

Vasodilator-stimulated phosphoprotein restricts cell-to-cell spread of *Shigella flexneri* at the cell periphery

Soo Young Lee,¹ Frank B. Gertler² and Marcia B. Goldberg^{1,3}

Correspondence

Marcia B. Goldberg
marcia.goldberg@
mgh.harvard.edu

¹Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

²Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

³Division of Infectious Diseases, Massachusetts General Hospital, Cambridge, MA 02139, USA

Shigella spp. are intracellular bacterial pathogens that cause diarrhoeal disease in humans. *Shigella* utilize the host actin cytoskeleton to enter cells, move through the cytoplasm of cells and pass into adjacent cells. Ena/VASP family proteins are highly conserved proteins that participate in actin-dependent dynamic cellular processes. We tested whether Ena/VASP family members VASP (vasodilator-stimulated phosphoprotein), Mena (mammalian-enabled) or EVL (Ena-VASP-like) contribute to *Shigella flexneri* spread through cell monolayers. VASP and EVL restricted cell-to-cell spread without significantly altering actin-based motility, whereas Mena had no effect on these processes. Phosphorylation of VASP on Ser153, Ser235 and Thr274 regulated its subcellular distribution and function. VASP derivatives that lack the Ena/VASP homology 1 (EVH1) domain or contain a phosphoablative mutation of Ser153 were defective in restricting *S. flexneri* spread, indicating that the EVH1 domain and phosphorylation on Ser153 are required for this process. The EVH1 domain and Ser153 of VASP were required for VASP localization to focal adhesions, and localization of VASP to focal adhesions and/or the leading edge was required for restriction of spread. The contribution of the EVH1 domain was from both the donor and the recipient cell, whereas the contribution of Ser153 phosphorylation was only from the donor cell. Thus, unlike host proteins characterized in *Shigella* pathogenesis that promote bacterial spread, VASP and EVL function to limit it. The ability of VASP and EVL to limit spread highlights the critical role of focal adhesion complexes and/or the leading edge in bacterial passage between cells.

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INTRODUCTION

Shigella spp. are Gram-negative bacteria that cause bacillary dysentery in humans by infection of the colonic epithelium (Labrec *et al.*, 1964). Once internalized into host cells, the disease process depends on the ability of *Shigella* to spread from one cell into adjacent cells (Sansone *et al.*, 1991). *Shigella*, like the intracellular pathogens *Listeria monocytogenes*, *Rickettsia* spp., *Burkholderia* spp. and *Mycobacterium marinum* (Kespichayawattana *et al.*, 2000; Stamm *et al.*, 2003; Teyssie *et al.*, 1992; Tilney *et al.*, 1989), escapes

the uptake vacuole and polymerizes host actin at the end of the bacterial body. Actin polymerization propels the bacterium to the cell periphery, where it pushes out against the plasma membrane to form a thin membrane-bound protrusion that is engulfed by membranes of the adjacent cell, resulting in bacterial engulfment within a secondary double-membrane vacuole. The pathogen then lyses the double-membrane vacuole, thereby releasing it into the cytoplasm of the adjacent cell, where the process is repeated (Goldberg, 2001; Ireton, 2013).

Members of the Ena/VASP family are highly conserved proteins implicated in actin-dependent dynamic cellular processes (Krause *et al.*, 2003). The vertebrate members of the Ena/VASP family are VASP (vasodilator-stimulated phosphoprotein), Mena (mammalian-enabled) and Ena-VASP-like (EVL). Ena/VASP proteins localize to sites of active actin rearrangements, including focal adhesions, the leading edge of lamellipodia, the tips of filopodia, cell-to-cell contacts and stress fibres, where they contribute

Abbreviations: COCO, coiled-coil; Dia, diaphanous formin; EVH1, Ena/VASP homology 1; EVH2, Ena/VASP homology 2; EVL, Ena-VASP-like; FAB, F-actin binding; Mena, mammalian-enabled; mDia, mammalian diaphanous formin; PRO, region, proline-rich region; VASP, vasodilator-stimulated phosphoprotein; WAVE, Wiskott–Aldrich syndrome verprolin homology protein.

Supplementary methods and three supplementary figures are available with the online Supplementary Material.

to actin assembly and cell motility (Krause *et al.*, 2003). They promote actin filament elongation by delaying binding of capping proteins and by recruiting profilin/G-actin to sites of actin polymerization (Bear & Gertler, 2009; Breitsprecher *et al.*, 2011; Hansen & Mullins, 2010). In addition, Ena/VASP proteins bind F-actin and tetramerize, facilitating bundling of actin filaments (Bear & Gertler, 2009).

VASP, EVL and Mena share three functional domains, a N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich (PRO) region and a C-terminal Ena/VASP homology 2 (EVH2) domain (Krause *et al.*, 2003) (Fig. S1, available in the online Supplementary Material). The EVH1 domains target Ena/VASP proteins to sites of actin rearrangements via interactions with proteins containing the proline-rich motif (D/E)(F/L/W/Y)PPPPX(D/E)(D/E) (designated FPPPP), including zyxin and vinculin at focal adhesions and lamellipodin at the leading edge (Hoffman *et al.*, 2006; Krause *et al.*, 2004; Niebuhr *et al.*, 1997). EVH1 domains also bind to diaphanous formin (Dia) and mammalian diaphanous formin 2 (mDia2), inhibiting actin nucleation and elongation (Barzik *et al.*, 2014; Bilancia *et al.*, 2014), and recent data suggest that VASP activates WAVE (Wiskott–Aldrich syndrome verprolin homology protein) via EVH1-mediated interactions (Chen *et al.*, 2014; Havrylenko *et al.*, 2015). The PRO regions bind to profilin and to SH3 and WW domain-containing proteins. The EVH2 domains harbour a coiled-coil (COCO) motif that mediates Ena/VASP protein tetramerization, a G-actin-binding site and an F-actin-binding (FAB) site.

VASP is phosphorylated at Ser153, Ser235 and Thr274 of the murine protein (corresponding to human VASP residues Ser157, Ser239 and Thr278) by the serine/threonine kinases cAMP-dependent protein kinase and cGMP-dependent protein kinase (Blume *et al.*, 2007; Butt *et al.*, 1994; Gertler *et al.*, 1996; Lambrechts *et al.*, 2000; Zhuang *et al.*, 2004). VASP is also a substrate for protein kinase D1, which phosphorylates it at Ser153 and Ser318 (which corresponds to human VASP Ser322) (Döppler *et al.*, 2013). Ser153, which is located between the EVH1 and PRO regions (Fig. S1), is the only phosphorylation site conserved amongst VASP, Mena and EVL. Phosphorylation at Ser153 is associated with increased VASP localization to focal adhesion-like structures and the plasma membrane (Benz *et al.*, 2009; Döppler *et al.*, 2013).

During *L. monocytogenes* infection, Ena/VASP proteins bind directly to the bacterial surface protein ActA, leading to recruitment of the actin nucleator Arp2/3, and modulating the speed and directionality of bacterial movement through the cell cytoplasm and into adjacent cells (Auerbuch *et al.*, 2003; Chakraborty *et al.*, 1995; Welch *et al.*, 1997, 1998).

As for *L. monocytogenes*, Ena/VASP proteins localize to the *Shigella* actin tail (Ally *et al.*, 2004; Chakraborty *et al.*, 1995; Gouin *et al.*, 1999). However, Mena is not required for actin tail assembly or intracellular motility of *S. flexneri*

(Ally *et al.*, 2004). The contribution of Ena/VASP proteins to *Shigella* cell-to-cell spread has not been assessed; in the present study, we sought to determine whether Ena/VASP proteins are required for this process. We demonstrated that VASP and EVL restrict *S. flexneri* spread in cell monolayers. Furthermore, the VASP EVH1 domain, phosphorylation of VASP Ser153 and subcellular localization of VASP are required for efficient restriction of spread.

METHODS

Plaque assay. Cells were plated to confluence on fibronectin-coated plates. *S. flexneri* were added to cell monolayers using m.o.i. 25 : 1 or 100 : 1, centrifuged at 2000 r.p.m. for 10 min to bring the bacteria into contact with the monolayer and incubated at 37 °C for 90 min. Infected monolayers were then washed and overlaid with 0.5 % agarose in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15 % FBS and 25 µg gentamicin ml⁻¹. After ~48 h, monolayers were stained for 4–6 h at 37 °C with neutral red in 0.7 % agarose in DMEM supplemented with 15 % FBS and 25 µg gentamicin ml⁻¹, and then imaged using an Epson Perfection 4990 Photo desktop scanner and Adobe Photoshop Elements software or Image Capture. Areas of individual plaques were measured in a blinded manner using iVision software (<http://www.biovis.com/ivision.html>).

Infected cell lift-off or adherence. Semi-confluent monolayers of MV^{D7} cells or MV^{D7}-EGFP-VASP cells were plated on fibronectin-coated plates. The cells were infected as above with *S. flexneri* pBR322-mCherry for 1 h, washed and incubated overnight in growth media containing 25 µg gentamicin ml⁻¹, which kills extracellular but not intracellular bacteria. The next day, infected monolayers were fixed with 3.7 % paraformaldehyde and imaged using a ×10 objective, which enabled the visualization of individual plaques. The perimeters of plaques were defined by the extent of mCherry signal from infecting bacteria. The area of cell lift-off within individual plaques was then measured in a blinded manner using the Region of Interest tools in iVision software.

Mixed cell spread assay. The mixed cell spread assay was performed essentially as described previously (Heindl *et al.*, 2010). Cell lines to be mixed were plated on fibronectin-coated plates to 40–60 % confluence. One of the cell lines to be tested was incubated with CellTracker Red dye (Invitrogen) (final concentration 2.5 µM) in pre-warmed serum-free media at 37 °C for 40 min, at which time the dye-containing media was replaced with pre-warmed normal growth medium for an additional 30 min. Stained and unstained cells were trypsinized, mixed at a ratio of 1 : 10, plated on fibronectin-coated coverslips, and allowed to adhere to plates overnight. Cells were then infected with *S. flexneri* at m.o.i. 1 : 10 as above. Following incubation at 37 °C for 1 h, infected monolayers were rinsed thoroughly with pre-warmed serum-free media and were incubated with media containing 25 µg gentamicin ml⁻¹ at 37 °C for an additional 1 h 40 min, until the total time of infection was just under 3 h. Infected monolayers were fixed with 3.7 % paraformaldehyde, rinsed with PBS and stained with DAPI. Within each focus of infection, for each primarily infected ('donor') cell, identified by the presence of greater than fivefold more bacteria in that cell than in adjacent cells, the corresponding number of infected adjacent ('recipient') cells was counted. The efficiency of spread was defined as the mean number of 'recipient' cells infected per focus of infection. Analysis was limited to foci that had three or more cells adjacent to the primarily infected cell. For each condition, a minimum of 10–15 foci was analysed.

Additional methods are described in the online Supplementary Material.

RESULTS AND DISCUSSION

VASP and EVL restrict *S. flexneri* spread through cell monolayers

During infection, *S. flexneri* spread through tissue by moving directly from one cell into the adjacent cell. The distance that the organism spreads over time is a measure of the efficiency of the composite of the steps involved in cell-to-cell spread, which include (1) the generation by the bacterium of an actin tail, which propels it to the cell periphery, (2) the generation by the bacterium of a protrusion of the plasma membrane at the periphery of the infected cell, and (3) the uptake of the bacterium-containing protrusion by the adjacent cell. In previous work, we demonstrated that *S. flexneri* actin-based motility was comparable in cells lacking all Ena/VASP proteins and isogenic cells in which the only Ena/VASP protein produced was Mena (Ally *et al.*, 2004). This prior study focused on Mena and did not address whether other members of the Ena/VASP protein family function in *S. flexneri* actin-based motility; nor did it address whether any Ena/VASP protein functions in *S. flexneri* cell-to-cell spread.

We tested whether EVL, Mena and/or VASP contributes to cell-to-cell spread of *S. flexneri* using mouse embryonic fibroblasts deficient in Ena/VASP family proteins (MV^{D7} cells) and stably reconstituted with each of EGFP-tagged EVL, Mena or VASP (designated 'EVL', 'Mena' or 'VASP' cells, respectively) (Bear *et al.*, 2000). The efficiency of bacterial spread was determined by the size of plaques formed by bacteria in confluent monolayers. Plaque size was decreased by 37 or 19 % in MV^{D7} cells reconstituted with EVL or VASP, respectively, compared with MV^{D7} cells lacking all three Ena/VASP proteins (designated 'null cells') (Fig. 1a). On Western blot analysis using antibody to the EGFP-tag, levels of the proteins (relative to the loading control glyceraldehyde 3-phosphate dehydrogenase) were similar, with Mena slightly less abundant than EVL or VASP (Fig. 1b).

Monolayers comprising cells that are of low density yet semi-confluent can lead to larger plaques than monolayers comprising cells at high density. In semi-confluent monolayers, we found no difference in cell density between any of the Ena/VASP cells and null cells (Fig. 1c). In addition, cell lift-off from the culture dishes can lead to plaques that appear larger than the actual area of bacterial spread. We determined the extent of cell lift-off within bacterial plaques by first defining the perimeter of individual plaques as the extent of the mCherry signal from infecting bacteria that carried pBR322-mCherry and then measuring within the plaques the area of adherent cells versus the area of cells that had lifted off. As our subsequent investigations focus on VASP cells (see below), we compared cell lift-off of VASP cells with that of null cells and found no difference (Fig. 1d), indicating that this was not contributing to the observed differences in plaque size between these cell lines. Thus, neither differences in cell density nor differences in

cell lift-off contributed to the observed effect of VASP and EVL on bacterial spread, consistent with a more direct effect of VASP and EVL on the process of *S. flexneri* cell-to-cell spread.

Ena/VASP proteins localize to actin tails, but likely do not contribute to the plaque phenotype via modulation of actin tail formation

S. flexneri move to the cell periphery by generation of actin tails that push the bacteria through the host cytoplasm. To test whether the observed VASP- and EVL-associated decreases in cell-to-cell spread were due to an effect on bacterial actin-based motility, we compared the efficiency of bacterial actin tail formation per se in the VASP, EVL and Mena cells with that in cells lacking all Ena/VASP proteins. Consistent with the possibility that these proteins might modulate this process, and as we and others have previously described for VASP and Mena (Ally *et al.*, 2004; Chakraborty *et al.*, 1995; Gouin *et al.*, 1999), each Ena/VASP protein co-localized with the polymerized actin in *S. flexneri* actin tails (Fig. S2). As actin tail length correlates with the rate of bacterial movement (Theriot *et al.*, 1992), we determined the length and frequency of actin tails as measures of the efficiency of actin-based motility.

The length of actin tails in cells producing VASP or EVL was similar to that in cells producing Mena or no Ena/VASP protein (Fig. 2a). Moreover, for the subset of actin tails that were within protrusions, actin tail lengths were similar (data not shown). In addition, the frequencies of bacteria associated with an actin tail were similar for cells producing VASP, EVL or Mena compared with null cells (Fig. 2b). Although a slightly higher frequency of bacteria formed actin tails in EVL and VASP cells compared with null cells, these differences did not reach statistical significance (Fig. 2b). Moreover, the distribution of these tails between the cell body and protrusions was similar amongst the cell lines (Fig. 2b). Of note, an increased frequency of actin tails would be predicted to be associated with increased spread through monolayers, whereas EVL and VASP cells support decreased spread, suggesting that the observed trends in actin tail frequency do not explain the spread phenotype. Thus, because the differences in actin tail lengths, frequency and distribution did not correlate with the observed differences in plaque size, they are unlikely to explain the plaque phenotype.

In contrast to our findings here and previously (Ally *et al.*, 2004) for *S. flexneri*, Ena/VASP proteins contribute to actin-based motility of the intracellular bacterial pathogens *L. monocytogenes* and *Burkholderia pseudomallei* (Breitbach *et al.*, 2003; Skoble *et al.*, 2001), both of which, like *Shigella* spp., move through the cell cytoplasm by generating propulsive actin tails behind the bacterium. In addition, VASP localizes throughout the actin tails of *Rickettsia rickettsii* and *Rickettsia conorii* (Gouin *et al.*, 1999; Van Kirk *et al.*, 2000), yet whether it is functional in actin tail assembly by these pathogens is unknown.

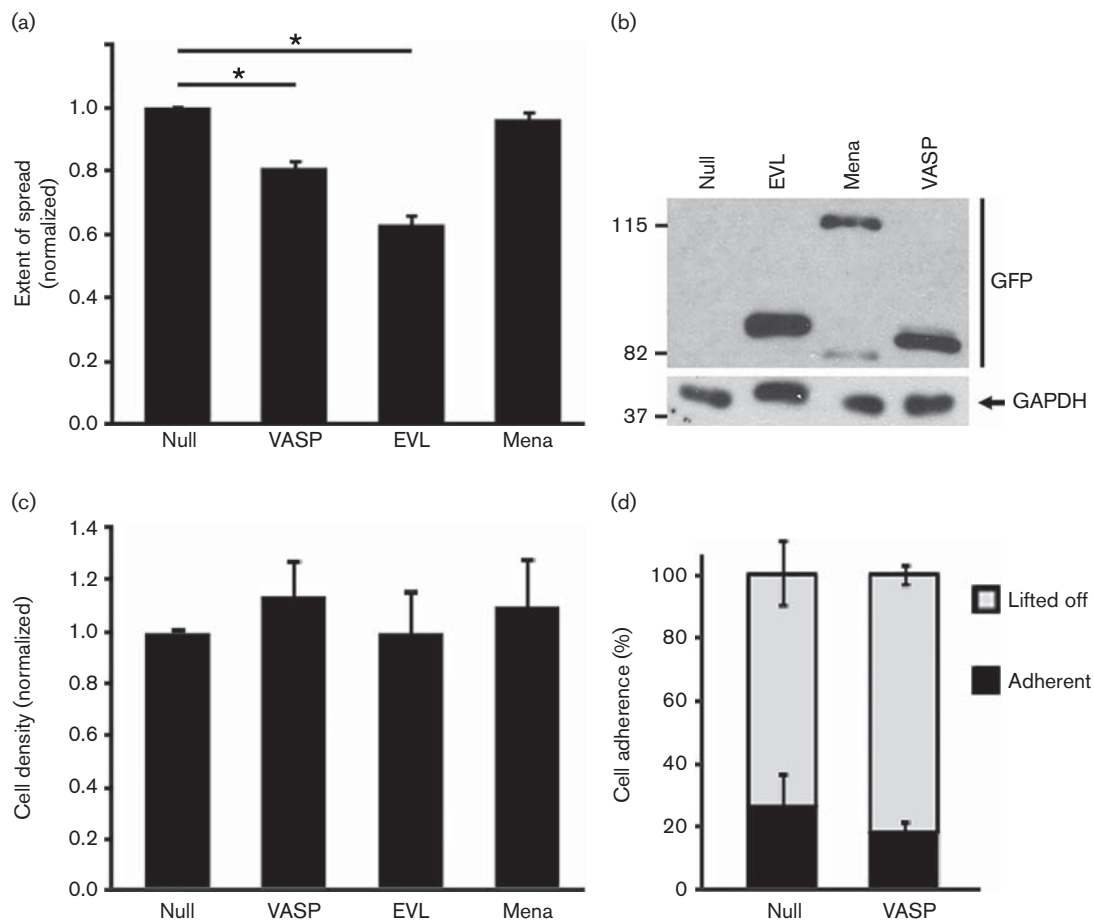


Fig. 1. Spread of *S. flexneri* through monolayers is restricted in the presence of EVL or VASP, but not in the presence of Mena. (a) Extent of spread, normalized to that in monolayers of Ena/VASP null cells. For each condition, a minimum of 30 plaques were measured per experiment. (b) Levels of EVL, Mena and VASP, each tagged with EGFP, in cell lysates. Western blot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), loading control. (c) Density of cells in monolayers used for these experiments, normalized to that of null cells. (d) Extent of lift-off of cells within bacterial plaques. Within the perimeter of individual plaques, area that contains adherent cells versus area in which cells have lifted off. Data are presented as mean \pm SD and represent a minimum of three independent experiments. * $P < 0.05$.

Taken together, the differences in *S. flexneri* cell-to-cell spread that we observed for Mena compared with VASP or EVL are not explained by variable effects of these proteins on actin-based motility per se. Therefore, we postulated that the observed differences in cell-to-cell spread might be due to distinct effects of these proteins on the processes of bacterial protrusion formation and protrusion uptake by adjacent cells. Of note, clathrin-mediated endocytosis, which is required for efficient uptake of the *S. flexneri* protrusion by the neighbouring cell (Fukumatsu *et al.*, 2012), depends on Mena, but not VASP (Vehlow *et al.*, 2013). A potential explanation that reconciles this observation with our findings is that whereas the contribution of Mena to clathrin-mediated endocytosis contributes positively to *S. flexneri* spread, counteracting this positive effect is a mechanism for limiting spread that is shared amongst Ena/VASP proteins, including Mena. The

net effect of Mena production in cells would be neutral, i.e. Mena would neither support spread nor inhibit it, leading to an effect that is approximately the same as that of cells that produce no Ena/VASP proteins. In contrast, the effect of VASP or EVL production would be to reduce spread more than cells that produce no Ena/VASP proteins. Of note, the LERER repeat region of Mena (Fig. S1), which is lacking in EVL and VASP, binds directly to the cytoplasmic tail of α_5 -integrins, and is important for fibronectin-triggered activation of $\alpha_5\beta_1$ -integrin-mediated signalling, cell spreading and cell migration on fibronectin (Gupton *et al.*, 2012), and the Mena EVH1 domain, but not that of EVL or VASP, binds Tes (Boëda *et al.*, 2007). We cannot eliminate the possibility that the Mena interaction with Tes or the contribution of Mena to integrin signalling contributes to the observed phenotype.

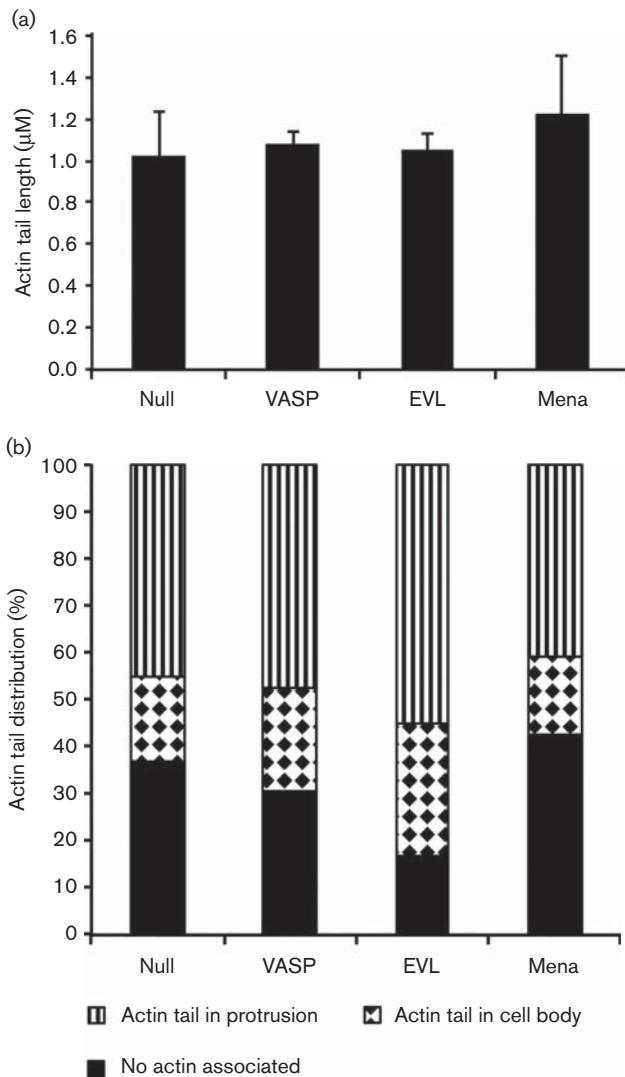


Fig. 2. Actin tail formation by *S. flexneri* in cells producing VASP, EVL or Mena, or no Ena/VASP protein (null). (a) The length of actin tails assembled on intracellular bacteria, including actin tails both in the cell body and in protrusions. For each condition, a minimum of 270 actin tails were measured. (b) The distribution of actin tails amongst intracellular bacteria as a function of location to the cell body, location to a protrusion or not associated with an actin tail. Data are presented as mean \pm SD and represent a minimum of three independent experiments.

EVH1 domain of VASP is required but not sufficient for restriction of *S. flexneri* spread

As Ena/VASP proteins are organized in three conserved domains with distinct functions (Figs. 3a and S1), functional insights can be gained by determining which domain(s) is (are) responsible for a phenotype. As VASP domain organization is conserved, its expression is relatively high in the colon (Kapushesky *et al.*, 2012) and reagents for the analysis of VASP were readily available, we selected VASP for use in all subsequent analyses.

To identify the domain(s) of VASP that contributes to restriction of *S. flexneri* cell-to-cell spread, we examined the efficiency of spread through monolayers of MV^{D7} cells stably producing VASP derivatives that each lack one of the following conserved domains: the N-terminal proline-rich ligand binding domain EVH1 (Δ EVH1'), the central PRO region that binds to SH3 domains (Δ PRO'), the FAB domain (Δ FAB'), the oligomeric binding motif (Δ COCO'), and the C-terminal domain EVH2 that contains the FAB and COCO domains (Δ EVH2') (Fig. 3b). Compared with cells producing WT VASP, the size of plaques in Δ EVH1 cells was increased 42 % (Fig. 3c), whereas the sizes of plaques in Δ PRO and Δ FAB cells were unchanged, and those in Δ EVH2 and Δ COCO cells were slightly but not significantly increased. The increase in the size of plaques in the Δ EVH1 cells was not due to instability of the Δ EVH1 protein, as it was more abundant than was the WT VASP construct in the WT VASP cells (Fig. S3a). Cells of the two cell lines displayed similar morphologies and were of similar density in the monolayers (Fig. S3b and data not shown). These data indicated that the EVH1 domain is required for restriction of *S. flexneri* spread. Moreover, the observation that the PRO and FAB domains were dispensable for restriction of *S. flexneri* spread suggests that the mechanism of VASP restriction of spread is independent of its ability to bind F-actin and to act as an anti-capping and elongation factor.

To determine whether the EVH1 domain was sufficient to restrict *S. flexneri* spread, we tested whether reconstitution of the null cells with the EVH1 domain alone would rescue plaque size. Plaques formed in cells producing the EVH1 domain alone (Δ EVH1') were larger than those formed in the Δ EVH1 cells (Fig. 3d), indicating that rather than restricting bacterial spread, production of the EVH1 domain alone enhances spread. The proteins were produced at similar levels and there was no difference in cell density (Fig. S3c, d). These findings indicate that the EVH1 domain alone is not sufficient to restrict *S. flexneri* spread and suggest that another region(s) of VASP is also required for restriction of bacterial spread. The reason for the increase in *S. flexneri* plaque size observed in cells producing the EVH1 domain alone is uncertain (Fig. 3d), but may be due to the function of the EVH1 domain in VASP-mediated activation of the WAVE regulatory complex (Chen *et al.*, 2014), which promotes actin polymerization.

VASP derivatives defective in phosphorylation at Ser153 are defective in restriction of *S. flexneri* spread

Activity of VASP is regulated in part by phosphorylation on serines and threonines (Benz *et al.*, 2009; Blume *et al.*, 2007; Döppler *et al.*, 2013; Lindsay *et al.*, 2007). Phosphorylation is known to occur on four residues, corresponding to Ser153, Ser235, Thr274 and Ser322 of the murine protein. To begin to test whether regulation of VASP by phosphorylation is required for its ability to limit *S. flexneri* cell-to-cell spread, we tested whether the

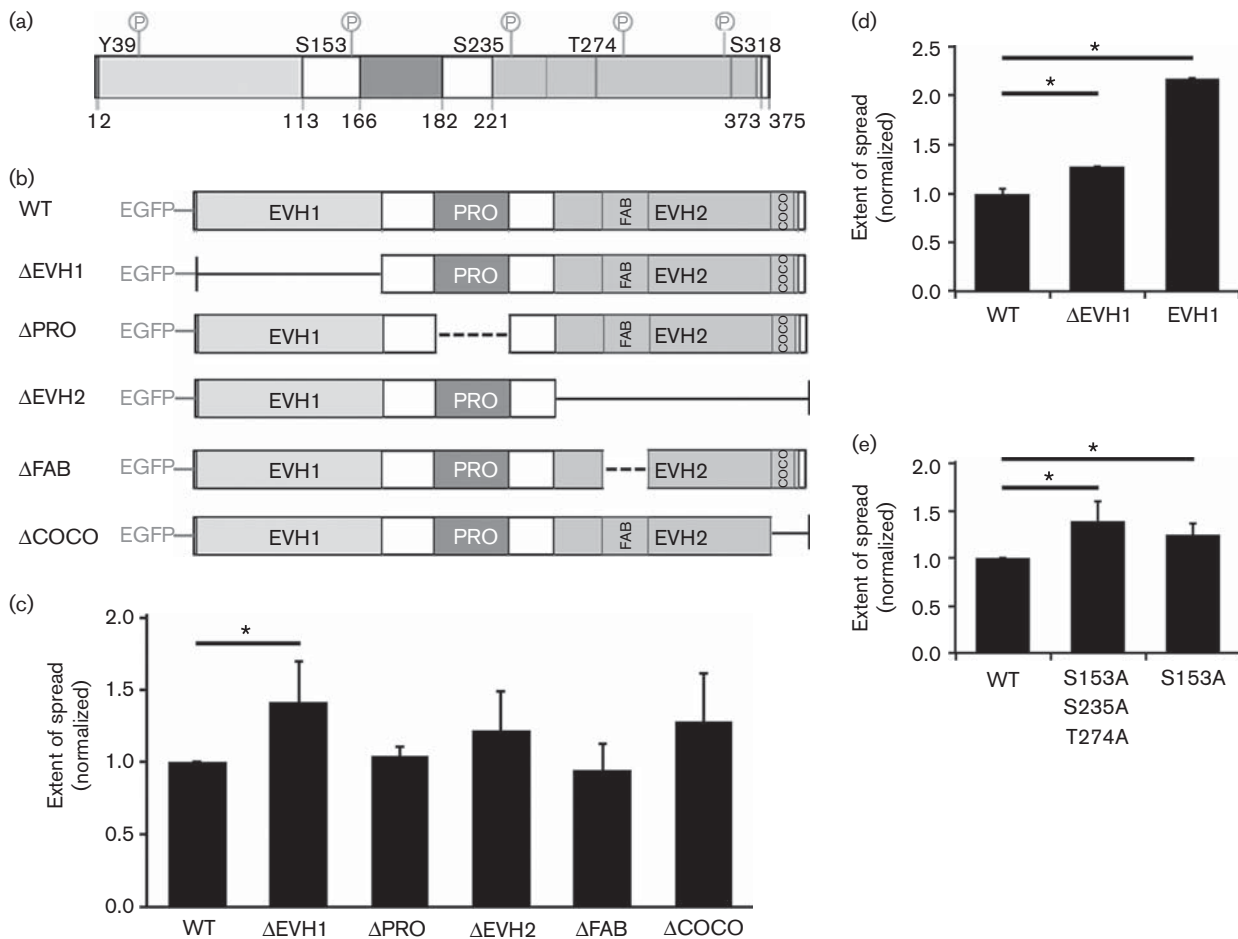


Fig. 3. VASP EVH1 domain is required for VASP-mediated restriction of *S. flexneri* spread. (a) Detailed diagram of VASP. Indicated are the N-terminal EVH1 domain, the central proline-rich core (PRO) and the C-terminal EVH2 domain, within which are located an FAB region and a COCO region that mediates oligomerization, as well as residues that are phosphorylated. Numbering of residues is that of murine VASP. (b) VASP deletion constructs used for these studies. (c) Extent of spread of *S. flexneri* through monolayers of cells producing each of the VASP constructs depicted in (b), normalized to that in monolayers of VASP WT cells. (d) Extent of spread of *S. flexneri* through monolayers of cells producing Δ EVH1 VASP or the VASP EVH1 domain alone, normalized to that in monolayers of VASP WT cells. (e) Extent of spread of *S. flexneri* through monolayers of cells producing the VASP phosphoablative derivative S153A/S235A/T274A or S153A, normalized to that in monolayers of VASP WT cells. * $P < 0.05$. Data are presented as mean \pm SD and represent a minimum of three independent experiments.

area of spread in a plaque assay was altered for cells producing a VASP derivative harbouring three phosphoablative mutations, Ser153, Ser235 and Thr274, each changed to Ala ('S153A/S235A/T274A'; Fig. 3a). Plaques formed in monolayers of S153A/S235A/T274A VASP cells were significantly larger than those formed in WT VASP cells (Fig. 3e). Moreover, plaques formed in cells producing a VASP derivative harbouring a single phosphoablative mutation at Ser153 ('S153A') were similar in size to those formed in S153A/S235A/T274A VASP cells and larger than those formed in WT VASP cells, with no difference in cell density (Fig. S3d), consistent with S153 being responsible for the phenotype of the S153A/S235A/T274A cells and with S153 phosphorylation being required for

restriction of bacterial spread. However, because the S153A/S235A/T274A VASP and S153A VASP derivatives were produced at higher levels than WT VASP (Fig. S3e), we cannot exclude the possibility that the phenotype observed in these cells is due to increased amounts of these VASP proteins.

Ability of VASP derivatives to restrict *S. flexneri* spread correlates with localization of the derivative to focal adhesions

Ena/VASP proteins are localized in regions of the cell that undergo dynamic actin rearrangement, including focal adhesions and the leading edge of lamellipodia (Krause

et al., 2003). Focal adhesions are dynamic complexes containing >50 proteins that actively participate in cell migration by connecting contractile cytoskeletal elements to the plasma membrane and transmitting the force that is generated to the extracellular matrix (Lo, 2006; Plotnikov & Waterman, 2013). The number and stability of focal adhesions contribute to cell-to-cell spread of *Shigella*, and components of focal adhesions are recruited to bacterial protrusions and sites of entry (Kim *et al.*, 2009; Mounier *et al.*, 1999).

To test whether the presence of VASP at these sites was associated with the ability of VASP to restrict *Shigella* spread, we determined the subcellular localization of each GFP-tagged VASP construct. In cells producing WT, Δ PRO or Δ FAB VASP, the VASP construct showed reproducible co-localization with vinculin, a marker of focal adhesions, whereas in cells producing Δ EVH1 or Δ EVH2 VASP, the VASP derivative did not clearly co-localize with vinculin (Table 1, Fig. 4a), consistent with previous reports that both the EVH1 and EVH2 domains are required for proper localization of VASP to focal adhesions, which is mediated through interaction of these domains with zyxin (Hoffman *et al.*, 2006). The Δ COCO VASP derivative and the EVH1 domain derivative also did not co-localize with vinculin (Table 1, Fig. 4a, b). The two phosphoablative derivatives S153A/S235A/T273A VASP and S153A VASP demonstrated reproducible co-localization with vinculin (Fig. 4c), which contrasts with a previous report that phosphorylation of VASP on Ser153 promotes its redistribution from stress fibres to focal adhesions and the plasma membrane (Benz *et al.*, 2009). No substantial redistribution of VASP localization was observed upon infection of these cell lines with *S. flexneri* (data not shown).

Thus, the ability of VASP derivatives to limit *S. flexneri* spread correlated directly with localization of the derivative

to focal adhesions. Aside from the phosphoablative mutants, those VASP derivatives that restricted bacterial spread, leading to plaques similar in size to those formed in WT VASP cells, Δ PRO and Δ FAB, each co-localized with focal adhesions, whereas those VASP derivatives that supported larger bacterial plaques, Δ EVH1 and EVH1, each displayed a nearly undetectable signal at focal adhesions (Table 1, Fig. 4). Moreover, the two VASP derivatives that enabled formation of bacterial plaques that trended towards being larger than those supported by WT VASP, Δ EVH1 and Δ COCO VASP were also undetectable at focal adhesions (Table 1, Fig. 4a). These observations suggested that VASP localization to focal adhesions might be necessary to restrict *S. flexneri* spread. The phosphoablative mutants each co-localized with vinculin, yet were defective in restricting bacterial spread, suggesting that localization to focal adhesions is not sufficient for restricting spread and that phosphorylation of critical residues is also required. As VASP proteins are obligate tetramers (Zimmermann *et al.*, 2002), it is also possible that EVL and VASP serve as scaffolds, which, via the COCO motif, bring together four EVH1-binding proteins, perhaps in conjunction with inhibition of mDia2. Similar clustering has been proposed as a mechanism contributing to EVH1-mediated VASP activation of WAVE (Chen *et al.*, 2014).

Localization of VASP to focal adhesions and/or the leading edge is required for restriction of *S. flexneri* spread

To directly test whether the role of VASP in restricting *S. flexneri* spread is due to the role of VASP at the focal adhesions per se, we assessed whether removing VASP from focal adhesions by sequestering it to mitochondria would alter plaque size. We generated cell lines that stably synthesized in WT VASP cells the EVH1-binding motif FPPPP fused to a mitochondrial targeting sequence and tagged with mCherry ('FP4-mito'), which targets WT

Table 1. Summary of subcellular localization of VASP and size of plaques formed by *S. flexneri*

Plaque size is a summary of the results shown in Figs 1 and 3: +, plaque size similar to that of cells producing WT VASP; ++, plaque size larger than that of cells producing WT VASP. VASP localization: +, co-localization with target site; -, absence of co-localization with target site or no EGFP signal.

VASP construct	Plaque size	VASP localization		Reference
		Focal adhesion	Leading edge	
WT	+	+	+	This study, Bear <i>et al.</i> (2000), Gertler <i>et al.</i> (1996)
Δ EVH1	++*	-	-	This study
Δ PRO	+	+	+	This study, Loureiro <i>et al.</i> (2002)
Δ EVH2	+	-	+/-	This study, Loureiro <i>et al.</i> (2002)
Δ FAB	+	+	+/-	This study, Loureiro <i>et al.</i> (2002)
Δ COCO	+	-	+/-	This study, Loureiro <i>et al.</i> (2002)
EVH1	++*	+	-	This study, Bear <i>et al.</i> (2000)
S153A/S235A/T274A	++*	+	+/-	This study, Benz <i>et al.</i> (2009)
S153A	++*	+	+/-	This study, Benz <i>et al.</i> (2009)

* $P < 0.05$ by Student's *t*-test.

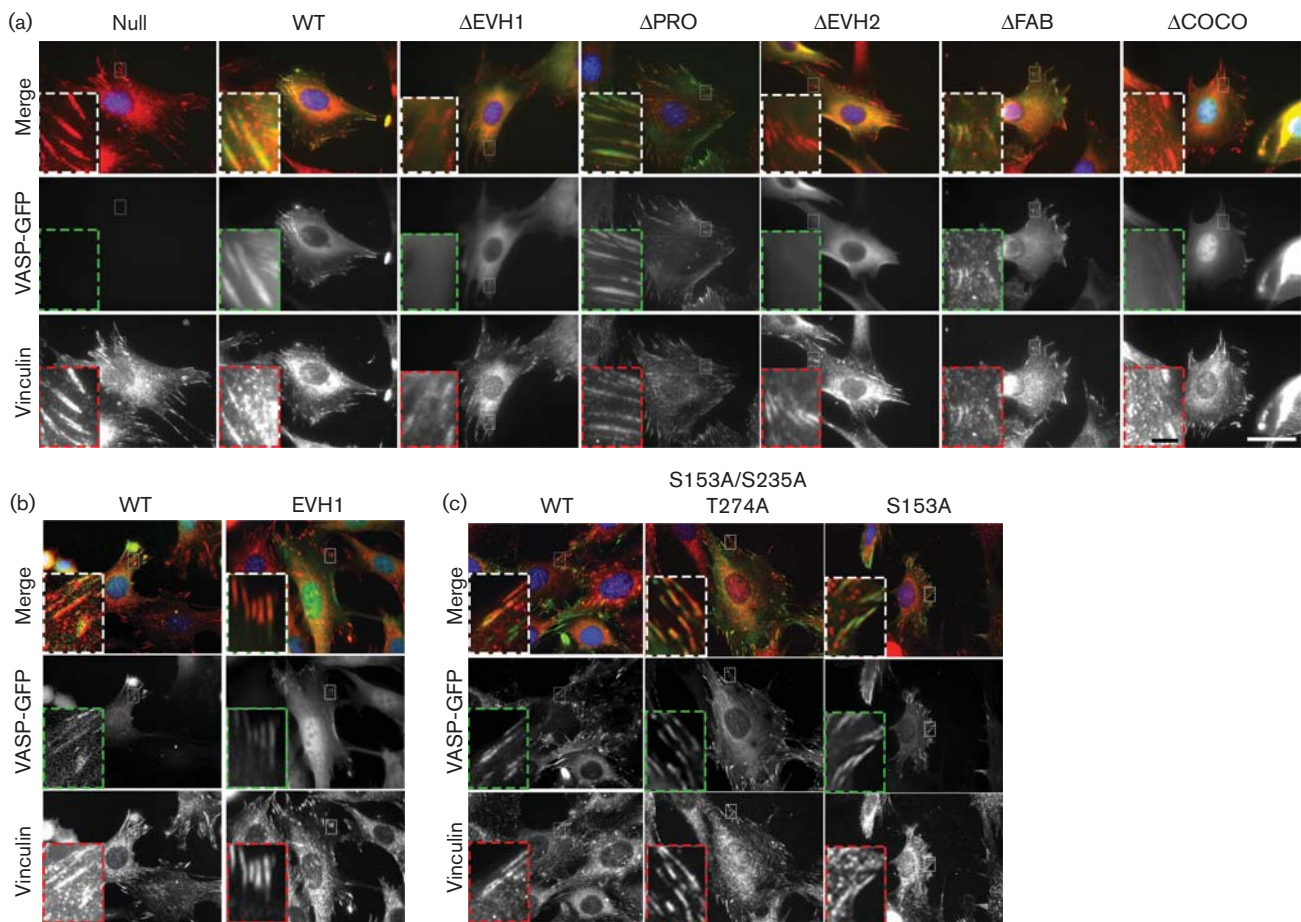


Fig. 4. Subcellular localization of VASP constructs. Signal from EGFP-tagged VASP constructs (VASP-GFP, green) and the focal adhesion protein vinculin (red) for (a) VASP deletion constructs, (b) EVH1 domain and (c) phosphoablative VASP mutants. Inset, 5.5-fold magnification of area indicated by white box, containing focal adhesions. Bar, 50 μm (main) and 5 μm (inset).

VASP to mitochondria via an EVH1-mediated interaction (Bear *et al.*, 2000). As a control, we generated a cell line that stably synthesized a construct in which the phenylalanine within FPPPP was mutated to alanine (APPPP), which prevents EVH1-domain interactions ('AP4-mito'). VASP redistribution to the mitochondria in FP4-mito cells was confirmed by immunofluorescence staining, as VASP co-localized with mitochondria (Fig. 5a, left panels, arrows) and was absent or only weakly visible at focal adhesions or in other subcellular compartments. In AP4-mito cells, VASP was localized to focal adhesions and the leading edge (Fig. 5a, right panels, arrowheads); its localization to these sites was indistinguishable from the localization of VASP to focal adhesions and the leading edge in cells in which AP4-mito was not produced. To test whether restriction of *S. flexneri* spread depended on localization of VASP to these sites, we compared plaque size in these two cell lines. Plaque size was increased twofold in FP4-mito cells compared with AP4-mito control cells (Fig. 5b). These results indicate that VASP localization to focal adhesions

and/or the leading edge is necessary to restrict *S. flexneri* spread in a cell monolayer.

VASP EVH1 domain restricts *S. flexneri* spread from both the donor and the recipient cell, whereas Ser153 phosphorylation contributes from the donor cell

The process of bacterial cell-to-cell spread depends on cellular factors both in the formation of a membrane-bound protrusion in the donor cell and in the uptake and resolution of the bacterium-containing protrusion in the recipient cell. To assess whether the role of VASP in restricting *S. flexneri* spread is in the donor cell and/or in the recipient cell, we measured the efficiency of bacterial spread into adjacent cells when the donor cell was one cell lineage and the recipient cell another cell lineage (modified from Heindl *et al.*, 2010; Sansonetti *et al.*, 1994). To address the place where VASP localization to focal adhesions is required, we mixed cells producing VASP lacking the

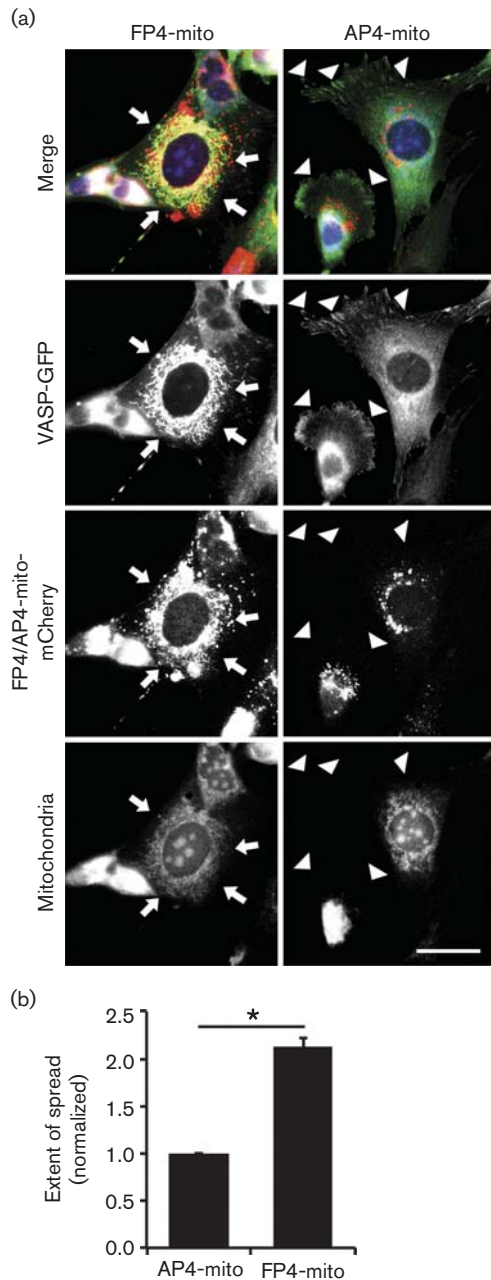


Fig. 5. Localization of VASP to focal adhesions and/or the leading edge is required for restriction of *S. flexneri* spread. (a) WT VASP cells that produce mCherry-tagged FP4-mito, which targets VASP to mitochondria (left), or the control construct mCherry-tagged AP4-mito, which has no effect on VASP localization (right). Signal from EGFP-tagged VASP constructs (VASP-GFP, green), mCherry-tagged FP4-mito or AP4-mito (red) and immunofluorescence to the mitochondrial protein TOM20 (blue). Arrows (left panels), VASP colocalized with mitochondria; arrowheads (right panels), VASP at focal adhesions and the leading edge. Bar, 50 μ m. (b) Extent of spread of *S. flexneri* through monolayers of cells shown in (a), normalized to that for cells producing the control construct AP4-mito-mCherry. * $P < 0.05$. Data are presented as mean \pm SD and represent a minimum of three independent experiments.

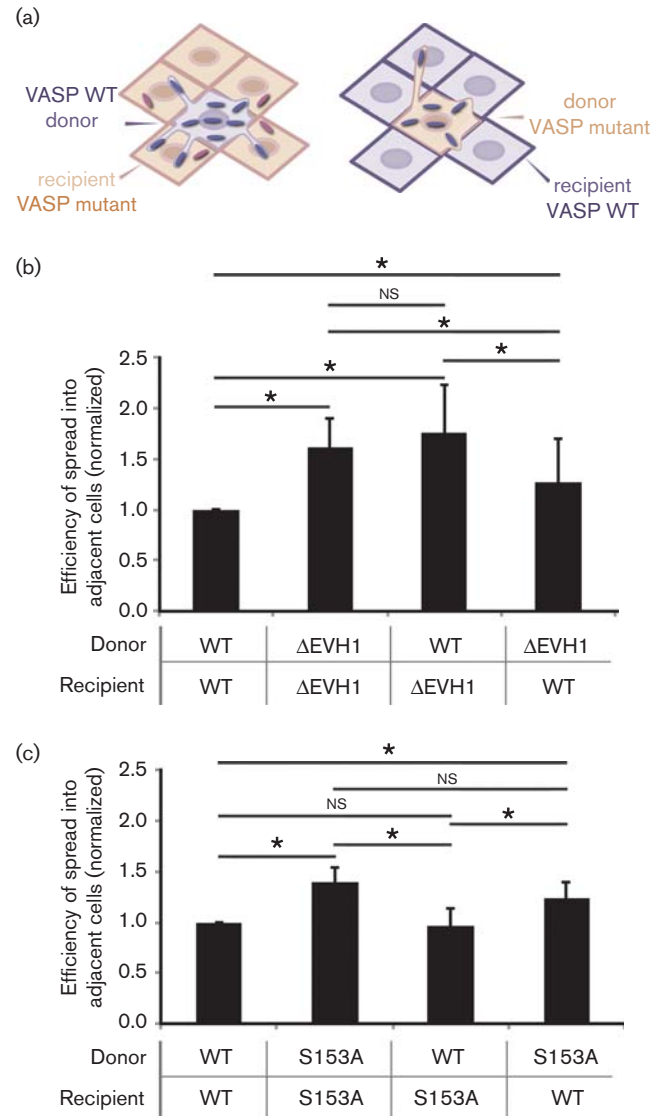


Fig. 6. Contributions of VASP from donor and recipient cells during *S. flexneri* spread. (a) Diagram depicting method used in the mixed cell spread assay. See Methods. (b, c) The efficiency of *S. flexneri* to spread from a primarily infected cell ('Donor') to an adjacent cell ('Recipient'), normalized to that of the condition where both donor and recipient cells were WT VASP cells. The efficiency of spread was defined as the mean number of 'recipient' cells infected per focus of infection. For each condition, a minimum of 10–15 foci was analysed. * $P < 0.05$; NS, not significant.

EVH1 domain (Δ EVH1) with cells producing WT VASP (Fig. 6a). *S. flexneri* spread was less efficient when the recipient cell produced WT VASP, regardless of which VASP derivative was produced by the donor cell (Fig. 6b), whereas bacterial spread was similar under conditions where the recipient cells produced Δ EVH1 VASP, regardless of whether the donor cells produced WT VASP or Δ EVH1 VASP (Fig. 6b), indicating that the EVH1 domain of VASP

restricts *S. flexneri* spread from the recipient cell. However, we also observed that when recipient cells produced WT VASP, but not when they produced Δ EVH1 VASP, donor cells contributed to restriction of cell-to-cell spread in a small but significant way (Fig. 6b), suggesting that in the presence of normal focal adhesion complexes in the recipient cell, the EVH1 domain contributes to restriction of spread from the donor cell as well from the recipient cell.

To determine whether the role of VASP phosphorylation of Ser153 in restricting *S. flexneri* spread is in the donor cell and/or in the recipient cell, we measured the efficiency of bacterial spread into adjacent cells in a parallel assay, mixing WT VASP cells and S153A VASP cells (Fig. 6a). *S. flexneri* spread was more efficient when the donor cell produced S153A VASP, regardless of which VASP derivative was produced by the recipient cell (Fig. 6c). In addition, bacterial spread was similar when the donor cells produced WT VASP, regardless of whether the recipient cells produced WT VASP or S153A VASP (Fig. 6c). These results indicate that VASP phosphorylation at Ser153 in the donor cell is necessary for VASP to restrict *S. flexneri* spread.

The defect observed for S153A VASP in donor cells further suggests that VASP is phosphorylated on Ser153 during *S. flexneri* infection. Infection with *Helicobacter pylori* and *Yersinia* spp. leads to phosphorylation of VASP (Ke *et al.*, 2015; Knauer *et al.*, 2008). The type IV secreted *H. pylori* effector CagA is associated with phosphorylation of VASP at Ser153, Ser235 and Thr274 (Knauer *et al.*, 2008), whereas the type III secreted *Yersinia* effector YpkA directly phosphorylates VASP at Ser153 (Ke *et al.*, 2015). *Shigella* spp. do not contain homologues of either CagA or YpkA (data not shown), indicating that whereas phosphorylation of VASP may be a common method employed by infecting bacterial pathogens, the specific mechanisms utilized are diverse.

Localization of VASP to focal adhesions is modulated both by interactions of the VASP EVH1 domain with other focal adhesion proteins and by phosphorylation of VASP, particularly on Ser153 (Applewhite *et al.*, 2007; Bear *et al.*, 2000; Benz *et al.*, 2009; Loureiro *et al.*, 2002). The association of VASP localization to focal adhesions with the ability to restrict *S. flexneri* spread raises the possibility that the dependence on the EVH1 domain is due exclusively to its role in subcellular localization. Our observation that the EVH1 domain contributes to *S. flexneri* spread from both the donor and the recipient cell, but that phosphorylation at Ser153 contributes only from the donor cell, implies that the roles of the EVH1 domain and of Ser153 phosphorylation in *S. flexneri* spread are at least partially distinct.

Cell-to-cell spread of *S. flexneri* is accompanied by phosphorylation on tyrosines of host proteins locally within bacterial protrusions (Dragoi & Agaisse, 2014). This tyrosine kinase signalling depends on the type III secretion system, suggesting that it is triggered by a type III secreted effector protein (Kuehl *et al.*, 2014). In contrast to serine phosphorylation of VASP described herein, which restricts

cell-to-cell spread of the pathogen, tyrosine kinase signalling promotes spread by contributing to the resolution of the bacterial protrusion into a vacuole within the recipient cell (Dragoi & Agaisse, 2014, 2015; Kuehl *et al.*, 2014).

Unlike host proteins characterized in *Shigella* pathogenesis that promote bacterial spread, VASP and EVL function to restrict it. The ability of VASP (and likely EVL) to restrict spread depends both on subcellular localization to focal adhesions and/or the leading edge and on phosphorylation at Ser153. The number and stability of focal adhesions contribute to cell-to-cell spread of *Shigella*, and components of focal adhesions are recruited to bacterial protrusions (Kim *et al.*, 2009; Mounier *et al.*, 1999). Our data demonstrate that localization of VASP to focal adhesions is critical to the role of VASP in restricting *Shigella* spread. We speculate that VASP-mediated modification of focal adhesions inhibits *Shigella* recruitment of critical focal adhesion components to protrusions. As the efficiency of *Shigella* spread is directly correlated with the stabilization of focal adhesions and reduction in focal adhesion turnover (Kim *et al.*, 2009), a mechanism that theoretically could contribute to the observed phenotype is a relative increase in focal adhesion turnover, associated with increased phosphorylation of focal adhesion kinase and paxillin, although whether VASP modulates focal adhesion turnover or stability is currently unclear. Mena, but not VASP, promotes clathrin-mediated uptake of *Shigella* protrusions in recipient cells (Fukumatsu *et al.*, 2012); our data indicating that the role of the VASP EVH1 domain in restriction of spread is largely in the recipient cell raise the possibility that VASP competitively inhibits Mena function in this process. The ability of VASP and EVL to limit spread highlights the critical role of focal adhesion complexes and/or the leading edge in bacterial passage between cells.

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