Localized PtdIns 3,5-P₂ synthesis to regulate early endosome dynamics and fusion

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Ikonomov, Ognian C., Diego Sbrissa, and Assia Shisheva. Localized PtdIns 3,5-P₂ synthesis to regulate early endosome dynamics and fusion. Am J Physiol Cell Physiol 291: C393–C404, 2006. First published March 8, 2006; doi:10.1152/ajpcell.00019.2006.—Perturbations in the intracellular PtdIns 3,5-P₂ pool or the downstream transmission of PtdIns 3,5-P₂ signals often result in a gradual development of gross morphological changes in the pleomorphic multivesicular endosomes, culminating with the appearance of cytoplasmic vacuoles. To identify the onset of PtdIns 3,5-P₂ functional requirements along the endocytic system, in this study we characterized the morphological changes associated with early expression of the dominant-negative kinase-deficient form (K183I) of the PtdIns 3,5-P₂-producing kinase PIKfyve, before the formation of cytoplasmic vacuoles in transfected COS cells. Enlarged PIKfyveK183I-positive vesicles co-localizing with dilated EEA1- and Rab5α WT-positive perinuclear endosomes were observed (WT, wild type). This was dependent on the presence of active forms of Rab5 and the generation of PtdIns 3-P-enriched platforms on early endosomes. Because PIKfyveWT did not substantially colocalize with EEA1- or Rab5-positive endosomes in COS cells, the dynamic PIKfyve-catalyzed PtdIns 3-to-PtdIns 3,5-P₂ switch was suggested to drive away PIKfyveWT from early endosomes toward later compartments. Late endosomes/lysosomes marked by LAMP1 or Rab7 were dislocated from their typical perinuclear position upon PIKfyveK183I early expression. Cytosols derived from cells stably expressing PIKfyveK183I stimulated endosome fusion in vitro, whereas PIKfyveWT-enriched cytosols had the opposite effect, consistent with PtdIns 3,5-P₂ production negatively regulating the endosome fusion. Together, our data indicate that PtdIns 3,5-P₂ defines specific endosome platforms at the onset of the degradation pathway to regulate the complex process of membrane remodeling and dynamics.

carrier vesicle; multivesicular bodies; PIKfyve; Rab5/EEA1/PtdIns3-P platforms; Rab7; LAMP1

IN MAMMALIAN CELLS, proteins and lipids from the cell surface or the Golgi apparatus are sorted to lysosomes for degradation via the endosome membrane system (for recent reviews, see Refs. 14, 16, 23, 28, 32, 36, 37). At the ultrastructural level, the endosomes in the degradation pathway are characterized by the presence of intralumenal membranes with a vesicular and/or lamellar appearance, and are referred to as multivesicular endosomes. While arising by a common mechanism of an inward invagination of the limiting membrane, the multivesicular endosomes in the degradation pathway comprise a system of heterogeneous membrane compartments that show significant functional and biochemical differences. Multivesicular bodies (MVBs) are a subset of multivesicular endosomes with characteristics of late endosome/prelysosomal compartments. They arise by detachment from (17) or maturation of early endosomes (37) and function as transport intermediates between early and late endosome compartments; hence, the acronym ECV for endosome carrier vesicles (14, 16), which is used herein, along with MVBs.

The formation of intralumenal endosome membranes is considered to be mechanistically coupled to protein sorting into the MVB pathway (for recent reviews, see Refs. 17, 23, 40, 47). Genetic studies in yeast reveal the requirement for a set of genes, the class E VPS, whose products, assembled in complexes ESCRT-I, -II, and -III, consecutively execute the selection of ubiquitylated membrane protein cargoes into the MVB pathway. A similar mechanism, at least for a subset of downregulated ubiquitylated receptors, likely operates in mammalian cells, where several ESCRT subunits and interactions have been functionally characterized (1–3, 51). In addition, an endosomal COP complex, ARF1, other accessory proteins, as well as lysobisphosphatidic acid (LBPA) have also been implicated in intralumenal vesiculation of mammalian ECV/MVBs or late endosomes (18, 24, 26), an increased complexity likely reflecting the elaborate heterogeneity of inward vesiculating endocytic compartments in mammalian cells. Detachment of ECV/MVBs from early endosomes is thought to use a protein machinery distinct from that required for intralumenal membrane formation with annexin II, characterized as one of multiple unidentified factors that regulate this complex process in mammalian cells (27).

Studies in both yeast and mammalian cells indicate that, in addition to the protein machinery, endosome membrane dynamics and protein sorting require the phosphorylated derivatives of phosphatidylinositol (PtdIns), and particularly PtdIns 3-P and PtdIns 3,5-P₂ (9, 11, 21, 34). PtdIns 3-P has been localized to membranes of early endosomes and ECV/MVB transport intermediates (13). Recent studies (35) implicate PtdIns 3-P in membrane receptor sorting and intralumenal vesicle formation but not in the biogenesis of the ECV/MVB transport intermediates in mammalian cells. Less clear is the distribution and the specific role of PtdIns 3,5-P₂ along the endocytic tract. The antibodies or bioreporters for PtdIns 3,5-P₂ selective binding and visualization in a cellular context

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are unavailable or inappropriate to address directly these questions (38). The intracellular distribution of PtdIns 3,5-P2 is therefore inferred from the localization of endogenous or ectopically expressed PIKfyve, the sole enzyme that synthesizes PtdIns 3,5-P2 from PtdIns 3-P in mammalian cells (reviewed in Ref. 44). Because at steady state, PIKfyve is largely absent form early/recycling endosomes, defined both biochemically and morphologically, but colocalizes, at least in part, with the late endosome/trans-Golgi network marker, cation-independent mannose 6-phosphate receptor (MPR) (45), PtdIns 3,5-P2 is, presumably, enriched in the later endocytic membranes. That PtdIns 3,5-P2 also functions along the late endocytic pathway is suggested on the basis of the ability of dominant-negative kinase-deficient PIKfyve mutants to induce diluted vesicles and cytoplasmic vacuoles, some positive for MPR, and to delay the later traffic of solutes (19, 20). However, the gross morphological changes induced on expression of these mutants, together with the heterogeneity of the multivesicular endosomes, make the initial compartment for a PtdIns 3,5-P2 functional requirement elusive. With the goal to identify the PtdIns 3,5-P2-sensitive endocytic compartment, we have examined the distribution of several endosome membrane markers under conditions of early expression of dominant-negative kinase-deficient PIKfyveK1831E, when vacuole formation and extensive changes of cellular architecture are still not visible. We found that unlike PIKfyveWT, expressed PIKfyveK1831E diluted and accumulated on the enlarged EEA1- or Rab5-positive perinuclear vesicles that likely have enlarged as a result of increased endosome fusion. Thus the onset of the PtdIns 3,5-P2 functional requirement appears to be earlier in the endocytic system than initially anticipated.

**MATERIALS AND METHODS**

**Cell cultures, cDNA constructs, fusion proteins, and primary antibodies.** COS-7 cells were maintained in DMEM containing 10% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin sulfate. Stably transfected doxycycline-inducible TetOn human embryonic kidney (HEK)-293 clonal cells expressing PIKfyve WT (clone 9) or kinase-deficient dominant-negative PIKfyveK1831E (clone 5), characterized elsewhere (41), as well as the parental control cell line, were maintained in DMEM, containing 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin sulfate, 100 μg/ml G418, and 125 μg/ml hygromycin B in the presence or absence of 1.0 μg/ml doxycycline. Baby hamster kidney cells (BHK21; C-13), were grown in MEM, supplemented with 10% FBS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and the antibiotics. pEGFP-hemagglutinin (HA)- or pcMV5-based HA- or myc-epitope-tagged PIKfyveWT, PIKfyveK1831E, and PIKfyveL99–473 constructs have been described previously (21, 43, 45, 46). The pEGFP-based Rab5 constructs (WT, S34N, and Q79L), and pEGFP-based Rab5 constructs have been described elsewhere (52, 43, 45, 46). pGEX-1-PIKfyveWT was used for the purification of PIKfyve fusion proteins.Western blot analysis was performed by using the HRP-conjugated anti-HA, anti-human PIKfyve, or anti-HRP antibodies. Proteins were visualized on the X-Omat AR film (Kodak). PtdIns 3,5-P2 levels were quantified by using a phosphoinositide assay kit (Cell Signaling, Beverly, MA). The concentration of PtdIns 3,5-P2 was determined by a sensitive and accurate assay (21). Western blots were scanned, and PtdIns 3,5-P2 content was determined using ImageJ software (National Institutes of Health). Protein levels were normalized to the levels of the corresponding actin band on the gel. These experiments were repeated twice with similar results.

**Transient cell transfection and microscopy.** COS-7 cells were seeded on 22 × 22-mm coverslips (35 mm dishes) and transfected with cDNA constructs indicated in the figure legends by LipofectAMINE (Invitrogen) as a transfection reagent. Twelve to sixteen hours posttransfection, the washed cells were fixed for 15 min at 25°C in 3% paraformaldehyde or 4% formaldehyde, rehydrated, and processed for fluorescence microscopy, as detailed elsewhere (21, 45). Labeling with anti-EEA1, anti-LBPA, or anti-LAMP1 antibodies was performed subsequent to paraformaldehyde fixation and cell permeabilization with 0.05% saponin in PBS. Labeling with anti-HA polyclonal or anti-myc monoclonal antibodies was performed subsequent to cell permeabilization with 0.5% Triton X-100 in PBS/1% FBS. Primary polyclonal antibodies were detected by CY3-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories), Texas red-conjugated rabbit anti-goat IgG and Alexa488-conjugated donkey anti-rabbit IgG (Molecular Probes). Primary monoclonal antibodies were detected with Alexa568-conjugated anti-mouse IgG (Molecular Probes). Coverslips were mounted on slides with the use of the Slow Fade Antifade Kit (Molecular Probes) and observed on a motorized inverted microscope (Olympus) with a ×60 1.4 NA objective lens, EGFP signals were captured by a standard green-fluorescence filter. Stacks of images were taken at a step of 1 μm and captured by a Retiga 1300 Cool Cooled Mono 12-bit digital charge-coupled device camera (QImaging). The stack of images was subjected to deconvolution analysis and the resulting images at one and the same Z-level, were used for colocalization analysis. The images were obtained using Image-ProPlus version 4.5.1 (Media Cybernetics), VolumScan, and Deconvolve software (VayTek). In some experiments (Fig. 1), the fluorescence analysis was performed with a confocal microscope (model LSM310, Zeiss) using a 63/1.4 oil-immersion lens.

**Cell-free endosome fusion assay.** In vitro endosome fusion was performed following previously published protocols (15, 29). Two sets of BHK21 cells grown to ~80% confluence (100-mm dishes) were incubated with 4.0 mg/ml avidin (Pierce) or 2.0 mg/ml biotinylated horseradish peroxidase (HRP; Pierce), respectively. Markers were allowed to continuously internalize for 5 or 25 min at 37°C. Cells were then placed on ice, washed, and homogenized at ~2,000 rpm (Heidolph Brinkmann 2101) in 250 mM sucrose, 10 mM Tris·HCl, pH 7.4. Cell homogenates were centrifuged for 10 min (1,000 g at 4°C) to yield postnuclear supernatants enriched in endocytosed avidin or biotinylated HRP. The postnuclear supernatants were mixed on ice with cytosols (0–1.5 mg/ml protein) prepared from noninduced or doxycycline-induced HEK-293 stable lines (clones 9 and 5, for PIKfyveWT or PIKfyveK1831E, respectively; Ref. 41), biotinylated immunosorbent (0.5 mg/ml), and an ATP regenerating mixture in an intracellular buffer as specified elsewhere (15). Control samples without cytosols were analyzed in parallel and showed no fusion. Where indicated, purified PIKfyve-GST fusion peptide fragments (PIKfyve 99–473 or PIKfyve 99–473Δ77–198) were mixed with cytosol from noninduced PIKfyve WT cell line and added to the fusion assay at a peptide concentration of 5 μg/ml. The assay mixtures were incubated for 45 min at 37°C and the membranes were then lysed with 10% Triton X-100/0.5% SDS for 30 min on ice. Lysates were clarified by centrifugation. Supernatants were used to immuno-precipitate avidin-biotin-HPR complexes with anti-avidin antibodies (1 mg/ml) for 18 h at 4°C. Protein A-Sepharose CL-4B beads were added in the last 90 min of incubation. HRP activity was quantified by reading the absorbance at 450 nm, subsequent to the addition of HRP substrate (1-StepTM TMB-ELISA kit; Pierce) and incubation for 20 min at 37°C.

In vitro membrane labeling of PtdIns and [3H]PtdIns 3,5-P2 detection. Total membrane fractions were isolated from the doxycycline-induced HEK-293-PIKfyveK1831E stable cell line (clone 5) and control parental HEK-293 cells by homogenization in “HES” buffer (20 mM HEPES/NaOH, pH 7.4, 255 mM sucrose, 1 mM EDTA, 1× protease, and 1× phosphatase inhibitor cocktails; Ref. 45), followed by two sequential centrifugations (2 min at 800 g and 30
showing massive cytoplasmic vacuolation of the PIKfyveK1831E-expressing cell but normal neighboring cells (transfection (9–15 h), a dilation of the PIKfyveK1831E-positive duration and level of protein expression. At earlier stages of complex morphological alterations, largely dependent on the kinase-deficient PIKfyveK1831E mutant in COS cells results in have revealed that ectopic expression of the dominant-negative structures conducted previously (21, 22) and in this study (Fig. 1) showing massive cytoplasmic vacuolation of the PIKfyveK1831E-expressing cell but normal neighboring cells (b), and the merge (c) of images in a and b, indicating a subpopulation of the cytoplasmic vacuoles accumulates PIKfyveK1831E on the limiting membrane. Bar, 10 μm.

RESULTS

Enlargement of EEA1- and Rab5-positive perinuclear endosomes in early PIKfyveK1831E expression. Confocal microscopy studies conducted previously (21, 22) and in this study (Fig. 1) have revealed that ectopic expression of the dominant-negative kinase-deficient PIKfyveK1831E mutant in COS cells results in complex morphological alterations, largely dependent on the duration and level of protein expression. At earlier stages of transfection (9–15 h), a dilation of the PIKfyveK1831E-positive vesicles could be observed. This is followed by the appearance of large translucent vacuoles, which may or may not contain PIKfyveK1831E on the limiting membrane (Fig. 1). The vacuoles are first seen at the perinuclear region, but 1–2 days posttransfection, they appear throughout the whole cytoplasm, with a tendency to increase in size and decrease in number, sometimes down to only 2–3 giant vacuoles (diameters of ∼5–10 μm) that occupy the whole cell volume (21, 22, and this study, data not shown). Under this exacerbated phenotype (Fig. 1) many proteins, even those with an exclusive cytosolic localization, concentrate onto or in the vicinity of the vacuole limiting membrane, likely as a result of increased membrane surface-to-cytosol volume ratio (Ikonomov O and Shisheva A, unpublished observations). The severely altered cell phenotype largely obscures morphological studies with the dominant-negative PIKfyveK1831E mutant. To overcome this problem and to make meaningful conclusions about the primary endosome membranes sensitive to PIKfyveK1831E expression and, hence, about the onset of PIKfyveK1831E expression.” Under these conditions, the majority of transfected cells show characteristic expansion of PIKfyveK1831E-positive vesicles particularly well seen at the perinuclear region, but not visible vacuoles. As illustrated in Fig. 2, a–c, the enlarged PIKfyveK1831E-positive vesicles positioned at the perinuclear region strongly colocalized (~40%) with vesicles positive for endogenous EEA1, a peripheral membrane protein that localizes on early endosomes (31). Intriguingly, the EEA1 endosomes positive for PIKfyveK1831E were also significantly enlarged, which was evident in ~80% of transfected cells. However, the shape of the two vesicle types did not always coincide identically (Fig. 2c), indicative for distinct early endosome microdomains populated by the two proteins. By contrast, ectopic expression of PIKfyveWT did not affect the size of the EEA1-positive vesicles nor was there any substantial colocalization between the two, as judged by the only occasional yellow color (~3%) on the merged images (Fig. 2, d–f).

The presence of the GTP-binding protein Rab5 is crucial for the EEA1 recruitment on early endosomes (12, 31). To understand whether early expression of PIKfyveK1831E induces similar changes in the early endosomes marked by Rab5, we coexpressed EGFP-Rab5AWT with myc-tagged PIKfyveK1831E
and inspected the status of Rab5a<sup>WT</sup>-positive endosomes relatively to those seen in the myc-PIKfyve<sup>WT</sup>-expressing COS cells. Similar to the EEA1-positive endosomes, early expression of PIKfyve<sup>K1831E</sup> substantially expanded the Rab5a<sup>WT</sup>-labeled perinuclear structures (Fig. 2, g–l). Likewise, a sub-population of the enlarged Rab5a<sup>WT</sup>-positive perinuclear membranes was found to strongly (~40%) colocalize with the expanded PIKfyve<sup>K1831E</sup>-positive vesicles. Importantly, as with EEA1, the Rab5a<sup>WT</sup>-positive endosomes did not colocalize to a substantial extent (~8%) with the PIKfyve<sup>WT</sup>-positive vesicles. These results demonstrate that, whereas the steady-state PIKfyve<sup>WT</sup> is only slightly seen on early endosomes marked by Rab5<sub>1</sub> and EEA1, or by other early endocytic markers examined in our previous studies (45), the dominant-negative PIKfyve<sup>K1831E</sup>, as monitored at early stages of expression, expands the EEA1- or Rab5-positive endosomes at the perinuclear area and largely accumulates on the enlarged structures.

Redistribution of late endosome/lysosome structures in early PIKfyve<sup>K1831E</sup> expression. To examine the effect of the perturbed PtdIns 3-P<sub>2</sub> transition due to PIKfyve<sup>K1831E</sup> expression on the late endocytic/lysosomal compartments, we next assessed the relationship between the enlarged PIKfyve<sup>K1831E</sup>-positive membranes and LAMP1- or Rab7<sup>WT</sup>-labeled structures. The latter two proteins localize on the limiting membrane of late endosomes and lysosomes and are well-established markers for these organelles (5, 37). Consistent with our previous observations (45) for a partial overlap between the PIKfyve<sup>WT</sup>-positive and MPR-labeled endocytic structures, double labeling for endogenous LAMP1 or ectopically expressed EGFP-Rab7<sup>WT</sup> in PIKfyve<sup>WT</sup>-transfected COS cells revealed a certain number of yellow vesicles (~10 –15%) upon merging the EGFP-PIKfyve<sup>WT</sup>/LAMP1 or the myc-PIKfyve<sup>WT</sup>/EGFP-Rab7<sup>WT</sup> images (Fig. 3, d–f and m–o). Importantly, PIKfyve<sup>WT</sup> expression did not alter the normal topology of LAMP1- or Rab7<sup>WT</sup>-positive late endosomes/lysosomes, and the latter were characteristically positioned in the perinuclear area of the cell much as in control nontransfected or singly Rab7<sup>WT</sup>-transfected COS cells (5, 6; Fig. 3, d–f, m–o, and data not shown). Intriguingly, upon early expression of PIKfyve<sup>K1831E</sup>, vesicles labeled by LAMP1- or

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**Fig. 2.** Expansion of EEA1- and Rab5-positive perinuclear endosomes and colocalization with PIKfyve<sup>K1831E</sup> in early PIKfyve<sup>K1831E</sup> expression. COS-7 cells were transfected with pEGFP-PIKfyve cDNAs (a–f) or cotransfected with wild-type pEGFP-Rab5<sup>WT</sup> and the pCMV5-Myc-PIKfyve constructs (g–l), as indicated. Twelve hours posttransfection, the cells were fixed and processed for indirect immunofluorescence with anti-EEA1 (a, d) or anti-myc antibodies (h, k), followed by detection with Texas red (TrR)-conjugated rabbit anti-goat and Alexa568-conjugated anti-mouse IgGs, respectively, as described in MATERIALS AND METHODS. b, e, g, j: expressed EGFP-fusion proteins were detected by the GFP fluorescence signals. c, f, i, l: overlays of the two left images processed by deconvolution analysis, as described in MATERIALS AND METHODS. Multiple enlarged EEA1- and Rab5<sup>WT</sup>-positive vesicles strongly colocalizing with dilated PIKfyve<sup>K1831E</sup>-positive vesicles are apparent at the perinuclear regions. Note the lack of significant colocalization of PIKfyve<sup>WT</sup> with EEA1- or Rab5-positive vesicles. Arrows and arrowheads (a–f) depict nontransfected and transfected cells, respectively. Bar, 10 μm.
Rab7WT were seen redistributed laterally from the perinuclear region toward the cell periphery in 70–80% of the transfected cells (Fig. 3, and data not shown). Noteworthy, in some cells (20%) coexpressing EGFP-Rab7WT and the pCMV5-Myc-PIKfyve constructs (g–o) as indicated. Twelve hours posttransfection, the cells were fixed and processed for indirect immunofluorescence with anti-LAMP1 (a, d), or anti-myc antibodies (h, k, n), followed by detection with Alexa568-conjugated anti-mouse IgG, as described in MATERIALS AND METHODS. b, e, g, j, m: expressed EGFP-fusion proteins were detected by the GFP fluorescence signals. c, f, i, l, o: overlay of the two left images processed by deconvolution analysis, as described in MATERIALS AND METHODS. Instead of a typical perinuclear distribution seen in PIKfyveWT-expressing cells, the LAMP1- and Rab7WT-positive late endosome structures in PIKfyveK1831E-expressing cells are seen rather dispersed, and sometimes dilated as observed in 20% of PIKfyveK1831E/Rab7WT-coexpressing cells (j–l). Arrows and arrowheads depict nontransfected and transfected cells, respectively. Bar, 10 μm.

**Fig. 3. Redistribution of late endosome/lysosome markers in early PIKfyveK1831E expression.** COS-7 cells were transfected with pEGFP-PIKfyve cDNAs (a–f) or cotransfected with pEGFP-Rab7WT and the pCMV5-Myc-PIKfyve constructs (g–o) as indicated. Twelve hours posttransfection, the cells were fixed and processed for indirect immunofluorescence with anti-LAMP1 (a, d), or anti-myc antibodies (h, k, n), followed by detection with Alexa568-conjugated anti-mouse IgG, as described in MATERIALS AND METHODS. b, e, g, j, m: expressed EGFP-fusion proteins were detected by the GFP fluorescence signals. c, f, i, l, o: overlay of the two left images processed by deconvolution analysis, as described in MATERIALS AND METHODS. Instead of a typical perinuclear distribution seen in PIKfyveWT-expressing cells, the LAMP1- and Rab7WT-positive late endosome structures in PIKfyveK1831E-expressing cells are seen rather dispersed, and sometimes dilated as observed in 20% of PIKfyveK1831E/Rab7WT-coexpressing cells (j–l). Arrows and arrowheads depict nontransfected and transfected cells, respectively. Bar, 10 μm.
but not those of ECV/MVB transport intermediates, have been shown to accumulate LBPA, a highly hydrophobic, poorly degradable phospholipid that is not found in other organelles (24).

To clarify whether the subpopulation of endosomes found positive for dominant-negative PIKfyve<sup>K1831E</sup> contain LBPA, we performed labeling for endogenous LBPA in EGFPIPKfyve<sup>K1831E</sup>-transfected cells when vacuole formation was still not manifested. As illustrated in Fig. 4, LBPA-positive signals were documented in a small fraction of PIKfyve<sup>K1831E</sup>-positive vesicles. The extent of colocalization (~10%) was however, comparable to that observed between the LBPA- and PIKfyve<sup>WT</sup>-positive membranes in the pEGFP-PIKfyve<sup>WT</sup>-transfected COS cells. Of note, although enlarged PIKfyve<sup>K1831E</sup>-positive vesicles were apparent, the LBPA-positive membranes did not increase in size due to PIKfyve<sup>K1831E</sup> expression, as judged by their analogous appearance in the PIKfyve<sup>WT</sup>- and PIKfyve<sup>K1831E</sup>-expressing or non-transfected cells (Fig. 4). The majority of enlarged PIKfyve<sup>K1831E</sup>-positive membranes, however, were devoid of LBPA signals, in agreement with the data detecting occasionally late endosome/lysosomal markers in the PIKfyve<sup>K1831E</sup>-positive membranes (Fig. 4). This result implies that intralumenal vesiculation of late endosome membranes, as monitored by LBPA, is not markedly affected by early PIKfyve<sup>K1831E</sup> expression.

**Arrest of PtdIns 3-P to PtdIns 3,5-P2 shift alters PIKfyve endosomal localization.** The results so far indicate that the major outcome of early expression of dominant-negative kinase-deficient PIKfyve<sup>K1831E</sup> in COS cells is enlargement of early endosomes and dislocation of the late endocytic compartment. There was also a major difference in the steady-state distribution of ectopically expressed PIKfyve<sup>WT</sup> vs. PIKfyve<sup>K1831E</sup> in that the wild type was only weakly detectable on early endosomes, whereas the dominant-negative mutant largely accumulated there. These observations led us to suggest that PIKfyve undergoes a cycle of transient recruitment to PtdIns 3-P-enriched microdomains on early endosomes via its FYVE domain (42), followed by a dynamic membrane retrieval driven by the PtdIns 3,5-P<sub>2</sub>-remodeled membrane sub-domain. Conceivably, due to the robust PIKfyve-directed PtdIns 3-P-PtdIns 3,5-P<sub>2</sub> conversion, early-endosome-associated PIKfyve is likely a short-lived intermediate stage in this cycle, a notion supported by the little PIKfyve<sup>WT</sup>-positive staining found on early endosomes at steady state (Ref. 45; and see Fig. 2). If this is correct, one would expect that the arrest in the PtdIns 3-P-to-PtdIns 3,5-P<sub>2</sub> shift on early endosomes will prolong PIKfyve’s retention time at this compartment and, thus will increase the propensity for detecting the here predicted intermediate stage of early endosome-associated PIKfyve.

To address this possibility, we conducted experiments in several directions. First, using EEA1 as a marker, we examined a plausible early endosome localization of PIKfyve NH<sub>2</sub>-terminal peptide fragment that harbors the FYVE finger localization module but is kinase inactive. It should be noted that the association of EEA1 with early endosomes is relatively stable, evidenced by its ability to largely sustain displacement by different FYVE finger domain probes (13). Likewise, endogenous EEA1 did not dissociate from membranes upon expression of EGFPIPKfyve1–286 in COS cells and the two signals largely colocalized (Fig. 5, a–c).

Next, we examined the localization of PIKfyve<sup>WT</sup> in COS cells that were cotransfected simultaneously with the dominant-negative PIKfyve<sup>K1831E</sup> mutant, reasoning that the latter, when expressed at higher levels vs. PIKfyve<sup>WT</sup>, will dominantly inhibit the wild-type enzymatic activity, and hence, the PtdIns 3-P-to-PtdIns 3,5-P<sub>2</sub> shift on early endosomes. To assure the selective detection of each form, PIKfyve<sup>WT</sup> and PIKfyve<sup>K1831E</sup> were tagged with myc- and HA-epitopes, respectively, and expressed from identical plasmids to better balance the expression levels. Remarkably, the vast majority (~80%) of PIKfyve<sup>WT</sup>-positive vesicles in cotransfected cells were found dilated and colocalizing with the enlarged PIKfyve<sup>K1831E</sup>-positive vesicles (Fig. 5). The reverse was also evident (Fig. 5), suggestive for the PIKfyve<sup>WT</sup> retention on PIKfyve<sup>K1831E</sup>-dilated endosomes under these conditions. When PIKfyve<sup>WT</sup>-positive/PIKfyve<sup>K1831E</sup>-negative structures were occasionally detected, they appear to display the same
characteristic fine puncta as the singly transfected PIKfyveWT (45; and Fig. 5). PIKfyveK1831E-positive/PIKfyveWT-negative structures were practically not observed (Fig. 5).

The data above are consistent with the notion that dominant-negative PIKfyveK1831E, by reducing membrane PtdIns 3,5-P2 levels, causes the PIKfyveWT early endosome retention. In the absence of appropriate PtdIns 3,5-P2 antibodies or bioreporters, direct experimental evidence that the endosome PtdIns 3,5-P2 levels are reduced under these conditions is currently unattainable. Similarly, given the low transient cotransfection efficiency of PIKfyve’s size, this key question could neither be approached by biochemical analysis. Therefore, to obtain experimental evidence that expression of dominant-negative PIKfyveK1831E impairs PtdIns 3,5-P2 production on membranes, we compared the ability of membrane fractions derived from a HEK-293 cell line inducibly expressing PIKfyveWT early endosome localization. The data presented above, implying retention of PIKfyveWT in the dilated PIKfyveK1831E-positive endosomes (f), Bar, 10 μm.

PIKfyve transient recruitment to Rab5-sensitive PtdIns 3-P pool on early endosomes and retrieval to later compartments. We have previously demonstrated that the steady-state localization of PIKfyveWT to intracellular membranes depends on the interaction between PIKfyve’s FYVE finger domain and PtdIns 3,5-P2. The data presented above, implying retention of kinase-deficient PIKfyve mutants on early endosomes, raise the question of whether, or not this recruitment engages early endosome-localized PtdIns 3-P (13). A great deal of the PtdIns 3-P pool on early endosomes has been related to the Rab5 GTP/GDP cycle (28, 53). Active GTP-bound forms of Rab5 have been demonstrated to interact directly with two types of PI 3-kinases (7, 33) and the formed Rab5/PtdIns 3-P platforms on early endosomes have been shown to recruit FYVE finger domain-containing proteins, including EEA1 (53). Therefore, we next examined a possible role for a Rab5/PtdIns 3-P-dependent mechanism in early endosome-recruitment of PIKfyve that is predicted here. We used the dominant-negative GDP-bound Rab5S34N mutant that has been shown to discriminate between the Rab5-dependent and -independent localization mechanism of the FYVE-finger domain proteins EEA1 and Hrs, respectively (39). Fluorescence microscopy analysis in COS cells cotransfected with the dominant-negative mutants myc-PIKfyveK1831E and EGFP-Rab5S34N detected almost no
membrane-associated PIKfyve<sup>K1831E</sup> in the Rab5<sup>S34N</sup>-expressing cells (Fig. 7, a–c). Remarkably, Rab5<sup>S34N</sup> expression not only rendered the PIKfyve<sup>K1831E</sup> distribution diffuse but also impaired the ability of PIKfyve<sup>K1831E</sup> to induce vacuoles even at 72 h posttransfection (Fig. 7, and data not shown), consistent with our previous findings detecting no vacuolar formation if the mutant was mislocalized through truncating the FYVE finger domain (21). Likewise, when coexpressed with EGFP-Rab5<sup>S34N</sup>, myc-PIKfyve<sup>WT</sup> was hardly seen membrane-associated (data not shown), thus implicating the role for active Rab5 on early endosomes in the PIKfyve recruitment to membranes of the endocytic system. This conclusion was further corroborated by the observations with the constitutively active GTPase-deficient EGFP-Rab5<sup>aQ79L</sup> mutant. Thus, coexpression of PIKfyve<sup>WT</sup> or PIKfyve<sup>K1831E</sup> with EGFP-Rab5<sup>aQ79L</sup>, which induces by itself endosome enlargement (48), resulted in a strong colocalization (>50%) of either PIKfyve<sup>WT</sup> or PIKfyve<sup>K1831E</sup> with Rab5<sup>aQ79L</sup> on the limiting membrane of the dilated endosome structures (Fig. 7, d–i). Importantly, both PIKfyve<sup>K1831E</sup> (Fig. 7, j–l) and PIKfyve<sup>WT</sup> (data not shown) were released from the Rab5<sup>aQ79L</sup>-positive membranes into the cytosol upon short treatment with the PI 3-kinase inhibitor wortmannin. These results imply a role for a Rab5-GTP-dependent mechanism and formation of Rab5/PhDs 3-P platforms in the here predicted early endosome intermediate stage of PIKfyve association. Together, the data presented in the last two sections are consistent with a model whereby PIKfyve, likely from the soluble pool, is transiently recruited to early endosomes in a Rab5/PhDs 3-P-dependent manner. The subsequent PIKfyve-catalyzed PhDns 3-P-to-PhDs 3,5-P<sub>2</sub> conversion on early endosomes drives PIKfyve retrieval to later endocytic compartments and release into the cytosol.

PIKfyve<sup>WT</sup> negatively regulates endosome fusion. The observed PIKfyve<sup>K1831E</sup>-induced expansion of endosome structures and the massive endomembrane vacuolation at longer expression times is associated with a gain of membranes. A potential cellular mechanism that could contribute to these effects is increased fusion efficiency of the endosome compartments. Therefore, we next examined the potency of cytosols, enriched in either PIKfyve<sup>WT</sup>- or PIKfyve<sup>K1831E</sup>-protein expression levels were increased by 10.220.33.4 on October 14, 2017 http://ajpcell.physiology.org/ Downloaded from
respectively, compared with cytosols from PIKfyveWT-noninduced cells. By contrast, cytosols from cells expressing PIKfyveK1831E increased the relative efficiency of the early or total endosome fusion to 140% and 180%, respectively, vs. cytosol from noninduced PIKfyveK1831E cells (Fig. 8). Control cytosols from parental cells did not show significant changes in endosome fusion as a function of the cell induction (data not shown).

Attempts were made to evaluate the relative proportion of the homotypic late-endosome fusion by pulse-labeling BHK21 cells with avidin or biotinylated HRP for 5 min, followed by additional 25 min of chase, conditions which should label predominantly late endosomes (15, 30). However, under our conditions, the labeling of late endosomes was too low, likely due to recycling of the markers (15); therefore, measurements of this type were not further pursued. Together, the data indicate that the endosome fusion efficiency is negatively regulated by a mechanism that involves PIKfyve enzymatic activity and, most likely, PtdIns 3,5-P2 production.

This notion was further corroborated by results from the cell-free fusion assay utilizing an NH2-terminal kinase-deficient peptide fragment of PIKfyve, PIKfyve99–473, that harbors the FYVE-finger domain for localization to membrane PtdIns 3-P. Experimental evidence obtained in a transfected cell system is consistent with a displacement of the endogenous PIKfyve from membranes under higher expression levels of the PIKfyve FYVE-finger peptide (42). As illustrated in Fig. 8, like the dominant-negative kinase-deficient PIKfyveK1831E mutant, purified GST-PIKfyve99–473 peptide increased the fusion activity of early endosomes. This effect was specific because the GST-PIKfyve99–473/H900477–198 mutant peptide, in which the basic PtdIns 3-P-interacting pocket within the FYVE-finger domain was truncated to abolish membrane association (42), had no effect (Fig. 8). Together these data suggest that a PtdIns 3-P targeting mechanism is essential in the PIKfyve-mediated negative regulation of endosome fusion.

DISCUSSION

Accumulating evidence suggests that impaired PtdIns 3,5-P2 synthesis, turnover, or downstream signaling in eukaryotic cells has a profound effect on the normal endosome membrane organization and dynamics. Thus direct inactivation of the enzymes producing PtdIns 3,5-P2 (i.e., Fab1, in yeast, or PIKfyve, in mammalian cells) is associated with related defects along the degradation pathway in the form of enlarged lyso-
somal (yeast) or MVB-like compartments (mammals), both reversed on restoration of PtdIns 3,5-P2 pools (19–21, 34). Next, mammalian cell treatment with the PI 3-kinase inhibitor wortmannin that decreases PtdIns 3-P and thus indirectly inactivates PIKfyve by depriving PIKfyve of both a substrate and a membrane localization target, results in a massive dilation of the endocytic membrane structures and cytoplasmic vacuolation, similarly to what is observed on ectopic expression of dominant-negative kinase-deficient PIKfyve mutants (9, 11, 19, 21). Furthermore, increased turnover of PtdIns 3,5-P2 in mammalian cells by ectopic expression of myotubulins, a conserved family of dual-specificity phosphatases that hydrolyze PtdIns 3,5-P2, has also been found to result in expanded endosomal compartiments and massive vacuolation (4, 49). Finally, a dysfunction of candidate downstream protein effectors of PtdIns 3,5-P2, such as Svp1 and Ent3 in yeast, or the ESCRT III subunit Vps24 in mammalian cells, besides a delay in protein sorting, results in enlarged endosomes or lysosomes (8, 10, 52). The aim of this study was to determine the onset of the PtdIns 3,5-P2 functional requirement within the endocytic system as well as the cellular mechanism underlying the endocytic membrane expansion under perturbations in the PtdIns 3,5-P2 pool. To this end, we made use of the ability of the dominant-negative kinase deficient PIKfyve mutant (K1831E) to induce biphasic morphological changes in transiently transfected COS cells, which could be clearly separated into an initial phase of vesicle dilation and a later stage of massive cytoplasmic vacuole formation (Fig. 1). We monitored cells during the first phase, thus avoiding possible formation of hybrid compartments due to acquisition of aberrant cellular architecture. Our studies unequivocally demonstrate that, under perturbed PtdIns 3-P-to-PtdIns 3,5-P2 conversion due to PIKfyveK1831E expression (Fig. 6), the kinase-deficient mutant, unlike the wild-type enzyme, is largely localized on expanded EEA1- and Rab5-labeled perinuclear endosomes (Fig. 2). This localization is dependent on the presence of active forms of Rab5 and PI 3-kinases (Fig. 7), mechanistically consistent with the formation of PtdIns 3-P-enriched microdomains by the Rab5-GTP-dependent recruitment of PI 3-kinases (7, 33, 53). We further demonstrate that, whereas PIKfyveWT is largely excluded from Rab5/EEA1-labeled early endosomes, its coexpression with PIKfyveK1831E renders PIKfyveWT accumulation on the diluted PIKfyveK1831E-positive endosomes. PIKfyveK1831E-enriched cytosols, as well as a PIKfyve-derived peptide fragment that lacks kinase activity but exhibits the FYVE finger domain for targeting membrane PtdIns 3-P, largely increased endosome fusion (Fig. 8). These data are consistent with the conclusion that the correct endosome organization and dynamics require the PIKfyve-catalyzed PtdIns 3-P-to-PtdIns 3,5-P2 conversion at early endosomes and that thereby produced PtdIns 3,5-P2 negatively regulates endosome membrane fusion activity.

**Place of PIKfyve action and steady-state localization.** That PtdIns 3,5-P2 action is required at early endosomes was quite unexpected because the steady-state distribution of PIKfyve, either the endogenous in 3T3-L1 adipocytes or ectopically expressed in COS and HEK-293 cells, largely skips over early/recycling endosomes. Thus, despite that the FYVE-finger domain directs PIKfyve to PtdIns 3-P (42) and that the early endosomes are enriched in PtdIns 3-P, endogenous PIKfyve or PIKfyveWT did not biochemically cofractionate with several early/recycling endosome markers, morphologically (43, 45), nor did they colocalize, with these markers to a substantial extent. The strong colocalization observed here between the dominant-negative kinase-deficient PIKfyveK1831E mutant and the early endosome markers (Fig. 2) is therefore consistent with the kinase activity being important for PIKfyve exclusion from early endosomes. Concordantly, when coexpressed with the dominant-negative PIKfyveK1831E, PIKfyveWT almost exclusively accumulated on the diluted endosome membranes populated by PIKfyveK1831E (Fig. 5), implying that the potency to convert PtdIns 3-P-to-PtdIns 3,5-P2 determines the residence time of PIKfyve on early endosomes. Further support for this conclusion comes from our observations with expressed GFP-PIKfyve(1–286) NH2-terminal peptide that harbors the FYVE-finger domain for PtdIns 3-P association but is kinase inactive...
PtdIns 3,5-P2 become available, we believe this hypothesis may be supported by the finding that the PIKfyve-catalyzed PtdIns 3-P-to-PtdIns 3,5-P2 conversion is part of still unidentified mechanisms for correct PtdIns 3,5-P2 remodeling at distinct regions of early endosomes. It is tempting to speculate that the PIKfyve-catalyzed PtdIns 3,5-P2 conversion is part of still unidentified mechanisms for correct PtdIns 3,5-P2 remodeling at distinct regions of early endosomes.

One important but still unresolved question in the present study is the nature of the later compartments of PIKfyve membrane retrieval predicted here, which most likely are the principal residence place of steady state PIKfyve. Membrane association of PIKfyve is dependent on PtdIns 3-P, indicated by the diffuse PIKfyve staining upon cell treatment with wortmannin (42, and this study) or expression of dominant-negative Rab5GTPase-deficient Rab5Q79L (Fig. 7). Together, these data support a model whereby the PtdIns 3-P microdomain on early endosomes represents a major entry site for the cytosolic pool of PIKfyve. Driven by its catalytic activity, PIKfyve is then rapidly retrieved from early endosomes to later compartments by a mechanism that involves local membrane remodeling in two ways, by decreasing PtdIns 3-P and increasing PtdIns 3,5-P2. The fact that PIKfyve WT is also visualized on endosomes positive for Rab5Q79L could be due to an abnormally high PtdIns 3-P-PtdIns 3,5-P2 ratio as a result of enhanced Rab5 recruitment at steady state. PIKfyve catalytic activity is therefore not restricted to early stages of the pathway. Our observations rather suggest that PIKfyve enzymatic activity is also involved in the mechanism that controls fusion steps in later compartments, although their precise identity was not determined in this study. Among others, these may include the intralumenal membrane fusion with the limiting membrane of multivesicular endosomes, which has been predicted to occur in mammalian cells (26, 48). Such a role for PIKfyve and PtdIns 3,5-P2 would be consistent with data from our transmission electron microscopy studies in a HEK-293 stable cell line expressing dominant-negative PIKfyveK1831E, demonstrating that while intralumenal endosome vesicles could be readily seen in dilated MVB-like structures, they are low in number vs. similar structures in control cells (19). It is conceivable that PtdIns 3,5-P2 is required to negatively regulate the rate of fusion at more than one place among the heterogeneous multivesicular endosomes, thus contributing to the dynamic properties of these membranes.

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REFERENCES

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\textbf{PrPds 3.5-P₂ in early endosome dynamics}


