FUNCTIONAL DISSECTION OF LIPID AND PROTEIN KINASE SIGNALS OF PIKfyve REVEALS THE ROLE OF PtdIns 3,5-P₂ PRODUCTION FOR ENDOMEMBRANE INTEGRITY

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Running title: Selective function of PtdIns 3,5-P₂ in endomembranes

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SUMMARY

PIKfyve enzymatic activity is required in maintaining late endocytic membrane integrity. PIKfyve is a dual-specificity enzyme that phosphorylates phosphatidylinositol (PtdIns) and PtdIns 3-P at the 5-hydroxyl and unidentified endogenous protein substrate(s). To distinguish between which of these activities (lipid versus protein kinase activity) is responsible for endomembrane homeostasis we analyzed a double mutant PIKfyveK1999/2000E. These substitutions in the putative lipid-substrate activation loop nearly completely abrogated the lipid kinase activity without any significant effect on the protein kinase activity of PIKfyveK1999/2000E. Expression of PIKfyveK1999/2000E in COS cells induced a dramatic dominant-negative effect in the form of endomembrane swelling and vacuolation. In addition, the lipid-substrate specificity of PIKfyve was modified by introducing single mutations in Lys1999 or Lys2000. This yielded proteins with preferentially abrogated synthesis of PtdIns 5-P (PIKfyveK2000E) or PtdIns 3,5-P2 (PIKfyveK1999E), of which only the PIKfyveK1999E mutant induced the characteristic endomembrane defects upon cell transfection. Furthermore, phosphoinositide microinjection into cells demonstrated a selective ability of PtdIns 3,5-P2 to correct the endomembrane defects induced by the dominant-negative PIKfyve lipid kinase-deficient mutants. Thus, PtdIns 3,5-P2 production by PIKfyve is crucial for endomembrane integrity and Lys1999 most likely directs the PIKfyve interactions with the 3-phosphate group in PtdIns 3-P.
In eukaryotic cells complex control mechanisms operate to ensure the temporal and spatial regulation of intracellular membrane trafficking pathways (for a recent review see Ref. 1). Although the primary role of proteins in the trafficking events is indisputable, multiple studies indicate that lipids, in particular the highly phosphorylated metabolites of phosphatidylinositol (PtdIns)\(^1\), collectively called phosphoinositides (PI) also play a fundamental role in this process (for recent reviews see Refs. 2-4). This has been first demonstrated by genetic studies of protein transport to the yeast vacuole (or lysosome in higher cells). One of the required genes, VPS34 appeared to be a PI 3-kinase that specifically phosphorylates PtdIns at position D-3 of the inositol ring to produce PtdIns 3-P (5). Subsequent genetic and biochemical studies both in yeast and mammalian cells have uncovered that many lipid kinases and phosphatases, presumably through generating specific PI signals are important in several cellular membrane transport processes such as clathrin-mediated endocytosis, GLUT4 vesicle translocation, formation of MVBs, autophagy, phagocytosis, micropinocytosis and biosynthetic trafficking events (2-4).

It should be emphasized that most of our knowledge related to the functions of PIs is based on studies involving stimulation and/or inactivation of the lipid kinases/phosphatases that produce them. To fully understand the roles of PIs and to obtain mechanistic insight in the context of live cells, specific cellular changes elicited by particular PI molecular species must be documented. Because the cell

\(^1\) **ABBREVIATIONS:** DMEM, Dulbecco's modified Eagle medium; PAGE, polyacrylamide gel electrophoresis; PI, phosphoinositide; PtdIns, phosphatidylinositol; P, phosphate; P\(_2\), bisphosphate; P\(_3\), trisphosphate; Ks, kinases; GFP, green fluorescent protein; HA, hemagglutinin; MVBs, multivesicular bodies; TxB; Texas-red.
membrane is impermeable to these anionic lipids, direct demonstration that a specific PI functions as an effector in a cellular context was illustrated in only few instances (6-9). Data based on lipid-derived studies are critical because of two reasons. First, in a cellular membrane micro-environment, a complex of factors such as substrate availability, accessibility and presentation, or enzyme regulation may affect the lipid substrate specificity, making the identity of the final PI effector unclear. Second, most members of the PIK family, including PI 3-Ks, PI 4-Ks and PI 5-Ks, are now found to display a dual specificity, i.e., act as both lipid and protein kinases (10). Physiologically relevant protein substrates and potential roles of these protein kinase activities in cellular regulation are still unknown for the majority of the dual specificity kinases. However, recent studies in which the lipid and protein kinase activities of PI 3-K\textgamma have functionally been separated (11) clearly demonstrated distinct signals triggering different cellular functions.

PIKfyve, a mammalian phosphoinositide 5-kinase containing a Fyve finger domain (12) is one of the best examples of such a perplexing situation. This kinase was found to display a dual specificity for both lipids and proteins (13). It is largely localized to MVBs suggesting a role of the phosphorylated products of the PIKfyve enzymatic activity in MVB function (14). In agreement with this prediction, expression of a kinase-dead point mutant, PIKfyve^{K1831E}, elicited a dramatic dominant-negative phenotype in a form of a progressive accumulation of multiple swollen vacuoles with endocytic origin (15). Based upon available structural studies of PIPK Type II\textbeta (16) and PI 3-K\textgamma (17), and the conservation of the catalytic
machinery between PIP-kinases and protein kinases (18), Lys1831 was determined as the putative amino acid that binds the \( \alpha \)-phosphate of ATP. Concordantly, both enzymatic activities were fully eliminated in the dominant-negative PIKfyve\( ^{K1831E} \) mutant (13). Furthermore, the PIKfyve lipid kinase activity was found to synthesize two phosphoinositide products, PtdIns 3,5-P\(_2\) and PtdIns 5-P (19). Thus, although the role of PIKfyve enzymatic activity in maintaining the endocytic membrane homeostasis was unequivocally established, the precise lipid and/or protein product responsible for this function remained to be determined. Therefore to distinguish between the lipid and protein kinase activities we analyzed several PIKfyve mutants that selectively display either one of the two enzymatic activities or altered PI-substrate specificity. In addition, microinjected PIKfyve lipid products were examined for correcting the intracellular membrane defects induced by the PIKfyve lipid kinase-deficient mutants. In this paper we demonstrate that PtdIns 3,5-P\(_2\) production is essential for endomembrane homeostasis.
EXPERIMENTAL PROCEDURE

Constructs and fusion proteins—Point or double substitutions of Lys1999 and Lys2000 to Glu in the PIKfyveS sequence were introduced by PCR. Initially, the HindIII-PstI fragment of clone K12 (12) that encompasses nt 5216 to 6462 of the PIKfyve sequence (nucleotide numbers correspond to the GenBank accession number AF102777) was amplified as 2 fragments and subsequently linked in the engineered XhoI site (CTCGAG replacing the original sequence C\textsuperscript{G\textsubscript{Lys1999}}\textsubscript{G\textsubscript{Lys2000}}\textsubscript{G\textsubscript{Lys1999/2000}} with no change in amino acids) positioned downstream of the mutations. The first fragment (~900 bp) was amplified with an external sense primer harboring the authentic HindIII site at nt 5216 (5'-GCCGAAGCTTCAGGAATGTGG-3') and an internal anti-sense primer specific for each mutant (5'-CGGCCTCGAGTTTTTGCTCCCATG-3' for K1999; 5'-CGGCCTCGAGTTTTTGCTCCCATG-3' for Lys2000 and CGGCCTCGAGTTTTTGCTCCCATG-3' for Lys1999/2000; the XhoI site and the base substitutions are underlined). The second fragment (~300 bp) was amplified with an internal sense primer identical for the three mutants with silent substitutions for the XhoI site (5'-CGGCCTCGAGATGGTTGTGAAG-3') and an external anti-sense primer harboring the PstI site from the 3' UTR of the PIKfyve sequence (5'-GCCGCTCGAGTCATGGATGTTGTGAAG-3'). The PCR products were digested with HindIII-XhoI or PstI-XhoI, respectively, and then ligated into the HindIII-PstI cloning sites of pBluescript II SK(+) to confirm the sequence and the desired mutation by sequencing and restriction analysis with XhoI. The HindIII-PstI fragment (aa 1694-2052) with the confirmed mutation together with the NcoI-
HindIII fragment (aa 1232-1694) of PIKfyve-pBluescript II SK(+) (nt 3833 to 5216) were inserted into the NcoI-PstI digest of pGEM-T vector (Promega). The NcoI-SalI fragment (aa 1232-2052) of the latter construct together with the KpnI-NcoI fragment of PIKfyve-pBluescript II SK(+) (nucleotides 2929 to 3833; aa 930-1232) were ligated into the KpnI-SalI digest of pBluescript II SK(+). Finally, to generate the full length of the mutants, N-termini-tagged with the HA epitope, the KpnI-SalI fragment (aa 930-2052) of the latter vector was inserted into the KpnI-SalI digest of pCMV5-HA-PIKfyve described previously (12). Mutants were subcloned into pEGFP-C2 (Clontech) by releasing the XbaI/SalI fragments from these pCMV5 constructs and subsequent ligation into the XbaISalI digest of pEGFP-HA-PIKfyveWT (14) to generate GFP-HA-tagged constructs. The expected organization of the mutants was confirmed by restriction endonuclease mapping.

**Cell cultures and transfections**-- COS-7 cells seeded at 750,000 cells per 100-mm plate (for biochemistry) or 125,000 cells per 35-mm plate (for fluorescence microscopy) were maintained in DMEM, containing 10% fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin sulfate. Cell transfection with cDNAs of HA-epitope tagged versions of PIKfyveWT and mutants either in pCMV5 or pEGFP expression vectors was performed by LipofectAmine as described previously (14). The two type of constructs were examined in both biochemical and fluorescence microscopy studies described below. Identical results were obtained for GFP-HA- vs HA-based constructs indicating that the GFP-epitope tag at the N-terminus did not affect the functions of the HA-PIKfyve constructs.
Cell Microinjection and Fluorescence microscopy – Synthetic dipalmitoyl PtdIns 3,5-P$_2$, PtdIns 4,5-P$_2$ PtdIns 3-P or PtdIns 5-P (a gift by Echelon Res. Lab., Inc) in chloroform-methanol-water (9:9:1 v/v) (0.5 mM) were evaporated to dryness under nitrogen and then hydrated in equal volume of injection buffer (100 mM KCl, 5 mM sodium phosphate, pH 7.4) by intermittent, vigorous vortexing (5 min at 40°C). Lipids were then sonicated (2 x 30 sec) and kept on ice. Fluorescent dextran (TxB-dextran, 70,000, Molecular Probes) was added at 0.5 mg/ml as an injection marker. The resultant mix was spun for 15 min at 4°C and back-loaded into microelectrodes (Femtotip, Eppendorf). Twenty h postransfection, COS-7 cells on 35 mm dishes were overlayed with L-15 media (Gibco, BRL) and microinjected in the cytoplasm with a semiautomatic microinjector (Eppendorf micromanipulator 5171 and Femtojet 5247), mounted on a Nikon Eclipse TE 300 inverted fluorescence microscope. Following injections of 100-250 cells (~1 h) the L-15 media was replaced with complete DMEM and cell cultures were maintained under standard conditions. Images of live cells were captured by SPOT RT Slider charge-coupled device camera (Diagnostic Instruments) mounted on the microscope and then processed by software SPOT 3.2.

Immunoprecipitation, Immunoblotting, Lipid and Protein Kinase assays

Forty eight hours posttransfection COS-7 cells, transiently transfected with HA-epitope-tagged PIKfyves constructs in either pCMV5 or pEGFP expression vectors, were lysed in RIPA buffer (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 1x protease inhibitor cocktail). Lysates were
immunoprecipitated with rabbit anti-HA polyclonal antibodies (kind gift from Mike Czech) that quantitatively immunoprecipitated HA-tagged PIKfyve as described elsewhere (13). Anti-HA-immunoprecipitates were immobilized on Protein A-Sepharose CL-4B beads and used in both protein and lipid kinase assays as described (13,19). Control immunoprecipitates on preimmune serum or anti-HA immunoprecipitates from control transfections with the empty vectors were run in parallel. Briefly, in the protein kinase assay the PIKfyve autokinase activity was evaluated (13) in a reaction composed of [γ-32P]ATP (3-6 μCi; 6000Ci/mmol, DuPont-NEN), 25 μM ATP, 2.5 mM MnCl₂ and 12 mM MgCl₂ in 50 mM HEPES, pH 7.4 and continued for 30 min (unless otherwise stated) at 25°C. Washed beads were boiled in Laemmli sample buffer and analyzed by SDS-PAGE (6% acrylamide). Resolved proteins, transferred onto Immobilon P were analyzed by autoradiography (Kodak X-OmatAR). Protein expression levels were controlled by parallel immunoprecipitation and immunoblotting with anti-HA antibodies. Detection by horseradish peroxidase-bound anti-rabbit IgG and chemiluminescence detection kit (DuPont-NEN) was described elsewhere (13).

For the lipid kinase assay, the HA immunoprecipitates immobilized on Sepharose beads were washed as previously described (19). The reaction (50 μl final volume) was carried out for 15 min at 30°C in the assay buffer (25 mM Hepes, pH 7.4, 120 mM NaCl, 2.5 mM MgCl₂ and 2.5 mM MnCl₂, 5 mM β–glycerophosphate and 1 mM DTT) supplemented with 50 μM ATP, [γ–32P]ATP (12.5 μCi) and 100 μM PtdIns (from soybean, Avanti Polar Lipids Inc) sonicated prior to use in 20 mM
HEPES, pH 7.5, 1 mM EDTA. Lipids were extracted, applied on TLC plates (Whatman, PE SIL G, 250 µm) and separated by the acidic solvent system as described previously (19). Generated radioactive products were detected by autoradiography and quantified by radioactive counting of the silica scrapings.
RESULTS AND DISCUSSION

In the absence of a resolved crystal structure of the PIKfyve catalytic domain, we compared the primary amino acid sequence of the PIKfyve-related proteins with the available structural characteristics of the PtdIns-3-P binding site within the FYVE finger domain in human EEA1 protein (20). Sequence comparison established that the seven known PIKfyve-related deduced protein sequences in S. cerevisiae, S. pombe, A. thaliana, C. elegans, D. melanogaster, M. musculus, and H. sapiens display a well conserved region within the catalytic domain, which is not present in the other PIP kinases (reviewed in Ref. 21). A consensus sequence, T(F/Y)T(W/L)DKLE(S/T/M)WVKXXG was proposed to define the specificity of the PtdIns 3-P-substrate activation loop (22). This motif displays a well-maintained pattern of hydrophobicity and three conserved basic residues, two of which are adjacent. Intriguingly, in the recent NMR studies of the FYVE finger domain of EEA1 in its PtdIns 3-P-bound state, basic clusters of the exposed hydrophobic loop were found to constitute the binding pocket for the inositol head group of PtdIns 3-P with two adjacent and one distal basic amino acids engaged in the ligation of 1- and 3-phosphate groups (20). Based on these data the two adjacent lysines of the consensus sequence, corresponding to Lys1999 and Lys2000 in PIKfyve, were selected for mutation with the premise that the lipid kinase but not the protein kinase activity will be compromised in the resulting PIKfyveK1999/2000E mutant.

To determine the effect of the double mutation on the protein and lipid kinase activity in vitro, COS-7 cells were transiently transfected with the HA-tagged
version of PIKfyve\textsuperscript{K1999/2000E}. Anti-HA-immunoprecipitates of the cell lysates were first analyzed in the lipid kinase assay along with HA-immunoprecipitates of COS cells transfected with PIKfyve\textsuperscript{WT} and the PIKfyve\textsuperscript{K1831E} kinase-defective mutant, that was shown to have no detectable lipid kinase activity (13). As expected, PIKfyve\textsuperscript{WT} displayed functional lipid kinase activity and synthesized PtdIns 5-P and PtdIns 3,5-P\textsubscript{2} from native PtdIns at a ratio 1:2.3 (Fig. 1A, lane 2). The lipid kinase activity of PIKfyve\textsuperscript{K1999/2000E} was dramatically reduced (Fig. 1A, lane 4) although not fully eliminated as with the PIKfyve\textsuperscript{K1831E} kinase-defective mutant (Fig. 1A, lane 3). At longer exposure times, a relatively small amounts of PtdIns 5-P and PtdIns 3,5-P\textsubscript{2} were detected. This corresponded to 9 \pm 1\% and 4 \pm 0.2\% (n=3) of the PtdIns 5-P and PtdIns 3,5-P\textsubscript{2} levels, respectively, produced by PIKfyve\textsuperscript{WT} at a similar level of protein overexpression (Fig. 1B). We next examined the ability of the double mutant to act as a protein kinase by monitoring the rate of its autophosphorylation activity. Importantly, whereas the lipid kinase activity of PIKfyve\textsuperscript{K1999/2000E} was largely abrogated, its protein kinase activity was identical to that of PIKfyve\textsuperscript{WT} (Fig. 1C and D). Consistent with our previous studies, lack of any detectable protein kinase activity was observed for the PIKfyve\textsuperscript{K1831E} mutant at expression levels equal to or higher than PIKfyve\textsuperscript{WT} (Fig. 1B and C). Importantly, examination of GFP-based HA-con structs showed protein and lipid kinase activities indistinguishable from the corresponding HA-tagged constructs, confirming that GFP-addition at the N-terminus of HA-PIKfyve did not alter the activity (Fig. 1D). Together, these results indicate that while PIKfyve protein and lipid kinase share many common
characteristics including the use of Lys1831 in ATP binding/catalysis (13), they utilize distinct substrate binding mechanisms. Furthermore, the fully preserved protein kinase activity of PIKfyve<sup>K1999/2000E</sup> demonstrates that these mutations did not result in a gross structural alterations in the kinase catalytic core.

The identification of a PIKfyve mutant, in which the protein and lipid kinase activities were differentially affected allowed us to test which of the two activities is important for the fundamental function of PIKfyve in the maintenance of endocytic membrane homeostasis (15). COS-7 cells transiently transfected with the pEGFP-PIKfyve<sup>K1999/2000E</sup> cDNA were examined for vacuolation phenotype. As illustrated in the images in Fig. 2a and b, the cells expressing PIKfyve<sup>K1999/2000E</sup> double mutant acquired the typical morphological defects identical to that of the PIKfyve<sup>K1831E</sup> mutant (15). Thus, 15 h post-transfection, dilation of the PIKfyve-containing vesicles was initiated followed by the formation of large vacuoles throughout the cytoplasm. Approximately 36 h post-transfection, all cells transfected with pEGFP-PIKfyve<sup>K1999/2000E</sup> cDNA developed vacuoles that highly resembled those developed in PIKfyve<sup>K1831E</sup>-expressing cells. These include the honeycomb appearance, giant 2-3 vacuoles of 5-10 μm in some cells, most probably resulting from an increased fusogenic activity, and co-localization of PIKfyve<sup>K1999/2000E</sup> fluorescence signals with the late endosomal marker cation-independent mannose 6-P receptors at the cell perinuclear region (15, and this study, data not shown). Taken together, these results unequivocally demonstrate that the function of PIKfyve in maintaining
Endomembrane homeostasis is mediated by phosphorylated signals generated by the PIKfyve lipid kinase but not by its protein kinase activity.

*In vitro* studies have defined PIKfyve as a kinase specific for position D-5 in both PtdIns 3-P and PtdIns substrates (19). PIKfyve-dependent intracellular production of PtdIns 3,5-P$_2$ has been confirmed in different cell types following metabolic labeling of cellular lipids with $^{32}$P orthophosphate (15). However, in these studies intracellular PtdIns 5-P levels were not detected, although their presence have been repeatedly reported in some cell types (reviewed in Ref. 21). Thus, it is possible that PIKfyve-originated PtdIns 5-P production is significant in the context of a given intracellular membrane microdomain and this could contribute to the observed dominant-negative effect of the PIKfyve$^{K1999/2000E}$ mutant. Therefore we next sought to design PIKfyve mutants with a selective specificity for PtdIns 5-P or PtdIns 3,5-P$_2$ production. Based on the largely compromised activity for both the PtdIns and PtdIns 3-P conversion documented above with the PIKfyve$^{K1999/2000E}$ mutant we rationalized that the adjacent lysines contribute differentially in the ligation of 1-and 3-phosphate groups of the inositol ring. If this assumption is correct, one would expect that the singly mutated PIKfyve proteins will display altered substrate specificity for PtdIns and PtdIns 3-P. To test this, HA versions of PIKfyve$^{K1999E}$ and PIKfyve$^{K2000E}$ were generated in pCMV5 vector and used to transiently transfected COS-7 cells. Expression of the PIKfyve proteins with expected size of ~200 kDa was confirmed by SDS-PAGE and Western blotting (Fig. 1B, lanes 5 and 6). Similarly to the PIKfyve$^{K1999/2000E}$ double mutant, the two point mutants displayed
protein kinase activity at a level and rate similar to that of the wild type (Fig. 1C, lanes 5 and 6, Fig. 1D and not shown). However, analysis of their \textit{in vitro} lipid kinase activity showed a markedly different pattern. The PIKfyve$^{K1999E}$ mutant displayed significantly compromised PtdIns 3,5-P$_2$ production, resulting in only $11 \pm 0.8\%$ of that produced by PIKfyve$^{WT}$. Conversely, the PtdIns 5-P production by this mutant was only reduced to $40 \pm 3\%$ compared to that of the wild type protein (Fig. 1A and Fig. 3A and B). The exact opposite functional activities were observed with PIKfyve$^{K2000E}$ mutant. This mutant displayed largely compromised PtdIns 5-P synthesis while PtdIns 3,5-P$_2$ production was much less affected (Fig. 1A and Fig. 3A and B). These data strongly suggest that Lys1999 is involved in the ligation of the 3-phosphate in PtdIns 3-P while Lys2000 serves to ligate the 1-phosphate in both PtdIns and PtdIns 3-P substrates. Hence, mutation in position 1999 results in a protein that still binds and converts a PtdIns substrate but is hardly effective with PtdIns 3-P. In contrast, the PIKfyve$^{K2000E}$ mutant can more efficiently utilize PtdIns 3-P as a substrate than PtdIns.

We therefore determined whether this altered lipid-substrate specificity of the point mutants was associated with manifestation of a different cell phenotype. The pEGFP version of these mutants together with PIKfyve$^{WT}$, PIKfyve$^{K1831E}$ and PIKfyve$^{K1999/2000E}$ were transiently expressed in COS cells. Transfected cells were examined relatively to the PIKfyve$^{K1831E}$ mutant for the appearance of the characteristic cell vacuolation phenotype between 10 and 48 hours posttransfection by fluorescence and phase-contrast microscopy. As illustrated in Fig. 2c and d, cell
expression of PIKfyveK2000E had essentially a normal phenotype. Conversely, cells expressing PIKfyveK1999E acquired vacuoles identical to those observed in cells transfected with the PIKfyveK1831E or PIKfyveK1999/2000E mutants (Fig. 2). These include a 15-h post-transfection period of the vacuole appearance, honeycomb shape, and 2-3 giant vacuoles in some cases comprising the majority of the cell volume. Quantitation of vacuolation potency of the mutants as well as their relative ability for PtdIns 3,5-P2 or PtdIns 5-P production from three independent experiments is shown in Fig. 3. These results demonstrate a correlation between the appearance of endomembrane defects/cytoplasmic vacuoles and the mutant’s attenuated ability for PtdIns 3,5-P2 rather than for PtdIns 5-P production. Together, these results infer that PtdIns 3,5-P2 but not PtdIns 5-P is the essential downstream mediator of the PIKfyve enzymatic activity in maintaining endomembrane homeostasis.

To further demonstrate a direct functional role of PtdIns 3,5-P2 we introduced PtdIns 3,5-P2, PtdIns 5-P, PtdIns 3-P or PtdIns 4,5-P2 by microinjection in single COS cells 20 h posttransfection with pEGFP-PIKfyveK1831E or pEGFP-PIKfyveK1999/2000E. Living cells were then observed over a period of 1-48 h post-injection by fluorescence/phase contrast microscopy. Expression and localization of the PIKfyve mutants was detected by the fluorescence signals of GFP, microinjected cells were detected by the fluorescence signals of Txr-dextran co-injected with the lipid, and the progression of the vacuolation phenotype was monitored by phase contrast. As illustrated in Fig. 4, microinjection of PtdIns 5-P or PtdIns 4,5-P2 in
combination with TxR-dextran did not alter the cell vacuolation phenotype of the transfected cells. Similarly, microinjected PtdIns 3-P was ineffective in correcting the abnormal phenotype (not shown). However, approximately 6 h post-injection of PtdIns 3,5-P$_2$, the vacuoles progressively diminished in diameter and virtually shrunk ~20 h after the lipid administration in practically all treated cells (Fig. 4a-c). Simultaneously, microinjected PtdIns 3,5-P$_2$ corrected the appearance of the dilated vesicles associated with the fluorescent signals of the PIKfyve mutants to fine punctate appearance seen with expressed PIKfyve$^{WT}$ (14, and Fig. 4a). Noteworthy, the ability of PtdIns 3,5-P$_2$ to reverse cell morphology back to normal was transient, that is 48h post-injection all the transfected cells that received PtdIns 3,5-P$_2$ reacquired the characteristic vacuolation phenotype consistent with a possible PtdIns 3,5-P$_2$ degradation/turnover. These results demonstrate that PtdIns 3,5-P$_2$ selectively and transiently corrects the abnormal endomembrane morphology.

In conclusion, we identify here PtdIns 3,5-P$_2$ as the key product of PIKfyve enzymatic activity that is required for maintenance of normal morphology of mammalian late endocytic membranes. Thus, the function of PtdIns 3,5-P$_2$ in mammalian cells appears to be analogous to that in yeast where the inactivation of the yeast PtdIns 3,5-P$_2$-producing enzyme Fab1p results in enlarged vacuoles (reviewed in Ref. 3). The identification of PtdIns 3,5-P$_2$ downstream effectors in both yeast and mammalian cells and how their function is related are important objectives that should shed further light on the molecular mechanism(s) maintaining endomembrane homeostasis.
REFERENCES


FIGURE LEGENDS

Fig. 1. Mutagenesis in a putative lipid substrate activation loop of PIKfyve dissociates the protein and lipid kinase activities and alters the lipid-substrate specificity. A-C. COS-7 cells on 100 mm plates were transiently transfected with cDNAs of pCMV5-HA-PIKfyveWT (lane 2), PIKfyveK1831E (lane 3), PIKfyveK1999/2000E (lane 4), PIKfyveK1999E (lane 5), PIKfyveK2000E (lane 6) or the empty vector (lane 1). Forty eight h post-transfection, parallel anti-HA immunoprecipitates (IP) were derived from cell lysates and analyzed in lipid kinase conditions (C) as described under “Experimental Procedures”. Shown are an autoradiogram of a plate with TLC-separated radiolabeled lipids (A), a chemiluminescence detection of the western blot probed with anti-HA antibodies (B) and autoradiogram of the autokinase reaction resolved by SDS-PAGE and transferred onto membranes (C). Shown are representative experiments out of three with identical results. Depicted are PtdIns 5-P and PtdIns 3,5-P2 products confirmed by HPLC analysis (Ref. 13), which quantitative evaluation is presented in Fig. 3. D. COS-7 cells were transiently transfected with cDNAs of pEGFP-HA-PIKfyveWT (open circles), PIKfyveK1999/2000E (filled circles), PIKfyveK2000E (triangles). Forty eight h post-transfection, anti-HA immunoprecipitates derived from cell lysates were subjected to autokinase assay for the indicated time intervals. The reactions were analyzed by SDS-PAGE and autoradiography as described under “Experimental Procedures”. Presented is a quantitation of the autokinase activity from the autoradiograms expressed as a percentage of the 60 min time point for each of the indicated proteins.
**Fig. 2.** Lipid kinase activity but not protein kinase activity, and Lys1999 but not Lys2000 are critical for inducing a dominant-negative phenotype in COS cells. Cells, transiently transfected with the indicated cDNA constructs engineered in pEGFP were observed live with a fluorescence microscope by the GFP fluorescence signals for expression (a, c and e) or under phase contrast for the vacuolation phenotype. Expression of PIKfyve^{K1999/2000E} and PIKfyve^{K1999E} but not PIKfyve^{K2000E} triggered the vacuolation phenotype as seen in the phase contrast images (b, d and f).

**Fig. 3.** Appearance of the vacuolation phenotype correlates with loss of production for PtdIns 3,5-P$_2$ but not for PtdIns 5-P. A and B. Lipid kinase activity of the indicated constructs was determined in HA-immunoprecipitates of transfected COS cells as described in the legend to Fig. 1A. Shown is quantitation of PtdIns 5-P (A) and PtdIns 3,5-P$_2$ production (B) from 3 independent experiments determined by counting of the radioactive spots indicated in Fig. 1A and expressed as percent of their synthesis by PIKfyve$^{WT}$. C. Fifty randomly selected cells transfected with the indicated cDNA constructs were counted for appearance of vacuolation phenotype, mean ± SD.

**Fig. 4.** Microinjected PtdIns 3,5-P$_2$ but not PtdIns 5-P selectively corrects cell vacuolation phenotype induced upon expression of lipid kinase-dead PIKfyve^{K1999/2000E}. COS-7 cells transfected with pEGFP-HA-PIKfyve^{K1999/2000E} cDNA (a, d and g) were microinjected 20 h post transfection with mixtures of TxR-dextran and the indicated PIs as described under “Experimental Procedures”. Twenty h post-
injection the cells were observed by fluorescence microscopy. Phase contrast images (c, f and i) show that only PtdIns 3,5-P2 microinjection reversed the vacuolation. Phenotype correction was observed in >90% of PtdIns 3,5-P2-injected cells and in <1% of PtdIns 5-P- or PtdIns 4,5-P2- injected cells based on 4 separate experiments counting 10 randomly selected cells per experiment. Arrows in c, f and i depict microinjected cells illustrated on b, e and h by the fluorescence signals of TxR-dextran.
Figure 1D

[Graph showing PIKfyve autokinase activity over time for different mutants: PIKfyveWT, PIKfyveK2000E, and PIKfyveK1999/2000E.]

PIKfyve autokinase activity vs. Time (min) with markers indicating each condition.
Figure 2

PIKfyve K1999/2000E

PIKfyve K2000E

PIKfyve K1999E
Figure 4

PIKfyve 1999/2000

Injected PIs

a

b PL 3, 5-P₂

c

H 5.00 µm

d

e PL 4, 5-P₂

f

H 5.00 µm

g

h PL 5-P

i
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