RESEARCH COMMUNICATION
Phosphatidylinositol 3-kinase activity is required for early endosome fusion

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The homotypic fusion between early endosomes from baby-hamster kidney cells is blocked by addition of the fungal metabolite wortmannin with an IC₅₀ of approx. 15 nM. Over this concentration range, wortmannin has been regarded as a specific inhibitor of phosphatidylinositol (PI) 3-kinase. Further confirmation of the participation of a PI 3-kinase in the fusion reaction has been obtained by demonstrating a sensitivity to an additional, structurally unrelated, PI 3-kinase inhibitor, LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]. Assays constructed such that only the membranous component has been incubated with wortmannin show in vitro fusion to be sensitive to treatment with the drug. Assays in which only the cytosolic component has been treated with wortmannin also showed inhibition of in vitro fusion, but to a lesser extent. PI 3-kinase action almost certainly involves direct regulation of membrane fusion, as no vesicular intermediate has been identified, despite previous extensive morphological examination of in vitro endosome fusions.

INTRODUCTION
Various in vitro assays have identified cytosolic factors which are almost universally required for intracellular membrane-fusion events, as well as factors which are specific for donor-acceptor pairs. In the present study we used an in vitro assay of homotypic early endosome fusion. Previous work with this type of assay has shown a requirement for the universal factors N-ethylmaleimide-sensitive factor (NSF) and α-SNAP (Soluble NSF Attachment Protein) [1,2], as well as an early endosome specific factor, the small GTP-binding protein Rab5 [3]. The involvement of other GTP-binding proteins has also been demonstrated [4,5]. We now demonstrate a further requirement for phosphatidylinositol (PI) 3-kinase activity to enable endosome fusion to proceed efficiently.

Wortmannin was originally identified as an inhibitor of myosin-light-chain kinase [6], but recent work has shown that it will specifically inhibit PI 3-kinase at 100-fold lower concentrations, suggesting that this enzyme is the physiologically relevant target of wortmannin [7–9]. In the present study we have also utilized a structurally unrelated inhibitor of PI 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-benzopyran-4-one (LY294002) [10], to corroborate our results obtained with wortmannin.

Inhibition of complex cellular events by wortmannin at concentrations less than 100 nM have been attributed to inhibition of PI 3-kinase activity. These include suppression of neutrophil outgrowth of PC12 cells (100 nM) [11], neutrophil stimulation (5–50 nM) [7,12,13] and insulin-stimulated GLUT4 translocation (50 nM) [14]. The canonical mammalian PI 3-kinase exists as a stable heterodimer comprising an 85 kDa subunit and a 110 kDa catalytic subunit [15]. The 85 kDa subunit binds the complex to membranes via interaction of SH2 domains with sequences containing a phosphorylated tyrosine residue. Intriguingly, the PI 3-kinase 110 kDa catalytic subunit has been shown to be related to the yeast Saccharomyces cerevisiae Vps34 protein, which is involved in sorting of proteins to the vacuole [16]. This observation led to the idea that mammalian PI 3-kinases might also control features of mammalian cell membrane traffic (reviewed by Liscovitch and Cantley [17]). Effects on internalization of platelet-derived-growth-factor (PDGF) receptor [18] and on fluid-phase endocytosis have recently been reported [19]. It has also been supported by the wortmannin-sensitivity of certain regulated exocytic events [8,14], but these may be unique in their contingency upon signal transduction from the plasma membrane.

METHODS
Materials
Cell-culture reagents were from Gibco, and bovine liver cytosol was prepared according to the method of Waters et al. [10]. Wortmannin was purchased from Sigma. Rabbit polyclonal anti-(PI 3-kinase) was purchased from Transduction Laboratories. This antibody was used for Western blotting at a dilution of 1:250 and detected by enhanced chemiluminescence (ECL; Amersham) following incubation with a horseradish-peroxidase (HRP)-coupled secondary antibody at a dilution of 1:1000. A 125I-labelled secondary antibody was also used for quantification by phosphorimaging.

Internalization and homogenization
The experiments were carried out essentially as described previously [3,21–23]. Briefly, baby-hamster kidney cells (BHK-21) grown on round Petri dishes were washed with PBS. Subsequently, either 3.2 mg/ml avidin or 1.8 mg/ml biotinylated HRP in Dulbecco’s PBS supplemented with 1 mM CaCl₂

Abbreviations used: BHK, baby-hamster kidney; PI 3-kinase, phosphatidylinositol 3-kinase; NSF, N-ethylmaleimide-sensitive-factor; HRP, horseradish peroxidase; PDGF, platelet-derived growth factor.

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and 1 mM MgCl₂ were incubated with the cells for 10 min at 37 °C. This incubation was followed by extensive washing at 4 °C, prior to removal of cells from the dish with a cell scraper and homogenization in a 250 mM sucrose/3 mM imidazole/HCl buffer, pH 7.4, by several passages through a 23-gauge needle. The cells were centrifuged at 4000 g for 15 min, and the resulting postnuclear supernatants containing HRP- or avidin-labelled endosomes were combined in a fusion assay.

Cell-free-fusion assay

Postnuclear fractions were combined in a mixture also containing salts, buffers, biotin–insulin and ATP-regenerating system as described by Gorvel et al. [3], to which had been added the various reagents used in this study. Alternatively, membranes were prepared by pelleting at 100 000 g for 30 min and combined in a fusion assay supplemented with bovine liver cytosol or with BHK cytosol. This mixture was then incubated at 37 °C for 30 min, before lysis on ice with 0.2 % Triton X-100. Fusion was measured by immunoprecipitation of avidin, followed by determination of bound HRP. The total precipitable signal was assayed from a sample lacking biotin–insulin as a competitive quencher. HRP activity was determined at 455 nm using o-dianisidine and H₂O₂ as substrates. This gives a measure of the efficiency of the fusion process.

RESULTS AND DISCUSSION

Inhibition of early endosome fusion by wortmannin

Wortmannin is a potent inhibitor of early endosome fusion (Figure 1). The IC₅₀ was approx. 15 nM. Maximal inhibition of the fusion assay typically approaches 80 %, inhibition of the ATP-dependent fusion signal when wortmannin is added directly to the assay mixture. In the configuration we have performed the experiments of Figure 1, wortmannin is added to an incubation mixture containing 2 mM ATP. Stephens and co-workers have shown that the IC₅₀ for wortmannin inhibition of a canonical PI 3-kinase containing fraction off a mono Q column is shifted from 6 nM to 17 nM when ATP levels are increased from 10 μM to 2 mM [24]. The likely target of the drug is therefore a PI 3-kinase.

The PI 3-kinase inhibitor LY294002 also inhibits the fusion assay over a concentration range, consistent with its inhibition of PI 3-kinase (Figure 2). The measured IC₅₀ of this drug in the endosome fusion assay was 4 μM. The IC₅₀ reported by Vlahos et al. for inhibition of PI 3-kinase activity is 1.4 μM [10].

We isolated membranes by centrifugation from postnuclear supernatants which had been incubated for 30 min at 4 °C with or without 50 nM wortmannin. These membranes were then combined with untreated fresh BHK or bovine liver cytosol (once frozen) in a fusion assay. The membrane-isolation procedure reduces free wortmannin to insignificant levels as the intact pellet is gently washed with homogenization buffer following centrifugation. Significant wortmannin-dependent inhibition (30 ± 3 %) is still observed (Table 1), consistent with the reported action of wortmannin via covalent modification of its target [8] and with a partial membrane localization of the target protein. The degree of inhibition observed was greater when bovine cytosol was used to support the fusion assay (46 ± 9 %). The generally reduced efficiency of inhibition could be due to the fact that incubation with wortmannin was carried out exclusively at 4 °C in this case, or that a cytosolic factor is able to partially substitute for the wortmannin-sensitive membrane factor. In support of this latter interpretation, without excluding the former as a contributory factor, is the inhibition of endosome fusion that we observed when the BHK cytosol alone is pretreated with wortmannin for 20 min at room temperature and desalted by spin-column chromatography prior to addition to the fusion assay mixture. Inhibition by cytosol treatment alone is smaller than inhibition with treated membranes (10 ± 1 %).

We have undertaken Western blotting using an anti-p85 polyclonal antibody which recognizes and α- and β-isoforms

Figure 1  Inhibition of early endosome fusion by wortmannin

Postnuclear supernatants prepared from avidin and biotin-HRP pulse-labelled cells were combined in a fusion assay incubation for 30 min at 37 °C. These incubated samples contained various concentrations of wortmannin. The dose response of the ATP-dependent endosome fusion assay signal to wortmannin is shown, where fusion activity in the absence of wortmannin has been set to 100 %. Error bars represent the S.E.M. associated with duplicate experimental points.

Figure 2  Inhibition of early endosome fusion by LY294002

Postnuclear supernatants prepared from avidin and biotin-HRP pulse-labelled cells were combined in a fusion assay incubation for 30 min at 37 °C. The incubated samples contained various concentrations of the PI 3-kinase inhibitor LY294002. The dose response of the ATP-dependent endosome fusion assay signal is shown, where fusion in the absence of LY294002 has been set to 100 %. Error bars represent the S.E.M. associated with duplicate experimental points.
Table 1  Inhibition of membrane fusion following treatments of different components of the endosome fusion assay with 50 nM wortmannin

Membranes treated with 50 nM wortmannin (+) were isolated by pelleting from a postnuclear supernatant which had been incubated for 30 min at 4 °C in the presence of the drug. These membranes were then combined with untreated fresh BHK cytosol (2 mg/ml final concn.) or bovine liver cytosol (5.4 mg/ml final concn.) which had been once frozen. In control experiments these concentrations of cytosol gave similar fusion efficiencies. BHK cytosol treated with 50 nM wortmannin was incubated for 20 min at room temperature in the presence of the drug, which was then removed by desalting on a (Bio-Rad) Bio-Spin chromatography column. This cytosol was then added to an incubation mixture containing untreated membranes and the fusion signal compared with that obtained using control cytosol. 50 nM wortmannin was also added to BHK postnuclear supernatants, together with other assay components and incubated at 4 °C for 20 min, prior to commencement of a fusion assay by incubation at 37 °C. In this case the wortmannin was not removed prior to the 37 °C incubation. Errors correspond to S.E.M. values for separate experiments for which duplicate data points were obtained. The conclusion drawn from the first two sets of data given is identical in that treatment of the membrane fraction alone is sufficient for significant inhibition by wortmannin.

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Cytosol</th>
<th>Postnuclear supernatant</th>
<th>Percentage inhibition of endosome fusion</th>
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<tbody>
<tr>
<td>+</td>
<td>− (BHK)</td>
<td>− (Bovine liver)</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>+</td>
<td>− (BHK)</td>
<td>+ (Bovine liver)</td>
<td>43 ± 7</td>
</tr>
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<td>−</td>
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<td>− (Bovine liver)</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>−</td>
<td>+ (BHK)</td>
<td>+ (Bovine liver)</td>
<td>72 ± 6</td>
</tr>
</tbody>
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Figure 3  Western blot with anti-p85 of fractions used in the fusion assay

Lane a, BHK membranes (200 μg); lane b, BHK cytosol (200 μg); lane c, bovine liver cytosol (200 μg). Samples were loaded on a 10% acrylamide mini-gel. Detection of an HRP-labelled secondary antibody was accomplished by using an enhanced chemiluminescence system (Amersham); the exposure time was 40 min.

In bovine liver cytosol we detected a single band (Figure 3). In bovine liver cytosol we detected a single band which we have assigned to 85 kDa on the basis of its migration relative to molecular-mass standards and on its co-migration with a single band which is recognized upon blotting a purified preparation of α-p85/p110 (results not shown). Equivalent protein concentrations of BHK cytosol revealed a more complex blotting pattern. At high exposure times, four bands were visible (Figure 3, lane b). Not all of these bands were detectable on a crude membrane fraction for which an equivalent amount of protein was loaded on the gel (lane a). However, a band migrating at about 85 kDa is present in both cytosol and membrane fractions. Phosphorimager analysis of this band using a 125I-labelled secondary antibody revealed it to be 3-fold enriched in BHK cytosol relative to membranes.

When cells are stimulated by insulin, two measurable responses are sensitive to wortmannin: translocation of GLUT4 and activation of p70-S6 kinase. Branching of the signalling pathways for these two events is evident from the sensitivity of the S6 kinase pathway to the immunosuppressive drug rapamycin, to which GLUT4 translocation is insensitive [25]. Intriguingly the target of rapamycin in yeast is homologous with PI 3-kinase and may itself be sensitive to wortmannin [26]. We have therefore tested the influence of rapamycin on endosome fusion. Inclusion of 30 ng/ml rapamycin in the fusion assay was without significant effect on the observed fusion efficiency (control fusion efficiency 43 ± 3 %; with rapamycin, 44 ± 2 %). The concentration of rapamycin we have used is greater than that shown to eliminate PDGF stimulation of p70-S6 kinase activity in HepG2 cells (20 ng/ml; [27]).

Sequence similarity between the Vps34 protein in yeast, which is essential for sorting to the vacuole, and the catalytic subunit of mammalian PI 3-kinase has suggested a role for PI 3-kinase in regulating the trafficking of membrane proteins. There are other data in the literature hinting at a more general role for phosphatidylinositol in membrane-trafficking events [28]. GLUT4 translocation is sensitive to wortmannin [14], the yeast sec14 protein important in intra-Golgi transport encodes a PI-transfer protein [29], and a factor implicated in the ATP-dependent priming of vesicles for regulated secretion in PC12 cells is also a PI-transfer protein [30].

The sensitivity to wortmannin of several complex intracellular events measured on intact cells has been attributed to inhibition of PI 3-kinase activity. Indeed, one study on the activation of neutrophils identified the 110 kDa catalytic subunit as the only discernible protein labelled by radioactive wortmannin over the relevant concentration range [13]. However, it has become clear that several isoforms of the kinase exist, and at least some of these are sensitive to wortmannin to various extents [24,25]. We have made initial attempts to reconstitute the wortmannin block of endosome fusion with p110 complexed to the p85 subunit, which have been unsuccessful. In the long term, the easy manipulation of this in vitro fusion system offers an opportunity to test for the role of specific PI 3-kinases as they become available.

We have described the first example of an in vitro assay of membrane trafficking dependent on PI 3-kinase activity. It seems likely that PI 3-kinase activities are important in multiple intracellular membrane trafficking events, which include trafficking to the vacuole in yeast [16], PDGF-receptor internalization [18] and endocytosis of fluid-phase marker [19]. Whilst the examples given above all possibly involve budding mechanisms, the case reported here almost certainly involves direct regulation of membrane fusion, as no vesicular intermediate has been identified, despite extensive morphological examination of in vitro endosome fusion [31,32]. As endosome fusion is dependent on the universal fusion factor NSF, it will be interesting to discover whether PI 3-kinase activity represents a conserved element of the fusion machinery or whether it is an endosome-specific regulator.

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REFERENCES


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