Structural and functional studies indicate that the EPEC effector, EspG, directly binds P21 activated kinase.

Katherine L. Germane[1], Benjamin W. Spiller[1,2]*.

Departments of [1]Microbiology and Immunology and [2]Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232

Supplemental Data

Experimental Procedures:

Expression and purification of EspG: **The 355 C-terminal residues of EspG were amplified from Enteropathogenic *E. coli* strain E2348/69 (accession FM180568) by PCR and cloned into pET-28a, incorporating a vector encoded N-terminal hexahistidine tag and thrombin site. This construct, Δ43EspG, was transformed into *E. coli* BL21(DE3) and grown in Luria Bertani medium containing 50 ug/ml kanamycin. Overnight cultures were diluted (1:50) and grown to an optical density of 0.6 (600 nm) at 37 ˚C. Cultures were moved to 20 ˚C, and protein expression was induced with a final concentration of 100 uM isopropyl Beta-D-1-thiogalactopyranoside for 16 h. Cells were harvested by centrifugation and lysed with a French Press in the presence of chicken egg white lysozyme, bovine pancreatic deoxyribonuclease I, 10 ug/mL leupeptin, 1 mM PMSF, 7 ug/mL pepstatin, and 1 mM tris(2-carboxyethyl)phosphine. EspG was purified by metal affinity chromatography and size exclusion chromatography.**

Expression and purification of GST and PBD-GST: **GST and PBD-GST (a kind gift from Gary M. Bokoch (1)) vectors were transformed into BL21 cells and grown in Luria Bertani medium containing 50 µg/ml ampicillin. Cultures were harvested and lysed as for EspG. GST and PBD-GST were purified using Glutathione sepharose 4 fast flow (GE Life Sciences).**

Crystallization and preparation of heavy atom derivatives: **EspG crystals were grown by vapor diffusion from a reservoir of 2.0-11.0% PEG8000 and 100 mM bis-tris pH 6.25-7.5. The NaBr derivative was prepared by quickly soaking (~30s) crystals in fresh drops of mother liquor containing 1M NaBr. Native and derivative crystals were cryoprotected with step-wise soaks in 10, 15, and 20% glycerol.**

Data collection and structure determination: **Diffraction data were collected from single crystals at 100K at sector 24-IC-C at the Advance Photon Source (Argonne, IL). Data were indexed, integrated and scaled with HKL2000 (2). Data collection statistics are given in supplemental Table 1. Heavy atom positions were located and refined using the Phenix program suite (3). Electron density was improved by automated density modification including solvent flipping and histogram matching as implemented in Phenix (3). The structure was traced with a combination of automated and manual tracing in Phenix and COOT (3, 4). Refinement was done using Phenix (3). TLS refinement was incorporated in the final rounds of refinement using TLS groups identified using the tlsmd webserver. The refined model consists of amino acids 44-158, 161-316, 321-395, and 427 water molecules.**

Stable cell lines expressing EspG: **EspG was amplified with a Flag tag from a pET28 vector encoding full-length EspG and cloned into the pcDNA5/FRT/TO vector (Invitrogen). This vector was subsequently mutated to create the DR-AA mutant. Inducible Flag-EspG and Flag-EspG_DR-AA HEK293 FlpIn T-Rex cell lines were produced by co-transfection of the pcDNA5/FRT/TO vectors with the recombinase expression plasmid pOG44 and selected with 150 µg/ml hygromycin according to the manufacturers instructions (FlpIn system, Invitrogen). Clones were verified by western blots and PCR. EspG and EspG_DR-AA expression was induced with 0.1 ug/ml of doxycycline 24 hours before each experiment. Cell lines were maintained in Dulbecco's modified Eagles medium (GIBCO) supplemented with 10% fetal bovine serum and 1% pen/strep. HEK293 FlpIn T-Rex (Invitrogen) cell medium was supplemented with 100 µg/ml zeocin and 15 µg/ml blasticidin.**

EspG-PBD binding assay using cellular lysates: **EspG and EspG_DR-AA stable cell lines were induced 24 hours prior to an experiment. Un-induced and induced cells were lysed with a non-denaturing PBS/triton based buffer from Cell Signalling (product 9803), supplemented with 10 ug/mL leupeptin, 1mM PMSF, and 7 µg/ml pepstatin. The lysate was clarified by centrifugation at 12,000 rpm for 5 minutes, and normalized for total protein level by densitometry. Purified PBD-GST and GST were added at 1.5 mg/ml to glutathione sepharose and bound for 1 hour at 4 °C prior to the experiment. The sepharose was washed 3 times with lysis buffer. Equal amounts of un-induced and induced lysates were added to a 35 µl slurry of 50% sepharose and incubated for 1 hour at 4 °C. The sepharose was washed 3 times with lysis buffer and boiled in SDS-PAGE buffer. The samples were subjected to SDS-PAGE and Western blotting with anti-flag (M2) antibody (Sigma) primary and goat anti-mouse secondary antibody (LI-COR Biosciences). Blots were developed with an Odyssey fluorescent scanner.**

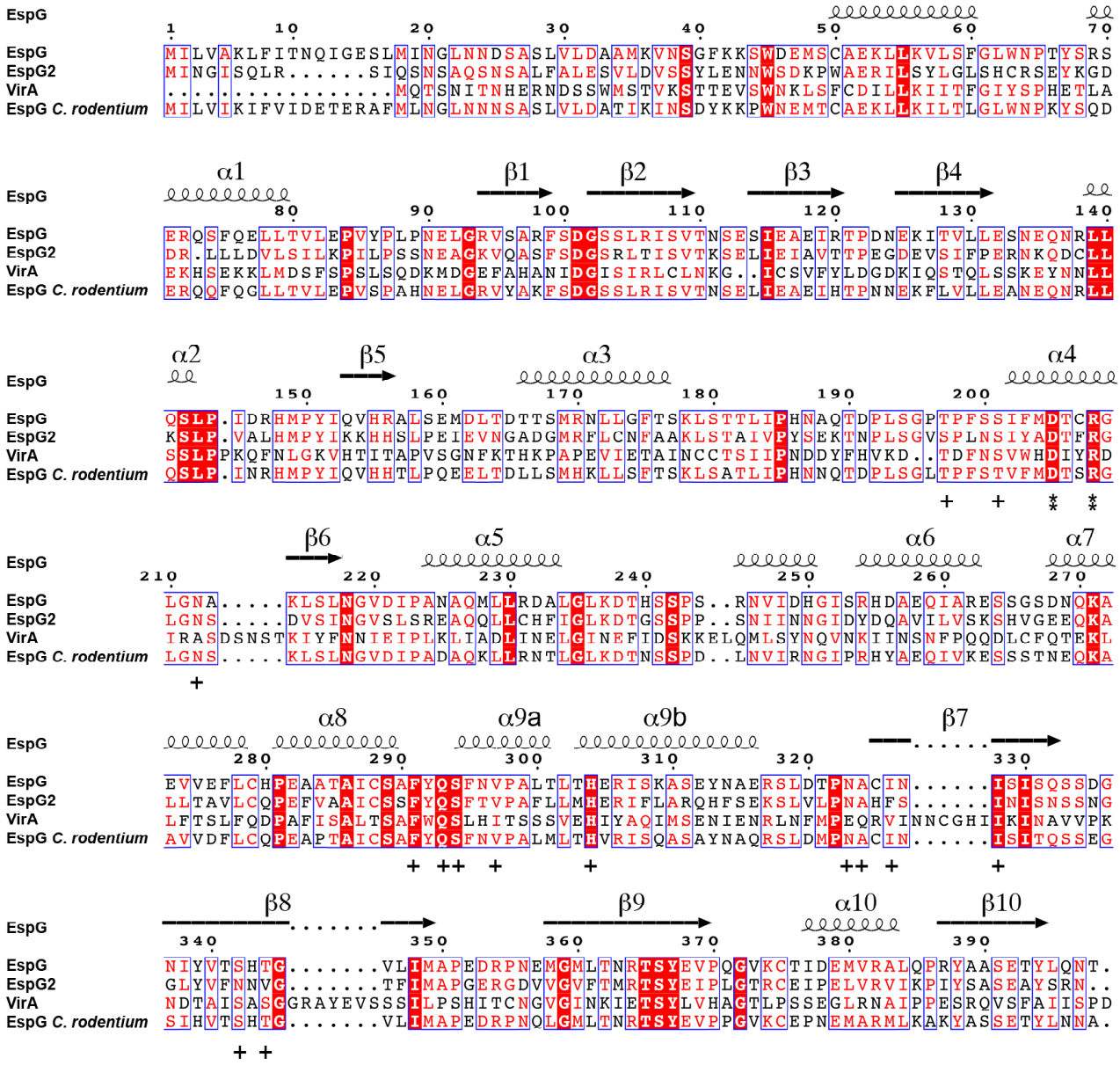
PAK1 activation/phosphoylation assay: **Cells were prepared and lysed as was done for the PBD binding assay, above. Extracts were probed for PAK1 phosphorylation by western blotting using an phospho-specific antibody that recognizing T423 of PAK1, the critical activation loop phosphorylation site. The primary antibody was from Cell Signalling, and the secondary antibody was from LI-COR Biosciences. Blots were developed with an Odyssey fluorescent scanner.**

EspG-PBD direct binding assay: **Purified His-tagged Δ43EspG was added to 35 µl of 50% slurry of GST or PBD-GST bound sepharose in the presence of PBS buffer with 1% triton-X100. The samples were incubated for 1 hour at 4°C. The agarose was washed 3 times with PBS/triton buffer and boiled in SDS-PAGE buffer and DTT. The samples were subjected to SDS-PAGE and Western blotting with an anti-hexahistidine primary antibody (Santa Cruz Biotechnology) and goat anti-rabbit secondary (LI-COR).**

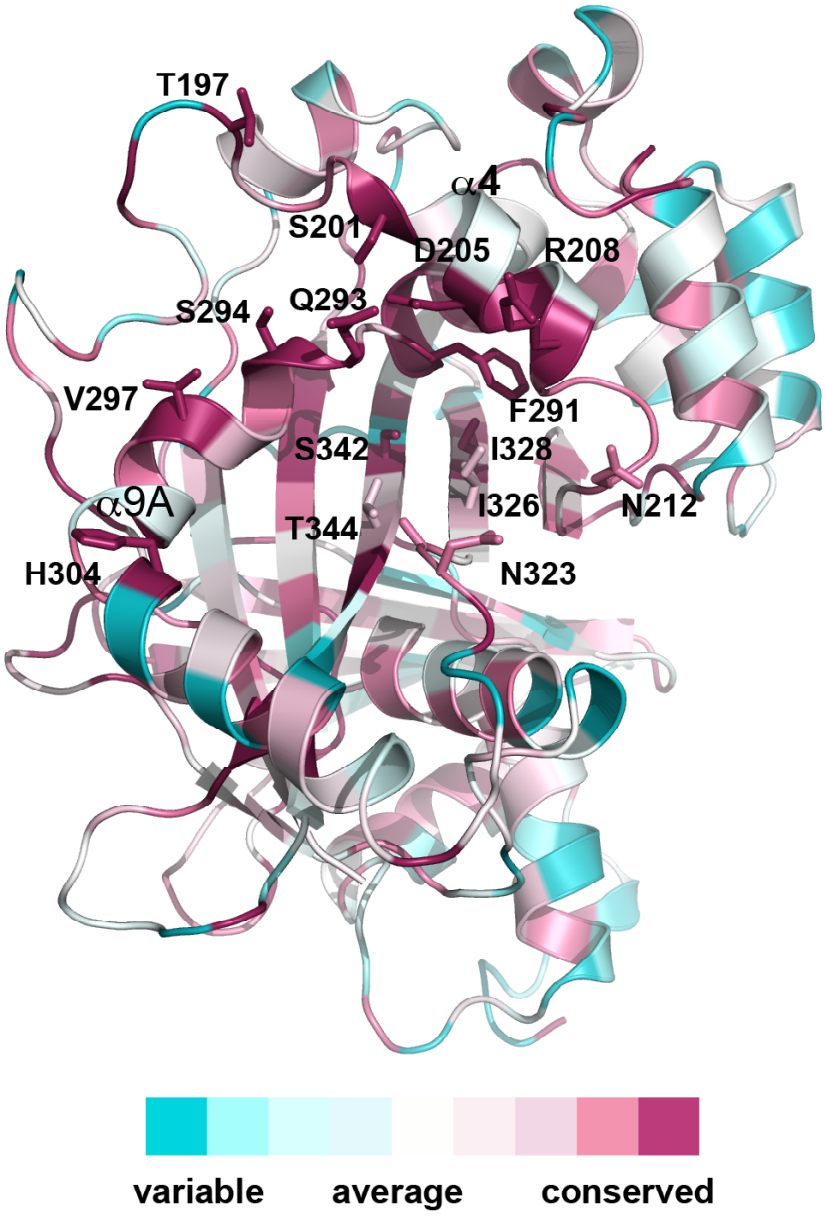
Structural analysis and figure preparation: **Pymol and COOT were used for structural analysis (4). PyMol was used for figure preparation. The sequence alignment was performed with ClustalW and ESPript (5, 6). Mapping of sequence conservation onto the structure was done using consurf (7) using an alignment of 19 sequences prepared in ClustalW (5).**

Table 1. Data collection and refinement statistics

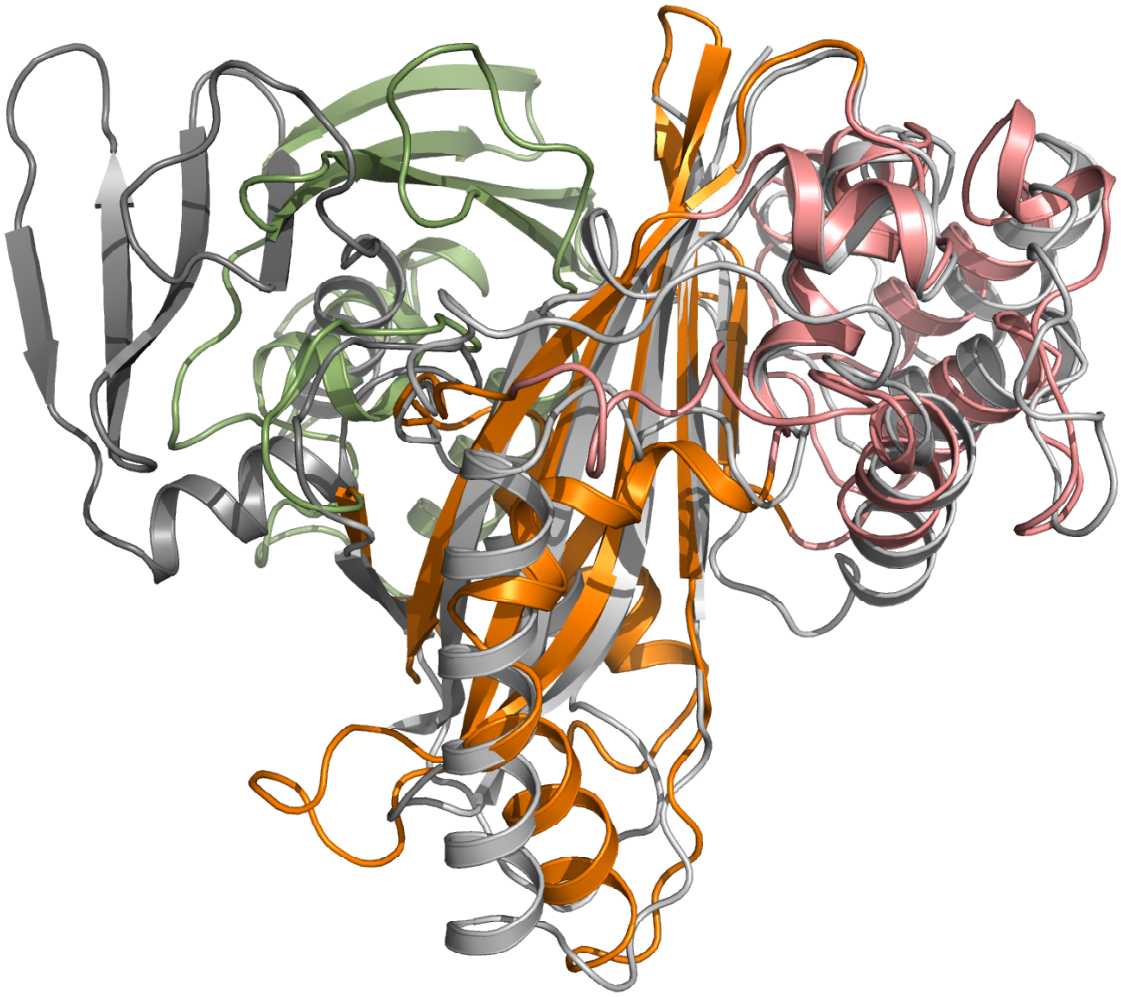
	Native	NaBr
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a, *b*, *c* (Å)	71.15, 74.38, 93.92	71.26, 74.6, 93.0
Wavelength	0.9795	0.9195
Resolution (Å)	50-1.6	50-2.2
Completeness (%)	50-1.6Å: 89.8(51.8) 50-1.8Å: 98.6(98.2)	99.5(99.3)
Redundancy	50-1.6Å: 4.8(4.2)	5.4(5.4)
I/sigmaI	50-1.6Å: 32.4(3.0)	16.8(3.7)
Figure of merit		Experimental: 0.37 DM: 0.66
Refinement		
Resolution (Å)	1.6Å	
No. reflections	58,526	
No. reflections free set	1,959	
R_{work} / R_{free} %	16.1/18.8	
R.m.s. deviations		
Bond lengths (Å)	0.006	
Bond angles (°)	1.004	
Ramachandran Plot	99.7% of residues in allowed regions	



Supplemental Figure 1: ***A multiple sequence alignment showing secondary structural elements as coils (α-helices) and arrows (β-strands). Conserved secondary structural elements are numbered according to the VirA structure. Asterisks denote residues mutated to alanine in EspG_DR-AA. Plus symbols denote conserved residues in or near the pocket described in the text. Regions of high conservation are boxed. Strictly conserved residues are colored white on a red background. Residues conserved in 3 of the proteins are colored red on a white background. Prepared with ESPript (6).***



Supplemental Figure 2: ***Secondary structure representation of EspG, oriented as in figure 2B, and colored by residue conservation. Secondary structural elements and conserved residues forming the pocket are highlighted.***



Supplemental Figure 3: ***Alignment of EspG and VirA, both oriented as in figure 1, with EspG colored by domain and VirA colored gray.***

References:

1. Benard, V., and Bokoch, G. M. (2002) Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods, *Methods Enzymol 345*, 349-359.
2. Otwinowski, Z. a. M., W. (1997) Processing of X-ray Diffraction Data Collected in Oscillation Mode, *Methods in Enzymology 276A*, 307-326.
3. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: building new software for automated crystallographic structure determination, *Acta Crystallogr D Biol Crystallogr 58*, 1948-1954.
4. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics, *Acta Crystallogr D Biol Crystallogr 60*, 2126-2132.
5. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res 22*, 4673-4680.
6. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript, *Bioinformatics 15*, 305-308.
7. Ashkenazy, H., Erez, E., Martz, E., Pupko, T., and Ben-Tal, N. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids, *Nucleic Acids Res 38 Suppl*, W529-533.