PIKfyve, a kinase that displays specificity for phosphatidylinositol (PtdIns), PtdIns 3-phosphate (3-P), and proteins, is important in multivesicular body/late endocytic function. Enzymatically inactive PIKfyve mutants elicit enormous dilation of late endocytic structures, suggesting a role for PIKfyve in endosome-to-trans-Golgi network (TGN) membrane retrieval. Here we report that p40, a Rab9 effector reported previously to bind Rab9-GTP and stimulate endosome-to-TGN transport, interacts with PIKfyve as determined by yeast two-hybrid assays, glutathione S-transferase (GST) pull-down assays, and co-immunoprecipitation in doubly transfected HEK293 cells. The interaction engages the PIKfyve chaperonin domain and four out of the six C-terminally positioned kelch repeats in p40. Differential centrifugation in a HEK293 cell line, stably expressing PIKfyve WT, showed the membrane-associated immunoreactive p40 co-sedimenting with PIKfyve in the high speed pellet (HSP) fraction. Remarkably, similar analysis in a HEK293 cell line stably expressing dominant-negative kinase-deficient PIKfyve K1831E demonstrated a marked depletion of p40 from the HSP fraction. GST-p40 failed to specifically associate with the PIKfyve lipid products PtdIns 5-P and PtdIns 3,5-P2 in a liposome binding assay but was found to be an in vitro substrate of the PIKfyve serine kinase activity. A band with the p40 electrophoretic mobility was found to react with a phosphoserine-specific antibody mainly in the PIKfyve WT-containing fractions obtained by density gradient sedimentation of total membranes from PIKfyve WT-expressing HEK293 cells. Together these results identify the Rab9 effector p40 as a PIKfyve partner and suggest that p40-PIKfyve interaction and the subsequent PIKfyve-catalyzed p40 phosphorylation anchor p40 to discrete membranes facilitating late endosome-to-TGN transport.

Multivesicular bodies (MVBs) (referred to also as prelysosomal compartment or late endosomes) are morphologically defined organelles that are a crossing point of several membrane trafficking pathways (reviewed in Refs. 1–8). Cell surface endocytosed materials destined for degradation enter MVBs en route to lysosomes. Next, newly synthesized acid hydrolases that function in lysosomes are diverted from the secretory pathway through binding to one of the two mannose 6-phosphate-receptor (MPR) types, and reach MVBs by the biosynthetic pathway. Finally, several transport routes that deliver cargo to the trans-Golgi network emanate from MVBs. To dispatch cargo in the correct direction, MVBs ought to perform numerous sorting functions, the molecular mechanisms of which are just beginning to be unraveled. PIKfyve enzyme, a large protein of 2052 amino acids, is one of the multiple molecules thought important in MVB morphogenesis and function (9). Encoded by a single-copy evolutionarily conserved gene on locus 2q34 in humans, mammalian PIKfyve is responsible for intracellular PtdIns 5-P and PtdIns 3,5-P2 biosynthesis (reviewed in Ref. 10). PIKfyve could also act as a protein kinase, but the physiologically relevant substrates are currently unknown (11). The enzyme is primarily cytosolic with ~30% associated with membranes (12). Immuno- and confocal fluorescence microscopy data co-localize a subset of membrane-bound PIKfyve with late-endosome markers consistent with a plausible role of PIKfyve in the function of this compartment. The first indication for such a role came from cell studies utilizing PIKfyve point mutants deficient in the lipid kinase activity (13, 14). Expression of these mutants in different cell types was found to induce a dramatic dominant-negative effect in the form of a progressive accumulation of dilated cytoplasmic vacuoles of endocytic origin indicating the role of PIKfyve in maintaining endosome membrane homeostasis. Recent ultrastructural studies identify the swollen compartment as MVBs, which, in addition to a significant gain of limiting membranes, display a lower number of internal vesicles (9). Ectopic expression of active PIKfyve at higher levels or cytoplasmic microinjection of the PIKfyve lipid product, PtdIns 3,5-P2, restores the normal cell morphology (13, 14). Together, these results specify the important role of PIKfyve PtdIns 3,5-P2-generating activity and yet-to-be identified PtdIns 3,5-P2 downstream effectors in MVB biogenesis and function.

Besides the C-terminally positioned catalytic domain, the PIKfyve molecule harbors three other evolutionarily conserved modules: a FYVE finger that interacts specifically with PtdIns 3-P, a DEP domain, found in other proteins important in membrane association, and a chaperonin domain, homologous to the TCP-1 complex that associates with and facilitates the folding of 6-phosphate receptor; HSP, high speed pellet; LSP, low speed pellet; IRAP, insulin-regulated aminopeptidase; nt, nucleotide(s); CMV, cytomegalovirus; HA, hemagglutinin.
DNA sequencing. (0.76 kbp) of the above vector was released and subcloned into the 3X. After confirmation of the correct orientation, the BamHI fragment anti-IRAP, a gift by Paul Pilch.

First, the N-terminal DNA fragment (from the ATG initiation codon up to the HindIII site) was obtained by PCR using BglII-flanked sense primers. The primer sequence (5'-CGGGAGATCTGCATTCTGAGAAACATC-3') and antisense 5'-CAGCTCCGTAGTTAGCTCCACTAC-3' with the restriction sites underlined. The PCR product was digested with HindIII, and the resulting two fragments of 180 bp (nt 150–329 of pEGFP-p40 cDNA) and 986 bp (nt 1298–1397) were subsequently cut with EcoRI and Xhol, respectively, and ligated into the EcoRI/Xhol digest of pEGFP-C2 (Clontech) using pQE31-p40 DNA as a template. The PCR product (~180 bp) was digested with BglII/HindIII and then ligated together with the HindIII fragment of pQE31-p40 (~1 kbp) into BlgII/HindIII-digested pEGFP-C2. The PCR-amplified sequence and the correct orientation of the construct were verified by sequencing and restriction endonuclease mapping.

Full-length p40 fused in-frame with GST was generated by first amplifying the coding sequence of p40 by PCR using primers with added EcoRI or Xhol restriction sites: sense 5'-CGGGGAATTCATGAAGCAAC-3' and antisense 5'-CAGCTCCGTAGTTAGCTCCACTAC-3' with the restriction sites underlined. The PCR product was digested with HindIII, and the resulting two fragments of 180 bp (nt 150–329 of pEGFP-p40 cDNA) and 986 bp (nt 1298–1397) were subsequently cut with EcoRI and Xhol, respectively, and ligated into the EcoRI/Xhol digest of pEGFP-C2 (Amersham Biosciences). Two C-terminal GST fusion peptides, residues 133–372, and 264–372, were also generated in pGEX5X-1 vector. For the first C-terminal peptide, the EcoRI/Xhol fragment (comprising nt 546–1297) derived from clone YA19 (isolated in the two-hybrid screen here, see below) was inserted into the corresponding digest of pGEX5X-1. For the second one, the Pest/Sll fragment (comprising nt 941–1297) from pEGFP-p40 was subcloned initially into the corresponding digest of pBluescript II SK+ and then released by Smal/Xhol digest for subcloning into Smal/Xhol sites of pGEX5X-1. An N-terminal GST fusion peptide of p40 (residues 1–264) was amplified by first subcloning the BglII/PstI fragment of pEGFP-p40 (nt 150–941) into pCMV5. The EcoRI/Sall fragment of the pCMV5-p40 construct was then ligated into EcoRI/Sall digest of pGEX5T-2 (Amersham Biosciences). The organization of the constructs was confirmed by restriction endonuclease digest and sequencing.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Human embryonic kidney (HEK) 293 or COS-7 cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, and antibiotics as described previously (13).

**Antibodies**—Anti-PIKfyve antibodies (R7069) were characterized earlier (19, 20). Monoclonal anti-GFP and anti-phospho-specific (IC8) antibodies were from Clontech and Calbiochem, respectively. The polyclonal antibodies were from the following sources: anti-HA and anti-GST, gifts from Mike Czech; anti-p40, a gift from Susan Pfeffer; and anti-IRAP, a gift from Paul Pilch.

**Plasmids for Yeast Two-hybrid System**—Three baits encompassing the evolutionarily conserved domains of the PIKfyve protein were inserted in-frame with the LexA into pNLexNLS vector for yeast two-hybrid screening. The first one constituted the FYVE finger plus the DEP domain. The second construct encompassed the FYVE finger plus the DEP plus the N-terminal kinase-deficient PIKfyve K1831E mutant (clone 5), or the latter two domains serve to interact with Rab9, a GTPase localized on late endosomes and facilitating late endosome-to-Golgi transport of MPR (16). Through selective binding to the GTP-loaded Rab9, p40 promotes the in vitro transport of MPR from late endosomes to TGN (16).

Because anti-p40 antibodies, but not the p40-depleted cytosols, inhibit this transport step, Pfeffer and collaborators (16) suggest the membrane-associates p40 is the active species that, together with the active Rab9, functions in the return of MPR to TGN. Here we demonstrate that PIKfyve chaperonin domain associates with p40 and that p40 membrane association is strictly dependent on PIKfyve enzymatic activity. Because p40 was found to be an in vitro substrate for the PIKfyve protein kinase activity, we speculate that p40-PIKfyve interaction and the subsequent PIKfyve-catalyzed p40 phosphorylation anchors p40 to discrete membranes facilitating late endosome-to-TGN transport.

**GST Protein Production and Purification**—All GST fusion proteins were produced in transformed XA-90 Escherichia coli strain. Bacteria were grown for 1.5 h and then stimulated with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for an additional 6 h. The cells were lysed with 1 mg/ml lysozyme in a buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were treated with DNase (0.1 mg/ml) in the presence of 10 mM MgCl₂. Purification of the GST fusion proteins was achieved on GSH-agarose beads (Sigma) as described previously (15). In some experiments, the GST proteins were eluted from the beads and dialyzed against 25 mM Hepes, pH 7.4. The concentrations of the purified proteins, bound to or eluted from the beads, were determined electrophoretically by the intensity of the Coomassie Blue-stained protein bands versus bovine serum albumin standard (Pierce).

**Transient Co-transfection and Subcellular Fractionation**—HEK293 cell stable lines inducibly expressing PIKfyve of PIKfyve-K1831E were made by transfecting 750,000 cells/100-mm plates with 10 μg of co-transfection plasmids containing pEGFP-p40 DNA using Lipofectamine as a transfection reagent. 24–40 h post-transfection, cells were rinsed in homogenization "HES + buffer" (20 mM Heps, pH 7.5, 1 mM EDTA, 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/methionine). The cells were homogenized by 5–10 strokes of a 25-gauge needle in 50 μl of the above buffer and then resuspended in the same buffer at 4 °C. The cell pellets were then homogenized in a motor-driven Teflon/glass homogenizer with 15 strokes at 1700 rpm. Homogenates were subjected to subcellu-
lar fraction following published protocols established for 3T3-L1 adipocytes (12) with modifications. Briefly, homogenates were first centrifuged at 9,000 × g for 20 min at 4 °C (Sorval Instrument Division, SS-34 rotor) to obtain the low speed pellet (LSP). The supernatant was then centrifuged in a TLA 100.3 rotor (Beckman Instruments Inc.) at 4 °C for 24 min at 200,000 × g to separate the high speed pellet (HSP) from the fraction of the soluble proteins (referred to herein as cytosol).

Proteins were reconstituted in “BESS++ buffer” to a protein concentration of ~2 mg/ml (bicinchoninic protein assay kit, Pierce, Rockford, IL). Fractions were analyzed by immunoblotting with the indicated antibodies. Cytosolic fractions were subjected to immunoprecipitation with anti-HA or anti-PIKfyve antibodies as described below.

In some experiments cultured cells were fractionated into total membrane pellets, equal volumes of these fractions were homogenized as above, and then fractionated by two sequential centrifugations at 800 × g for 5 min to eliminate the nuclear pellet and then at 200,000 × g for 20 min in a TLA 100.3 rotor, to obtain the total membrane and cytosolic fractions. Total membrane pellets were resuspended as above, and the fractions were analyzed by immunoblotting or immunoprecipitation.

Equilibrium Centrifugation in Self-formed Inositol Gradient—A total membrane fraction prepared from HEK293 cell stable lines inducibly expressing PIKfyveWT and reconstituted in HES (Hepes, 10 mM and 50 mM NaF) and PtdIns 5-P-enriched or PtdIns 3,5-P2-enriched liposomes. GST protein (5 μg) was used as a control.

RESULTS

Identification of p40 as a PIKfyve-binding Protein by Yeast Two-hybrid Interaction—To identify proteins that interact with PIKfyve, we constructed the chaperonin domain (residues 616–868) and DEP domain (residues 384–445) of PIKfyve in pNLexNLS vector for screening a HeLa cell cDNA library. Initial tests, however, revealed that the DEP domain construct produced a high background. A similar high background was seen with a DEP domain bait expanded with some upstream sequence encompassing the N-terminally positioned FYVE finger (residues 99–473). Therefore, both baits containing the DEP domain were not used for further screening. Screening of 10^6 cDNA library transformants by the mating version of the yeast two-hybrid system (21) with the chaperonin-like domain as a bait resulted in 27 clones as potential interactors. Four of these clones encoded the p40 transport factor. The remaining clones encoded three other proteins, which are under characterization and will be described elsewhere.

Pull-down and Immunoprecipitation Experiments Confirm PIKfyve-p40 Interaction—All the p40 clones isolated from our screening lacked the initial N-terminal part of the full-length molecule. The latter was generated by PCR amplification of the N-terminal amino acid segment using the full-length human p40 (16) as a template as described under “Experimental Procedures.” To confirm the interaction of p40 with PIKfyve, a GST-conjugated peptide corresponding to full-length human p40 was used in pull-down experiments. Western blot analysis, illustrated in Fig. 1A, reveals that a substantial fraction of the immunoreactive PIKfyveWT binds to GST-p40 following incubation of bacterially produced purified GST-p40 with cytosols derived from COS cells infected with adenovirus encoding HA-PIKfyveWT. Control pull-down experiments with GST protein immobilized on GSH beads alone showed undetectable immunoreactive bands with electrophoretic properties of HA-PIKfyve (Fig. 1A). The interaction of PIKfyveWT with p40 was further verified by co-immunoprecipitation experiments in a HEK293 stable cell line inducibly expressing HA-PIKfyveWT and transiently co-transfected with pEFGP-p40 cDNA (Fig. 1B). Western blot analysis with anti-GFP antibodies detected a small fraction of GFP-p40 only in the HA-immunoprecipitates of doxycycline-induced PIKfyveWT-expressing cells but not in the anti-HA-immunoprecipitates derived from non-induced HEK293 cells that did not express HA-PIKfyve (Fig. 1B). Furthermore, to confirm that p40-PIKfyve interaction proceeds via the PIKfyve chaperonin domain, a PIKfyve chaperonin-deletion mutant was transiently expressed in COS-7 cells and the isolated cytosol used in pull-down experiments. As illustrated in Fig. 2, Western blot analysis failed to immunodetect HA-PIKfyveCh following incubation of purified GST-p40 protein with cytosols from HA-PIKfyveCh-transfected cells. In contrast, a parallel incubation of GST-p40 affinity beads with cytosols derived from COS-7 cells transiently transfected with HA-PIKfyveWT demonstrated a clear-cut band of immunoreactive PIKfyveWT (Fig. 2), in agreement with the pull-down experiments using cytosols enriched in PIKfyveWT by adenosyl expression as shown in Fig. 1A. It should be emphasized that a lack of immunoreactive HA-PIKfyveCh in GST-p40 beads was observed despite the substantially higher transfection efficiency and protein expression levels of HA-PIKfyveCh versus...
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Interaction with PIKfyve—PIKfyve interacts with full-length p40. A, full-length p40 protein (5 μg) expressed as a GST fusion in XA 90 E. coli strain purified and immobilized on GSH-agarose beads was incubated (18 h at 4°C) with cytosols isolated from adenovirus-PIKfyve WT or mock-infected COS-7 cells (lanes 2 and 3). GST protein (5 μg) immobilized on GSH-agarose beads (lanes 4 and 5) or GSH-agarose beads alone (lanes 6 and 7) were incubated in parallel. Beads were washed with cytosol-wash buffer, and captured proteins were analyzed by SDS-PAGE (6% gel) and immunoblotting with anti-HA antibodies as detailed under “Experimental Procedures.” The input represents 10% of the input used for incubation. Shown is a chemiluminescence detection of a representative immunoblot out of four independent pull-down experiments with similar results. The arrowhead shows the PIKfyve band. B, PIKfyve WT, HEK293 stable cell line (clone 9) induced or not with doxycycline to express the protein was transiently transfected or not with pEGFP-p40 (lane 2) or pCMV5-HA-tagged versions of indicated constructs (d = Δ) or with the vector alone. Anti-HA immunoprecipitates derived from cell lysates were analyzed for protein expression (lanes 1–3), whereas the cytosols collected from the parallel dishes were used in pull-down experiments using GST-p40 immobilized on GSH beads (lanes 4–6). Following incubation (18 h at 4°C) beads were washed as detailed under “Experimental Procedures.” Proteins were resolved by SDS-PAGE and following their transfer onto nitrocellulose membranes, were analyzed by Western blotting with anti-HA antibodies. Shown are chemiluminescence detections of representative immunoblots out of two independent pull-down experiments with similar results.

The PIKfyve chaperonin domain is required for interaction with p40. COS-7 cells seeded on duplicate 60-mm dishes were transfected with pCMV5-HA-tagged versions of indicated constructs (d = Δ) or with the vector alone. Anti-HA immunoprecipitates derived from cell lysates were analyzed for protein expression (lanes 1–3), whereas the cytosols collected from the parallel dishes were used in pull-down experiments using GST-p40 immobilized on GSH beads (lanes 4–6). Following incubation (18 h at 4°C) beads were washed as detailed under “Experimental Procedures.” Proteins were resolved by SDS-PAGE and following their transfer onto nitrocellulose membranes, were analyzed by Western blotting with anti-HA antibodies. Shown are chemiluminescence detections of representative immunoblots out of two independent pull-down experiments with similar results.

Fig. 2. The PIKfyve chaperonin domain is required for interaction with p40. COS-7 cells seeded on duplicate 60-mm dishes were transfected with pCMV5-HA-tagged versions of indicated constructs (d = Δ) or with the vector alone. Anti-HA immunoprecipitates derived from cell lysates were analyzed for protein expression (lanes 1–3), whereas the cytosols collected from the parallel dishes were used in pull-down experiments using GST-p40 immobilized on GSH beads (lanes 4–6). Following incubation (18 h at 4°C) beads were washed as detailed under “Experimental Procedures.” Proteins were resolved by SDS-PAGE and following their transfer onto nitrocellulose membranes, were analyzed by Western blotting with anti-HA antibodies. Shown are chemiluminescence detections of representative immunoblots out of two independent pull-down experiments with similar results.

Fig. 1. PIKfyve interacts with full-length p40. A, full-length p40 protein (5 μg) expressed as a GST fusion in XA 90 E. coli strain purified and immobilized on GSH-agarose beads was incubated (18 h at 4°C) with cytosols isolated from adenovirus-PIKfyve WT or mock-infected COS-7 cells (lanes 2 and 3). GST protein (5 μg) immobilized on GSH-agarose beads (lanes 4 and 5) or GSH-agarose beads alone (lanes 6 and 7) were incubated in parallel. Beads were washed with cytosol-wash buffer, and captured proteins were analyzed by SDS-PAGE (6% gel) and immunoblotting with anti-HA antibodies as detailed under “Experimental Procedures.” The input represents 15% of the input used for incubation. Shown is a chemiluminescence detection of a representative immunoblot out of four independent pull-down experiments with similar results. The arrowhead shows the PIKfyve band. B, PIKfyve WT, HEK293 stable cell line (clone 9) induced or not with doxycycline to express the protein was transiently transfected or not with pEGFP-p40 as indicated. Cells were homogenized, and the cytosols were used to immunoprecipitate PIKfyve with anti-HA. Parallel immunoprecipitates were resolved by SDS-PAGE (10.5% left panel or 8% acrylamide, right panel) and immunoblotted with the indicated antibodies. Loading in lanes 1–4 represents 5% of the immunoprecipitated material. HA-PIKfyve WT band is detected just below a nonspecific band as indicated by the arrows in the right panel. Shown are chemiluminescence detections of a representative immunoblot out of five independent experiments with similar results.

HA-PIKfyve WT in the transiently transfected cells. This is evidenced here by immunoblotting the anti-HA immunoprecipitates derived from the transfected cells with anti-HA antibodies (Fig. 2). Together these results demonstrate that p40 interacts with PIKfyve and this interaction requires the PIKfyve chaperonin domain.

A Substantial Portion of C-terminal p40 Is Engaged in the Interaction with PIKfyve—The p40 sequence is comprised almost entirely of a single domain of approximately 1,000 residues in length, known as a kelch repeat domain (16). Kelch repeats are found in proteins that participate in many aspects of cell function (23). They are predicted to form a four-stranded β-sheet corresponding to a single blade of a propeller-like barrel structure. This type of structure is thought to contain multiple protein-protein contact sites. To define the portion of the p40 protein that interacts with PIKfyve we generated several p40-truncation mutants as GST fusions and expressed them in E. coli (Fig. 3A). Purified p40 peptide fragments, depicted in the schematic diagram in Fig. 3B, were then used to pull-down PIKfyve WT under conditions similar to those described in Fig. 1A. A p40 C-terminal peptide fragment, expressing residues 133–372, which retains at least three out of the six kelch repeats, was found to bind PIKfyve equally as well as the full-length p40 (Fig. 3B). In contrast, a GST construct, in which two additional kelch repeats positioned downstream were eliminated, GST-p40 (264–372), failed to pull-down PIKfyve (Fig. 3B). Similarly, a GST-p40-N-terminal peptide fragment comprising the first two kelch repeats did not interact with PIKfyve (Fig. 3B). These results indicate that PIKfyve interaction requires a C-terminal portion of p40, whereas the N-terminal part of the p40 molecule seems to be less important. Thus, the first two kelch repeats seem to not affect the binding to PIKfyve, as evidenced here by both the pull-down experiments and the two yeast hybrid analysis, where all of the p40 clones were without the first one or two kelch repeats.

Decrease of Membrane-bound p40 in the Presence of Catalytically Inactive PIKfyvek1831E Mutant—Work by others demonstrated that the endogenous p40 in HeLa cells is mainly cytosolic with ~30% associated with membranes (16). We have confirmed a similar partitioning of the endogenous p40 between the total membrane and cytosolic fractions derived from both COS-7 (~40% membrane-associated) and HEK293 cells (~30% membrane-associated) by Western blotting with anti-p40 antibodies (Fig. 4A). To further characterize the localization of p40 in the context of PIKfyve distribution, we used HEK293 cells inducibly expressing PIKfyve WT. PIKfyve WT partitioned at a ratio of 1:1 between the cytosol and total membrane fractions (not shown). Differential centrifugation of cell
homogenates combined with Western blotting of the fraction proteins revealed that, similarly to our previous observations with endogenous PIKfyve in 3T3-L1 adipocytes (12), the majority of the membrane-bound PIKfyveWT pool in the HEK293 stable line is recovered predominantly in the high speed pellet (HSP; 200,000 × g), whereas the low speed pellet (LSP; 9,000 × g) composed of plasma membranes, nuclei, mitochondria, and possibly other heavier elements as ER, contains a minor fraction of PIKfyveWT (not shown). To examine whether a subfraction of membrane-bound p40 pool partitions together with PIKfyveWT in HSP, we performed Western blotting of these fractions using anti-p40 antibodies. This analysis revealed that

**Fig. 3. Schematic diagram of p40 and truncated forms, their expression and interaction with PIKfyveWT determined by GST pull-down experiments.** A, indicated GST-p40 constructs, expressed, and purified from *E. coli* as detailed under “Experimental Procedures” were separated by SDS-PAGE to estimate their quality and concentration relatively to a bovine serum albumin protein standard. Shown is Coomassie Blue staining of gels from a typical protein production of the indicated constructs. B, schematic presentation of the six kelch repeats and the large loop domain of p40. Indicated GST fusions (5 μg), purified and immobilized on GSH-agarose beads, were incubated (18 h at 4 °C) in the presence of cytosol isolated from Ad-PIKfyveWT or mock-infected COS-7 cells. GST protein (5 μg) immobilized on GSH-agarose beads was incubated in parallel. Beads were washed with cytosol-wash buffer, and captured proteins were analyzed by SDS-PAGE (6% gel) and immunoblotting with anti-HA antibodies as described in the legend to Fig. 1A. The fusion proteins that scored positively for PIKfyve interaction displayed a clear band for immunoreactive PIKfyve, whereas those that scored negatively lacked such a band even on overexposed blots. The data stem from two to five experiments for each protein.
indicated doxycycline-induced TetOn control, PIKfyve WT-expressing procedures.

Previously and confirmed here (18, and not shown), this stable cell line (clone 5) in the absence (lane 3 versus 4 – lane 2, was not reproduced from experiment to experiment and the band was likely unspecific. In contrast, PIKfyve WT and PIKfyve K1831E proteins, Western blotting of the subcellular fractions with anti-p40 demonstrated a dramatic diminution of endogenous p40 in the HSP fraction. Thus, as demonstrated in Fig. 4B (lane 6), in contrast to the PIKfyve WT-expressing or control HEK293 cell lines, the HSP fraction of the PIKfyve K1831E-expressing cells was almost devoid of the immunoreactive p40 band. Quantitation from four independent cell fractionation experiments indicated that p40 associated with the HSP fraction of PIKfyve K1831E-expressing cells was substantially decreased, corresponding to 38 ± 6% of p40 found in the HSP fraction of PIKfyve WT-expressing cells. This result becomes particularly compelling if one compares the nonspecific bands in this fraction cross-reacting with the anti-p40 antibodies, which show practically identical intensities in all three cell lines. The levels of p40 in the LSP fraction did not show significant changes due to PIKfyve K1831E expression and were found to be practically identical among the three cell lines (not shown). We have observed a trend of a slight increase of the p40 immunoreactive band in the cytosol derived from PIKfyve K1831E-expressing cells versus the PIKfyve WT-expressing or the control cells (Fig. 4B), suggestive for a p40 cytosolic redistribution upon expression of the kinase-deficient mutant. However, a quantitation of this increase is challenged due to the larger amount of cytosolic versus HSP-associated p40 (70 versus 7.5% of the total p40).

Unlike in the cytosolic fraction, the anti-p40 antibodies cross-react with multiple proteins in the membrane fractions as evidenced here by the detection of numerous nonspecific bands on the immunoblot presented in Fig. 4B. Therefore, we sought to confirm the p40 membrane dissociation phenomenon observed in the presence of inactive PIKfyve K1831E by a complementary approach. We co-transfected HEK293 cells stably expressing PIKfyve WT or PIKfyve K1831E with pEGFP-p40 and performed a similar subcellular fractionation of the cell homogenates. As shown in Fig. 4C, Western blot analyses of the cytosolic and HSP fractions with either anti-p40 or anti-GFP antibodies demonstrated substantially lower amounts of EGFP-p40 associated with the membranes derived from PIKfyve K1831E-expressing versus the PIKfyve WT-expressing cells (Fig. 4C, lane 3 versus 4). This diminution was observed at similar levels of GFP-p40 expression in the cytosols of the two stable cell lines (Fig. 4C, lane 1 versus 2) consistent with the specific PIKfyve K1831E-dependent decrease of membrane-associated EGFP-p40. Together, these results demonstrate, first, that a subfraction of membrane-bound p40 co-fractions with PIKfyve in HSP and second, that expression of enzymatically inactive PIKfyve K1831E induces p40 release from the HSP membrane fraction, consistent with the idea that PIKfyve enzymatic activity plays a role in p40 membrane attachment.

p40 is Phosphorylated in Vitro by PIKfyve but Does Not Interact with the PIKfyve Lipid Products—PIKfyve is a dual specificity kinase, both activities being eliminated in the PIKfyve K1831E mutant. Therefore, either one of its enzymatic activities could have played a role in the predicted PIKfyve-dependent p40 membrane tethering. p40 protein sequence displays two clusters of basic residues positioned prior to the first kelch repeat (KPR1831E and KRK1831E). Because basic pockets of this type are found to interact with acidic phospholipids, we

![Image of membrane fractionation experiments](image_url)
anti-GST antibodies (B). Shown are autoradiograms (A and C) and a chemiluminescence detection (B) of two representative experiments out of three with identical results.

First, examined whether the two PIKfyve lipid products, PtdIns 5-P or PtdIns 3,5-P2, could interact specifically with purified GST-p40 fusion protein immobilized on GSH beads. The GST-p40 lipid interaction was tested in a reaction constituted of liposome enriched in PtdIns 3,5-P2 (2%) or PtdIns 5-P (2%), and the observed binding was compared with that obtained for the control GST protein. The conditions of the liposome binding assay were similar to those we used previously to document a PIKfyve-dependent GST phosphorylation under these conditions (Fig. 5A). Upon washing, aliquots of the immunoprecipitates were resolved by SDS-PAGE on 6% (A, shows phosphorylated PIKfyve and GST-p40) or 10.5% gels (C, shows lack of phosphorylation on GST). After electrotransfer, the nitrocellulose membranes were analyzed by autoradiography (A and C). The membrane in A was probed with anti-GST antibodies (B). Shown are autoradiograms (A and C) and a chemiluminescence detection (B) of two representative experiments out of three with identical results.

We next tested whether p40 could be a plausible substrate of the PIKfyve protein kinase activity. In these experiments we used immunopurified PIKfyve immobilized on protein A-Sepharose beads as an enzyme source, purified GST-p40 as a substrate, and [γ-32P]ATP as a phosphate donor and applied in vitro phosphorylation conditions that previously demonstrated a selective transphosphorylation activity of PIKfyve for exogenously added histone (11). We were able to make two important observations from the in vitro phosphorylation assay. First, as illustrated in Fig. 5B, a fraction of the exogenously added GST-p40 was immunodetected by anti-GST antibodies only in the PIKfyve immunoprecipitates but not in immunoprecipitates with the preimmune serum in agreement with our pull-down or co-immunoprecipitation experiments presented above. Second, and more importantly, the GST-p40 detected in PIKfyve immunoprecipitates was found to be specifically phosphorylated as evidenced by the appearance of a radioactive phosphate fragment of PIKfyve (15). Data from a repeated implementation of the liposome binding assay demonstrated no specific binding of GST-p40 protein to either the PtdIns 3,5-P2 or PtdIns 5-P-enriched liposomes (data not shown).

Fig. 6. Equilibrium gradient sedimentation analysis detects a portion of p40 reactive with phosphoserine-specific antibody and co-fractionating with PIKfyve. Total membrane fractions derived from a HEK293 cell line induced to express PIKfyveWT were subjected to equilibrium sedimentation in 30% iodixanol, as described under "Experimental Procedures." Fractions were collected from the bottom of the gradient. A, aliquots were analyzed by SDS-PAGE on 6% (PIKfyve and IRAP) or 10.5% gels (p40) and immunoblotting with the indicated antibodies. B, after SDS-PAGE (10.5% gel) and electrophoresis the blot was first probed with anti-phosphoserine and after stripping, reprobed with anti-p40 antibodies. Alignment of the exposures showed an exact overlap of the p40 and the phosphoserine bands. The relatively lower intensity of fractions 3 and 4 in the p40 blot in B versus corresponding fractions in p40 blot in A is unexplained due to the interference of the phosphoserine antibody and was not observed upon direct blotting with anti-p40 as depicted in A. The results shown are chemiluminescence detections of blots from a representative experiment out of three independent fractionations with similar results.

PhosphoSer-p40 Co-fractionates with PIKfyve in Density Gradients—Previous fractionation studies with 3T3-L1 adipocyte membranes utilizing equilibrium sedimentation in density gradients combined with Western blotting recovered PIKfyve in a distinct population of denser structures, clearly segregated from recycling endosome membrane markers, including transferrin receptors and IRAP (12). Consistent with these studies, similar analysis with membranes derived form the stable HEK293 cell line recovered immunoreactive overexpressed PIKfyveWT in the denser bottom half of the 30% iodixanol gradient, whereas the recycling protein IRAP was found prim-
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...in the top lighter fractions (Fig. 6A). Under these conditions, immunoreactive p40 was found across almost the whole gradient but was particularly abundant in the fractions enriched in PIKfyve (fractions 2–5; Fig. 6A).

Because the data presented in Figs. 4 and 5 predicted a PIKfyve-catalyzed phosphorylation for membrane-associated p40, we next assessed whether phosphorylated forms of p40 could be detected in the gradient fractions. We performed immunoblotting of the SDS-PAGE-resolved fraction protein with an anti-phosphoserine-specific antibody, because PIKfyve protein kinase has been previously shown to phosphorylate seryl residues (11). A strong chemiluminescent signal was observed with the anti-phosphoserine antibody that aligned exactly with the p40 band, detected upon stripping and reprobing the same membrane with anti-p40 antibodies (Fig. 6B). These results are consistent with the notion that a subfraction of p40 co-fractionating with PIKfyve is phosphorylated on seryl residues.

**DISCUSSION**

Since the initial discovery that p40 is a Rab9 effector required in endosome-to-TGN transport (16), studies related to this protein have been surprisingly sparse. As a result we know little about p40 intracellular regulation and partners other than Rab9. The present report provides, for the first time, experimental evidence that p40 interacts specifically with the PtdIns 3,5-P2/PtdIns 5-P-generating enzyme PIKfyve and that the p40 membrane association requires the presence of the active enzyme. This interaction was identified here by a yeast two-hybrid screen and was subsequently confirmed both in vitro, using recombinant GST-p40 (Figs. 1A and 3B) as well as by co-immunoprecipitating p40 with PIKfyve from cytosols of HEK293 cell lines (Fig. 1B). The interaction involves the chaperonin domain of PIKfyve and requires a substantial portion of the p40 molecule positioned C-terminally (Figs. 2 and 3). Biochemical fractionation indicated that p40 membrane association requires active PIKfyve, because the high speed membrane fraction, derived from a cell line stably expressing the kinase-inactive dominant-negative PIKfyveK183I mutant demonstrated a profound decrease in the immunoreactive p40 levels with a proportional increase of the soluble p40 forms (Fig. 4, B and C). Because recombinant GST-p40 was found as a 32P-labeled phosphoprotein specifically in anti-PIKfyve immunoprecipitates (Fig. 5) but did not associate with the PIKfyve lipid products PtdIns 5-P/PtdIns 3,5-P2, we suggested that p40 phosphorylation by the PIKfyve protein-serine kinase activity is an important mechanism to anchor p40 to yet-to-be-identified membrane structures where its function is required. This conclusion was substantiated further by observing an immunoreactive-phosphoserine band that aligned identically with the band of p40 in the density gradient fractions containing PIKfyve (Fig. 6B). The combined results are consistent with a model whereby the PIKfyve chaperonin domain plays a role in p40 membrane recruitment and a subsequent serine phosphorylation by PIKfyve protein kinase promotes phospho-p40 membrane tethering.

An important point of the current study is the quantitative aspect of p40–PIKfyve interaction and the intracellular place of this association. Our co-immunoprecipitation using cytosols from doubly transfected cells documented ~1% of the GFP-p40 populations in PIKfyve immunoprecipitates, indicating that only a small fraction of p40 might be engaged in PIKfyve interaction that takes place in cytosol. This conclusion is in agreement with the data from the gel filtration analysis of K562 cytosols, identifying monomeric p40 soluble forms (16). Therefore, the functional association is likely to take place on membranes, consistent with the prediction of the membrane-bound p40 being the active species in stimulating late endosome-to-TGN transport of MPR (16). In line with this notion we found a substantial overlap (20–35%) between the sedimentation profiles of membrane-bound forms of PIKfyveWT and p40 by both subcellular fractionation and equilibrium sedimentation in iodixanol density gradients in a HEK293 stable cell line expressing PIKfyveWT (Fig. 6A, and not shown).

The physiological significance of PIKfyve-p40 interaction and the predicted PIKfyve-catalyzed p40 phosphorylation for membrane tethering are currently unknown. However, several lines of evidence suggest that this interaction may be necessary in the late endosome-to-TGN transport. First, expression of dominant-negative kinase-deficient PIKfyveK183I mutant is associated with a dramatic MVB enlargement (9). Because the transport to lysosomes through the biosynthetic or endocytic pathway remains largely intact under these conditions (9), the gain of MVB membrane is consistent with a block in the retrograde transport to TGN. Furthermore, PIKfyve is found to co-localize with MPR (12), predicting a yet-to-be identified functional relationship. Finally, p40 has been found to promote the return of MPR from the prelysosomal compartment to TGN, a step that requires in addition Rab9, TIP47, and mapmodulin (24–26). Because the same domain of p40 shown to bind Rab9-GTP interacts with PIKfyve (Ref. 16 and this study) we suggest that membrane-bound PIKfyve through the chaperonin domain recruits p40, whereby subsequent PIKfyve-catalyzed serine phosphorylation tethers phospho-p40 to membranes, releasing it from the complex with PIKfyve to promote the interaction with Rab9 and the late endosome-to-TGN transport. Future work should be able to test whether this hypothesis is correct and to elucidate other molecular interactions relevant for late endosome-to-TGN transport in mammalian cells.

There are no obvious homologs of p40 and Rab9 in S. cerevisiae. However, genetic studies in yeast have identified a subset of other gene products assembled in a complex termed the retromer complex, which retrieves proteins selectively from prevacuolar compartment and transports them to the Golgi (27). Although the operation of a similar molecular mechanism in mammalian cells has yet to be elucidated, it should be emphasized that recent studies support the conclusion for multiple and distinct pathways between endosomes and the Golgi complex in mammals that most likely use different machinery for cargo selection and vesicle formation (28). It is essential to note, however, that the yeast retromer complex requires a specific endosomal pool of PtdIns 3-P, produced by the phosphatidylinositol 3-kinase Vps34, for its recruitment (29). Although the role of the yeast PIKfyve ortholog, Fab1p, in this process has not yet been demonstrated, phenotypes in Fab1p-defective mutants, characterized by enormously enlarged vacuoles, could be explained, at least in part, with an arrest of the retrograde transport to earlier compartments (30, 31). Our data evolve the concept that PIKfyve uses its chaperonin domain and protein kinase activity to assemble and tether complexes relevant to late endosome-to-TGN transport. Whether such a mechanism is conserved in evolution remains to be seen.

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