The dual specificity mammalian enzyme PIKfyve phosphorylates in vitro position D-5 in phosphatidylinositol (PtdIns) and PtdIns 3-P, itself or exogenous protein substrates. Here we have addressed the crucial questions for the identity of the lipid products and the role of PIKfyve enzymatic activity in mammalian cells. CHO, HEK293, and COS cells expressing PIKfyveWT at high levels and >90% efficiencies increased selectively the intracellular PtdIns 3,5-P2 production by 30–55%. In these cell types PtdIns 5-P was undetectable. A kinase-deficient point mutant, PIKfyveK1831E, transiently transfected into these or other cells elicited a dramatic dominant phenotype. Subsequent to a dilation of the PIKfyve-containing vesicles, PIKfyveK1831E-expressing cells progressively accumulated multiple swollen lucent vacuoles of endosomal origin, first in the perinuclear cytoplasm and then toward the cell periphery. Despite their drastically altered morphology, the PIKfyveWT-expressing cells were viable and functionally active, evidenced by several criteria. This phenotype was completely reversed by introducing PIKfyve WT into the PIKfyveK1831E-transfected cells. Disruptions of the localization signal in the PIKfyve kinase-deficient mutant yielded a PIKfyveK1831E-expressing cells incompetent to vacuolate cells, implying that an active PIKfyve enzyme at distinct late endocytic membranes is crucial for normal cell morphology. This was further substantiated by examining the vacuolation-induced potency of several pharmacological stimuli in cells expressing high PIKfyveWT levels. Together, the results indicate that PIKfyve enzymatic activity, possibly through the generation of PtdIns 3,5-P2, and/or yet to be identified endogenous phosphoproteins, is critical for cell morphology and endosome homeostasis.

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‡ The abbreviations used are: PtdIns, phosphatidylinositol; CI-MPR, cation-independent mannose 6-phosphate receptor; GFP, green fluorescent protein; EGFP, enhanced GFP; CH, chaperonin-like region; HA, hemagglutinin; GroPIns, glycerophosphoinositol; PI, phosphoinositide; P, phosphate; P2, bisphosphate; CMV, cytomegalovirus; HPLC, high performance liquid chromatography; WT, wild type.
remain unknown. In this study we report the essential role of PIKfyve enzymatic activity in cell morphology and intracellular membrane integrity by characterization of a PIKfyve kinase-dead point mutant. Mammalian cells expressing PIKfyve<sup>K1831E</sup> display a dominant phenotype characterized by progressive, but reversible, PIKfyve-vesicle dilation and vacuolation of membranes with endocytic origin. Because high levels of PIKfyve<sup>WT</sup> increase selectively the endogenous PtdIns 3,5-P<sub>2</sub> production in these cells, we conclude that PIKfyve enzymatic activity, likely through PtdIns 3,5-P<sub>2</sub> production, plays an essential role to maintain cell morphology by regulating late endocytic membrane homeostasis.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Antibodies, and Transfection Constructs—** COS-7 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. CHO-T cells were maintained in F-12 media supplemented as described above. Differentiated 3T3-L1 adipocytes were obtained as described previously (15). Rabbit polyclonal anti-PIKfyve antiserum (R7069; directed against the N terminus of PIKfyve), anti-HA monoclonal (12CA5; Babco), or polyclonal antibodies were characterized elsewhere (15, 16).

Generation of pEGFP-HA-PIKfyve<sub>WT</sub>, or the HA-tagged PIKfyve<sub>WT</sub> (PIKfyve<sub>WT</sub>), PIKfyve<sub>K1831E</sub>, and PIKfyve<sub>K1831E</sub> cDNA constructs in pCMV5 vector was described previously (15–17). EGFP-HA-PIKfyve<sub>WT</sub> was generated by replacing the full-length PIKfyve<sub>WT</sub> released as a XhoI fragment from pEGFP-HA-PIKfyve<sub>WT</sub> and the corresponding fragments from pCMV5-HA-PIKfyve<sub>WT</sub> and pCMV5-HA-PIKfyve<sub>K1831E</sub>, respectively. EGFP-HA-PIKfyve<sub>K1831E</sub> and pEGFP-HA-PIKfyve<sub>K1831E</sub> were generated by replacing the KpnI/SalI fragment in pEGFP-HA-PIKfyve<sub>WT</sub> and the XbaI/SalI insert in pEGFP-HA-PIKfyve<sub>WT</sub> with the corresponding fragments from pCMV5-HA-PIKfyve<sub>K1831E</sub> cDNA.

**Transient Transfection, Fluorescence Microscopy, and Western Blotting—** COS-7 cells, seeded on 22 × 22-mm coverslips were transfected with the cDNA constructs indicated in the figure legends by LipofectAMINE (Life Technologies, Inc.). Cells were then processed for fluorescence microscopy at the indicated posttransfection time or further treated as described below. Cells transfected with PIKfyve constructs in pCMV5 were detected with anti-HA monoclonal or polyclonal antibodies and Texas red-coupled goat anti-mouse or CY3-coupled goat anti-rabbit IgG, respectively, followed by avidin (dye-dehyde) and washing as described previously (17). Cells transfected with pEGFP constructs were detected by the GFP fluorescence signal following cell fixation or directly in live cells. HEK293 cells were transfected with PIKfyve constructs in an adheroviral plasmid (see below). Coverslips were mounted on slides using the Slow Fade Antifade Kit (Molecular Probes). Fluorescence analyses were performed with a confocal microscope (Zeiss LSM 310) using a 63/1.4 oil or 40/0.75 water immersion lens and a standard green fluorescence filter (for GFP). The levels of overexpressed EGFP-PIKfyve constructs were compared with that of the endogenous PIKfyve by Western blotting with anti-PIKfyve antibodies as described previously (17).

**Preparation of Recombinant Adenovirus and Cell Infection—** Recombinant adenoviruses, expressing HA-tagged PIKfyve<sub>WT</sub>, PIKfyve<sub>K1831E</sub>, and GFP (AdPIKfyve<sub>K1831E</sub>), or GFP alone (AdEmpty), were generated by the AdEase system (kindly provided by Dr. B. Vogelstein, Ref. 19). Briefly, HA-PIKfyve<sub>WT</sub> was first cloned into pAdTrack-CMV shuttle vector, engineered with two separate CMV promoters for expression of GFP, and for expression of PIKfyve<sub>WT</sub>, respectively. For this purpose the N-terminal part of PIKfyve<sub>WT</sub>, released by BglII-KpnI from pEGFP-HA-PIKfyve<sub>WT</sub> and the KpnI/SalI C-terminal part taken from pCMV5-HA-PIKfyve<sub>WT</sub>, were ligated into a BglII-SalI-digested shuttle vector. The resultant shuttle construct or the "empty" shuttle vector was linearized by PacI and co-transformed with pAdEasy1 adenoviral backbone plasmid into COS-7 cells. Selected recombinants were confirmed by restriction mapping, linearized with PucI, and used to transfect a HEK293 adenovirus packaging cell line with LipofectAMINE. Two weeks after the cells were harvested. The viruses were extracted by freeze-thaw and subsequent centrifugation. This extract was used for further viral propagation in HEK293 cells. Viral stocks were purified by ultracentrifugation in two discontinuous CsCl gradients and subsequent passage through a Naper 10 column (Sephadex G25, Amersham Pharmacia Biotech). Purified viral stocks were titrated, and the lowest dilution resulting in 100% infection of HEK293 cells 18 h postinfection (monitored by the GFP signals) was defined as multiplicity of infection = 1. The adenovirus-mediated overexpression and enzymatic activity of PIKfyve were confirmed by Western blotting and lipid kinase assays, performed under previously specified conditions (15).

**Treatments of Transfected Cells—** COS-7 cells transfected with pcMV5 PIKfyve<sup>K1831E</sup> cDNAs were infected with recombinant adenoviruses expressing PIKfyve<sub>WT</sub> and GFP, or control GFP. Twenty-four to 48 h postinfection the cells were fixed, processed for fluorescence microscopy, and observed under a 63/1.4 oil immersion lens. In some experiments COS-7 cells transfected with pEGFP-HA-PIKfyve<sub>WT</sub> were treated with NHCl (20 μM) or Wortmannin (800 nM) for 30 min at 37 °C and then examined live by confocal microscopy with a 40/0.75 water immersion lens.

**Labeling of Cellular Phospholipids with [32P]P and HPLC Analysis—** COS-7 cells seeded on 60-mm dishes were transfected with pcMV5-HA-PIKfyve<sup>WT</sup> or only pcMV5 cDNAs by electroporation (Bio-Rad Gene Pulser II electroporator), a technique reaching ~90% cell transfection efficiency (20). CHO-T or HEK293 cells (60-mm dishes) were infected with adenoviruses expressing PIKfyve<sup>WT</sup> and GFP, or only GFP at multiplicity of infection = 1. Fluorescence microscopy of the infected cells indicated ~100% efficiency 24 h posttransfection. Forty-eight hours posttransfection or 24 h postinfection, the cells were washed in phosphate-free Dulbecco's modified Eagle's medium, and then labeled with 5 mM EDTA and 5 mM tetrathylammonium sulfate, as a reagent increasing PI extraction (22). Extracted radiolabeled lipids were deacylated and analyzed by HPLC with [H]-labeled GroPIns 3-P, GroPIns 4-P, GroPIns 5-P, and GroPIns 3,4-P<sub>2</sub> internal or [32P]-labeled GroPIns 3,4-P<sub>2</sub> and GroPIns 3,5-P<sub>2</sub> external standards under previously specified conditions (15).

**RESULTS AND DISCUSSION**

To determine the function of PIKfyve enzymatic activity in mammalian cells we have used a catalytically deficient HA-tagged form of PIKfyve, bearing a single point mutation at Lys<sup>21831</sup> proposed as a candidate ATP-binding site (16). Lack of both lipid and protein kinase activity for this PIKfyve<sup>K1831E</sup> point mutant was demonstrated in vitro by the complete inability of the HA immunoprecipitates derived from lysates of PIKfyve<sup>K1831E</sup>-transfected COS cells to synthesize PtdIns 5-P and PtdIns 3,5-P<sub>2</sub> or to autophosphorylate (16). Transient transfection in COS cells with HA-PIKfyve<sup>K1831E</sup> cDNA engineered in mammalian expression vectors pCMV5 or pEGFP resulted in a drastic dominant phenotype, readily seen upon observation of live cells under a light microscope. Approximately 15 h posttransfection, large vacuoles appeared as round structures, initially at the perinuclear region and 24 h posttransfections, throughout the whole cytoplasm with a tendency to increase in size and decrease in number within the observation period (72 h posttransfections). In some cells expressing PIKfyve<sup>K1831E</sup> at higher levels, two to three giant vacuoles could be seen 2 days posttransfection, most likely having arisen as a result of fusion, reaching diameters of 5–10 μm and comprising the majority of the cell volume. The intracellular appearance of PIKfyve<sup>K1831E</sup> also underwent a dramatic change. The first alterations, which actually preceded the formation of large vacuoles were associated with dilution of the PIKfyve<sup>K1831E</sup>-containing vesicles as revealed by fluorescence signals associated with anti-HA antibodies (Fig. 1) or GFP (data not shown). With the progression of cell vacuolation, the signals associated with anti-HA antibodies (Fig. 1) or GFP could be seen 2 days posttransfection, most likely having arisen upon the GFP signals detected PIKfyve<sup>K1831E</sup>-containing dilated vesicles as revealed by fluorescence signals associated with anti-HA antibodies (Fig. 1) or GFP. Twenty-four to 48 h postinfection the cells were fixed, processed for fluorescence microscopy, and observed under a 63/1.4 oil immersion lens. In some experiments COS-7 cells transfected with pEGFP-HA-PIKfyve<sub>WT</sub> were treated with NHCl (20 μM) or Wortmannin (800 nM) for 30 min at 37 °C and then examined live by confocal microscopy with a 40/0.75 water immersion lens.
vacuoles was free of PIKfyveK1831E and appeared as an empty, lucent space in phase contrast images from a confocal microscope (Fig. 1). The cell vacuolation was striking and unequivocal and was detected even in cells for which the HA immunofluorescence or GFP fluorescence signals of expressed PIKfyveK1831E were only slightly above the background level. Western blotting with anti-PIKfyve antibodies of lysates from transiently transfected COS cells showed equal intensities for the endogenous PIKfyve (−200 kDa) and GFP-PIKfyveK1831E (230 kDa) bands (data not shown). Given the transfection efficiency in these experiments (~40%) and the variability of overexpression in individual cells, these results support the notion that even at a low ratio (1:1) of the mutant to the endogenous PIKfyve, the PIKfyveK1831E point mutant induces the appearance of dilated PIKfyve-containing vesicles and abnormal vacuoles. Parallel immunofluorescence and phase contrast microscopy in COS cells transiently expressing HA-PIKfyveWT revealed no morphological changes (Fig. 1). Consistent with our previous observations (17), a distinct peripheral vesicular pattern of the fluorescence staining associated with expressed PIKfyveWT, along with a diffuse staining could be observed (Fig. 1). Because PIKfyveWT as well as PIKfyveK1831E largely co-localize with the late endosomal marker CI-MPR, but not with marker proteins for Golgi, early/recycling endosomes or end lysosomes (Fig. 2, Ref. 17, and this study, data not shown), it is conceivable that the PIKfyveK1831E-induced vacuoles originate from the late endosomal compartment.

The dramatic and unequivocal dominant effect of PIKfyveK1831E in inducing cell vacuolation was observed in other cell types. Thus, transient transfection of HIRcB or 3T3-L1 fibroblasts with the PIKfyveK1831E cDNA in either pCMV5 or pEGFP vectors resulted in the appearance of the characteristic phenotype of multiple empty vacuoles in the PIKfyveK1831E-expressing cells (data not shown). Next, transient transfection of HEK293 cells with PIKfyveK1831E engineered in pAdTrack CMV vector with an independent promoter as described under “Experimental Procedures” (a and d, with b and e being the respective phase contrast images). Shown are three PIKfyveK1831E-transfected COS cells, in which different phases of cell vacuolation could be observed (a and b) and see “Results and Discussion”). HEK293 cells were visualized live 48 h posttransfection by the fluorescence signals of GFP expressed together with the indicated constructs under independent promoter as described under “Experimental Procedures.” Phenotype is observed only upon expression of PIKfyveK1831E but not PIKfyveWT in both cell types. Bar, 10 μm.
suggesting that the vacuoles originate from the late endocytic compartment. Shown are three PIKfyveK1831E-expressing cells and the induced cell vacuolation in the phase contrast image (b, arrows). A control merged image in c shows the large overlap between PIKfyveWT and CI-MPR as reported previously (17). Bar, 10 μm.

Fig. 2. PIKfyveK1831E-induced vacuoles originate from late endocytic/multivesicular body compartment. COS-7 cells were transiently transected with pCMV5 HA-PIKfyveK1831E (a) or pCMV5 HA-PIKfyveWT cDNAs (c). Double staining with monoclonal anti-HA and polyclonal anti-CI-MPR antibodies (a and c) was performed 24 h posttransfection as described under “Experimental Procedures”, resulting in green and red fluorescence signals, respectively. The merged image, shown in a, indicates a substantial overlap between PIKfyveK1831E and CI-MPR (yellow), suggesting that the vacuoles originate from the late endocytic compartment. Shown are three PIKfyveK1831E-expressing cells and the induced cell vacuolation in the phase contrast image (b, arrows). A control merged image in c shows the large overlap between PIKfyveWT and CI-MPR as reported previously (17). Bar, 10 μm.

effect of PIKfyveWT overexpression on cellular phosphoinositide levels. COS-7, HEK293, and CHO-T infected with adenovirus expressing PIKfyveWT, or transfected by electroporation at an efficiency of infection/transfection >90%, were subsequently labeled with [32P]orthophosphate and the extracted radioactive lipids analyzed by HPLC. Heterologous expression of PIKfyveWT in these cell types increased the [32P]PtdIns 5-P and PtdIns 3,5-P2 (15), only the latter is synthesized that although PIKfyve has the potential to generate both others, for which a role of the PIKfyve enzymatic activity in NIH-3T3 fibroblasts (23), 3T3-L1 adipocytes (24), and possibly PtdIns 5-P is produced only in certain mammalian cells such as not shown), our inability to detect32P accumulation into PtdIns 5-P (Ref. 24 and data otherwise enzymatically active PIKfyve (16) was typically characterized the localization pattern of the PIKfyve WT produced the characteristic peripheral vesicular pattern, while the FYVE finder exhibit a similar vesicular pattern, while the FYVE finger-deleted protein fragments did not (13), we assigned the FYVE finger in PIKfyve as a necessary determinant for intracellular localization, similarly to the observations with other FYVE finger-containing mammalian proteins (5, 7). This assumption was confirmed in experiments in which we have characterized the localization pattern of the PIKfyveWT truncated mutant versus PIKfyveWT or other PIKfyve mutants, harboring deletions in different regions of the molecule. As illustrated in Fig. 4, when expressed in COS cells, PIKfyveWT and PIKfyveΔ FYVE produced the characteristic peripheral vesicular puncta scattered throughout the cytoplasm, while the protein with a FYVE finger truncation produced a dramatically different staining pattern consisting of mainly perinuclear and diffuse appearance; scattered puncta were practically undetected. Introduction of the kinase-dead point mutation into otherwise enzymatically active PIKfyveWT (16) was typically without phenotype. The absence of endomembrane vacuolation in COS cells expressing PIKfyveK1831EΔ FYVE is depicted in Fig. 4. Immunofluorescence detection of PIKfyveK1831EΔ FYVE confirmed its mistargeting to the cell perinuclear region. Together, these
results indicate that membrane attachment to late endosomes through the FYVE finger is required for the dominant-negative phenotype of PIKfyve\(^{K1831E}\).

It should be emphasized that a deletion of the chaperonin-like domain abrogated the in vitro lipid kinase activity associated with expressed PIKfyve\(^{ACCH}\) (Ref. 15 and this study, data not shown). The fact that PIKfyve\(^{ACCH}\) failed to induce a dominant phenotype in COS cells, despite its correct intracellular targeting (Fig. 4), could be taken as an indication that possibly slight PIKfyve enzymatic activity at the right site in the cellular context could prevent cell vacuolation. This notion is consistent with studies in yeast demonstrating that replenishment of as low as 10% of the PtdIns 3,5-P\(_2\) pool reverses the vacuolar phenotype in \(\textit{fab1}\)-deficient strains (9). An alternative interpretation could view the chaperonin-like domain as essential in recruiting/assembly of additional molecular elements, whose coordinated action in the absence of PtdIns 3,5-P\(_2\) induces cell vacuolation.

Progressive and reversible mammalian cell vacuolation can be induced by different pharmacological treatments including weak bases (27), inhibition of the PI 3-kinase family members by wortmannin and LY294002 (28–30), intoxication with the \textit{Helicobacter pylori} VacA cytotoxin (31, 32), or under other conditions (for a recent review, see Ref. 33). Similarly to the cell vacuolation induced by PIKfyve\(^{K1831E}\), the cytoplasmic vacuoles observed in the above experimental paradigms have been shown to originate from swollen vesicles of the postendosomal/late endocytic pathway (28–31). While the molecular mechanism(s) responsible for the vacuolar formation in these instances is largely obscure, we rationalized that if the vacuolation phenomenon depends on the PIKfyve pathway and membrane-localized PtdIns 3,5-P\(_2\) production, then an intracellular overproduction of PIKfyve\(^{WT}\) should prevent cell vacuolation induced by weak bases. Conversely, overproduced PIKfyve\(^{WT}\) should be inefficient for rescue of the wortmannin-induced vacuolation due to a direct dependence of the PtdIns 3,5-P\(_2\) biosynthesis on cellular PtdIns 3-P levels (25), shown to undergo a dramatic depletion (70%) upon acute cell treatment with wortmannin (28, 34, 35). Consistent with our prediction, heterologously expressed PIKfyve\(^{WT}\) prevented the COS cell vacuolation induced by short treatment with NH\(_4\)Cl (20 mM; Fig. 5, a and b). By contrast, PIKfyve\(^{WT}\)-transfected cells vacuolated just as well as the nontransfected cells upon wortmannin treatment (800 nM; Fig. 5, c and d). These results suggest that cell vacuolation, at least in the experimental paradigms examined here, is likely induced by common mechanisms, operating probably through PtdIns 3,5-P\(_2\) production. With the reservation regarding the broad specificity in wortmannin inhibition, it is worth emphasizing that the most dramatic cell vacuolation is produced by high wortmannin concentrations (5–10 \(\mu\)M; Ref. 28 and this study, data not shown), doses that cause a complete inhibition of not only the PI 3-kinase activity but that of PIKfyve (15) as well.

The results presented in this study indicate that the kinase-

![PIKfyve\(^{K1831E}\)-induced cell vacuolation is rescued by overproduction of PIKfyve\(^{WT}\).](Image)

**Fig. 3.** PIKfyve\(^{K1831E}\)-induced cell vacuolation is rescued by overproduction of PIKfyve\(^{WT}\). COS-7 cells transfected with pCMV5-HA-PIKfyve\(^{K1831E}\) DNA (a and c) were transduced 24 h posttransfection with either PIKfyve\(^{WT}\) adenovirus harboring GFP under independent promoter (c; PIKfyve\(^{WT}\)-Ad) or only GFP (a; Ad) at multiplicity of infection = 1. Thirty-six hours postinfection the cells were fixed and observed on a confocal microscope as described under “Experimental Procedures.” The expression of PIKfyve\(^{K1831E}\) was monitored by the phenotypic changes of cell vacuolation seen by phase contrast (b and d), while the expressions of PIKfyve\(^{WT}\)-Ad or Ad were monitored by the fluorescence signals of GFP (a and c). The phenotype was rescued in all transfected cells transduced with PIKfyve\(^{WT}\)-Ad but remained unaffected by control Ad transduction. Shown are: in a and c, three PIKfyve\(^{K1831E}\)-transfected/PIKfyve\(^{WT}\)-Ad-infected cells, with shrunken or missing vacuoles and one PIKfyve\(^{K1831E}\)-transfected cell, which remained noninfected with PIKfyve\(^{WT}\)-Ad (no fluorescence in c) and displayed the vacuolar phenotype; in a and b, a PIKfyve\(^{K1831E}\)-transfected/Ad-infected control cell with unaltered vacuolar phenotype. Bar, 10 \(\mu\)m.

**Table 1.** PIKfyve\(^{WT}\) selectively increases the \(^{32}\)P accumulation into PtdIns 3,5-P\(_2\)

| PI species | COS-7 cells | HEK293 cells | CHO-T cells |
|------------|-------------|--------------|-------------|
|            | Basal       | PIKfyve\(^{WT}\) | Basal       | PIKfyve\(^{WT}\) | Basal       | PIKfyve\(^{WT}\) |
| PtdIns 3-P  | 8,350       | 105 ± 8      | 7,420       | 55 ± 6        | 1,210       | 85 ± 9        |
| PtdIns 3,5-P\(_2\) | 1,760       | 155 ± 12     | 624        | 155 ± 10      | 970        | 130 ± 6      |
| PtdIns 4-P  | 397,000     | 100 ± 5      | 42,500     | 105 ± 9       | 60,100     | 100 ± 8      |
| PtdIns 3,4-P\(_2\) | 7,980       | 92 ± 5       | 1,030      | 110 ± 9       | 1,060      | 105 ± 6      |
| PtdIns 4,5-P\(_2\) | 688,500     | 100 ± 5      | 82,000     | 100 ± 4       | 80,800     | 100 ± 3      |
| PtdIns 5-P  | Undetected  | Undetected   | Undetected | Undetected    | Undetected | Undetected   |
signals (b cells transfected with pEGFP-HA-PIKfyve WT cDNA were treated 24 h
with the indicated constructs in COS cells transfected with pEGFP-HA vector were fixed and pro-
cessed for fluorescence as described under “Experimental Procedures.” A punctate
pattern is seen in PIKfyveWT- and PIKfyveK1831E-transfected cells and diffuse
staining upon elimination of the FYVE finger in PIKfyveWT and PIKfyveK1831E. Absence of vacuola-
tion phenotype for PIKfyveK1831E is shown in the phase contrast image. Bar, 10 μm.

Fig. 4. Membrane attachment through an intact FYVE finger is re-
quired for the PIKfyveK1831E-induced cell vacuolation. COS cells transfected

with the indicated constructs in pEGFP-HA vector were fixed and pro-
cessed for fluorescence as described under “Experimental Procedures.” A punctate
pattern is seen in PIKfyveWT- and PIKfyveK1831E-transfected cells and diffuse
staining upon elimination of the FYVE finger in PIKfyveWT and PIKfyveK1831E. Absence of vacuola-
tion phenotype for PIKfyveK1831E is shown in the phase contrast image. Bar, 10 μm.

endosomes/multivesicular bodies to store endocytosed mem-
branes as intraluminal vesicles, thereby causing formation of
large vacuoles (30). Inhibition of the retrograde transport out of
lysosomes is proposed to yield the enlarged vacuoles docu-
menced in Δfab1 yeast strains, in which PtdIns 3,5-P2 produc-
tion is fully suppressed (9). An increase of the endosomal mem-
brane permeability to anions and accumulation of osmotically
active species, that cause an osmotic imbalance of late endo-
somes with subsequent vacuole formation, have been proposed
for Vca-induced cell vacuolation (36). Based on the results
presented herein and the above considerations we propose the
following model to explain the PIKfyveK1831E-induced dilation
of late endosomal membranes and cell vacuolation. Lack of
PIKfyve enzymatic activity and, most likely, the PtdIns 3,5-P2
production at specific sites within late endosomes/multivesicu-
lar bodies changes membrane composition and induces a dilu-
tion of PIKfyve endocytic compartment, due to a block in mem-
brane outward/inward budding or an increase in its fusogenic
activity. This event, alone or in conjunction with the loss of
the PtdIns 3,5-P2 pool and the subsequent changes in endocytic
membrane composition, induces alterations in endomembrane
ion permeability that lead to accumulation of ions, increases
in osmotic pressure, and a subsequent vacuole formation. It is
worth emphasizing that while this model predicts an essential
function of the PtdIns 3,5-P2 products of PIKfyve, a contribu-
tion of the protein products phosphorylated by PIKfyve protein
kinase activity (16) could not be ruled out. Whether this model
is correct requires testing of different aspects of the proposed
steps. In any case the present results demonstrate for the first
time the essential role of PIKfyve enzymatic activity in main-
taining mammalian cell morphology and endocytic membrane
homeostasis. The studies also add functional similarity to the
structural homology between PIKfyve and yeast Fab1p, indi-
cating functional conservation in these two proteins.

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