stability and Association Properties of Tir and C-Tir on SDS-PAGE**—The anomalous migration of Tir (apparent molecular mass of 78 kDa, predicted molecular mass of 57 kDa) on SDS gels was also reproduced by its C-terminal domain, (apparent molecular mass of 30 kDa, with a predicted molecular mass of 20 kDa) (Fig. 3). This indicates that at least some of the structural properties of Tir responsible for anomalous migration reside in the C-terminal domain. Moreover, these observations indicate that the proteins retain some structure on SDS-PAGE.

### Table II

**Molecular parameters of the C-terminal domain of Tir**

Native molecular masses in 20 mM Tris-HCl, 50 mM NaCl (pH 7.5) were determined by analytical ultracentrifugation and evaluated according to single-species fit by non-linear-squares fitting of the combined data at three protein concentrations (5, 10, 20 µM) and at three separate run speeds: 17,000, 22,000, and 28,000 rpm. The theoretical molecular mass of C-Tir (residues 385–550) was calculated using ExPASy Web software ProtParam with the addition at the C-terminal end of HSV/His fusion tags and in close agreement with the experimental value determined using MALDI-TOF mass spectrometry (in parentheses is the value of C-Tir dimer). The calculated mass of phosphorylated C-Tir assumes the addition of one phosphate group at the low PKA concentration. The PI value of C-Tir was estimated by two dimensional gel electrophoresis with the theoretical pI of 5.45 determined using ExPASy web software ProtParam. Also given are the PI values of C-Tir after phosphorylation at low and high (in parentheses) PKA concentrations as described in the text.

| Protein               | Molecular mass | $K_d$ (Monomer-dimer) | PI values |
|-----------------------|----------------|-----------------------|-----------|
|                       | $cm^3 g^{-1}$ | Da                    | µM        | Calc. | Exp. | Calc. | Exp. |
| C-Tir                 | 0.7109         | 19,606                | 27,562    | 37    |      | 5.45  | 5.54 |
| Phosphorylated C-Tir  | 0.7109         | 19,886                | ND*       | 46,601|      | 5.26 (5.11) |

* ND, not determined.
PKA-mediated Phosphorylation of EPEC Tir

PKA-mediated Phosphorylation Affects the Self-association Properties of the C-terminal Domain of Tir—Figs. 3 and 4A reveal that Tir migrates as monomers, dimers, and higher oligomers on SDS-PAGE gels when SDS is omitted from the sample application buffer. PKA phosphorylation of Tir (1:1, PKA/Tir, mol/mol), as expected, induces shifts in the apparent molecular mass of the protein (78–85 kDa), while the distribution of monomeric, dimeric, and higher oligomeric phosphorylated forms remained unaffected (Fig. 4A). Fig. 4B shows that phosphorylation of C-Tir, like Tir (14), induces equivalent shifts in apparent molecular mass with these forms designated C-Tir' and C-Tir". Low concentrations of PKA (1:300, PKA/C-Tir, mol/mol) resulted in the majority of C-Tir being converted to the C-Tir" form with only low levels of C-Tir' evident. At higher PKA levels (1:15 and 1:3, PKA/C-Tir, mol/mol) all C-Tir is converted to C-Tir". This result differs from that previously reported, and reproduced in this study (not shown), where Tir is only modified to the T' form at low PKA concentration (1:100, PKA/Tir, mol/mol) (11). The apparent enhancement in phosphorylation efficiency may be attributed to enhanced accessibility to PKA of serine residues in the isolated C-terminal domain.

To further assess the analytical ultracentrifugation data, unmodified C-Tir was examined under native gel conditions and the detection of two distinct forms further supports an equilibrium (monomer/dimer) model (Fig. 4C). In vitro phosphorylation of C-Tir at low PKA concentration shifted this equilibrium predominately to a dimeric form (D1), as also suggested by analytical ultracentrifugation (Fig. 2), with two monomeric species (M1/M2) evident that presumably equate to the C-Tir' and C-Tir" forms observed on SDS gels. The pI of unmodified C-Tir and the phosphorylated forms generated at low PKA concentration (i.e. M1/M2) were determined by two-dimensional gel electrophoresis revealing values of 5.54, 5.26, and 5.1 respectively (Table II). The pI value of 5.54 for the unmodified C-Tir is in close agreement with the theoretical pI of 5.45 predicted by the ExPASy Web software ProtParam. The pI of the two phosphorylated forms of C-Tir (M1/M2) confirms that both these forms are distinguished from the unmodified C-Tir by a significant difference in net charge.

At higher PKA concentrations (1:15 or 1:3, PKA/C-Tir, mol/mol) phosphorylation results in decreased level of the dimeric form of C-Tir as the protein is now predominately in a monomeric form (M3). This monomeric species now migrates more rapidly compared with the two phosphorylated forms observed following modification at the lower PKA concentration (M1/M2).

The detection of this M3 form is surprising as only two phosphorylated states are detected by SDS-PAGE analysis and further work including mass spectrum analysis is required to establish the number of residues that are phosphorylated in Tir. It is possible that PKA has low affinity for the serine residue involved in this modification perhaps as it is inaccessible due to location in a dimerization interface. To test this possibility the ionic strength of the phosphorylation buffer was altered from 50 to 150 mM NaCl (physiological ionic strength) to destabilize possible electrostatic interactions, and the effect on protein modification at low PKA concentration was assessed. This change in ionic strength resulted in the detection of the M3 form, in contrast to the detection of only M1/M2 forms following modification at low ionic strength.

Denaturation of C-Tir by Guanidine Hydrochloride—The CD data indicate a high content of undefined structure within the C-terminal domain of Tir. To assess the extent to which C-Tir displays properties of a globular protein, including co-operative unfolding transitions, guanidine hydrochloride unfolding was carried out. This experiment revealed a two-step unfolding transition (Fig. 5). The first transition occurring at very low concentrations of denaturant (0–1 M GdnHCl) was ascribed to dimer dissociation since ultracentrifugation and native gel electrophoresis demonstrate that C-Tir is largely dimeric at the protein concentration used in these experiments. The second transition was ascribed to unfolding of the monomer and occurs through a single unfolding step between 1 and 5 M GdnHCl. From the data the transition midpoints, the free energy of unfolding (ΔG(U)) at 25 °C, and the change in free energy of unfolding with GdnHCl concentration (m) were calculated as previously described (20, 21). The free energy associated with the first transition (dimer dissociation) was determined to be -0.5 kcal/mol with a transition midpoint of 0.1 M GdnHCl and m value of 4.7 indicating weak dimeric interactions. The free energy of unfolding of the second transition (unfolding of monomer) was determined to be -3.8 kcal/mol with a transition midpoint of 2.8 M GdnHCl and m value of 2.6. The free energy of the native state relative to the unfolded state, (ΔG(U)) and the small denaturant dependence for unfolding (m) are each smaller than expected for compact, dimeric proteins with a well packed hydrophobic core (22, 23), and support the CD data of a partially disordered or loosely packed domain.

DISCUSSION

Structural Composition of Tir and C-Tir—In this study biophysical methods have been used to characterize the effect of PKA-mediated phosphorylation on the conformation and self-association properties of Tir in order to address the physiolog-
ical relevance of this modification. The CD spectra of Tir, and in particular that of its C-terminal domain, contain contributions from both ordered and disordered structure. Tir has a significant helical content (around 25% of the total protein) consistent with the expectation that hydrophobic (and probably helical) domains are inserted into the membrane during translocation of the intimin-binding domain to the exterior of the host cell plasma membrane. However, the isolated C-terminal domain has little regular secondary structure. Since this domain undergoes a co-operative denaturation transition, displays self-association properties and phosphorylation-induced shifts on native and SDS-PAGE gels, which parallel those of the native protein, it is unlikely that most or all of the disordered structure calculated from spectral deconvolution is equivalent to random coil. This is supported by preliminary data using small angle x-ray scattering techniques which reveal a globular and compact conformation for the C-terminal domain.\footnote{A. Hawrani, C. E. Dempsey, M. J. Banfield, D. J. Scott, and B. Kenny, unpublished data.} The CD data presented here also indicate that serine phosphorylation

![Figure 4](image_url)

**Figure 4.** Effect of PKA phosphorylation on Tir and C-Tir as determined by SDS and native PAGE (10%). Purified Tir and C-Tir were analyzed by SDS or native PAGE followed by Coomassie Blue staining. Tir migrates as monomers, dimers and higher oligomers when SDS is omitted from the sample application buffer (A). Phosphorylation of Tir (1:1, PKA/Tir, mol/mol) induces shifts in the apparent molecular mass of the protein with the relative distribution of monomeric, dimeric, and higher oligomeric phosphorylated forms of the protein unaffected. Phosphorylation of C-Tir (B), with increasing concentrations of PKA (0.05 units of PKA is equivalent to 1:300, PKA/C-Tir, mol/mol) induces equivalent shifts in apparent molecular mass as those previously described with Tir on SDS gels (11). Arrows indicate the position of unmodified C-Tir, the two PKA-modified forms (C-Tir\(^\text{H11032}\) and C-Tir\(^\text{H11033}\)) as well as the modifying enzyme PKA. On native gels (C), phosphorylation of C-Tir promotes dimerization (D1) at low PKA concentrations and dissociation to a monomeric state at higher PKA concentrations (M1–M3). Efficient modification of C-Tir to the monomeric form could also be produced at low PKA concentrations by increasing the ionic strength to physiological levels. NaCl alone does not affect the monomer/dimer equilibrium. \(+/–\) indicates the presence/absence of the reagent in the sample application buffer.

![Figure 5](image_url)

**Figure 5.** Equilibrium folding profile of C-Tir. An equilibrium folding profile of C-Tir using guanidine hydrochloride was monitored using tryptophan fluorescence emission spectra and an excitation wavelength of 290 nm. Fluorescence is plotted as a function of the molar activity of GdnHCl as described previously (20, 21) to allow a more reliable extrapolation of the data and evaluation of the rate and equilibrium constants in water.
per se does not promote structural enhancement of unstructured regions within C-Tir, although this may occur in the context of protein interactions within the host cytoplasm. On the other hand, serine phosphorylation of Tir and C-Tir results in significant changes in mobility on native and SDS gels and for the C-terminal domain in self-association properties. It seems either that any secondary structural changes induced upon phosphorylation are subtle and not easily detected by CD spectroscopy, or that phosphorylation affects interactions among domains whose structures are not significantly affected. The limited secondary structure in the C-terminal domain observed by CD does not therefore preclude interactions, some of which are mediated by phosphorylation that may be functionally relevant in the context of the native protein. Indeed, a number of proteins involved in bacterial pathogenicity have been found to have partially disordered domains (24, 25). It is suggested that structural disorder affords a degree of functional plasticity that could both facilitate translocation in a partially folded form and promote interactions with host proteins. Moreover, atomic force microscopy techniques indicate that secretion of effector proteins through the translocation apparatus is dependent on partially unfolded forms of these proteins (26).

Phosphorylation-induced Changes on SDS and Native Gels—The migration of C-Tir, and its phosphorylated forms, on SDS-PAGE and native PAGE appears to be inconsistent at first sight, since the phosphorylated forms migrate with apparently higher molecular mass in SDS, and with lower molecular mass on native gels. These apparently conflicting observations may result, either from phosphorylation-induced changes in the hydrodynamic properties of the proteins or may simply be due to charge effects. For example, a more compact or structured phosphorylated state will have increased migration on native gels, but decreased migration on SDS gels if the compact state has reduced binding sites for SDS (27). The pI values of the two phosphorylated forms of C-Tir confirmed that both these forms are distinguishable from the unmodified C-Tir by a significant difference in net charge. As no significant differences were observed in the circular dichroism spectra of the phosphorylated and non-phosphorylated Tir and C-Tir proteins, it is therefore more likely that phosphorylation-induced changes in the assembly-state of C-Tir may involve modulation of electrostatic interactions rather than large scale conformational changes.

PKA-mediated Phosphorylation Effects on the Self-association Properties of C-Tir—Analytical ultracentrifugation data shows that unphosphorylated C-Tir exists in a dynamic equilibrium of monomers and dimers that is modulated in vitro by PKA-mediated phosphorylation. This is consistent with the observations from native gel electrophoresis. Upon phosphorylation of C-Tir with low concentrations of PKA (1:300, PKA/C-Tir, mol/mol) there is an increase of the dimeric form. It therefore appears that phosphorylation at this level of PKA activity promotes dimerization of C-Tir. The dissociation constant of 37 μM, calculated from the sedimentation equilibrium data set (Fig. 2B), suggest that at physiological concentrations of protein (1 μM to 1 μM, Ref. 22) C-Tir is likely to exist in a monomeric form within host cells, with in vivo phosphorylation leading to dimerization. Such a mechanism has been reported both in vitro and in vivo for the Bacillus subtilis transactivator SpOa, which also displays phosphorylation-mediated shifts from monomers to an activated dimer conformation (28).

In previous studies, in vitro phosphorylation of Tir using high concentrations of protein kinase-A (1:1 PKA/Tir, mol/mol) reproduced the shifts in electrophoretic mobility (as assessed by SDS gels) that are displayed by Tir isolated from membrane fractions of EPEC-infected host cells (11). These shifts are also displayed by C-Tir (phosphorylated) using high concentrations of PKA (Fig. 4B). Interestingly, at these higher concentrations of PKA, C-Tir (as assessed by native gels) is now almost completely dissociated to a monomeric form compared with the dimeric state detected at low PKA concentrations (Fig. 4C). This monomeric form of C-Tir (M3) could also be generated using low concentrations of PKA and physiological ionic strength, perhaps due to salt-induced exposure of an inaccessible serine that is destabilized at higher salt concentrations. Although the relevance of this observation in the context of native Tir remains to be determined, PKA-mediated dissociation of domain contacts may function to expose binding sites for interaction with host proteins, for example the tyrosine kinase that mediates the final phosphorylation of Tir on tyrosine 474 (2). A role for phosphorylation-induced dimer destabilization is illustrated by studies with ezrin, a membrane cytoskeleton linker protein. Threonine phosphorylation of ezrin is shown to weaken the interactions between its C- and N-terminal domains through both electrostatic and steric effects (29). Similarly, phosphorylation of the sporulation response regulator Spo0A is viewed as altering an equilibrium between an inactive (monomeric) and active (dimeric) forms of the protein (28). Protein kinase A phosphorylation has also been found to induce the association of ADP-ribosylation factor 1 to Golgi membranes (30).

In conclusion, we have shown that the C-terminal domain of Tir exists in an equilibrium (monomer/dimer) that is modulated by in vitro phosphorylation. PKA-mediated phosphorylation induces changes in the association properties of the C-terminal domain that may facilitate interaction with host proteins in vivo, such as the tyrosine kinase that phosphorylates Tyr-474 (6, 7) and/or other proteins involved in Tir-intimin mediated actin rearrangements. However, the assembly state of Tir is not as clearly defined, and this is evident from our analytical ultracentrifugation and gel electrophoresis data. It may be the case that oligomerization of Tir is promoted by contacts in addition to those of its C-terminal domain. This is consistent with the observed dimerization of the intimin-binding domain bound to intimin (9), though this Tir-Tir interaction would presumably be a later step that occurs following intimin-binding domain insertion across the plasma membrane.

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