Inactivation of Two Dictyostelium discoideum Genes, DdPIK1 and DdPIK2, Encoding Proteins Related to Mammalian Phosphatidylinositol 3-kinases, Results in Defects in Endocytosis, Lysosome to Postlysosome Transport, and Actin Cytoskeleton Organization

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Abstract. Phosphatidylinositol 3-kinases (PI 3-kinases) have been implicated in controlling cell proliferation, actin cytoskeleton organization, and the regulation of vesicle trafficking between intracellular organelles. There are at least three genes in Dictyostelium discoideum, DdPIK1, DdPIK2, and DdPIK3, encoding proteins most closely related to the mammalian 110-kD PI-3 kinase in amino acid sequence within the kinase domain. A mutant disrupted in DdPIK1 and DdPIK2 (Δddpik1/ddpik2) grows slowly in liquid medium. Using FITC-dextran (FD) as a fluid phase marker, we determined that the mutant strain was impaired in pinocytosis but normal in phagocytosis of beads or bacteria. Microscopic and biochemical approaches indicated that the transport rate of fluid-phase from acidic lysosomes to non-acidic postlysosomal vacuoles was reduced in mutant cells resulting in a reduction in efflux of fluid phase. Mutant cells were also almost completely devoid of large postlysosomal vacuoles as determined by transmission EM. However, Δddpik1/ddpik2 cells functioned normally in the regulation of other membrane traffic. For instance, radiolabel pulse-chase experiments indicated that the transport rates along the secretory pathway and the sorting efficiency of the lysosomal enzyme α-mannosidase were normal in the mutant strain. Furthermore, the contractile vacuole network of membranes (probably connected to the endosomal pathway by membrane traffic) was functionally and morphologically normal in mutant cells. Light microscopy revealed that Δddpik1/ddpik2 cells appeared smaller and more irregularly shaped than wild-type cells; 1–3% of the mutant cells were also connected by a thin cytoplasmic bridge. Scanning EM indicated that the mutant cells contained numerous filopodia projecting laterally and vertically from the cell surface, and fluorescent microscopy indicated that these filopodia were enriched in F-actin which accumulated in a cortical pattern in control cells. Finally, Δddpik1/ddpik2 cells responded and moved more rapidly towards cAMP. Together, these results suggest that Dictyostelium DdPIK1 and DdPIK2 gene products regulate multiple steps in the endosomal pathway, and function in the regulation of cell shape and movement perhaps through changes in actin organization.

Vesicle transport is a cellular process important in the trafficking of lipid and proteins between intracellular organelles. The regulation of vesicle traffic is critical to ensure the biogenesis, as well as the functional and structural integrity of endomembrane compartments.

Genetic and biochemical approaches in yeast and mammalian cells have identified a large number of proteins that are involved in the various steps of vesicle transport including the production of vesicles from donor compartments, the transport and docking of these vesicles, and the fusion of vesicles with acceptor membranes (Rothman, 1994). Among these proteins are the Rab family of small M, Ras-like GTPases, numbering over 30 members (Pfeffer, 1994), as well as Arfs, Cops, Snares, and NSF (Rothman, 1994). In addition, some recent evidence indicates that phosphatidylinositol 3-kinases (PI 3-kinases) may play a role in vesicle transport (see below).
The phosphatidylinositol (PI) 3-kinase family contains a group of biochemically diverse forms (Liscovitch and Cantley, 1995). The canonical PI 3-kinase, p85/p110, is a heterodimer consisting of an 85-kD regulatory subunit and a 110-kD catalytic subunit that associates with activated receptor tyrosine kinases (Schlessinger and Ullrich, 1992). Activation of the lipid kinase leads to the production of phosphoinositide 3,4,5-trisphosphate (PI(3,4,5)P3), an acidic phospholipid that acts as a second messenger (Downes and Carter, 1991). This lipid acts to stimulate cell proliferation (Cantley et al., 1991), regulate actin cytoskeleton changes, and induce membrane ruffling (Wenstrom et al., 1994). Recent evidence suggests that the lipid kinase may activate Rac GTPases that are responsible for the observed changes in the plasma membrane (Ridley et al., 1992; Hawkins et al., 1995).

PI 3-kinases have also been implicated in the regulation of membrane traffic. For instance, mutations in the PDGF receptor that inactivate binding of the p85/p110 PI 3-kinase result in a block in transport of these receptors to a compartment involved in degradation (Joly et al., 1994). Also, inactivation of PI 3-kinase (possibly p85/p110) with the inhibitor, wortmannin (Arcaro and Wymann, 1993), results in a 50% decrease in pinocytosis in baby hamster kidney cells (Clague et al., 1995; Li et al., 1995), an inhibition of endosomal fusion in vitro (Jones and Clague, 1995) and blockage of transport of glucose transporters to the cell surface in response to insulin (Okada et al., 1994; Cheatham et al., 1994).

The most compelling evidence implicating a PI 3-kinase in vesicle trafficking comes from genetic and biochemical studies in yeast examining the targeting of soluble hydrolases to the lysosome-like vacuole (Stack et al., 1995). The vps34 gene encodes a PI 3-kinase with a catalytic domain related in amino acid sequence to the mammalian 110-kD subunit (Stack and Emr, 1994; Hiles et al., 1992; Schu et al., 1993); however, this enzyme only phosphorylates the 3′ position of PI. Deletion of the vps34 gene or mutations in the lipid kinase domain result in missorting of soluble hydrolases (Schu et al., 1993; Herman et al., 1991). A mammalian gene encoding a protein closely related to Vps34p has been cloned (Stephens et al., 1994), and two recent studies have indicated that inactivation of PI 3-kinase(s) with specific inhibitors like wortmannin and LY294002 result in missorting of newly synthesized lysosomal enzymes (Brown et al., 1995; Davidson, 1995). However, PI 3-kinase independent lysosomal enzyme targeting pathways have been reported to operate in plant cells (Matsuoka et al., 1995).

Therefore, given the demonstrated importance of PI 3-kinases in membrane trafficking and other cellular processes in mammalian cells and yeast cells, we used a genetic approach to examine the function of two genes encoding PI 3-kinase-like products. The simple eukaryote, Dictyostelium discoideum, is an organism that has proven to be a useful system in which to investigate the regulation of membrane traffic according to the secretory and endosomal pathways leading to lysosomes (reviewed in Cardelli, 1993); as both of the pathways have been well characterized. For instance, the Dictyostelium clathrin heavy chain (CHC) has been demonstrated to play a role in hydrolase sorting, secretion of mature enzymes and in endocytosis (O’Halloran and Anderson, 1992; Ruscetti et al., 1994). CHC minus cell lines pinocytose fluid phase markers very inefficiently implying that fluid phase enters cells via small clathrin-coated vesicles (O’Halloran and Anderson, 1992; Ruscetti et al., 1994); however, other mechanisms of internalization based on regulation of the actin cytoskeleton probably exist (Novak et al., 1995; Temesvari et al., 1996).

Within 15 min of formation, pinosomes fuse and merge with larger (0.5–1.0 microns), acidic, hydrolase-rich lysosomal vacuoles (Nolta et al., 1995; Padh et al., 1993; Aubry et al., 1993; Cardelli et al., 1989). Fluid phase material is then transported to larger (>2 µm in diameter), near-neutral pH, postlysosomal vacuoles (Padh et al., 1993; Aubry et al., 1993); a trafficking step regulated by a Rab4-like GTPase (Bush et al., 1996). Finally, fluid phase material is exocytosed from the postlysosomal vacuoles ~45–60 min after internalization. No efficient early endosomal recycling compartment has been identified.

Five putative PI-kinase encoding genes have been cloned from Dictyostelium discoideum (Zhou et al., 1995). Three of these genes, DdPIK1, DdPIK2, and DdPIK3, encode products that are closely related to the mammalian 110-kD PI 3-kinase. DdPIK5 encodes a protein most closely related to the yeast Vps34p protein in amino acid sequence while DdPIK4 encodes a protein most related to mammalian PI 4-kinases. Dictyostelium DdPIK5 partially compensates for the absence of the S. pombe Vps34 homologue in a null mutant, suggesting that the Dictyostelium gene may encode the Dictyostelium Vps34 homologue. Strains in which both DdPIK1 and DdPIK2 genes have been disrupted showed growth and developmental defects (Zhou et al., 1995) while single knockout mutants showed no observable phenotypes. Double null mutant cells were smaller than wild-type cells and grew more slowly in liquid medium when attached to plastic; they did not grow in suspension in axenic medium.

In the current study, we have examined the importance of the protein products encoded by DdPIK1 and DdPIK2 in vesicle transport in Dictyostelium by analyzing trafficking along the endosomal pathway and the secretory pathway to lysosomes in double null mutants (Δddpik1/Δddpik2). We have also examined the function of the contractile vacuole (CV) complex in mutant cells, an organelle involved in osmotic regulation and perhaps connected to the endosomal pathway via membrane trafficking (Bush et al., 1996; Padh and Tanjore, 1995). The results from this study demonstrate that disruption of DdPIK1/DdPIK2 results in defects in multiple steps of the endosomal pathway. In addition, the double knockout mutant cells were altered in shape and actin cytoskeleton organization, but were normal in sorting of lysosomal enzymes, phagocytosis, and the regulation of the CV.

Materials and Methods

Organism and Materials

The D. discoideum wild-type strain Ax3 and the double null mutant, Δddpik1/ddpik2, were grown axenically in HL5 medium attached to plas-
tic T175 tissue culture flasks. The construction of the mutant strain is detailed elsewhere (Zhou et al., 1995).

**PI 3-Kinase Assays**

Wild-type and Δddpik1/ddpik2 cells (3 × 10^4) were solubilized in 10 µl of 0.1% Triton X-100 containing 50 µg/ml leupeptin, 1 mM PMSF, 2 mM benzamidine, and 10 µg/ml pepstatin. 1 µl (3 × 10^4) cells was used in each reaction. PI 3-kinase assays were performed basically as described (Whitman et al., 1998; Stack et al., 1995). The 50-µl reactions contained 20 mM Hepes, pH 7.5; 60 µM ATP, 10 mM MgCl2, 0.2 mg/ml sonicated PI, PI(4)P or PI(4,5)P2, and 0.2 µCi/ml (γ-32P)-ATP. The reactions were incubated at 25°C for 5 min and stopped by the addition of 80 µl of 1 M HCl. Lipids were extracted by the addition of 160 µl of CHCl3:MeOH (1:1) and vortexing for 30 s. The organic phase was removed and 10-µl aliquots were dried under vacuum. The dried lipids were resuspended in 10 µl chloroform and spotted on a silica 60 TLC plate. Samples were separated using the borate buffer system as described (Walsh et al., 1991).

**Endocytosis Assays**

Fluid phase uptake, exocytosis, and flux was measured using FITC-dextran (FD) as previously described (Temesvari et al., 1996b). Measurement of pH was performed also as described (Padh et al., 1993) using FD.

**Phagocytosis Assays**

Fluorescently labeled (crimson), carboxylate modified, latex beads (1 µm diameter; Molecular Probes, Eugene, OR) were added to cells in growth media at a concentration of 100 beads/cell. At the times indicated, 1 ml of acetone for 3–5 minutes and air dried. FITC-labeled phalloidin solution (Molecular Probes) was evaporated under vacuum, resuspended in 200 µl of PBS and pipetted onto the coverslips which were incubated at room temperature for 20 min. The coverslips were then washed twice with PBS and the cells were extracted in a solution of cold (−20°C) acetone for 3–5 minutes and air dried. FITC-labeled phalloidin solution (Molecular Probes) was evaporated under vacuum, resuspended in 200 µl of PBS and pipetted onto the coverslips which were incubated at room temperature for 20 min. The coverslips were then washed twice with PBS and mounted on glass slides using one drop of mounting media (10 ml PBS, 90 µl glycerol with 100 mg p-phenylenediamine) to prevent quenching of fluorescence. Photographs were taken with an Olympus BX50 fluorescence microscope using T-MAX 400 speed film.

**Scanning Electron Microscopy**

Sterile, one centimeter square, glass coverslips were inserted into T25 tissue flasks and submerged in 10 ml of HLG growth medium. Cells were seeded in the flask at an initial titer of 10^5 cell/ml and were grown for 24 h to allow cell attachment. The coverslips with attached cells were removed and fixed in 1.25% glutaraldehyde buffered in 50 mM sodium cacodylate at pH 6.8. The cells were dehydrated in a graded acetone series and critical point dried from liquid carbon dioxide. The coverslips with adhering dried cells were mounted on specimen stubs, sputter coated with 10 nm of gold and viewed with a ISI DS 130 scanning electron microscope.

**Transmission Electron Microscopy**

Wild-type and mutant cells were pulsed for 2 h with iron dextran (prepared as described; Rodriguez-París et al., 1993), washed, and fixed overnight at 4°C in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 6.8). After fixation in glutaraldehyde, the cells were washed with 0.1 M cacodylate buffer, postfixed for 2 h on ice in 2% osmium tetroxide buffered with 0.1 M cacodylate (pH 6.8), washed again with 0.1 M cacodylate buffer, and dehydrated in a graded series of ethanol solutions. The dehydrated samples were infiltrated overnight in a 1:1 mixture of 100% ethanol and Polybed 812 (Polysciences, Warrington, PA) and then embedded in Polybed 812 by standard procedures. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips CM-10 transmission electron microscope.

**Results**

**Levels of Functional Phosphatidylinositide 3-kinase(s)**

*Are Not Altered in Δddpik1/ddpik2 Strains*

In vitro PI phosphorylation assays were performed to measure the relative intracellular concentrations of different PI kinases in wild-type Ax3 and Δddpik1/ddpik2 cells. Cells grown in tissue culture flasks were harvested by centrifugation, and lysed in 0.1% Triton X-100. Extracts prepared from the equivalent of 3 × 10^5 cells were mixed with PI and γ-labeled ATP. After incubation at 25°C for 5 min, the PI lipids were extracted and resolved by TLC. The only reaction products identified using protein from Ax3 extracts were PI(3)P and PI(4)P (Fig. 1; lane 1). Even with longer exposures times, no labeled PI(4,5)P2 or PI(3,4,5)P3 were detected. These later PI products were also not observed by modifying reaction times, by changing the concentration of protein added from the cell extracts, or by adding PI(4)P or PI(4,5)P3 substrates. In addition, the level of PI 3-kinase activity was not altered in wild-type or mutant strains by generating extracts by freeze thaw in the absence of detergent; detergent has been reported to effect PI 3-kinase activity. Interestingly, the TLC profile of *Dictyostelium* phosphorylated PIs was nearly identical to the yeast phosphorylated PI generated in vitro; only PI(3)P and PI(4)P were observed using PI as a substrate (Fig. 1; lanes 2 and 3). Finally, no significant reduction in the levels of PI(3)P and PI(4)P was observed using extracts from Δddpik1/ddpik2 cells (lanes 4 and 5). We conclude that disrupting *DdPIK1* and *DdPIK2* does not alter the measurable levels of functional PI 3-kinase or PI 4-kinase using PI as a substrate. Because we cannot detect PI (3,4)P2 or PI (3,4,5)P3 in our assays, we cannot address the effect of removing DdPIK1 and DdPIK2 on the synthesis of these lipids.

**Fluid Phase Influx and Efflux Are Reduced in Δddpik1/ddpik2 Cells**

Δddpik1/ddpik2 cells do not grow axenically in shaken sus-
pension culture, and only grow slowly in liquid medium while attached to solid surfaces such as plastic (Zhou et al., 1995). The slow growth rate of this strain indicates a possible defect in endocytosis; therefore, we examined the role of the DdPIK1 and DdPIK2 gene products in the endocytic pathway. To measure pinocytosis, logarithmically growing wild-type and mutant cells were exposed to FD for 60 min. At the times indicated in Fig. 2A, cell samples were removed, washed, lysed with detergent, and the intracellular fluorescence measured. The level of internalized FD was standardized to total cell protein to correct for differences in cell size. DΔdppik1/ddpik2 cells internalized FD at a greatly reduced rate compared to wild-type Ax3 cells. For instance, after 60 min (internalization was linear over this period), the control cells internalized 3–4-fold higher levels of FD than that internalized by mutant cells. The rate of uptake of FD was reduced over the first 5 min of internalization (results not shown). Taken together, these results suggest that the DdPIK1 and DdPIK2 gene products may regulate the earliest steps in pinocytosis.

An alternative explanation for the lower levels of fluorescence in the mutant cells is that the FD was internalized at the same rate in all cultures but the fluid phase marker was exocytosed at a faster rate from mutant cells as compared to wild-type cells. To test this possibility, we measured the rate of release of FD from cells loaded with this fluid phase marker to steady state (influx rates equal efflux rates). Wild-type and mutant cells were exposed to FD for 3 h, washed, and resuspended in FITC-free media and at various times, cell samples were removed and processed for fluorimetry as described in the Materials and Methods section. After 90 min, ~25% of the internalized FITC-dextran was retained in the control cells whereas the mutant cells retained 70% of the FITC-dextran, indicating a block in exocytosis (Fig. 2B). Therefore, the reduced levels of fluorescence observed in the pinocytosis assays cannot be accounted for by an increased rate of exocytosis from the cells. Instead, the data indicate that DdPIK1 and

Figure 1. The functional levels of PI 3-kinase and PI 4-kinase are identical in Δdppik1/ddpik2 and wild-type cells. The levels of PI kinases were measured in vitro as described in the Materials and Methods section.

Figure 2. Endocytosis and exocytosis of fluid phase are reduced in Δdppik1/ddpik2 phagocytosis is normal. Influx, efflux, and phagocytosis measurements were done as described in Materials and Methods. (A) Cells were centrifuged and resuspended in growth media containing FD at 2 mg/ml. At the times indicated, 1 ml of cells were removed, washed twice with growth medium, and once with buffered 100 mM sucrose. The cells were lysed with Triton X-100 and the fluorescence measured as described in the Materials and Methods section. (B) Cells were allowed to internalize FD for 3 h, washed, and resuspended in fresh growth medium. At the times indicated in the figure, cells were collected by centrifugation, washed and lysed in Triton X-100. The percent of FD that was retained within the cells was calculated by dividing the intracellular fluorescence measurement at the time indicated by the fluorescence measurement at 0 min. Symbols are described above. (C) Cells were centrifuged and resuspended in growth medium containing 1 micron crimson beads. At the times indicated in the figure, cells were collected, washed, and lysed in Triton X-100 and fluorescence measurements were made at Ex 625 nm and an Em 645 nm.
DdPIK2 encoded products regulated an early step in pinocytosis and perhaps a later step in the endocytic pathway leading to the efflux of fluid phase.

**The Rate of Phagocytosis Is Normal in Mutant Cells**

The mammalian p85/p110 PI 3-kinase has been reported to play a role in phagocytosis in mammalian cells (Nomiyama et al., 1994). To determine if Δddpik1/ddpik2 cells were normal in phagocytosis in D. discoideum, exponentially growing wild-type cells and mutant cells were exposed to crimson-labeled 1-μm latex beads for 90 min. At various times, cells were removed, washed, lysed, and the intracellular fluorescence was measured. Δddpik1/ddpik2 cells internalized beads (on a per protein basis) at a rate that was higher than wild-type cells throughout the time course; however, these rates were not significantly different (Fig. 2 C). A similar result was observed when FITC-labeled bacteria were substituted for beads (results not shown). The phagocytosis results were somewhat surprising since this process, like pinocytosis, is a form of endocytosis, and pinocytosis was severely reduced in the Δddpik1/ddpik2 strains. Therefore, these results indicate that the processes of phagocytosis and pinocytosis may be independently regulated processes in Dictyostelium.

**Fluid Phase Transport from Lysosomes to Postlysosomes Is Reduced in Δddpik1/ddpik2 Cells**

The results presented in the previous section suggest that DdPIK1 and DdPIK2 gene products regulated an early step in pinocytosis and a later step in the endosomal pathway. In D. discoideum, fluid phase reaches acidic lysosomes 15–20 min after internalization, and begins to be exocytosed from nonacidic postlysosomal vacuoles after a 45–60-min lag; no early recycling compartment has been identified in Dictyostelium. If DdPIK1 and DdPIK2 regulate fluid phase traffic at a stage like the lysosome to postlyso-

![Image](image_url)
with other smaller iron dextran-containing lysosomes. Greater than 90% of the wild-type Ax3 cells contained at least one large postlysosomal vacuole and 20–30% of the cells contained 3–6 postlysosomes. In contrast, greater than 90% of the mutant cells completely lacked the large postlysosomal vacuoles found in the control cells and instead contained an increased number of smaller iron positive vesicles (Fig. 4, bottom panels). The absence of postlysosomes and the presence of numerous, smaller lysosomes in the mutant cells, supports the hypothesis that DdPIK1 and DdPIK2 proteins regulate the formation of postlysosomes from lysosomes.

Figure 4. Δddpik1/ddpik2 cells lack large postlysosomal vacuoles. Cells were fed iron dextran for 2 h and processed for thin section electron microscopy as described in the Materials and Methods section. Control cells with the contractile vacuole (open arrowhead) and a postlysosome (arrow) indicated. Iron positive vesicles (closed arrowhead) in mutant cells. The bar is equivalent to 1.5 μm.
**The Processing and Sorting of Lysosomal α-Mannosidase Are Normal in Mutant Cells**

α-Mannosidase is synthesized as a 140-kD precursor that is processed to an 80-kD intermediate polypeptide and a 58-kD mature subunit; the 80-kD form is processed in lysosomes to the other 60-kD subunit (Cardelli, 1993). Mature lysosomal enzymes are eventually secreted from postlysosomal vacuoles. To determine if trafficking from the secretory pathway to lysosomes (the biosynthetic targeting pathway) was normal in Δddpik1/ddpik2, radiolabel pulse-chase experiments were performed. Logarithmically growing cells were pulsed with [35S]Met for 20 min, centrifuged, and resuspended in label free medium. At various times, samples were removed and separated into cell pellets and extracellular supernatants by centrifugation. α-Mannosidase was immunoprecipitated from both cells and supernatants using the monoclonal antibody, 2H9, and the immunoprecipitates were separated by SDS-PAGE and analyzed by fluorography. As shown in Fig. 5 a and b, the newly synthesized 140-kD α-mannosidase precursor was processed to the 60- and 58-kD mature forms in wild-type and mutant cultures with a half time of ~30–40 min. Since the final proteolytic processing event occurs in lysosomes, this result suggests that DdPIK1 and DdPIK2 do not play a role in regulating transport of hydrolases from the ER to lysosomes. Furthermore, in both control cells and the mutant cells <5% of the α-mannosidase precursors were secreted from either strain. This result suggests that DdPIK1 and DdPIK2 did not regulate hydrolase targeting to lysosomes. The newly synthesized precursors that were secreted, exited control and mutant cells by 10 min of chase and by 300 min of chase, 70% of the enzyme had been exocytosed (Fig. 5 a). In contrast, by 300 min of chase <5% of the mature radiolabeled enzyme was secreted from the mutant cells (Fig. 5 b). This result supports the fluid phase exocytosis data presented above and suggests that the processed mature lysosomal enzymes may not reach the secretory postlysosomal vacuoles in mutant cells.

**The Structure and Function of the Contractile Vacuole System Is Normal in Δddpik1/ddpik2 Cells**

The vacuolar proton pump populates both the endocytic pathway and the contractile vacuole (CV) system of membranes in *Dictyostelium* (Temesvari et al., 1994; Bush et al., 1994; Fok et al., 1994; Heuser et al., 1993; Nolta and Steck, 1994). The CV complex consists of a reticular network of membranes and a few large associated bladder-like vacuoles (Bush et al., 1994; Nolta and Steck, 1994). Recent evidence also suggests that the vacuolar proton pump regulates the structure and function of the CV and endosomal pathway and that these two pathways may be functionally connected (Bush et al., 1996; Temesvari et al., 1996b). Conceivably, PI 3-kinases could regulate membrane flow between the CV system and endosomal pathway. Therefore, to determine if the organization of the CV network was normal in Δddpik1/ddpik2 cells, permeabilized wild-type and mutant cells were incubated with monoclonal antibodies directed against the 100-kD subunit of the V H⁺-ATPase followed by the addition of secondary antibodies coupled to FITC. Fluorescence microscopy indicated that all cells contained a reticular network enriched in the 100-kD proton pump subunit (Fig. 6, B and D). In addition to the reticular network, large proton pump positive vacuoles were seen in control and mutant cells (Fig. 6). We have also observed that mutant cells were normal in osmotic regulation (results not shown) suggesting that DdPIK1 and DdPIK2 do not regulate the function of the CV network. Together, these results suggest that DdPIK1 and DdPIK2 proteins do not play a major role in the regulation of the organization or function of the CV complex.

**The Regulation of Cell Shape and the Distribution of Actin in the Cytoskeleton Is Altered in Mutant Cells**

A number of recent reports have implicated the PI 3-kinase

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**Figure 5.** Processing and sorting of lysosomal α-mannosidase is normal in mutant cells. Control cells (a) and mutant cells (b) were pulsed with 35S-met for 20 minutes, washed and resuspended in fresh growth medium. At the indicated times, α-mannosidase was immunoprecipitated from cells and supernatants, and subjected to SDS-PAGE followed by fluorography.
p85/p110 in controlling membrane ruffling and cell shape (Wennstrom et al., 1994), perhaps through activation of Rac GTPases (Hawkins et al., 1995) which in turn regulate changes to the actin cytoskeleton. The following experiments provide evidence that in Dictyostelium, the putative PI 3-kinases DdPIK1 and DdPIK2 may also regulate these processes. First, control cells grown overnight on plastic appeared amoeboid in shape when examined by phase contrast microscopy (Fig. 7A). In contrast, the mutant cells appeared as small, irregularly shaped cells with jagged edges around their periphery (Fig. 7B). Interestingly, 1–3% of the mutant cells contained long cellular bridges (inset Fig. 7B) that connected cells. These structures were stable for at least 1 h and probably represent cellular material shared by cells unable to efficiently complete fission. It remains to be demonstrated that these mutant cells are actually defective in cytokinesis.

Secondly, scanning electron micrographs of control cells revealed amoeboid-like cells with a smooth ruffled exterior (Fig. 8A) similar to what has been observed previously (Novak et al., 1995). Some filopodia were seen but these structures were usually short (< 2 μm in length) and mainly found in areas where the cell was attached to the surface. In contrast, scanning EM of mutant cells demonstrated that these cells were smaller than wild-type and more irregularly shaped (assuming a “puzzle piece” morphology), and the cells contained numerous filopodia (Fig. 8B) extending along both the plane of attachment of cells to the surface (closed arrowhead) and outward from the surface of cells (open arrowhead).

To determine the distribution of F-actin in control and mutant cells, we stained detergent permeabilized cells with FITC-labeled phalloidin, a compound that tightly binds F-actin. As seen in Fig. 9, A and B, F-actin in the control cells distributed primarily in a cortical pattern (open arrowhead) with only a few actin rich filopodia being observed (Fig. 9B). In mutant cells (Fig. 9, C and D), there was a decrease in the overall staining of cortical actin and...
a striking increase in the amount of actin-rich filopodia. Most of the remaining F-actin was found in patches around the cells and in the long cellular bridges described earlier (results not shown). In summary, compared to wild-type cells, mutant cells were smaller, more irregularly shaped and contained long cellular bridges that connected cells in the process of division. In addition, these cells contained numerous, actin-rich filopodia coupled with a decrease in cortical actin staining. Taken together, these results suggest DdPIK1 and DdPIK2 regulate cell morphology and organization of the actin cytoskeleton in Dictyostelium.

**Δdppik1/ddpik2 Cells Are Altered in Chemotaxis**

During the aggregation phase of development, Dictyostelium cells chemotax toward cAMP, resulting in the formation of a multicellular organism containing up to $10^5$ cells. This process is regulated through G protein–coupled, cell surface receptors that activate downstream effector pathways, including the activation of guanylyl cyclase, adenylyl cyclase, and aggregation-stage gene expression (Firtel, 1995; Parent and Devreotes, 1996). To determine if chemotactic movement was altered in Δdppik1/ddpik2 cells, we examined the ability of aggregation-competent cells to chemotax toward a micropipette containing the chemoattractant CAMP (Gerisch et al., 1995) (see legend to Fig. 10 for details). As can be observed by comparing the movement of Δdppik1/ddpik2 null to wild-type cells in Fig. 10 A, Δdppik1/ddpik2 cells chemotax significantly more rapidly toward a pipette tip emitting cAMP than do wild-type cells. To examine this in more detail, we followed the ability of individual cells to undergo chemotaxis toward cAMP and then to reverse direction as the position of the micropipette was changed. Fig. 10 B shows this anal-
ysis for \( \Delta dd pik1/dd pik2 \) cells. As is observed, \( \Delta dd pik1/dd pik2 \) cells rapidly respond both to the initial insertion of the micropipette (Fig. 10 B, a–e) and when the position of the tip was placed on the other side of the cells (Fig. 10 B, f–i). Comparison of these images to the movement of wild-type cells toward the pipette (Fig. 10 C, a–f) and as the position of the needle is moved (Fig. 10 C, g–i) shows that the wild-type cells respond and move more slowly. (Note \( \Delta dd pik1/dd pik2 \) cell images are every 1 min while those of the wild-type cells are every 2 min on the initial movement. See legend to Fig. 10 for details.) \( \Delta dd pik1/dd pik2 \) cells show rapid reorientation and movement when the tip is moved, while, within the similar period of time, wild-type cells had just started to reorient themselves. These results indicate that both the response to the chemoattractant and the rate of chemotaxis are faster in the double knockout strain.

**Discussion**

In this report, we used cell biological and biochemical approaches to demonstrate that the \( Dd PI K1 \) and \( Dd PI K2 \) PI 3-kinase–related gene products regulated multiple processes in *Dictyostelium discoideum*. \( \Delta dd pik1/dd pik2 \) cells internalized fluid phase at 20–25% of the rate of wild-type cells; the efflux rate of internalized fluid phase was also greatly reduced in the mutant cells. The lysosome to postlysosome transport step appeared to be delayed in \( \Delta dd pik1/dd pik2 \), accounting for the reduction in exocytosis of fluid phase in mutant cells. Phagocytosis, lysosomal enzyme targeting, and CV structure and function were normal in mutant cells demonstrating the specificity of action of the enzymes. Finally, \( Dd PI K1 \) and \( Dd PI K2 \) also appeared to play a role in regulating cell shape and cell movement perhaps by regulating the actin cytoskeleton.
DdPIK1 and DdPIK2, Proteins Related to the Mammalian p110 PI 3-kinase May Regulate Pinocytosis, but Do Not Play a Major Role in Phagocytosis

Five genes encoding proteins related to PI kinases on the basis of amino acid sequence homology have been cloned from *Dictyostelium*. Three of these genes, DdPIK1, DdPIK2, and DdPIK3, encode proteins most related to the mammalian 110-kD PI 3-kinase subunit (Zhou et al., 1995). The strain characterized in this report Δdppik1/dppik2 is disrupted in two of these genes, DdPIK1 and DdPIK2, and grows slowly in axenic medium when attached to plastic; the strain does not grow at all in suspension in axenic medium (Zhou et al., 1995). Single knockout strains grow normally with no observable defects. This suggests that the DdPIK1 and DdPIK2 gene products have overlapping or redundant functions, therefore, requiring that both genes be disrupted before an effect is observed. The rate of fluid phase uptake was much less in the mutant cells as compared to wild-type cells. This might account for the growth defects observed for the mutant since nutrients are internalized from growth medium by pinocytosis in *Dictyostelium*.

Disruption of the DdPIK1 and DdPIK2 genes did not significantly reduce the levels of functional enzymes capable of phosphorylating PI in vitro to generate PI(3)P. The levels of mRNA encoding DdPIK1 and DdPIK2 are extremely low, and it is possible that the loss of these enzymes in the mutant would not be detected even if these enzymes use PI as a preferred substrate. Furthermore, using PI(4)P and PI(4,5)P2 as substrates we were still only able to identify PI(3)P and PI(4)P products following in vitro kinase assays, therefore, we cannot determine if the levels of PI 3-kinase enzymes recognizing PI(4)P and PI(4,5)P were reduced in Δdppik1/dppik2. Based on amino acid sequence homology, however, DdPIK1 and DdPIK2 are more closely related to mammalian p85/p110 PI 3-kinase than to yeast Vps34 (only recognizing PI as a substrate). Therefore, we speculate that disruption of DdPIK1 and DdPIK2 reduce the levels of PI 3-kinase enzymes capable of recognizing substrates like PI(4,5)P2 and that reduced levels of PI(3,4,5)P3 may account for the phenotypic changes presented here.

The reduction in the rate of fluid phase internalization was evident at the earliest time points examined suggesting that a very early step in pinocytosis (perhaps the internalization step) was regulated by DdPIK1 and DdPIK2. Consistent with our results, two recent reports have indicated that a PI 3-kinase sensitive to low levels of wortmannin (hypothesized to be p85/p110) also played a role in pinocytosis in mammalian cells (Li et al., 1995; Clague et al., 1995). Although it has been reported that the clathrin heavy chain is involved in pinocytosis in *Dictyostelium* (O’Halloran and Anderson, 1992; Ruscetti et al., 1994), recent studies examining endocytosis in yeast (Kubler and Riezman, 1993) and in *Dictyostelium* (Temesvari, L., and J. Cardelli, unpublished results) indicate that actin may also be critical in regulating pinocytosis. In support of this, influx of fluid phase is regulated by select myosin I proteins that are known to interact with and regulate the actin cytoskeleton (Luna and Condeelis, 1990; Novak et al., 1995; Temesvari et al., 1996a).

It has been reported that the p85/p110 PI 3-kinase regulates phagocytosis in mammalian cells (Ninomiya et al., 1994); however, phagocytosis of beads and bacteria were normal in the mutant cells. It is possible though that other *Dictyostelium* PI 3-kinases, like DdPIK3 or DdPIK5, regulate phagocytosis. As discussed below, Δdppik1/dppik2 cells contained an increased number of filopodia, structures that may play an important role in binding beads and bacteria as the first step of phagocytosis (Maniak et al., 1995). Conceivably, increased filopodia may partially compensate for the reduction of cortical actin in the mutant cells; the organization of cortical actin has been suggested to be important in phagocytosis (Maniak et al., 1995). Regardless, these observations suggest that the mechanisms regulating pinocytosis and phagocytosis may be overlapping but they are not identical, a possibility further supported by recent studies that indicate cells lacking functional heterotrimeric G proteins (Wu et al., 1995) are normal in pinocytosis but defective in phagocytosis (Buczynski, G., and J. Cardelli, unpublished results).

Δdppik1/dppik2 Cells Are Defective in Transport of Material from Lysosomes to Postlysosomes but Are Normal in Transport, Proteolytic Processing, and Targeting of Lysosomal Enzymes

Endocytosis in *Dictyostelium* differs from endocytosis in mammalian cells in at least three fundamental ways. First, no early fluid phase endosomal recycling compartment has been identified in *Dictyostelium*, second, nearly 100% of internalized fluid phase is secreted from *Dictyostelium* cells (Padh et al., 1993; Aubry et al., 1993) and third, large vacuoles exist located downstream from lysosomes along the endosomal pathway. These vacuoles termed postlysosomes are greater than 2.5 μm in diameter, contain less vacuolar proton pumps and have lumens that are much less acidic than lysosomes (Nolta et al., 1995). Postlysosomes may arise from fusion of lysosomes (Rodriguez-Paris et al., 1991). Mammalian cells do not contain a compartment analogous to postlysosomes although this type of vacuole may exist in other free living amoebae including the human pathogen *Entamoeba histolytica* (Aley et al., 1984). Our results demonstrate that DdPIK1 and DdPIK2 played an important role in regulating traffic between lysosomes and postlysosomes. A combination of biochemical and microscopic approaches revealed that FD accumulated in mutant cells in small, acidic, protease positive vesicles (0.5–1.0 μm in diameter) that have all the characteristics of *Dictyostelium* lysosomes (Temesvari et al., 1994; Rodriguez-Paris et al., 1993). The block in transport to postlysosomes may account for the reduction in rates of fluid phase exocytosis. Electron microscopy also indicated a nearly complete absence of postlysosomal vacuoles in mutant cells supporting the model proposed by others (Nolta et al., 1995) that postlysosomes are not stable compartments but instead form from the fusion of smaller lysosomes and are later consumed during exocytosis. It has been reported recently that a late step in transcytosis is regulated by PI 3-kinase sensitive to low concentrations of wortmannin (Hansen et al., 1995), consistent with our data supporting a role for DdPIK1 and DdPIK2 later in the endosomal pathway.
It has been demonstrated that low concentrations of wortmannin inhibit endosomal fusion in mammalian cells (Jones and Clague, 1995; Li et al., 1995), and Stahl and co-workers have interpreted this to mean that a PI 3-kinase might regulate this event (Li et al., 1995). They further propose that this PI 3-kinase regulates the fusion of endosomal vesicles by activation of Rab5 (Li et al., 1995), a small Mr GTPase previously demonstrated to play an important role in fusion of early endosomes (Bucci et al., 1992). The Rab GTPases are members of the Ras superfamily of G proteins, and Rab proteins have been demonstrated to regulate vesicle trafficking and fusion in a variety of systems (Pfeffer, 1994). Although a number of Dictyostelium Rab proteins have been characterized (Bush et al., 1993, 1994; Bush and Cardelli, 1995), no Rab5 encoding gene has been isolated from this organism. However, we have recently determined that a Rab4-like GTPase (named RabD) regulated lysosome to postlysosome transport (Bush et al., 1996), and conceivably PI 3-kinase may activate or regulate this Rab protein, or other Rab proteins such as a Rab7-like GTPase and a novel GTPase (RabB), also localized to the endosomal pathway (Bush and Cardelli, 1995; Buczynski, G., and J. Cardelli, unpublished results).

\( \Delta ddpik1/ddpik2 \) cells were normal in sorting and processing of lysosomal \( \alpha \)-mannosidase, suggesting that DdPIK1 and DdPIK2 were not critical in the regulation of the biosynthetic targeting pathway to lysosomes. In contrast another PI 3-kinase, Vps34p, plays a role in the sorting of hydrolases to the yeast vacuole (Stack et al., 1995) but this enzyme is more related to the Dictyostelium PI 3-kinase, DdPIK5, than to DdPIK1, DdPIK2, or DdPIK3 (Zhou et al., 1995). Also, DdPIK5 can partially compensate for the absence of Vps34 in \( S. \ pombe \) suggesting that DdPIK5 may be a Dictyostelium Vps34 homologue. Sorting of hydrolases may be normal in \( \Delta ddpik1/ddpik2 \) because another PI 3-kinase (perhaps DdPIK5) is responsible for this process. Therefore, it remains to be tested whether lysoso-
mal α-mannosidase is targeted by a PI 3-kinase independent pathway. Others, however, studying the targeting of certain plant proteins have concluded that PI 3-kinase independent lysosomal targeting pathways do exist.

Two proteins, RabD (Bush et al., 1996) and the vacuolar proton pump (Temesvari et al., 1996b), play important roles in regulating endocytosis and the structure and function of the CV in Dictyostelium. Based in part on this evidence, it has been proposed that the CV and the endosomal pathway are physically connected by membrane trafficking (Bush et al., 1996; Padh and Tanjore, 1995). In contrast, the Dictyostelium DdPIK1 and DdPIK2 gene products, although important in endocytosis, did not play a role in regulating the structure and function of the CV network, suggesting that only a subset of the proteins regulating endocytosis also regulate the structure and function of the CV network.

**DddPIK1/ddPIK2 Mutants Are Altered in Regulation of the Actin Cytoskeleton and in Chemotactic Responses**

1–3% of the Dddpik1/ddpik2 cells growing attached to plastic were connected by thin cytoplasmic bridges that lasted for 1–2 h suggesting defects in cytokinesis. Furthermore, defects in cytokinesis could account for the inability of Dddpik1/ddpik2 cells to grow in shaking suspense culture. Cytokinesis is regulated by a variety of proteins including myosin II (Knecht and Loomis, 1987; De Lozanne and Spudich, 1987), myosin essential light chain (Chen et al., 1995), and calmodulin (Liu et al., 1992). All of these proteins regulate the function of actin and/or myosin, and acto-myosin complexes are important in controlling the retraction of the contractile ring during cytokinesis leading to the fission of newly replicated cells. Therefore, given the proposed role for PI 3-kinases in regulating the actin cytoskeleton, it is possible but remains to be formally tested that DdPIK1 and DdPIK2 regulate cytokinesis.

Dddpik1/ddpik2 cells were also morphologically different from wild-type cells. Mutant cells were smaller than wild-type cells and were more irregular in shape. Light microscopy and scanning electron microscopy also indicated that these cells contained significantly more actin-rich filopodia than wild-type cells and less cortical actin. Membrane ruffles were almost never seen in mutant cells consistent with the demonstrated role of p85/p110 PI 3-kinase in regulating membrane ruffling in mammalian cells. The altered distribution of actin may be responsible for the de-
crease observed in pinocytosis although phagocytosis was normal.

It is not yet clear how the putative PI 3-kinases, DdPIK1 and DdPIK2, regulate the distribution of F-actin or why reductions in the level of these enzymes would lead to an increase in filopodia formation. It is conceivable that these putative PI 3-kinase(s) normally function to repress the formation of filopodia and the loss of these proteins results in an increased number of filopodia. However, in mammalian cells, PI 3-kinase has been shown to activate Rac GTPases that, in turn, stimulate actin redistribution and induce membrane ruffling (Wennstrom et al., 1994; Ridley et al., 1992). Therefore, we consider it more likely that formation of filopodia may be part of a signal transduction pathway that triggers the activation of DdPIK1 and DdPIK2 which in turn stimulates changes in F-actin distribution leading to the formation of plasma membrane lamellipodia “webs” that fill the space between the filopodial “fingers” (Nobes and Hall, 1995a).

Our results also indicate that there is a significant alter-

Figure 10. Effect of disruption of ΔDdPIK1/DdPIK2 on chemotaxis towards cAMP. Log phase vegetative cells grown on monolayer on plastic Petri dishes were washed and pulsed with 30 nM cAMP every 6 min for 4 h to maximize the number of cAMP receptors and other components of the aggregation-stage cAMP receptor signal transduction pathway (aggregation-competent cells) (Devreotes et al., 1987; Mann et al., 1994). Cells were then washed and plated at low density in plastic Petri dishes in 12 mM phosphate buffer (pH 6.2) allowed to adhere. 200 μM cAMP was loaded into Eppendorf femtotips and the location of the tip positioned using an Eppendorf micro-manipulator. Cells were visualized using a Nikon phase contrast inverted microscope with a 20× (A) or 40× (B and C) objective. Movement of the cells was followed using a time-lapse video microscopy. Individual frames were then grabbed onto a Macintosh computer using NIH image 1.59 and a Scion imaging board. (A) Chemotaxis of wild-type and Δdppik1/dppik2 cells to cAMP. Images a–f are of Δdppik1/dppik2 cells and are taken 4 min apart. (B) Movement of Δdppik1/dppik2 cells toward the pipette tip at higher magnification and lower cell density. The micropipette was inserted and the movement of the cells recorded. Images a–e are taken 1 min apart, compared to 2 min for the wild-type cells. Image f shows the position of a micropipette with cells migrating toward it. Immediately after image f, the micropipette was moved to the right. Image g is 30 s later. Images h and i are 1 min apart after image g. Note that the Δdppik1/dppik2 cells have changed shape and initiated movement. (C). Movement of wild-type cells toward the pipette tip at higher magnification at lower density. Images a–e are 2 min apart. Image f is 1.5 min after image e. Image g is 4 min later. Immediately after image g, the micropipette was moved to the left. Image h is 30 s after g and image i is 1.5 min after h.
ation in the ability of aggregation-competent cells to move toward the chemotactrant cAMP. Unexpectedly, DdPIK1 and DdPIK2 encode PI-3 kinases that the phospholipid products may be involved in modulating the effect of chemotactrant on the ability of the cells to move, possibly by regulating pathways controlled by members of the Rho/Rac family that affect membrane ruffling, filopod extension, etc. (Nobes and Hall, 1995). Alternatively, the ability of the cells to respond and move more rapidly may be the result of our observed changes in the actin cytoskeleton in resting cells.

In summary, we have found that DdPIK1 and DdPIK2, genes that encode proteins homologous to p110-like PI 3-kinases, play diverse but select roles in regulating endocytosis, cell shape, and cell motility in Dictyostelium. Future studies will be directed towards determining the substrate specificity of these proteins as well as the nature of the effector molecules they interact with.

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