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A *Rickettsia* WASP-like protein activates the Arp2/3 complex and mediates actin-based motility

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Summary

Spotted fever group *Rickettsia* are obligate intracellular pathogens that exploit the host cell actin cytoskeleton to promote motility and cell-to-cell spread. Although other pathogens such as *Listeria monocytogenes* use an Arp2/3 complex-dependent nucleation mechanism to generate comet tails consisting of Y-branched filament arrays, *Rickettsia* polymerize tails consisting of unbranched filaments by a previously unknown mechanism. We identified genes in several *Rickettsia* species encoding proteins (termed RickA) with similarity to the WASP family of Arp2/3-complex activators. *Rickettsia rickettsii* RickA activated both the nucleation and Y-branching activities of the Arp2/3 complex like other WASP-family proteins, and was sufficient to direct the motility of microscopic beads in cell extracts. Actin tails generated by RickA-coated beads consisted of Y-branched filament networks. These data suggest that *Rickettsia* use an Arp2/3 complex-dependent actin-nucleation mechanism similar to that of other pathogens. We propose that additional *Rickettsia* or host factors reorganize the Y-branched networks into parallel arrays in a manner similar to a recently proposed model of filopodia formation.

Introduction

Rickettsia are obligate intracellular pathogens that cause a range of potentially severe diseases including Rocky Mountain spotted fever and epidemic typhus. Species of the spotted fever group (Teysseire *et al*., 1992; Heinzen *et al*., 1993) are among an expanding group of pathogens, including *Listeria monocytogenes* (Tilney and Portnoy, 1989) and *Shigella flexneri* (Bernardini *et al*., 1989), that enter the host cell cytoplasm and induce actin polymerization at their cell surface. The force from actin assembly propels the bacteria through the cytoplasm and into neighbouring cells, facilitating cell-to-cell spread (Goldberg, 2001).

The molecular mechanisms of actin polymerization by some of these pathogens, particularly *L. monocytogenes* and *S. flexneri*, have been well-studied, and primarily involve activation of the Arp2/3 complex, an actin nucleator that can initiate the polymerization of new actin filaments and organize filaments into Y-branched arrays (Mullins *et al*., 1998; Welch *et al*., 1998; Blanchoin *et al*., 2000). In host cells, the Arp2/3 complex is, in turn, activated by nucleation promoting factors including members of the Wiskott–Aldrich Syndrome protein (WASP) family of proteins (Higgs and Pollard, 2001; Welch and Mullins, 2002). In general, intracellular pathogens have adopted two strategies for activating the Arp2/3 complex: they express surface proteins that recruit host-family WASP proteins (e.g. *S. flexneri* IcsA), or they express functional mimics of WASPs (e.g. *L. monocytogenes* ActA) (Goldberg, 2001).

By contrast, several observations have suggested that the molecular mechanism of *Rickettsia* motility is different from that of these other pathogens. First, the Arp2/3 complex has not been detected in *Rickettsia* tails as it has been for other pathogens (Gouin *et al*., 1999; Harlander *et al*., 2003). Second, whereas *L. monocytogenes* tails consist of short, highly branched filament networks characteristic of those organized by the Arp2/3 complex (Cameron *et al*., 2001), *Rickettsia* tails consist of long, unbranched filament arrays (Gouin *et al*., 1999; Van Kirk *et al*., 2000). Finally, an attempt to inhibit the Arp2/3 complex by expressing dominant negative N-WASP truncations in cultured cells had less of an effect on *Rickettsia* motility than on *S. flexneri* motility (Harlander *et al*., 2003). Although these observations were interpreted to suggest a novel Arp2/3 complex-independent mechanism of motility, the limited experimental techniques available with *Rickettsia* slowed the identification of any molecular components involved in this process.

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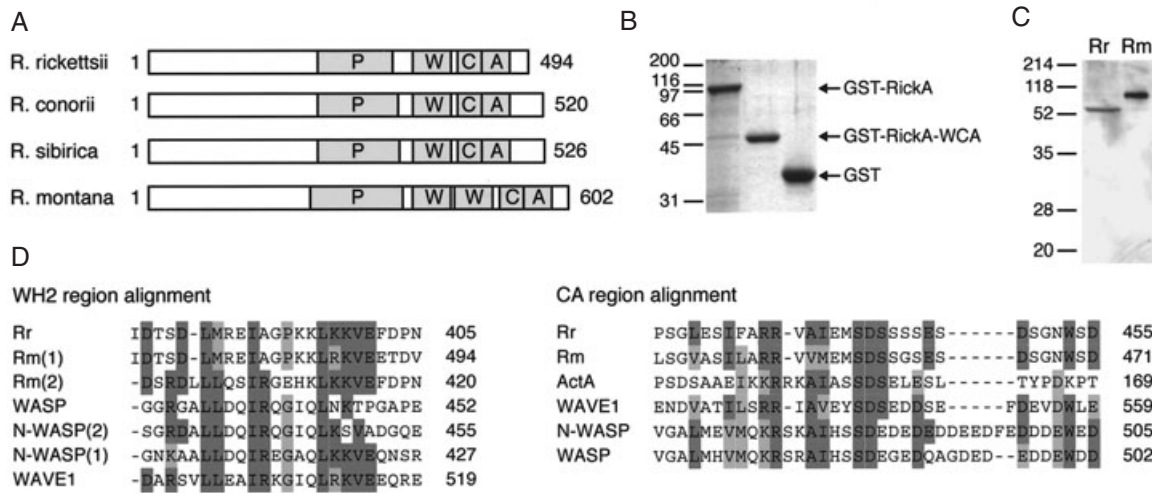

Fig. 1. RickA proteins in spotted fever group *Rickettsia*.
A. Diagram of RickA proteins from different *Rickettsia* species.
B. Coomassie-stained SDS-PAGE gel of the purified GST-RickA proteins used in this study.
C. Western blot of *R. rickettsii* and *R. montana* extracts probed with an antibody raised against the *R. rickettsii* RickA.
D. Alignment of the WH2 and CA regions of RickA and WASP-family proteins generated using CLUSTAL X. Highlighted residues are identical (dark grey) or similar (light grey) among at least three proteins including one rickettsial and one non-rickettsial protein.

Here, we report the identification of a WASP-like protein expressed by *Rickettsia* termed RickA that is likely to be involved in actin-based motility. The identification of RickA proteins was also recently reported in another study (Gouin *et al*., 2004). We find that *R. rickettsii* RickA activates the actin-nucleating and Y-branching activities of the Arp2/3 complex, and is sufficient to direct actin-based motility and the formation of Y-branched actin arrays in *Xenopus laevis* egg extracts. These data suggest that *Rickettsia* use an Arp2/3 complex-dependent actin-nucleation mechanism similar to that of other pathogens and that other factors are involved in reorganizing Arp2/3 complex-generated Y-branched networks into parallel arrays during bacterial motility.

Results

Rickettsia *express a WASP-like protein*

To identify molecular components involved in *Rickettsia* actin-based motility, we searched bacterial genome sequence databases for genes encoding proteins with similarity to the WASP family of Arp2/3 complex activators, which are implicated in actin nucleation by other bacterial pathogens. We identified genes in four spotted fever group *Rickettsia* – one in *R. conorii* that was initially noted by the genome sequencing project (Ogata *et al*., 2001) and others in *R. rickettsii*, *R. sibirica* and *R. montana* – that encode WASP-like proteins, termed RickA (Fig. 1A; *Supplementary material* Fig. S1). The RickA paralogues contain several regions at their carboxyl-termini that are characteristic of WASP proteins, including a proline-rich (P) domain as well as WASP-homology 2 (WH2), central (C), and acidic (A) domains (Fig. 1A and D), collectively called the WCA region. In general, RickA proteins have a single WH2 domain, similar to vertebrate WASP, although the *R. montana* RickA has two WH2 domains, similar to vertebrate N-WASP. Unlike the carboxyl-terminal region, the amino-terminal sequence of RickA does not contain significant similarity to any other proteins and does not contain predicted signal sequences or transmembrane domains that would indicate the mode of secretion and localization of RickA.

Genes encoding RickA proteins are present only in spotted fever group *Rickettsia* species, which exhibit actin-based motility, and RickA is encoded by a single gene (RC0909) in the sequenced genome of *R. conorii* (Ogata *et al*., 2001). In contrast, a gene encoding RickA is absent from the sequenced genome of *R. prowazekii* (Andersson *et al*., 1998; Gouin *et al*., 2004), which is a member of the typhus group and does not exhibit actin-based motility. Thus RickA proteins appear to be limited to species that exploit the host actin cytoskeleton during infection. Surprisingly, the genome of *R. typhi*, a *Rickettsia* species in the typhus group that exhibits erratic actin-based motility (Teysseire *et al*., 1992; Van Kirk *et al*., 2000; Heinzen, 2003), also does not appear to contain a gene encoding RickA (Walker and Weinstock, 2003), suggesting *Rickettsia* may possess multiple mechanisms for initiating actin polymerization.

To evaluate the expression and biochemical function of RickA, we isolated its gene (ORFB4) (Andersson and Andersson, 2001) by PCR from *R. rickettsii,* the causative

agent of Rocky Mountain spotted fever, and used it to express the RickA protein in *E. coli* as a fusion protein with glutathione-S-transferase (GST-RickA) (Fig. 1B). We raised a rabbit polyclonal antibody against GST-RickA that recognized a single band of approximately 60 and 75 kDa in immunoblots of *R. rickettsii* and *R. montana* whole cell extracts respectively (Fig. 1C). This is similar to the predicted molecular masses of the full-length proteins (56 kDa and 67 kDa, respectively), indicating that the bacteria express RickA. We also generated a truncation of RickA consisting only of the WCA region fused to GST (GST-RickA-WCA) because in other WASP-family proteins the WCA region is the minimal region that is sufficient to activate the Arp2/3 complex.

RickA activates the Arp2/3 complex

All WASP family proteins examined thus far stimulate two activities of the Arp2/3 complex, nucleation of actin filaments and organization of filaments into Y-branched arrays (Welch and Mullins, 2002), so we tested the ability of RickA to stimulate these activities. Both GST-RickA and GST-RickA-WCA activated the nucleation activity of the Arp2/3 complex in a concentration-dependent manner (Fig. 2). Concentrations of GST-RickA or GST-RickA-WCA exhibiting saturating levels of activity (100 nM GST-RickA or 20 nM GST-RickA-WCA) accelerated the polymerization rate by nine- and eightfold, respectively, relative to the Arp2/3 complex or actin alone at half-maximal polymerization. This was virtually identical to the 10-fold acceleration caused by saturating concentrations (100 nM) of the *L. monocytogenes* ActA protein, a potent Arp2/3-complex activator (Welch *et al.*, 1998). Full-length GST-RickA alone also had a minor effect on the kinetics of polymerization, although it did not appear to stimulate nucleation because the polymerization rate was virtually identical to actin alone at half-maximal polymerization. These observations indicate that RickA is a potent activator of the Arp2/3 complex, and that its activity is mediated by the WCA region.

The observations that actin filaments in *Rickettsia* tails are unbranched (Gouin *et al.*, 1999; Van Kirk *et al.*, 2000) suggested that RickA might behave differently than other WASP family proteins in its ability to activate the Y-branching activity of the Arp2/3 complex. However, both GST-RickA and GST-RickA-WCA were able to stimulate the formation of actin Y-branches at a comparable frequency to the GST human WASP-WCA control (Fig. 3). In all cases, branches were stable over a 10 min timecourse (data not shown), suggesting that RickA does not inhibit branch stability. Although the full-length RickA was slightly less active than the carboxyl-terminal RickA-WCA fragment, this effect was likely the result of differences in optimum assay conditions for the two proteins rather than a specific influence of the amino-terminal region of RickA on branching activity because the amino-terminal domain had no effect on the branching activity of GST-RickA-WCA when added in *trans*, and furthermore had no effect on the branching activity of the human WASP-WCA region *in cis* when joined to it in a fusion protein (data not shown).

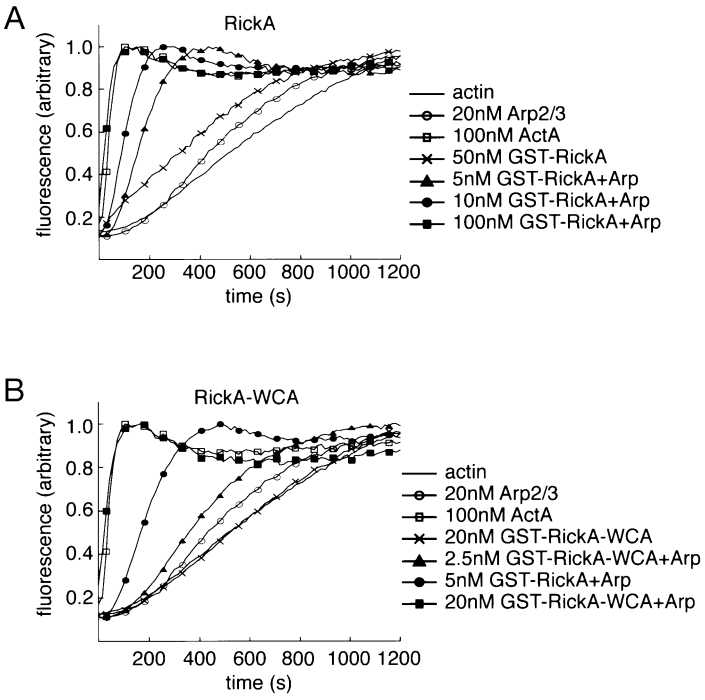

Fig. 2. Effect of RickA on the nucleation activity of the Arp2/3 complex. Nucleation activity was assessed by monitoring polymerization kinetics using the pyrene actin polymerization assay with 1 μM actin (10% pyrene-labelled). Graphs plot fluorescence versus time in the presence of the indicated concentrations of the Arp2/3 complex and (A), GST-RickA or (B), GST-RickA-WCA. *L. monocytogenes* ActA was also tested as a standard reference.

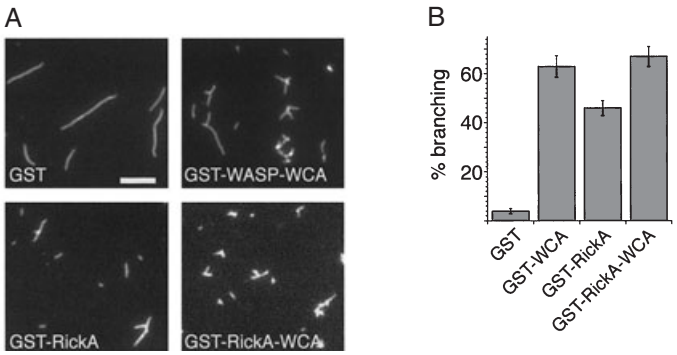

Fig. 3. Effect of RickA on the branching activity of the Arp2/3 complex.
A. Actin filament branching activity was assessed by visualizing filaments stained with rhodamine-phalloidin using fluorescence microscopy after initiating polymerization of 1 μM actin in the presence of 20 nM Arp2/3 complex and 200 nM GST, GST-WASP-WCA, GST-RickA, or GST-RickA-WCA. Scale bar = 5 μm.
B. Graph plots percentage of branched structures formed under the conditions described in (A). For each condition 100–300 individual structures were counted in three separate experiments. Error bars indicate the standard deviation.

These data indicate that RickA behaves like other WASP-family proteins in its ability to activate both the nucleation and branching activities of the Arp2/3 complex, and are consistent with earlier findings that the nucleation and branching activities of the Arp2/3 complex are tightly coupled (Blanchoin *et al*., 2000).

RickA-coated beads move in cell-free extracts

The biochemical activities of RickA suggest that it represents the factor that nucleates actin polymerization at the *Rickettsia* surface to initiate actin-based motility in infected cells. *Rickettsia* lack genetic tools to probe the function of RickA in bacteria, and we have been unable to observe bacterial motility in cell-free extracts. Therefore, to test whether RickA is sufficient to direct actin-based motility, we used an assay in which bacteria are replaced by microscopic beads that are coated with RickA and introduced into cell-free extracts made from *Xenopus laevis* eggs (Cameron *et al*., 1999; Yarar *et al*., 1999). Beads coated with GST-RickA were able to polymerize actin, generate actin comet tails and move at a mean rate of 3.2 μm min^{-1} (SD = 1.5 μm min^{-1}, $n = 62$, eight separate experiments) (Fig. 4A, *Supplementary material,* Video S2), approximately the same as the rate exhibited by *Rickettsia* in infected cells (Gouin *et al*., 1999; Heinzen *et al*., 1999),

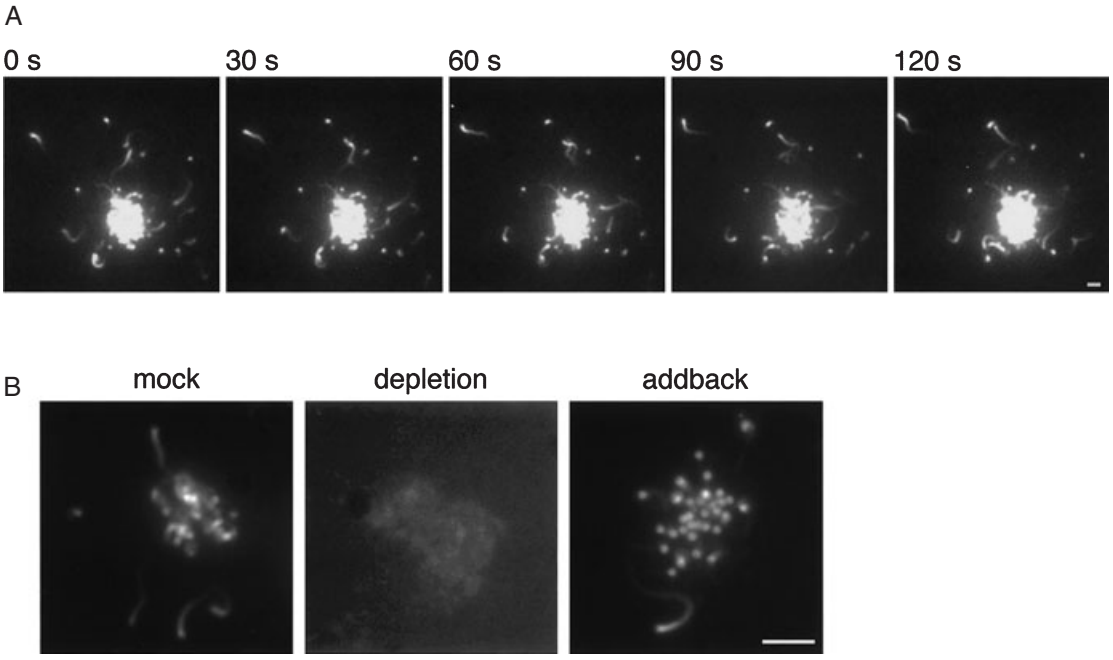

Fig. 4. Behaviour of RickA-coated beads in *Xenopus* egg extract.
A. Time-lapse image sequence of actin structures formed by GST-RickA-coated beads in *Xenopus* egg extracts supplemented with rhodamine-labelled actin and visualized by fluorescence microscopy. A video of the moving beads is included in Supplementary movie 1.
B. Actin structures formed by GST-RickA coated beads in mock depleted extract, Arp2/3 depleted extract, and Arp2/3 depleted extract supplemented with 0.8 μM purified Arp2/3 complex. Scale bars = 5 μm.

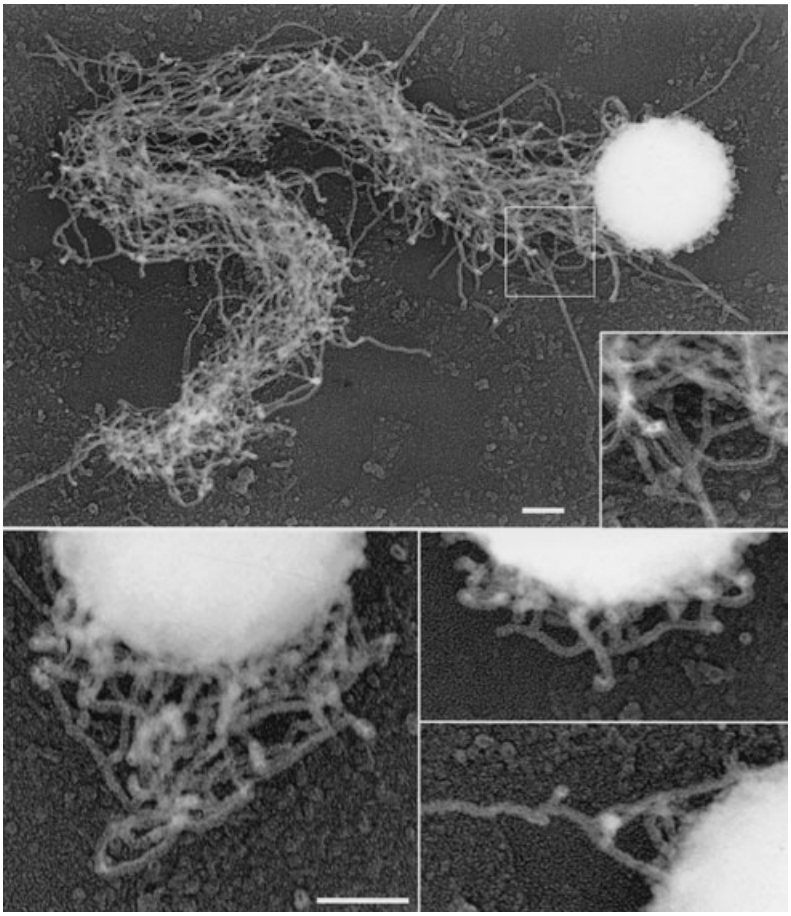

Fig. 5. Platinum-replica electron microscopy images of actin filaments in structures formed by RickA-coated beads in *Xenopus* egg extracts. Inset in upper panel is a magnified view of the indicated area. Scale bars = 100 nm.

and similar to motility rates that have been reported for beads coated with human WASP family proteins (Yarar *et al*., 1999) and *L. monocytogenes* ActA (Cameron *et al*., 1999) in *Xenopus* egg extracts.

To assess whether RickA-mediated bead motility is dependent on the Arp2/3 complex, *Xenopus* egg extracts were depleted of Arp2/3 complex (to approximately 50% of endogenous levels) using sepharose beads coated with the Arp2/3-binding CA fragment of human WAVE1/Scar1 (GST-Scar-CA), or mock depleted using control GST sepharose beads. GST-RickA-coated beads generated tails and moved in control depleted extracts (2.6 ± 0.8 μm min^{-1}, $n = 7$), but no actin polymerization or motility was observed in extracts depleted of Arp2/3 complex (Fig. 4B). Actin tail formation and motility (1.3 ± 0.4 μm min^{-1}, $n = 21$) was restored after the addition of recombinant Arp2/3 complex to the depleted extract. Together, these results demonstrate that RickA is sufficient to direct motility in cell cytosol, and that motility requires the Arp2/3 complex.

To determine whether RickA-coated beads generate tails with the unbranched filament architecture characteristic of tails formed by *Rickettsia* in infected cells, or with a branched architecture, we visualized actin filaments in comet tails generated by GST-RickA-coated beads in *Xenopus* egg extracts using platinum-replica electron microscopy (Svitkina and Borisy, 1998) (Fig. 5). Tails contained a dense meshwork of actin filaments throughout their lengths, generally similar to the networks generated by *L. monocytogenes* and ActA-coated beads in *Xenopus* egg extracts (Cameron *et al*., 2001). In regions of long tails with sparse filament density, or in areas of short tails with well-spaced filaments, Y-branches were clearly visible. Branches exhibited the 70° angle characteristic of assemblies generated by the Arp2/3 complex *in vitro* (Mullins *et al*., 1998; Blanchoin *et al*., 2000) and in the lamellipodia of tissue culture cells (Svitkina and Borisy, 1999). These results indicate that RickA can generate Y-branched actin arrays in cell cytoplasm, and suggest that other *Rickettsia* or host factors contribute to organizing filaments into the unbranched arrays assembled by *Rickettsia* in infected cells.

Discussion

There has been great interest in understanding the molecular mechanism of *Rickettsia* actin-based motility, in part because these bacteria generate actin comet tails that

consist of unbranched actin filaments arrays in contrast with the branched actin filament networks found in the comet tails made by other pathogens. However, the molecular mechanism underlying *Rickettsia* motility has been difficult to dissect because of the lack of available experimental tools. Together with another recent study (Gouin *et al*., 2004), we report the identification of RickA, a protein expressed by *Rickettsia* that is similar to members of the WASP family of Arp2/3 complex activators, providing the first molecular insight into the mechanism of *Rickettsia* actin-based motility. Consistent with a role in motility, the gene encoding RickA is present in spotted fever group *Rickettsia* species, which exhibit actin-based motility, but is absent from the typhus group *Rickettsia* species, which do not exhibit actin-based motility. Surprisingly, although a gene encoding RickA is not present in the genome of *R. typhi*, a typhus group member, this species exhibits erratic actin-based motility. This suggests that *Rickettsia* use multiple redundant mechanisms for polymerizing actin, and that additional molecular players involved in actin polymerization remain to be identified.

The sequence of RickA exhibits similarity with WASP-family proteins in two domains, the proline-rich and WCA regions, both of which are key to the function of these proteins. In other WASP-family members, the WCA region binds to and activates the Arp2/3 complex (Higgs and Pollard, 2001), and we have shown that the RickA WCA region is similarly sufficient to activate Arp2/3 complex in nucleation and branching assays. Interestingly, the organization and primary sequence of the RickA WCA region is more similar to that of eukaryotic WASP proteins than is the corresponding region of ActA (Skoble *et al*., 2000; Zalevsky *et al*., 2001), the *L. monocytogenes* protein that is functionally similar to RickA. This suggests that *Rickettsia* may have acquired the gene encoding a WASP-like protein by gene transfer from the eukaryotic host more recently than *L. monocytogenes*, or that *Rickettsia* and *L. monocytogenes* WASP-like proteins have different evolutionary origins. Unlike the WCA region, the function of the proline-rich (P) region of RickA has not yet been addressed, although by analogy with other WASP family members, it may interact with proteins such as profilin (Miki *et al*., 1998; Suetsugu *et al*., 1998) and VASP (Castellano *et al*., 2001) that promote actin polymerization and actin-based motility (Theriot *et al*., 1994; Marchand *et al*., 1995; Egile *et al*., 1999; Loisel *et al*., 1999; Mimuro *et al*., 2000; Castellano *et al*., 2001; Geese *et al*., 2002; Yarar *et al*., 2002). The possibility that RickA binds to VASP is especially intriguing because VASP reduces the frequency of branching by the Arp2/3 complex (Skoble *et al*., 2001; Bear *et al*., 2002; Samarin *et al*., 2003), although the presence of VASP is apparently insufficient to alter the predominant Y-branched architecture of actin filaments in cellular lamellipodia and *L. monocytogenes* comet tails. In contrast with the carboxy-terminal P and WCA regions of RickA, the amino terminus differs significantly from other WASP family proteins and lacks recognizable motifs including predicted signal sequences or transmembrane domains that would indicate the mechanism of RickA secretion and mode of tethering to the bacterial surface.

We have demonstrated that the biochemical activity of RickA is very similar to that of other WASP proteins – it stimulates the actin-nucleating and Y-branching activities of the Arp2/3 complex, directs actin-based motility of microscopic beads, and generates comet tails consisting of Y-branched actin arrays in *Xenopus* egg extracts. Moreover, RickA was recently found to be localized to the surface of *Rickettsia*, where actin polymerization takes place (Gouin *et al*., 2004). These observations suggest that RickA is a key factor that nucleates actin polymerization with the Arp2/3 complex to promote *Rickettsia* motility during infection and that *Rickettsia* have evolved a mechanism for exploiting the host actin-nucleating machinery that is fundamentally similar at the molecular level to that of other bacterial pathogens like *L. monocytogenes* and *S. flexneri* (Goldberg, 2001).

The proposal that *Rickettsia* employ the Arp2/3 complex to nucleate actin assembly like other pathogens raises the question, why are the actin filaments in *Rickettsia* tails organized into unbranched arrays that differ from those formed by the Arp2/3 complex? One possibility is that RickA activates the Arp2/3 complex in a fundamentally different manner than other activators, as was suggested by Gouin *et al*. (2004), who observed that RickA was a relatively weak Arp2/3 complex activator. However, that model is inconsistent with our data which indicates that RickA is as robust as other WASP-family proteins in activating the Arp2/3 complex, and that it can direct the formation of Y-branched actin networks. Instead, we propose another model that is consistent with our observations as well as previous reports. In this model, the initiation of actin polymerization at the bacterial surface is dominated by the activity of RickA and the Arp2/3 complex, generating a transient Y-branched array of actin filaments. During the transition to sustained motility, elongation of the barbed ends of existing filaments in the network may then replace Arp2/3 complex-mediated nucleation as the predominant polymerization mechanism, resulting in the conversion of the branched network into an unbranched array. The transition from nucleation- to elongation-dominant polymerization may be directed by *Rickettsia* proteins other than RickA and/or host factors that are not present in *Xenopus* egg extracts, thus explaining the difference in organization between comet tails generated by RickA-coated beads *in vitro* and by *Rickettsia* in infected cells. This model of a transient activation of Arp2/3 complex may also explain conflicting results from previous studies. For example, in some reports the Arp2/3 complex was not

observed associated with bacteria in infected cells (Gouin *et al*., 1999; Harlander *et al*., 2003), whereas in another it was found to be localized to the bacterial surface (Gouin *et al*., 2004). In addition, attempts to inhibit the Arp2/3 complex have had varying effects on *Rickettsia* motility (Harlander *et al*., 2003; Gouin *et al*., 2004). Different levels of Arp2/3 complex activity at the bacterial surface at different times during motility may explain these disparate observations.

Our model is also appealing because it is similar to the recently proposed convergent-elongation model of filopodia formation (Svitkina *et al*., 2003; Vignjevic *et al*., 2003) which postulates that the parallel bundles that make up filopodia cores arise from the Y-branched network in lamellipodia by a similar conversion from nucleation- to elongation-dominant polymerization. The proteins involved in reorganizing filament arrays in *Rickettsia* tails and in filopodia may be similar, suggesting that *Rickettsia* motility may represent a powerful model for dissecting filopodia formation.

Experimental procedures

Gene isolation and bacterial strains

DNA encoding RickA was amplified by PCR from *R. rickettsii* Norgaard strain genomic DNA with primers oRJ1 (GCG GAA TTC GTT AAA GAA ATA GAT ATA)/oRJ2 (GCG GTC GAC TTA TCT AAC AAA TGA TGG) and cloned into the *Eco*RI-*Sal*I sites of pGAT2 (Amersham Pharmacia Biotech) to generate the plasmid pRJ4 encoding GST-RickA. The RickA-WCA truncation composed of amino acids 356–494 of RickA was amplified by PCR of plasmid pRJ4 with the primers oRJ17 (GCG GAA TTC ATG GCT CCG GTT AAG ACA)/oRJ2 and cloned into the *Eco*RI-*Sal*I site of pGAT2 to create plasmid pRJ16. Total cell extracts for immunoblots were prepared from *R. rickettsii* strain HLP and *R. montana* strain M/5–6.

Protein expression and purification

Recombinant GST-RickA and GST-RickA-WCA were expressed in *E. coli* BL21-CodonPlus-RP cells (Stratagene). Proteins were purified by glutathione-sepharose (Amersham Pharmacia Biotech), followed by cation exchange (Mono S, Amersham Biosciences) and gel filtration chromatography (Superdex 200, Amersham Biosciences). All protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as a standard.

Arp2/3 complex used in the pyrene-actin polymerization assays was purified from human platelets as previously described (Welch and Mitchison, 1998). Recombinant Arp2/3 complex used in the depletion/addback experiment was purified from insect cells using a baculovirus expression system (Gournier *et al*., 2001) to express Arp2/3 complex with p41 (ARPC1b) tagged with His_6 and Flag epitope tags. The recombinant Arp2/3 complex was isolated on Ni-NTA resin (Qiagen) followed by cation-exchange chromatography (HiTrap SP, Amersham Pharmacia Biotech) and gel filtration (Superdex 200, Amersham Pharmacia Biotech) as described previously (Gournier *et al*., 2001). His-tagged ActA was purified as described previously (Welch *et al*., 1998; Skoble *et al*., 2000).

Pyrene-actin polymerization assay

Preparation of rabbit skeletal muscle actin and pyrene-labelled actin, and pyrene-actin polymerization assays were performed as described previously (Yarar *et al*., 2002).

Actin branching assay

Actin filament branching was assayed as previously described (Blanchoin *et al*., 2000; Gournier *et al*., 2001) except that polymerization was initiated under the same conditions used in the pyrene-actin polymerization assay. Each trial consisted of 200 nM of each protein and 20 nM recombinant Arp2/3 complex, and polymerization was allowed to proceed for 6 min before the addition of rhodamine-labelled phalloidin. For each set of conditions, all filaments longer than a standard reference length (above which branches could be easily distinguished) were counted in 6–10 fields (100–300 total filaments) in at least three separate experiments.

Bead motility assays

Beads (0.5 μm) (Polysciences) were coated with protein, and motility assays were performed as previously described (Yarar *et al*., 2002). To determine rates of movement, fluorescence images were recorded every 10 s and rates of movement were determined by averaging the distance moved in a 60 s time interval using Metamorph software (Universal Imaging).

Arp2/3 complex depletion/addback

To deplete Arp2/3 complex, 100 μl of *Xenopus* egg extract was incubated with 30 μl glutathione-sepharose (Amersham Pharmacia Biotech) bound with either GST-Scar-CA or GST for 1 h at 4°C. Beads were then pelleted in a microfuge for 5 min and the supernatant was used directly in motility assays. Approximately 50% of the Arp2/3 complex was depleted following a single round of depletion.

Electron microscopy

Actin tails associated with protein-coated beads were generated in *Xenopus* egg extract as described above except that 4 μl samples were squashed in a glass slide chamber using Teflon tape as spacers as previously described (Cameron *et al*., 2001) and incubated for 30 min. Samples were prepared for platinum-replica electron microscopy as described previously (Svitkina and Borisy, 1998). Images were assembled using Adobe Photoshop software (Adobe Systems).

Acknowledgements

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/cmi/cmi402/cmi402sm.htm

Fig. S1. Amino acid sequence alignment of RickA from *R. rickettsii* (Rr), *R. sibirica* (Rs), *R. conorii* (Rc), and *R. montana* (Rm). The proline-rich regions are highlighted in blue, WH2 regions in green, and CA regions in yellow. The GenBank/EMBL accession numbers for the genes encoding RickA proteins are AJ293314 (Rr), ZP00142939 (Rs), NP360546 (Rc), and AJ293315 (Rm).

Fig. S2. Movie of actin structures assembled by RickA-coated beads in *Xenopus* egg extract that was supplemented with rhodamine-labelled actin and visualized by fluorescence microscopy. 100 ′ actual speed.

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