Core Protein Machinery for Mammalian Phosphatidylinositol 3,5-Bisphosphate Synthesis and Turnover That Regulates the Progression of Endosomal Transport

**NOVEL SAC PHOSPHATASE JOINS THE ArPIKfyve-PIKfyve COMPLEX**

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The phosphorylated derivatives of phosphatidylinositol (PtdIns)4, called phosphoinositides (PI), are eukaryotic cell membrane-anchored phospholipids, whose cytosol-exposed hydrophilic inositol headgroup is phosphorylated at positions D3, D4, and/or D5 to yield seven PIs (1, 2). The ability to undergo acute and reversible phosphorylation by kinases and phosphatases makes the PIs indispensable and versatile membrane-anchored signals that, by recruiting distinct effector proteins, govern diverse and essential cellular processes, such as intracellular membrane trafficking and signaling (3–9). PtdIns(3,5)P2, one of the seven PIs, is widespread in eukaryotes but is present only in minute quantity, comprising as little as 0.8% of total PIs (10, 11). Identified first in smooth muscle cells ~17 years ago (12), steady-state PtdIns(3,5)P2 levels are now detected in all mammalian cell types examined as well as in yeast and plants (13). PtdIns(3,5)P2 appears to be up-regulated by various stimuli, the most prominent of which is the hypertensive stress in *Saccharomyces cerevisiae*, plant cells, or mouse 3T3-L1 adipocytes (13, 14).

The enzymes that make PtdIns(3,5)P2 comprise a family of evolutionarily conserved proteins, all products of a single copy gene (7). Although the mechanism is still elusive, the action of the PtdIns(3,5)P2-synthesizing enzymes is apparently indispensable in multicellular organisms, as evidenced by the recent findings for embryonic lethality of the loss-of-function *Caenorhabditis elegans* and *Drosophila melanogaster* mutants (15, 16). In cellular contexts, *S. cerevisiae* Fab1 and mammalian PIKfyve are the most intensively studied PtdIns(3,5)P2-producing enzymes (7, 8, 13, 17). Their close functional relationship is indicated by the similar morphological changes in the form of diluted endosomes and swollen endocytic organelles associated with inactivation of *FAB1* in yeast, and expression of dominant-negative kinase-deficient PIKfyve**K1831E** or ablation of PIKfyve in mammalian cells (10, 18–21). Cellular studies documenting a similar phenotype of enlarged compartments along the endosomal/endo-cytic system in the fruit fly and *C. elegans* PIKfyve mutants...
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(15, 16) are consistent with evolutionary conservation of PtdIns(3,5)P$_2$ in endosome-related functions. The main difference among the species studied so far is related to the identity of the endosomal compartment where PtdIns(3,5)P$_2$ function is required. Thus, whereas in lower organisms, i.e. S. cerevisiae and C. elegans, the loss of Fab1/PIKfyve function affects the later stages of the endocytic pathway, at the level of the lysosomes (15, 18), in the higher eukaryotes (fruit fly and mammals) the defect arises earlier in the endocytic pathway, at the level of the multivesicular endosomes (15, 20–22).

In mammalian cells, multivesicular endosomes (MVEs) constitute the majority of the endosome membrane system of the degradation pathway (23). They are so called for their distinctive ultrastructure, characterized by numerous intraluminal membranes with a vesicular and/or lamellar appearance. MVE is therefore a generic term for any early, intermediate, or late endocytic compartment in the degradation pathway as proposed previously (23). It is now clear that intraluminal membrane invaginations in MVEs are mechanistically coupled to protein sorting into the degradation pathway. Components of the molecular machinery, first identified in yeast and found conserved in mammals, form three protein complexes, ESCRT I, II, and III, which act sequentially in cargo inclusion in the internal vesicles of MVEs (24–27). In addition to protein sorting, MVEs possess the ability to emanate cargo-loaded endosome transport intermediates. According to the vesicle-transport model, endosome carrier vesicles (ECV) or multivesicular bodies (MVBs) arise by budding/detachment from early endosomes (23, 28–30). An alternative model views early endosome maturation as a means of cargo transport (31–33). A recent study appears to reconcile both models by adapting elements of each one (34). Regardless of their mode of biogenesis, there is a consensus that endosome transport intermediates (for which we retain herein the acronym ECV/MVBs) are a subpopulation of endosome vesicles with characteristics distinct from both early and late endosomes (23). Although the underlying molecular mechanism of ECV/MVB biogenesis is still elusive, it appears that, at least in mammalian cells, it is distinct from the inward invagination of the MVE limiting membrane. Specifically, membrane receptor sorting into the MVE pathway is affected by perturbations in membrane PtdIns(3)P or depletion of annexin1, whereas the ECV/MVB formation remains intact under these conditions (35, 36). By contrast, ECV/MVB formation/detachment, but not the inward invagination of the MVE limiting membrane, is reportedly dependent on annexin2 (37).

Whether the enlarged size of MVEs observed in cell models with loss-of-function or expressing dominant-negative mutants of PIKfyve (10, 16, 19, 20) is associated with arrested ECV/MVB formation/detachment (or maturation) because of perturbed PtdIns(3,5)P$_2$ endosome membrane remodeling has never been examined.

The evolutionary conservation of the PtdIns(3,5)P$_2$ pathway is further substantiated by the recent findings for structural and functional homology between yeast and mammalian Vac14, also known as ArPIKfyve (14, 38). They both activate Fab1 and PIKfyve, respectively, and in the case of mammalian cells, this is by a physical association (38–40). ArPIKfyve and Vac14 are essential for both steady-state and hyperosmotically elevated PtdIns(3,5)P$_2$ in cultured adipocytes and yeast, respectively (14, 38–40). It should be emphasized, however, that regulation of PtdIns(3,5)P$_2$ levels could occur by both synthesis and turnover. Concordantly, a Sac domain-containing 5-phosphatase, Fig4, has been recently characterized in budding yeast and found to turn over PtdIns(3,5)P$_2$ to PtdIns(3)P both in vitro and in vivo (11, 18, 41). Reportedly, Fig4 directly interacts with Vac14, which promotes its localization to the site of PtdIns(3,5)P$_2$ synthesis (11, 40). It is unknown whether a similar coordination of PtdIns(3,5)P$_2$ synthesis and turnover operates in mammalian cells and whether the uncharacterized mammalian Sac domain phosphatase, Sac3, or KIAA0274 (42), is the true Fig4 ortholog. In the present study we have characterized Sac3 as the mammalian counterpart of the yeast PtdIns(3,5)P$_2$-specific phosphatase Fig4. Sac3 assembles with PIKfyve and ArPIKfyve in a stable ternary complex and controls PtdIns(3,5)P$_2$ levels. We further demonstrate a key function for each of the three proteins in the biogenesis of ECV/MVB transport intermediates from early endosomes. These data indicate a tight control of mammalian PtdIns(3,5)P$_2$ levels, which is coordinated through a physical association of a core protein machinery for PtdIns(3,5)P$_2$ synthesis and turnover to regulate membrane exit from early endosomes.

**Experimental Procedures**

**Human Sac3 and Other Antibodies**—Rabbit polyclonal Sac3 antibodies were directed against the C-terminal region of human Sac3 (amino acids 610–907). Polyclonal anti-PIKfyve (R7069, directed against the N terminus) and anti-ArPIKfyve (WS047, directed against the C terminus) were described elsewhere (38, 43). The antibodies were used for immunoprecipitation and Western blotting either as crude antiserum or after affinity purification on the corresponding GST fusion peptides as described previously (38). Anti-Myc monoclonal antibody was produced by 9E10.2 hybridoma cells (ATCC). Goat anti-EEA1 (N-19) and anti-GFP polyclonal antibodies (Ab290) were from Santa Cruz Biotechnology and AbCam, respectively. Monoclonal anti-α-tubulin, anti-β1/2-adaptin, and anti-γ-adaptin antibodies were from Sigma. Polyclonal anti-HA (R4289), anti-IRAP, anti-GRP94, anti-Rab4, and monoclonal anti-transferrin receptor antibodies were gifts by Drs. Mike Czech, Paul Pilch, Steve Cala, Ira Melman, and Ian Traubridge and used under previously specified conditions (38, 43–46).

**Sac3 and Other Constructs**—Human cDNA clone KIAA0274 representing full-length hSac3 was obtained from Kazusa DNA Research Institute. Myc-Sac3 was generated by introducing full-length Sac3 cDNA into pEF-Bos-Myc vector by blunt-end cloning. Sac3 cDNA was ligated into Xhol-Kpn1 digest of pEGFP-C3 (Clontech) to generate eGFP-Sac3WT. A phosphatase-deficient mutant, eGFP-Sac3C$_{N888A}$, was generated by the QuikChange site-directed mutagenesis kit (Stratagene). Expression of Myc- or GFP-tagged Sac3 proteins was confirmed by Western blotting with anti-Myc and anti-GFP antibodies. pRSETb-His$_6$-hArPIKfyve and pRSETb-His$_6$-mGDI2 were described previously (38, 47). Proteins were produced in *Escherichia coli* BL21(DE3) strain and purified as described (47). Construction of pCMV5-HA-hArPIKfyve, pEGFP-HA-
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hArPIKfyve, or pEGFP-HA-mPIKfyveWT was detailed elsewhere (10, 38, 43).

Tissues and Cell Cultures—Tissues were dissected from pregnant female mice (Swiss-Wistar) and rinsed in PBS prior to homogenization. Stable HEK293 (TetOn) cell lines, inducibly expressing PIKfyveWT (clone 9) or PIKfyveK1831E (clone 5), were generated and maintained as described previously (44). HEK293, COS7, and PC12 cells were cultured under conditions described in our previous studies (38, 43, 44, 48). BHK21 cells were maintained in Glasgow minimum essential medium, supplemented with 5% fetal bovine serum, 10% tryptose phosphate broth, and 1 mM glutamine as described (30). Culturing and differentiation of 3T3-L1 fibroblasts to adipocyte phenotype were described elsewhere (14).

siRNAs and Cell Transfection—Smart Pool™ siRNA duplexes targeting human (M-019141-01) or mouse Sac3 (M-052024-00) and human PIKfyve (M-005058-03) were designed and synthesized by Dharmacon on a fee-for-service basis. Human ArPIKfyve, mouse ArPIKfyve, mouse PIKfyve, and cyclophilin B siRNA pools (Dharmacon) were characterized previously (38, 49). HEK293 cells were transiently transfected with human-specific siRNA duplexes (100 nM) by Oligo-fectamine (Invitrogen), Lipofectamine 2000 (Invitrogen), or electroporation and used 72 h post-transfection. 3T3-L1 adipocytes were transiently transfected with mouse-specific siRNA duplexes (0.2–0.4 nmol/5 × 10⁶ cells) by electroporation and used 72 h post-transfection. HEK293 or COS7 cells were transfected with the indicated cDNAs by Lipofectamine 2000 or Lipofectamine (Invitrogen), for biochemical and immunofluorescence microscopy studies, respectively.

Confocal and Light Microscopy—For confocal microscopy, COS7 cells grown on coverslips were transfected with the constructs indicated in the figure legends. Twenty four hours following transfections, cells were washed, fixed, permeabilized, and stained with monoclonal anti-Myc or polyclonal anti-EEA1 antibodies as described elsewhere (21) and specified in the legend to Fig. 7. Detection of anti-Myc was achieved with Alexa568- or fluorescein isothiocyanate-coupled goat anti-mouse IgG (Molecular Probes), whereas anti-EEA1 was detected with CY3-coupled rabbit anti-goat IgG (Sigma). Coverslips were mounted on slides using the Slow Fade antifade kit (Molecular Probes), and observed on a motorized inverted confocal microscope (model 1X81, Olympus, Melville, NY) by a 60× UPlanApo objective. GFP signals were captured by a standard green fluorescence filter. Images were captured by a cooled charge-coupled device 12-bit camera (Hamamatsu). In some experiments fluorescence microscopy in live COS cells was performed by Nikon Eclipse TE200 (Tokyo, Japan) using a ×40 objective. In this case, images were captured by a SPOT RT Slider charge-coupled device camera (Diagnostic Instruments) and processed by SPOT 3.2 software.

Immunoblotting and Immunoprecipitation—Cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) supplemented with 1× protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM benzamidine) and 1× phosphatase inhibitor mixture (25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, and 2 mM NaVO₃). Mouse tissues were homogenized in “HES ++ buffer” (20 mM HEPES/NaOH, pH 7.5, 1 mM EDTA, 255 mM sucrose, supplemented with 1× protease and 1× phosphatase inhibitor mixtures) by a motorized homogenizer (Heidolph) and then lysed in RIPA buffer. Cell or tissue lysates were clarified by centrifugation (30 min, 14,000 rpm; 4 °C). Immunoblotting with the antibodies was performed subsequent to protein resolution by SDS-PAGE and electrotransfer onto nitrocellulose membranes as described (14, 38, 43, 44). A chemiluminescence kit (Pierce) was used to detect the horseradish peroxidase-bound secondary antibodies. Endogenous PIKfyve, ArPIKfyve, Sac3, and their HA- or Myc-tagged forms were immunoprecipitated from RIPA lysates (supplemented with 1× protease and 1× phosphatase inhibitor mixtures) of tissues and cells using polyclonal anti-PIKfyve, anti-ArPIKfyve, anti-Sac3 or anti-HA antibodies, and the monoclonal anti-Myc antibody. Control immunoprecipitates with preimmune/nonimmune sera were run in parallel. Immunoprecipitations were carried out for 16 h at 4 °C, with protein-A-Sepharose CL-4B added in the final 1.5 h of incubation. Immunoprecipitates were washed with RIPA buffer plus the inhibitor mixtures and then processed by Western blotting.

Subcellular Fractionation and Equilibrium Centrifugation in Iodixanol Gradient—HEK293 stable cells induced to express PIKfyveWT were homogenized in HES ++ buffer at 4 °C and fractionated into total membranes and cytosols as described previously (38, 48). Total membrane fractions resuspended under HES ++ buffer were mixed with iodixanol (OptiPrep; Sigma) in a Quick-Seal centrifuge tube to 30% iodixanol and 128 mM sucrose. A self-generating gradient was performed by centrifugation to equilibrium as specified previously (38, 48). Fractions, collected from the bottom of the tube, were analyzed for protein concentration and immunoblotted with the indicated antibodies.

[^2P]Orthophosphate Cell Labeling, Lipid Extraction, and HPLC—HEK293 cells, transfected with Sac3 or control cyclophilin B siRNA duplexes, were labeled in phosphate/serum-free Dulbecco's modified Eagle's medium for 2.5 h at 37 °C with [^2P]orthophosphate as described previously (10, 38). Longer labeling times with [^2P]orthophosphate (6 h) did not affect the relative amount of individual[^2P]PIs. Cells were washed in the presence of 1× phosphatase inhibitors and scraped with CH₃OH, 1 M HCl (1:1). Extracted lipids were deacylated and analyzed by HPLC on a Whatman 5-micron Partisphere SAX column eluted with a shallow ammonium phosphate gradient as detailed elsewhere (10, 14, 38, 43). The radioactivity was analyzed with an on-line flow scintillation analyzer (Radiomatic 525TR, Packard Instrument Co.).[^3H]GroPins-4-P,[^3H]GroPins-4,5-P₂, and[^3H]GroPins-3-P deacylated from[^3H]PtdIns(4)P (PerkinElmer Life Sciences),[^3H]PtdIns(4,5)P₂ (PerkinElmer Life Sciences), and[^3H]PtdIns(3)P, respectively, were co-injected as internal HPLC standards.[^2P]GroPins-3,5-P₂ and[^3P]GroPins-3,4-P₂ external standards were deacylated from[^3P]PtdIns(3,5)P₂ and[^3P]PtdIns(3,4)P₂ that were synthesized with PIKfyve and PI 3-kinase as described previously (14, 38, 43). FLO-ONE radiochromatography software (Packard Instrument Co.) was used for data evaluation. Individual peak radioactivity was quantified by area integration and is pre-
sent as a percentage of the combined radioactivity from the $^{32}$P-labeled GroPIns-3-P, -4-P, -3,5-P$_2$, -3,4-P$_2$, and -4,5-P$_2$ peaks (“total radioactivity”).

**In Vitro Phosphatase Assay**—Sac3 hydrolysing activity toward the different PI substrates was determined in vitro by the malachite green-based assay that measures the released inorganic phosphate (50). Briefly, RIPA buffer lysates derived from COS cells transfected with Myc-Sac3, eGFP-Sac3, eGFP-Sac3$^{D488A}$, or empty vectors were subjected to immunoprecipitation in the presence of 1× protease and 1× phosphatase inhibitor mixtures as described above. Protein A-Sepharose beads were washed three times with the same buffer, then six times with the phosphatase “assay buffer” (50 mM Tris/HC1, pH 7.4, 1 mM MgCl$_2$, and 1 mM dithiothreitol), and finally resuspended in the assay buffer (final volume 55 µl), containing one of the seven di-C8 PI lipids (75 µM final concentration) (Echelon Generonic Phosphatase assay kit). Phosphatase reactions were incubated at 37 °C for 60 min and were terminated by adding 35 µl of cold assay buffer at 4 °C. The supernatants were mixed with the malachite green reagent, and after 30 min, the absorbance was read at 660 nm.

**In Vitro ECV/MVB Formation Assay from Early Endosomes**—Formation of ECV/MVBs from donor early endosomes was determined exactly as described previously (51, 52). The assay uses horseradish peroxidase (HRP) activity as a measure of ECV/MVB formation from early endosomes. To load the early endosome compartments, baby hamster kidney cells (18 × 100-mm dishes/experiment) were allowed to internalize HRP (5 mg/ml, Sigma) for 7 min at 37 °C. All subsequent procedures were performed at 4 °C. Cells were washed three times with PBS, then scraped in PBS, sedimented by centrifugation at 175 × g for 5 min, resuspended in homogenization buffer (250 mM sucrose and 3 mM imidazole), and centrifuged again. Cells were resuspended in homogenization buffer (twice the cell volume) and homogenized through a 1.0-ml syringe with 22-gauge needle (five strokes). A postnuclear supernatant was obtained by centrifugation at 1355 × g for 10 min. The sucrose concentration of the isolated postnuclear supernatant was adjusted to 40.6% with a 62% sucrose solution. The postnuclear supernatant (0.5 – 0.6 ml) was placed at the bottom of a centrifuge tube and overlaid with 1.5 ml of 35% sucrose, followed by 1.0 ml of 25% sucrose. The tube was filled up with homogenization buffer (8% sucrose). The resulting flotation gradient was centrifuged in an SW60 rotor (Beckman) at 35,000 rpm for 60 min. Early endosomes were collected from the 35/25% interface and used immediately in the ECV/MVB formation assay. Early endosomes (60–80 µg of protein) were incubated (30 min, 37 °C) in the presence of ATP-regeneration systems (creatinine phosphate + creatine phosphokinase + ATP) or ATP-depletion systems (apryase; Sigma) and cytosols (3.5–5.0 mg of protein/ml) derived from the following sources: (i) doxycycline-induced HEK293 parental, HEK293-PIKfyveWT, or HEK293-PIKfyveK1831E stable cell lines; (ii) HEK293 cells that were transfected with pEF-Bos-Myc-Sac3, pEGFP-Sac3$^{WT}$, or pEGFP-Sac3$^{D488A}$ cDNA constructs or with empty vectors as specified in the figure legends. The ECV/MVBs formed from early endosomes were then separated by centrifugation (35,000 rpm/60 min) in a discontinuous sucrose gradient (25/8%). Because of their different flotation density, the ECV/MVBs were recovered from the 8% interface, whereas the early endosomes were pelleted. ECV/MVBs were finally sedimented by centrifugation at 100,000 × g for 30 min. HRP activity was measured in both the ECV/MVB and early endosome fractions using 1-Step Ultra TMB-enzyme-linked immunosorbent assay (Pierce), following the manufacturer’s protocol. HRP activity in the ECV/MVB fraction was then calculated as a percent of that determined in early endosomes. Where indicated, values were normalized as a percentage of the corresponding controls.

**Others**—Protein concentration was determined by bicinchoninic protein assay kit (Pierce). Protein levels were quantified from the intensity of the bands by a laser scanner (Microteck) and UN-SCAN-IT software (Silk Scientific). Several films of different exposure times were quantified to ensure the signals were within the linear range. Statistical analysis was performed by Student’s t test with p < 0.05 considered as significant.

**RESULTS**

**The Evolutionarily Conserved Sac3 Protein, Detection and Distribution**—The Sac domain, an N-terminal ~400-amino acid region that exhibits phosphatidylinositol polysaccharide phosphatase activity, occurs in proteins separately, as in yeast Sac1p, or in conjunction with a second C-terminally positioned 5-phosphatase-activity domain, as in synaptojanins (53, 54). Three proteins with homology to yeast Sac1p are identified in the mammalian cDNA data bases as follows: Sac1, Sac2, and Sac3 (Fig. 1). rSac1 and hSac2 have been characterized previously (42, 55), but Sac3 awaited characterization. Whereas rSac1 is the mammalian counterpart of yeast Sac1p, hSac2 has no obvious yeast counterparts (42, 55). hSac3, a 3089-bp clone with an estimated open reading frame of 2721 bp encoding a 907-residue protein (GenBank™ accession number NM_014845), displays sequence homology to the 879-residue Sac3 molecule (Fig. 1).

The consensus C$_X$R(T/S) active site, invariant in mammalian Sac domain containing phosphatases, is positioned within the sixth motif with a sequence 486CVCLKDTN. Accordingly, the Sac3$^{D488A}$ mutant, similarly to a corresponding mutation in Fig4 (11, 41), is devoid of phosphatase activity as measured in vitro by the malachite green assay (see below). Unlike the Sac1 proteins, yeast Fig4 and mammalian Sac3 do not display a putative transmembrane sequence, and besides the regions of the Sac phosphatase domain and low complexity, there are no other obvious conserved domains along the Fig4/Sac3 molecule (Fig. 1).

Scansite analysis of the hSac3 protein sequence identifies multiple phosphorylation sites for Ser/Thr or Tyr kinases, including cAMP-dependent protein kinase, the protein kinase
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![Schematic diagram of the Sac domain proteins with a single phosphatase domain. Combined analyses performed by Swiss-Prot, Smart, Prosite, and ScanSite data bases. Accession numbers are as follows: S. cerevisiae (S.c.) Sac1, P32368; Rattus norvegicus (R.n.) Sac1, Q9ES21; Homo sapiens (H.s.) Sac2, Q5W136; S. cerevisiae (S.c.) Fig4, P42837; Arabidopsis thaliana (A.t.) Fig4, Q7XZU3; Homo sapiens (H.s.) Sac3, Q92562. Asterisk, D488A mutation within the sixth motif of the Sac catalytic domain. See text for details.](Image 60x464 to 396x733)

C isoforms, calmodulin-dependent-kinase 2, Akt, platelet-derived growth factor, or insulin receptor tyrosine kinases, indicating a possible regulation by phosphorylation. Analysis by Eukaryotic Linear Motif predicts several putative trafficking motifs displayed by proteins that interact with the endocytic machinery. These include the following: 351DPF and 656F DXXF motifs (Fig. 1), found in Epsin, Eps15, and synaptojanin to bind the α subunit of AP2 (56, 57); a clathrin box (48LVIID) found in AP, GGA, and other endocytic accessory adapter proteins to bind the β-propeller structure of the clathrin heavy chain; several consensus Tyr-based signals that interact with the AP2 μ subunit; and three acidic dileucine sorting signals found in the cytoplasmic portion of receptor proteins, which interact with the GGA adapters to target proteins from the TGN to the endosome/lysosomal system (57). These features suggest a plausible role of Sac3 in endosomal operations.

The electrophoretic mobility of endogenous Sac3 was assessed relative to the mobility of ectopically expressed Myc-hSac3 in HEK293 cells by Western blotting analysis with anti-hSac3 antibodies, generated in rabbits and directed against the hSac3 subunit of AP2 (56, 57); a clathrin box (48LVIID) found in RIPA lysates derived from HEK293 (Fig. 3B). The electrophoretic mobility of endogenous Sac3 was selectively detected by anti-hSac3 in both nontransfected and transfected HEK293 cells (Fig. 2A). This band was positioned just below the Myc-hSac3 band that was detected by both anti-Myc and anti-Sac3 antibodies, consistent with the subtle mobility up-shift of Myc-Sac3 versus endogenous Sac3 (Fig. 2A). The authenticity of the 97-kDa band as endogenous Sac3 in mammalian cells was further confirmed by siRNA-mediated gene silencing. Under equal protein loading, we observed selective depletion (65–70%) of the 97-kDa immuno-reactive protein band in lysates of human HEK293 cells (Fig. 2B, lanes 1 and 2) or mouse 3T3-L1 fibroblasts (Fig. 2C) upon transfection with the corresponding species-specific Sac3 siRNA pools. Concordantly, anti-Sac3 antibodies, but not control IgG, immunoprecipitated a 97-kDa band that was significantly reduced upon siRNA-directed sac3 gene silencing in HEK293 cells (Fig. 2B, lanes 3 and 4). The immunoprecipitation was specific as abundant proteins such as α-tubulin were not detected (Fig. 2B). Together these data demonstrate that the electrophoretic mobility of endogenous Sac3 is in the range of the predicted molecular weight of 103,627 for the full-length protein, thus defining the 97-kDa band as the endogenous Sac3. Of note, close inspection of overexposed Western blots reveals that the Sac3 immunoreactive band often appears as a broad band composed of a closely spaced doublet or triplet, with all forms ablated by cell treatment with Sac3 siRNAs (Fig. 2C). Because the data base information is inconsistent with the presence of alternatively spliced forms, the broad Sac3 band likely indicates post-translational modifications.

Western blotting with anti-hSac3 antibodies detected Sac3 protein expression in all mouse tissues tested, including white fat, skeletal muscle, mammary gland, brain, liver, kidney, heart, lung, and spleen (Fig. 2D). Despite this widespread distribution, however, considerable variations in Sac3 expression levels were noted (up to 10-fold), with highest levels observed in brain fat or lung and the lowest found in heart (Fig. 2D).

Physical Association of Sac3 and ArPIKfyve—In yeast, Fig4 has been recently found to interact directly with Vac14 in two-hybrid and coimmunoprecipitation assays (11, 40). To test whether the mammalian counterparts ArPIKfyve and Sac3 are physically associated, we performed coimmunoprecipitation analysis for both the endogenous and ectopically expressed epitope-tagged proteins using antibodies specific for the two proteins or their epitopes. We have observed unequivocal coimmunoprecipitation of endogenous ArPIKfyve with anti-Sac3 and, vice versa, of endogenous Sac3 with anti-ArPIKfyve from RIPA lysates derived from HEK293 (Fig. 3A). Control proteins of high abundance, including α-tubulin, β- and γ-adaptins, EEA1, and IRAP (Fig. 3A, and not shown), were not coimmunoprecipitated, substantiating the specificity in the Sac3/ArPIKfyve codection under the experimental conditions. Likewise, specific coimmunoprecipitation of Sac3 with ArPIKfyve, and vice versa, was documented in other mammalian cell types, including COS, PC12, and 3T3-L1 fibroblasts (see below). Concordantly, in transiently transfected COS cells...
coexpressing HA-hArPIKfyve and Myc-hSac3, the anti-Myc antibody coimmunoprecipitated HA-ArPIKfyve and, vice versa, the anti-HA antibodies coimmunoprecipitated Myc-Sac3 (Fig. 3B). A control nonimmune serum failed to pull down the overexpressed proteins (Fig. 3B). These data indicate that ArPIKfyve and Sac3 physically associate, like the yeast counterparts Vac14 and Fig4.

**PIKfyve-ArPIKfyve-Sac3 Ternary Complexes**—We have demonstrated previously that PIKfyve and ArPIKfyve physically interact in mammalian cells (38). These data taken

**FIGURE 2. Sac3 phosphatase is a widespread 97-kDa protein.** A, HEK293 cells were transiently transfected with pEF-Bos-Myc-hSac3 cDNA (+) or left untransfected (−) as indicated. RIPA lysates were collected 24 h post-transfection. Equal protein amounts (140 µg) were analyzed by SDS-PAGE and immunoblotting with anti-hSac3 or anti-Myc antibodies, with a stripping step in between. Depicted are the 97-kDa endogenous Sac3 and Myc-Sac3, whose mobility is slightly above the endogenous Sac3 (forced arrowhead). B, HEK293 cells were transfected by Oligofectamine with siRNA duplexes targeting human Sac3 (+) or control cyclophilin B (−). Ninety hours post-transfection, cell lysates were immunoprecipitated with anti-Sac3 antibodies, affinity-purified on a C-terminal GST-Sac3 peptide. Irrelevant antiserum affinity purified on a GST peptide was used as a control (nonimm). Immunoprecipitates (lanes 3–5) together with the input (120 µg; 6.4% of immunoprecipitated lysates) were resolved by SDS-PAGE and immunoblotted with anti-Sac3 antiserum and, following stripping, with anti-α-tubulin monoclonal antibody. Endogenous Sac3 and its selective ablation by siRNAs (lanes 1 versus 2 and lanes 3 versus 4) is depicted by an arrowhead. C, 3T3-L1 fibroblasts were transfected by electroporation with Sac3 (+) or control cyclophilin B siRNA duplexes (−) directed to the mouse sequences. Seventy two hours post-transfection, cell lysates (150 µg protein/lane) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies with a stripping step in between. Arrowheads depict endogenous Sac3 and ArPIKfyve. Sac3 band was incompletely stripped and is still visible in the anti-ArPIKfyve blot (both anti-rabbit antibodies). At the exposures illustrated, the bands for low abundance ArPIKfyve and Sac3 in inputs are not visible and therefore are not shown. To confirm specificity of the coimmunoprecipitation, the blot was further stripped and reprobed with anti-β-adaptin or anti-α-tubulin monoclonal antibodies as indicated, where the duplicate inputs are presented (lanes 4 and 5 in lower panels). D, COS7 cells were transfected with pEF-Bos-Myc-Sac3 cDNA and, 4 h later, cotransfected with pCMV5-HA-ArPIKfyve for an additional 4 h. Equal amounts of cell lysates, collected 24 h post-transfection, were immunoprecipitated with the preimmune serum described in A, anti-Myc monoclonal or anti-HA polyclonal antibodies as indicated. Immunoprecipitates and the input (4.5% of the immunoprecipitated lysate) were resolved by SDS-PAGE and immunoblotted as indicated, with a stripping step in between. Arrowheads depict Myc-Sac3 and HA-ArPIKfyve coimmunoprecipitated with the reciprocal antibody. Given 2% immunoprecipitation efficiencies of both antibodies under the high overexpression levels, we calculate ~30% of each overexpressed protein is engaged in the Myc-Sac3-HA-ArPIKfyve complex under the conditions of the experiment. A and B, shown are chemiluminescence detections of immunoblots from representative experiments out of two to five independent experiments with similar results. WB, Western blot.
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together with our novel observation for a Sac3-ArPKfyve association, documented above, suggest that the three proteins may exist in a common complex. To test this possibility we conducted further coimmunoprecipitation analysis with the endogenous proteins, including now anti-ArPKfyve antibodies in the immunoprecipitation reactions. Examination of fresh RIPA lysates derived from a number of mammalian cell types, such as COS7 cells (Fig. 4A), PC12 cells (Fig. 4B), 3T3-L1 adipocytes (Fig. 4C), and HEK293 (Fig. 4D), documented coimmunoprecipitation of both Sac3 and ArPKfyve with the anti-ArPKfyve antibodies. Accordingly, the reciprocal immunoprecipitation with anti-ArPKfyve or anti-Sac3 antibodies pulled down the other two proteins from freshly prepared lysates of each cell line (Fig. 4, A–D). Importantly, control coimmunoprecipitations with preimmune/nonimmune sera (with or without affinity purification) combined with a Western blotting analysis were negative under each condition (Fig. 4, A–D). Calculations based on at least 15 immunoprecipitation/coimmunoprecipitation experiments in the above-mentioned cell types determined substantial fractions of the three proteins versus total cellular amounts engaged in the ternary complex. Thus, normalizing for immunoprecipitation efficiency for each antibody (50–90% depending on the total protein amount subjected to immunoprecipitation in a given experiment), we have estimated that at least 40% of total Sac3 or ArPKfyve and at least 20% of total PIKfyve are engaged in the complex, with only slight variations depending on the cell type tested. We believe that these numbers might be underestimated because of the presence of detergents (1% Nonidet P-40 and 0.5% deoxycholate) during coimmunoprecipitation assays and washes. Of note, a freezing step prior to immunoprecipitation analyses largely diminished the amounts of the coimmunoprecipitated but not immunoprecipitated PIKfyve, further substantiating the specificity of the codetection. Collectively, these data unequivocally demonstrate that in a cellular context endogenous PIKfyve, ArPIKfyve, and Sac3 assemble in a remarkably stable ternary complex that can sustain the detergents normally present in RIPA buffer.

**Sac3 Displays a PI Phosphatase Activity in Vitro and Controls PtdIns(3,5)P$_2$ in Vivo**—Our findings for a stable assembly of Sac3 phosphatase with the PtdIns(3,5)P$_2$-synthesizing enzyme PIKfyve and its upstream regulator ArPKfyve implicate Sac3 in PtdIns(3,5)P$_2$ turnover. This is also suggested by studies in yeast, which demonstrated an in vitro or in vivo specificity of the Fig4 phosphatase for position 5 in PtdIns(3,5)P$_2$ substrate and a marked up-regulation of PtdIns(3,5)P$_2$ upon expression of Fig4 mutants with substitutions within the Sac3 phosphatase domain (11, 18, 41, 58). To examine whether Sac3, like Fig4, hydrolyzes PtdIns(3,5)P$_2$, we conducted experiments both in vitro and in a cell context. We first explored anti-Myc immunoprecipitates of MycSac3$^{3\text{WT}}$-expressing or control cells and assayed the in vitro activity by malachite green assay with all di-C8 PIs. Myc-Sac3$^{3\text{WT}}$ hydrolyzed all three D5-phosphorylated polyphosphoinositide substrates in the order PtdIns(4,5)P$_2$ > PtdIns(3,5)P$_2$ > PtdIns(3,4,5)P$_3$ but was inactive with monophosphorylated PIs or PtdIns(3,4)P$_2$ (Fig. 5A). To reveal whether the hydrolyzing activity is intrinsic to Sac3$^{3\text{WT}}$, rather than associated, we next performed similar analysis with eGFP-Sac3$^{3\text{WT}}$ versus eGFP-Sac3$^{3\text{DNAK}}$, both proteins expressed in COS cells to equal levels (Fig. 5B). This analysis confirmed the Sac3 specificity for the three D5 polyphosphorylated PI. In this case, however, the highest

**FIGURE 4. Endogenous Sac3, ArPKfyve, and PIKfyve form a ternary complex.** A and B, equal protein amounts of fresh RIPA lysates derived from COS7 cells (A) or PC12 cells (B) were immunoprecipitated with the preimmune serum of the PIKfyve antibody production, anti-Sac3 or anti-ArPKfyve antisera as indicated. Washed immunoprecipitates and the Myc-Sac3 molecular size markers (lane 1 in A and B) were resolved by SDS-PAGE. The membrane was cut at the 110-kDa protein marker and immunoblotted with anti-Sac3 and anti-PIKfyve antisera, or affinity purified anti-ArPKfyve, with a stripping step in between, as indicated. C and D, equal protein amounts of fresh RIPA lysates derived from 3T3-L1 adipocytes (1.7 mg, C) or HEK293 cells (2.0 mg, D) were immunoprecipitated with irrelevant, anti-PIKfyve or anti-ArPKfyve antibodies, all affinity-purified on the corresponding GST peptides as described under "Experimental Procedures." Washed immunoprecipitates and the Myc-Sac3 and HA-PIKfyve molecular size markers (lanes 1 in C and D) or HA-PIKfyve (1.3 mg, lane 2 in D) were resolved by SDS-PAGE. Membranes were cut at the 110-kDa protein marker and immunoblotted with anti-Sac3 and anti-PIKfyve antisera, or affinity-purified anti-ArPKfyve, with a stripping step in between, as indicated. A–D, shown are chemiluminescence detections of immunoblots from representative experiments out of two to four independent experiments with similar results. Immunoprecipitation of the other two proteins (arrowheads) is seen with each of the antibodies used. WB, Western blot.
hydrolyzing activity was observed with the PtdIns(3,5)P2 substrate (3.2-fold), followed by PtdIns(3,4,5)P3 (2.2-fold) and PtdIns(4,5)P2 (1.5-fold) (Fig. 5C), thus attributing at least a portion of the in vitro measured PtdIns(4,5)P2 hydrolysis to phosphatase(s) associated with the Myc-Sac3 immunoprecipitates. Together, the data demonstrate that Sac3 is a D5 polyphosphoinositide phosphatase, and although displaying preferences for PtdIns(3,5)P2, it is capable of hydrolyzing PtdIns(3,4,5)P3 and PtdIns(4,5)P2, at least in vitro.

To assess whether the Sac3 phosphatase displays the ability to turn over PtdIns(3,5)P2 in intact cells, we conducted experiments in two directions. First, we have directly examined the phosphoinositide levels by HPLC inositol headgroup analysis in 32P-labeled HEK293 cells depleted of endogenous Sac3 by siRNA-mediated gene targeting. As demonstrated above, in this cell type this approach yielded ~70% knockdown in Sac3 protein expression (see Fig. 2B). Importantly, under these conditions the [32P]PtdIns(3,5)P2 accumulated levels were slightly (~20%) increased (Fig. 5D). However, if Sac3 was eliminated together with PIKfyve, whose siRNA-mediated depletion resulted in ~90% protein ablation (Fig. 5E), the [32P]PtdIns(3,5)P2 accumulation remained reduced to levels similar to those seen by the PIKfyve ablation alone (Fig. 5D). One explanation of this modest effect on PtdIns(3,5)P2 may lie in our observation that, for reasons that appear to be unspecific (see ‘Discussion’), the siRNA-mediated Sac3 depletion resulting in a concomitant ablation of the ArPIKfyve protein (data not shown). Reduced ArPIKfyve protein expression is associated with decreased synthesis of PtdIns(3,5)P2 from PtdIns(3)P as we have demonstrated previously (38). Therefore, the concomitant ablation of ArPIKfyve may explain, at least in part, why in the absence of Sac3 the steady-state PtdIns(3,5)P2 was only marginally increased or remained unchanged under PIKfyve knockdown. Consistent with this idea, under Sac3 depletion alone we have measured higher, rather than the expected lower, [32P]PtdIns(3)P levels (11, 18), indicative of a perturbed normal PtdIns(3,5)P2 synthesis from PtdIns(3)P along with the blunted PtdIns(3,5)P2 turnover. Noteworthy, under these conditions, no increased accumulation of [32P]PtdIns(4,5)P2 was detected (data not shown), and in fact, there was a trend for a diminution by 6–8% observed in four independent experiments. These data indicate that although Sac3 may hydrolyze PtdIns(4,5)P2 in vitro, such activity is not expressed in intact cells under the conditions of the experiment. As expected for quiescent cells, cellular PtdIns(3,4,5)P2

**FIGURE 5.** Sac3 is a D5 polyphosphoinositide phosphatase in vitro, whose intracellular knockdown elevates [32P]PtdIns(3,5)P2. A, anti-Myc immunoprecipitates (500 μg of protein), derived from Myc-Sac3WT-transfected (++) or nontransfected COS7 cells (--), were washed and analyzed for phosphatase activity with the indicated synthetic di-C8-Pls using a malachite green assay as detailed under “Experimental Procedures.” The absorbance at 660 nm was measured, and the released inorganic phosphate was quantified by a standard curve run in parallel in each experiment. Reactions were carried out in duplicate and are presented as the mean ± S.E. of three independent experiments; * indicates different versus nontransfected controls, p < 0.05. B and C, equal protein amounts of COS7 cells transiently expressing eGFP-Sac3WT or eGFP-Sac3D488A at equal levels revealed by the immunoblot shown in B were immunoprecipitated with anti-GFP. Immunoprecipitates were washed, and the phosphatase activity was tested as described in A. The assay was conducted in triplicate, and the quantitation in C reflects two independent experiments. D, HEK293 cells were transfected with the siRNAs derived from the human sequences of Sac3, PIKfyve ( singly or in combination), or cyclophilin B (control), as indicated. On day 4 post-transfection, cells were labeled with [32P]orthophosphate. Lipids were extracted, deacylated, and coinjected on an HPLC column with 3H-labeled internal or 32P-labeled external HPLC standards as described under “Experimental Procedures.” Fractions were monitored for 3H and 32P radioactivity by an on-line flow scintillation analyzer. 32P radioactivity was plotted, and the counts within the elution times corresponding to the [32P]GroPIns peaks determined by the above 3H/32P-labeled standards were summed (total PI radioactivity). The [32P]PtdIns(3,5)P2 and [32P]PtdIns(3)P were then calculated as a percentage of total PI radioactivity and expressed relatively to the PtdIns(3)P or PtdIns(3,5)P2 values of the control in each experiment (mean ± S.E., four independent experiments). E, silencing of PIKfyve under HEK293 cell transfection with siRNA duplexes targeting PIKfyve or PIKfyve + Sac3, as described in D. Detection is achieved by immunoblotting with anti-PIKfyve antibodies and chemiluminescence. Shown is a typical immunoblot out of four with similar results. WB, Western blot.
Coupled PtdIns(3,5)P₂ Synthesis and Turnover

![Image](eGFP-Sac3WT.png)
![Image](eGFP-Sac3D488A.png)

**FIGURE 6. Sac3 WT but not Sac3 D488A protein expression renders cells susceptible to vacuolation.** HEK293 cells were transfected with pEFGP-Sac3 WT (a and a') or pEFGP-Sac3 D488A (b and b') constructs as indicated. Forty-eight hours post-transfection, cells were treated with NH₄Cl (10 mM) for 40 min at 37 °C and then observed live in a fluorescence microscope (TE200, Nikon) at ×40. Shown are images of live cells from two independent experiments captured by a SPOT RT Slider camera. NH₄Cl treatment induced multiple cytoplasmic vacuoles in cells expressing eGFP-Sac3 WT, seen in >80% of transfected cells but only in <8% of eGFP-Sac3 D488A-expressing cells. Cytoplasmic vacuoles were seen in <3% of nontransfected cells (not shown).

levels were undetectable, precluding conclusions about the Sac3 specificity toward this substrate. Collectively, the data from the HPLC inositol headgroup analysis are consistent with the notion that in a cellular context Sac3 can turn over the steady-state PtdIns(3,5)P₂ levels.

In the second approach, we verified the specificity of the Sac3 phosphatase for PtdIns(3,5)P₂ hydrolysis by taking advantage of the morphological changes in the form of cytoplasmic vacuoles seen upon perturbations in PtdIns(3,5)P₂. This phenomenon was observed previously upon expression of dominant-negative PIKfyve mutants or knockdown of PIKfyve/ArPIKfyve (10, 19–21, 38) and, more recently, in cell systems of the PIKfyve knock-out animal models (15, 16). HEK293 cells ectopically expressing eGFP-hSac3 WT or the phosphatase-deficient eGFP-hSac3 D488A mutant did not display obvious changes in their normal morphology 24–72 h post-transfection (not shown). However, the eGFP-hSac3 WT-, but not the eGFP-hSac3 D488A-expressing cells, were highly susceptible for developing cytoplasmic vacuoles upon short treatment with low concentrations of weak bases (NH₄Cl, 10 mM; 40 min; Fig. 6). Consistent with previous data (38), these mild conditions of NH₄Cl treatment were ineffective in inducing phenotypic changes in control HEK293 cells. These data indicate that overexpression of eGFP-hSac3 WT renders cells prone to developing a dilated endomembrane phenotype seen typically upon manipulations that perturb normal PtdIns(3,5)P₂ production. Clearly, combined biochemical and morphological data are consistent with the notion that in a mammalian cell context, Sac3 turns over PtdIns(3,5)P₂.

**Sac3 Cofractionates and Colocalizes with PIKfyve and ArPIKfyve**—The observation that Sac3 forms a ternary complex with PIKfyve and ArPIKfyve predicts that the three proteins will colocalize. We have previously demonstrated that significant subpopulations (40–50%) of ArPIKfyve and PIKfyve reside on membranes (38, 49). Likewise, fractionation of HEK293 cells to total membranes and cytosol found about one-half of total Sac3 in a membrane-associated form (data not shown). To obtain more detailed information about the distribution of membrane-bound Sac3 relative to ArPIKfyve and PIKfyve, we used an equilibrium density gradient sedimentation of membranes isolated from HEK293-PIKfyve WT stable cells. Our studies in this cell line conducted previously and in this study documented a strong cofractionation of PIKfyve WT and ArPIKfyve in the denser part of the gradient, where protein markers of the cellular cytoskeleton (α-tubulin), ER (GRP94), or TGN elements (γ-adaptin) are predominantly detected (38 and Fig. 7A). Endosomal proteins such as IRAP, transferrin receptor, and Rab4 were recovered predominantly in the top lighter fractions, but small amounts could also be found in the denser fractions where PIKfyve/ArPIKfyve were detected (Fig. 7A). Importantly, examination of the gradient by immunoblotting with anti-Sac3 antibodies detected the membrane-bound Sac3 exclusively in the PIKfyve/ArPIKfyve-containing fractions (Fig. 7A). These data, combined with the fact that PIKfyve-ArPIKfyve-Sac3 complexes were detected in both the cytosolic and the solubilized membrane fraction (data not shown), indicate that a subpopulation of the PIKfyve/ArPIKfyve-Sac3 ternary complex is associated with membranes.

This point was further elaborated by confocal microscopy in COS7 cells ectopically expressing Myc-hSac3 WT together with pEGFP-HA-hVac14 WT or pEGFP-HA-mPIKfyve WT. It should be noted that the in situ detection of endogenous Sac3 or ArPIKfyve in cells is currently precluded because of relatively low protein levels and inadequate antibodies for immunofluorescence microscopy. However, at least in the case of PIKfyve, the localization of ectopically expressed PIKfyve WT likely reflects that of the endogenous protein, as we have concluded previously based on data obtained in 3T3-L1 adipocytes, where the endogenous PIKfyve was successfully detected (46). Immunofluorescence microscopy with anti-Myc antibody revealed that the majority of COS7 cells expressing Myc-hSac3 alone displayed diffuse and perinuclear staining (80–85%). However, ∼15% of the Myc-hSac3-expressing COS7 cells exhibited a clear-cut vesicular pattern. Importantly, when coexpressed with eGFP-PIKfyve or eGFP-ArPIKfyve, the percentage of cells with a Myc-Sac3 vesicle appearance increased to 30–35% of the cotransfected cells. There was a considerable colocalization (>80%) between the Myc–Sac3 vesicles and the eGFP-PIKfyve or eGFP-ArPIKfyve-positive vesicles (Fig. 7B). These data indicate that ArPIKfyve, PIKfyve, or both may facilitate Sac3 localization to membranes. Intriguingly, Myc-Sac3/eGFP-PIKfyve- or Myc-Sac3/eGFP-ArPIKfyve-positive vesicles appeared significantly enlarged as compared with the fine puncta seen typically upon expression of the eGFP-PIKfyve protein alone (Fig. 7B) (10, 19, 21). These data are consistent with the notion that Sac3 localizes onto ArPIKfyve/PIKfyve sites and induces vesicle enlargement because of increased rate of PtdIns(3,5)P₂ turnover.

The identity of the Sac3-positive vesicles was addressed by immunostaining the eGFP-hSac3-transfected COS7 cells for...
endogenous EEA1, a marker for early endosomes (4). As illustrated in Fig. 7C, almost all eGFP-Sac3-marked vesicles were positive for EEA1 as seen on the merged images. The fraction of the EEA1-positive vesicles that overlapped with the eGFP-Sac3 WT-positive vesicles was ~30%. Intriguingly, close inspection of the images revealed that the EEA1 endosomes positive for the eGFP-Sac3 signals were considerably enlarged compared with the eGFP-Sac3-negative endosomes seen in the same cell or in the neighboring nontransfected cells (Fig. 7C). Together, these data indicate that ectopically expressed Sac3 WT, much like the dominant-negative kinase-deficient PIKfyve K1831E mutant but unlike PIKfyve WT and Sac3 D488A (21 and data not shown), resides on a subpopulation of EEA1-marked early endosomes where it elicits a vesicle enlargement.

**Effect of Sac3, PIKfyve, and ArPIKfyve in ECV/MVB Formation/Detachment in Vitro**—As demonstrated above with ectopically expressed Sac3 WT, and in our previous studies with the kinase-deficient PIKfyve K1831E mutant (21), increased turnover rate or perturbed normal synthesis of PtdIns(3,5)P2 is associated with an enlargement of the early endosome membranes. A potential cellular mechanism that could explain the gain of endosome membranes is a defect in the mechanism that controls membrane traffic progression from early endosomes to later compartments in the degradation pathway and/or retrograde transport to the TGN. Although it is not exactly clear whether the transport step(s) are achieved by means of ECV/MVB intermediates that form/detach from early endosomes, early endosome maturation, or both, the currently existing

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**FIGURE 7. Sac3 cofractionates and colocalizes with PIKfyve and ArPIKfyve.** A, HEK293 cell line stably expressing PIKfyve WT was fractionated into total membranes and cytosol. The membrane fraction was subjected to equilibrium sedimentation in 30% iodixanol, as described under “Experimental Procedures.” Fractions were collected and analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Shown are chemiluminescence detections of blots from a representative fractionation out of three independent fractionations with similar results. B, COS7 cells were cotransfected with pEF-Bos-Myc-Sac3 WT and either pEGFP-HA-ArPIKfyve WT or pEGFP-HA-PIKfyve WT. Twenty four hours post-transfection cells were fixed in formaldehyde (4%) and permeabilized (Trion X-100, 0.5%). Expression of Myc-Sac3 was detected with anti-Myc monoclonal antibody and Alexa568-conjugated anti-mouse IgG. Expression of eGFP-PIKfyve and eGFP-ArPIKfyve was visualized by the GFP fluorescence. Bar, 10 μm.

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reconstitution assay, known as in vitro ECV/MVB biogenesis, quantifies the transport intermediates from donor early endosomes irrespective of their formation mode. The assay is well established and applied in numerous studies (35, 51, 52). It reconstitutes the biogenesis of ECV/MVB transport intermediates from HRP-labeled donor early endosome membranes in the presence of cytosol and an ATP regeneration system. Therefore, we examined whether alterations in PtdIns(3,5)P₂ levels, achieved by modulating the protein levels of Sac3, PIKfyve, and ArPIKfyve or by dominantly interfering with the PIKfyve kinase activity, affect the normal biogenesis of ECV/MVB transport intermediates on early endosomes.

We first assessed the effect of cytosols isolated from the HEK293 stable cell line inducibly expressing the dominantly-negative kinase-deficient PIKfyveK1831E mutant in the in vitro ECV/MVB assay. Expression of this mutant exerts a powerful dominant-negative effect and results in substantially reduced PtdIns(3,5)P₂ levels on membranes (10, 19, 21). As illustrated in Fig. 8A, the presence of cytosol derived from control HEK293 cells readily supported the formation of ECV/MVB. A slight increase was observed with cytosols from a HEK293 cell line stably expressing PIKfyveWT (Fig. 8A). By contrast, the presence of cytosols derived from a HEK293-PIKfyveK1831E-expressing cell line completely abolished the ECV/MVB formation, as judged by measuring only a background HRP activity (Fig. 8A). These data, taken together with the reduced amounts of PtdIns(3,5)P₂ on membranes of the HEK293-PIKfyveK1831E stable cell line (21), are consistent with the idea that PtdIns(3,5)P₂ is central to the cellular mechanisms that control the formation/detachment (or maturation) of ECV/MVB transport intermediates on early endosomes.

To further validate this conclusion, we next examined the potency of HEK293 cytosols depleted or enriched in Sac3, PIKfyve, or ArPIKfyve in the ECV/MVB reconstitution assay. Protein depletion was achieved by siRNA-mediated knockdown in HEK293 cells or cytosol immunoabsorption on affinity beads, whereas protein enrichment was produced by ectopic transfection with cDNAs or addition of purified recombinant proteins. The data are summarized in Fig. 8B and are presented as a percentage normalized to the corresponding control values of each condition, as specified in the figure legends. HEK293 cytosols with ArPIKfyve or PIKfyve proteins reduced by 80~90% (Fig. 5E) (Fig. 1C in Ref. 38) markedly suppressed the ECV/MVB formation versus control cytosols (Fig. 8B). This effect was highly specific because the purified His₆-PIKfyve protein, but not His₆-GDI2 (47), added to the ArPIKfyve-depleted cytosol rescued the ECV/MVB formation (Fig. 8B). The specificity of the effect was further substantiated by documenting a similar arrest in the ECV/MVB formation if the PIKfyve protein was depleted by immunoabsorption of HEK293 cytosols on anti-PIKfyve antibodies (Fig. 8B). By contrast, the siRNA-mediated loss of the Sac3 phosphatase (~70% decrease, Fig. 2B) produced a gain of the ECV/MVB formation (Fig. 8B). The specific requirement for Sac3 in the ECV/MVB biogenesis was further validated by documenting decreased HRP activity if cytosols from Myc- or GFP-Sac₃WT-expressing HEK293 cells were added to the ECV/MVB formation assay (Fig. 8B).

**DISCUSSION**

In this study we have biochemically and functionally characterized Sac3, an evolutionarily conserved Sac domain-containing phosphatase that is structurally related to the yeast...
PtdIns(3,5)P_2-specific phosphatase Fig4. Immunoreactive Sac3 migrates with an electrophoretic mobility of 97 kDa and is expressed at various levels in all mammalian cells and tissues tested. Endogenous Sac3 forms a stable ternary complex with the PtdIns(3,5)P_2-producing enzyme PIKfyve and its activator ArPIKfyve in a number of mammalian cell types. Sac3^{WT} displays an intrinsic phosphatase activity in vitro with specificity for D5-phosphorylated PI and preferences for PtdIns(3,5)P_2. Depletion of endogenous Sac3 increased PtdIns(3,5)P_2 steady-state levels, whereas ectopic expression of Sac3^{WT} enlarged early endocytic structures and rendered cells susceptible to formation of cytoplasmic vacuoles, similar to those seen upon perturbation of PtdIns(3,5)P_2 by dominant-negative kinase-deficient PIKfyve^{K1831E}. These data implicate the Sac3 phosphatase in PtdIns(3,5)P_2 turnover in mammalian cells and uncover a mechanism whereby the tight control of PtdIns(3,5)P_2, homeostasis is coordinated through a physical association of the core enzymes, PIKfyve and Sac3 executing PtdIns(3,5)P_2 synthesis and turnover. Using an in vitro reconstitution assay, we demonstrate a central function for each component of the core protein machinery for PtdIns(3,5)P_2 synthesis and turnover in the formation/detachment (or maturation) of transport vesicle intermediates from early endosomes.

One intriguing yet not unexpected observation in our study was the modest elevation of PtdIns(3,5)P_2 under depletion of endogenous Sac3 phosphatase. Moreover, this effect was manifested on the steady-state PtdIns(3,5)P_2 levels, but not those reduced by PIKfyve protein knockdown (Fig. 5D). These data raise the question as to the extent to which Sac3 antagonizes PIKfyve activity. Although the exact answer is currently unknown, it seems likely that other phosphatases turn over PtdIns(3,5)P_2, either as a normal or a compensatory mechanism under Sac3 loss. This notion is supported by findings in yeast where strikingly higher PtdIns(3,5)P_2 has been observed only by the combined loss of Fig4 and two synaptojanin-like Sac phosphatases Sjl2 and Sjl3, whereas the singly eliminated Fig4 results in only a 20% increase (18). Mammalian phosphatases of the myotubulin and synaptojanin families are found to hydrolyze PtdIns(3,5)P_2 (53, 54, 59–61), and thus it remains to be identified whether they act in conjunction with Sac3 in antagonizing PIKfyve action in a cell context.

One issue that was enlightened by our work here was whether or not the individual depletion of PIKfyve, ArPIKfyve, or Sac3 affects the expression levels of the remaining two proteins and, if so, whether the protein off-target effect is specific or due to the siRNA nature and/or delivery mode (62). We concluded that the observed ArPIKfyve reduction upon Sac3 knockdown in HEK293 cells (Fig. 5D) was inconsistent with a plausible specific effect because, first, this was manifested by lipid-based siRNA delivery but not by electroporation and, second, such changes were not reproduced in electroporated mouse 3T3-L1 adipocytes (data not shown) that received a different siRNA pool to knock down the mouse sequences. By contrast, we found a consistent off-target reduction in Sac3 protein levels upon ArPIKfyve knockdown with both mouse or human siRNA pools under either lipid-based or electroporation-based methods of siRNA delivery (data not shown). Thus, combined data from mouse 3T3-L1 adipocytes and human HEK293 cells are consistent with the notion that the off-target effect on Sac3 expression levels upon ArPIKfyve ablation is specific rather than related to the delivery method and/or the nature of the siRNAs. It is worth noting that, for reasons still not completely understood, yeast mutants with vac14 deletion show significantly lower levels of Fig4 (58). Whether and how ArPIKfyve controls Sac3 protein expression and/or stability in yeast and mammals is important objectives in future studies.

Sac3 localization appears to be dependent, at least in part, on ArPIKfyve and PIKfyve, which likely localize the phosphatase to sites of PtdIns(3,5)P_2 production. This is supported by the morphological data of pairwise ectopic expression, where the number of Sac3^{WT}-positive cells displaying a vesicular pattern increased above 2-fold in the background of coexpressed PIKfyve^{WT} or ArPIKfyve^{WT}. Noteworthy, the Sac3^{WT}/PIKfyve^{WT} or Sac3^{WT}/ArPIKfyve^{WT}-positive vesicles appeared substantially enlarged versus vesicles of singly expressed PIKfyve^{WT} (Fig. 7B). This effect was strikingly pronounced if Sac3^{WT} was expressed from the pEF-Bos vector that carries a powerful promoter derived from the transcription factor EF-1α gene (42). As endosome enlargement is a hallmark of perturbed PtdIns(3,5)P_2 production (10, 16, 19–22, 38), these data further substantiate our conclusion for the role of Sac3 in PtdIns(3,5)P_2 turnover. Of note, endosome vesicle dilation was more pronounced in the background of ArPIKfyve^{WT} versus PIKfyve^{WT} coexpression (Fig. 7B). This observation is consistent with the PIKfyve activity partially antagonizing the Sac3 action by increasing the rate of PtdIns(3,5)P_2 synthesis. Also, although the mechanism is unknown, a dual role of yeast Vac14 in upregulating both Fab1 and Fig4 has recently been suggested (58).

In light of these findings, the more pronounced vesicle dilation upon Sac3^{WT}/ArPIKfyve^{WT} coexpression in COS cells as observed here may reflect this regulatory mechanism and require further investigation.

Our previous studies with the dominant-negative kinase-deficient PIKfyve^{K1831E} mutant revealed that normal PtdIns(3)P-to-PtdIns(3,5)P_2 conversion is required in several endosome-related events (10, 19, 21, 22, 63). Thus, expression of PIKfyve^{K1831E} resulted in early endosome enlargement, high colocalization with early endosome markers, vacuole formation, accelerated rate of endosome fusion, and reduced number of intraluminal vesicles in MVB-like structures, with changes largely depending on the duration of expression (10, 21). Noteworthy, some of these changes are remarkably similar to those recently observed by PIKfyve protein depletion (20) and reduced PtdIns(3)P-to-PtdIns(3,5)P_2 conversion that is now measured in our study (Fig. 5D). Therefore, elevated expression of the Sac3 phosphatase, which should decrease PtdIns(3,5)P_2 in favor of a PtdIns(3)P increase, is expected to resemble the endosome defects seen by PIKfyve^{K1831E} expression. Our observation for enlarged EEA1-positive early endosomes in the background of Sac3^{WT} expression is consistent with this prediction (Fig. 7C). Because EEA1 is a fusogenic protein that binds PtdIns(3)P (4, 64, 65), the endosome enlargement in Sac3^{WT}-expressing cells may reflect, at least in part, the increased endosome fusion due to EEA1 recruitment onto elevated PtdIns(3)P. Although the early endosome EEA1 was not directly quantified
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under these conditions, the striking increase in the intensity of the EEA1-positive endosomes upon Sac3 WT expression (Fig. 7C) supports this notion.

Recent studies in higher eukaryotes (15, 16, 20) together with our observations about the effects of PIKfyve on the performance of trafficking pathways (10, 19, 21, 22, 49, 63) provide a basis for definitive conclusions. Thus, there is a consensus that perturbed PtdIns(3,5)P₂ (by dominant-negative PIKfyve K1831E or PIKfyve protein depletion) in mammalian cells does not affect to a significant degree the receptor internalization, recycling, trafficking to lysosomes and degradation, or cargo sorting in the biosynthetic pathway (20, 22). By and large, similar conclusions emerge from cell studies in D. melanogaster and C. elegans PIKfyve mutants, except that receptor degradation in the fruit fly shows a greater sensitivity to the PIKfyve WT reduction (15, 16). Perturbed PtdIns(3,5)P₂ synthesis, however, delays the fluid-phase transport to lysosomes at a post-early endosomal step in both mammalian and fruit fly Garland cells (16, 22).

In addition, in mammalian cells PIKfyve protein depletion was found to impair the early endosome-to-TGN traffic and proper endosome processing of GLUT4-containing vesicles in response to insulin (20, 49, 63). These data, together with the unconditional endosome processing of GLUT4-containing vesicles in response to insulin (20, 49, 63) provide a basis for definitive conclusions. Thus, there is a consensus that perturbed PtdIns(3,5)P₂ (by dominant-negative PIKfyve K1831E or PIKfyve protein depletion) in mammalian cells does not affect to a significant degree the receptor internalization, recycling, trafficking to lysosomes and degradation, or cargo sorting in the biosynthetic pathway (20, 22). By and large, similar conclusions emerge from cell studies in D. melanogaster and C. elegans PIKfyve mutants, except that receptor degradation in the fruit fly shows a greater sensitivity to the PIKfyve WT reduction (15, 16). Perturbed PtdIns(3,5)P₂ synthesis, however, delays the fluid-phase transport to lysosomes at a post-early endosomal step in both mammalian and fruit fly Garland cells (16, 22).

In conclusion, the biochemical, morphological, and functional characterization of Sac3, presented herein, indicates that Sac3 is a PtdIns(3,5)P₂ phosphatase physically interacting with the PtdIns(3,5)P₂-synthesizing complex PIKfyve-ArPIKfyve. Thus, our data uncover a core machinery for PtdIns(3,5)P₂ synthesis and turnover, whose protein components, assembled in a stable complex, are targeted to the endosomal system. Fission and fusion events in the endosomal network seem to correlate with PtdIns(3,5)P₂ synthesis and turnover, respectively, thus mechanistically coupling PIKfyve type-Sac3 action and the dynamic changes in PtdIns(3,5)P₂ with early endosome dynamics in the course of cargo transport.

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