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Structural and functional studies indicate that the EPEC effector, EspG, directly binds p21 activated kinase

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Abstract

Bacterial pathogens secrete effectors into their hosts that subvert host defenses and re-direct host processes. EspG is a type three-secretion effector with a disputed function that is found in enteropathogenic *E. coli.* Here we show: that EspG is structurally similar to VirA, a Shigella virulence factor; that EspG has a large, conserved pocket on its surface; that EspG binds directly to the amino-terminal inhibitory domain of human p21 activated kinase, (PAK); and that mutations to conserved residues in the surface pocket disrupt the interaction with PAK.

Enteropathogenic *E. coli*, EPEC, are a leading bacterial cause of diarrhea, and the leading cause of diarrhea in infants (1). Infection occurs when, following ingestion by the host, EPEC adhere to intestinal epithelial cells and form attaching and effacing (A/E) lesions (2). The formation of A/E lesions requires the locus of enterocyte effacement (LEE), which encodes a type three-secretion system (TTSS) and multiple effector molecules (3). *Shigella*, a related genus of pathogens, use a similar TTSS to promote internalization, rather than A/E lesions (4). In both cases, effector proteins orchestrate a complex series of poorly understood molecular events to subvert host cell defenses, thereby preventing or delaying infection clearance.

EPEC and *Shigella* express the homologous effector proteins, EspG and VirA, respectively. (EPEC also express EspG2, a distant, redundant homolog of EspG that is not LEE encoded (5).) These proteins are thought to have roles in cytoskeletal remodeling (6), although the mechanism of action and molecular targets are unknown. Recent reports indicate that these proteins do not act as papain family proteases (7,8) as was originally described (9–12). Despite ambiguity regarding the functional roles of EspG and VirA, it is clear that VirA has a role in cell-to-cell spread (13), and that EspG can reverse the spread phenotype of a *virA* null (14). The structure of VirA led to the identification of an inter-domain cleft and an inter-domain pocket (7,8). A goal for the present study was to determine which if either of these features was conserved in EspG and to gain insight into how these effectors modulate the host cytoskeleton.

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Note Added as proof: While this manuscript was under review another paper (Selyunin *et al*.) (27) demonstrated that EspG binds PAK2 as well as ADP ribosylation factor 6 ARF6), resulting in activation of PAK2, inactivation of ARF6, and localization of the complex to the golgi. Our study agrees with their findings.

SUPPORTING INFORMATION

Supporting information includes detailed experimental methods, crystallographic statistics, a sequence alignment, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

We have determined the X-ray crystal structure of EspG (PDB 3Q1C) and compared it to that of VirA (pdb 3EB8). As with VirA and other TTSS effectors, the amino-terminus of EspG is likely disordered (7,8,15). While we were unable to obtain crystals of full-length EspG, crystals of Δ43EspG diffracted to 1.6Å. The EspG structure was determined by single wavelength anomalous scattering, using data from a NaBr derivative, and density modification (16). The final structure was refined to 1.8Å, and includes residues 44–158, 161–316, and 321–395. Data collection and refinement details are given in Table 1 of the Supporting Information.

Despite only ~17% sequence identity (supplemental figure 1), EspG and VirA are structurally similar (figure 1). Both proteins contain a central six-stranded sheet, flanked by an amino-terminal domain, composed of a 4-stranded beta sheet, and a helical domain. The individual domains of the two proteins are fairly similar, with rms differences of 2.3Å for the amino-terminal domain (espG residues 70–141), 4.6Å for the central sheet (EspG residues 153–187, 213–220, 294–371, 386–395), and 3.3Å for the helical domain (EspG residues 194–212, 221–292, 374–384). The principal differences are a ~60º rotation of the amino-terminal domain between EspG and VirA, and a ~90º kink in helix 9 (figure 1).

The structural analysis of VirA revealed a prominent inter-domain cleft (figure 1B) (8). The alternate position of the amino-terminal domain observed in EspG largely fills this cleft (figure 1A). Although this could represent the closed conformation of a mobile domain, the lack of sequence conservation indicates that this cleft is not required for EspG function (figure 2). There is, however, a deep pocket revealed in the structure of EspG (figure 1A) that appears to be highly conserved between EspG and VirA (figure 2). This pocket is ~18Å long, ~11Å wide, and ~20Å deep, and the residues surrounding it are well conserved among all EspG, EspG2, and VirA sequences.

The rim of the ~20Å deep pocket is lined by 12 well-conserved residues, primarily from helix 4, helix 9, and the loop preceding strand 7 (figure 2 and supplemental figure 2). Two isoleucines, 328 and 330, from strand 7 and two hydrophilic residues, S342 and T344, from strand 8 form the base of the pocket (supplemental figure 2). Helix 4, which forms one rim of the pocket as shown in figure 2, uses conserved hydrophilic and charged residues: S201, D205, and R208. An orthogonal rim of the pocket is formed by residues from the kinked helix 9: Q293, S294, and V297. D205 and R208 are notable because they are the only conserved charges that line the entrance to the pocket. To evaluate the importance of D205 and R208, they were mutated to alanine and the double mutant (EspG_DR-AA) was stably expressed in Hek cells.

Although the functional roles of EspG and VirA in EPEC and *Shigella* infection may be different, the common need to modulate cytoskeletal structure at the membrane led us to investigate the activation of small G-proteins. Activation of Rho, Rac, and Cdc42 is important in regulation of the cytoskeleton (17), and EspG and VirA have been reported to modulate these proteins (12,18). Using inducible cell lines expressing flag-tagged EspG and the EspG_DR-AA mutant, we assessed Rho, Rac, and Cdc42 activation (19,20). In brief, binding domains for GTP-bound states of Rho and Rac/Cdc42 (from rhotekin and p21 activated kinase-1, PAK1, respectively) (19,20) were incubated with induced and non-induced EspG-containing cells. While we did not observe significant levels of G-protein activation (data not shown), we were surprised to observe EspG bound directly to beads containing the Rac/Cdc42 (p21) binding domain of PAK1 (PBD beads) (figure 3). EspG_DR-AA bound substantially weaker to the beads (figure 3), consistent with a role in binding for the large conserved patch. EspG and EspG_DR-AA are expressed at similar levels, indicating that the failure of EspG_DR-AA to bind PBD is not caused by gross changes in EspG stability. Paradoxically, EspG expression results in a loss of active

(phosphorylated) PAK1 (figure 3), presumably due to degradation or trafficking to an insoluble cellular compartment (see note added in proof).

PAK1 is a Ser/Thr kinase with a two-lobed catalytic subunit and an activation loop that is phosphorylated in the active form (21,22). Additionally, PAK1 contains an amino-terminal inhibitory domain that binds GTP-bound Rac or Cdc42 (20). Binding to Rac or Cdc42 reorganizes the inhibitory domain, such that it no longer binds the catalytic subunit, at which point phosphorylation of the activation loop is sufficient to activate PAK (21). The inhibitory domain includes the PBD, and binding to the PBD is sufficient to release the PBD from the catalytic subunit and relieve inhibition. Subsequently, activated PAK1 auto-phosphorylates the inhibitory domain, which prevents future inactivation. Thus, PAK1 can be considered fully active when phosphorylated both on the activation loop and on the inhibitory domain (21).

The specific functional roles played by EspG, EspG2, and VirA during infections have been controversial. Our results show that EspG is structurally similar to VirA and contains a functionally important conserved surface patch that binds the PBD of PAK1. These results support a role in infection in which EspG simultaneously recruits PAK to the sites of EPEC attachment and obviates the requirement for G-protein activation. These observations immediately afford opportunities to identify the specific molecular pathways that these virulence factors target during infection. PAK principally regulates the actin cytoskeleton, although there are numerous other targets, and over-expression of active PAK promotes formation of lamellipodia, filopodia, and membrane ruffles (23–25). The observation that EspG binds directly to the inhibitory domain of PAK, mimicking a small GTPase, implies that EspG's primary role during pathogenesis is to promote actin remodeling. Rather than recruiting a small GTPase, EPEC circumvent the standard PAK activation mechanism, consistent with a previous finding that inactivation of host small GTPases did not alter EPEC's ability to form actin rich pedestals (26).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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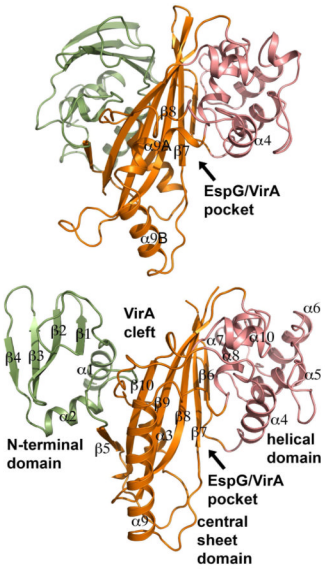

Figure 1.

EspG (A.) and VirA (B.) shown as ribbon diagrams with the NT domain colored green, the central sheet orange, and the helical domain salmon. The structures are oriented so that the central sheet and helical domain align and highlights the rotation of the N-terminal domain. Secondary structural elements, and domains are labeled in (B.). Features of EspG described in the text are labeled in (A.).

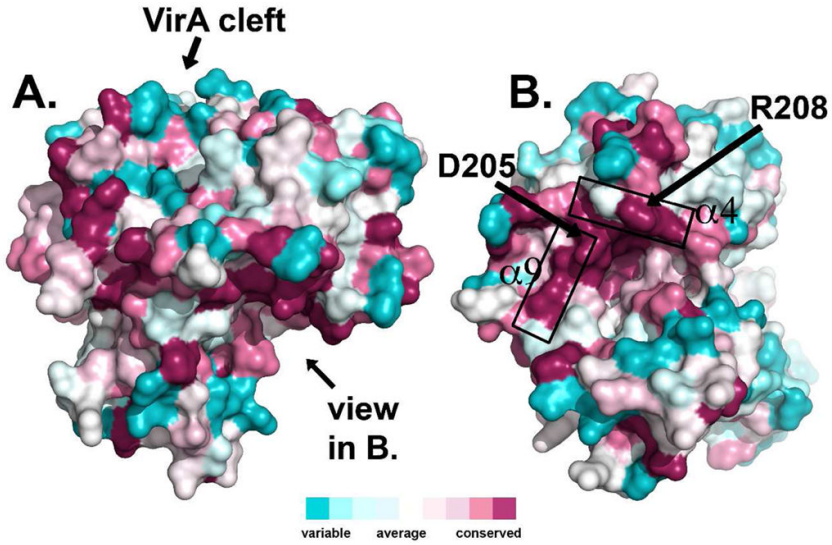

Figure 2.

Surface rendering of EspG, colored by residue conservation as shown. Panel (A.) shows EspG oriented as in figure 1, the residues that align to the VirA cleft are highly variable amongst EspG, EspG2, and VirA family members. (B.) EspG viewed along the arrow in panel (A.). A continuous stripe of highly conserved residues are observed in (A.) and continued into (B.). A deep, highly conserved pocket, the rim of which is formed by residues from α4 and α9, is seen in (B.). Residues D205 and R208 form much of the entrance to the pocket and are strictly conserved.

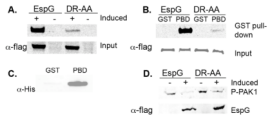

Figure 3.

PAK-PBD pull-down assay. (A.) Cells expressing equivalent amounts of flag-tagged EspG and EspG_DR-AA were incubated with PBD-beads. EspG remained bound to PBD after 3 washes, whereas EspG_DR-AA was predominately washed off. (B.) Neither EspG nor EspG_DR-AA bind to GST-beads. (C.) Direct binding between purified EspG and GST-PBD indicates that no other cellular proteins are involved in mediating the EspG-PBD interaction. (D.) Expression of EspG, and to a lesser extent EspG_DR-AA, results in loss of active (phosphorylated) PAK1.