p110-related PI 3-kinases regulate phagosome-phagosome fusion and phagosomal pH through a PKB/Akt dependent pathway in *Dictyostelium*

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SUMMARY

The *Dictyostelium* p110-related PI 3-kinases, PIK1 and PIK2, regulate the endosomal pathway and the actin cytoskeleton, but do not significantly regulate internalization of particles in *D. discoideum*. Bacteria internalized into Δddpik1/ddpik2 cells or cells treated with PI 3-kinase inhibitors remained intact as single particles in phagosomes with closely associated membranes after 2 hours of internalization, while in control cells, bacteria appeared degraded in multi-particle spacious phagosomes. Addition of LY294002 to control cells, after 60 minutes of chase, blocked formation of spacious phagosomes, suggesting PI 3-kinases acted late to regulate spacious phagosome formation. Phagosomes purified from control and drug treated cells contained equivalent levels of lysosomal proteins, including the proton pump complex, and were acidic, but in drug treated cells and Δddpik1/ddpik2 cells phagosomal pH was significantly more acidic during maturation than the pH of control phagosomes. Inhibition of phagosomal maturation by LY294002 was overcome by increasing phagosomal pH with NH4Cl, suggesting that an increase in pH might trigger homotypic phagosome fusion. A pkbA null cell line (PKB/Akt) reproduced the phenotype described for cells treated with PI 3-kinase inhibitors and Δddpik1/ddpik2 cells. We propose that PI 3-kinases, through a PKB/Akt dependent pathway, directly regulate homotypic fusion of single particle containing phagosomes to form multi-particle, spacious phagosomes, possibly through the regulation of phagosomal pH.

Key words: *Dictyostelium discoideum*, Phagosomal maturation, Phagosome fusion, Phosphoinositiide 3-kinase, Protein kinase B

INTRODUCTION

Phagocytosis is the process by which particulate matter is internalized into cells (for review see Aderem and Underhill, 1999; Allen and Aderem, 1996; Brown, 1995; Swanson and Baer, 1995). It often consists of the engagement of specific receptors at the cell surface by ligands associated with particulate matter. The cross-linked cell surface receptors stimulate signal transduction pathways that cause local protrusion of the plasma membrane via remodeling of the actin cytoskeleton resulting in engulfment of the particle. Neutrophils and macrophages utilize this process to internalize and degrade invading pathogens and remove cell debris from damaged or apoptotic cells. Once a phagosome is formed, rapid modification of the phagosome ensues through membrane fission and fusion processes with the endolysosomal system, while cell membrane proteins are recycled back to the cellular surface. These processes are essential for the formation of an acidic phago-lysosome, the destruction of pathogenic organisms and the degradation of cellular debris.

Despite the importance of phagosomal maturation only a small number of proteins that regulate this process have been characterized. The small molecular mass GTPase, Rab5, plays an important role in regulating fusion of early endosomes with phagosomes (Alvarez-Dominguez et al., 1996). Treatment of macrophages with interferon (INF)-γ results in cellular activation and increased levels of mRNA encoding Rab5a and Rab5a protein (Alvarez-Dominguez and Stahl, 1998), suggesting that Rab5a plays an important role in the macrophage’s ability to internalize and destroy pathogens when stimulated by INF-γ. Many other proteins such as Rab7, and annexins have been localized to phagosomal membranes, but little is known about the function these proteins have during phagosomal maturation (Desjardins, 1995). Furthermore, a number of intracellular pathogens have evolved to evade destruction, upon engulfment by a professional phagocyte, by disrupting the process of phagosomal maturation and actively forming an environment conducive to their survival (Sinai and Joiner, 1997; Haas, 1998). Thus, understanding the molecular mechanisms of phagosomal maturation may enable the discovery of new treatments for diseases caused by intracellular pathogens.

Though the lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase), is required for internalization of particles in some cells (Araki et al., 1996; Cox et al., 1999), it has not been shown to play a role in phagosomal maturation. PI 3-kinases catalyze the phosphorylation of phosphatidylinositol (PI) at the D-3 position of the inositol ring (Vanhaesebroeck and
Waterfield, 1999). First shown to play a role in membrane trafficking from the yeast Golgi to the vacuole (DeWald et al., 1997), PI 3-kinases have also been implicated in macropinocytosis (Araki et al., 1996), receptor mediated endocytosis and receptor trafficking (Gilllooly et al., 1999; Brunskill et al., 1998; Joly et al., 1994), GLUT4 trafficking (Elmendorf and Pessin, 1999), and early endosome homotypic fusion (Mills et al., 1999). PI 3-kinases probably mediate these events through the regulation Rab GTases (Mills et al., 1999) and ARF GTases (ADP ribosylation factor) (Chavrier and Goud, 1999).

One of the known downstream effectors of PI 3-kinases is the protein kinase, PKB/Akt. This enzyme controls a variety of processes in mammalian cells, including apoptosis, protein synthesis, glucose metabolism, and endocytosis (reviewed by Downward, 1998; Kandel and Hay, 1999). The enzyme contains a pleckstrin homology domain that binds to PIP3, which serves as a target for membrane localization and also enables activation of PKB through phosphorylation of thr308 by PDK1 (PIP3-dependent kinase 1) and ser473 by PDK2 (identity unknown) (Downward, 1998; Kandel and Hay, 1999). The activity of both PDK1 and PDK2 is increased by the presence of PIP3.

Three genes encoding PI 3-kinases have been cloned in Dictyostelium (Zhou et al., 1995); an organism suitable for the study of phagocytic processes because it can be analyzed genetically and biochemically, and the single cell amoebae demonstrate properties comparable to neutrophils and macrophages. These PI 3-kinases, PIK1, PIK2, and PIK3, have similar domain structures and show the highest amino acid identity to mammalian p110-kD PI 3-kinase proteins when compared with class III VPS34 homologues and PI 4-kinases (Zhou et al., 1995). These genes may be redundant in function since genetic disruptions of any one gene alone revealed no phenotype. However, a cell line with a genetic disruption of both dpik1 and dpik2 (Δdpik1/Δdpik2) was defective in growth and development, and cellular levels of PI(3,4)P2 and PI(3,4,5)P3 were reduced at least 75% as compared to levels in control cells (Zhou et al., 1995; Zhou et al., 1998).

The Δdpik1/Δdpik2 mutant was also defective in multiple membrane trafficking events, indicating a role for PIK1 and PIK2 in pinocytosis, macropinocytosis (A. Rupper and J. Cardelli, unpublished results), a late step in the endosomal pathway, and in secretion of lysosomal enzymes (Buczynski et al., 1997b). In Δdpik1/Δdpik2 cells, acidic lysosomes accumulated, while in wild-type cells, fluid phase was transferred to nonacidic vacuoles termed postlysosomes (a terminal secretory compartment) (Buczynski et al., 1997b). As for phagocytosis, the Δdpik1/Δdpik2 cells were not apparently defective in internalization of particulate matter, but these cells grew slowly on bacterial lawns, suggesting a potential defect in phagosomal maturation (Buczynski et al., 1997b; Zhou et al., 1995). In Dictyostelium, phagosomal maturation consists of a series of fission and fusion events that result in (1) the delivery of membrane proteins and soluble contents of endosomes and lysosomes to phagosomes (Rezabek et al., 1997b), (2) rapid acidification of the phagosome (Rupper et al., 1998), and (3) homotypic fusion of phagosomes to form spacious multi-particle containing phagosomes (Rupper et al., 1998).

In addition, a PKB homolog, pkba, has recently been described in Dictyostelium and the activity of PKB was controlled by PIK1 and PIK2 (Meili et al., 1999). Meili et al. demonstrated that PKB was transiently activated by the chemoattractant cAMP and that the activation was dependent on PI 3-kinase activity. A pkba null cell line was defective in chemotaxis towards cAMP gradients and had other developmental defects.

In this paper, we will present data supporting a role for PI 3-kinases in the regulation of late phagosome-phagosome fusion and phagosomal pH. We present evidence that PKB may act as an effector of PI 3-kinase to regulate homotypic phagosome fusion and phagosomal pH changes; and we suggest that phagosomal pH may be a trigger for homotypic phagosome fusion.

MATERIALS AND METHODS

Cells and culture conditions

D. discoideum, strain Ax3, was grown axenically at 18°C in HL5 growth medium (1% Oxoid proteose peptone, 1% glucose, 0.5% yeast extract, 2.4 mM Na2HPO4, and 8.8 mM KH2PO4, pH 6.5) either in shaking suspension or in tissue culture flasks. Δdpik1/Δdpik2 cells and pkba null cells were grown axenically in T175 tissue culture flasks in HL5 growth medium. Construction of the mutant strain Δdpik1/Δdpik2 was as described (Zhou et al., 1995). Construction of the mutant cell line pkba null as well as the myr-PKB (myristylated PKB) overexpressing cell line was as described (Meili et al., 1999).

Endocytosis assays

Fluid phase influx and efflux was measured as described (Temesvari et al., 1996) using FITC-dextran M, 70,000 (Sigma, St Louis, MO) as a fluid phase marker. Cells treated with Wortmannin (10 μM stock resuspended in DMSO, Sigma, St Louis, MO) or LY294002 (50 μM stock resuspended in DMSO, Calbiochem, La Jolla, CA) or 0.01% v/v DMSO (control) were pre-treated with the drug for 10 minutes in HL5 and then the assays were performed in the presence of the drug.

Secretion of lysosomal α-mannosidase

Radiolabel pulse-chase analysis and immunoprecipitation of α-mannosidase was performed as described (Temesvari et al., 1996).

Light and fluorescence microscopy

F-actin was labeled as described using FITC labeled phalloidin (Molecular Probes, Eugene, OR) (Buczynski et al., 1997b). Drug-treated cells were treated for 15 minutes prior to fixation and staining. Morphology of the endo/lysosomal system in control cells, cells treated with wortmannin and Δdpik1/Δdpik2 cells was determined by loading cells for 3 hours with FITC-dextran, washing the cells in growth medium and allowing them to attach to coverslips. Drug-treated cells were incubated in the presence of drug 10 minutes before addition of FITC-dextran and drug was present during the loading period. Photographs were taken with an Olympus BX50 fluorescence microscope using Kodak T-MAX 400 speed film for black and white prints.

Fluorescence microscopy of phagosomal maturation

Cells were fed FITC-dextran labeled E. coli (see below) for a pulse period and then chased in the absence of bacteria following attachment to coverslips. Cells were photographed using an Olympus epifluorescence microscope and Kodak T-Max 400 speed film.

Fluorescent labeling of bacteria

E. coli from a stationary phase culture were washed 1x with 0.1 M NaHCO3, pH 9.0, and resuspended in 0.1 M NaHCO3, pH 9.0, with

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1 mg/ml FITC Isomer 1 (Sigma) for 1 hour at 25°C with gentle stirring. The labeled E. coli were then washed with PBS until the supernatant was free of visible FITC. The bacteria were resuspended in a suitable volume of PBS and stored at 4°C until needed.

### Confocal microscopy

To demonstrate that late phagosomes fuse to give rise to spacious phagosomes, control cells, \( \Delta ddpik1/ddpik2 \) cells, and cells pre-treated with LY294002 (20 \( \mu M \)) were allowed to internalize a mixture of FITC-labeled and Texas Red-labeled E. coli (Molecular Probes, Eugene, OR) for 10 minutes in shaking suspension. The cells were then washed 2 times with growth medium and chased for various times in shaking suspension. 10 minutes prior to each time point the cells were harvested and allowed to attach to glass coverslips. At various time points, the cells were fixed with 1% formaldehyde in HL5 for 3 minutes, washed with HL5 and images were captured with a Bio-Rad MRC-1000 confocal microscope. Laser lines of 488 nm and 568 nm of the Krypton/Argon laser were used at 10% laser power to sequentially excite FITC and Texas Red, respectively. FITC fluorescence was imaged after passing through a 522±35 nm filter and Texas Red fluorescence was imaged after passing through a 605±32 nm filter. The images were analyzed with IPLAB software (Scanalytics, Inc., Fairfax, VA).

### Quantitation of spacious phagosomes

Cells were resuspended at 3\( \times 10^5 \) cells/ml in conditioned medium (HL5 in which the cells had been grown) pulsed with FITC-labeled bacteria for ten minutes and then washed free of bacteria with fresh HL5. At time zero, cells contained 30-50 bacteria. The cells were then chased in conditioned medium for 90 minutes in shaking suspension. Cells were removed and allowed to attach to polycarbonate coverslips within a silicone perfusion chamber for ten minutes, at which time the chamber was mounted to a glass slide and filled with conditioned medium. The cells were then chased to a total of 135 minutes before being viewed. Cells treated with LY294002 were pretreated for 10 minutes, during bacterial uptake, and drug was present during the chase period. Concannamycin A (CMA, Sigma) was resuspended in 100% ethanol. Fluorescence and DIC images were captured using the 40x lens of an Olympus AX70 fluorescent microscope equipped with a 16 bit CCD camera using neutral density filters to attenuate the fluorescent light source to 25% light transmittance. Though spacious phagosomes could be found in multiple planes of focus, a plane was chosen which gave the highest number of spacious phagosomes in focus for each field of view. For each experiment, 150-200 cells were analyzed with 50-70 cells/field. Because of the large number of cells/field, it was not necessary to image multiple planes of focus to achieve a representative average.

Images were analyzed with IPLAB software. Objects were considered spacious phagosomes if they had a diameter of 1.5 \( \mu m \) or greater, were roughly circular, with a pixel intensity of no less than 45% of the image’s maximum pixel intensity. All objects in control cells that we considered spacious phagosomes were included by these parameters. The definition of spacious phagosomes was the same for images of cells treated with LY294002, \( \Delta ddpik1/ddpik2 \) cells, and \( \Delta pkh1 \) null cells, except that the minimum pixel intensity allowed for a spacious phagosome was set at the minimum pixel intensity allowed in control cells for that experiment. Exposure times used for images of drug treated cells, \( \Delta ddpik1/ddpik2 \) cells, and \( \Delta pkb4 \) null cells were the same as the times used for control cells (usually 0.5 seconds). These parameters allowed us to define spacious phagosomes by size, shape, and intensity. Intensity was an important parameter since FITC fluorescence is quenched in acidic environments and spacious phagosomes in control cells were less acidic than similar objects in drug treated or \( \Delta ddpik1/ddpik2 \) cells, thus making spacious phagosomes in control cells much brighter than less mature phagosomes. We used these parameters to determine the total number of spacious phagosomes/cell in a given image. Only cells with FITC-bacteria visible in the fluorescence image were counted.

### Transmission electron microscopy

Control cells, \( \Delta ddpik1/ddpik2 \) cells, and cells treated with wortmannin were allowed to internalize 1 \( \mu M \) latex beads or E. coli for ten minutes in shaking suspension, washed 2 times with HL5, and chased in HL5 in shaking suspension for 120 minutes. Fixation and preparation of the cells for transmission electron microscopy was performed as described previously (Buczynski et al., 1997b).

### Purification of latex bead containing phagosomes

AX3 cells were harvested during log phase growth between 3-5\( \times 10^5 \) cells/ml, pelleted at 1000 \( g \) for 5 minutes and resuspended at 2.5\( \times 10^7 \) cells/ml in HL5. The cells were allowed to recover 10 minutes and in the case of drug treatment were incubated with drug (LY294002 at 25 \( \mu M \) or control 0.01% DMSO) for 15 minutes prior to addition of latex beads. Latex beads (0.8 \( \mu m \) diameter, blue-dyed; Sigma, St Louis, MO) were added at a concentration of 100 beads/cell and incubated with the cells for 5 minutes. The cells were washed 4 times with cold HL5 by centrifugation at 1000 \( g \) for 5 minutes. The cells were then resuspended at 2.5\( \times 10^7 \) cells/ml in HL5 and chased to various time points in shaking suspension. In the case of treatment with LY294002, drug was included during the chase period. At each time point, 5.6\( \times 10^5 \) cells were harvested, washed 1 time in cold homogenization buffer (100 mM sucrose, 5 mM phosphate pH 8.5) and the pellet was kept on ice. The cell pellets were resuspended in 2.8 ml of cold homogenization buffer supplemented with protease inhibitors (0.1 mM TLCK, 5 \( \mu g/ml \) leupeptin, 5 \( \mu g/ml \) pepstatin A, 5 \( \mu g/ml \) aprotinin and 0.5 mM phenylmethylsulphonyl fluoride, final concentrations; Sigma, St Louis, MO), and the cells were broken by passage through double, 5 \( \mu m \) pore size, polycarbonate filters (Osmonics, Inc.). Protease inhibitors were not included in preparations where protease activity was to be measured. The cell homogenate was centrifuged at 500 \( g \) for 3 minutes to remove unbroken cells and nuclei (post-nuclear supernatant (PNS)) and mixed with 4.8 ml of 62% sucrose (all sucrose solutions were w/v in 5 mM glycine, pH 8.5). The sample was layered onto a 1 ml cushion of 62% sucrose and overlayed with 8 ml of 35% sucrose, 12 ml of 25% sucrose, and 7 ml of 10% sucrose in 25\( mm \) centriufuge tubes. The gradients were centrifuged 1 hour at 100,000 \( g \) in an SW28 rotor (Beckman Instruments, Palo Alto, CA). The phagosomes were gently collected from the 10-25% sucrose interface with a transfer pipette, mixed with an equal volume of 4x Laemmli buffer and heat denatured for 10 minutes at 65°C (Laemmli, 1970). When the samples were to be used to measure protease activity, they were not heated.

### Western blot and protease activity analysis

PNS and phagosome protein samples were loaded with equal protein loads and separated by SDS-PAGE using the Laemmli discontinuous buffer system (Laemmli, 1970). Proteins were electrobotted to nitrocellulose membranes in Towbin buffer at 100 volts for 1 hour (Towbin et al., 1979). The membranes were blocked overnight with TBSTG buffer (10 mM Tris base, 150 mM NaCl, 0.05% (w/v) Tween-20, 0.1% (v/v) gelatin). All antibodies were diluted with TBSTG buffer system (Laemmli, 1970). When the samples were to be used to measure protease activity, they were not heated.
phenotypically indistinguishable from kinase inhibitors wortmannin or LY294002, were demonstrate that control cells, treated with the PI 3-later) difficult. We, therefore, felt it was worthwhile to medium making biochemical analysis (to be described labeled determined by measuring the fluorescence ratio of FITC-pH by extrapolation from an in vitro standard curve nm was calculated and used to determine the intraphagosomal fluorescence was measured with a Hitachi fluorimeter (Model F-4010) at 520 nm following excitation at 450 nm and 495 nm. The ratio of 495 nm/450 nm fluorescence emission at 520 cold HL5 and one time with ice-cold 50 mM MES (pH 6.5) buffer. The cells were resuspended in 2 ml of MES buffer and 4.0) with 1 mM dithiothreitol for 16 hours. The cells were then stained with Coomassie Brilliant Blue (0.25% (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid) and destained with a solution of Polyacrylamide gel. Following electrophoresis, the gel was incubated in 2.5% Triton X-100 (v/v) with mild agitation for 30 minutes at room temperature, and then in 0.1 M sodium acetate/acetic acid buffer (pH 4.0) with 1 mM dithiothreitol for 16 hours. The gels were then stained with E64 inhibited protease activity completely except in the top two bands visible in the gel suggesting that a majority of the visible activity was due to cysteine proteinases.

Measurement of phagosomal pH
Phagosomal pH was measured using FITC-labeled E. coli as a pH sensitive phagocytic probe. Ax3 cells were harvested in log phase growth in shaking suspension and resuspended at 5X10^6 cells/ml in HL5 and allowed to recover 10 minutes. Control cells (0.01% DMSO) and cells pre-treated with LY294002 (25 μM) for 15 minutes were pulsed with FITC-labeled E. coli for 10 minutes in shaking suspension. In experiments with Δddpik1/ddpik2 cells and pkbA null cells, control cells were not treated with 0.01% DMSO. The cells were then diluted with ice cold HL5 and centrifuged at 1000 g at 4°C, this was repeated 3 times, followed by resuspension at 5X10^6 cells/ml in room temperature HL5. At each time point 5X10^6 cells were harvested and washed 2 times with ice cold HL5 and one time with ice-cold 50 mM MES (pH 6.5) buffer. The cells were resuspended in 2 ml of MES buffer and fluorescence was measured with a Hitachi fluorimeter (Model F-4010) at 520 nm following excitation at 450 nm and 495 nm. The ratio of 495 nm/450 nm fluorescence emission at 520 nm was calculated and used to determine the intraphagosomal pH by extrapolation from an in vitro standard curve determined by measuring the fluorescence ratio of FITC-labeled E. coli in buffers from pH 4-7.

RESULTS
The PI 3-kinases PIK1 and PIK2 represent the primary targets for the drugs wortmannin and LY294002
Δddpik1/ddpik2 cells grow very slowly in axenic medium making biochemical analysis (to be described later) difficult. We, therefore, felt it was worthwhile to demonstrate that control cells, treated with the PI 3-kinase inhibitors wortmannin or LY294002, were phenotypically indistinguishable from Δddpik1/ddpik2 cells. Accordingly, control cells were treated with a range of concentrations of LY294002 (1 μM to 50 μM) or wortmannin (0.05 μM to 2 μM) and analyzed for a variety of phenotypic changes. All of the phenotypic changes to be described were observed with concentrations of wortmannin as low as 0.2 μM and of LY294002 of 5 μM suggesting that these drugs were acting through PI 3-kinases.

As indicated in Fig. 1, cells exposed to wortmannin contained fine surface filopodia (Fig. 1C) and displayed numerous cytokinesis bridges (Fig. 1F). In addition, drug treated cells pulsed with FITC dextran for 1 hour contained no fluorescent vacuoles larger then 0.2 μm suggesting the accumulation of this fluid phase marker in lysosomes (Fig. 1I).

Each of these phenotypic changes was also observed for Δddpik1/ddpik2 cells (Fig. 1B,E,H). In contrast, control cells lacked abundant surface filopodia (Fig. 1A), showed no cytoplasmic bridges (Fig. 1D) and contained 3-5 large postlysosomes (2-3 μm in diameter) after a 60 minute pulse with FITC dextran (Fig. 1G).

The data in Table 1 summarized experiments that

Fig. 1. Δddpik1/ddpik2 cells and cells treated with PI 3-kinase inhibitors have the same morphological phenotype. Control cells (A,D,G), Δddpik1/ddpik2 cells (B,E,H), and cells treated with 2 μM wortmannin (C,F,J). (A,B,C) FITC-phalloidin stain of cellular F-actin as described in Materials and Methods. (A) Arrowhead denotes cortical actin staining in control cells. (B and C) Arrows denote characteristic filopodia seen in cells deficient in PI 3-kinase activity. (D,E,F) Phase-contrast images of cells in tissue culture flasks. (E and F) Arrows denote cytokinesis bridges seen in 1-3% of cells deficient in PI 3-kinase activity (Buczynski et al., 1997b). (G,H,I) The endo/lysosomal system of cells loaded with FITC-dextran as described in Materials and Methods. (G) Arrows denote large postlysosomes absent from cells deficient in PI 3-kinase activity. Bar, 2 μm.
infect FITC conjugated cells and cells treated with 20 μM LY294002 were allowed to ingest FITC labeled E. coli for ten minutes. The cells were then washed and chased for 30, 60, 90, and 135 minutes in conditioned growth medium, and finally examined with a fluorescent microscope. At early time points, 0-30 minutes of chase, 30-50 FITC-bacteria were seen within the control cells (Fig. 2A and A’). These bacteria appeared to be internalized singly and were found in phagosomes with tightly opposed membranes (see Fig. 3). After 60 minutes of chase, some cells have phagosomes containing multiple bacteria with tightly opposed membranes (Fig. 2B and B’). Then, by 90 minutes of chase, the number of cells with this type of phagosome increased dramatically (Fig. 2C and C’). After 135 minutes of chase, these multi-particle containing phagosomes became swollen and were filled with both bacteria and diffuse fluorescent staining, suggestive of bacteria being degraded. We termed these phagosomes, spacious phagosomes. Cells treated with 20 μM LY294002 and Δddpik1/ddpik2 cells (results not shown) were blocked from the formation of spacious phagosomes, even after 135 minutes of chase, while numerous FITC-bacteria were still apparent. It was clear that spacious phagosomes in wild-type cells often contained multiple bacteria, suggesting they had arisen from phagosome-phagosome fusion events. These experiments suggested that PI 3-kinase activity and the PI 3-kinases, PIK1 and PIK2, were necessary for both the degradation of bacteria and the formation of late, spacious phagosomes.

In order to confirm the results we obtained using light microscopy, we used EM to visualize the ultrastructure of bacteria-containing spacious phagosomes formed in control cells, cells treated with wortmannin and Δddpik1/ddpik2 cells. Using the same pulse chase strategy as described above, we observed that after 120 minutes of chase, control cells contained large, spacious phagosomes often enclosing multiple bacteria in a state of degradation (Fig. 3A and B). Since the majority of bacteria were phagocytosed as single particles, spacious phagosomes containing multiple particles must have arisen from phagosome-phagosome fusion events. Cells treated with wortmannin (Fig. 3C) and Δddpik1/ddpik2 cells (Fig. 3D) did not form numerous spacious phagosomes containing multiple bacteria. Bacteria-containing vacuoles in these cells had tightly opposed membranes with few phagosomes containing more than one bacterium, suggesting that PIK1 and PIK2 activity was necessary for homotypic phagosome fusion. In addition, it was apparent that drug treated and Δddpik1/ddpik2 cells did not contain large postlysosomes that were observed in control cells, confirming previously reported results (Buczynski et al., 1997b), nor did they contain macropinosomes, suggesting a defect in macropinocytosis.

To confirm the hypothesis that the formation of spacious phagosomes was due to phagosome-phagosome fusion, we developed a phagosome fusion assay based on imaging by laser scanning confocal microscopy (LSCM). In this assay, control cells in growth medium were fed a mixture of FITC-labeled and Texas Red-labeled bacteria for 10 minutes, washed and then chased to various time points. The cells were allowed to adhere to glass coverslips for 10 minutes prior to every time point and fixed with 1% formaldehyde in growth medium at the time point. The cells were viewed using a LSCM and images were captured in both red and green channels. Fig. 4 depicts cells imaged at 120 minutes of chase. The arrows on Fig. 4C point to spacious phagosomes stained brightly with both FITC and Texas Red-labeled bacteria. Since FITC and Texas Red-labeled bacteria were ingested individually (results not shown), spacious phagosomes that were stained brightly with both fluorophores were indicative of one or more phagosome-phagosome fusion events. Though every spacious phagosome may be the result of phagosome-phagosome fusion, not every phagosome was expected to be brightly

Table 1. The PI 3-kinase drugs wortmannin and LY294002 have comparable effects on cells as the absence of DdPIK1 and DdPIK2

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Wort*</th>
<th>LY**</th>
<th>Δpik1/2</th>
<th>Δpik1/2-drug‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influx rate - 60 min§</td>
<td>0.39</td>
<td>0.13</td>
<td>0.10</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>Efflux rate (T1/2 in mins¶)</td>
<td>48</td>
<td>115</td>
<td>90</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Secretion (% at 300 mins∥)</td>
<td>70</td>
<td>10</td>
<td>-</td>
<td>9</td>
<td>-</td>
</tr>
</tbody>
</table>

See Materials and Methods for details concerning the assays described here.

*Wortmannin at a final concentration of 0.2 μM.

**LY294002 at a final concentration of 10 μM.

†Wortmannin at a final concentration of 0.5 μM.

§The numbers represent μl of fluid phase internalized per μg of cellular protein.

¶The numbers represent the time required to efflux 50% of preinternalized fluid.

∥The numbers represent % of total radiolabeled α-mannosidase secreted.

PIK1 and PIK2 regulate the homotypic fusion of phagosomes to form spacious multi-particle phagosomes

To test the hypothesis that a defect in phagosomal maturation caused Δddpik1/ddpik2 cells to grow slowly when utilizing bacteria as a food source, phagosomal maturation was visualized using a fluorescent microscopic technique. Control cells and cells treated with 20 μM LY294002 were allowed to ingest FITC conjugated E. coli for ten minutes. The cells were then washed and chased for 30, 60, 90, and 135 minutes in conditioned growth medium, and finally examined with a fluorescent microscope. At early time points, 0-30 minutes of chase, 30-50 FITC-bacteria were seen within the control cells (Fig. 2A and A’). These bacteria appeared to be internalized singly and were found in phagosomes with tightly opposed membranes (see Fig. 3). After 60 minutes of chase, some cells have phagosomes containing multiple bacteria with tightly opposed membranes (Fig. 2B and B’). Then, by 90 minutes of chase, the number of cells with this type of phagosome increased dramatically (Fig. 2C and C’). After 135 minutes of chase, these multi-particle containing phagosomes became swollen and were filled with both bacteria and diffuse fluorescent staining, suggestive of bacteria being degraded. We termed these phagosomes, spacious phagosomes. Cells treated with 20 μM LY294002 and Δddpik1/ddpik2 cells (results not shown) were blocked from the formation of spacious phagosomes, even after 135 minutes of chase, while numerous FITC-bacteria were still apparent. It was clear that spacious phagosomes in wild-type cells often contained multiple bacteria, suggesting they had arisen from phagosome-phagosome fusion events. These experiments suggested that PI 3-kinase activity and the PI 3-kinases, PIK1 and PIK2, were necessary for both the degradation of bacteria and the formation of late, spacious phagosomes.

In order to confirm the results we obtained using light microscopy, we used EM to visualize the ultrastructure of bacteria-containing spacious phagosomes formed in control cells, cells treated with wortmannin and Δddpik1/ddpik2 cells. Using the same pulse chase strategy as described above, we observed that after 120 minutes of chase, control cells contained large, spacious phagosomes often enclosing multiple bacteria in a state of degradation (Fig. 3A and B). Since the majority of bacteria were phagocytosed as single particles, spacious phagosomes containing multiple particles must have arisen from phagosome-phagosome fusion events. Cells treated with wortmannin (Fig. 3C) and Δddpik1/ddpik2 cells (Fig. 3D) did not form numerous spacious phagosomes containing multiple bacteria. Bacteria-containing vacuoles in these cells had tightly opposed membranes with few phagosomes containing more than one bacterium, suggesting that PIK1 and PIK2 activity was necessary for homotypic phagosome fusion. In addition, it was apparent that drug treated and Δddpik1/ddpik2 cells did not contain large postlysosomes that were observed in control cells, confirming previously reported results (Buczynski et al., 1997b), nor did they contain macropinosomes, suggesting a defect in macropinocytosis.

To confirm the hypothesis that the formation of spacious phagosomes was due to phagosome-phagosome fusion, we developed a phagosome fusion assay based on imaging by laser scanning confocal microscopy (LSCM). In this assay, control cells in growth medium were fed a mixture of FITC-labeled and Texas Red-labeled bacteria for 10 minutes, washed and then chased to various time points. The cells were allowed to adhere to glass coverslips for 10 minutes prior to every time point and fixed with 1% formaldehyde in growth medium at the time point. The cells were viewed using a LSCM and images were captured in both red and green channels. Fig. 4 depicts cells imaged at 120 minutes of chase. The arrows on Fig. 4C point to spacious phagosomes stained brightly with both FITC and Texas Red-labeled bacteria. Since FITC and Texas Red-labeled bacteria were ingested individually (results not shown), spacious phagosomes that were stained brightly with both fluorophores were indicative of one or more phagosome-phagosome fusion events. Though every spacious phagosome may be the result of phagosome-phagosome fusion, not every phagosome was expected to be brightly
stained by both FITC and Texas Red. Some cells ingested mostly Texas Red-labeled bacteria while others ingested mostly FITC-labeled bacteria. Since a spacious phagosome may arise from the fusion of two phagosomes containing FITC-bacteria or by fusion with varying numbers of phagosomes containing Texas Red-bacteria or FITC-bacteria, we saw some variability in the amount of mixing that was evident, but mixing was clearly occurring.

A simple assay was employed to quantitate the formation of spacious phagosomes in control cells, cells treated with PI 3-kinase inhibitors, and Δddpk1/ddpk2 cells. The cells in question were allowed to internalize FITC-labeled bacteria for ten minutes in the absence or the presence of drugs, washed free of bacteria and then chased in bacteria free medium for 90 minutes. The cells were then allowed to adhere to coverslips for ten minutes and further chased for 35 minutes making the total chase time 135 minutes. Drug treated cells were chased in the presence of drug. At the end of the chase, the cells were viewed with a fluorescent microscope and images were captured with a 16 bit CCD camera. Since spacious phagosomes were large and non-acidic, they could be defined by size and by the intensity of FITC-bacteria fluorescence within them. IPLAB software was used to help count the number of spacious phagosomes in an image based on predetermined standards of size and intensity (see Materials and Methods for details). We considered this assay to be a test of both phagosome-phagosome fusion and formation of spacious phagosomes. We determined, through studying fluorescence images (Figs 2 and 4) and EM data (Fig. 3), that less than 5% of the spacious phagosomes arose without undergoing fusion events with other phagosomes. The use of EM to image the cells in a similar assay may have proved more quantitative with respect to phagosome fusion data, but the cost of using EM as a read out in time, labor and materials was much greater than the assay we chose to use.

Spacious phagosomes in control cells were 1.5-3 microns in diameter and very bright. On average control cells contained 0.21±0.026 (mean ± s.e.m.) spacious phagosomes per cell (Fig. 5), while cells treated with 20 μM LY294002, and Δddpk1/ddpk2 cells were significantly reduced in the formation of spacious phagosomes containing 0.0057±0.0057 and 0.011±0.0092 spacious phagosomes per cell, respectively. Spacious phagosomes could be found in more than one plane of focus using a ×40 microscopic lens, thus the quantitative results from the images underestimated the actual number of spacious phagosomes per cell. Since many fields of LY294002 treated cells and Δddpk1/ddpk2 cells had no spacious phagosomes, the aforementioned fact made the differences between control cells and cells reduced in PI 3-kinase activity even more profound when viewing the cells by eye.

Fig. 2. Cells treated with 20 μM LY294002 do not form spacious phagosomes. Control cells (A,B,C,D; A',B',C',D'), and cells treated with 20 μM LY294002 (E,E') were allowed to internalize FITC-bacteria for ten minutes, washed and then chased for various times in growth medium without bacteria (see Materials and Methods).

(A) Control cells chased 30 minutes; (A') DIC image of same cell; arrow indicates single bacterium. (B) Control cells chased 60 minutes, (B') DIC; arrow indicates rare example of multi-particle phagosome at this time of chase. (C) Control cells chased 90 minutes, (C') DIC; arrow indicates multi-particle phagosome with apparently tight membrane. (D) Control cells chased 135 minutes, (D') DIC; arrow indicates multi-particle spacious phagosome. (E) Cells treated with 20 μM LY294002, (E') DIC; arrow indicates single bacterium. Images were taken by fluorescence and DIC microscopy using a CCD camera. Bar, 3 μm.
Phagosomal maturation in Dictyostelium PI 3-kinases do not regulate the delivery to phagosomes of multiple endo/lysosomally localized proteins, and appear to act late to regulate the formation of spacious phagosomes

To test the hypothesis that PI 3-kinases might regulate the delivery of proteins necessary for homotypic phagosome fusion and degradation of bacteria from the endo/lysosomal system to phagosomes, latex bead-containing phagosomes were purified from cells treated with 25 μM LY294002 or control cells using a pulse-chase format as described previously (Rezabek et al., Fig. 3. Δddpik1/ddpik2 cells and cells treated with wortmannin do not form spacious phagosomes containing multiple bacteria. Control cells (A and B), cells treated with 2 μM wortmannin (C) and Δddpik1/ddpik2 cells (D) were allowed to internalize E. coli for ten minutes, washed and chased for 60 minutes in growth medium without bacteria. The cells were then prepared for transmission electron microscopy (see Materials and Methods). White arrows in A and B denote spacious phagosomes containing multiple bacteria. Black arrows in C and D denote single bacterium containing phagosomes with tightly opposed membranes. Bar, 1 μm.

Fig. 4. Spacious phagosomes arise by homotypic phagosome fusion. Wild-type cells were pulsed with FITC-labeled bacteria and Texas Red-labeled bacteria for ten minutes, washed and chased for 90 minutes in growth medium without bacteria. The cells were fixed on coverslips with 1% formaldehyde and imaged by LSCM as described in Materials and methods. Images representing FITC fluorescence (A), Texas Red fluorescence (B), A and B merged (C) and transmitted light (D) are shown. Arrows denote spacious phagosomes positive for both FITC and Texas Red fluorescence. Bar, 2 μm.
Transmission electron microscopy revealed that following a 60 minute chase, control cells contained spacious multiple particle latex bead containing phagosome, while drug-treated and mutant cells contained primarily single particle latex bead phagosomes with tightly opposed membranes (Fig. 6). This result suggests that PI 3-kinases are important in the maturation of phagosomes containing bacteria or latex beads.

Control cells and cells treated with 20 μM LY294002 were pulsed with latex beads for 10 minutes, washed free of non-internalized beads, and then chased in fresh growth medium for 10 or 85 minutes. We found no decrease in the delivery of the lysosomal proteins: DdLIMP, 100 and 41 kDa subunits of the V-ATPase, Rab7, α-mannosidase, CPP36 and cysteine proteinase activity to phagosomes in LY294002 treated cells after 10 or 85 minutes of chase (Fig. 7). In addition, phagosomes from control and drug treated cells contained identical profiles of proteins as revealed by silver staining of gels. We conclude that PI 3-kinase activity was not necessary for delivery of proteins from the endo/lysosomal system to phagosomes. This suggested that the defect in homotypic phagosome fusion in Δdppk1/Δdppk2 cells and cells treated with PI 3-kinase inhibitors was not due to a defect in vesicle trafficking between phagosomes and the endo/lysosomal system, but instead, PI 3-kinases might be directly involved in the homotypic phagosome fusion event.

Since endo/lysosomal proteins were rapidly delivered to newly formed phagosomes (data not shown; Rezabek et al., 1997b; Souza et al., 1997), and PI 3-kinase activity was apparently not required for this, it was reasonable to hypothesize that PI 3-kinase activity was required late during the maturation process to regulate the formation of spacious phagosomes. To test this, the phagosomal maturation assay was performed as described above (Fig. 5) with the exception that 20 μM LY294002 was added to the cells after 60 minutes of chase. Phagosome fusion measurements were made as described above. The addition of 20 μM LY294002, even just prior to the beginning of phagosome fusion, completely prevented fusion from occurring, suggesting that PI 3-kinases were acting directly to initiate the process of fusion.

**PI 3-kinases regulate changes in phagosomal pH**

Previously, Buczynski et al. demonstrated that fluid phase markers accumulated in acidic, lysosome-like vesicles in Δdppk1/Δdppk2 cells, suggesting a defect in homotypic lysosome fusion and/or a defect in the regulation of lysosomal
Phagosomal maturation in Dictyostelium acidity (Buczynski et al., 1997b). Since a similar step in phagosomal maturation seemed to be regulated by PI 3-kinases, we measured phagosomal pH at different time points during phagosomal maturation by using FITC-conjugated bacteria as a pH sensitive probe. In wild type cells, nascent phagosomes were rapidly acidified to an average pH of 5.2 followed by an increase in pH until it reached a plateau of an average pH of 6.1 after 60 minutes of chase (Fig. 8A). Nascent phagosomes were also rapidly acidified in cells treated with 20 μM LY294002, however, the pH increased slowly as these phagosomes matured. By 60 minutes of chase, the pH of phagosomes from LY294002 treated cells remained significantly lower than the pH of phagosomes from wild type cells. We obtained very similar data measuring phagosomal pH in Dddpik1/ddpik2 cells (Fig. 8B). Nascent phagosomes in Dddpik1/ddpik2 cells were also rapidly acidified, but like phagosomes in cells treated with LY294002, the phagosomes remained significantly more acidic during maturation than phagosomes in control cells.

pkbA null cells duplicate the phagosomal maturation defects observed in Dddpik1/ddpik2 It has been demonstrated (Meili et al., 1999) that PI 3-kinase activity was required for cyclic AMP activation of the protein kinase PKB; a known downstream effector of PI 3-kinases in mammalian cells (Kandel and Hay, 1999). We, therefore, tested the role of PKB, a potential effector of PI 3-kinase activity in Dictyostelium, in phagosomal maturation. We quantitated the ability of pkbA null cells to form spacious phagosomes using the same procedure as described for Dddpik1/ddpik2 cells and cells treated with LY294002 in Fig. 5. pkbA null cells containing FITC-labeled bacteria had the same phagosomal morphology as Dddpik1/ddpik2 cells and cells treated with LY294002. Internalized FITC-labeled bacteria appeared dim suggesting that the FITC fluorescence was quenched in an acidic environment, and spacious phagosomes never formed. After a long chase period, significantly fewer spacious phagosomes were observed in pkbA null cells (0.005±0.0018, mean spacious phagosomes/cell ± s.e.m.; see Fig. 5) as compared to Ax3 control cells (0.21±0.026).

We further characterized the phenotype of pkbA null cells by measuring phagosomal pH changes during phagosomal maturation as described above (see Fig. 8B). Very similar to the
results for \( \Delta dlpk1/dlpk2 \) cells, nascent phagosomes in \( pkba \) null cells were rapidly acidified, and the phagosomes remained significantly more acidic at comparable time points during the chase period than phagosomes from control cells (Fig. 8B). These results suggested that PKB/Akt might act as a downstream effector of PI 3-kinases in the regulation of changes in vacuolar acidification and fusion, perhaps through the phosphorylation of proteins that regulate or mediate these processes.

**An artificial increase in phagosomal pH rescues the formation of spacious phagosomes in cells treated with the PI 3-kinase inhibitor LY294002**

Temesvari et al. demonstrated that treatment of *Dictyostelium* cells with 5 \( \mu \)M CMA caused an increase in the average pH of the endo/lysosomal system from 5.5 to 6.5 and induced fusion between endolysosomal vesicles (Temesvari et al., 1996). Therefore, we employed our phagosome maturation assay to test the hypothesis that PI 3-kinases and PKB regulated the formation of spacious phagosomes by controlling phagosomal pH, and that increases in pH in control cells might trigger phagosomal fusion. Two methods were used to raise the pH of phagosomes in cells treated with LY294002. First, we inhibited the V-ATPase with Concaminycin A (CMA) (Temesvari et al., 1996) and secondly, we used the weak base NH\(_4\)Cl to raise phagosomal pH (Cardelli et al., 1989). The experimental procedure was as described for Fig. 5. Cells were pulsed with FITC bacteria for 10 minutes, washed, and chased in the presence of 20 \( \mu \)M LY294002. Either 5 \( \mu \)M CMA or 40 mM NH\(_4\)Cl was added at 60 minutes of chase. The cells were imaged at 135 minutes of chase and the images were processed as described in Materials and Methods. We found that treatment of *Dictyostelium* cells with 5 \( \mu \)M CMA at 60 minutes of chase, in cells where PI 3-kinases were inhibited by the presence of 20 \( \mu \)M LY294002, resulted in partial rescue of the LY294002 induced phenotype (Fig. 9A). Although, CMA was a strong inhibitor of spacious phagosome formation by itself, treatment of cells with 20 \( \mu \)M LY294002 followed by addition of 5 \( \mu \)M CMA resulted in rescue of the observed LY294002 inhibition levels to the level observed in cells treated with CMA alone. Significantly more spacious phagosomes were found in cells treated with LY294002 followed by CMA (0.077±0.011 mean spacious phagosomes/cell; mean ± s.e.m.) than in cells treated with LY294002 alone (0.0057±0.0057). This suggested that an increase in phagosomal pH caused by inhibition of the V-ATPase was sufficient to rescue the LY294002 induced block of spacious phagosome formation, but that activity of the V-ATPase might also be required for efficient formation of spacious phagosomes.

Inhibition of the V-ATPase could have changed more than just the internal pH of phagosomes. Membrane potential and ion fluxes were presumably also altered, and these things might have played an important role in phagosome-phagosome fusion events. We, therefore, used NH\(_4\)Cl as another method to increase phagosomal pH. Free NH\(_3\) molecules can cross the phagosomal membrane, become protonated, and thus accumulate in acidic compartments and raise the pH of the compartment (Mellman et al., 1986). Previous experiments (Cardelli et al., 1989) demonstrated that treatment of *Dictyostelium* cells with 40 mM NH\(_4\)Cl was sufficient to raise the average internal pH of the endo/lysosomal system from 5.4 to 6.4.

Compared to control cells, phagosomal maturation was not affected by the addition of 40 mM NH\(_4\)Cl (Fig. 9B). When PI 3-kinases were inhibited by 20 \( \mu \)M LY294002, the addition of 40 mM NH\(_4\)Cl rescued the formation of spacious phagosomes (Fig. 8B). In contrast, addition 40 mM NaCl did not rescue the formation of spacious phagosomes in cells inhibited by LY294002, and the addition of 40 mM NaCl at 60 minutes of chase to control cells inhibited spacious phagosome formation significantly (Fig. 9B). This suggested that the effect of NH\(_4\)Cl on spacious phagosome formation was due to an increase in phagosomal pH and not due to an effect of increased salt concentration in the cell medium. Thus, an increase in phagosomal pH appeared to be both necessary and sufficient for the formation of spacious phagosomes.
DISCUSSION

In this report, we have demonstrated that the PI 3-kinases, PIK1 and PIK2, and the protein kinase, PKB, are required for the formation of multi-particle spacious phagosomes in *D. discoideum*. Δdppik1/ddpik2 cells, ΔpkbA cells, and cells treated with two different inhibitors of PI 3-kinase activity were defective in spacious phagosome formation, an event that corresponds in time to the degradation of bacteria. We speculate that spacious phagosomes arise from homotypic fusion events between phagosomes of similar age and maturation stage. The defects in spacious phagosome formation did not appear to arise from a block in vesicle trafficking between phagosomes and elements of the endo/lysosomal system, since phagosomes received a full complement of proteins from the endo/lysosomal system in cells treated with PI 3-kinase inhibitors. This suggested that PI 3-kinase might act later to directly regulate homotypic phagosome fusion. Inhibitor timed addition experiments revealed that PI 3-kinases acted just prior to the appearance of spacious phagosomes, suggesting that the product of PI 3-kinase activity, PIP₃, was directly involved in regulating the formation of spacious phagosomes. PI 3-kinases and PKB were also required for the increase in phagosomal pH we observed concomitant with spacious phagosome formation. Experiments, which used CMA or NH₄Cl to increase the pH of phagosomes in cells where PI 3-kinases had been inhibited by LY294002, suggested that an increase in phagosomal pH might trigger homotypic phagosome fusion. Based on these results, we speculate that PI 3-kinase activity is required directly for mature phagosomes to fuse, most likely by regulating an increase in phagosomal pH.

To our knowledge this is the first study that implicates PI 3-kinases and PKB in phagosomal maturation, particularly homotypic phagosome fusion, and we propose that PKB plays an important role in this process. There is ample evidence to support a role for PKB as a downstream effector of PI 3-kinases (Kandel and Hay, 1999). In *Dictyostelium*, the cAMP stimulated activity of PKB depends on PIK1 and DdPIK2 (Meili et al., 1999) and PKB has been demonstrated to bind with high specificity to PIP₃, the product of PIK1 and DdPIK2 activity (Tanaka et al., 1999). Based on our early results suggesting a role for PI 3-kinase in phagosomal maturation, we hypothesized that two mechanisms could account for how these lipid kinases regulate phagosome fusion: (1) PI 3-kinase activity could be required to regulate the activity or trafficking of proteins that directly regulate the fusion process. (2) PI 3-kinases could regulate an increase in phagosomal pH or a change in phagosomal membrane potential that could trigger homotypic phagosome fusion.

Regarding the first possibility, PI 3-kinases might regulate vesicle trafficking through the generation of PIP₃, which acts as a second messenger by serving as a binding site for proteins containing pleckstrin homology domains (PH) specific for PIP₃ (Toker and Cantley, 1997). Some PIP₃ binding proteins serve as ARF guanine nucleotide exchange factors (GRPs, cytohesin-1, and ARNO), suggesting PI 3-kinases can regulate the activation state of ARFs (Chavrier and Goud, 1999). These proteins are localized to the plasma membrane where they may activate Arf6 and regulate endosomal recycling and actin cytoskeleton changes through Arf6 effectors (Chavrier and Goud, 1999).

In the case of early endosome fusion, it has been demonstrated that the PI 3-kinases, hVPS34 and p110β may be effectors of Rab5. Both Rab5 and the lipid product of hVPS34 were necessary for binding of EEA1 to the endosomal membrane and subsequent endosome-endosome fusion (Christoforidis et al., 1999). In another study, PKB was required for the stimulation of endocytosis by expression of activated Ras in NIH3T3 cells (Barbieri et al., 1998). It was suggested that PKB might stimulate guanine nucleotide exchange on Rab5, since a kinase dead form of PKB blocked early endosome fusion and could only be rescued by expression of constitutively active Rab5, but not by wt Rab5 (Barbieri et al., 1998).

A similar mechanism might regulate homotypic phagosome fusion in *Dictyostelium*. PI 3-kinases, perhaps through PKB, might activate Rab7 or another Rab and thus regulate homotypic phagosome fusion. However, in the case of Rab7, inhibition of PI 3-kinases did not prevent recruitment of Rab7 to maturing phagosomes, although the activation state of this GTPase was not measured. In addition, the lack of PI 3-kinase activity did not prevent the transport of other endo/lysosomal proteins to phagosomes, including DdLIMP, cathepsin D, cysteine protease and subunits of the V-ATPase. It remains to be determined if PI 3-kinases regulate the association of fusogenic proteins, that we have not yet identified, with phagosomal membranes.

Our results suggest that PI 3-kinases and PKB might regulate phagosome fusion by regulating the internal pH of phagosomes. We found that the internal phagosomal pH failed to increase to the levels observed in control cells during phagosomal maturation in *pkbA* null and Δdppik1/ddpik2 cells, as well as cells treated with PI 3-kinase inhibitors, suggesting that changes in phagosomal pH might stimulate homotypic phagosome fusion. Furthermore, we found that increasing phagosomal pH by inhibiting the V-ATPase with CMA or treatment of cells with NH₄Cl was sufficient to overcome the LY294002 block of spacious phagosome formation, supporting the idea that an increase in phagosomal pH may be both necessary and sufficient to trigger homotypic phagosome fusion and formation of spacious phagosomes.

These results are consistent with a number of studies that have suggested that changes in phagosomal or endosomal pH might regulate fusion of those organelles. For instance, treatment of macrophages, which have phagocytosed *Leishmania amazonensis* and *Leishmania mexicana*, with weak bases (NH₄Cl and chloroquine) caused an increase in the transfer of particles (zymosan, latex beads) to these parasitophorous vacuoles, and in the case of *L. mexicana* containing parasitophorous vacuoles, stimulated transfer of previously excluded particles (Veras et al., 1992; Collins et al., 1997). Ammonium chloride has been shown to have effects upon phagosome fusion with both endosomes and lysosomes (Gordon et al., 1980; Hart et al., 1983; Hart and Young, 1991). These experiments suggest that an increase in the pH of early endosomes, and compartments that communicate with early endosomes, stimulates fusion events between those compartments, but little is known about the mechanism through which this occurs.

Our results suggested that even though an increase in
Potassium conductance in exchange for H+ ions could also have been speculated to maintain electrical neutrality across the membranes. Membrane potential affects the ability of the V-ATPase to pump protons since it is an electrogenic pump. The rate of endosome-endosome fusion, in vitro, of endosomal fractions from K-562 cells was stimulated by various reagents that increase endosomal pH (NH4Cl, monensin, nigericin, chloroquine; Pless and Wellner, 1996). The effect of these reagents may have been due to an accumulation of cations (these reagents exchange cations for protons or become protonated in the endosome) in the endosomes, since their effect could be blocked by inhibition of the V-ATPase with Bafilomycin A1. In a different study, homotypic fusion, in vitro, of subapical endosomes from rat renal cortex was blocked by inhibition of the V-ATPase with bafilomycin A1, but not inhibited by disruption of the proton gradient with nigericin (Hammond et al., 1998). Furthermore, the K+ ionophore, valinomycin, inhibited endosome fusion, suggesting that membrane potential, not a pH gradient, was necessary for homotypic fusion of subapical endosomes. It has also been reported that endosome fusion was stimulated by activation of cystic fibrosis transmembrane conductance regulator (CFTR) in fibroblasts transfected with CFTR (Biwersi et al., 1996). Though activation of CFTR through stimulation of PKA by forskolin did not change endosomal pH, it may have altered endosomal membrane potential or ionic content and thus stimulated endosomal fusion (Biwersi et al., 1996). Thus, we speculate that direct regulation of the V-ATPase or an ion channel by PI 3-kinases and/or PKB could regulate homotypic phagosome fusion by altering the proton gradient or the membrane potential.

PI 3-kinases and PKB could regulate counter ion conductance across phagosomal membranes, and thus pH or membrane potential, by controlling the delivery or removal of ion channels. Membrane potential affects the ability of the V-ATPase to pump protons since it is an electrogenic pump (Harvey, 1992) and membrane conductance of chloride ions has been speculated to maintain electrical neutrality across endosomal and lysosomal membranes and to enable generation of a low intra-luminal pH by the V-ATPase (Harvey, 1992). Potassium conductance in exchange for H+ ions could also achieve electrical neutrality across these membranes (Nelson and Harvey, 1999). An example of this is the Na+/H+ exchanger, NHE3. In CHO cells, it has been demonstrated that PI 3-kinase activity is required for trafficking of NHE3 to the plasma membrane from endolysosomal compartments where it plays an important role in regulating cytoplasmic pH (Kurashima et al., 1998). PI 3-kinases have also been implicated in Na+ uptake by renal epithelial cells (Blazer-Yost et al., 1999), acid efflux in osteoblasts (Santhanagopalam and Dixon, 1999), coupling of Cl− channels to M2 muscarinic receptors (Wang et al., 1999), and K+ efflux in polarized epithelial cells (Eckmann et al., 1997), suggesting PI 3-kinases play an important role in the regulation of ion fluxes at the plasma membrane.

We conclude that PI 3-kinases (PKI and PIK2) probably regulate homotypic phagosome fusion and the alkalinization of late phagosomes through a direct mechanism dependent upon PKB. We hypothesize that these PI 3-kinases generate PIP3 on maturing phagosomal membranes and that this phosphorylated lipid in turn recruits PKB. The accumulation of phosphorylated lipids and proteins (the result of PKB activity) could recruit proteins that regulate fusion, and might directly or indirectly regulate phagosomal pH. In current studies we are using biochemical and microscopic approaches to determine if PIP3 accumulates on maturing phagosomes, to discover the nature of the proteins recruited in a PI 3-kinase dependent fashion, and to determine the mechanism regulating pH changes in late phagosomes.

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