Bacterial and Host Determinants of MAL Activation upon EPEC Infection: The Roles of Tir, ABRA, and FLRT3

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Abstract

Infection of host cells by pathogenic microbes triggers signal transduction pathways leading to a multitude of host cell responses including actin cytoskeletal re-arrangements and transcriptional programs. The diarrheagenic pathogens *Enteropathogenic E. coli* (EPEC) and the related *Enterohemorrhagic E. coli* (EHEC) subvert the host-cell actin cytoskeleton to form attaching and effacing lesions on the surface of intestinal epithelial cells by injecting effector proteins via a type III secretion system. Here we use a MAL translocation assay to establish the effect of bacterial pathogens on host cell signaling to transcription factor activation. MAL is a cofactor of Serum response factor (SRF), a transcription factor with important roles in the regulation of the actin cytoskeleton. We show that EPEC induces nuclear accumulation of MAL-GFP. The translocated intimin receptor is essential for this process and phosphorylation of Tyrosine residues 454 and 474 is important. Using an expression screen we identify FLRT3, C22orf28 and TESK1 as novel activators of SRF. Importantly we demonstrate that ABRA (actin-binding Rho-activating protein, also known as STARS) is necessary for EPEC-induced nuclear accumulation of MAL and the novel SRF activator FLRT3, is a component of this pathway. We further demonstrate that ABRA is important for structural maintenance of EPEC pedestals. Our results uncover novel components in pathogen-activated cytoskeleton

factor that controls the expression of many immediate early, muscle-specific and cytoskeletal genes [5,6]. The activity of SRF is primarily controlled by its interaction with signal-regulated or tissue-specific regulatory cofactors. Two families of signal-regulated cofactors have been identified: the ternary complex factor (TCF) family, which are activated by mitogen activated protein (MAP) kinase phosphorylation [7], and the myocardin-related transcription factors (MRTFs). The MRTFs include Myocardin, MAL (also known as MRTF-A, BSAC or MKL1) and MRTF-B (also called MKL2 or MAL16). Rho-family GTPases and monomeric actin regulate the activity of MAL and MRTF-B [8,9]. Rho family-mediated changes in actin dynamics are sensed by MAL, which contains G-actin-binding RPEL motifs at the N-terminus. Stimulation of Rho family-GTPases releases MAL from an inhibitory complex with G-actin and strongly activates SRF-regulated transcription [9,10].

further 24 hours and then infected in DMEM containing 0.3%

of MAL-GFP (Figure 4A and B). Similar to COS-7 cells infected with wild-type EPEC (60.08%6 1.03% nuclear). Therefore the formation of the F-actin-rich pedestal is clearly necessary for EPEC induced MAL-GFP accumulation in the nucleus. This is supported by the fact that no SRF target genes were induced by infection with EPEC Dtir (Figure 3).

addition we, identified three novel genes that could induce nuclear accumulation of MAL-GFP by overexpression. These genes are FLRT3 (32.2% nuclear), TESK1 (54.3% nuclear) and C22orf 28 (35.59% nuclear, Figures 6A and S3).

ABRA is an actin binding protein that can induce nuclear

FRT3 and ABRA localize to the EPEC pedestal and ABRA is necessary for maintenance of discreet pedestals

We next sought to establish the cellular localization of each of the candidate proteins during EPEC infection to determine

appearance akin to a ruffle. This suggests that maintenance of discreet pedestals is lost in the ABRA knockdown, with single pedestals merging into one large structure. We therefore suggest that ABRA is necessary for proper pedestal formation, which in turn, is necessary for SRF activation.

Discussion

cytoplasm and the nucleus. Perhaps MAL has a higher binding affinity for SRF than G-actin, and upon entering the nucleus, preferentially complexes with SRF and is retained in the nucleus.

Transcription of SRF is controlled by SRF its self [47], the overexpression of SRF may be interpreted by the cell as activation of the pathway, leading to an upregulation of SRF target genes

To generate constructs for mammalian expression of ABRA, SRF, FLRT3, TESK1 and C22orf28 the coding sequences were amplified from cDNA clones in pCMV-SPORT6 obtained from Open Biosystems. Coding sequences were amplified using the primers in Table 1.

After digestion with the appropriate restriction enzymes, the coding sequence was subcloned into N-terminally tagged pCMV-3xFlag or -3xMyc vectors derived from the pCMV-Myc vector (Clontech, catalog no. 631604).

Transfections and knockdowns

COS-7 cells were plated onto 18 mm glass coverslips in 12-well plates at a density of $6 \cdot 10^4$ cells per well. After 24 h cells were transfected in antibiotic-free medium with MAL-GFP plus additional myc- or Flag- tagged constructs (in a modified pCMV vector, Clontech, USA), where noted, at a 1:1 ratio, using GeneJuice (Novagen, UK), according to the manufacturers instructions. 18 h post-transfection cells were washed twice in PBS and incubated in DMEM 0.3% FCS for a further 18 h, prior to bacterial infections.

RNA interference

COS-7 cells were plated onto 18 mm glass coverslips in 12-well plates at a density of $6 \cdot 10^4$ cells per well. After 24 h, 20 pmol of modified RNA oligoduplexes (Stealth RNAi; Invitrogen), were transfected into each well using X-tremeGENE (Roche), according to the manufacturers instructions. siRNA silencing sequences are shown in Table 2.

Cells were serum starved 48 h post-transfection as described above and infected, fixed and stained as described below.

Reverse transcription and real-time PCR

C22ORF28 siA	
C22ORF28 siC	CGUGUUGCCUCA
FLRT3 siC	

guidelines. Cells were infected as described above. 3.5 hours post infection samples were washed once in PBS, scraped and lysed with a bent 21 gauge needle in LAS2 lysis buffer. F-actin was then separated from G-actin by centrifugation at 100,0006 g for 60 min at 37uC. The F-actin-containing pellet was resuspended in LAS2 buffer containing 2 m

10. Vartiainen MK, Guettler S, Larijani B, Treisman R (2007) Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL.