Cdc42 is required for membrane dependent actin polymerization in vitro

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Received 6 March 1998; revised version received 6 April 1998

Abstract In vitro actin based motility assays with bacterial pathogens have provided powerful systems to both understand and dissect actin dynamics as well as cell motility. Taking advantage of endogenous membrane vesicles in *Xenopus* extracts we have developed an in vitro assay to study membrane dependent actin polymerization. Our results demonstrate that membrane dependent actin polymerization, in contrast to *Listeria* stimulated actin filament assembly, is dependent on small GTPases of the Rho family. Using a combination of depletion and reconstitution experiments we have shown that Cdc42 but not Rac or Rho is required to stimulate actin polymerization from membranes. The in vitro system we have described here is amenable to identification of the downstream effectors of Cdc42 required for membrane dependent actin polymerization.

Key words: Cdc42; Membrane; Actin polymerization

1. Introduction

During the last five years it has become clear that the Rho family of small GTPases, Cdc42, Rac and Rho, are instrumental in regulating actin dynamics, acting as a link between membrane receptor signalling and the cytoskeleton [1–3]. Initial observations from micro-injection studies in Swiss 3T3 fibroblasts demonstrated that constitutively active Rho induces the formation of actin stress fibers and focal adhesions [4], while constitutively active Rac and Cdc42 induce actin rich membrane ruffles and filopodia respectively [5,6]. Based on these observations it was proposed that Rho family members work in a hierarchical cascade in Swiss 3T3 fibroblasts in which Cdc42 activates Rac, which in turn activates Rho to control cell motility [5]. Since these initial observations subsequent studies in a variety of systems have confirmed that Rho proteins are potent regulators of many different actin dependent processes in addition to cell motility including establishment, cell-cell contacts in epithelial cells [7,8], control of bud formation and morphogenesis in *S. cerevisiae* [9,10] and *S. pombe* [11], neurite outgrowth [12,13], as well as cytokinesis in HeLa cells and *Xenopus* embryos [14,15].

While analyses of Rho proteins and their downstream effectors together with studies on the mechanism of actin based motility of pathogens has provided great insights into the regulation of the actin cytoskeleton and cell motility, our understanding of membrane dependent actin polymerization is much more limited. Dissection of the actin-membrane interface is critical if we are to understand the events at the plasma membrane that occur during signalling and motility. The in vitro actin based motility assay of *Listeria* in *Xenopus* egg extracts first described by Theriot et al. [16] has enabled biochemical dissection of *Listeria* motility [17] and offers a potential novel system with which to analyse actin-membrane interactions. Indeed, Marchand et al. [18] have previously reported that endogenous vesicles in extracts are occasionally capable of nucleating actin polymerization suggesting that components required for this process are present in the extract. Using such an in vitro motility assay we show here that activation of Cdc42 but not Rac or Rho is required for membrane dependent actin assembly in vitro. The system we describe here will greatly facilitate the complete biochemical dissection of the components involved in actin-membrane interactions.

2. Materials and methods

2.1. Reagents and protein expression

Butanedione monoxime (BDM), Cytochalasin D, nocodazole and wortmannin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protein kinase inhibitor herbimycin A, specific for tyrosine kinases was obtained from Calbiochem (Bad Soden, Germany). Staurosporine was a gift from Dr. A. Nebreda (EMBL, Heidelberg, Germany). Recombinant constitutively active Rho proteins (L63Rho, L61Rac and L61Cdc42) and C3 exoenzyme expressed in *E. coli* were a generous gift from Dr. A. Hall (MRC, London, UK). These proteins have been successfully used for micro-injection experiments in the laboratory of Alan Hall. Purified toxin B from *C. difficile*, which specifically inhibits the Rho family members was provided by Professor K. Aktories (Freiburg, Germany) while active RabGDI and Cdc42 was provided by Dr. V. Rybin and R. Vincentelli respectively (EMBL, Heidelberg). The GST-RhoGDI and GST-L61Cdc42 expression constructs were provided by Dr. M. Zerial (EMBL, Heidelberg) and Dr. A. Hall (MRC, London) respectively. Both constructs were expressed in the *E. coli* strain XL1-Blue and purified by affinity chromatography on glutathione Sepharose.

2.2. Preparation of Xenopus egg extract

Xenopus egg extracts were prepared as described previously [16]. Briefly, the meiotically arrested *Xenopus laevis* eggs are dejellyed in 2% cysteine, pH 7.8, washed with XB (100 mM KCl, 10 mM K-HEPES pH 7.8, 50 mM sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and transferred into buffer XB-CSF (1 mM $MgCl_2$, 5 mM EGTA, 10 μg/ml leupeptin, pepstatin and aprotinin in XB). The eggs are crushed at 10 000 rpm in a Sorvall HB-4 rotor at 16°C for 15 min. The cytoplasmic layer was removed and 1/40 volume of energy mix (300 mM creatine phosphate, 40 mM ATP, 2 mM EGTA and 40 mM $MgCl_2$) was added. The crude extract is obtained after a centrifugation of 15 min at 14 000 rpm in a microfuge at 4°C. Aliquots were frozen in liquid nitrogen and stored at −80°C.

2.3. Motility assay and extract depletion

Actin based motility was performed in 4 μl of *Xenopus* egg extract containing a final concentration of 3 μM rhodamine labelled G-actin. GTPγS (Boehringer-Mannheim, Germany) and vanadate were added to a final concentration of 0.5 mM and 1 mM respectively. For the *Listeria* motility assay, we used SLCC-5764 strain kindly provided by Dr. Matt Welch (UCSF, San Francisco, USA) prepared as described previously [19]. Depleted extracts for motility assays were prepared by incubating *Xenopus* egg extracts with GST, GST-RhoGDI or GST-L61Cdc42 on glutathione beads overnight at 4°C with rotation. The

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PII S0014-5793(98)00443-8

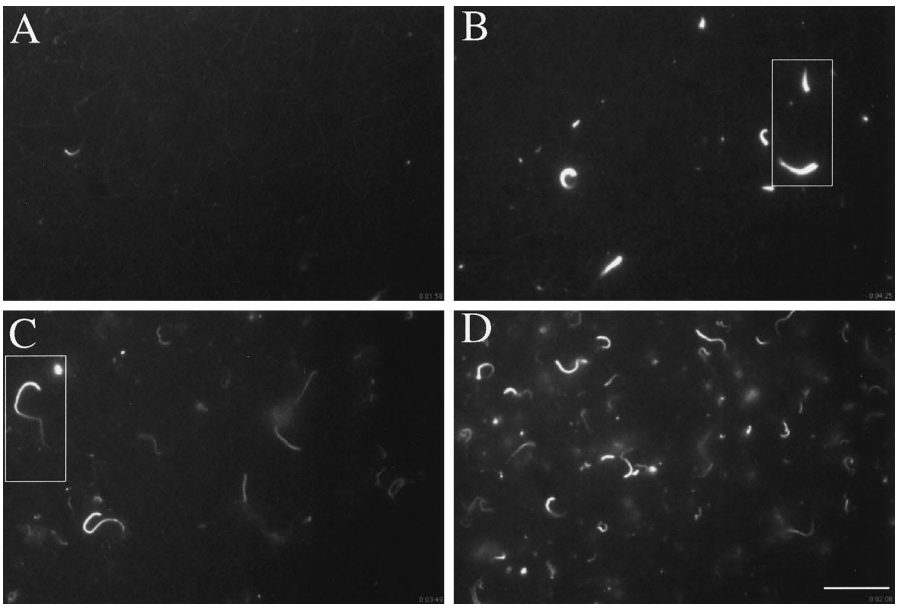

Fig. 1. GTPγS or vanadate induce actin tail formation in *Xenopus* extracts. A: Control extract; B: GTPγS treated extract; C: vanadate treated extract and D: GTPγS and vanadate treated extract. Boxed areas in B and C indicate actin tails shown in video sequences shown in Fig. 2. Scale bar: 12 μm.

beads were subsequently spun down and the depleted extract used for motility assays. Video sequences from motility assays were collected using a COHU high performance CCD camera (San Diego, CA, USA) in conjunction with NIH image (Version 1.59) at a rate of one picture every 3 s. Actin tail length and rates of motility were measured using a NIH image macro written by Denis Chretien (EMBL, Heidelberg). Single still images were collected using a Colour Coolview camera (Photonic Science, Millham, UK) in conjunction with IPLab Spectrum software (Signal Analytics Corp., Vienna, VA, USA). All digital images were subsequently cropped and annotated using the Adobe 3.0 Software package.

2.4. R18 staining

Octadecyl rhodamine B chloride (R18) (Molecular Probes, Eugene, USA) was added to the crude extract to a final concentration of 0.1 μM. Motility assays were performed as described above, except that FITC labelled G-actin (Cytoskeleton, Denver, USA) was substituted for rhodamine labelled G-actin. Images were recorded as described above using dual pass filters to ensure alignment.

3. Results

3.1. GTPγS and vanadate stimulate membrane dependent actin polymerization

In crude *Xenopus* egg extracts supplemented with rhodamine G-actin occasional actin tails are observed (Fig. 1A). Although the abundance of these actin tails is variable from extract to extract, their number is in general small (<1 per field). We found that addition of GTPγS or vanadate to extracts resulted in a consistent stimulation of the number of endogenous actin tails (Fig. 1B and C), while addition of both reagents resulted in massive stimulation (Fig. 1D). Actin tails moved at an average rate of 7.2 μm/min (range 5.3–15.5) and 14.6 μm/min (range 8.6–22.6) for GTPγS and vanadate respectively (Fig. 2). We also observed that actin tails induced by GTPγS were always shorter than those induced by vanadate, average lengths were 7.0 μm (range 2.9–11.0) and 20.0 μm (range 13.0–39.0) respectively. Stimulation of endogenous actin tails by both reagents was extremely rapidly, occurring within 2 min. This is in contrast to nucleation of actin tails by *Listeria* which takes 10–15 min under the conditions of our assay (data not shown). The presence of GTPγS or vanadate in the extract did not affect the ability or timing of *Listeria* to nucleate actin tails (data not shown). To determine whether GTPγS and vanadate stimulated actin tail formation required membrane components we added R18, a lipophilic marker to the extract. In these samples we observed that each actin tail

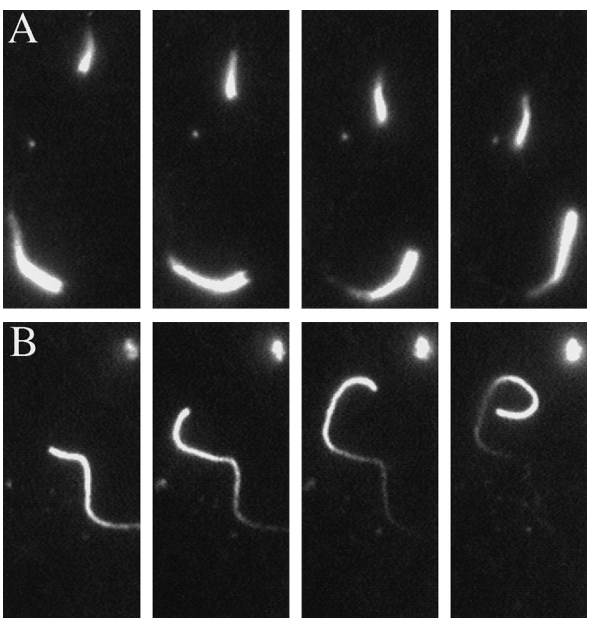

Fig. 2. Examples of the motility of GTPγS and vanadate induced actin tails. Video sequence stills are 30 s apart and correspond to the actin tails boxed in Fig. 1B and C. GTPγS induced tails (A) tend to be short and move at slower rates than vanadate induced tails (B). In A the upper actin tail is 4.9 μm long and moving at a rate of 5.8 μm/min while the lower tail is 8.8 μm long and moving at a rate of 7.1 μm/min while in B the actin tail is 22.2 μm long and moving at a rate of 12.2 μm/min.

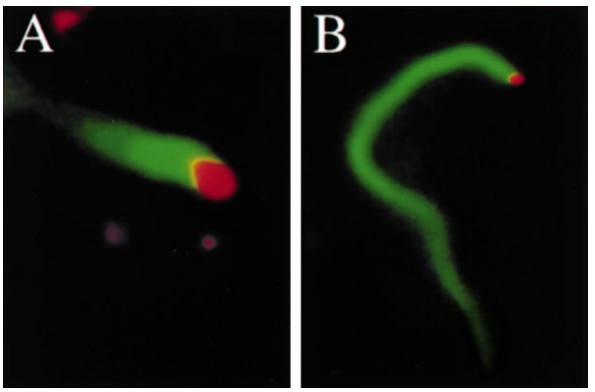

Fig. 3. R18 staining reveals that GTPγS (A) and vanadate (B) stimulate actin tails from endogenous vesicles in *Xenopus* extracts.

was indeed nucleated from a membrane bound vesicle which varied considerably in size (0.5–2.5 μm) (Fig. 3). Furthermore, addition of Triton X-100 to the extract resulted in the complete loss of actin tail stimulation by GTPγS or vanadate (data not shown). Taken together these results show that actin tail assembly is membrane dependent.

3.2. Vesicle actin tail nucleation requires small GTPases of the Rho family

GTPγS stimulation of actin tails by vesicles is strongly indicative of the involvement of small GTPases of the Rho family. To investigate this possibility we examined the effects of addition of two small GTPase inhibitors, toxin B from *C. difficile* and the guanine nucleotide dissociation inhibitor RhoGDI on vesicle actin tail formation. Pre-incubation of extracts with either toxin B or RhoGDI completely inhibits GTPγS and vanadate stimulation of actin tails from endogenous vesicles (Fig. 4). Extracts pre-treated with toxin B or RhoGDI are however still competent for actin polymerization as judged from the formation of actin networks in the background (Fig. 4B and C) and the ability of *Listeria* to still induce actin tails (data not shown). Addition of guanine nucleotide dissociation inhibitor RabGDI, which is specific for the Rab proteins, had no effect on actin tail formation by endogenous vesicles or *Listeria* indicating that rab proteins are not required for actin tail assembly (data not shown). In addition BDM, an inhibitor of myosin II and V [20] as well as the protein kinase inhibitors staurosporine, wortmannin and herbimycin A did not effect GTPγS or vanadate stimulated vesicle actin tail formation.

3.3. Cdc42 is required for vesicle actin tail nucleation

To further distinguish which Rho protein(s) are involved in vesicle actin tail assembly, we treated the extract with C3 transferase which specifically inactivates RhoA-C. Pre-incubation of extracts with C3 did not inhibit GTPγS or vanadate stimulated actin tails indicating that RhoA-C is not required for vesicle actin tail nucleation (data not shown). To identify whether Rac or Cdc42 was involved in actin tail formation we examined if activated forms of Rac and Cdc42 could induce actin tails from endogenous vesicles in the absence of GTPγS or vanadate. Extracts depleted of Rho proteins by incubation with GST-RhoGDI beads show no GTPγS or vanadate stimulated vesicle actin tail formation but are still competent to nucleate actin polymerization as judged by the formation of actin networks in the background and by the ability of *Listeria* to nucleate actin tails (Fig. 5). When dominant active forms of Rho, Rac and Cdc42 are added to Rho protein depleted extracts only activated Cdc42 is able to induce vesicle actin tail formation (Fig. 5). Actin tails stimulated by L61Cdc42 often looked ragged and mis-formed compared to controls but moved at a similar speed, the average rate was 10.3 μm/min. By contrast, addition of Cdc42 pre-loaded with GTPγS was able to stimulate vesicle actin tails that more closely resembled those seen in controls. The reason for this difference in appearance remains obscure but presumably reflects the efficiency of the nucleation process on the vesicle. There was no difference in the number of actin tails stimulated by activated Cdc42 in the presence or absence of activated Rac and Rho suggesting that only Cdc42 is required to stimulate actin tail nucleation.

4. Discussion

While the mechanism by which actin is spatially and temporally regulated is far from understood it is clear that small GTPases of the Rho family are instrumental in regulating actin dynamics through the action of numerous downstream effectors [1–3]. Our results now demonstrate that the small G-protein Cdc42 but not Rac or Rho is involved in stimulating actin nucleation from membrane bound vesicles in vitro. Cdc42 has also recently been shown to induce actin polymerization in a cell-free system of polymorphonuclear leukocytes and *D. discoideum* amoeba extracts [21]. To date a limited number of downstream effectors of Cdc42 have been implicated in rearrangements of the actin cytoskeleton. These include phosphatidylinositol-3-OH kinase (PI3-kinase) [22,23], IQGAP [24,25], CIP4 [26], Pak1 [27,28] and WASP/N-

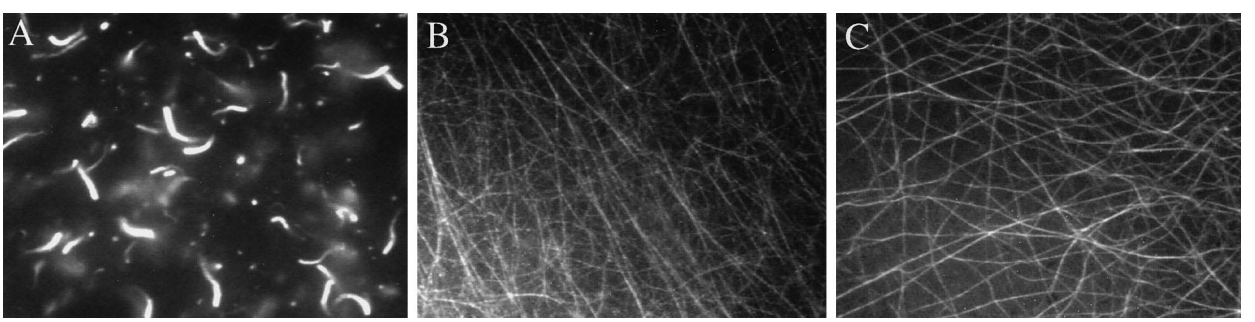

Fig. 4. Small GTPases of the Rho family are required for GTPγS and vanadate stimulation of actin tails. A: Extract stimulated with GTPγS and vanadate; B: extract pre-incubated with toxin B stimulated with GTPγS and vanadate and C: extract pre-incubated with RhoGDI stimulated with GTPγS and vanadate. Scale bar: 12 μm.

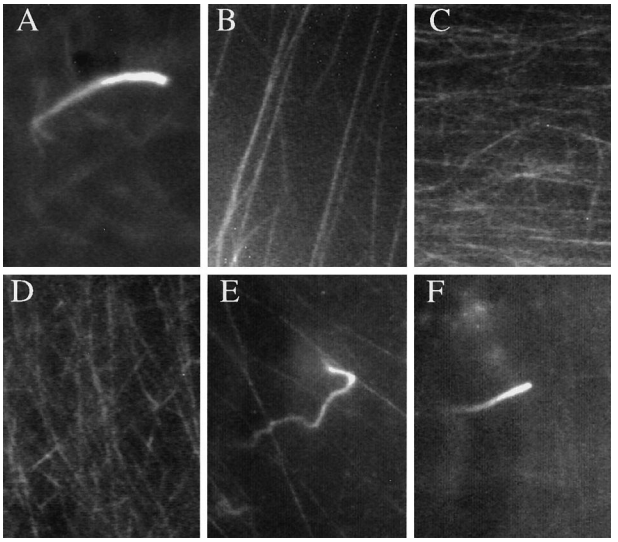

Fig. 5. Activated Cdc42 can restore vesicle induced actin tails in Rho protein depleted extracts. While *Listeria* (A) is able to nucleate actin tails in Rho protein depleted extracts, GTPγS and vanadate (B) cannot stimulate vesicle induced actin tails. Similarly L63Rho (C) and L61Rac (D) do not stimulate actin tails in Rho protein depleted extracts while L61Cdc42 (E) and GTPγS charged Cdc42 (F) are able to stimulate vesicle induced actin tails.

WASP [29–31]. Based on our observations with wortmannin we can however rule out the possible involvement of PI3-kinase which induces alterations in the actin cytoskeleton that lead to cell motility when activated by Cdc42 [23] as well as Pak1 which is also inhibited by wortmannin [28]. While the mechanism by which Cdc42 is able to stimulate actin polymerization from vesicles remains to be elucidated our observations with vanadate suggest that tyrosine phosphorylation plays an important role in the process.

The involvement of Cdc42 in vesicle actin tail nucleation is in complete contrast to that of *Listeria* where there is no requirement of small GTPases in vitro. In addition while the rate of vesicle motility and *Listeria* in our assay are comparable the kinetics of actin tail assembly are quite different. Under the conditions of our assay *Listeria* requires 10–15 min to nucleate actin tails whereas vesicles are more immediate requiring 1–2 min. Taken together these observations suggest that the mechanism of actin nucleation from membrane bound vesicles is different from that of *Listeria*. Indeed, the mechanism of actin tail nucleation by *Listeria* is quite different from *Shigella*, another bacterial pathogen which also induces actin tails during infection [32]. Thus while bacterial systems provide excellent model systems to dissect actin dynamics and cell motility they may not identify all the components involved in the mechanism of actin polymerization at the leading edge of motile cells as the nature of the nucleation site is fundamentally different. In contrast the system we have described here will facilitate biochemical dissection of the components involved in mediating actin-membrane interactions and will provide further insights into the role of Cdc42 in remodelling of the actin cytoskeleton during cell motility.

5. Note added in proof

During the review process of this paper, Ma et al. have described similar observations with synthetic lipid vesicles in *Xenopus* egg extracts (Ma et al. (1998) J. Cell Biol. 140, 1125–1136).

Acknowledgements: We would like to thank Professor K. Aktories (Freiburg, Germany), Dr. A. Hall (MRC, London), Dr. M. Welch (UCSF, USA), Drs. A. Nebreda, V. Rybin, R. Vincentelli, M. Zerial (EMBL, Heidelberg) for generously providing numerous reagents. We would like to thank Dr. D. Chretien for the NIH image macro used for measurements and T. Wittman for help with *Xenopus* egg extracts. Finally we would like to thank Dr. Laura Machesky (MRC, London) and Dr. Carlos Dotti (EMBL, Heidelberg) for critically reading the manuscript and Dr. Le Ma (Harvard, USA) for communicating work in press.

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