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Phosphoinositides Regulate Membrane-dependent Actin Assembly by Latex Bead Phagosomes

Hélène Defacque,*†‡ Evelyne Bos,*‡ Boyan Garvalov,* Cécile Barret,§ Christian Roy,§ Paul Mangeat,§ Hye-Won Shin,* Vladimir Rybin,* and Gareth Griffiths*‖

*European Molecular Biology Laboratory, 69012 Heidelberg, Germany; §Université Montpellier II, CNRS UMR 5539, CC 107, 34095 Montpellier Cedex 05, France

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Actin assembly on membrane surfaces is an elusive process in which several phosphoinositides (PIPs) have been implicated. We have reconstituted actin assembly using a defined membrane surface, the latex bead phagosome (LBP), and shown that the PI(4,5)P_2-binding proteins ezrin and/or moesin were essential for this process (Defacque *et al.*, 2000b). Here, we provide several lines of evidence that both preexisting and newly synthesized PI(4,5)P_2, and probably PI(4)P, are essential for phagosomal actin assembly; only these PIPs were routinely synthesized from ATP during in vitro actin assembly. Treatment of LBP with phospholipase C or with adenosine, an inhibitor of type II PI 4-kinase, as well as preincubation with anti-PI(4)P or anti-PI(4,5)P_2 antibodies all inhibited this process. Incorporation of extra PI(4)P or PI(4,5)P_2 into the LBP membrane led to a fivefold increase in the number of phagosomes that assemble actin. An ezrin mutant mutated in the PI(4,5)P_2-binding sites was less efficient in binding to LBPs and in reconstituting actin assembly than wild-type ezrin. Our data show that PI 4- and PI 5-kinase, and under some conditions also PI 3-kinase, activities are present on LBPs and can be activated by ATP, even in the absence of GTP or cytosolic components. However, PI 3-kinase activity is not required for actin assembly, because the process was not affected by PI 3-kinase inhibitors. We suggest that the ezrin-dependent actin assembly on the LBP membrane may require active turnover of D4 and D5 PIPs on the organelle membrane.

INTRODUCTION

A significant fraction of the de novo nucleation of actin in cells occurs on the cytoplasmic surface of eukaryotic cell membranes, especially the plasma membrane (Tilney, 1976; Carraway and Carraway, 1989; Small *et al.*, 1995; Mitchison and Cramer, 1996), and a role for phosphoinositides in this elusive process has been widely discussed (Divecha and Irvine, 1995; Martin, 1998; Caroni, 2001). However, the precise function of these lipids is still not clear and is likely to be quite complicated. In several cellular systems that show rapid actin assembly in response to extracellular ligands, synthesis of phosphoinositides, especially phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2], and in some cases phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3], coincides precisely with the transient burst of actin assembly (Eberle *et al.*, 1990; Dobos *et al.*, 1992; Apgar, 1995; Hartwig *et al.*, 1995; Gachet *et al.*, 1997). In addition, overexpression of phosphatidylinositol-4-phosphate [PI(4)P] 5-kinase in cells leads to a significant polymerization of actin (Shibasaki *et al.*, 1997). However, in other systems, the synthesis of PI(4,5)P_2 as well as PI(3,4,5)P_3 coincides more with actin depolymerization, after a transient assembly of F-actin (Apgar, 1995; Gratacap *et al.*, 1998).

One important clue to the functions of phosphoinositides in actin assembly/disassembly is that these lipids can bind in vitro to an increasing number of actin-binding proteins (ABPs). Interestingly, two different behaviors of these ABPs have been described. First, many ABPs, such as profilin,

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‖ Corresponding author. E-mail address: griffiths@embl-heidelberg.de.
† Present address: Observatoire Oceanologique de Banyuls, UMR CNRS 7628, Banyuls/Mer, France
‡ These authors contributed equally to this study.

Abbreviations used: ABP, actin-binding protein; ERM, ezrin/radixin/moesin; HPLC, high-pressure liquid chromatography; LBP, latex bead phagosome; N-WASP, neural Wiskott-Aldrich syndrome protein; PI, phosphatidylinositol; PIP, phosphoinositide; PI(4)P, phosphatidylinositol-4-phosphate; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate.

gelsolin, and cofilin, lose their affinity for actin when bound to PI(4,5)P_2 (Lassing and Lindberg, 1985; Janmey *et al.*, 1987; Yonezawa *et al.*, 1990; Janmey *et al.*, 1999). The second class, which includes vinculin, talin, and the ezrin/radixin/moesin (ERM) family, appear to be able to bind PI(4,5)P_2 and actin simultaneously (Niggli *et al.*, 1995; Gilmore and Burridge, 1996; Isenberg and Niggli, 1998; Nakamura *et al.*, 1999). Lipid vesicles containing PI(4,5)P_2 have been shown to recruit ABPs and other regulatory factors (e.g., N-WASP, which recruits Arp2/3 and Cdc42) from cytosolic extracts. When bound to the vesicles, these proteins somehow coordinate the insertion of actin monomers into filaments such that the vesicles are propelled by actin comets (Ma *et al.*, 1998; Rozelle *et al.*, 2000; Taunton *et al.*, 2000). To the best of our knowledge, no systematic attempt using a defined biological membrane system in vitro has been made to address the role of phosphoinositides in membrane-bound actin assembly.

Phagosomes from J774 macrophages can be prepared by internalizing 1-μm latex beads for up to 1 h followed by various times of chase up to 36 h. During this intracellular period, the latex-bead phagosomes (LBPs), which are de novo–assembled membrane organelles, mature, and as they age, show significant changes in their protein and lipid composition (Desjardins *et al.*, 1994a; Desjardins *et al.*, 1994b; Diakonova *et al.*, 1997; Claus *et al.*, 1998; Jahraus *et al.*, 1998), as well as in their ability to interact with microtubules (Blocker *et al.*, 1996; Blocker *et al.*, 1997), to fuse with endocytic organelles (Desjardins *et al.*, 1994a; Desjardins *et al.*, 1994b; Jahraus *et al.*, 1998, 2001), and to assemble actin de novo (Defacque *et al.*, 2000b). LBPs also bind in vitro to F-actin (Al-Haddad *et al.*, 2001) in a process that is distinct from actin nucleation. A recent proteomic analysis of purified LBPs has identified ~150 of the estimated maximum 500 proteins present on this organelle (Garin *et al.*, 2001).

We have recently established two related assays to monitor in vitro actin assembly on this membrane surface, using either fluorescence microscopy (Defacque *et al.*, 2000b) or flow cytometry analysis (Defacque *et al.*, 2000a). Importantly, neither GTP nor cytosolic proteins are added to this system, which depends on the intrinsic capacity of the phagosomal membrane. As the phagosomes mature in the cell, they fluctuate in their ability to assemble actin, both in vivo and in vitro (Defacque *et al.*, 2000b), and this cyclical pattern of assembly activity correlates strongly with the phosphorylation state of many (still to be identified) phagosomal proteins (Emans *et al.*, 1996). The PI(4,5)P_2-binding proteins ezrin and moesin were shown to be essential for the actin assembly process on LBPs (Defacque *et al.*, 2000b).

Here, we provide many lines of evidence that both preexisting and newly synthesized D4 and D5, but not D3 PIPs are essential for the ezrin-dependent process of de novo actin assembly by the phagosomal membrane. This implies that phosphoinositide turnover may be essential for this process to occur.

MATERIALS AND METHODS

Reagents

Phospholipids were obtained from Sigma (St. Louis, MO), except phosphatidylinositol-3,4-bisphosphate [PI(3,4)P_2] and PI(3,4,5)P_3, which were from Matreya Inc., State College, PA; PI(4)P and PI(4,5)P_2 were from Calbiochem (San Diego, Ca). Recombinant wild-type and mutant ezrins were expressed in bacteria as previously described (Roy *et al.*, 1997). The ezrin mutant K63N, K64N, K253N, K254N, K262N, K263N is described in Barret *et al.* (2000).

J774 Cell Culture, Phagosome Purification, and Treatment

Phagosomes containing 1-μm latex beads were prepared in J774A.1 mouse macrophages as described previously (Jahraus *et al.*, 1998). "Salt stripping" of the phagosomes with 1.3 M NaCl and recovery of the peripheral proteins were performed as described (Defacque *et al.*, 2000b). The protein concentration of the salt-stripped extracts was typically ~160 μg/ml as determined by use of BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL). Of this, ~1.5 μg/ml (22 nM) is ezrin (Defacque *et al.*, 2000b).

For phospholipase C treatment, phosphatidylinositol-specific phospholipase C from *Bacillus cereus* (PI-PLC; Sigma) was reconstituted in PLC buffer (10 mM PIPES, pH 6.8, 200 mM sorbitol, 150 mM KCl, 0.5 mM $MgCl_2$), stored at −20°C, and used within 2 wk. Purified phagosomes were pretreated for 15 min at 37°C with 0, 0.1, or 0.6 U/ml PLC in a minimal volume of PLC buffer supplemented with protease inhibitors and 0.5 mM dithiothreitol. They were then immediately diluted 1:20 in the actin/Tβ4 mix and assayed for their actin assembly activity.

Actin Assembly (Nucleation) Assay by Fluorescence Microscopy

This assay was described in detail by Defacque *et al.* (2000b). Briefly, glass slides were coated with 0.5% fish-skin gelatin in water and air-dried before the experiment. A constant number of phagosomes (Blocker *et al.*, 1997) was incubated between a slide and a coverslip in P buffer (20 mM HEPES, pH 7.0, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.03% fish-skin gelatin, and protease inhibitors) with 2 μM rhodamine G-actin, 6 μM thymosin β4, and an antifade reagent (Blocker *et al.*, 1997) at room temperature for 15 min. The percentage of positive phagosomes was determined with a Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany). In all experiments described, the errors reported are the SDs from counts from at least three different microscope slides.

For lipid treatments, the PI(4)P and PI(4,5)P_2 stock lipids were dissolved in water or chloroform (1 mg/ml). For the preincubations, the phagosomes were mixed for 15–30 min with the respective lipid (or antibody) at concentrations indicated for each experiment and then mixed with sucrose to a final concentration of 35%, placed at the bottom of a tube, and overlaid with a 25%/8% sucrose step gradient. After ultracentrifugation, the phagosomes with the bound lipid float up to the 25%/8% interface, whereas unbound lipid floats to the top. The refloated phagosomes are used immediately in the actin assembly assay.

Reconstitution Assay

For ezrin binding to phagosomes, the indicated amount of recombinant ezrin was incubated with previously salt-stripped phagosomes in D buffer (10 mM HEPES, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, protease inhibitors) with 0.3% fish-skin gelatin for 15 min at 25°C. For each condition, 100 μl (binding experiment) or 6 μl (microscopy assay) of a phagosome preparation (OD_{600} ~0.4) (Blocker *et al.*, 1997) was used. The phagosomes were then repurified on a sucrose gradient as previously described (Jahraus *et al.*, 1998) before the fluorescence microscopy actin assembly assay was performed. Alternatively, for the ezrin-binding experiments, recovered phagosomes were diluted with 4 volumes of S/J buffer (25 mM HEPES-KOH, 115 mM potassium acetate, 25 mM magnesium acetate, protease inhibitors, pH 7.4) and pelleted by centrifugation at 12,000 rpm for 10 min in a TLS55 rotor (Beckman TL100 ultracentrifuge). The pellets were resuspended in

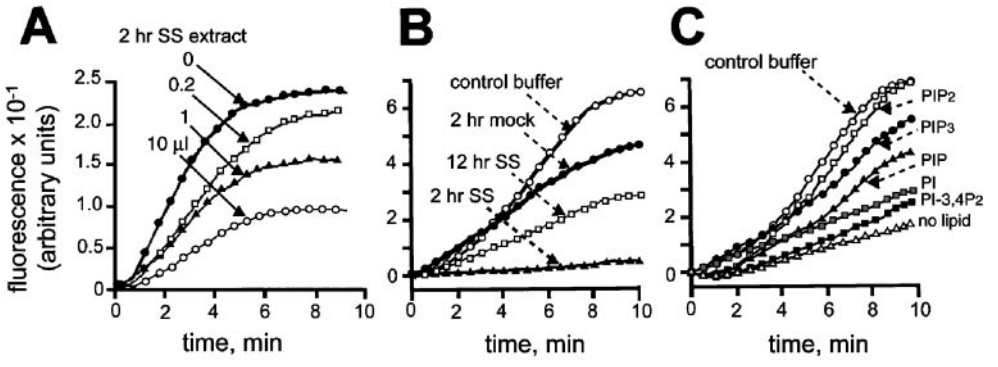

Figure 1. Inhibition of actin polymerization by a salt-stripped (SS) extract of 2-h phagosomes can be restored by PI(4,5)P_2 and PI(3,4,5)P_3. (A) Unlabeled F-actin seeds were preincubated in a total final volume of 50 μl with a control buffer (●), 0.2 μl (□), 1 μl (▲), or 10 μl (○) of a 2-h salt-stripped extract (containing ~160 μg/ml total protein) for 5 min at 25°C. After preincubation, 1 μM pyrene G-actin was added. (B) 1 μM pyrene G-actin was preincubated for 5 min at 25°C with a 2-h salt extract fraction (▲), a 2-h mock extract (●), a 12-h salt extract (□), or control buffer (○). (C) In the absence of membranes, 1 μM pyrene G-actin was preincubated for 5 min at 25°C with a control buffer alone (○) or a 2-h salt extract without (●) or with 50 μM PI(4,5)P_2 (□), PI(3,4,5)P_3 (●), PI (▨), PI(3,4)P_2 (■), or PI(4)P (▲). PI(4,5)P_2 itself had no effect on pyrene actin polymerization (data not shown). In all experiments, after preincubation, actin polymerization was triggered with 50 mM KCl and 2 mM $MgCl_2$ and followed by spectrofluorometry. Fluorescence increase was expressed in arbitrary units. All extracts, corresponding to ~30 ng/ml protein, were prepared with same numbers of phagosomes (see Blocker *et al.*, 1997).

Laemmli buffer (Laemmli, 1970), heated at 95°C for 5 min, and separated by SDS-PAGE. Western blotting onto polyvinylidene difluoride membranes was performed with a polyclonal anti-ezrin antibody (Andreoli *et al.*, 1994). Before solubilization of proteins in Laemmli buffer, we checked that the amounts of phagosomes recovered in all the pellets were constant by measuring the OD at 600 nm.

Fluorometric Assay of Actin Polymerization

Polymerization of G-actin (10% pyrenyl-labeled) was carried out exactly as described by Cooper (1992) in the presence of 10 nM unlabeled F-actin seeds when indicated in the legend to Figure 1. Nucleation and polymerization of pyrene G-actin (in the absence of F-actin) was performed in P buffer at 25°C for 10 min immediately after addition of salts (50 mM KCl, 1 mM $MgCl_2$). An increase of fluorescence was followed in an Aminco-Bowman Series 2 Luminescence Spectrometer (SLM-Aminco Inc., Northampton, MA). Excitation and emission wavelengths were 365 and 407 nm, respectively.

Specificity of the KT10 Anti-PI(4,5)P_2 Antibody

The highly specific mAb KT10 against PI(4,5)P_2 has been shown to be effective in blocking PI(4,5)P_2-regulated functions in many systems (Fukami *et al.*, 1988; Matuoka *et al.*, 1988; Uno *et al.*, 1988; Gilmore and Burridge, 1996; Mayer *et al.*, 2000). According to the manufacturer's data (Assay Designs, Inc., Ann Arbor, MI; Fukami *et al.*, 1988; Matuoka *et al.*, 1988), the KT10 anti-PI(4,5)P_2 antibody gives <0.2% cross-reactivity for phosphatidylinositol, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, cardiolipin, cholesterol, or diacylglycerol. It cross-reacts with PI(4)P at similar low levels but has slightly higher cross-reactivity with phosphatidic acid (<5%). We also set up an ELISA assay by coating each lipid on ELISA plates. A strong signal was seen with PI(4,5)P_2, but there was no significant detection of antibody binding to PI(4)P. A low degree of cross-reactivity was obtained with PI(3,4)P_2 (results not shown). As additional evidence of antibody specificity in the fluorescence-activated cell sorter (FACS) analysis (see below), the phagosome labeling was abolished when the antibody was preincubated with PI(4,5)P_2. A similar preincubation with PI(4)P or PI showed no difference in the signal relative to control phagosomes (results not shown). Collectively, these data indicate that the KT10 antibody is highly specific for PI(4,5)P_2.

Immunofluorescence Labeling and FACS Analysis of Phosphoinositides on Phagosomes

Phagosome preparations were incubated for 5 min at room temperature with monoclonal anti-PI(4,5)P_2 antibody KT10 (1:25 diluted; Assay Designs, Inc., Ann Arbor, MI) in a minimal volume of PBS, 0.03% fish-skin gelatin, and protease inhibitors, followed by 5 min of incubation with a fluorescein-labeled antimouse IgG (Dianova, Hamburg, Germany) in the same buffer. In parallel, a control sample was prepared by incubating phagosomes under the same conditions but without the primary antibody. The samples were then gently fixed in the same tube with 1% paraformaldehyde/PBS, and FACS analysis (Becton Dickinson, San Jose, CA) was performed by acquisition of 10,000 events. Quantification of positive phagosomes corresponded to the percentage of individual phagosomes incubated with both antibodies and having a fluorescence intensity higher than that of phagosomes incubated with the secondary antibody alone. The errors reported are the population SDs from at least three separate reactions. PLC-treated and mock-treated phagosomes were also assessed for their PI(4,5)P_2 content by indirect immunofluorescence microscopy as described above, but without fixation.

^{32}P-Labeling of Phospholipids

Phagosomes were incubated as in the actin assembly assay, except that the ATP concentration in the P buffer was lowered to 0–20 μM and 10 μCi of γ-^{32}P[ATP] (10 mCi/ml, Amersham) was added. After the incubation, the volume was adjusted up to 50 μl with buffer before addition of 50 μl 1N HCl and 200 μl methanol/chloroform (1:1, vol/vol). The organic phase was collected and washed with an equal volume of 1 M HCl/chloroform (1:1, vol/vol). The sample was dried under vacuum and dissolved in chloroform/methanol/water (75:25:2, vol/vol). Subsequently, ^{32}P incorporation into the lipids was quantified in a scintillation counter, or the lipids were separated by TLC on Silica Gel G60 plates [pretreated with 1% potassium oxalate/2 mM EDTA in methanol/water (1:1, vol/vol)] using a solvent mixture of chloroform/acetone/methanol/glacial acetic acid/water (80:30:26:24:14, vol/vol) (Norris and Majerus, 1994). Phospholipid standards [PI, PI(4)P, PI(4,5)P_2, PI(3,4,5)P_3, and PA] were stained with iodine. The quantification of ^{32}P-labeled phospholipids separated by TLC was performed with a Fujifilm Imaging Plate and Fujifilm Fluorescent Image Analyzer FLA-2000 equipment (Fujifilm, Elmsford, NY).

Deacylation of Phosphoinositides and High-Pressure Liquid Chromatography Analysis

Spots scraped from TLC plates were incubated with 1.5 ml methylamine reagent (5.77 ml 25% methylamine in water, 6.16 ml methanol, and 1.54 ml 1-butanol) at 53°C for 50 min. The samples were subsequently dried under vacuum, and the lipids were redissolved in 1 ml water and redried. The residue was subsequently dissolved in 600 μl water and extracted with 700 μl 1-butanol/petroleum ether/ethyl formate (20:4:1, vol/vol). The upper phase, containing the fatty acids, was discarded, and the lower phase was washed twice in 700 μl of the above solvent mixture, dried under vacuum, and dissolved in water for SAX high-pressure liquid chromatography (HPLC) analysis.

RESULTS

Membrane-associated Proteins from Salt-Stripped Phagosomes Inhibit Actin Assembly, Which Can Be Rescued by PI(4,5)P_2

We previously showed both in vitro and in vivo that 2-h phagosomes (1-h pulse of beads, followed by another 1-h chase) were active in the process of in vitro actin assembly, but 12-h LBPs (1-h pulse, followed by an 11-h chase) were inactive, whereas 24-h phagosomes regain a high activity (Defacque *et al.*, 2000b). The 2-h, active LBPs lost most of their actin-assembling capacity when treated with 1.3 M NaCl. When we added the ensuing phagosome-derived salt extract to the 2-h salt-stripped phagosomes, actin assembly was restored (Defacque *et al.*, 2000b). Here, we show that when the salt extract of 2-h phagosomes was mixed with pyrene G-actin and F-actin seeds in the absence of membranes, actin polymerization was inhibited in a dose-dependent manner (Figure 1A). As shown in Figure 1B, the extract from 2-h phagosomes could completely inhibit the polymerization of actin from seeds, whereas the extract from 12-h phagosomes, which is inactive in rescuing actin assembly on salt-stripped 2-h LBPs (Defacque *et al.*, 2000b), inhibited actin polymerization only partially. Thus, the 2-h salt extract, whose components behave as a positive effector of actin assembly in the presence of membranes, inhibits actin polymerization when free in solution.

The LBP membrane is known to contain many ABPs (Desjardins *et al.*, 1994a; Dermine *et al.*, 2001; Garin *et al.*, 2001), and a number of these ABPs, such as ezrin, moesin, gelsolin, capping proteins, profilin, and cofilin, are known to bind PI(4,5)P_2 and other PIPs (Isenberg *et al.*, 1996). We therefore tested the effects of different phosphoinositides in combination with the 2-h salt-stripped extract on pyrene actin assembly (in the absence of membranes). As seen in Figure 1C, micelles of some pure phosphoinositide lipids could completely rescue the ability of pyrene actin to elongate from seeds. In the presence of PI(4,5)P_2 or PI(3,4,5)P_3, the rate of polymerization and relative levels of total polymerized actin were similar to those measured with actin alone (Figure 1C). PI(3,4)P_2, PI(4)P, and PI were much less efficient in the restoration of actin polymerization. The above data indirectly suggest that ABPs that can bind PI(4,5)P_2 or PI(3,4,5)P_3 must be present on phagosomal membranes.

Effects of Inhibitors of PI 3- and 4-Kinases

Because PI(3,4,5)P_3 rescued the salt-stripped extract–induced inhibition of pyrene actin assembly (Figure 1C), we tested the effects of wortmannin and LY294002, two inhibitors of PI 3-kinases. As determined by fluorescence microscopy, preincubation of 2-h phagosomes with such inhibitors (at concentrations up to 1 μM), or adding them during the assay, had no effect on actin assembly on LBPs (our unpublished results). This argues that the synthesis of the products of PI 3-kinases is not necessary for actin assembly on phagosomal membranes. However, the addition of 200 μM adenosine, an inhibitor of type II PI 4-kinase (Fruman *et al.*, 1998; Barylko *et al.*, 2001), to the LBP actin assembly assay led to an ~60% inhibition in actin assembly (Figure 2A). This suggests that the synthesis of PI(4)P or its downstream product PI(4,5)P_2 is necessary for actin assembly on the phagosomal membrane. That adenosine had the expected inhibitory effect on PIP synthesis is shown below by TLC analysis.

Labeling of Phagosomes with Antibodies against PI(4,5)P_2 and PI(4)P

We next investigated the presence of PI(4,5)P_2 on LPB using a well-characterized monoclonal anti-PI(4,5)P_2 antibody (see MATERIALS AND METHODS). We also compared the labeling of LBPs of different ages, because the ability of phagosomes to assemble actin fluctuates with their maturation state in the cell (see INTRODUCTION) (Defacque *et al.*, 2000b). As determined by flow cytometry, there was a relatively low percentage of PI(4,5)P_2-labeled phagosomes at the 30-min time point, with a significant rise at the 2-h stage (Figure 2B). Both the 12-h and 24-h LBPs were poorly labeled (Figure 2B). The rise and fall over the first 12 h coincides well with the ability of phagosomes to assemble actin (Defacque *et al.*, 2000b). The absence of labeling on 24-h LBPs may be a result of epitope inaccessibility (see below). Flow cytometry analysis of 2-h phagosomes also showed that the treatment of phagosomes with 1.3 M NaCl had no effect on their labeling with anti PI(4,5)P_2, arguing that the lipid is not extracted by the salt treatment (our unpublished results).

Blocking PI(4)P or PI(4,5)P_2 with Antibodies Inhibits Phagosomal Actin Assembly

We next attempted to functionally inhibit phosphoinositide function, or synthesis, in the context of actin assembly. The phagosome system has the advantage that reagents can be preincubated with the organelles, followed by a reisolation via flotation to remove unbound reagent (Defacque *et al.*, 2000a,b). We took advantage of this approach, in combination with antibodies against PI(4)P and PI(4,5)P_2.

Preincubation of 2-h phagosomes with the highly specific monoclonal anti-PI(4,5)P_2 antibody (see MATERIALS AND METHODS) followed by flotation of the organelles gave a significant inhibition of phagosomal actin assembly, whereas the anti-PI(4)P antibody gave a lesser degree of inhibition (Figure 2C). A similar inhibition was obtained with a rabbit anti-PI(4,5)P_2 antibody (a gift of Dr. T. Yoshioka; our unpublished results). This result suggests that a preexisting pool of PI(4,5)P_2, and/or its more abundant precursor PI(4)P (see below), is involved in LBP actin assembly.

PI-PLC (Divecha and Irvine, 1995) is known to significantly decrease total PI(4,5)P_2 levels in mammalian cells (Eberhard *et al.*, 1990; Ross *et al.*, 1992) and to block both

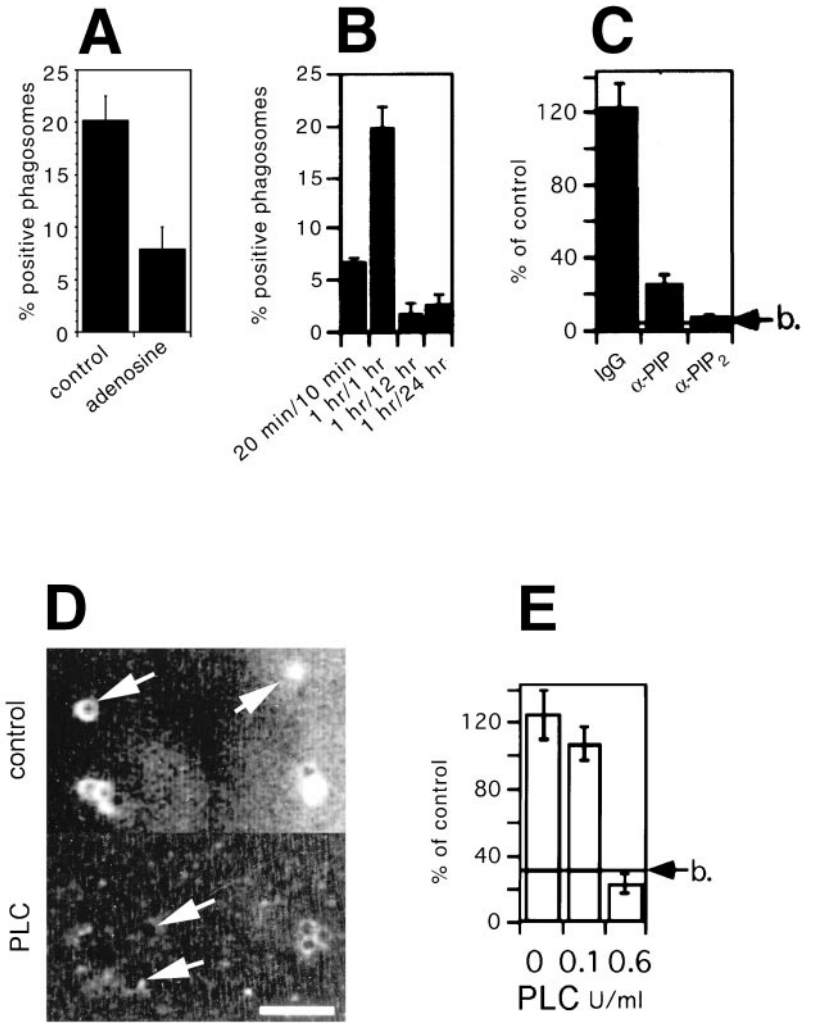

Figure 2. Involvement of PI(4,5)P_2 in actin assembly. (A) Actin assembly on 2-h phagosomes in the absence (control) or presence of 200 μM adenosine. (B) Phagosomes purified after different pulse/chase times of beads in cells were analyzed for their PI(4,5)P_2 content, via antibody labeling, followed by flow cytometry analysis. The same treatment of noninternalized beads, Triton X-100–pretreated phagosomes, or incubation of phagosomes with the secondary antibody alone gave no detectable signal (data not shown). (C) 2-h phagosomes were preincubated for 5 min at 25°C with an irrelevant antibody (IgG), anti-PI(4)P antibodies (α-PIP), or anti-PI(4,5)P_2 antibodies (α-PIP$_2$). Actin assembly on phagosomes after antibody preincubation was assayed by microscopy. Results show the percentage of positive phagosomes for each sample relative to control phagosomes. (D) 2-h phagosomes were pretreated with 0 (control) or 0.6 U/ml bacterial PI-PLC and immediately labeled for PI(4,5)P_2 by indirect immunofluorescence microscopy. Arrows show individual phagosomes seen by phase-contrast microscopy. Bar, 5 μm. (E) In parallel experiments, 2-h phagosomes were pretreated in the absence or presence of 0.1 or 0.6 U/ml bacterial PI-PLC. Actin assembly on subsequently reisolated phagosomes was then assayed by microscopy. RESULTS show the percentage of positive phagosomes for each sample relative to control (untreated) phagosomes. b indicates the value obtained with fish-skin gelatin–coated beads.

PI(4,5)P_2 synthesis and vacuole fusion in yeast (Mayer *et al.*, 2000). Pretreatment of phagosomes with this phospholipase drastically lowered their PI(4,5)P_2 content, as seen by immunofluorescence microscopy (Figure 2D). After their reisolation, PLC-treated 2-h phagosomes (Figure 2E) as well as 24-h phagosomes (our unpublished results) completely lost their ability to polymerize actin. These data provide further evidence for a role for PI(4)P and/or PI(4,5)P_2 in the actin assembly process.

In Vitro Synthesis of PI(4)P and PI(4,5)P_2

To investigate whether active synthesis of phospholipids, and in particular PI(4,5)P_2, accompanied phagosomal actin assembly, isolated phagosomes or latex beads were incubated with 10 μCi γ-^{32}P–labeled ATP in P buffer (see MATERIALS AND METHODS) and up to 20 μM unlabeled ATP. The standard actin assembly assay contains 0.2 mM ATP. To achieve efficient incorporation of ^{32}P, we routinely reduced the concentration of unlabeled ATP to 10–20 μM. At this concentration, the level of phagosomal actin assembly was modestly reduced relative to the standard 200 μM (~30%; our unpublished results). At 5 μM, however, no polymerization was observed (our unpublished results).

The incubation of latex beads alone at room temperature or phagosomes at 4°C with [γ-^{32}P]ATP resulted in a low nonspecific binding of label, whereas phagosomes incubated at room temperature for 15 min incorporated much higher amounts of ^{32}P into the lipid fraction (Figure 3A), suggesting that lipids in the phagosomal membrane were indeed phosphorylated during the assay. This phosphorylation of lipids was further shown to be time-dependent and formed a plateau at 15 min, a time corresponding to the end of the actin assembly assay (our unpublished results). When the actin/Tβ4 mixture was omitted from the assay, no significant change in the amount of ^{32}P incorporated into lipids was observed (Figure 3A). Most of the subsequent experiments were carried out without the actin/Tβ4 mixture.

Analysis of the ^{32}P-labeled phosphoinositides using TLC revealed several labeled species, including an abundant spot that comigrated with PI(4)P and a less dense spot that comigrated with PI(4,5)P_2 (Figure 3B). Of the ^{32}P-labeled phos-

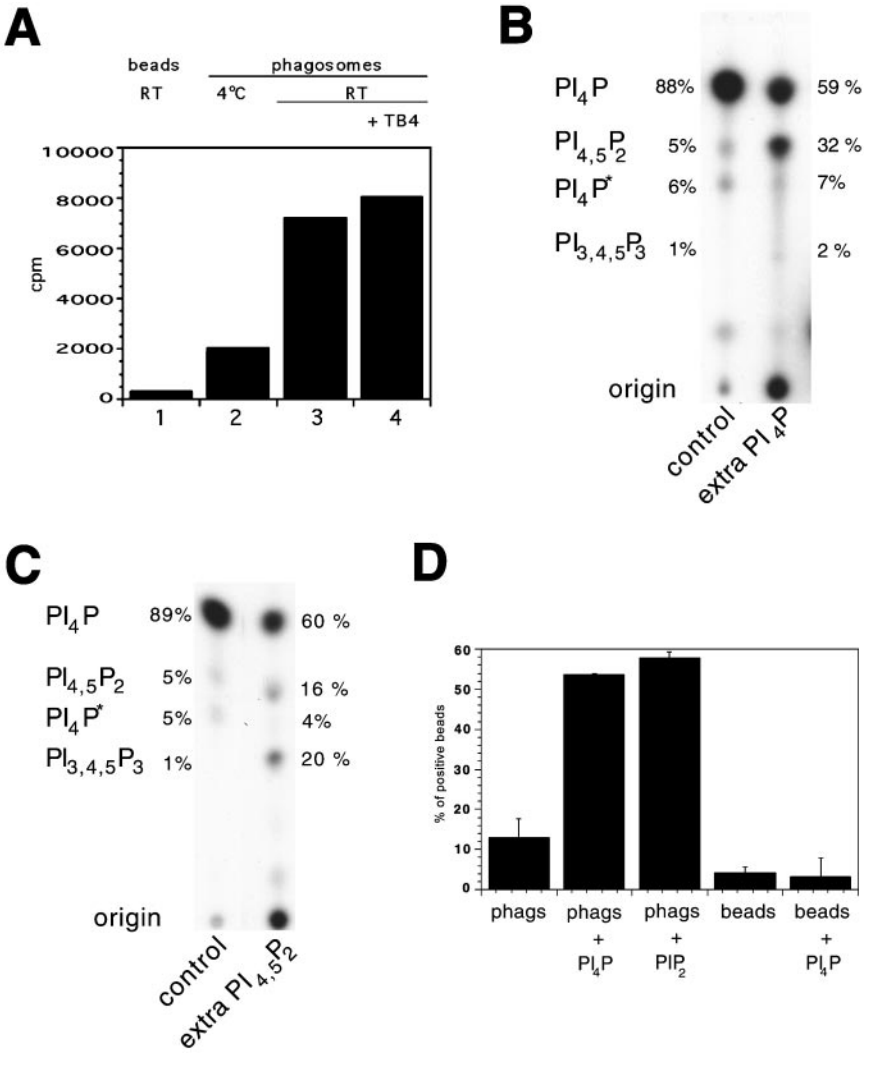

Figure 3. Phosphorylation of phagosomal lipids. (A) Phagosomes (lanes 2–4) or beads (lane 1) were incubated with [γ-^{32}P]ATP. The reactions were either kept on ice (lane 2) or kept at room temperature (lanes 1, 3, and 4) for 15 min. Phagosomes were incubated either without (lanes 1–3) or with actin/thymosin β4 (lane 4). Lipids were extracted as described in MATERIALS AND METHODS, and ^{32}P incorporation was quantified in a scintillation counter. Although the absolute values we observed varied greatly between experiments, the pattern shown in this experiment was seen consistently (5 experiments). In B and C, the ^{32}P-labeled lipids were separated by TLC on Silica Gel G60 plates, followed by autoradiography. Unlabeled PI(4)P (0.2 mg/ml) (B) or PI(4,5)P_2 (0.2 mg/ml) (C) were coincubated with phagosomes. The identity of the spots, as determined by HPLC, is indicated on the left. The relative amounts of radioactivity in each lipid spot were quantified by use of a Fujifilm Imaging Plate and Fluorescent Image Analyzer and expressed as a percentage of the total counts in the PIP species (indicated next to the lanes). PI_4P* indicates the second form of PI(4)P. The total counts for the signal detected in the PIPs are (B) control, 7800; extra PI_4P, 5100; (C) control, 47,700; extra $PI_{4,5}P_2$, 32,700. (D) Phagosomes (phags) or beads were coincubated with PI(4)P or PI(4,5)P_2 (0.2 mg/ml) and tested, along with untreated phagosomes/beads, in the actin assembly assay. The signal per phagosome was also distinctly higher in the LBPs preincubated with PI(4)P or PI(4,5)P_2.

pholipids that were detected in the total lipid fraction, ~90% were PIPs, and of this pool, ~90% were in PI(4)P, whereas ~5% comigrated with a PI(4,5)P_2 standard. The remaining 5% of total label in the PI(4)P fraction went into a spot that migrated more slowly than the PI(4,5)P_2 spot [subsequently identified by HPLC as being another variant of PI(4)P; see below]. A low signal in a species comigrating with phosphatidic acid was usually detected. In a few experiments, we also detected a minor amount of label in a species that comigrated with PI(3,4,5)P_3; in most experiments however, this species was not observed. When the labeled ATP was mixed with 0.2 mM ATP (the concentration used in the actin assembly assay), after a longer exposure, again the only PIPs detected were PI(4)P and PI(4,5)P_2, at ratios similar to those seen at low ATP (our unpublished results).

To identify more definitively the species of phosphoinositides that were synthesized in the phagosomal membrane, the relevant lipids were scraped from the TLC plates, deacylated, and subjected to HPLC analysis (Figure 4A). Only two phosphoinositides were observed under standard conditions. The major species synthesized after 10 min was PI(4)P (~90%). The two different TLC spots [indicated as PI(4)P and PI(4)P* in Figure 3B] were resolved as a single species with HPLC. These two TLC species must therefore differ in their fatty acid tails, which affect the migration on TLC but are removed for the HPLC analysis. Importantly, no significant label was incorporated into PI(3)P, a lipid that is essential for endosome (and probably phagosome) fusion (Simonsen *et al.*, 1998).

The second major synthesized species seen by TLC was confirmed by HPLC to be PI(4,5)P_2 (Figure 4A), representing ~6% of the total label, again consistent with the TLC results. PI(3,4)P_2 and PI(3,5)P_2 were not detected. The triple phosphorylated species PI(3,4,5)P_3 was again detected occasionally in a few experiments as a minor species (results not shown).

In conclusion, these data show that under actin assembly conditions, only two phosphoinositides were routinely synthesized by phagosomal membranes, with ~94% being incorporated into the two isoforms of PI(4)P and ~6% into PI(4,5)P_2. These three species represented the bulk of total phospholipid synthesized from ATP in

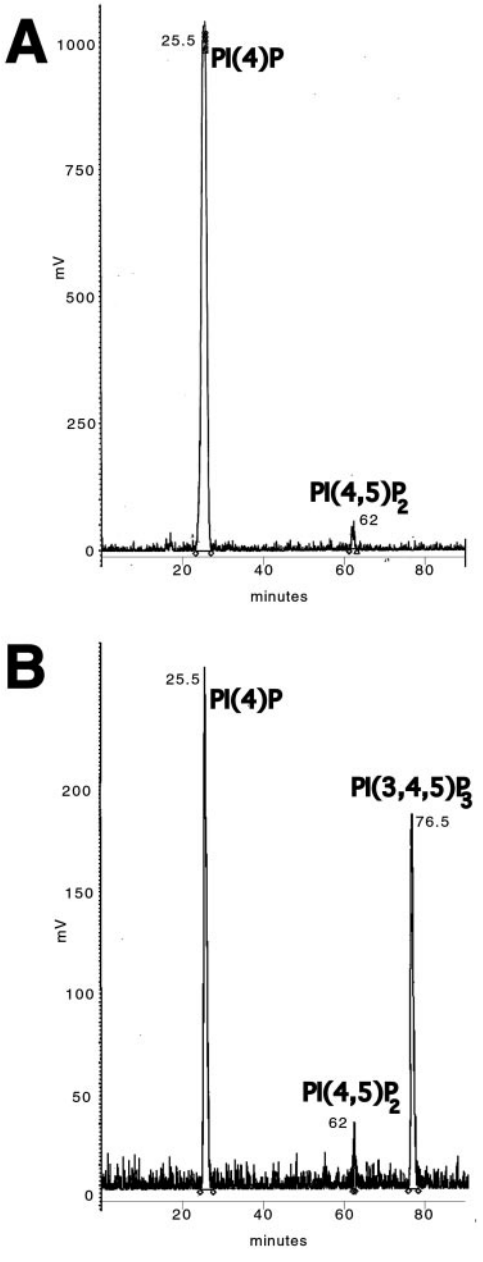

Figure 4. HPLC analysis of ^{32}P-labeled lipids. All the spots comigrating with phosphoinositide standards were scraped together from TLC plates of 2-h phagosomes (A) and 2-h phagosomes with the addition of unlabeled $PI(4,5)P_2$ (B), and after deacylation, they were analyzed by HPLC. The peak elution times of the different phosphoinositides [PI(4)P, $PI(4,5)P_2$ and $PI(3,4,5)P_3$] are indicated at the peaks.

vitro. The routine absence of newly synthesized $PI(3,4)P_2$ is significant, because a transient rise in this lipid has been correlated with actin assembly in some systems (Apgar, 1995; Gratacap *et al.*, 1998). Our experiments show clearly that PI kinases are present and active on the LBP membrane in the absence of GTP or cytosolic components.

Coincubation of Phagosomes with PI(4)P or $PI(4,5)P_2$ During Actin Assembly: Effects on Actin Assembly and on PI(4)P Synthesis

Because phagosomes synthesized both PI(4)P and $PI(4,5)P_2$ during the actin assembly assay, we next investigated the effects of adding extra amounts of these PIPs to the assay. Strikingly, the addition of PI(4)P resulted in a dramatic increase (approximately fivefold) in the percentage of phagosomes that assembled actin (Figure 3D). When LBPs that had been preincubated with PI(4)P were labeled with [γ-^{32}P]ATP, TLC analysis revealed a significant incorporation of the γ phosphate from ATP into $PI(4,5)P_2$ (Figure 3B). When the LBPs were preincubated with $PI(4,5)P_2$, the levels of actin assembly were even higher than with the PI(4)P preincubation (Figure 3D); under this condition, TLC analysis showed a significant increase in the synthesis of $PI(3,4,5)P_3$. Thus, incorporation of $PI(4,5)P_2$ into the LBP membrane must have activated a PI 3-kinase. The increase in $PI(4,5)P_2$ after preincubation with PI(4)P (data not shown) and of $PI(3,4,5)P_3$ after preincubation with $PI(4,5)P_2$ (Figure 4B) was confirmed by HPLC analyses.

Effects of PI 3- and 4-Kinase Inhibitors on Phosphoinositide Synthesis

To gain information on the type of PI 4-kinases that are active on the phagosomal membrane, we tested the effects of wortmannin and adenosine on PI(4)P and $PI(4,5)P_2$ synthesis. At a concentration of 1 μM, wortmannin inhibits not only PI 3-kinases but also certain type III PI 4-kinases (Downing *et al.*, 1996; Gehrmann and Heilmeyer, 1998). At this concentration of the drug, we observed only a minor inhibition of the level of PI(4)P and $PI(4,5)P_2$ synthesis ($<$10%; our unpublished results), suggesting that type III PI 4-kinases are not significantly active in our assay.

The other class of PI 4-kinases, type II PI 4-kinases, are generally inhibited by adenosine (Barylko *et al.*, 2001; Endemann *et al.*, 1987). As described above, this drug inhibits actin assembly on phagosomal membranes by 60% (Figure 2A). The addition of 200 μM adenosine to our TLC assay inhibited the synthesis of PI(4)P $>$ 95% (Figure 5A), suggesting that a type II PI 4-kinase is present and active on the LBP membrane. The synthesis of $PI(4,5)P_2$ was also significantly reduced after this treatment ($\sim$75%). Because adenosine inhibits both actin assembly and PIP synthesis, it provides further evidence for a correlation between these two processes on phagosomal membranes.

Both Phagosome Maturation and Salt Stripping Affect the Levels of Phagosomal PI(4)P and $PI(4,5)P_2$ Synthesis

We next investigated PIP synthesis under conditions in which actin assembly on phagosomal membranes is poor. As mentioned, phagosomes fluctuate in their ability to assemble actin during maturation. We therefore compared the amount of PIPs produced during the actin assembly assay by 2-h (active) and 12-h (inactive) LBPs. Equal amounts of phagosomes (determined by OD_{600}; Blocker *et al.*, 1997) of the two different time points were included in the assay. TLC analysis showed that the synthesis of PI(4)P and

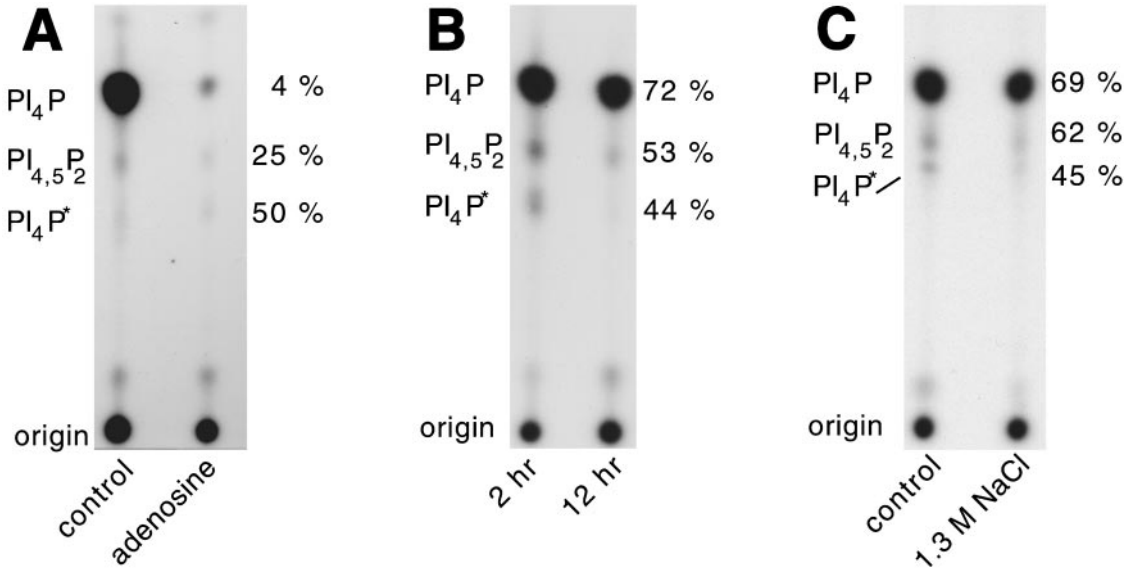

Figure 5. Correlation between actin assembly and PI(4,5)P_2 synthesis. (A) 2-h phagosomes were ^{32}P-labeled in the presence of 200 μM adenosine. Lipids were analyzed by TLC. (B) Equal amounts of 2-h and 12-h phagosomes were analyzed and quantified. (C) 2-h phagosomes were salt-stripped and subsequently repurified on a sucrose gradient. Equal amounts of control phagosomes and salt-stripped phagosomes were analyzed. In all panels, the lipid species (as determined by HLPC) are indicated on the left. Shown on the right is the level of ^{32}P incorporation into each lipid species compared with control (2-h) phagosomes, quantified as in Figure 3. The total counts for the signal detected in the PIPs are (A) control, 76,000; adenosine, 4100; (B) 2 h, 33,900; 12 h, 23,100; (C) control, 90,200; 1.3 M NaCl, 60,900.

PI(4,5)P_2 was significantly reduced on the 12-h phagosomes (Figure 5B).

As previously shown (Defacque *et al.*, 2000b), salt stripping of phagosomal membranes with 1.3 M NaCl results in a 70% reduction of actin assembly. When 2-h phagosomes were salt-stripped, incubated with [γ-^{32}P]ATP, and subsequently analyzed by TLC, we observed a slight (~30%) reduction in the amount of both PI(4)P and PI(4,5)P_2 synthesized (Figure 5C). This result has two implications. First, a large fraction of the phagosomal membrane-bound PI 4- and 5-kinases are not removed by the salt treatment (which removes the bulk of ezrin and moesin). Second, the finding that two conditions (salt stripping and ageing) that strongly inhibit actin assembly only modestly inhibit PI(4)P and PI(4,5)P_2 synthesis suggests that a relatively high threshold concentration of these lipids may be required (perhaps as a local patch) on the LBP membrane for the actin assembly process to be switched on. An alternative possibility is that there may be distinct PIP-dependent and -independent processes that assemble actin on the membrane.

An Ezrin Mutant Defective in PI(4,5)P_2 Binding Nucleates and Binds Poorly to Phagosomal Membranes

We recently reported that ezrin and moesin are essential for actin assembly on phagosomes (Defacque *et al.*, 2000b). Ezrin binds in vitro to PI(4,5)P_2 via its N-terminal domain (Niggli *et al.*, 1995), and it can also bind to protein receptors in the membrane, as well to adaptors, which themselves are associated with membrane components (Hirao *et al.*, 1996; Tsukita and Yonemura, 1997; Heiska *et al.*, 1998; for review, see Bretscher, 1999; Mangeat *et al.*, 1999). In this study, an ezrin mutant with a significantly reduced affinity for PI(4,5)P_2 (Barret *et al.*, 2000) bound much less to salt-stripped phagosomes than did wt ezrin (Figure 6A). The ezrin mutant was also much less efficient at reconstituting actin assembly on the salt-stripped LBPs (Figure 6B), presumably a consequence of its lower affinity for phagosomes. These results suggest that in the standard LBP actin assembly assay, ezrin/moesin needs to interact with PI(4,5)P_2 for the membrane-dependent actin assembly process to proceed optimally.

DISCUSSION

We show here, using a defined in vitro membrane system, that the presence of a preexisting pool, as well as active synthesis of PI(4,5)P_2 and probably also PI(4)P, is essential for efficient actin assembly induced by phagosomal membranes, thus extending a large body of evidence that has strongly implicated these phosphoinositides in actin assembly (Lassing and Lindberg, 1985; Eberle *et al.*, 1990; Gilmore and Burridge, 1996; Gachet *et al.*, 1997; DiNubile, 1998; Isenberg and Niggli, 1998; Ma *et al.*, 1998; Janmey *et al.*, 1999; Rozelle *et al.*, 2000). Collectively, our data argue that an active turnover of D4 and D5 PIPs may be required for the ezrin/moesin–facilitated process of actin assembly to proceed on the LBP membrane. An unexpected finding in our studies was that PI 3-, PI 4-, and PI 5-kinases are present on the LBP membrane and can be activated by a low level (0.2 mM) of ATP, even in the absence of GTP or cytosolic factors. However, the PI 3-kinases are not essential for LBP actin

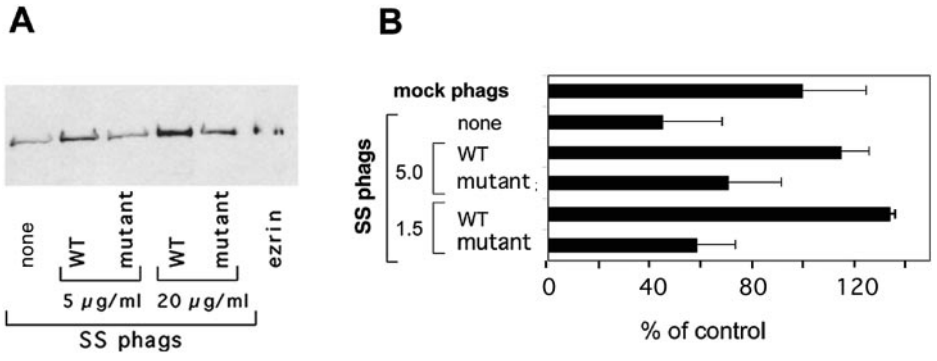

Figure 6. Actin assembly on phagosomes requires PI(4,5)P_2/ezrin interactions. (A) 2-h salt-stripped phagosomes were preincubated with a control buffer (none), or with wild-type (WT) or a mutant ezrin defective in PI(4,5)P_2-binding at the indicated concentrations. After preincubation the phagosomes were repurified and the amount of ezrin bound to phagosomes was analyzed by Western blotting. Densitometric quantitation of the ezrin signal on phagosomes gave the following values for the different lanes (in percent, from left to right): 100%, 143%, 87%, 199%, 125%. (B) Actin assembly activity was assayed by fluorescence microscopy. The results show the percentage of positive phagosomes for each sample relative to control (mock) phagosomes (phags). The addition of 5 or 1.5 μg of WT or mutant ezrin to the salt-stripped phagosomes is indicated. The errors reported are the SDs from at least three independent experiments. Higher amounts of ezrin and phagosomes were used in (A) [100 μl vs. 6 μl in (B)], because ezrin could not be detected by Western blot below 5 μg/ml.

assembly, because inhibitors of these enzymes had no effect on the process.

The actin assembly by LBPs that we analyzed is a specific process that probably requires many components besides ezrin/moesin and PIPs to assemble the machinery on the membrane. We recently found that the sphingolipids, sphingomyelin, ceramide, sphingosine, and sphingosine-1-phosphate in the LBP membrane are also major regulators of this process (Bos *et al.*; manuscript submitted). That the membrane environment is important for actin assembly to occur on the LBP membrane is further supported here by our findings that the salt extract of active 2-h LBPs, which allows actin to assemble on salt-stripped phagosomes, strongly inhibited the polymerization of pure actin free in solution. We suggest that this inhibition is caused by phagosome-derived PI(4,5)P_2-binding, actin-capping proteins, two of which, CapG and CapZ, are detected on LBPs (Garin *et al.*, 2001); such a proposal would be in agreement with a similar scenario put forward by DiNubile (1998). Consistent with this notion, the addition of PI(4,5)P_2 to the extract allowed pyrene actin polymerization to proceed at the same rate as that of actin alone, presumably by inactivating the actin barbed-end capping function of proteins present in the extract.

As pointed out in the INTRODUCTION, one can classify two groups of ABPs that bind PIPs and could be important for phagosomal actin assembly. Gelsolin is a good candidate among the first category of ABPs that do not bind PIPs and actin simultaneously. The N-terminal three domains of gelsolin (G1–3) can sever and cap, but not nucleate, actin filaments in the absence of calcium (Way *et al.*, 1989). When phagosomes were preincubated with G1–3 and then reisolated on gradients, their subsequent ability to polymerize actin was significantly enhanced (Defacque *et al.*, 2000a). It is possible that gelsolin can bind to and influence the activity of signaling molecules, such as phospholipases C and D (Steed *et al.*, 1996; Baldassare *et al.*, 1997; Sun *et al.*, 1997), that regulate the actin assembly process; pharmacological evidence suggests that these enzymes are also present and active on isolated phagosomes (our unpublished data). However, preincubation or coincubation of LBPs with gelsolin G1–3 had no effects on the synthesis of PI(4)P, P(4,5)P_2, or phosphatidic acid by phagosomes (results not shown). Gelsolin has also been found to be phosphorylated by Src in the presence of PI(4,5)P_2 (De Corte *et al.*, 1997).

Of the second category of PI(4,5)P_2-binding proteins (which can bind PIPs and actin simultaneously), ezrin and moesin are clearly essential for actin assembly by phagosomes (Defacque *et al.*, 2000b). The ERM proteins, as well as talin, bind in vitro to PI(4,5)P_2 via their N-terminal domains (Niggli *et al.*, 1995), and this interaction, along with phosphorylation, has been proposed to induce an open conformation of the molecule, as is the case for vinculin (Gilmore and Burridge, 1996). In addition, talin has been shown to nucleate actin when bound to PI(4,5)P_2 vesicles (Isenberg *et al.*, 1996). Both PI(4,5)P_2 binding and phosphorylation have been proposed to facilitate stabilization of these proteins or a more efficient binding to their various transmembrane receptors, such as CD44 (which by immune electron microscopy is present in J774 cells and in small amounts on LBPs) or intercellular adhesion molecule-ICAM-1 and -2 (Hirao *et al.*, 1996; Heiska *et al.*, 1998; Legg and Isacke, 1998; Nakamura *et al.*, 1999). It also remains to be established whether ezrin and/or moesin phosphorylation plays any role in the LBP system. However, neither genistein (an inhibitor of tyrosine kinases) nor staurosporine (an inhibitor of protein kinase C and other kinases) had any effect on the standard phagosomal actin assay (our unpublished data).

An ezrin mutant unable to bind PI(4,5)P_2 binds poorly to phagosomes and is a less potent stimulator of actin assembly on phagosomal membranes. This PI(4,5)P_2-independent binding of the mutant ezrin to LBP is probably a result of interactions with membrane receptors, and it seems logical to suggest that binding of newly synthesized PI(4,5)P_2 to already receptor-bound ezrin/moesin may help to transiently stabilize an active conformation of these proteins on the phagosomal membrane, a process essential for the membrane-dependent actin assembly process. That binding of proteins to PI(4,5)P_2 can change their structure is well established (Raghunathan *et al.*, 1992; Lu and Chen, 1997; Tuominen *et al.*, 1999; for review, see Janmey *et al.*, 1999). It should be noted that in vitro ERM proteins bind significantly better to PI(4,5)P_2 than to PI(4)P (Niggli *et al.*, 1995). This fact induces us to believe that in our system, the PI(4,5)P_2 that is

synthesized may be more crucial for the ezrin-dependent actin assembly than is PI(4)P. Nevertheless, because of the many interacting components available and the relatively large amounts of PI(4)P synthesized, we consider it likely that this lipid is also an important player in our system, perhaps bound to different ABPs relative to $PI(4,5)P_2$.

Because preincubation of LBP with anti-PI(4)P and $PI(4,5)P_2$ antibodies blocked their ability to assemble actin, we conclude that a preexisting pool of PIPs may be necessary for the process to occur. This is also consistent with the less efficient binding to phagosomes of an ezrin mutant defective in $PI(4,5)P_2$ binding (Figure 6). However, significant amounts of $PI(4,5)P_2$ and PI(4)P were also synthesized by the LBPs upon incubation with ATP, and this pool seems to be required for efficient actin assembly, because adenosine, an inhibitor of type II PI 4-kinase, could inhibit LBP actin assembly by 60% and the synthesis of PI(4)P and $PI(4,5)P_2$ by 75–90%. The simplest explanation for these results is that these PIPs need to be dynamically synthesized and degraded for actin assembly on the LBPs to occur. A speculative scenario is that the ezrin would remain bound to the phagosomal membrane mostly via relatively stable interactions with protein receptors. In contrast, the binding to $PI(4,5)P_2$ may be more dynamic; conceivably, it may involve on–off interactions controlled by cycles of alternating PIP synthesis and breakdown via PIP 4- and 5-phosphatase activities. Such a scenario may be linked to the complex process by which actin monomers are inserted into the growing actin filaments that are somehow also attached to the membrane surface. The complexity of this process is evident from the fact that not a single model exists in the literature that can incorporate all the necessary steps in this process.

We speculate that $PI(4,5)P_2$ may exist in raft-like microdomains on the LBP after isolation. On activation of cells with agonists or addition of ATP to the in vitro actin assay, PIPs are rapidly synthesized and may aggregate laterally into larger raft domains that may now become intimately associated with the ezrin/receptor complexes (Oliferenko *et al.*, 1999). The rafts may thus provide a platform for the proteins and lipids necessary for actin assembly to occur locally on the LBP membrane. Rafts are now known to be enriched not only in cholesterol and sphingomyelin but also in $PI(4,5)P_2$ (Pike and Miller, 1998; Toomre *et al.*, 2000), and recent studies are increasingly connecting these domains to dynamic actin processes on membranes (Rozelle *et al.*, 2000; Toomre *et al.*, 2000; Caroni, 2001). Pretreatment of LBP with the cholesterol-depleting reagents methyl-β-cyclodextrin or digitonin led to ~50% inhibition of the actin assembly process (E.B., unpublished data). Further, Dermine *et al.* (2001) recently showed that raft subdomains are also present on LBPs prepared identically to the LBPs used in our study; interestingly, in the latter publication, ~20 ABPs were found to be enriched in these Triton X-100–resistant fractions. Whether ezrin or moesin is in this fraction remains to be determined, but it is interesting to note that the amount of Triton X-100–nonextractable ezrin is higher on cell (ezrin) activation (Berryman *et al.*, 1995; Lamb *et al.*, 1997). Finally, type IIIα PI 4-kinase and a PI-phosphatase have been found in raft fractions (see Payrastre *et al.*, 2001).

The Arp2/3 complex, N-WASP, Cdc42, and partners that undoubtedly nucleate actin under some conditions (Machesky and Gould, 1999; Cooper and Schafer, 2000; Pollard *et al.*, 2000) are present on phagosomes (Garin *et al.*, 2001; our unpublished data), but this whole complex is unlikely to be involved in the LBP actin assay, because no GTP is present. Further, GTPγS, toxin B, and C3 toxin (which cleave or inactivate Rho proteins), as well as the N-WASP WA domain (a potent regulator of the Arp2/3 system, which stimulated cytosolic actin assembly in our hands) had no effect (our unpublished data). We suggest that in the cell, the ezrin/moesin–facilitated process is responsible for the primary membrane nucleation of actin on phagosomes, whereas the Arp2/3 system might drive secondary nucleation (branching) from the sides of these primary actin filaments (see Amann and Pollard, 2001, and references therein).

Actin assembly by the phagosomal membrane is likely to be a highly complex process, even as it occurs in a technically simple, GTP-free in vitro system. The results shown here nevertheless highlight an important role for two phosphoinositides in the regulation of this process. Our more recent data extend this complexity by showing that a large cascade of signaling lipids and enzymes communicate with the PIPs and sphingolipids in the LBP membrane. An eventual understanding of this process will require a more complete deciphering of all the relevant protein–protein, protein–lipid, and lipid–lipid interactions, as well as a detailed structural analysis of these components on their specific membrane subdomains. It will also require signaling network analysis, which is now in progress.

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