Molecular characterization of mammalian homologues of Class C Vps proteins that interact with syntaxin-7

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Summary

Vesicle-mediated protein sorting plays an important role in segregation of intracellular molecules into distinct organelles. Extensive genetic studies using yeast have identified more than forty VPS genes involved in vesicle transport to vacuoles. However, their mammalian counterparts are not fully elucidated. In this study, we identified two human homologues of yeast Class C VPS genes, human VPS11 (hVPS11) and human VPS18 (hVPS18). We also characterized the subcellular localization and interactions of the protein products not only from these genes but also from the other mammalian Class C VPS homologue genes, hVPS16, and rVPS33a. The protein products of hVPS11 (hVps11) and hVPS18 (hVps18) were ubiquitously expressed in peripheral tissues, suggesting that they have a fundamental role in cellular function. Indirect immunofluorescence microscopy revealed that the mammalian Class C Vps proteins are predominantly associated with late endosomes/lysosomes. Immunoprecipitation and gel filtration studies showed that the mammalian Class C Vps proteins constitute a large hetero-oligomeric complex that interacts with syntaxin-7. These results indicate that, like their yeast counterparts, mammalian Class C Vps proteins mediate vesicle trafficking steps in the endosome/lysosome pathway.
Introduction

Eukaryotic cells contain highly specialized intracellular membrane bound compartments. Vesicle trafficking between these organelles is very important for the maintenance of cell homeostasis (1-2). Although numerous proteins and protein complexes have been characterized to have a role in intracellular vesicle transport and protein sorting, their precise mechanisms of involvement are not yet elucidated. Genetic studies using yeast mutants have identified more than 40 vacuolar protein sorting (VPS) genes coding for proteins required for vacuolar proteins transports (3-5). These vps mutants are categorized into six classes, A-F, with respect to their morphology and acidification defects (6, 7). The Class C vps mutants are characterized by remarkable abnormalities in vacuole morphology, accumulations of multivesicular bodies, temperature sensitive growth defects, osmotic sensitivity, reduced amino acid pools and sporulation defects (5, 6, 8-11).

There are four Class C VPS genes, VPS11, VPS16, VPS18 and VPS33. The Yeast VPS11 and VPS18 genes are also known as END1/PEP5/VAM1 (8-10) and PEP3/VAM8, respectively (9,11). The protein products of VPS11 and VPS18 contain a characteristic cysteine-rich RING-H2 finger domain in their C-terminal regions (10-13). The RING-H2 finger domain is a subfamily of the RING finger motif utilized by numerous proteins for diverse cellular functions including oncogenesis, cell differentiation, signal transduction, and membrane vesicle trafficking (14, 15).
The Class C Vps proteins, Vps11p, Vps16p, Vps18p, and Vps33p, exist as a large detergent-insoluble HOPS (homotypic fusion and vacuole protein sorting) complex that also contains Vps39p and Vps41p. This Class C Vps/HOPS complex associates with Vam3p involved in regulating both vesicle docking/fusion and vacuole-to-vacuole fusion (12, 16-19).

Previous studies have reported that Vps18p and Vps33p share significant homology with the *Drosophila* gene products, *deep orange* (*dor*) and *carnation* (*car*), respectively. These proteins are associated into a large complex that localizes to the endosomal compartment and is required for membrane trafficking to lysosomes and pigment granules in *Drosophila* eyes (20, 21). Therefore it appeared likely that mammalian homologues of the yeast Class C Vps proteins are also involved in protein sorting steps. Additionally, syntaxin-7, a Vam3p related protein was recently identified in mammals (22-25). Although there is some discrepancy regarding the precise intracellular localization of syntaxin-7, it is clear that syntaxin-7 is an essential factor for the fusion of late endosomes with lysosomes, lysosome homotypic fusion, and endocytic trafficking to late endosomes (24-26).

The functional roles of Class C Vps proteins have been extensively investigated in yeast. In contrast, relatively little is known about their mammalian counterparts. Therefore, we sought to identify and characterize Vps proteins that may control intracellular vesicle trafficking events in mammalian cells. In the present study, we identify two human VPS gene homologues, hVPS11 and
hVPS18, and characterize biochemical features and intracellular localizations of Class C Vps proteins. We show that mammalian Class C Vps proteins exist as a hetero-oligomeric complex primarily associated with late endosomes/lysosomes, and that the complex interacts with syntaxin-7 suggesting that it has a role in SNARE complex assembly.

**Experimental procedures**

*Isolation of hVPS11 and hVPS18*---- The GenBank™ database of human expressed sequence tags (ESTs) was searched using the *S. cerevisiae* VPS11/PEP5/END1 sequences (8, 10). One of the human EST clones (accession number: AA385518) had a distant homology to the RING-H2 finger domain region of *S. cerevisiae* VPS11/PEP5/END1. Two oligonucleotide PCR primers (5'`-AGCAGATTGCACAGGATGAG-3'` and 5'`-CAGAGTCAATTTGTTGAAAA-3'`) were designed and used to amplify a 395 bps fragment using a HeLa cell cDNA template for polymerase chain reaction (PCR) under standard conditions. Amplified fragments were subcloned into pGEM-T Easy vector (Promega, Madison, WI) and subsequently sequenced. The *EcoRI* digested insert fragment from pGEM-T Easy vector was used as a probe for screening the human brain cDNA library constructed in lambda ZAPII (gift from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan). Ten positive clones were obtained from 1 X 10⁶
plaques screened. The clone carrying the longest insert was sequenced from both strands.

hVPS18 was identified by searching the GenBank™ database using *S. cerevisiae* VPS18/PEP3 sequences (11, 13) and *Drosophila* deep orange sequences (20, 21). A novel cDNA, designated KIAA1475 (accession number: AB040908) containing an open reading frame of 976 amino acids was found to be 34% identical to deep orange and 26% identical to *S. cerevisiae* VPS18/PEP3. The KIAA1475 clone was obtained from the KAZUSA DNA institute (Chiba, Japan).

**Northern Blot Analyses**--- A hVPS11 cDNA fragment was excised using the BamHI restriction enzyme (1,808 bps), and a hVPS18 cDNA fragment was excised using the SacI restriction enzyme (972 bps). Fragments were random-primed radiolabeled (Life Technologies, Rockville, MD) and hybridization was carried out at 42°C overnight using human multiple tissue northern (MTN) blots (Clontech, Palo Alto, CA) as described previously (27).

**Expression Vectors**--- Epitope-tagged, full-length hVPS11 and hVPS18 and rVPS33a were prepared using PCR with custom designed oligonucleotide primers containing the appropriate restriction enzyme sites. To insert full length hVPS11 downstream of the Myc- and HA-tag sequence at the N-terminal end, a forward primer containing the *EcoRI* site upstream of the initiation codon (5’-CGGAATTCAAATGGCGGCCTACCTGCA-3’) and a reverse primer including the
XhoI site downstream of stop codon (5’-CCTCGAGTTAAGTGCCCCTCCTGGA-3’) were used to amplify PCR products from a pBluescript SK(+)-hVPS11 template. PCR products were subcloned into the pGEM-T Easy vector and subsequently sequenced. The EcoRI/XhoI digested full-length hVPS11 was inserted into the EcoRI and XhoI site of the pMyc-CMV and pHA-CMV mammalian expression vectors (Clontech) to generate pMyc- and pHA-hVPS11, respectively. The N-terminally Myc- and HA-tagged hVPS18 constructs were also generated by PCR using oligonucleotide primers, 5’ primer-CGGAATTCCCATGGCGTCCATCCAT, 3’ primer-CCGCTCGAGCTACAGCCAACTGAGC using pBluescript II SK(+) -KIAA1475 clone as a template. EcoRI and XhoI digested full-length hVPS18 was inserted into the EcoRI and XhoI site of the pMyc-CMV and pHA-CMV mammalian expression vectors to generate pMyc- and pHA-hVPS18, respectively. A full-length rat VPS33a (accession number: U35244) was amplified by PCR from rat total brain cDNAs. A forward primer containing the XhoI site upstream of the initiation codon (5’-CAGATCTCGAGCGATGGCGGCTCACCT) and a reverse primer containing the EcoRI site downstream of the stop codon (5’-CAGAATTCCTAGAAAGGCTTTTCCATGA) were used to amplify PCR products. The XhoI and EcoRI digested full-length rVPS33a was inserted into the XhoI and EcoRI site of the pEGFP-C1 mammalian expression vector (Clontech) to generate GFP-rVPS33a. The N-terminally Myc-tagged full-length human syntaxin-7 (accession number: U77942) construct was generated by PCR using custom
designed oligonucleotide primers. The 5'-primer (CGGAATTCCATGTCTTACACTCCA) and the 3'-primer (AATGC GGCCGCTCAGTGGTTCAATC) were used to amplify PCR products from a human cDNA brain library. All constructs were verified by DNA sequencing.

Cell Culture and Transfection----- COS-7, HeLa, HEK293, NRK, and BHK cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum (Nippon Bio-Supply Center, Tokyo, Japan), 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified incubators with 5% CO₂ at 37°C. Plasmid DNAs were transfected into COS-7 cells using FuGENE 6 transfection regents (Roche Molecular Biochemicals, Mannheim, Germany).

Antibodies----- Peptides corresponding to the internal 20 amino acids of hVps11 (222IVSRDRKVSPKSEFTSRDSQ 241) and 14 amino acids of hVps18 (426RPDSLLEERVWEY 439) were synthesized. The peptides were coupled to m-maleimidobenzoyl-N-hydroxysuccinimideester (MBS) activated keyhole limpet hemocyanin (KLH) and used as immunogens in rabbits. The polyclonal antiserum was affinity purified. Briefly, the immunogen peptides were linked to activated CH Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) and polyclonal antiserum was affinity-purified by binding and elution from the Sepharose column. A GST-fusion protein containing aa 599 to 839 of hVPS16 was expressed in
bacteria, purified and used to raise antisera in rabbits. The polyclonal antiserum was affinity purified using the GST-hVps16 (599-839) fusion protein. The monoclonal antibodies for human early endosome antigen 1 (EEA1) were purchased from Transduction Laboratories (Lexington, KY). The mouse monoclonal antibodies recognizing the transferrin receptor (CD71) and GFP (B-2) were purchased from Santa Cruz Biotechnology (Sanat Cruz, CA). The rabbit polyclonal antibodies recognizing GFP were purchased from Clontech. A mouse monoclonal anti-Myc epitope (9E10) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). A rat monoclonal anti-HA (3F10) antibody was purchased from Roche Molecular Biochemicals. The mouse monoclonal antibody for Lamp-1 was a gift from Dr. M. Fukuda (The Burnham Institute, La Jolla, CA). The polyclonal antibodies for syntaxin-7 were kindly provided by Dr. Y. Wada (Osaka University, Osaka, Japan). FITC-conjugated affinity isolated goat anti-mouse IgG secondary antibody was purchased from Biosource (Camarillo, CA, USA). Texas-Red-conjugated affinity isolated donkey anti-rabbit IgG secondary antibody was purchased from Amersham Pharmacia Biotech.

**Indirect Immunofluorescence Analyses**--- For immunofluorescence microscopy, COS-7 cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then permealized in 0.2% saponin (WAKO Co. Ltd, Tokyo, Japan) for 20 min, and nonspecific antibody binding sites were blocked with PBS containing 0.2% BSA, 0.2% saponin, and 1% normal goat serum for 30 min at
room temperature. Cells were then incubated with 2 µg/ml affinity purified anti-hVps11, hVps16 or hVps18 antibodies diluted in PBS containing 0.2% BSA, 0.2% saponin and 1% normal goat serum for 2 hr. After rinsing three times with PBS, cells were incubated with secondary antibodies for 1 hr and then rinsed five times with PBS. Cells were mounted in Perma Fluor (Immunon, Pittsburgh, PA) and viewed under the confocal laser-scanning microscope CLSM2010 (Amersham Pharmacia Biotech).

*Western Blot Analyses*---- The cultured cells were washed twice with ice-cold PBS then scraped into ice-cold homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and Complete™ protease inhibitor mixture (Roche Molecular Biochemicals). The cells were rotated at 4°C for 30 min to lyse the cells. The cells were centrifuged at 10,000 X g for 15 min and the supernatants collected and protein quantitated. Supernatants were diluted into equal volumes of 2 X SDS gel sample buffer (300 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 10% β-mercaptoethanol) and boiled for 5 min. Samples were electrophoresed on 4/20% gradient SDS-polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), and electroblotted onto Imobilon-P membrane (Millipore, Bedford, MA). The blots were incubated with anti-hVps11, hVps16, and hVps18 antibodies for 2 hr at room temperature and then incubated in HRP-conjugated secondary
antibodies. Antibody binding was detected using the ECL protein detection kit (Amersham Pharmacia Biotech) according to the manufacturer specifications.

Subcellular Fractionation---- HEK293 cells grown in 10 cm dishes were washed twice with ice-cold PBS, scraped into 200 µl of PBS, and lysed by sonication. Nuclei and unlysed cells were removed by spinning the lysate at 6,000 X g for 10 min. The postnuclear supernatant was then centrifuged at 100,000 X g for 30 min at 4°C to separate the cytosolic (supernatant) and membrane fractions (pellet). The pellets were resuspended in 200 µl of PBS containing 1% Triton X-100, incubated on ice for 1 hr then centrifuged at 100,000 X g for 30 min. Equal portions of the cytosol and membrane fractions were separated by SDS-PAGE and analyzed by immunoblotting.

Treatment of Membranes with Various Detergents---- Membrane preparations were performed as described previously (28). Briefly, three 10 cm culture dishes of confluent HEK 293 cells were scraped into ice-cold PBS, washed once and the cells were spun down by brief centrifugation. The cells were resuspended in 1ml of ice-cold homogenization buffer (0.25 M sucrose, 20 mM HEPES, pH 7.0, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, Complete™ protease inhibitor mixture) and homogenized by brief sonication. Unlysed cells and nuclei were removed by centrifugation at 1,000 X g for 15 min. Postnuclear supernatants were divided into four aliquots (200 µl each) and
centrifuged at 12,000 X g for 15 min. The pellets were resuspended in homogenization buffer containing 1.5 M NaCl; 0.2 M Na₂CO₃, pH 11.4; 5 M urea; or 1% Triton X-100, respectively. These suspensions were incubated on ice for 30 min then centrifuged at 100,000 X g for 15 min. After centrifugation, equal portions of each supernatant and pellet were diluted into equal volumes of 2 X SDS gel sample buffer and SDS-PAGE and immunoblotting were carried out as described above.

**Immunoprecipitation**--- For immunoprecipitation, the cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM PMSF, Complete™ protease inhibitor mixture). Total cell lysates were clarified by centrifugation at 10,000 X g for 10 min and protein concentrations were determined. Identical amounts of protein from each sample were precleared by incubation with protein A/G Sepharose 4 fast flow (Amersham Pharmacia Biotech) for 30 min at 4°C. After the removal of protein A/G Sepharose by brief centrifugation, the solution was incubated with 2 µg each of anti-hVps11, hVps16, hVps18, polyclonal anti-GFP, monoclonal anti-Myc antibodies, or control IgGs for overnight at 4°C. Immunoprecipitation of the antigen-antibody complex was accomplished by adding 40 µl of protein A/G Sepharose for 1 hr at 4°C. Sepharose bound proteins were solubilized in 40 µl of 1X SDS gel sample buffer. Samples were separated and analyzed by 4/20% gradient SDS-polyacrylamide
gels and then transferred onto Immobilon-P membranes. Western blots were detected with ECL detection kits.

Gel Filtration Chromatography--- COS-7 cells transiently cotransfected with expression constructs encoding Myc-hVps11, HA-hVps18, and GFP-rVps33a. After 24 hr for transfection, cells were washed ice-cold PBS and scraped into 1 ml of ice-cold homogenization buffer (20mM HEPES-KOH, pH7.4, 2mM EDTA, 1mM MgCl₂, and Complete™ protease inhibitor mixture). Cells lysed by 15 passage through a 25-gauge needle. Nuclei and unlysed cells were removed by spinning the lysate at 6,000 X g for 10 min. The postnuclear supernatants were centrifuged at 100,000 X g for 30 min at 4°C. The 0.5 ml of resulting supernatant was loaded onto a Superose 6 gel filtration column (HR 10/30) equilibrated in homogenization buffer prior to load. The column was run at a flow rate of 1.0 ml/min using ÄKTA explorer 10S (Amersham Pharmacia Biotech) and 1.0 ml of each fraction was collected and analyzed by SDS-polyacrylamide gel followed by Western blot analyses. The blots were incubated with anti-Myc, anti-HA, and anti-GFP antibodies for 2 hr at room temperature and then incubated in HRP-conjugated secondary antibodies. Molecular weight was estimated by HMW Marker Kit (Amersham Pharmacia Biotech).

Results
Cloning of human homologues of Yeast VPS11 and VPS18--- To identify mammalian homologues of the yeast Class C VPS genes, we searched the expressed sequence tag (EST) database using the BLAST (National Center for Biotechnology Information, Bethesda, MD) with the *S. cerevisiae* Vps11p as a query. A human EST clone (accession number: AA385518) fragment encoding a RING–H2 finger domain was identified. This cDNA fragment was used to probe a human brain cDNA library. Among the ten positive clones, the longest ORF was 2,823 bp encoding a 941-amino acid polypeptide (Fig. 1A). A BLAST search for the NR protein database using this ORF amino acid sequence as the query retrieved not only *S. cerevisiae* Vps11p but also homologues of other species at the high score range. We hereafter refer to this protein as human Vps11 (hVps11).

The hVps11 shares 25% overall identity of amino acid sequence with *S. cerevisiae* Vps11p. Additionally, hVps11 shows a distant homology to proteins of unknown function from *Arabidopsis thaliana* (accession number: AC007018), *Neurospora crassa* (accession number: AL355933), and *C. elegans* (accession number: Z46794) with 39, 34, and 26% identity, respectively.

The human protein homologue of yeast Vps18p, hVps18, was identified by searching the sequence database using *S. cerevisiae* Vps18p and the *Drosophila dor* protein as a query. The KIAA1475 cDNA (accession number: AB040908), was found contain an open reading frame of 976-amino acids (Fig. 1B), which displays 34% overall identity *Drosophila dor* protein and 26% identity with *S. cerevisiae*.
Furthermore, hVps18 is homologues to the *Arabidopsis thaliana* T12C242 protein (accession number: AC025417), the *C. elegans* Pep3-related protein (accession number: U23522), and the *Candida albicano* Vps18-related protein (accession number: AJ289080) with identity of 30, 25, and 20%, respectively.

*hVPS11 and hVPS18 are ubiquitously expressed in all tissues examined---* Northern blot analyses were performed to determine tissue distribution of hVPS11 and hVPS18 mRNAs. The hVPS11 mRNA (an ~3.2 kb transcript) was ubiquitously expressed with the lowest levels in the lung and liver (Fig. 2A). The mRNA expression pattern of hVPS18 (an ~4.0 kb transcript) was similar to that of hVPS11 (Fig. 2B).

*hVps11, hVps16, and hVps18 are expressed in cell lines from several species---* To determine cellular expression patterns for hVps11, hVps16, and hVps18, western blot analyses were performed on lysates from the following cell lines: HeLa, HEK293, COS-7, NRK, BHK. Anti-hVps11 and hVps18 antibodies recognized major bands of approximately 112 and 116 kDa, respectively, in lysates from all the cell lines tested (Fig. 3A, B). The anti-hVps18 antibody recognized an additional higher molecular weight band in COS-7 and BHK cells and an approximately 88 kDa protein band in HeLa, HEK293, COS-7 and NRK cell lines. This 88 kDa band may represent a hVps18 degradation product since it was not detected in membrane fractionation experiments (see Fig. 4A). The anti-hVps16
antibody recognized a major protein band migrating at approximately 97kDa in lysates from all cell lines tested (Fig. 3C).

*hVps11, hVps16 and hVps18 localize to both cytosolic and membrane compartments*--- Yeast Class C Vps proteins have been reported to be associated with the cytosolic face of vacuolar membranes (10-13). To determine intracellular localizations of hVps11, hVps16 and hVps18, postnuclear supernatants from HEK 293 cells were fractionated into cytosolic and membrane components, and were subjected to western blot analyses using antibodies recognizing the three proteins. The analyses showed that all the three proteins exist in both the cytosolic and membrane fractions (Fig. 4A). In control experiments, EEA1 was also detected in both cytosolic and membrane fractions from HEK 293 cells in agreement with published reports (29), whereas the transmembrane protein, syntaxin-7, was detected in the membrane fraction (23). These results suggest that the human Class C Vps proteins cycle between the cytosolic and membrane-bound pools.

*hVps11, hVps16 and hVps18 are membrane-associated*--- Although hVps11, hVps16 and hVps18 lack a putative transmembrane region, they are found to be in the membrane fractions from HEK293 cells. To elucidate the molecular mechanism of their membrane association, membrane fractions from HEK293 cells were further extracted with 1.5 M NaCl, 5 M urea, 0.2 M sodium bicarbonate at pH 11.4, or 1% Triton X-100, respectively. The extracts were
Western blotted for hVps11, hVps16, and hVps18. All three proteins were highly solubilized with urea, sodium bicarbonate and Triton X-100, but only partially extracted with in 1.5 M NaCl (Fig. 4B). These results suggest that the proteins are primarily soluble and associated weakly with the cytosolic face of membranes.

**hVps11, hVps16, and hVps18 associate with late endosomes/lysosomes**--- To further determine the intracellular localizations of hVps11, hVps16, and hVps18, immunocytochemistry was performed on COS-7 cells. As shown in Fig. 5, all three proteins showed a vesicular and cytosolic staining. Staining was eliminated when antibodies were preincubated with the respective antigen (data not shown). Essentially the same stainings were observed in HeLa, NRK and BHK cells (data not shown). We then compared the immunostaining patterns for hVps11, hVps16, and hVps18 with those of EEA1, transferrin receptor (TfR), and lysosome associated membrane protein-1 (Lamp-1). EEA1, TfR and Lamp-1 were selected for comparison as standard markers for early endosomes (29, 30), recycling endosomes/plasma membranes (31, 32), and late endosomes/lysosomes (33), respectively. The staining patterns of hVps11, hVps16, and hVps18 overlapped significantly with that of Lamp-1 (Fig. 5). These observations indicate that human Class C Vps proteins are associated primarily with late endosomes/lysosomes, and are compatible with the fact that their yeast counterparts are involved in the late step transport to vacuoles.

*Human Class C Vps proteins interact with each other in vivo*---
Previous studies have shown that yeast Class C Vps proteins form a complex within cells (12, 16-18). To test whether such a complex is formed also in mammalian cells, interactions of endogenous human Class C Vps proteins were examined by immunoprecipitation experiments. As shown in Fig. 6A, anti-hVps11 antibody co-immunoprecipitated hVps18 and hVp16. Similarly, antibodies against hVps18 and hVps16 co-immunoprecipitated the other two proteins (Fig. 6B and C, respectively). These data unequivocally indicate that those three proteins interact with each other in vivo.

hVps11, hVps18, and rVps33a are constitute a large oligomeric complex-- We also analyzed whether the other mammalian Class C Vps protein, rVps33a is included in the complex. For this purpose, expression vectors for Myc-, HA- and GFP tagged mammalian Class C Vps proteins were cotransfected into COS-7 cells. HA-hVps18 and GFP-rVps33a efficiently co-immunoprecipitated with Myc-hVps11 (Fig. 7A). Similarly, Myc-hVps11 and hVps18 co-immunoprecipitated with GFP-rVps33a (Fig. 7B).

To verify the co-immunoprecipitation data, GFP-rVps33a transfected COS-7 cells were immunostained anti-hVps11, hVps18, and hVps16 antibody. GFP-rVps33a demonstrated a vesicular staining pattern and colocalized with hVps11 immunoreactivity (Fig. 7C). Similar colocalization was observed for hVps18 and hVps16 (data not shown).

In order to analyze whether mammalian Class C Vps proteins constitute a
complex in vivo, COS-7 cells expressing myc-hVps11p, HA-hVPS18p, and GFP-rVps33a were subjected to gel filtration analyses. In cytosolic fraction of transfected COS-7 cells, these three molecules migrated to the fractions corresponding to high molecular masses (>670 kDa), suggesting that they form a large oligomeric protein complex (Fig. 7D).

Taken together with the data in Fig. 6 and Fig. 7, these results indicate that mammalian Class C Vps proteins constitute a hetero-oligomeric complex in vivo, and play roles for late endosome/lysosomal trafficking pathway.

*hVps11 and hVps18 are associated with syntaxin-7----* Previous reports have described that the Class C Vps complex binds to Vam3p but not Vam7p or Vti1 suggesting that it may function prior to trans-SNARE pairing and is required for vesicle docking/fusion reactions (16, 19). It has also been reported that the mammalian counterpart of yeast Vam3p, syntaxin-7, is responsible for mediating endocytic trafficking to late endosomes as well as late endosome-lysosome and lysosome hetero/homotypic fusion (24-26). To examine whether this interaction is conserved between yeast and mammals, Myc-tagged human syntaxin-7 (Myc-hSyn-7) and either HA-tagged hVPS11 or hVPS18 were transfected into COS-7 cells and subjected to immunoprecipitation analyses. Myc-hSyn-7 was co-immunoprecipitated with both HA-hVps11 and HA-hVps18 (Fig. 8A). Similarly, when the inverse immunoprecipitation was performed, HA-tagged hVps11 and hVps18 were found to co-immunoprecipitate with Myc-hSyn-7 (Fig. 8B).
To verify this result, Myc-hSyn-7 transfected COS-7 cells were immunostained anti-hVps11, hVps18, and hVps16 antibody. Myc-hSyn-7 showed a vesicular staining pattern and colocalized with hVps11 immunoreactivity in some vesicle structures (Fig. 8C). Similar localization was observed for hVps18 and hVps16 (data not shown). This interaction of mammalian Class C Vps proteins and syntaxin-7 indicate that mammalian Class C Vps complex functions are at the late endocytic trafficking and membrane docking/fusion reactions of late endosomes/lysosomes.

**Discussion**

The yeast Class C Vps proteins form a large hetero-oligomeric protein complex and mediate the delivery of vacuolar hydrolases to vacuoles and regulates homotypic vacuole fusion through interactions with Vam3p (12, 16-19). In the present study, we identified two human homologues of yeast Class C Vps proteins, hVps11 and hVps18. Furthermore, we showed that these proteins along with other Class C Vps proteins, hVps16 and rVps33a, constitute a hetero-oligomeric complex, interact with the Vam3p homologue, syntaxin-7, and associate *en bloc* with membranes of late endosomes/lysosomes.

A search of the database revealed the presence of homologues of these proteins in a variety of eukaryotic organisms, including yeasts, fungi, fly, nematode, plants and mammals. Furthermore, these proteins are highly conserved across
eukaryotic species not only in the primary structure but also in the domain organization, suggesting that they share common functions in membrane trafficking. For example, both hVps11 and hVps18 and their counterparts in other species contain a C-terminal RING-H2 finger domain, a variant of the RING finger domain. The RING-H2 finger domain differs from the RING finger by the presence of a second histidine at the corresponding position of the fourth cysteine in the RING finger domain (Fig. 1C). These domains are found in various proteins that form multiprotein complexes, including those with known roles in membrane trafficking (14,15). EEA1 has a specific lipid-binding domain, FYVE finger, which is also a variant of the RING finger domain. The FYVE finger domain binds to PtdIns(3)P on early endosome membranes with high specificity and is required for early endosome localization of EEA1 (34-37). It is likely that the RING-H2 finger domains of hVps11 and hVps18 are also involved in protein-protein and/or protein-lipid interactions. In addition, hVps11 and hVps18 are predicted to form one or more α-helical coiled-coil domain (38). The coiled-coil domain is conserved in a broad spectrum of membrane fusion proteins, suggesting a similar function for this domain in the Class C Vps proteins (39). Finally, hVps11 and hVps18 have a highly conserved sequence related to a region of the clathrin heavy chain repeat (CHCR) domains required for clathrin heavy chain self assembly, and light chain binding and trimerization (Fig. 1A, B). The CHCR domains are also found in other proteins implicated in vacuole protein sorting, such as
Vam2p/Vps41p and Vam6p/Vps39p (40, 41), suggesting that the domains are responsible for their complex formation. We are currently investigating the roles of these domains in the complex formation and association with their target membranes of the mammalian Class C Vps proteins.

Northern blot and immunoblot analyses of the mammalian Vps proteins showed that they are expressed in a wide variety of tissues and cell lines, suggesting that these proteins may have common physiological functions. Previous studies in yeast demonstrated that Class C Vps proteins cofractionated with vacuolar membranes (12). Our cellular fractionation and subcellular localization studies showed that mammalian Class C Vps proteins are soluble proteins that are weakly associated with the cytosolic face of endosome/lysosome membranes. Furthermore, our immunoprecipitation and gel filtration analyses unequivocally demonstrated that all the mammalian Class C Vps proteins together constitute a hetero-oligomeric protein complex like the yeast counterparts. Homologues of Vps18p and Vps33p have been also identified in Drosophila; Dor and Car, respectively, whose mutations cause defects in eye pigmentation (20, 21). Car and Vps33p belong to the family of Sec1p-related proteins, which are essential for vesicle docking and fusion by binding to the syntaxin like SNAREs (21,42). biochemical studies have demonstrated that Dor and Car are part of a large protein complex associated with endosomal membranes. Phenotypic characterization of the dor and car mutants has indicated defects in the lysosomal
delivery of internalized ligands and the biogenesis of the pigment granules, a compartment related to the vacuole/lysosomes (21). Collectively, the organization and functions of the Class C Vps protein complex appear to be conserved from yeast through Drosophila to mammals.

Several lines of evidence have suggested that, in yeast, the molecular complex including Class C Vps proteins contain two additional regulators of vacuolar fusion and docking, Vam2p/Vps41p and Vam6p/Vps39p. This complex was termed HOPS (homotypic fusion and vacuole protein sorting) complex and is essential for homotypic vacuole fusion and vacuole protein sorting (17, 18). Although it is not entirely clear whether mammalian Class C Vps proteins interact with the counterparts of Vam2p/Vps41p and Vam6p/Vps39p, the structural similarities between the yeast and mammalian Class C Vps proteins suggest that this may be the case.

Syntaxin-7 shares 24% identity with yeast Vam3p and is ubiquitously expressed in multiple tissues tested (22). It is localized to the late endosomes and is required for late endosome and lysosome fusion. Induced expression of mouse syntaxin-7 lacking the transmembrane domain block endocytic transport from early endosome to late endosome (24,25). Likewise, microinjection into cells of bacterially expressed syntaxin-7 lacking the transmembrane domain or of anti-syntaxin-7 antibodies inhibits homotypic lysosome fusion and heterotypic late endosome/lysosome fusion but has no affect on homotypic early endosome fusion
(26). Although there is some discrepancy regarding the localization of mammalian syntaxin-7, the syntaxin-7 expression pattern is very similar to that of hVps11 and hVps18, and both hVps11 and hVps18 interact with Myc-tagged human syntaxin-7. Taken together, it seems likely that mammalian Class C Vps proteins are also required for late endosome/lysosome fusion and the endocytic transport pathway.

Many questions still remain regarding the roles of mammalian Class C Vps proteins. Our results suggest that Class C Vps proteins are structurally and functionally conserved among yeast, Drosophila and mammals. Understanding the interaction of mammalian Class C Vps proteins with syntaxin-7 will likely provide significant clues for studying membrane docking and fusion events in mammalian cells.

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**Footnotes**

The nucleotide sequence of hVPS11 has been submitted to GenBank™/EBI/DDBJ database with accession numbers AB027508 and NM_021729.
Abbreviations: PMSF, phenylmethylsulfonyl fluoride; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; PtdIns(3)P, phosphatidylinositol-3-phosphate; SNARE, soluble N-ethylmaleimide sensitive factor attachment protein receptor
Legends for Figures

Fig. 1. Amino acid sequences and structures of human Vps11 and Vps18. (A, B) Amino acid sequences of human Vps11 and Vps18. The carboxyl-terminal RING-H2 finger domain is boxed (schematic representation is shown in green) with conserved cysteine and histidine residues represented as bold within the box. The clathrin heavy chain repeat (CHCR) domain is shown in bold (schematic representation is shown in blue), and the coiled-coil domain is thick-underlined and bold-oblique (schematic representation is shown in purple). The ATP/GTP binding motif is thin-underlined and bold (schematic representation is shown in black). (C) Amino acid alignments of RING-H2 finger domains are shown. Conserved cysteine and histidine residues are boxed with purple and blue, respectively.

Fig. 2. Analyses of hVPS11 and hVPS18 mRNA expression in human multiple tissues. Northern blot analysis of hVPS11 (A) and hVPS18 (B) in selected human tissues was performed. Two micrograms per lane of poly(A)+ RNA isolated from different human tissues (Clontech) were hybridized to radiolabeled hVPS11 and hVPS18 fragments. Expression of the housekeeping gene, ubiquitin, was used as an internal loading control (bottom). The exposure shown for hVPS11 was overnight (A), while that for hVPS18 was 5 days (B).
Fig. 3. **Expression of human homologues of Class C Vps proteins in several cell lines.** Western blotting of 30 mg of lysates from HeLa, HEK293, COS-7, NRK, and BHK cells (Lane1-5) was performed using anti-hVps11(A), hVps18 (B), and hVps16 (C) polyclonal antibodies. Antibody specificity was confirmed by preincubation of anti-hVps11, hVps18, and hVps16 with their antigens (lane 6-10).

Fig. 4. **Subcellular distribution of human homologues of Class C Vps proteins.** (A) The cytosol and total membrane fractions derived from HEK 293 cells were resolved by SDS-PAGE and processed for immunoblot analysis using antibodies against hVps11, hVps18, hVps16, EEA1, and syntaxin-7. (B) Postnuclear membrane pellet fractions from HEK 293 cells were extracted with various disruptive agents and centrifuged 100,000 X g and the resulting supernatants (S) and pellets (P) were analyzed by SDS-PAGE and immunoblotted with anti-hVps1, hVps18, and hVps16 antibodies.

Fig. 5. **Immunolocalization of hVps11, hVps18, and hVps16.** Double labeling of hVps11 (panel a, d, g), hVps18 (panel j), and hVps16 (panel m) with endosomal/lysosomal markers in COS-7 cells was performed. hVps11 immunoreactivity (Texas red; a, d, g) was compared with immunofluorescence (FITC) of TfR (panel b), EEA1 (panel e) and Lamp-1 (panel h). hVps18 and hVps16 immunoreactivities were compared with immunoreactivity for Lamp-1.
Arrowheads indicate examples of structures positive for hVps11 (panel g), hVps18 (panel j), hVps16 (panel m), and Lamp-1 (panel h, k, n). Merged images are shown in panels c, f, i, l, o. Arrows indicate examples of colocalized vesicles. *Bar* = 10µm.

Fig. 6. **Co-immunoprecipitation of human Class C Vps proteins in HEK 293 cells.** 1% NP-40 soluble fractions were immunoprecipitated from postnuclear supernatants from HEK 293 cells using affinity-purified polyclonal antibodies recognizing hVps11, hVps18, and hVps16 (control rabbit IgG was used as a negative control). Immunoprecipitates were analyzed by SDS-PAGE using the indicated antibodies: (A) hVps11 immunoprecipitation blotted with anti-hVps18 and hVps16 antibodies. (B) hVps18 immunoprecipitation blotted with anti-hVps11 and hVps16 antibodies. (C) hVps16 immunoprecipitation blotted with anti-hVps11 and hVps18 antibodies.

Fig. 7. **Subcellular localization of GFP-rVps33a and Gel filtration analyses of mammalian Class C Vps proteins in transfected COS-7 cells.** COS-7 cells were transiently cotransfected with combinations of Myc-hVps11, HA-hVps18, GFP alone, and GFP-rVps33a. Immunoprecipitations were performed from lysates using (A) anti-hVps11 and (B) anti-GFP. Immunoprecipitates were Western blotted using anti-HA antibody, anti-GFP antibody, and anti-Myc antibody. Rabbit
IgG immunoprecipitates were used as negative controls. Total cell lysates were Western blotted to assess expression of (A) Myc-hVps11, HA-Vps18, GFP-Vps33a and (B) GFP alone, GFP-rVps33a, Myc-hVps11, and hVps18. (C) GFP-rVps33a expressing COS-7 cells were immunostained using anti-hVps11 antibody to assess colocalization. Bar = 10 µm. (D) Cytosolic fraction prepared from COS-7 cells transiently cotransfected with expression vectors encoding Myc-hVps11, HA-hVps18, and GFP-rVps33a was chromatographed, and analyzed with SDS-PAGE. The immunoblots were probed with an anti-Myc, anti-HA, and anti-GFP antibodies to detect the proteins.

Fig. 8. **hVps11 and hVps18 interact with human syntaxin-7.** COS-7 cells were cotransfected with indicated combinations of HA-hVps11 or hVps18, Myc-hSyn-7. After 48 hr, total cell lysates were prepared and immunoprecipitated with anti-hVps11 (A, lane 2), anti-hVps18 (A, lane 4), or anti-Myc antibodies (B, lane 2, 4). Rabbit IgG immunoprecipitates served as negative controls (A, lane 1, 3; B, lane 1, 3). Immunoprecipitates were Western blotted using anti-Myc and anti-HA antibodies to detect co-precipitated proteins. Total cell lysates were Western blotted with anti-HA and anti-Myc antibodies to assess expression of HA-hVps11, HA-hVps18, and Myc-hSyn-7. (C) Myc-hSyn-7 expressing COS-7 cells were immunostained using anti-hVps11 antibody to assess localization. Arrowheads indicate examples of structures positive for hVps11 and Myc-hSyn-7. Arrows
indicate examples of colocalized vesicles. *Bar* = 10µm.
Fig. 2

(A) 

| tissue   |
|----------|
| heart    |
| brain    |
| placenta |
| lung     |
| liver    |
| muscle   |
| kidney   |
| pancreas |

(B) 

- ubiquitin
Fig. 3

A

|        | HeLa | HEK293 | COS-7 | NRK | BHK |
|-------|------|--------|-------|-----|-----|
| 1     |      |        |       |     |     |
| 2     |      |        |       |     |     |
| 3     |      |        |       |     |     |
| 4     |      |        |       |     |     |
| 5     |      |        |       |     |     |

Blot: α-hVPS11

α-hVPS11 + antigen

B

|        | HeLa | HEK293 | COS-7 | NRK | BHK |
|-------|------|--------|-------|-----|-----|
| 1     |      |        |       |     |     |
| 2     |      |        |       |     |     |
| 3     |      |        |       |     |     |
| 4     |      |        |       |     |     |
| 5     |      |        |       |     |     |

Blot: α-hVPS18

α-hVPS18 + antigen

C

|        | HeLa | HEK293 | COS-7 | NRK | BHK |
|-------|------|--------|-------|-----|-----|
| 1     |      |        |       |     |     |
| 2     |      |        |       |     |     |
| 3     |      |        |       |     |     |
| 4     |      |        |       |     |     |
| 5     |      |        |       |     |     |

Blot: α-hVPS16

α-hVPS16 + antigen
Fig. 7A, B, and C

A

|                | 1 | 2 | 3 | 4 | 5 | 6 |
|----------------|---|---|---|---|---|---|
| Myc-hVPS11     | + | + | + | + | + | + |
| HA-hVPS18      | - | + | + | - | + | - |
| GFP-rVPS33a    | - | - | - | + | + | + |

Blot: α-HA

Blot: α-GFP

IP: α-hVPS11

Control IgG

B

|                | 1 | 2 | 3 | 4 |
|----------------|---|---|---|---|
| GFP-alone      | + | + | - | - |
| GFP-rVPS33a    | - | - | + | + |
| Myc-hVPS11     | + | - | + | + |
| Myc-hVPS18     | - | + | + | - |

IP: α-GFP(poly)

Blot: α-Myc

total cell lysates

1 2 3 4

Myc-hVPS11

HA-hVPS18

GFP-rVPS33a

GFP-alone

GFP-rVPS33a

C

hVPS11

GFP-rVPS33a

Merged
Molecular characterization of mammalian homologues of Class C Vps proteins that interact with syntaxin-7
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