The Coiled-coil Domain of EspA Is Essential for the Assembly of the Type III Secretion Translocon on the Surface of Enteropathogenic Escherichia coli

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Enteropathogenic E. coli (EPEC) utilize a type III secretion system to deliver virulence-associated effector proteins to the host cell. Four proteins, EspA, EspB, EspD, and Tir, which are integral to the formation of characteristic “attaching and effacing” (A/E) intestinal lesions, are known to be exported via the EPEC type III secretion system. Recent work demonstrated that EspA is a major component of a filamentous structure, elaborated on the surface of EPEC, which is required for translocation of EspB and Tir. The carboxyl terminus of EspA is predicted to comprise an α-helical region, which demonstrates heptad periodicity whereby positions a and d in the heptad repeat unit abedefg are occupied by hydrophobic residues, indicating a propensity for coiled-coil interactions. Here we demonstrate multimeric EspA isoforms in EPEC culture supernatants and EspA:EspA interaction on solid phase. Non-conservative amino acid substitution of specific EspA heptad residues generated EPEC mutants defective in filament assembly but which retained the ability to induce A/E lesions; additional mutation totally abolished EspA filament assembly and A/E lesion formation. These results demonstrate a similarity to flagellar biosynthesis and indicate that the coiled-coil domain of EspA is required for assembly of the EspA filament-associated type III secretion translocon.

Most bacterial virulence-associated determinants are either surface located or are secreted from the bacterium. There are only a limited number of ways by which Gram-negative bacteria can transport proteins across their unique double membrane (reviewed in Ref. 1). The type III secretion system, found in many Gram-negative pathogens (reviewed in Ref. 2), is responsible for delivery of virulence-associated factors involved in subversion of the host-cell signal transduction pathways required for bacterial adhesion, invasion, and disease. A particularly good example of bacterial pathogens that employ a multi-stage infection strategy involving a type III secretion system is provided by enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC) (reviewed in Ref. 3). EPEC is an established etiological agent of human diarrhea, and EHEC is an emerging food borne cause of acute gastro-enteritis and hemorrhagic colitis (reviewed in Ref. 4). Subversion of epithelial cell function by EPEC and EHEC leads to the formation of distinctive “attaching and effacing” (A/E) lesions, characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to the host cell membrane, and the formation of an actin-rich underlying pedestal-like structure in the host cell (reviewed in Ref. 3). All the genes necessary for the A/E effect map to a pathogenicity island termed the locus for enterocyte effacement or LEE (5), which includes structural components of the secretion apparatus (6), the adherence molecule intimin (7, 8), secreted proteins EspA, EspB, EspD (reviewed in Ref. 3), and Tir (9), and their respective chaperones (10, 11).

The type III secretion apparatus comprises approximately 20 mainly inner membrane-associated proteins, which demonstrate broad functional conservation across bacterial species (2, 12). Additionally, many of these components demonstrate similarity to proteins involved in bacterial flagellar biosynthesis (2). EspA is secreted via the type III secretion apparatus and is required for the translocation of EspB and Tir into the host cell cytosol (9, 13, 14). It has recently been identified as a major component of large extracellular filamentous structures, which appear on the surface of EPEC at an early stage of infection prior to intimate attachment (14).

The assembly of a number of eukaryotic proteins into filamentous structures is based upon interactions between dimeric coiled-coil domains (15), and similarly predicted coiled-coil segments lie within regions of functional significance in a number of bacterial proteins. Coiled-coil segments have been predicted to occur in many proteins associated with type III secretion systems (16), where they have a potential role in mediating the formation of large multi-protein complexes or more discrete interactions that may facilitate protein translocation. Notably, bacterial flagellins contain coiled-coil domains at the amino and carboxyl termini of the protein corresponding to regions deemed important in the polymerization of flagellin into the flagellar filament (17, 18). EspA demonstrates discrete sequence similarity to flagellins in the carboxyl-terminal region of the protein which is predicted with high probability to adopt a coiled-coil conformation (16, 19).
The similarity between type III secretion components and proteins of the flagellar export machinery, particularly in regions predicted to form coiled-coils prompted us to investigate the importance of the predicted coiled-coil domain of EspA for the assembly and function of the EspA filaments. In this study we provide evidence, based on a rational site-directed mutagenesis approach, that mutation of specific residues predicted to be critical in coiled-coil conformation have dramatic consequences for the assembly of the EspA filament and subversion of host cell signal transduction pathways leading to A/E lesion formation. Considering the similarities between different type III secretion systems, coiled-coil interactions may represent a common feature of their function.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The bacterial strains used in this study were generated by vector complementation of the espA gene in the espA mutant strain UMD872 (20). Complementation was achieved by transformation of plasmid pMSD2 containing the functional EPEC espA gene (20). In addition, strains UMD864 (espB) and UMD870 (espD), and the prototype (parent) EPEC E2348/69 were used. Mutant strains were generated by site directed mutagenesis of pMSD2. Bacteria were grown in an aerated stationary phase at 37 °C in t broth, or Dulbecco’s modified Eagle’s medium (DMEM), with the addition of chloramphenicol to a final concentration of 30 µg/ml as appropriate.

**Sequence Analysis**—Coiled-coil predictions were carried out using a revised version of the computer program described by Lupas et al. (21) available via the World Wide Web. Predictions were based on a window size of 28 residues and weighting the algorithm in favor of hydrophobic residues at positions a and d of the heptad repeat.

**Construction of GST-EspA Fusion Protein and Solid Phase Binding Assay**—For analysis of EspA monomeric interactions using GST-EspA fusion protein, a BamHI-flanked espA polymerase chain reaction fragment (primer pair; 5'GGGATCCATGGATACATCAACTAC-3' and 5'-CGGATCTTTATTACAAACGATTTCC-3', temperature cycling as follows; 1 cycle of 95 °C, 5 min, then 25 cycles of 95 °C, 30 s, 55 °C, 45 s, 72 °C, 30 s, with a final extension step of 72 °C, 5 min) was cloned into the BamHI site of the gene fusion vector pGEX-2T, creating a translational fusion with glutathione S-transferase (generating plasmid pCC34). The GST-EspA fusion was produced in XL1-Blue by induction of a 2-h log phase culture with 1 µg isopropyl-1-thio-

**RESULTS**

**Identification of Multimeric EspA in Supernatants of EPEC Cultures**—Secreted proteins from mutant EPEC strains UMD870 (espB), UMD864 (espB), UMD872 (espA), and the prototype wild type strain E2348/69 were separated in 12% native gels, blotted, and probed for EspB (lanes 1–4, respectively). The shifted bands imply multimeric forms of EspA, however, due to the non-gradient nature of the gel it cannot be determined which specific multimeric forms are represented. Significantly, EspD and EspB mutant strains demonstrate the same laddered profiles as the wild type EPEC strain.

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**FIG. 1. EspA Multimerization in the Supernatants of EPEC Cultures**—Secreted proteins from mutant EPEC strains UMD870 (espB), UMD864 (espB), UMD872 (espA), and the prototype wild type strain E2348/69 were separated in 12% native gels, blotted, and probed for EspB (lanes 1–4, respectively). The shifted bands imply multimeric forms of EspA, however, due to the non-gradient nature of the gel it cannot be determined which specific multimeric forms are represented. Significantly, EspD and EspB mutant strains demonstrate the same laddered profiles as the wild type EPEC strain.
pMSD2 containing a cloned espA gene (results not shown). These results demonstrate that EspA is a major component of the EspA filament assembly, and as such, monomers would be expected to possess structural regions that would mediate hetero- and/or homo-oligomeric interactions. Importantly, multimerization of EspA was not affected in the background of espD and espB mutant strains and appeared identical to the wild type strain E2348/69 (Fig. 1, lanes 1 and 2, respectively).

**Solid-phase EspA-EspA Binding Assays**—As a preliminary demonstration of EspA:EspA interactions, we assessed the ability of purified EspA fusions to associate in an ELISA based assay. Purified His-tagged EspA was used to coat wells of a 96-well plate, then serial dilutions of purified GST-EspA were added. GST-EspA immobilized in wells by association with His-EspA was detected by anti-GST antibody followed by alkaline phosphatase-conjugated anti-goat antiserum. GST-EspA immobilized in wells by association with His-EspA indicating EspA:EspA association on the solid phase. In the absence of competing His-EspA, however, binding of GST-EspA is substantially reduced, suggesting competition for EspA interactions. GST alone does not demonstrate significant binding to immobilized His-EspA indicating EspA:EspA association on the solid phase. In the presence of competing His-EspA, however, binding of GST-EspA to immobilized His-EspA indicating EspA:EspA association on the solid phase. In the presence of competing His-EspA, however, binding of GST-EspA is substantially reduced, suggesting competition for EspA interactions. GST alone does not demonstrate significant binding to immobilized His-EspA (C).

**Table I**

| Mutations in the Coiled-coil Domain of EspA Do Not Affect Protein Expression and Secretion—EspA multimerization, EspA filament assembly, and the ability to support A/E lesion formation.

The EspA derivatives were constructed by site-directed mutagenesis using plasmid pMSD2. Mutant derivatives were introduced into UMD872, and biological functions of the mutated EspA were characterized in terms of Esp protein secretion, EspA multimerization, EspA filament assembly, and the ability to support A/E lesion formation.

**Mutations in the Coiled-coil Domain of EspA Do Not Affect Protein Expression and Secretion—Western blots of bacterial supernatants and whole cell extracts were used to confirm that the mutations introduced into the coiled-coil domain of EspA did not affect LEE-encoded protein expression or secretion. All of the single and double espA mutants secreted EspA at overall levels comparable to wild type in both L-broth and DMEM cultures (Fig. 4A), although some culture to culture variation between experiments was observed (Fig. 4A). In contrast, the triple espA mutant UMD872/pICCC24 produced an insoluble EspA that was only detected in the bacterial pellets and migrated with a higher molecular weight compared with the native EspA (data not shown). Consequently, the triple EspA mutant was not investigated further.
Mutagenesis of espA had no significant effect on secretion of EspB and although, similarly to EspA, some variations between experiments was observed, similar levels of secreted EspB were detected in culture supernatants of all strains (Fig. 4B); expression of intimin, an outer membrane EPEC adhesion molecule, by all mutant strains was similarly unaffected (data not shown). These results show that the mutations in the coiled-coil domain of EspA do not have pleotropic effects on expression or secretion of LEE-encoded virulence genes, including EspA itself.

**Mutations in the Coiled-coil Domain Affect Multimerization of EspA**—An indication of the impact of position a substitutions on the ability of EspA to polymerize into higher structures was obtained by analysis of Western blots of DMEM-secreted proteins separated under non-denaturing conditions. Mobility shifts were demonstrated in all mutant EspAs as a consequence of slight charge differences between arginine and the residues substituted and likely conformational changes induced by the a position substitutions. Additionally, single and double mutations, with the exception of Arg163 (pICC25), appeared to affect the multimerization potential of the protein, as evidenced by depletion of some oligomeric forms (Fig. 5).

**Mutations in the Coiled-coil Domain Affect EspA Filament Formation**—The results of the immunostaining of the EspA filaments is summarized in Table II and illustrated in Fig. 6. This revealed greatly shortened surface protrusions, which appeared as ‘bubbles’ or stumps around the cell in UMD872(pICC25) and UMD872(pICC26) compared with UMD872(pMSD2). In contrast, and as suggested by the altered mobility pattern on native gels, the double position a amino acid mutants lacked any EspA filament structure. The observation that all double substitutions at different positions along the length of the predicted coiled-coil domain in EspA resulted in the same mutant phenotype (Table II) provides a strong indication that the underlying basis for the inability of the EspA mutant to assemble into a filament involves disruption of a structural domain rather than resulting from more serious perturbations in the predicted a-helical secondary structure of the protein. The fact that the double mutant EspAs are soluble and secreted further support this suggestion (data not shown).

**A/E lesion Formation on Cultured HEp2 Cells**—In order to identify possible functional effects of the coiled-coil mutagenesis, we investigated the ability of the different strains to induce A/E lesions on cultured HEp-2 cells using the FAS test as a marker for lesion formation (23). Single amino acid substitution by arginine at position Leu149 or Met163 was insufficient to inhibit formation of A/E lesions by UMD872(pICC26) or UMD872(pICC25), respectively, whereas double mutants, even after extended incubations (5 h), were unable to initiate host cytoskeletal rearrangements and A/E lesion formation (Table II, Fig. 6).

**Conservative Reversion of Arginine Mutations**—To demonstrate that the mutant phenotypes were solely a consequence of the specific substitutions introduced at the carboxyl terminus, we targeted the non-permissive Arg149 residue in pICC26 for substitution with more conservative amino acids methionine, phenylalanine, and valine producing plasmids pICC30, pICC31, and pICC32, respectively. A similar methionine substitution was also introduced into the double mutant pICC27 at the same position generating plasmid pICC33.

The FAS phenotype and EspA filament assembly were restored in UMD872(pICC31) and UMD872(pICC32) (Table II; Fig. 6), while UMD872(pICC30) remained deficient of wild type.
Coiled-coil Domain Required for EPEC EspA Filament Assembly

Fig. 6. EspA fluorescence (column 1), actin fluorescence (column 2), and corresponding phase contrast (column 3) micrographs illustrating production of EspA filaments and A/E activity (actin accumulation at sites of bacterial attachment) of strains. A, E2348/69 (wild type EPEC); B, UMD872 (espA\(^-\)) C, UMD872pMSD2 (espA); D, UMD872pICC25 (Arg\(^{149}\)); E, UMD872pICC31 (Phe\(^{149}\)); F, UMD872pICC27 (Arg\(^{149}\)/Arg\(^{163}\)). Strains E2348/69 (A), UMD872pMSD2 (C), and UMD872pICC31 (E) produced mature EspA filaments and exhibited A/E activity, strain UMD872pICC25 (D) produced only vestigial EspA filaments but still exhibited A/E activity, and strains UMD872 (B) and UMD872pICC27 (F) did not produce EspA filaments and were negative for A/E activity. Magnification: columns 1, \(\times 2,000\); columns 2 and 3, \(\times 1,300\).

Discussion

Coiled-coils form important structural domains in diverse proteins mediating oligomerization of monomers via association of two or more \(\alpha\)-helices. Sequences that are capable of forming coiled-coils are characterized by heptad repeats comprising seven amino acids designated a–g, where the first (a) and fourth (d) positions are occupied by hydrophobic amino acids, and the remaining positions (b, c, e, f, and g) by polar amino acids. The a and d position residues form a hydrophobic interface between helices which are buried in the core of the assembled coiled-coil complex. The hydrophobic interactions form the basis for the stability of the complex.

The prevalence of predicted coiled-coils among proteins of type III secretion systems (16) hints at a potential mechanism by which proteins may interact in the formation of multimeric complexes or processes involved in the translocation of effector proteins. The prediction of a coiled-coil segment in a region of EspA that demonstrates significant similarity to a well-characterized coiled-coil region in flagellins from a number of bacterial species introduces the possibility that coiled-coil interactions may contribute to EspA filament assembly.

In addition to flagellins, the assembly of a number of eukaryotic proteins into filamentous structures is based upon interactions between dimeric coiled-coil domains (15). However, only one coiled-coil domain is predicted in the carboxyl terminus of EspA, which presents the possibility that this region mediates homo- and/or hetero-oligomeric interactions between different polypeptides (16). Our results suggest that EspA:EspA interaction is impaired by mutation at an essential carboxyl-terminal domain, although some oligomeric forms of EspA are still evident in the more extreme double mutants (Fig. 5). This is perhaps not unexpected since other molecular interactions in addition to the potential contribution of coiled-coils are likely to be involved in EspA association. Using GST-EspA and His-EspA, we demonstrated EspA-EspA interaction on solid phase. Unfortunately, due to low expression level of the double mutant GST-EspA-EspA fusion, we were unable to determine the effect of the mutations on EspA-EspA interaction using this binding assay.

Carboxyl-terminal mutants are, however, unable to produce surface EspA filaments. This is a direct effect of mutation within the predicted coiled-coil domain and may be a consequence of the destabilizing effect incurred by coiled-coil disruption on homo-oligomeric interactions, or alternatively, abrogation of EspA interactions with additional protein(s) more integral to filament assembly or structure. Mutations affecting flagellar presentation and shape are similarly located in a coiled-coil region of the flagellin (17, 26).

Unlike double substitution mutants where EspA:EspA interaction and EspA filament assembly was totally abrogated, single substitution mutants produced a vestigial EspA filament. In this situation, alterations within an essential domain may not be sufficient to prevent EspA:EspA interaction but appear to be sufficient to prevent polymerization into long flagella. Interestingly, the vestigial filaments produced by the single substitution mutants are reminiscent of the filament stumps produced by an \(espD^-\) strain UMD870 (14). We have provided evidence that neither EspD nor EspB is required for EspA multimerization, although it is clear that EspD is integral to assembly and or elaboration of the filament on the bacterial cell surface (14). It has recently been determined that EspD is transported to the host cell membrane where it remains exposed and solvent accessible (27). EspD has two predicted coiled regions, spanning residues 147–174, and 336–368 (16), which could potentially mediate structural interactions with EspA and/or other proteins, presenting a potential mechanism by which the EspA filament could be anchored at the host cell surface.

In the light of this recent information, EspA filament assembly may require the contribution of EPEC proteins, which perhaps reflect further the function of flagellar biogenesis components, such as the flagellar capping protein FliD, which is essential for polymerization of flagellin monomers at the tip of the growing flagellum (28). Similarly, a second gene in the \(fliD\) operon of \(Salmonella\) typhimurium, \(fliS\), encodes a protein that appears to facilitate the export of flagellin through the growing filament channel (29). Mutation at \(fliS\) affects the filament elongation step, resulting in short flagella with a concomitant decrease in the amount of excreted flagellin. Both these effects are mirrored, with respect to EspA, by mutation of the EPEC \(espD\) gene (14). Alternatively, aberrant surface presentation of the filament may be due to a failure of a more mechanical...
nature involving proteins that anchor the filament at the bacterial cell surface.

Demonstration of the structural integrity of the mutant EspA proteins is an important consideration if inferences are to be made concerning the effect of mutations in an uncharacterized domain. The ability to restore wild type EspA function by reversion of a functionally non-permissive mutation to a more conservative residue (Arg to Val), but one that is still different from wild type (Leu), provides an indication that the altered phenotypic effects were the consequence of aberrant structural interactions, since low tolerance for substitution at α positions comprising the hydrophobic core in coiled-coils is well documented (30). The smaller Val and Phe residues are less likely to grossly deform a coiled-coil in the way that the larger Met residue at the same position might. It is also possible that Met interacts differently with residues from neighboring peptides.

The results presented herein provide the first analysis of EspA filament assembly assessed in the context of culture supernatants, which in the prototypic strain E2348/69 are known to contain several proteins secreted by the type III secretion system (31). Future studies using purified wild type E2348/69 derivatives and pMDS2.

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