

SOL-1::GFP. To clear excess antibody from the pseudocoelomic space, we allowed injected worms to recover for 6 h on NGM plates seeded with the *E. coli* strain OP50. All labelling experiments were repeated at least four times. Epifluorescence and GFP images were acquired using a LSM 510 confocal imaging system (Zeiss).

#### Tissue culture and immunoprecipitation

COS-7 cells were cultured in DMEM nutrient medium with 10% bovine fetal serum. The cells were transiently transfected by using Lipofectamine2000 (Invitrogen), collected after 36 h, and lysed in ice-cold IP buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail (Roche)). The lysates were incubated with agarose-conjugated antibodies (Santa Cruz Biotech) in IP buffer overnight at 4°C. After three washes in ice-cold IP buffer, the samples were boiled in SDS-PAGE sample buffer for 2 min, and the precipitated proteins were resolved on a 7% SDS-PAGE gel, probed with peroxidase-conjugated antibodies (Jackson ImmunoResearch) and analysed with the Supersignal Chemiluminescence kit (Pierce).

#### Behavioural and electrophysiological studies

Drug delivery was done by pressure application using a Picospritzer II (General Valve Corporation). We made electrophysiological recordings of ligand-gated currents in the AVA interneurons using standard patch-clamp technology as described<sup>16</sup>. All electrophysiological experiments were repeated 3–8 times. Behavioural assays for the nose touch response, osmotic avoidance and the mean duration of forward and backward movement have been described<sup>12</sup>. Statistical significance was determined using the standard Student's *t*-test. Error bars represent the s.e.m.

Received 23 September; accepted 20 November 2003; doi:10.1038/nature02244.

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Supplementary Information accompanies the paper on [www.nature.com/nature](http://www.nature.com/nature).

**Acknowledgements** We thank M. Vetter and members of the Maricq laboratory for comments on the manuscript; L. Jack for generating transgenic strains; C. Walker, N. Strutz, M. Francis and A. Ebens for discussions; C. Rongo and J. Kaplan for the *nuls25* strain; and A. Gottschalk and W. Schafer for help with immunolabelling live worms. Some strains were provided by the *Caenorhabditis* Genetics Center. This research was supported by the Burroughs Wellcome Foundation, and by a grant from the NIH.

**Competing interests statement** The authors declare that they have no competing financial interests.

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## The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex

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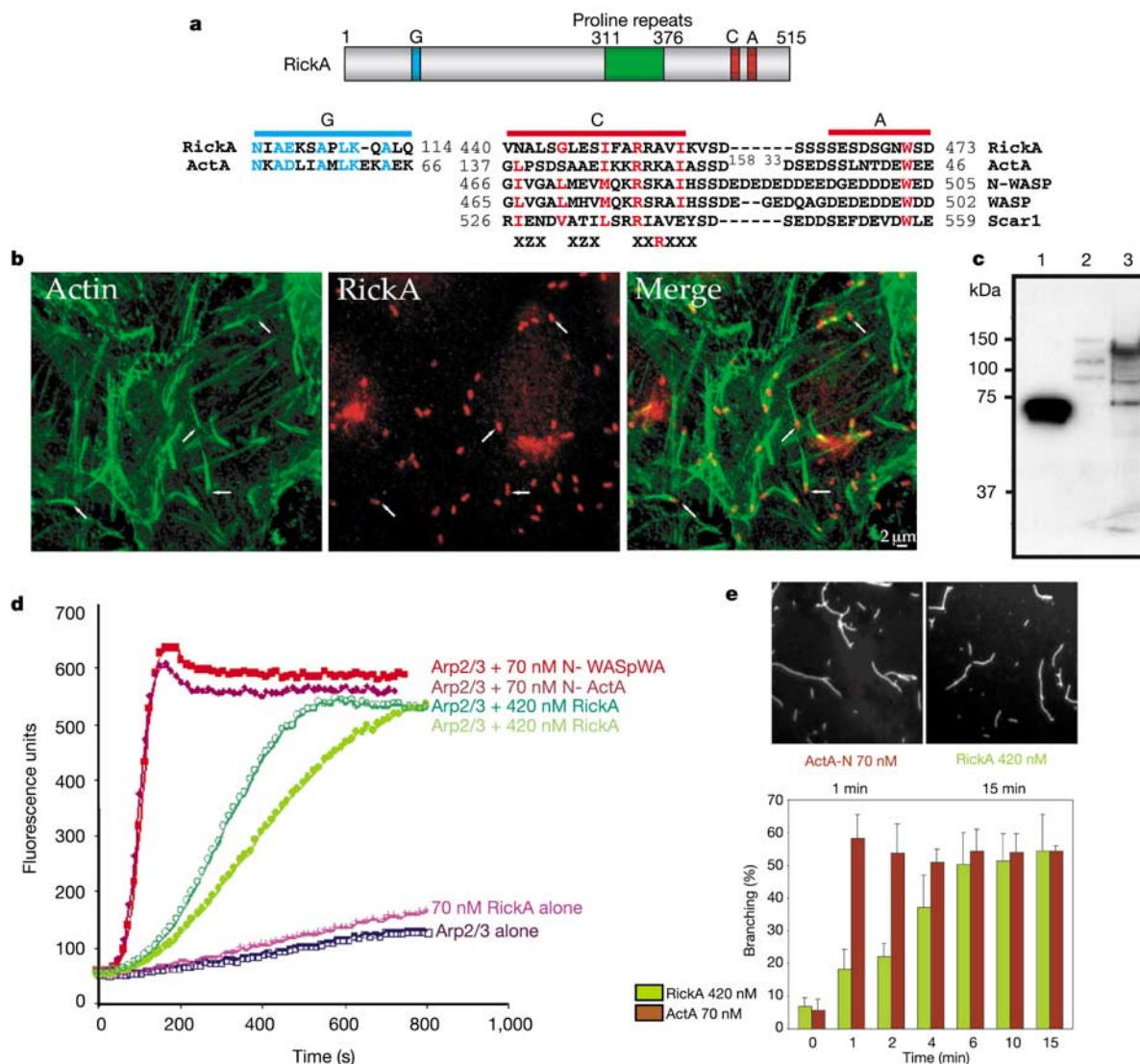
Actin polymerization, the main driving force for cell locomotion, is also used by the bacteria *Listeria* and *Shigella* and vaccinia virus for intracellular and intercellular movements<sup>1,2</sup>. Seminal studies have shown the key function of the Arp2/3 complex in nucleating actin and generating a branched array of actin filaments during membrane extension and pathogen movement<sup>3</sup>. Arp2/3 requires activation by proteins such as the WASP-family proteins or ActA of *Listeria*. We previously reported that actin tails of *Rickettsia conorii*, another intracellular bacterium, unlike those of *Listeria*, *Shigella* or vaccinia, are made of long unbranched actin filaments apparently devoid of Arp2/3 (ref. 4). Here we identify a *R. conorii* surface protein, RickA, that activates Arp2/3 *in vitro*, although less efficiently than ActA. In infected cells, Arp2/3 is detected on the rickettsial surface but not in actin tails. When expressed in mammalian cells and targeted to the membrane, RickA induces filopodia. Thus RickA-induced actin polymerization, by generating long actin filaments reminiscent of those present in filopodia, has potential as a tool for studying filopodia formation.

Converging studies on actin-based motility have led to the establishment of the essential role of Arp2/3 in this process<sup>1–3</sup>. Arp2/3 activates the nucleation of actin polymerization and induces the continuous formation of a network of short and highly branched filaments in lamellipodia and pathogen actin tails<sup>1–3</sup>. Arp2/3 is normally inactive and has to be activated by proteins of the WASP/N-WASP/Scar/Wave-family proteins in eukaryotic cells or the ActA protein at the surface of *L. monocytogenes*<sup>1,3–8</sup>. The *Shigella* protein VirG/IcsA and the vaccinia viral protein A36R are not directly involved in the activation of nucleation<sup>2</sup>. They recruit N-WASP and then Arp2/3, respectively directly and indirectly. Other known activators of the Arp2/3 complex include yeast

Beel/Las 17 (ref. 9) and cortactin<sup>10,11</sup>. In spite of major advances in our understanding of lamellipodia formation and the movements of cells and pathogens, it is still unknown how long and unbranched actin filaments are generated during filopodia formation<sup>12</sup>. Here we address how another bacterial pathogen *Rickettsia conorii* polymerizes actin. An earlier study had revealed that *Rickettsia* actin tails are different from *Listeria* or *Shigella* tails; that is, they are much less dense and are made of long unbranched filaments, reminiscent of those present in filopodia<sup>4</sup>. We report that *R. conorii* expresses on its surface a previously unknown type of Arp2/3 activator that might prove useful in studying filopodia formation.

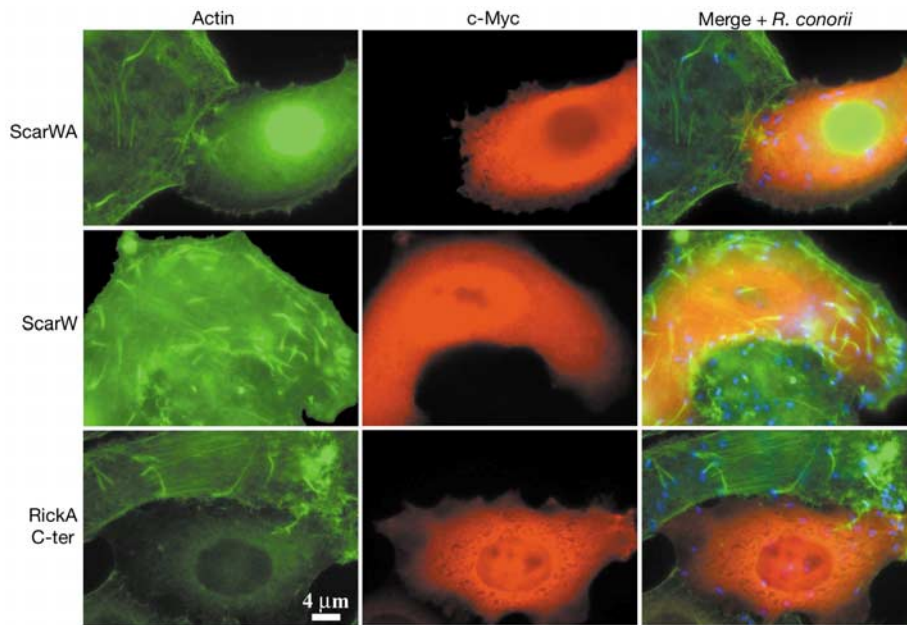
Members of the genus *Rickettsia* are obligate intracellular bacteria that grow in the cytoplasm of their eukaryotic host<sup>13</sup>. They are responsible for several serious human diseases including epidemic typhus (*Rickettsia prowazekii*) and spotted fevers (*Rickettsia conorii*,

*Rickettsia montana* and *Rickettsia rickettsii*) and are transmitted by arthropod vectors such as ticks and fleas. Bacteria of the spotted fever group, in contrast to most of the typhus group, have the capacity to move intracellularly and intercellularly, as do *Listeria* and *Shigella*<sup>4,14</sup>. However, in contrast to these organisms, *Rickettsia* are not genetically manipulatable, and genetic approaches such as those used to discover the genes responsible for the actin-based motility in *Listeria* and *Shigella* are not possible. The genome sequences of *R. prowazekii* and *R. conorii* were determined recently<sup>15,16</sup>. Through genome comparison, we identified a 2-kilobase region that is present in the *R. conorii* genome and is absent from that of *R. prowazekii*. It encodes a 517-amino-acid protein, RickA, that can be arbitrarily divided into three parts: a central proline-rich region (amino acids 311–376) consisting of repeats that are different from the ActA repeats (D/E)FPPPPX(D/E)(D/E)



**Figure 1** The RickA protein of *R. conorii* activates Arp2/3. **a**, Schematic representation of RickA and alignment of the G, C and A regions of RickA, ActA, N-WASP, WASP and Scar1. **b**, Vero cells infected with *R. conorii* and labelled with anti-β-actin (clone AC-15; Sigma) and anti-RickA antibodies (R80). **c**, Western blot analysis of RickA using R80: lane 1, purified RickA; lane 2, extracts from non-infected cells; lane 3, *R. conorii* extracts. **d**, Pyrenyl-actin polymerization assays: Arp2/3 (30 nM alone, blue), RickA (70 nM alone,

pink) or Arp2/3 (30 nM) in the presence of RickA (70 and 420 nM, green), N-WASP-WA (70 nM, purple) or N-ActA (70 nM, red). **e**, Branching assays were performed as described<sup>19</sup> with 60 nM Arp2/3 and 2 μM actin, plus 70 nM N-ActA or 420 nM RickA. Samples were taken at various intervals before the addition of 2 μM rhodamine-phalloidin. Maximal branching was obtained at 60 s for ActA and at 900 s for RickA.



**Figure 2** Transfection with ScarWA or the C-terminal part of RickA inhibits actin polymerization. Hep-2 cells were infected with *R. conorii* 24 h before transfection with the plasmids encoding ScarWA, ScarW or RickA-C-ter (the fragment containing residues 377–517 from RickA was cloned in pRK5Myc (ref. 28), resulting in plasmid pRC19 (strain

BUG1998)). The cells were processed for immunofluorescence 24 h after transfection with fluorescein isothiocyanate-conjugated phalloidin for F-actin, an anti-c-Myc monoclonal antibody for transfected cells and the polyclonal R47 antibody<sup>4</sup> for *R. conorii*.

known to allow binding of the Ena/VASP proteins, surrounded by an amino-terminal domain with a potential G-actin-binding site, similar to that of thymosin  $\beta$ 4 (ref. 17), and a carboxy-terminal region that shares similarities with the CA domains of WASP-family proteins (Fig. 1a and Supplementary Fig. 1), including the conserved hydrophobic domain and the positively charged region that would form the predicted amphipathic helix able to bind the Arp2/3 complex<sup>18</sup>. The amino-acid sequence of RickA does not display any signal sequence or a C-terminal motif that could act as a membrane anchor. Interestingly, the RickA paralogues in *R. montana*, *R. rickettsii* and *R. siberica* have the same general organization as RickA but with some variation in the number and the amino acid sequences of the proline-rich repeats (data not shown).

To study the function of the protein, we produced, although with very low yields, a recombinant protein in *Escherichia coli* and also raised antibodies against a peptide present in the N-terminal region of RickA. As shown by immunofluorescence (Fig. 1b), the RickA protein is highly expressed on the surface of all *Rickettsiae*, in infected cells. In addition, western blot analysis showed that the RickA protein, present in *Rickettsia* extracts and absent from extracts of non-infected cells, migrated as both a protein of 70 kDa (as does the recombinant protein) and a protein of 140 kDa, indicating that RickA can dimerize (Fig. 1c).

Actin polymerization assays *in vitro* with the N-terminal part of ActA (N-ActA) as a control showed that RickA alone was unable to stimulate actin polymerization but was able to activate the Arp2/3 complex, in a dose-dependent manner and as efficiently as ActA (Fig. 1d). However, in contrast to ActA, RickA did not reduce the lag period preceding the exponential phase of actin nucleation.

To investigate further the unexpected role of RickA in Arp2/3 activation we also used the technique<sup>19</sup> that analyses the capacity of activated Arp2/3 to induce branch formation in actin filaments. Again taking N-ActA as a control, we showed that in the presence of actin and Arp2/3 the RickA protein promotes filament branching (Fig. 1e). However, a similar percentage of branching, at equivalent concentrations of Arp2/3 and actin, required sixfold more RickA

than ActA and was reached after 15 min for RickA but after only 1 min for ActA (Fig. 1e).

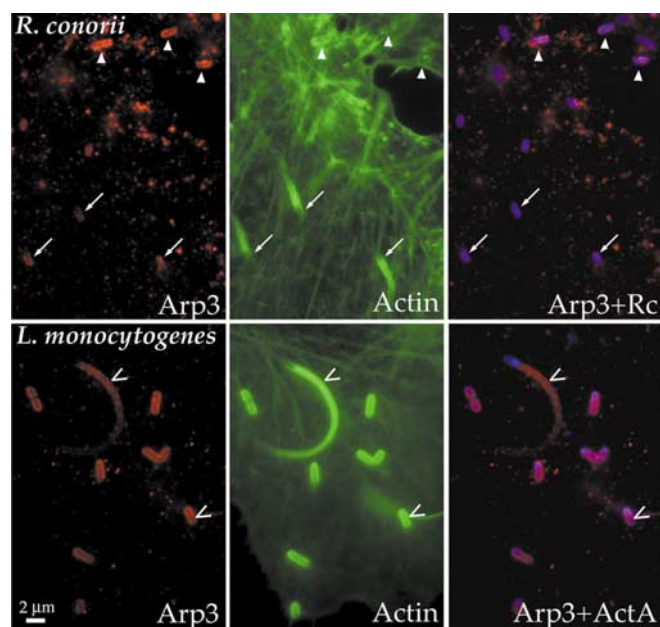
Having shown that RickA is sufficient to activate Arp2/3, and thereby actin nucleation *in vitro*, it was of interest to clarify whether the Arp2/3 complex has a function in the formation of the *Rickettsia* actin tail and in the actin-based motility process *in vivo*, and also whether it is present in the tails.

We therefore first transfected cells with plasmids expressing either the C-terminal part of Scar (WA)—which can recruit and sequester Arp2/3 and acts as a dominant-negative fragment with respect to the function of the Arp2/3 complex—or a truncated version (W), and infected these cells with *Rickettsia* (Fig. 2), using *Listeria* as a control (data not shown)<sup>8</sup>. Clearly, *Rickettsia* and *Listeria* actin tails were both detected in ScarW-transfected cells. In contrast, the number of bacteria (*Rickettsia* or *Listeria*) recruiting actin was greatly decreased in ScarWA-expressing cells, further indicating that the Arp2/3 complex might be used by *Rickettsia*, as it is in *Listeria*, to induce actin polymerization and movement.

We next re-examined Arp2/3 recruitment in the tails. All previous attempts to detect the Arp2/3 complex in *Rickettsia* tails had failed<sup>4,20</sup>. Using a new batch of affinity-purified antibodies, we were able to detect Arp3 around *Rickettsia*. Strikingly, Arp3 localized with cytoplasmic bacteria, whether with or without actin tails (Fig. 3). However, for those without actin tails (arrowheads in Fig. 3, top panels), Arp3 was detected around the whole bacterial bodies, and the strong labelling indicates that it was probably abundantly recruited. In contrast, when bacteria were polymerizing actin (arrows in Fig. 3, upper panels), Arp3 was barely detectable at the base of the tail and was absent from the tail. This distribution of Arp3 is therefore different from that in *Listeria* tails (arrowheads in Fig. 3, lower panels), in which Arp3 localizes with actin and is present both at the base and all along the tail. These observations are in complete agreement with the *in vitro* data and strongly indicate that RickA can recruit and activate the Arp2/3 complex to induce actin polymerization in infected cells.

If RickA recruits the Arp2/3 complex, it could behave as



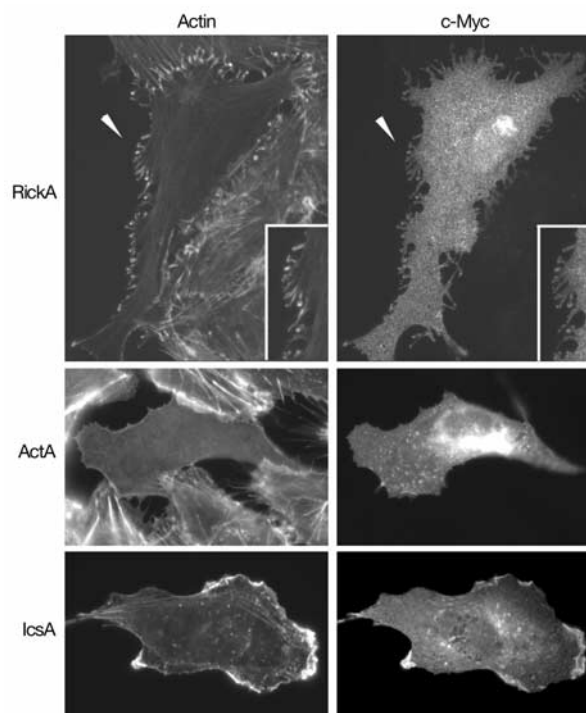


**Figure 3** Arp3 localizes with the bacteria *R. conorii* and with *L. monocytogenes* comet tails in Hep-2-infected cells. Upper panels, Hep-2 cells infected with *R. conorii*; lower panels, Hep-2 cells infected with *L. monocytogenes*. A new batch of affinity-purified polyclonal anti-Arp3 (0.94 mg ml<sup>-1</sup>), described in ref. 29, was used. *L. monocytogenes* cells were labelled with monoclonal anti-N-ActA A16 (ref. 6).

ScarWA when overexpressed in cells. To test this hypothesis we expressed the C-terminal part of RickA, which is similar to the VCA domain of N-WASP, in cells and infected them with *Listeria* or *Rickettsia* (Fig. 2). The results were similar to those obtained with ScarWA: transfection with the C-terminal part of RickA impaired *Rickettsia* tail formation. For *Listeria*, bacterial entry, which is known to be dependent on Arp2/3, was even more strongly inhibited than with ScarWA. Collectively, both the *in vitro* and *in vivo* data demonstrate that RickA is a previously unknown Arp2/3 activator.

Because the known Arp2/3 activators have been involved in cell motility or the formation of plasma membrane protrusions, we were interested to analyse how RickA would behave when expressed at the plasma membrane. We therefore transfected cells with a plasmid expressing a RickA-CAAX construct designed to drive expression of the protein at the inner face of the plasma membrane, as previously performed for ActA<sup>21</sup>. As shown in Fig. 4, after RickA expression, Vero-transfected cells displayed thin filopodia that were absent from non-transfected cells. These filopodia, whose length varied with the cell line used for the transfection, were different from the lamellipodia-like structures obtained when ActA, or even more strikingly IcsA, was transfected in mammalian cells, reinforcing the hypothesis that RickA is a novel type of activator of actin nucleation.

In conclusion, *Rickettsia conorii* cells express a surface protein, RickA, which recruits Arp2/3, activates it and induces actin polymerization. It is unknown how RickA is addressed to the bacterial surface and whether the type IV secretion system predicted by the genome sequence is involved in targeting to the surface. The actin filaments present behind intracellular bacteria in the tails are long and unbranched (Supplementary Fig. 2). These filaments might form bundles, in particular when bacteria spread from cell to cell, as shown previously<sup>4</sup>. Accordingly, as filopodia, *Rickettsia* tails contain the bundling protein fascin (Supplementary Fig. 3)<sup>22</sup>. How could such bundles form? In agreement with the well-established findings that activated Arp2/3 initiates actin polymerization on previously



**Figure 4** Transfection of mammalian cells with RickA-CAAX induces filopodia-like structure. Vero cells were transfected with plasmids expressing RickA-CAAX, ActA-CAAX or IcsA-CAAX, and processed for immunofluorescence 24 h later. Arrowheads point to regions shown in the inserts.

formed actin filaments, generating a branch, it is possible that RickA initiates actin polymerization in such a way that actin filaments nucleated by Arp2/3 are then elongated at only one barbed end, just as in filopodia<sup>12</sup>. Could VASP have a function in this process? VASP is present all along the *Rickettsia* tail<sup>4</sup> but is restricted to the base of the *Listeria* tails, where it binds directly to ActA through its EVH1 domain<sup>23</sup>. We infected D7 cells, which are completely devoid of Mena, VASP and EVL<sup>24</sup>, with *Rickettsia* or *Listeria* and compared the bacterial motility in these cells with that observed in D7 cells transfected with Mena. *Rickettsia* cells were less motile in the D7 cells, as were *Listeria* cells, suggesting that VASP-family proteins are required for maximal movement in *Rickettsia*. In both lamellipodia<sup>25</sup> and ActA-induced actin assembly<sup>26</sup>, VASP decreases branch density and, as proposed, could compete with capping proteins at the barbed ends<sup>25</sup> and thus increase filament length. This could also occur in RickA-induced actin assembly. In *Shigella*, Ena/VASP proteins are located throughout the tails, but reconstitution experiments suggest that Ena/Vasp proteins are dispensable for *Shigella* motility<sup>7</sup>. The role of VASP in *Rickettsia* motility therefore seems different from that in *Listeria* and *Shigella* motilities.

Taken together, the *Rickettsia* protein RickA is a previously unknown bacterial activator of actin nucleation that requires Arp2/3 to initiate an actin polymerization process similar to that leading to filopodia<sup>12</sup>. Whether proteins thought to contribute to filopodia formation and/or predicted to be present at the tip of the filopodia are also involved in *Rickettsia* movement is unknown. It is possible that post-translational modifications of RickA such as phosphorylations at various positions including Ser 459, a position corresponding to Ser 484 in WASP<sup>27</sup>, controls its activity. Whether another *Rickettsia* protein also participates in the actin-based motility has to be addressed. Thus, RickA is a bacterial actin nucleator that is most closely related to WASP/N-WASP-family

proteins. It is unknown whether RickA is of eukaryotic origin or has been transferred from *Rickettsia* to eukaryotic cells. □

## Methods

### Expression and purification of recombinant proteins

RickA, the ActA-N fragment (residues 1–233) and the WA fragment of N-WASP (residues 392–505) were obtained as His-tagged proteins. The *rickA* gene was amplified by polymerase chain reaction with *Rickettsia conorii* (Malish strain) chromosomal DNA as template and the primers 5′-CCatggttaagaagaatagataataaaa-3′ and 5′-caagctttctacaaatgatgggtttg-3′. This fragment was digested with *NcoI* and *HindIII* and cloned into these sites in pET28b. The DNA fragment encoding the WA fragment of N-WASP was cloned into the *EcoRI* and *XhoI* sites of pET28a to generate an N-terminal His<sub>6</sub>-tagged protein. Proteins were expressed in *E. coli* BL21(DE3) and affinity purified with Talon resin (Clontech).

### Actin polymerization assays

Polymerization assays were performed with the change in pyrenyl-actin fluorescence. Pyrene-labelled rabbit muscle actin (10% pyrene-labelled) in G buffer (0.2 mM CaCl<sub>2</sub>, 0.2 mM DTT, 0.2 mM ATP, 1 mM MgCl<sub>2</sub>, 5 mM Tris-HCl pH 7.5) was precleared at 250,000 g for 30 min. MgATP-actin (1.5 μM, 10% pyrene-labelled) was polymerized in 1 × KMET (50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM Tris-HCl pH 7) supplemented with 0.5 mM ATP and RickA (70 nM) or Arp2/3 (30 nM) alone or in the presence of N-WASP-WA (70 nM), N-ActA (70 nM) and RickA (70 and 420 nM). Measurements were made with a Varian Eclipse Spectrofluorometer at 25 °C, with excitation and emission wavelengths of 350 and 390 nm respectively.

### Membrane targeting of RickA-CAAX, ActA-CAAX and IcsA-CAAX

To express RickA at the plasma membrane, an approach similar to that used in ref. 21 was taken with plasmid pRK5Myc (ref. 28), which allows the expression of N-terminal Myc-tagged and C-terminal CAAX-tagged protein. DNA fragments encoding ActA (1–580), RickA (1–517) and IcsA (53–758) were cloned in the *BamHI* and *EcoRI* sites of pRK5Myc-CAAX. The resultant plasmids were transiently expressed in Vero cells with Lipofectamine 2000.

### Antibodies

Anti-RickA antibodies (R80) were generated by immunizing rabbits against the peptide CQNTEKELEKEHNRS and affinity purified. Anti-*Rickettsia* mouse polyclonal antibodies (S1) were obtained by immunizing BALBc mice with formalin-killed bacteria purified from infected yolk sac.

Received 28 October; accepted 23 December 2003; doi:10.1038/nature02318.

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Supplementary Information accompanies the paper on [www.nature.com/nature](http://www.nature.com/nature).

**Acknowledgements** We thank L. Blanchoin for help and discussions, L. Machesky for the gift of plasmids Scar-WA and ScarW, and P. Renesto and all members of the Cossart laboratory for discussions and suggestions. This work was supported by the Pasteur Institute, the Ministère de la Recherche et de la Technologie (Programme PRFMMIP) and the Direction Générale des Armées (DGA). C.E. is supported by a Human Frontier Science programme fellowship. P.C. is an international scholar from the Howard Hughes Medical Institute.

**Competing interests statement** The authors declare that they have no competing financial interests.

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## The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore

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A sudden increase in permeability of the inner mitochondrial membrane, the so-called mitochondrial permeability transition, is a common feature of apoptosis and is mediated by the mitochondrial permeability transition pore (mtPTP). It is thought that the mtPTP is a protein complex formed by the voltage-dependent anion channel, members of the pro- and anti-apoptotic BAX-BCL2 protein family, cyclophilin D, and the adenine nucleotide (ADP/ATP) translocators (ANTs)<sup>1,2</sup>. The latter exchange mitochondrial ATP for cytosolic ADP and have been implicated in cell death. To investigate the role of the ANTs in the mtPTP, we genetically inactivated the two isoforms of ANT<sup>3–5</sup> in mouse liver and analysed mtPTP activation in isolated mitochondria and the induction of cell death in hepatocytes. Mito-