Identification of Mammalian Vps24p as an Effector of Phosphatidylinositol 3,5-Bisphosphate-dependent Endosome Compartmentalization

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Phosphatidylinositol 3,5-bisphosphate is a membrane lipid found in all eukaryotes so far studied but downstream effector proteins of this lipid have yet to be identified. Here we report the use of cDNA phage libraries in conjunction with synthetic biotinylated derivatives of phosphatidylinositol 3,5-bisphosphate in the identification of a mammalian phosphatidylinositol 3,5-bisphosphate-binding protein, mVps24p. This protein is orthologous to the Saccharomyces cerevisiae protein, Vps24p, a class-E vacuolar protein-sorting protein. Using in vitro liposome binding and competition assays, we demonstrate that mVps24p selectively binds to phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 3,4-bisphosphate in preference to other phosphoinositides tested. When expressed in cultured mammalian cells, full-length mVps24p is cytosolic. However, when cells expressing the full-length mVps24p are co-transfected with a mutated form of mVps4p (which is defective in ATP hydrolysis), or when a N-terminal construct of mVps24p is expressed, the class-E cellular phenotype with swollen vacuoles is induced and mVps24p is membrane-associated. Furthermore, the accumulation of the N-terminal mVps24p construct on the swollen endosomal membranes is abrogated when phosphatidylinositol 3,5-bisphosphate synthesis is blocked with wortmannin. These data provide the first direct link between phosphatidylinositol 3,5-bisphosphate and the protein machinery involved in the production of the class-E cellular phenotype. We hypothesize that accumulation of Vps24p on membranes occurs when membrane association (dependent on interaction of phosphatidylinositol 3,5-bisphosphate with the N-terminal domain of the protein) is uncoupled from membrane dissociation (driven by Vps4p).

Phosphoinositides are phosphorylated derivatives of phosphatidylinositol (PtdIns) that have been implicated in diverse cellular functions such as cell growth, apoptosis, transcription, cytoskeletal organization, and membrane trafficking (1, 2). The most recent addition to the repertoire of phosphoinositides found in eukaryotic cell membranes came with the discovery of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂) in yeast and fibroblasts (3, 4). PtdIns(3,5)P₂ is synthesized by the Pt-
dlns-3-phosphate 5-kinase Fab1p and is involved in regulating vacular homeostasis in yeast (5–8). One of the major defects in fab1Δ cells, which are unable to make PtdIns(3,5)P₂, is the failure to sort some proteins into multivesicular bodies (MVBs) (7, 8), but the molecular basis for this defect is poorly understood. A mammalian orthologue of Fab1p, named PIKfyve, was originally identified in insulin-sensitive tissues using a differential display screen (9). PIKfyve is largely localized to late endosomal membranes (10) where the local synthesis of PtdIns(3,5)P₂ may be regulated by insulin action in some cell types (11, 12). Expression of a kinase-dead mutant of PIKfyve in mammalian cells results in the dilation and vacuolation of endosomal membranes, drastically altering cell morphology (13). This phenotype is consistent with the fab1Δ phenotype seen in yeast, suggesting a conserved role for PtdIns(3,5)P₂ in MVB formation throughout the evolution of eukaryotic organisms. The sorting of membrane proteins into MVBs, often leading to their hydrolysis in lysosomal compartments, is an important process in regulating their cellular levels. Degradation of membrane proteins may have particular importance in the control of cell signaling, by mediating the down-regulation of activated receptors such as the EGF receptor (14).

Most phosphoinositide species have known effector molecules with modular phosphoinositide binding domains such as pleckstrin homology (PH), FYVE, or PX domains (15). There are presently, however, only two reports of proteins binding specifically to PtdIns(3,5)P₂: centaurin-β2, which has a PH domain with binding specificity for this phosphoinositide (16) and sorting nexin 1, which localizes exclusively to early or sorting endosomes (17). The aim of this study has been to identify PtdIns(3,5)P₂-binding proteins in rat adipocytes. These have elevated levels of PIKfyve compared with non-insulin-sensitive tissues and should therefore be a good source of PtdIns(3,5)P₂ (9, 12).

Previous methods for isolating novel phosphoinositide-binding proteins have been based on the affinity purification of proteins from cell/tissue extracts (18, 19). However, a screen utilizing in vitro coupled transcription/translation system has
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recently been used for the same purpose, demonstrating the power of combining cDNA library technology with affinity isolation techniques (20). The screen described here also combines affinity purification with cDNA libraries but is based on phage display technology. This technology has been used in the selection of proteins able to bind to target ligands such as IgE (21, 22), RNA (23), and small natural product molecules (24). These targets are not readily amenable to the two-hybrid technologies commonly used in the identification of interactions between two proteins.

We report the use of a phage display screen in the isolation of PtdIns(3,5)P2-binding proteins from a complex library containing a vast excess of nonspecific proteins. In addition we report the characterization of one of the selected proteins, mVps24p, and demonstrate that it is selective in its binding to PtdIns(3,5)P2. Furthermore, we show that the subcellular localization of mVps24p and cellular phenotype associated with expression of mVps24p derivatives is consistent with it being a PtdIns(3,5)P2 effector in vivo.

EXPERIMENTAL PROCEDURES

Construction of Adipocyte Phage Display Library—Epididymal fat pads were removed from male Wistar rats that were fasted for 24 h and frozen in liquid nitrogen. Total RNA was extracted from the tissues using TRIzol (Invitrogen, UK) following the manufacturer’s instructions. Polyadenylated mRNA was purified using the PolyATract mRNA isolation system (Promega, UK) and quantified by measuring absorbance at 260 nm. Randomly primed cDNA was synthesized from 4 μg of adipocyte mRNA and directionally cloned into the T7Select10-3 vector arms using the TransPhat T7 kit (Promega, UK). The cloned cDNA was packaged into phage particles, and a serial dilution was made of a small proportion (5%) of the packaged phage to determine the primary size of the library. To make an amplified phage lysate for biopanning, the remainder of the phage were used to infect and lyse E. coli strain BL21(DE3)pLysS and purified using Talon (Clontech) metal affinity resin using standard methods. Purified protein was dialyzed extensively against 50 mM Hepes, pH 7.2, 100 mM NaCl, 0.5 mM EDTA and stored at 4°C. For expression in mammalian tissue culture cells VPS24 was subcloned into pET15b (Novagen). His6-mVps24p was expressed in E. coli strain BL21(DE3)pLysS and purified using Talon (Clontech) metal affinity resin using standard methods. Purified protein was dialyzed extensively against 50 mM Hepes, pH 7.2, 100 mM NaCl, 0.5 mM EDTA and stored at 4°C. For expression in mammalian tissue culture cells, VPS24 was subcloned into pFLAG-CMV12 (BD Biosciences, San Diego, CA).

Rat VPS4 cDNA was cloned by PCR amplification using oligonucleotide primers designed against the mouse VPS4 cDNA sequence. A Marathon (Clontech) cdNA library constructed according to the manufacturer’s instructions from mRNA isolated from rat adipocytes was used as the template. The amplified PCR product was purified and then added to the blunt cloning vector pT7-Blue (Promega). The correct sequence was confirmed by DNA sequencing. The QuickChange mutagenesis protocol (Strategene) was used to make the Vps4 E235Q mutation. The coding region of Vps4 WT and Vps4 E235Q was subcloned into the mammalian expression vector pEGFP-C1 (Clontech) to express these proteins with enhanced green fluorescent protein GFP at their N termini.

Antibodies—An anti-mVps24 antibody was raised in rabbits, with His6-mVps24p as an antigen. The antibody was affinity-purified against the same antigen covalently coupled to Reacti-Gel (6×)(Pierce). The rabbit anti-rat cIM6PR was as described previously (26) and was a gift of Dr. J. P. Luzio, University of Cambridge; the mouse anti-FLAG antibody was purchased from Sigma; and the AlexaFluor 488 goat anti-mouse IgG, AlexaFluor 546 goat anti-rabbit IgG, and AlexaFluor 633 goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR).

Biotinylation of PtdIns(3,5)P2—Beads—Biotinylated PtdIns(3,5)P2 (typically 1 nmol for phosphoinositide competition experiments or amount indicated in individual experiments) was incubated with 25 μl of NeutrAvidin beads that had been pre-equilibrated in binding buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, 0.5 mM EDTA) for a total volume of 500 μl for 30 min. The beads were washed twice with 1 ml of binding buffer and then resuspended in 500 μl of the same buffer. Recombinant His8-mVps24p (5 μg) was added to the NeutrAvidin beads and incubated at 18°C for 2 h. The beads were washed three times with 1 ml of detergent buffer (100 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol), and the final pellet was resuspended in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Samples were analyzed for mVps24p by 12% SDS-PAGE and immunoblotting. The bead competition assays followed the same format described above except that the His6-mVps24p was mixed with competing phosphoinositides (typically 4 nmol of competitor was used) prior to incubation with the NeutrAvidin-PtdIns(3,5)P2 beads.

Liposome Binding—Phosphatidylethanolamine/phosphatidylcholine liposomes containing the amounts of phosphoinositide indicated in the legend were prepared as described by Park et al. (27). Liposomes (100 μg of total lipid) in 100 μl of binding buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, 0.5 mM EDTA) were incubated with 5 μg of His6-mVps24p at 18°C for 30 min. Samples were cooled on ice and centrifuged at 20,000 × g for 15 min at 4°C. The supernatants were removed, and SDS-PAGE sample buffer (30 μl) was added to the pellets. Samples were analyzed for His6-mVps24p by 12% SDS-PAGE and immunoblotting.

Affinity Isolation of mVps24p from Rat Adipocyte Cytosol—Neutra- avidin-PtdIns(3,5)P2 beads were prepared as described previously for the PtdIns(3,5)P2 bead binding experiments except that 2.5 nmol of bio- tinylated PtdIns(3,5)P2 was bound to each 25 μl of beads. Control beads were treated without the addition of biotinylated PtdIns(3,5)P2. For each experiment, 25 μl of beads was incubated with 1 mg of cytosol from rat adipocytes in binding buffer for 1 h at 18°C. The beads were washed five times with 1 ml of the binding buffer, and after the final wash the beads were resuspended in 30 μl of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and by immunoblotting for the enrichment of mVps24p using anti-mVps24 affinity-purified polyclonal antibodies.

Cell Culture, Transfection, and Immunofluorescence Microscopy—

COS-7 cells were grown in Dulbecco’s modified essential medium (Invitrogen) containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated onto coverslips and grown to 60% confluence prior to transfection using GeneJuice transfection reagent (Novagen) according to manufacturer’s instructions. Cells grown on glass coverslips were transfected 18–24 h prior to fixation and processing for immunofluorescence microscopy. Cells were rinsed with PBS and fixed in 2% paraformalde-
hyde in PBS for 15 min prior to permeabilizing with 0.1% Triton X-100 for 5 min at room temperature. Nonspecific binding sites were blocked with 10% fetal calf serum for 20 min prior to addition of primary antibodies. Double labeling of Vps24p and M6PR was carried out using mouse anti-FLAG (10 μg/ml final) and rabbit anti-rat cM6PR followed by washing and addition of Alexa 488 goat anti-mouse IgG and Alexa 546 goat anti-rabbit IgG at 5–8 μg/ml in 10% fetal calf serum-PBS. Coverslips were washed and mounted in Mowiol (Calbiochem, San Diego, CA). In cells expressing GFP-Vps4E235Q, rabbit anti-M6PR was detected using Alexa 546 goat anti-rabbit IgG and Vps24-FLAG using Alexa 633 goat anti-mouse IgG. The labeled cells were viewed, and images were obtained on a Zeiss LSM510 laser scanning confocal microscope or on a Nikon Eclipse fluorescent microscope.

RESULTS

Identification of PtdIns(3,5)P₂ Binding Proteins Using Phage Display—The affinity ligand (biotin-PtdIns(3,5)P₂) (Fig. 1) was immobilized on NeutrAvidin beads. A T7Select10-3 adipocyte library was constructed that contained 2 × 10⁶ independent phage. Phage selected at random from the un-amplified library all contained cDNA inserts over 200 base pairs in length. Following the first round of biopanning against the NeutrAvidin PtdIns(3,5)P₂ beads, the titer of the eluted phage was 2 × 10⁶. The eluted titer was 1.5 × 10⁷ in the second round and 5 × 10⁷ in the third, a 25-fold overall increase. This was an indicator of successful biopanning, because the enrichment of phage that bind the target molecule during early rounds usually results in increases in titer for recovered phage in later rounds (T7-Select manual, Novagen). Following the third round of biopanning, individual plaques were picked, and their cDNA inserts were amplified by PCR and then sequenced. Sequencing of 12 plaques revealed only two different cDNA inserts, indicating a highly specific enrichment from the initial library, potentially containing 2 × 10⁶ different clones. Only one of the cDNA inserts is considered here, encoding for an open reading frame (ORF) corresponding to 230 amino acids, in-frame with the T7-capsid protein (Fig. 2A). The ORF shared a high degree of sequence identity (30%) with Saccharomyces cerevisiae Vps24p (Fig. 2B). S. cerevisiae Vps24p is a vacuolar protein-sorting protein of the class-E type (28, 29). We therefore named the protein mammalian Vps24 protein (mVps24p). The sequence of mVps24p lacks any previously identified phosphoinositide binding domains. It is predicted to be a 223-amino acid (7 amino acids in the 230-amino acid ORF were upstream of the start methionine), 25-kDa hydrophilic protein (pI = 5.5) that has no long hydrophobic stretches of amino acids. One noticeable feature is that the charge distribution of the protein is extremely biased, with the N-terminal half being very basic (pI = 10.2) and the C-terminal half being acidic (pI = 3.9).

The similarity between S. cerevisiae Vps24p and mVps24p, which was selected on its ability to bind PtdIns(3,5)P₂ in the phage display screen, was intriguing. Deletion mutants of Vps24 in S. cerevisiae have a swollen vacuolar phenotype (class-E Vps phenotype), similar to the fab1Δ phenotype of cells that cannot produce PtdIns(3,5)P₂, suggesting a possible functional connection. This functional connection, implicating one or more of the Vps class-E proteins as possible PtdIns(3,5)P₂ effectors, had been made previously (7), but to date there has not been any direct evidence to substantiate this link.

mVps24p Binds Selectively to Phosphoinositides—The coding region of mVps24p was cloned into the pET15b vector and protein was expressed and purified from E. coli as a hexahis-
FIG. 2. Sequence analysis of PtdIns(3,5)P$_2$ binding phage. A, nucleotide and deduced amino acid sequence of the cDNA insert in a pT7 phage selected by its ability to bind PtdIns(3,5)P$_2$ beads. The first three amino acids are derived from the phage capsid protein. The predicted start codon and first methionine are shown in \textbf{boldface type}. Amino acids in the ORF that are upstream of the start codon and that are predicted not to be present in the natural protein are \underline{underlined}. B, alignment of the deduced amino acid sequence (Rat) and \textit{S. cerevisiae} Vps24p (S.cer). Identical amino acids are \textbf{shaded} in black; conserved amino acids are gray.
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Fig. 3. Adsorption of mVps24p by immobilized NeutrAvidin-PtdIns(3,5)P₂. A, recombinant His₆-mVps24p (5 μg) was incubated with NeutrAvidin beads having the indicated amounts of biotinylated-PtdIns(3,5)P₂ coupled to their surface. The beads were washed to remove unbound His₆-mVps24p. Recovery of His₆-mVps24p bound to the beads was assessed by SDS-PAGE and immunoblotting with affinity purified anti-mVps24p antibody. B, cytosolic extracts (1 mg of protein) from rat adipocytes were incubated with NeutrAvidin beads (NeutrAvidin) or NeutrAvidin beads coupled to biotinylated-PtdIns(3,5)P₂ (NeutrAvidin-PtdIns(3,5)P₂). The beads were washed to remove unbound proteins. Recovery of mVps24p bound to the beads was assessed by SDS-PAGE and immunoblotting with affinity purified anti-mVps24p antibody. Representative results from three separate experiments are shown.

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Fig. 4. Analysis of the lipid binding specificity of His₆-mVps24p. Analysis of interaction by competition assays. Recombinant His₆-mVps24p (5 μg) was mixed with the indicated competitor phosphoinositide or phosphatidylserine (PS). Liposomes were pelleted by centrifugation and recovery of His₆-mVps24p bound to the liposomes was assessed by SDS-PAGE and immunoblotting with affinity purified anti-mVps24p antibody. A, competition with C₆ phosphoinositides and inositol phosphate head groups. B, competition with C₁₆ phosphoinositides. Representative results from three and six experiments are shown for A and B, respectively. Analysis of binding of mVps24p to liposomes. Recombinant His₆-mVps24p (5 μg) was incubated with liposomes (100 μg) consisting of PE:PC and the indicated phosphoinositide, prepared as described under “Experimental Procedures.” The liposomes were pelleted by centrifugation and recovery of His₆-mVps24p bound to the liposomes was assessed by SDS-PAGE and immunoblotting with affinity purified anti-mVps24p antibody. C, liposomes reconstituted with 5 mol % of the indicated phosphoinositide or phosphatidylserine (PS). D, liposomes reconstituted with 0, 1, 2, 3, 4, and 5 mol % of PtdIns(3,5)P₂. Representative results from three and two experiments are shown for C and D, respectively.
Fig. 5. Mutation of a conserved positively charged amino acid residue reduces PtdIns(3,5)P2 binding. A, sequence alignment of rat/mouse CHMPs. Sequences were aligned using ClustalX (default parameters), and amino acids conserved in greater than 50% of sequences were shaded using BOXSHADE. GenBankTM accession numbers for the mouse and rat proteins are: CHMP1 (mouse), NP_663581; CHMP2 (mouse), NP_081161; CHMP3 (rat, this study), AY150169; CHMP4 (rat), XP_231625; CHMP5 (mouse), NP_084090; and CHMP6 (mouse), BB610671. The conserved positively charged amino acid that was mutated in mVps24p/CHMP3 is marked with an asterisk. B, recombinant His6-mVps24p (WT) or mutant His6-mVps24p K49D (1 μg) was incubated with liposomes (100 μg) consisting of PE/PC or PE/PC spiked with 2 mol% of PtdIns(3,5)P2, prepared as described under “Experimental Procedures.” The liposomes were pelleted by centrifugation. His6-mVps24p bound to the liposomes pellet (P) and that remaining in the supernatant (S) was assessed by SDS-PAGE and immunoblotting with affinity purified anti-mVps24p antibody. Representative results from three separate experiments are shown.

binding of His6-mVps24p to PtdIns(3,5)P2 and PtdIns(3,4)P2 has been observed, with little or no binding to other phosphoinositides tested.

A Conserved Lysine Residue Is Important for PtdIns(3,5)P2 Binding—Previous work on PH, FYVE, and PX domains has revealed that specific binding to phosphoinositides requires electrostatic interactions between the negatively charged phosphates on the lipid and positively charged amino acids in the protein. In the case of mVps24, the majority of the positively charged amino acids are in the N-terminal half of the protein, making this region the most probable to be involved in phosphoinositide binding. mVps24p belongs to a family of proteins termed charged multivesicular body proteins (CHMPs) that also have basic N termini (31). Sequence alignment of the mammalian CHMPs reveals an invariant positively charged amino acid residue (Lys-49 in Vps24p) (Fig. 5A). To investigate the possible role of the N terminus and this amino acid in particular in PtdIns(3,5)P2 binding, it was changed to asparagine-6-phosphate receptors (M6PR), in cells expressing either mVps24p-FLAG or GFP-Vps4p WT (Fig. 6D) localize perinuclearly in small puncta, a similar distribution to that seen in untransfected cells (Fig. 6, B and D, arrows). As expected expression of GFP-Vps4p E235Q induced large vacuolated structures, analogous to the class-E compartment in yeast (Fig. 6, E–H). When mVps24p-FLAG was co-expressed with the GFP-Vps4p E235Q, mVps24p-FLAG also accumulated in the swollen structures as did M6PR, verifying that they correspond to late endosomes/multivesicular bodies. GFP-Vps4p E235Q-induced vacuoles also have the early endosomal protein EEA1 on the limiting membranes (data not shown), suggesting that they could be hybrid organelles (34). These data are consistent with mVps24 being a mammalian class-E Vps protein, because its membrane association is regulated in a Vps4-dependent manner in the same way as its yeast orthologue.

The N Terminus of mVps24p Associates with Class-E Compartment—The full-length mVps24 is predominantly cytosolic, but probably cycles on and off membranes in a Vps4-dependent manner. Therefore, when membrane disassociation is prevented by the co-expression of GFP-Vps4p E235Q, it accumulates on membranes. We therefore hypothesized that it might be possible to uncouple membrane association from membrane disassociation by expression of a truncated mVps24p. This would be expected to retain the ability to bind phosphoinositides but to lack a possible Vps4 interaction domain. To investigate this possibility we generated a construct to express the N-terminal 112 amino acids of mVps24p (the probable phosphoinositide binding domain, see lipid binding section above and under “Discussion”) fused to GFP (NTmVps24p-GFP).

Expression of NTmVps24p-GFP in COS-7 cells resulted in the transfected cells becoming vacuolated with a morphology...
those induced by a dominant negative Vps4 (34). NTmVps24p-GFP-expressing cells had an apparently normal TGN, because the localization of TGN46 was unaffected.2

These data are consistent with the hypothesis that the class-E endosomal phenotype induced by NTmVps24p-GFP expression is due to prolongation of its binding to PtdIns(3,5)P2. This effectively masks the lipid, preventing its normal molecular interactions and thus function. Membrane budding reactions from the compartment (and in the case of MVB formation, internal budding reactions) are prevented, and the compartment vacuolates. These functions would normally be dependent on full-length mVps24p being acted upon by Vps4. To further test this possibility, cells were treated with the phosphatidylinositol 3-kinase inhibitor wortmannin, which reduces the amount of PtdIns 3-phosphate, the precursor to PtdIns(3,5)P2.

Cells transiently transfected with NTmVps24p-GFP were either treated with 100 nm wortmannin for 3 h or mock treated. As expected NTmVps24p-GFP-transfected cells in the mock treated control exhibited the swollen endosomal phenotype with NTmVps24p-GFP localized on the limiting membranes of the swollen class-E endosomes (Fig. 8A). In wortmannin-treated cells, however, although swollen endosomes were present, NTmVps24p-GFP was not localized to them in any of the transfected cells (Fig. 8B). These results are consistent with dependence of NTmVps24p-GFP localization to swollen endosomal membranes on the presence of PtdIns(3,5)P2.

**DISCUSSION**

A requirement for PtdIns(3,5)P2 and class-E Vps proteins for the efficient sorting of proteins into MVBs and/or endosomal function seems to have been conserved throughout the evolution of eukaryotic organisms. Here we report the first evidence that a mammalian class-E Vps protein orthologue, mVps24p, can interact directly with PtdIns(3,5)P2. Because mVps24p lacks any previously characterized lipid binding domains, this protein would not have been identified with bioinformatics approaches, using homology searches for known phosphoinositide binding domains. Our results therefore demonstrate the usefulness of *de novo* approaches such as phage display in the identification of novel interactions.

His6-mVps24p showed a high degree of selectivity for PtdIns(3,5)P2 in lipid binding experiments, although the specificity was not exclusive, because it also bound to PtdIns(3,4)P2. This *in vitro* promiscuity does not discount PtdIns(3,5)P2 from being the relevant phosphoinositide ligand for mVps24p in a cellular situation. The localization of mVps24p on endosomal membranes, the proposed site of synthesis of PtdIns(3,5)P2 (12), places mVps24p in the expected location to be an effector of this lipid. However, we cannot rule out a role for PtdIns(3,4)P2, a phosphoinositide that is thought to be produced at the plasma membrane (1), in mVps24p function, although we have no data to suggest mVps24p is localized to the plasma membrane. In addition to the demonstration of recombinant protein binding to PtdIns(3,5)P2, we were able to affinity-isolate mVps24p from a rat adipocyte cytosolic fraction using PtdIns(3,5)P2 beads, showing that endogenous mVps24p also binds PtdIns(3,5)P2.

The observation that short-chain water-soluble phosphoinositides failed to compete for binding of His6-mVps24p to biotinylated PtdIns(3,5)P2 could have several possible explanations as outlined below. It is possible that hydrophobic interactions may contribute to the strength of PtdIns(3,5)P2 binding and that short-chain monomeric phosphoinositides cannot compete as efficiently as long-chain (hydrophobic) phosphoinositides presented in a more membrane-like environment. Hydro-
phobic interactions have been proposed to strengthen the membrane association of PX and FYVE domains (35–37). Alternatively, multiple interactions may be necessary for His6-mVps24 binding to PtdIns(3,5)P2, and these may only be possible for long-chain lipids present in micelles. Multiple interactions may involve multiple binding sites in the mVps24p monomer or may be due to oligomerization of the protein. PtdIns(3,5)P2 immobilized on beads would also provide multiple binding sites on the same surface. The phenomenon of inefficient binding of short-chain phosphoinositides in comparison to long-chain phosphoinositides has been reported for other protein domains such as the FYVE domain of Hrs-1 (30).

The cell-based data presented in this study are consistent with mVps24 acting as a PtdIns(3,5)P2 effector protein in vivo. Although mVps24p-FLAG is predominantly cytosolic and nuclear, it becomes stably associated with membranes containing endosomal markers when co-expressed with an ATPase-defective Vps4p that is unable to disassemble class-E Vps complexes (29, 32). Because the proposed site of PtdIns(3,5)P2 synthesis is late on endosomal membranes (13), these observations place both protein and lipid at the same subcellular location. The nuclear localization of mVps24p is intriguing, because CHMP1, which shares some sequence similarities with mVps24p (Fig. 5A), has been proposed to have a nuclear function in addition to a class-E Vps function (39) and other unrelated Vps class-E proteins such as TSG101 are also found in the nucleus (40, 41). Consideration of a possible nuclear function of Vps24p is, however, beyond the scope of this present study.

Because the localization data alone do not sufficiently establish whether a direct interaction between protein and lipid is

**Fig. 7.** NTmVps24p-GFP accumulates on swollen endosomal compartments. COS-7 cells were transiently transfected with a plasmid encoding NTmVps24p-GFP and incubated for 24 h in media containing 10K Texas Red dextran (B–D) or normal growth media (A and E–I). Cells were washed and processed for immunofluorescence analysis with antibodies against EEA1/Alexa 546 (F) and M6PR/Alexa 546 (I). Top and left-hand panels (A, B, E, and H) show NTVps24p-GFP fluorescence. The middle panels show detection of Texas Red dextran (C), EEA1 (F), and M6PR (I) in the same cells as in the corresponding left-hand panel. Right-hand panels show a merged image of the left and middle panels (GFP (green) and Texas Red/Alexa 546 (red)). These results are representative of three separate experiments.

**Fig. 8.** Membrane association of NTmVps24p-GFP is abrogated by wortmannin treatment. COS-7 cells were transiently transfected with a plasmid encoding NTmVps24p-GFP. Cells were incubated for 16 h during which NTmVps24p-GFP expression occurred and were then mock treated or treated with 100 nM wortmannin for 3 h. Fresh serum-free media containing 100 nM wortmannin or with no additions (as appropriate) was changed every hour during treatment. Following treatment, cells were washed and processed for detection of GFP immunofluorescence. A representative cell from both mock treated (A) and wortmannin-treated cells (B) is shown. Association of NTmVps24p with vacuolated membranes was seen in the majority of transfected mock treated, but in none of the wortmannin-treated cells. These results are representative of three separate experiments.
taking place, further supporting evidence is required. Other studies with phosphoinositide-binding proteins have made use of drugs that perturb lipid synthesis and/or site-directed mutagenesis of critical phosphoinositide binding residues to interfere with membrane localization in vivo (17, 42). In the case of mVps24p, the probable transient nature of its membrane association does not make studies of this protein amenable to such approaches. However, we hypothesized that it may be possible to uncouple phosphoinositide binding from membrane dissociation by expressing only the phosphoinositide binding domain of mVps24p. When NTmVps24p-GFP was expressed in COS-7 cells, the protein became stably membrane-associated and caused vacuolation of endosomal compartments. Furthermore, the membrane association of NTmVps24p-GFP was blocked by the incubation of transfected cells with the phosphatidylinositol 3-kinase inhibitor wortmannin. This treatment reduces PtdIns(3,5)P2 in addition to PtdIns 3-phosphate levels (43) indicating that the membrane localization of the N terminus of mVps24 is 3-phosphoinositide-dependent. The class E-like vacuolation phenotype induced by NTmVps24p-GFP expression is similar, if not identical to the phenotype induced by a kinase-dead PIKfyve that causes a reduction in cellular PtdIns(3,5)P2 levels (13). It is likely, therefore, that vacuolation induced by NTmVps24p-GFP expression occurs through essentially the same mechanism as that occurring when PtdIns(3,5)P2 is depleted.

Recent studies on the S. cerevisiae ESCRT-III complex, which contains Vps24p and other CHMPs, suggests that ESCRT-II facilitates ESCRT-III assembly. Thus, membrane association of Vps24p appears to require an interaction with proteins in addition to, instead of, or prior to lipid binding. We favor a model in which PtdIns(3,5)P2 binding occurs following assembly of mVps24p into a membrane-associated complex. Our model builds on the data from studies by others (reviewed in Ref. 44) and incorporates new aspects derived from our observations as follows. (i) Direct binding of cytosolic full-length Vps24p to membrane lipid is prevented through intramolecular interactions, which maintain low affinity binding for PtdIns(3,5)P2. An interaction between the N and C termini of mVps24 is proposed (see below). (ii) Vps24p associates with the appropriate membrane via an association with other pre-assembled ESCRT components. (iii) This association places mVps24p (and other CHMPs) on an endocytic compartment where there is a high local concentration of PtdIns(3)P and where PtdIns(3,5)P2 can be synthesized by PIKfyve. The high concentration of PtdIns(3,5)P2 now leads to avid binding of the N-terminal half of mVps24p leading its disassociation from the C terminus. This conformational change initiates further events such as membrane invagination and budding to form MVBSs.

In the proposed model, PtdIns(3,5)P2 production influences downstream events coordinated by an assembled ESCRT-III complex. Pre-assembly of ESCRT-III prior to PtdIns(3,5)P2 binding explains why Snf7p (an S. cerevisiae CHMP) can associate with membranes in a fab1 vaseΔ background (45). Similarly we have observed that mVps24p-FLAG remains membrane-associated when co-expressed with GFP-Vps4p E235Q in wortmannin-treated COS-7 cells.2 However, when the C terminus of the protein is removed, there is nothing preventing the N terminus associating with PtdIns(3,5)P2 independently of ESCRT-III assembly and causing the vacuolated phenotype described above.

Although we do not as yet have conclusive evidence for an interaction between the N and C termini of mVps24p, there is a striking charge distribution within the protein and all other CHMPs. The N terminus is extremely basic (pI = 10.2), and the C terminus is extremely acidic (pI = 3.9). We therefore propose the nature of the interaction to be electrostatic. This interaction possibly masks (at least partially) the true affinity of the N terminus for PtdIns(3,5)P2 in the full-length protein (Kd ≈ 50 μM), although it is in the range that occurs in weak phosphoinositide-binding proteins such as many of the PX domain-containing proteins from S. cerevisiae (46). Quantitative measurements of the binding affinity of a range of N-terminal constructs of mVps24p will establish whether masking by the C terminus affects its PtdIns(3,5)P2 binding affinity. Masking of phosphoinositide binding domains by intramolecular interactions is not unique and has recently been reported for a number of PX domain containing proteins such as Fish (47) and p47phox (38).

Clarification of whether PtdIns(3,5)P2 production coordinates an outward budding from endosomes/lysosomes, an invagination of vesicles to form multivesicular bodies, or a regulation of a membrane fusion step is still a topic for further study. However, the results presented here are the first to reveal the existence of a direct connection between PtdIns(3,5)P2 class E Vps proteins, and endosomal trafficking machinery. Functional studies on mVps24p and other CHMPs may provide us with further insight into the role or roles of PtdIns(3,5)P2 in endocytic trafficking pathways.

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