# Recognition of Two Classes of Oligoproline Sequences in Profilin-mediated Acceleration of Actin-based *Shigella* Motility

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*Abstract.* The gram negative rod *Shigella flexneri* uses it surface protein IcsA to induce host cell actin assembly and to achieve intracellular motility. Yet, the IcsA protein lacks the oligoproline sequences found in ActA, the surface protein required for locomotion of the gram positive rod *Listeria monocytogenes*. Microinjection of a peptide matching the second ActA oligoproline repeat (FEFPPPPTDE) stops *Listeria* locomotion (Southwick, F.S., and D.L. Purich. 1994a. *Proc. Natl. Acad. Sci. USA.* 91:5168–5172), and submicromolar concentrations (intracellular concentration 80–800 nM) similarly arrest *Shigella* rocket-tail assembly and intracellular motility. Coinjection of a binary solution containing profilin and the ActA analogue increased the observed rates of intracellular motility by a factor of three (mean velocity $0.09 \pm 0.07$ μm/s, SD $n = 16$ before injection vs $0.3 \pm 0.1$ μm/s, $n = 33$ postinjection, intracellular concentration = 80 nM profilin plus 80 nM ActA analogue). Recent evidence suggests the ActA analogue may act by displacing the profilin-binding

protein VASP (Pistor, S.C., T. Chakaborty, V. Walter, and J. Wehland. 1995. *Curr. Biol.* 5:517–525). At considerably higher intracellular concentrations (10 μM), the VASP oligoproline sequence (GPPPPP)$_3$ thought to represent the profilin-binding site (Reinhard, M., K. Giehl, K. Abel, C. Haffner, T. Jarchau, V. Hoppe, B.M. Jockusch, and U. Walter. 1995. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1583–1589) also inhibited *Shigella* movement. A binary mixture of the VASP analogue and profilin (each 10 μM intracellular concentration) led to a doubling of *Shigella* intracellular migration velocity ($0.09 \pm 0.06$ μm/s, $n = 25$ preinjection vs $0.18 \pm 0.10$ μm/s, $n = 61$ postinjection). Thus, the two structurally divergent bacteria, *Listeria* and *Shigella*, have adopted convergent mechanisms involving profilin recognition of VASP oligoproline sequences and VASP recognition of oligoproline sequences in ActA or an ActA-like host protein to induce host cell actin assembly and to provide the force for intracellular locomotion and cell–cell spread.

THE gram-negative rod *Shigella flexneri* and the gram-positive rod *Listeria monocytogenes* are biochemically and structurally divergent intracellular pathogens. Yet, these two bacteria have developed convergent solutions for parasitizing host cells: both enter the host via the gastrointestinal tract by phagocytosis, and they then produce hemolysins allowing escape into the host cell's cytoplasm. They subsequently usurp the host cell's cytoskeleton to induce actin filament rocket tails for intracellular migration to the peripheral cytoplasm, and they ultimately form outward membrane projections, or filopods, which can be ingested by adjacent cells. This permits *Shigella* and *Listeria* to avoid the harmful consequences of humoral factors such as antibody, complement, as well as those antibiotics that poorly penetrate host cells. Understanding how these pathogens accomplish the above

tasks promises to provide new insights into the pathogenesis of *Listeria* and *Shigella* infections, and to reveal how nonmuscle cells remodel their actin cytoskeleton during normal motile functions such as chemotaxis and phagocytosis.

*Listeria*-associated actin assembly has been studied by time-lapse video microscopy which demonstrated that this bacterium can migrate at 0.02–0.4 μm/s in the PtK2 renal tubular epithelial cell (Dabiri et al., 1990; Sanger et al., 1992; Theriot et al., 1992). Microinjection of fluorescently labeled actin monomers proved that the actin filament tails progressively lengthen as the bacterium migrates through the cytoplasm (Sanger et al., 1992). The rate of monomer incorporation directly correlates with the velocity of movement, while the depolymerization rate is independent of migration velocity and remains constant throughout the tail (Sanger et al., 1992; Theriot et al., 1992). Together, these features define the properties of a Brownian ratchet model for bacterial propulsion (Peskin et al. 1993). Transposon mutation experiments indicate that the *Listeria* surface protein ActA is required for *Liste-*

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ria-induced actin assembly. Inactivation of the ActA gene blocks actin rocket-tail formation, intracellular movement, and cell–cell spreading of Listeria (Kocks et al., 1992; Domann et al., 1992). The ActA protein does not bind directly to actin (Kocks et al., 1992), and ActA does not stimulate polymerization of purified actin (Tilney et al., 1990). The ActA molecule may enhance actin assembly via a series of four nonidentical oligoproline repeats 235-DFPPPPTDE, 269-FEFPPPPPTDE, 304-FEFPPPPTED, and 350-DFPPIPTEE, which are thought to be the binding site for the host actin regulatory protein vasodilator-stimulated phosphoprotein (VASP).[1] VASP is normally concentrated in host cell focal contacts, but during Listeria infection becomes concentrated on motile bacteria at the bacterial-actin tail interface (Chakraborty et al., 1995). The ActA oligoproline repeats are very similar to sequences found in the actin regulatory protein vinculin found in the focal adhesion plaques of nonmuscle cells (Kocks et al., 1992; Domann et al., 1992). Vinculin, therefore, may serve to concentrate VASP at sites of focal adhesion in host cells.

Microinjection of a synthetic peptide (CFEFPPPPTDE) analogue of the second ActA repeat into bacteria-laden PtK2 cells rapidly and completely blocks Listeria-induced actin assembly at a final intracellular peptide concentration of ~80 nM (Southwick and Purich, 1994a). We have also demonstrated that microinjection of mosquito oostatic factor, the freely occurring decapeptide YDPAPPPPPP, inhibits Listeria locomotion (Southwick and Purich, 1995). In a similar concentration range and time frame, both of these peptides also result in loss of the host cell's normal peripheral actin filament architecture as well as retraction of the peripheral membrane. Microinjection of a third peptide analogue DFPPPPTDEELRL derived from first oligoproline repeat in ActA also results in membrane retraction and loss of the normal actin filament architecture. These changes were associated with the dissociation of VASP from focal adhesion plaques and redistribution throughout the cytoplasm (Pistor et al., 1995). These peptides, therefore, are likely to block VASP binding to an ActA-like host protein (possibly vinculin) as well as block VASP binding to the oligoproline regions of ActA.

In addition to binding to ActA, VASP also binds to profilin (Reinhard et al., 1995). Profilin is the only actin-regulatory protein known to bind to poly-L-proline (Tanaka and Shibata, 1985), and VASP contains a series of oligoproline repeats consisting of a glycine and five prolines. VASP and profilin colocalize in the peripheral lamellae of locomoting fibroblasts (Reinhard et al., 1995) and are both found at bacterial-actin tail interface of intracellular Listeria (Theriot et al., 1994; Chakraborty et al., 1995). Profilin, therefore, also is likely to be the key host cell component responsible for Listeria locomotion. Depletion of profilin from Xenopus egg extracts, using beads with covalently bound poly-L-proline, blocked in vitro movement of Listeria and readdition of profilin partially restored motility (Theriot et al., 1994). Profilin enhances the exchange of ATP on actin monomers (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991) and may produce higher in-tracellular concentrations of the more polymerization-competent ATP-actin species at sites immediately adjacent to the bacterium/rocket-tail interface (Southwick and Purich, 1994b). In addition, in the presence of the monomer sequestering protein thymosin $\beta_4$, profilin may interact with the barbed ends of actin filaments to lower the critical concentration for actin assembly (Pantaloni and Carlier, 1993).

Although the first descriptions of actin filaments being associated with intracellular bacteria were reported with Shigella-infected cells (Bernardini et al., 1989), video microscopy experiments similar to those designed to explore actin-based motility in Listeria have not been performed in live cells infected with Shigella. We have now performed time-lapse studies which reveal that Shigella moves at rates and trajectories similar to Listeria, suggesting these two bacteria stimulate actin based motility by similar mechanisms. Shigella like Listeria has an outer cell wall protein, IcsA, which is necessary for actin-based motility (Bernardini et al., 1989; Goldberg et al., 1993) and is sufficient to support actin-based movement in Xenopus egg extracts (Goldberg and Theriot, 1995). This 120-kD protein, however, shares no sequence identity with the Listeria ActA protein and lacks oligoproline sequences which might recruit host cell components to facilitate actin filament assembly. To test the possibility that the IcsA protein attracts a host cell oligoproline-containing protein to serve in place of ActA, we examined intracellular Shigella motility after the microinjection of two oligoproline analogues derived from ActA and VASP amino acid sequences. Cellular ActA analogue concentrations necessary to inhibit Listeria movement (i.e., in the range of 80–800 nM) blocked Shigella motility as well. The introduction of an oligoproline peptide based on the VASP sequence, $(GPPPPP)_3$, at considerably higher intracellular concentrations (10 μM) also blocked Shigella movement. Microinjection of an equimolar binary solution of profilin with the ActA or the VASP analogue neutralized the inhibition of Shigella movement. Even more surprisingly, the binary solutions caused a 200–300% increase in the velocities of intracellular bacterial migration. These findings provide evidence for a shared mechanism involving certain oligoproline-containing proteins and profilin in actin-based motility of both Shigella and Listeria; they also suggest that a similar mechanism may regulate actin filament assembly at the cytoskeleton-membrane interface of actively moving nonmuscle cells.

## Materials and Methods

### Materials

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1. Abbreviation used in this paper: VASP, vasodilator-stimulated phosphoprotein.

## Tissue Culture Methods and Infection Procedures

The PtK2 cell line (derived from the kidney epithelium of the kangaroo rat *Pororous tridactylis*) was seeded at a concentration of $10^6$ cells per coverslip in 35-mm culture dishes in 3 ml of culture media (MEM with 10% FCS, 1% penicillin-streptomycin) and incubated for 72 h at 37°C and 5% $CO_2$. *Shigella flexneri* M90T wild-type strain was inoculated into brain heart infusion (Difco, Detroit, MI) and grown overnight at 37°C. Bacteria were harvested at mid-log phase and resuspended in MEM without antibiotics to give a final concentration of $10^7$ or a ratio of 10 bacteria per host cell. Bacteria in 3 ml of culture media were added to each dish followed by centrifugation at 400 *g* at room temperature for 10 min and then incubation for 45 min at 37°C and 5% $CO_2$. After incubation, extracellular bacteria were removed by washing three times with Hank's balanced salt (GIBCO BRL, Gaithersburg, MD). The culture media containing gentamicin sulfate (10 μg/ml) was added back to prevent extracellular growth of bacteria. The monolayers were then incubated for 1–4 h during which microinjection and video microscopy were performed.

## Microscopy and Microinjection

A Nikon Diaphot inverted microscope was equipped with a charge-coupled device camera (Dage-MTI, Michigan City, IN), and the microscope stage temperature was maintained at 37°C with a MS-200D perfusion microincubation system (Narishige, Tokyo). Digital images were obtained and processed, using an Image-1 computer image analyzer (Universal Imaging, West Chester, PA). Velocities of bacterial movement were determined by comparing the images at two time points and measuring the distance traveled by each bacterium using the measure curve length function (Image I/AT program). Distances were calibrated using a Nikon micrometer. Differences in migration velocities were analyzed using the unpaired Student's *t* test or the Mann-Whitney nonparametric test. For each bacterium, velocity was determined for 3–4 time points before and 3–4 time points after each microinjection. One to two bacteria were analyzed for each injected cell. In each experiment *n* indicates the number of velocity measurements. Individual cells were microinjected with peptide using a micromanipulator and microinjector (models 5171 and 5242; Eppendorf, Inc. Madison, WI), as previously described (Southwick and Purich, 1994a).

Immunofluorescence staining using anti–α-actinin antibodies was performed as previously described (Dabiri et al., 1990). In experiments requiring phallicidin staining, PtK2 cells were fixed with 3.7% (vol/vol) formaldehyde in phosphate-buffered saline for 15 min at 25°C followed by treatment with 0.4% Triton X-100 and $1.7 \times 10^{-7}$ M bodipy-phallacidin (Molecular Probes) for 10 min at 37°C. The relative fluorescence intensities of the bodipy-phallacidin stained tails were measured with the Image-1 system using a Genesis I image intensifier (Dage-MTI) in the linear response range. Gain settings were identical for both the *Shigella* and *Listeria* rocket tails. The relative intensity was measured at different locations on the tail with a fixed square template (2 × 2 pixels, brightness function; Image-I/AT). Fluorescence intensity of an identical area adjacent to the actin rocket tail within the cell was measured and subtracted from each value.

## Results

### Characteristics of Shigella Movement and Actin Rocket-Tail Formation in PtK2 Cells

Like *Listeria*, *Shigella* moves at relatively rapid velocities through the cytoplasm. Although their larger size might be expected to resist migration in a viscous medium, the observed mean rates of *Shigella* movement in PtK2 cells (0.17–0.05 μm/s) were comparable to *Listeria* (0.15–0.05 μm/s) (Southwick and Purich, 1994a; Southwick and Purich, 1995). The maximal velocities of 0.4 μm/s attained by *Shigella* are rarely seen in *Listeria*-infected PtK2 cells. As observed with *Listeria* infections, the mean rate of migration varied considerably from day to day. These differences appear to be related to age of the tissue culture cells at the time of infection, and in all microinjection experiments pre- and posttreatment rates were compared in the same cells.

Intracellular movement of *Listeria* in PtK2 cells is usually associated with the formation of phase-dense rocket-tails on phase contrast micrographs (Sanger et al., 1992; Southwick and Purich, 1994a, 1995). On the other hand, motile *Shigella* are infrequently associated with phase-dense tails (Fig. 1 *A*). Bacteria migrating in regions near or within the cell nucleus often display phase-lucent tails (Fig. 1 *B*). However, the majority of *Shigella* that migrate through the cytoplasm fail to produce phase-dense or phase-lucent rocket tails (Fig. 6 *B*).
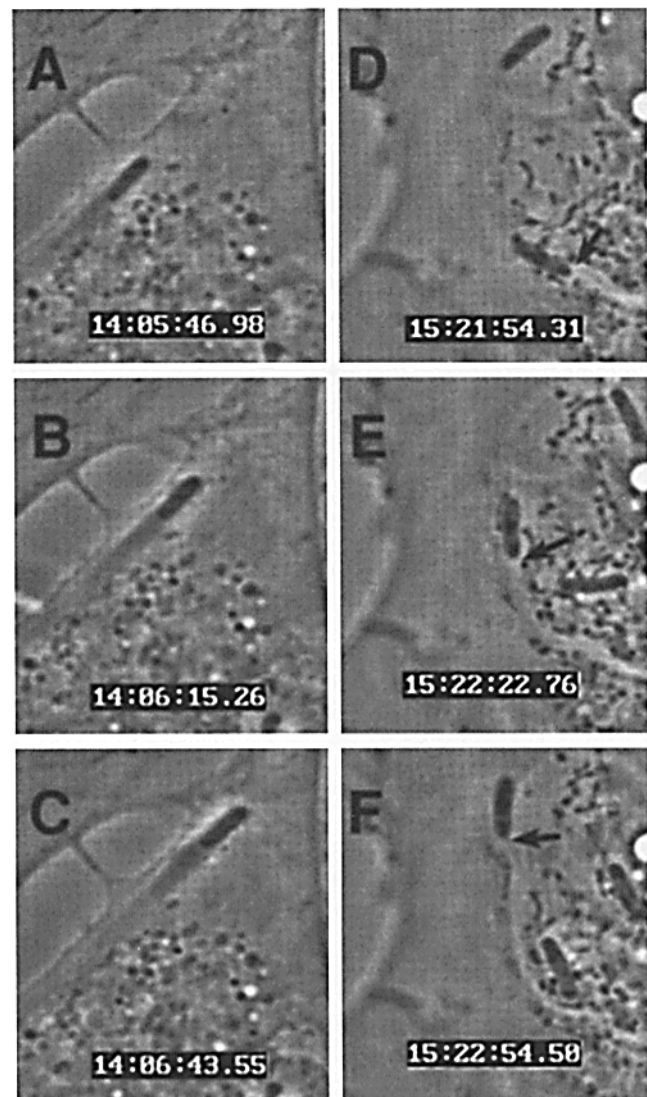


*Figure 1.* (*A–C*) Formation of a phase dense rocket tail as a *Shigella* bacterium migrates upward and to the right through a thin region of the cytoplasm in a PtK2 host cell. Images are taken at ~30-s intervals as indicated by the time stamp. (*Top to bottom*) (*D–F*) Formation of a phase lucent actin rocket tail as the bacterium in the lower right hand corner of *D* migrates through the perinuclear region of a PtK2 cell. The arrow points to the back of the moving bacterium. This bacterium has turned to the right in *E* and *F*, and is migrating toward the top of the micrograph. A thin clear area that displaces subcellular organelles trails behind the bacterium and is best seen in *D*, just below and to the right of the arrow. Length of time stamp bar, 12 μm.
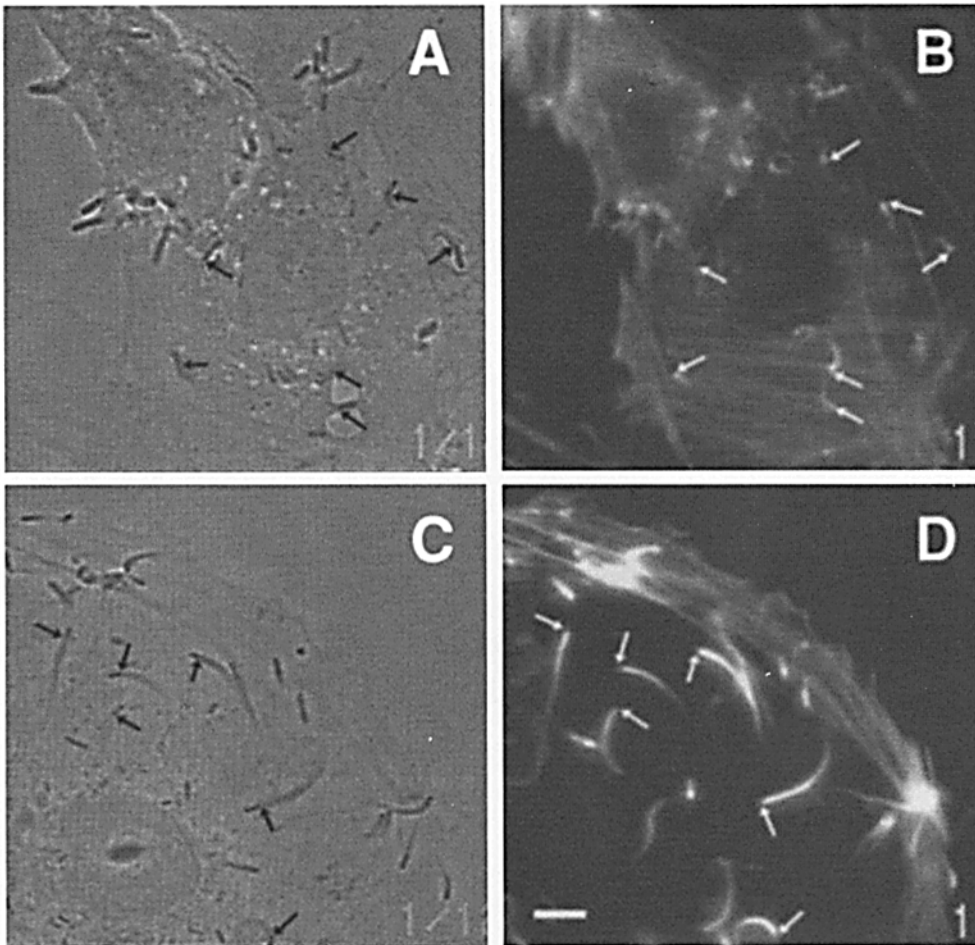
*Figure 2.* Comparison of bodipy-phallacidin staining of *Shigella* and *Listeria* actin filament rocket tails. Simultaneous phase (*A*) and fluorescent micrographs (*B*) of an intracellular *Shigella* are shown. Arrows point to the bacterial-actin rocket tail interfaces. Note the faint fluorescence of the actin rocket tails (*B*) which extend from the back of many of the bacteria. The rocket tails are thin and demonstrate relatively low fluorescence intensity as compared to *Listeria* actin rocket tails (*D*). In the phase micrograph of *Listeria*, phase dense actin rocket tails can be readily visualized (*C*), and the tails exhibit highest bodipy-phallacidin fluorescence in the region nearest each bacterium (*D*). Infections were performed simultaneously using the same stock of cells and stained in parallel. Gain settings were identical for both fluorescence images (*B* and *D*). The relative fluorescence intensities (*vertical axis*) of various regions of a representative *Shigella* and *Listeria* tail were measured digitally and graphically depicted in *E*. Horizontal axis represents the distance from the back of the bacteria of each intensity measurement along the tail (see Materials and Methods). In this example, the relative intensity was 5–6 times higher in the *Listeria* as compared to the *Shigella* rocket tail. Bar, (*left lower corner, D*) 10 μm.
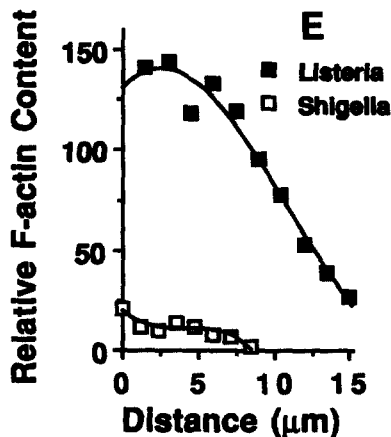
## Fluorescence Staining of Actin and α-Actinin in Shigella Rocket-Tails

Comparisons of bodipy-phallacidin staining of the actin filament tails reveal that the *Shigella*-associated structures (Fig. 2 *B*) have significantly lower fluorescence intensities than *Listeria* ($P < 0.001$, $n = 16$) (Fig. 2, *D* and *E*). This observation suggests that *Shigella* rocket tails have a lower actin filament content than *Listeria*. As observed in *Listeria* (Dabiri et al., 1990), the actin filament bundling protein and cross-linking protein α-actinin also localizes to the *Shigella* rocket tails (Fig. 3).

## Arrest of Shigella Intracellular Movement by the Second Oligoproline Repeat Analogue in Listeria Act-A Protein

Bacterial motility ceases within 30 s after injection of the ActA analogue (800 nM needle concentration, estimated intracellular concentration = 80 nM)(Fig. 4, *A–D*). Phase-dense actin tails present before injection also disappear within 30 s. Similar results are shown graphically in Fig. 5 *A*. Microinjection of this concentration of peptide consistently blocks *Shigella* movement (mean preinjection rate of 0.06 ± 0.03 μm/s, SD $n = 47$ vs a mean postinjection rate of 0.004 ±
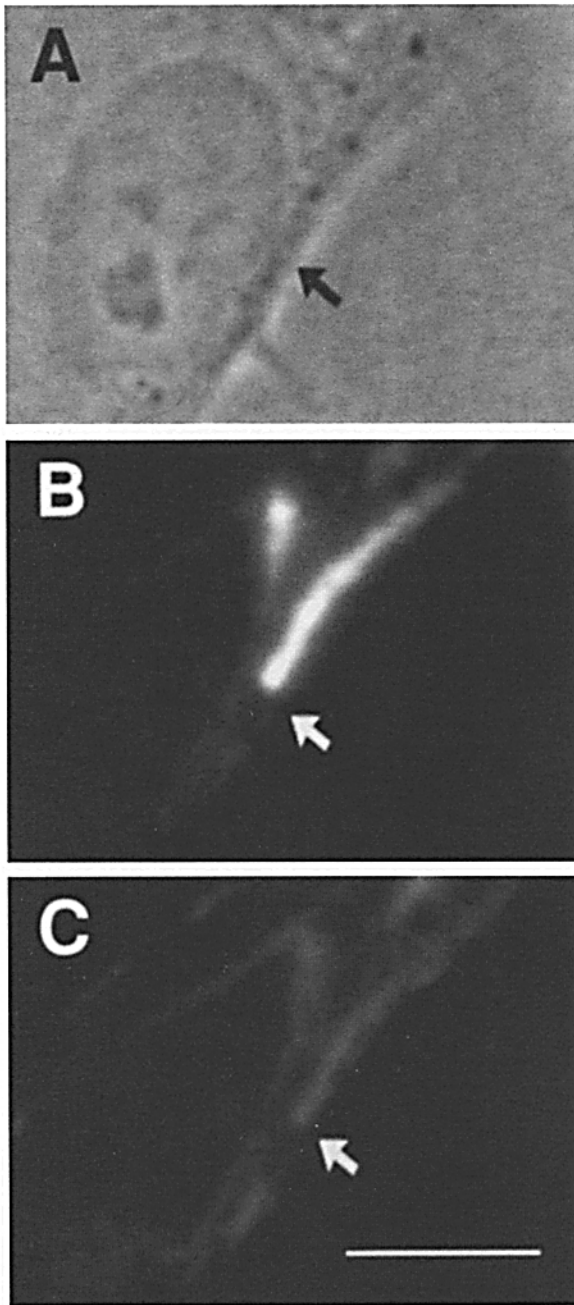
Figure 3. Simultaneous phase-contrast (A), anti-α-actinin immunofluorescence (B) and bodipy-phallacidin stained fluorescence (C) images of a *Shigella* rocket tail. Arrow points to the back of the bacterium which in the phase-contrast image refracts poorly in this region of the cell. Note the bright anti-α-actinin fluorescence as compared to that associated with phallacidin staining (both images were captured with gain settings in the linear response range of the image intensifier). Bar, 10 μm.

0.01 μm/s, $n = 85$ velocity measurements) (Table I). At this low intracellular concentration the inhibitory effects of the ActA analogue are not always permanent (Fig. 5 A). One quarter of the bacteria resume migration 2–4 min after microinjection. The rates of movement, however, are in all instances 25–30% of the velocities measured before injection (0.01–0.02 μm/s). The inhibitory effects of the

ActA are concentration dependent (Fig. 5 B). A lower intracellular concentration (8 nM) of ActA fails to inhibit, while higher intracellular concentrations (400–800 nM) consistently block intracellular movement. In some cells these higher concentrations also cause membrane retraction.

### Effect of Microinjecting a Binary Solution of Profilin and ActA Analogue on Shigella Intracellular Movement
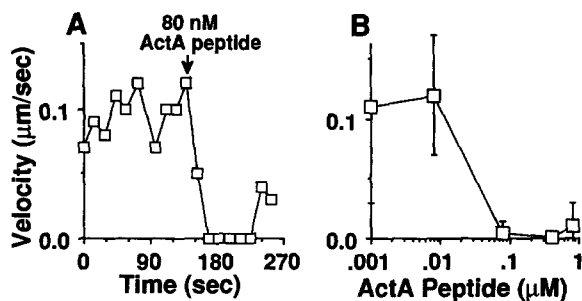
Although high intracellular concentrations of profilin (10 μM, see below) can markedly inhibit *Shigella* movement, microinjection of an 80-nM intracellular concentration of profilin does not significantly effect *Shigella* locomotion (Table I). Nonetheless, microinjection of equimolar binary solutions of the ActA peptide analogue and profilin (needle concentration = 0.8–1.0 μM, corresponding to estimated intracellular concentrations of 80–100 nM) not only neutralizes the analogue's inhibition but significantly increases the velocities by a factor of three (mean rate of movement before microinjection $0.09 \pm 0.07$ μm/s, $n = 16$ vs $0.3 \pm 0.1$ μm/s, $n = 33$ postinjection)(Fig. 6 A and Table I). The differences in velocities pre- and postinjection were highly significant on a statistical basis ($P < 0.0001$).

Velocities increased to nearly 0.5 μm/s in some instances. Introduction of the binary solution also frequently activated stationary bacteria to move at rapid rates (Fig. 6 A). If the stationary bacteria were included in pre- and postinjection velocity comparisons, the differences were also highly significant (mean pretreatment velocity $0.06 \pm 0.07$ μm/s, $n = 25$ vs mean posttreatment velocity $0.25 \pm 0.12$ μm/s, $n = 49$, $P < 0.0001$). The dramatic effects of the binary solution are also illustrated in the time-lapse micrographs (Fig. 6 B). A bacterium can be seen to rapidly accelerate in response to microinjection of a final intracellular concentration of 100 nM of the binary mixture. We have found no other treatment to evoke such a marked enhancement of the bacterial motility. Microinjection of a lower concentration of this equimolar mixture (20 nM) caused a statistically insignificant acceleration of *Shigella* velocity. (Table I).

### Effects of Microinjection of a VASP Oligoproline Analogue Alone and in Combination with Profilin on Shigella Intracellular Motility

Introduction of the VASP analogue (GPPPPPGPPPPGP-PPPP) can also inhibit *Shigella* motility without causing significant membrane retraction (Fig. 7 A and Table I). This effect is concentration dependent (Fig. 7 B), complete inhibition being seen at intracellular concentrations of 10 μM, while lower concentrations (2 and 6 μM) cause variable inhibition (note the large standard deviation bars at these two concentrations, Fig. 7 B). Introduction of poly-L-proline also causes a dose-dependent slowing of bacterial velocity (Fig. 8 A). Microinjection of the same concentration of an unrelated peptide derived from the sequence of MAP-2 had no effect on *Shigella* migration (Table I). As previously observed with *Listeria* (Sanger et al., 1995), microinjection of profilin also causes a concentration-dependent inhibition of *Shigella* movement, intracel-
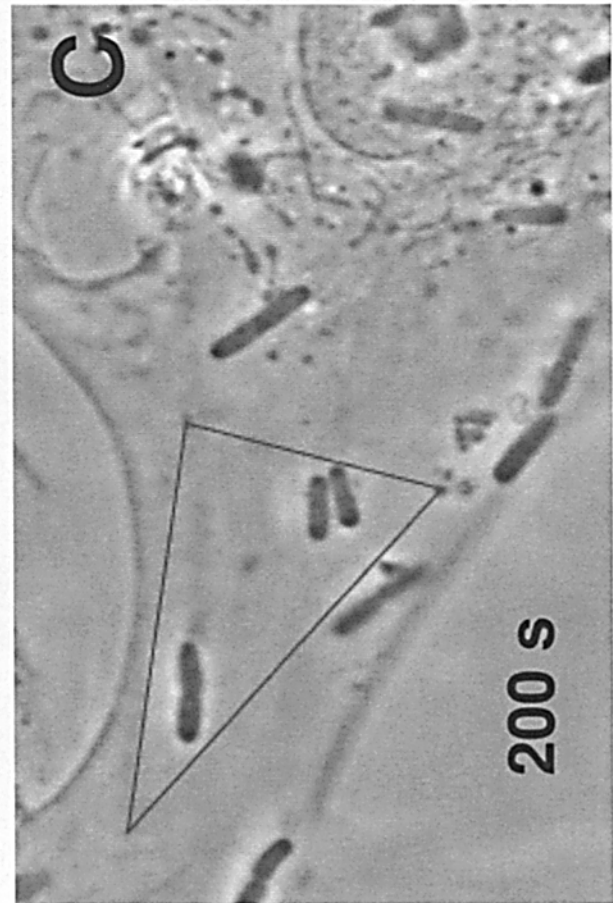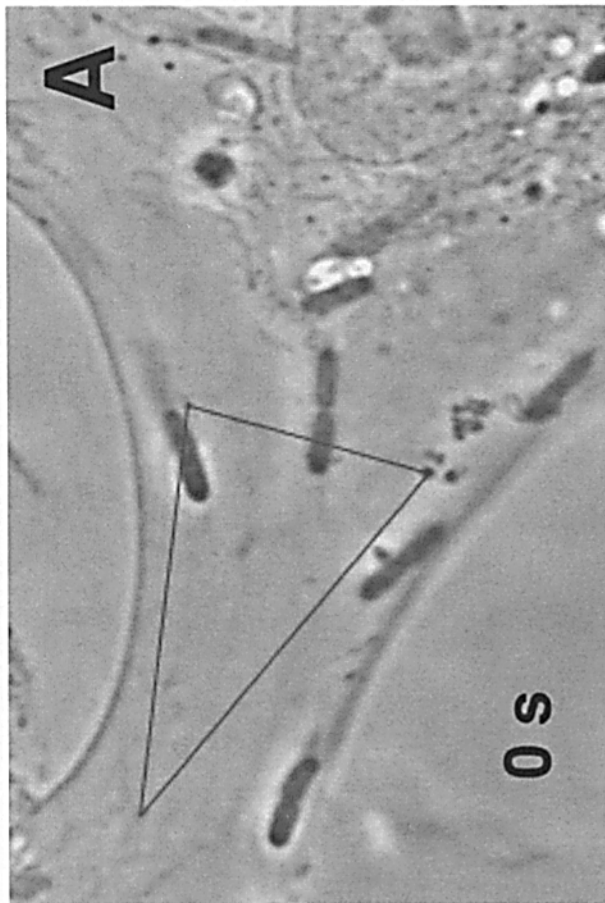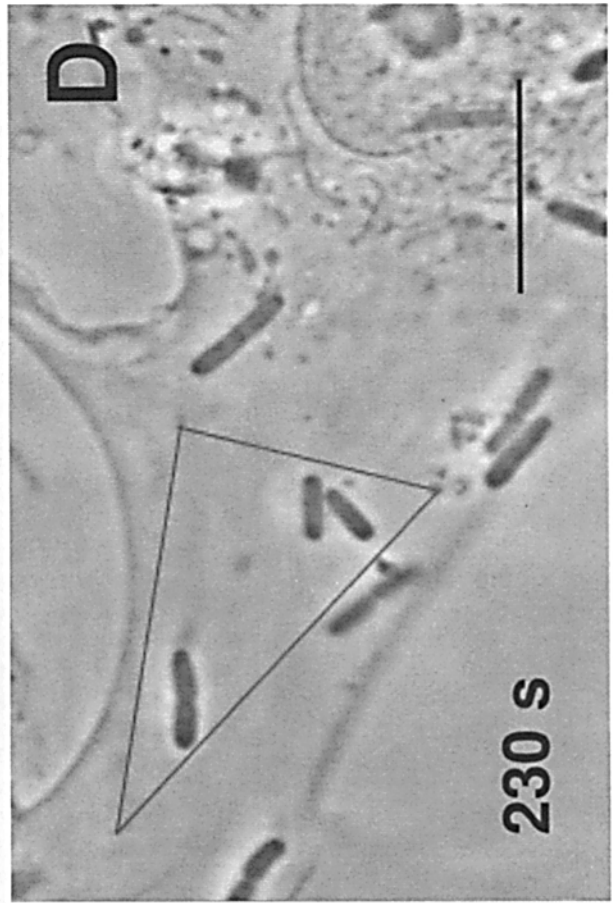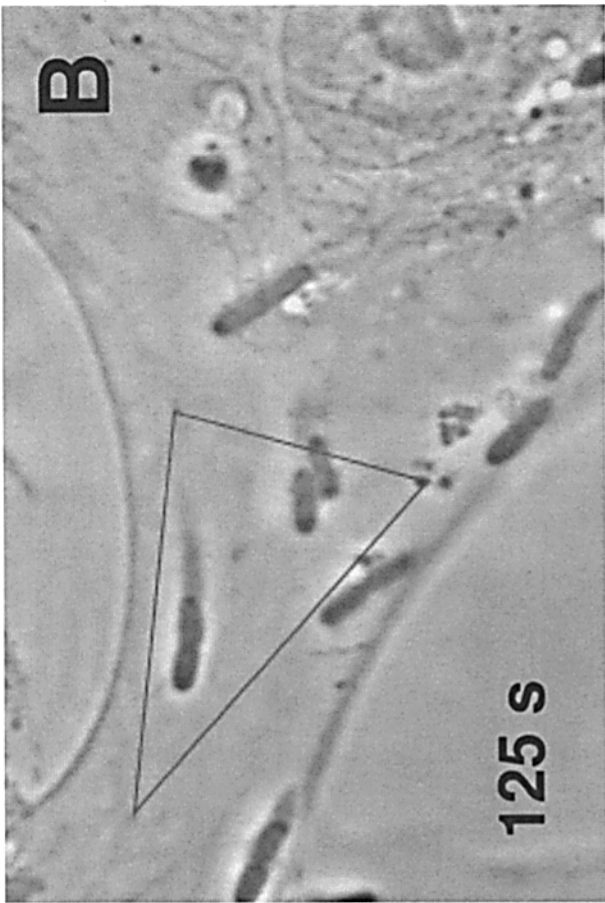
*Figure 5.* (*A*) Velocity of a single *Shigella* bacterium in a PtK2 cell before and after microinjection of the ActA analogue (estimated intracellular concentration 80 nM (needle concentration 0.8 μM). The arrow marks the time point at which the peptide was introduced. The graph corresponds to the bacterium shown within triangle of the micrograph shown in Fig. 4. (*B*) Effect of varying intracellular concentrations of the ActA analogue on *Shigella* intracellular velocity. Horizontal axis is in a log scale. Intracellular concentrations of 8, 80, 400, and 800 nM were studied. Bars represent the standard deviation of the mean for 30–80 velocity determinations per concentration.

lular concentrations of 10 μM causing nearly total inhibition (Fig. 8 *B* and Table I). Curiously, introduction of an intermediate intracellular concentration of profilin (6 μM) resulted in a bimodal behavior. 60% of the bacteria stopped moving. The remaining 40% accelerated their velocity, attaining mean migration rates of 0.19 ± 0.08 μm/s (*n* = 17). These postinjection velocities were significantly higher than the bacteria's preinjection velocities of 0.14 ± 0.05 (*n* = 28, *P* = 0.039).

The effect of microinjecting a binary mixture of profilin and the VASP oligoproline analogue was also examined (Fig. 8 *C*). In vitro experiments employing profilin tryptophan fluorescence have recently demonstrated that the (GPPPPP)$_3$ peptide binds to profilin with a $K_d$ in the $10^{-5}$ M range (Kang, F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation). Based on these findings, high equimolar concentrations (10 μM intracellular concentrations) of both profilin and the VASP oligoproline analogue when microinjected (barring interference from other intracellular constituents) should exist as a complex in the cell. We predicted that such a complex might neutralize the inhibitory activity of the two components. In fact, microinjection of this binary mixture accelerates *Shigella* movement, velocities increasing by a mean of 100% (preinjection mean velocity: 0.09 ± 0.05 μm/s, *n* = 25 vs postinjection mean velocity: 0.18 ± 0.10, *n* = 61, *P* < 0.0001) (Table I). Introduction of an equivalent binary mixture of poly-L-proline and profilin inhibits *Shigella* movement (Fig. 8 *D* and Table I). Microinjection of a lower equimolar concentration of the VASP analogue and profilin (1 μM intracellular concentrations) fails to accelerate *Shigella* migration (Table I).

## Discussion

Dynamic remodeling of the actin cytoskeleton must be exquisitely controlled (Stossel, 1993; Condeelis, 1993), and bacterial pathogens must use these regulatory processes to achieve actin-based motility in host cells in their efforts to evade host defense mechanisms. To gain further insights into bacterial as well as nonmuscle cell actin-based motility, we compared the mechanisms underlying *Listeria* and *Shigella* movement in PtK2 host cells. While *Shigella* rocket tails have a lower F-actin content than *Listeria*, the average velocities of both pathogens are quite similar. As observed with *Listeria*, we now find that *Shigella* rockettails also contain the actin bundling and cross-linking protein α-actinin shown to be critical for *Listeria* motility (Dold et al., 1994). These similarities raised the possibility that these two biochemically distinct pathogens may be adopting convergent mechanisms to subvert the host cell's actin regulatory system to allow their locomotion within cells and their spread from cell to cell. To explore this possibility, the inhibitory effects of oligoproline peptides based on the sequences in the ActA protein and VASP were examined in cells infected with *Shigella*. Over the same concentration range that inhibited *Listeria* intracellular motility (Southwick and Purich, 1994*a*), the ActA analogue likewise blocked *Shigella* movement.

We originally hypothesized that the ActA analogue acted by competitively inhibiting profilin binding to bacterial cell wall ActA protein; however, in vitro experiments failed to demonstrate any binding of the ActA oligoproline analogue to profilin (Kang, F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation). The discovery that a second host cell actin regulatory protein VASP may serve to link profilin to ActA now provides a self-consistent explanation for our results (Reinhard et al., 1995). It is likely that the ActA oligoproline analogue FEFPPPPTDE dissociates VASP from both *Listeria* and *Shigella*. Based on the estimates of Reinhard et al. (1992), the concentration of VASP tetramer in platelets is ∼0.5–1 μM. The content of VASP in other cells is considerably lower (i.e., ∼100 nM). The latter value is quite close to the estimated intracellular concentrations of ActA analogue (80 nM) found to arrest *Shigella* motility. It is noteworthy that *Listeria* intracellular movement is inhibited by both the ActA analogue and oostatic factor in the identical concentration range. This behavior would be predicted if the peptides interact directly with the limited intracellular pool of VASP.

Dissociation of VASP from the surface of the bacteria would be expected to prevent the concentration of profilin at the bacterial-actin tail interface blocking further actin assembly at this site, thereby preventing bacterial movement (Fig. 9). Based on our recent studies demonstrating that profilin binds directly to a contiguous triad of GPP-PPP repeats spanning positions 172-189 in VASP (Kang,

*Figure 4. Shigella* movement and actin rocket tail formation in PtK2 host cells before and after microinjection of the synthetic ActA peptide. Before injection the bacteria are seen to move at 0.12 μm/s, and maximum tail length is 6.0 μm (*A* and *B*). After injection of an estimated intracellular concentration of 80 nM of ActA analogue (needle concentration 0.8 μM ActA peptide) at 160 s, bacterial movement stops and the actin tails almost completely disappear (*C* and *D*). Times (indicated in seconds) are included in the lower left corner of each micrograph. The triangle (drawn by connecting three small phase-dense granules in the cytoplasm) served as a stable reference point. Solid bar, 10 μm.

*Table I. Effects of Microinjected Peptides on Shigella Intracellular Motility*

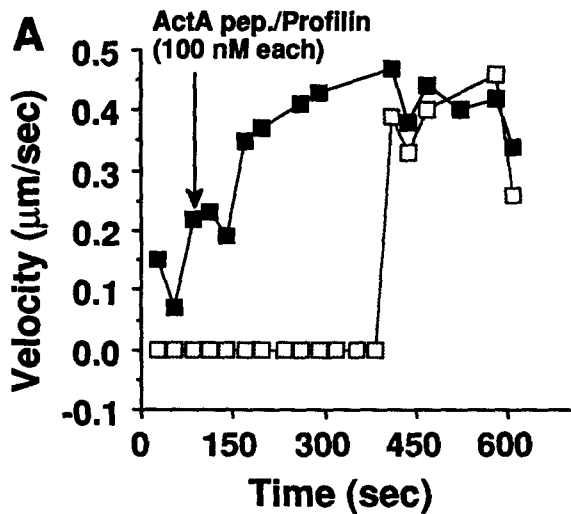| Additions | Intracellular concentration | Preinjection velocity | Postinjection velocity | Postinjection/ preinjection velocity | P value |
|---|---|---|---|---|---|
| | | *mean, μm/s, SD* | | | |
| Act A peptide CFEFPPPPTDE | 80 nM | 0.06 ± 0.03 ($n = 47$) | 0.004 ± 0.01 ($n = 85$) | 0.07 | < 0.001 |
| Profilin | 80 nM | 0.14 ± 0.04 ($n = 16$) | 0.12 ± 0.06 ($n = 21$) | 0.85 | NS* |
| ActA peptide and Profilin | 80 nM/80 nM | 0.09 ± 0.07 ($n = 16$) | 0.30 ± 0.11 ($n = 33$) | 3.33 | < 0.001 |
| | 20 nM/20 nM | 0.13 ± 0.05 ($n = 15$) | 0.17 ± 0.08 ($n = 12$) | 1.31 | NS |
| VASP peptide (GPPPPP)$_3$ | 10 μM | 0.13 ± 0.05 ($n = 40$) | 0.02 ± 0.05 ($n = 65$) | 0.15 | < 0.001 |
| Profilin | 10 μM | 0.07 ± 0.03 ($n = 31$) | 0.02 ± 0.05 ($n = 21$) | 0.28 | < 0.001 |
| VASP peptide and profilin | 10 μM/10 μM | 0.09 ± 0.06 ($n = 25$) | 0.18 ± 0.10 ($n = 61$) | 2.00 | = 0.002 |
| | 1 μM/1 μM | 0.12 ± 0.06 ($n = 6$) | 0.07 ± 0.04 ($n = 16$) | 0.58 | NS |
| Poly-L-proline and profilin | 2.5 μM/10 μM | 0.14 ± 0.08 ($n = 29$) | 0.06 ± 0.11 ($n = 45$) | 0.43 | < 0.001 |
| MAP-2 peptide VKSKIGSTDNIKYZPKGG | 10 μM | 0.15 ± 0.05 ($n = 17$) | 0.15 ± 0.07 ($n = 44$) | 1.00 | NS |

*NS, not significant.

F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation), we predicted that microinjection of a synthetic peptide containing this 18 residue triad would block profilin localization at the bacterial actin interface and prevent bacterial induced actin filament assembly and intracellular movement. Our experiments confirmed this expectation. The intracellular concentrations of peptide required to achieve inhibition of motility were considerably higher than the ActA analogue (10 μM GPPPPPGP-PPPPGPPPPP vs 80 nM FEFPPPPTDE), reflecting the higher concentrations of profilin likely to be present in PtK2 cells as compared to VASP and/or a lower affinity of profilin for the VASP oligoproline sequence. It is of interest that other investigators have recently demonstrated that the same VASP analogue can dissociate profilin from VASP in vitro (Reinhard et al., 1995), providing further biochemical support for our inferences about the mechanism of action of the VASP analogue in *Shigella*-infected cells. We also find that this same VASP analogue inhibits *Listeria* intracellular movement at identical concentrations (Kang, F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation). Therefore both *Shigella* and *Listeria* are likely to use VASP and profilin to induce actin assembly in host cells. While all of our results are consistent with the above interpretation, these synthetic peptides may not be entirely specific for the proposed targets, and impaired bacterial movement could represent a nonspecific side effect. Our other findings argue against such an interpretation. First, introduction of high intracellular concentrations of an unrelated peptide fail to impair motility, excluding a nonspecific toxic effect of synthetic peptides. Second, the ability of equimolar concentrations of profilin to totally reverse the inhibitory effects of the
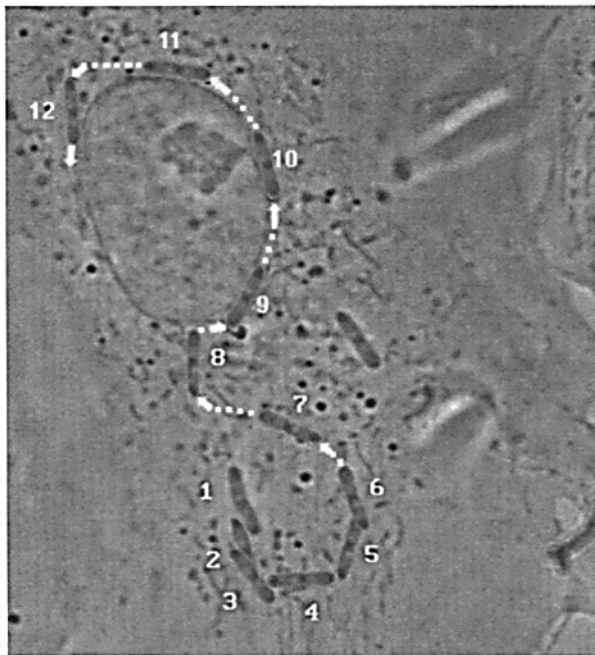
peptides suggests specific protein–protein interactions are responsible for the observed inhibitory effects. Our observations, however, do not exclude the possibility that other host cell actin regulatory proteins in addition to VASP and profilin may play roles in *Listeria* and *Shigella* intracellular motility.

What then can be said about the results of our experiments with binary solutions containing profilin and either of the aforementioned oligoproline sequences? Simultaneous introduction of a profilin and ActA analogue or profilin and the VASP analogue binary solution did more than simply neutralize the inhibitory action. In fact, we were surprised to find that coinjection actually stimulated *Shigella* to move at rates that were two to three times greater than their usual velocities. Introduction of the binary solutions even occasionally caused previously quiescent bacteria to commence moving, and these bacteria often reached maximal velocity. This stimulation of movement was observed following the addition of only 80–100 nM concentrations of profilin and the ActA analogue, the same concentration range where microinjection of ActA analogue alone evoked maximal inhibition of both *Listeria* and *Shigella* movement. Binding experiments monitoring tryptophan fluorescence of profilin fail to detect binding of the ActA analogue to profilin at concentrations of 100 μM (Kang, F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation). Therefore, it is unlikely that these two polypeptides alone form a binary complex before or after microinjection into the cell. They are more likely to form ternary complex with a third host cell protein, possibly VASP, and this complex in turn could stimulate actin assembly. In vitro binding experiments indicate that the VASP analogue and profilin will
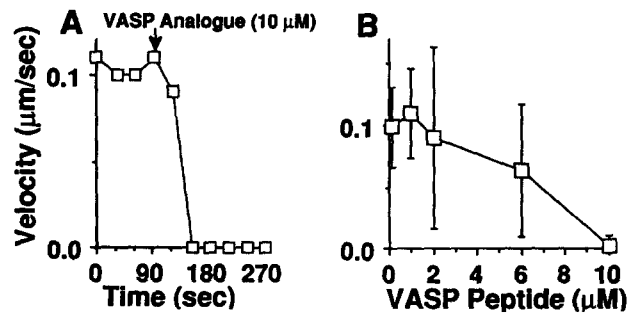
Figure 7. (A) The effect of introducing a 10-μM intracellular concentration of the VASP peptide analogue on *Shigella* motility in a PtK2 cell. Arrow represents the approximate time of the microinjection. (B) Effect of varying intracellular concentrations of the VASP analogue on *Shigella* intracellular velocity. The estimated intracellular concentrations of the microinjected peptide are plotted on the horizontal axis. Bars represent the standard deviation of the mean for $n = 20$–$40$ velocity measurements per concentration.



Figure 6. (A) Velocities of two bacteria in a PtK2 cell before and after the microinjection of an ActA analogue/profilin binary solution (100 nM intracellular concentrations of both reagents, shown in parentheses; needle concentrations, 1 μM). The vertical arrow indicates the approximate time when the mixture was injected. This individual experiment is representative of numerous experiments (see Results section and Table I). (B) Time-lapse phase micrographs of *Shigella* motility in a PtK2 before and after microinjection of the ActA/profilin binary solution. This composite photograph depicts the path and distances covered by a single bacterium before and after microinjection of ActA/profilin in an equal molar ratio (estimated intracellular concentration 100 nM, needle concentration 1 μM). Images show the position of the bacterium at 30 s intervals and are numbered sequentially. The cell as microinjected with the binary solution between images 3 and 4 of the composite. After microinjection, note the progressive increase in the distance traveled by the bacteria after each time interval. The same information is depicted graphically as the upper curve of Fig. 6 A. The moving bacterium is 5 μm in length.

associate at the concentrations used in our experiments ($10^{-5}$ M range, Kang, F., P. Passaro, M. Bubb, D. Purich, and F. Southwick, manuscript in preparation). Therefore, the acceleration of *Shigella* motility by the binary mixtures of VASP and profilin suggests that the profilin-VASP complex can enhance actin assembly in nonmuscle cells. Although further experiments will be required to fully characterize these interactions, the present studies do indicate that under the appropriate conditions profilin can stimulate actin assembly.
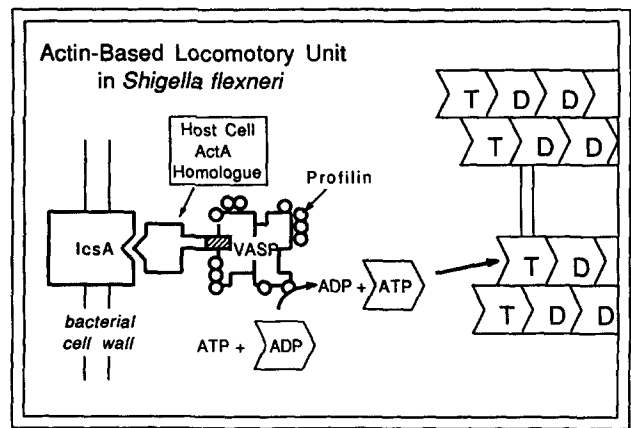
Based on our current findings, a working model of how *Shigella* induces actin assembly in host cells can be constructed (Fig. 9). Because the IcsA surface protein of *Shigella* possesses no ActA oligoproline VASP-binding sequence, IcsA protein probably attracts a host cell VASP-binding protein to the bacterial surface to concentrate VASP which in turn binds profilin. Profilin stimulates actin filament assembly behind the bacterium, and this polymerization process propels the bacterium through the host cell cytoplasm. The mechanism(s) by which profilin stimulates actin assembly in cells remain(s) ill-defined. In the presence of the monomer sequestering protein, thymosin $\beta_4$, profilin can lower the critical concentration of actin filaments (Pantaloni and Carlier, 1993). Profilin also enhances nucleotide exchange on actin monomers (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991). Under the rapid assembly conditions, 40–200 monomers per second, associated with *Shigella* locomotion at rates of 0.1–0.5 μm/s, nucleotide exchange could prove to be the rate limiting step for new actin assembly and profilin could serve to accelerate this process. In the present model we have illustrated ATP - ADP exchange on actin monomers as the most likely explanation for profilin's ability to stimulate host cell actin assembly. While additional biochemical experiments promise a rigorous test of this scheme, a key finding in support of the model is the recent immunofluorescence study demonstrating VASP localization on intracellular *Shigella* (Chakraborty et al., 1995).

The observation that the ActA analogue can block both *Shigella* and *Listeria* actin-based motility suggests that *Shi-*
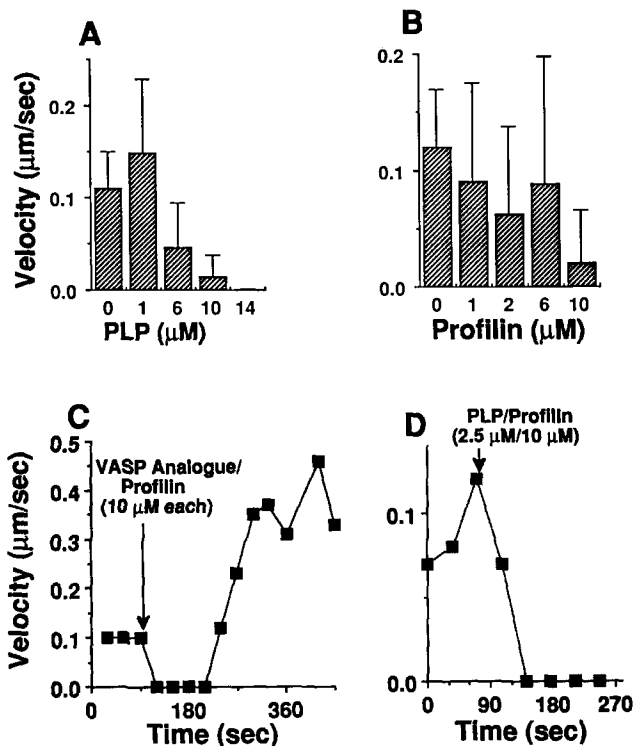
**Figure 8.** Dose dependence of (A) poly-L-proline and (B) profilin inhibition of *Shigella* intracellular motility. The mean velocities of *Shigella* intracellular migration in PtK2 cells are shown following the microinjection of increasing intracellular concentrations of the two polypeptides. Each point represents the mean of 20–40 velocity measurements. Introduction of an estimated intracellular concentration of 6 µM profilin (needle concentration 60 µM) resulted in a bimodal behavior, 40% of the bacteria accelerating their velocity while 60% stopped moving (see Results). (C) The velocities of a bacterium migrating through a PtK2 cell before and after the microinjection of a VASP analogue/profilin binary solution and (D) before and after the microinjection of binary solution of poly-L-proline and profilin. The values in parentheses are the estimated intracellular concentrations of the two reagents. Vertical arrows indicate the time when each solution was injected. These individual experiments are representative of numerous experiments for each condition (see Results and Table I).



**Figure 9.** Working model showing the primary components likely to be involved in the actin-based locomotory unit of *Shigella*. *Shigella* contains on its surface the 120-kD protein IcsA that is likely to attract an ActA-like mammalian protein homologue onto the bacterial surface. This ActA-like protein contains one or more VASP-binding sequences (designated as a hatched region) responsible for attracting VASP to the bacterial surface. Because of its tetrameric structure, VASP is capable of binding up to 16 profilin molecules, serving to highly concentrate profilin at the bacterial-actin tail interface. Profilin may promote actin filament assembly by increasing the rate of ADP-ATP exchange on actin monomers (chevrons) or profilin may usher actin subunits onto the barbed ends of actin filaments. Microinjection of the ActA peptide FEFPPPPTDE is thought to disrupt VASP binding to the ActA homologue on *Shigella* and microinjection of the VASP peptide (GPPPPP)₃ would be expected to dissociate profilin from VASP. Both peptides act at different steps in *Shigella*-induced actin assembly and disperse locomotory elements (VASP and/or profilin) from the bacterial surface, thereby blocking actin rocket tail formation and bacterial motility.

*gella* probably recruits to its surface a host cell protein that contains an ActA-like oligoproline sequence. Kadurugamuwa et al. (1991) recently suggested that vinculin, itself an oligoproline-containing actin-binding protein, might serve in place of ActA in *Shigella* actin-based motility. When *Shigella* infects host cells, vinculin is lost from focal adhesion plaques and could be concentrated on the bacterial surface. Although we clearly observed immunolocalization of vinculin at focal adhesion contacts, we could not demonstrate any accumulation of this protein on the cell wall of intracellular *Shigella* (data not shown). Such observations do not completely exclude vinculin as the candidate ActA-like host protein because the amount of vinculin needed on the bacterial surface may be below our detection limit. Alternatively, yet another oligoproline-containing host cell protein may fulfill the requirement for an oligoproline recognition site. Determining the identity of this protein will be of great interest because this ActA-

homologue is likely to play a key role in the generation of new actin filaments required for the extension of lamellipods and pseudopods in nonmuscle cells.

In conclusion, our finding that the ActA analogue arrests *Shigella* motility indicates that its locomotion requires the presence of an oligoproline-containing protein that binds to the bacterium's surface in a manner mimicking the action of *Listeria* ActA protein. Moreover, we have demonstrated for the first time that microinjection of a mixture of profilin and the ActA sequence FEFPPPPTDE (or the GPPPP triad from VASP) can markedly accelerate actin-based motility in living cells. This represents an unprecedented finding that factors introduced by microinjection can actually stimulate directional intracellular actin assembly. These in vivo experiments emphasize the importance of a discrete pool of profilin that is likely to be responsible for stimulating new actin filament assembly. *Shigella* and *Listeria*, two bacterial pathogens with structurally unrelated membrane surface proteins, have thus managed to subvert the host's contractile system to generate force needed for intracellular movement, an evolutionary achievement that allows these pathogens to spread from cell to cell and cause disease. This same system is likely to play a role in promoting localized actin assembly necessary for dynamic remodeling of the leading edge during chemotaxis and phagocytosis.

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