The Role of LIP5 and CHMP5 in Multivesicular Body Formation and HIV-1 Budding in Mammalian Cells*

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We examined the function of LIP5 in mammalian cells, because the yeast homologue Vta1p was recently identified as a protein required for multivesicular body (MVB) formation. LIP5 is predominantly a cytosolic protein. Depletion of LIP5 by small inhibitory RNA (siRNA) does not affect the distribution or morphology of early endosomes, lysosomes, or Golgi but does reduce the degradation of internalized epidermal growth factor receptor (EGFR), with EGFR accumulating in intracellular vesicles. Depletion of LIP5 by siRNA also decreases human immunodeficiency virus type 1 (HIV-1) budding by 70%. We identify CHMP5 as a LIP5-binding protein and show that CHMP5 is primarily cytosolic. Depletion of CHMP5 by siRNA does not affect the distribution or morphology of early endosomes, lysosomes, or Golgi but does result in reduced degradation of the EGFR similar to silencing of LIP5. Surprisingly, CHMP5 depletion results in an increase in the release of infectious HIV-1 particles. Overexpression of CHMP5 with a large carboxyl-terminal epitope affects the distribution of both early and late endocytic compartments, whereas overexpression of LIP5 does not alter the endocytic pathway. Comparison of overexpression and siRNA phenotypes suggests that the roles of these proteins in MVB formation may be more specifically addressed using RNA interference and that both LIP5 and CHMP5 function in MVB sorting, whereas only LIP5 is required for HIV release.

Endosomes play a crucial role in transporting molecules from the plasma membrane to intracellular compartments as well as transporting molecules from the biosynthetic apparatus to their site of action (1–3). Several different endosomal compartments have been described based on morphology, constituents, and role within the endocytic apparatus. The multivesicular body (MVB)1 is an endosomal compartment that serves to sort membrane proteins destined for degradation or routing to the lysosome (4–6). These proteins are internalized into vesicles that form by the invagination of the limiting membrane (forming intravesicular vesicles). The contents of the MVB are then transferred to lysosomes. The physiologic importance of MVBs has been shown through studies in organisms as diverse as yeast and humans (5, 7–11). In yeast, the role of the MVB has been defined through the identification of mutations that alter protein sorting through the MVB (12). A specific class of sorting mutants, class E vacuolar protein sorting (VPS) mutations, results in the accumulation of transport cargo in a prevacuolar compartment and a selective deficit in the delivery of that cargo to vacuoles (7, 13–16). The class E phenotype is described as an enlarged late endosomal compartment, which presumably arises because of an inability to invaginate the limiting membrane that would normally form the MVB. In higher eukaryotes, studies on the internalization and degradation of growth factor receptors as well as studies on viral budding have helped to elucidate the importance of MVBs, because many enveloped RNA viruses utilize components of the MVB to bud from cells (8, 11).

Recently, Vta1p was shown to play a role in MVB sorting in Saccharomyces cerevisiae, and it was shown that Vta1p interacts with Vps4p (17, 18). Analysis of mammalian Vps4p/SKD1-interacting proteins identified a protein, SBP1 (19). Examination of genomic data bases showed that SBP1 (or the data base gene name DRG-1) was homologous to Vta1p, with most of the homology in the carboxyl terminus of the reported sequence. A yeast two-hybrid screen identified LIP5 as a protein that interacted with CHS1/Lyst (20). LIP5 and DRG-1 are identical with the exception that the carboxyl terminus of LIP5 is distinct from that of DRG-1. Our sequencing of LIP5 cDNA is in accord with the suggestion of Fujita et al. (19) that LIP5 is, in fact, the same as DRG-1. The sequence of LIP5/DRG-1 is homologous to Vta1p; the two proteins show 20.2% identity and 48.9% similarity. In this paper we demonstrate that LIP5 is homologous to Vta1p and like Vta1p functions in MVB sorting. Depletion of LIP5 by siRNA reduces the degradation of epidermal growth factor receptor (EGFR) and reduces HIV-1 budding. We further show that LIP5 specifically interacts with CHMP5/Hspc177, the homologue of the yeast protein Vps60p (21). Depletion of CHMP5 by RNA interference alters the degradation of EGFR, but in contrast to LIP5, CHMP5 silencing leads to an increase in HIV particle release. Finally, we show that there are marked differences on the behavior of the endocytic apparatus in cells depleted for CHMP5 compared with cells in which CHMP5 with a large epitope is overexpressed.
LIP5 and CHMP5 in the Endocytic Pathway

DNA oligonucleotides

| DNA oligonucleotides | Sequence |
|----------------------|----------|
| LIP5pET              | CCGCTCGAGATGGGGGGCTTGAGGCCC |
| LIP5pRSET            | CGGGATCCATATGCCTCTGGGTTTCA |
| LIP5F                | GAAGATCTTTTCTGGCAGGCTG |
| Chmp2F               | CAATGACTGCTTGATAGTGAG |
| Chmp2RFLAG           | CGGGTCTACTTGTTCATGGCAGG |
| Chmp4F               | GAAGATCTTTTCTGGCAGGCTG |
| Chmp4RFLAG           | CGGGTCTACTTGTTCATGGCAGG |
| Chmp5R               | CGGGTCTACTTGTTCATGGCAGG |
| Chmp5RFLAG           | CGGGTCTACTTGTTCATGGCAGG |
| RNA oligos           | DNA and RNA oligonucleotides used in this study |

**MATERIALS AND METHODS**

**Cells and Reagents**—HeLa cells, fibroblasts, HeK293T cells, and Cos-7 cells were maintained in Dulbecco's minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Alexa 594-dextran, Alexa 594-transferrin, Alexa 594-phal.

**Structures**—LIP5 and CHMP5 were PCR-amplified from I.M.A.G.E. Consortium clone 3961637 and GST-CHMP fusion clone (22). All other primers used in generating constructs are listed in Table I. All of the clone sequences were verified at the University of Utah Cores Sequencing Facility.

**Antibody Production**—LIP5 was cloned into pET-16 (Stratagene, La Jolla, CA) and expressed as a LIP5-HIS fusion protein in E. coli as per the manufacturer's instructions. LIP5 protein was purified on nickel-nitrioltriacetic acid columns (Qiagen). CHMP5 was cloned into pGEX-2T (Amerham Biosciences), expressed in E. coli as a CHMP5-GST fusion protein and purified without the GST as per the manufacturer’s instructions. Purified LIP5 or CHMP5 proteins were emulsified with Freund’s complete adjuvant (Sigma) and injected into host animals. Following subsequent immunizations with recombinant protein, serum was obtained and tested for immunoreactivity by Western analysis.

**Fluorescence Microscopy**—Transiently transfected Cos-7 cells, HeLa cells, or mouse fibroblasts expressing GFP or DsRed epitope-tagged proteins were imaged live using an epifluorescence microscope with a 100× oil immersion objective. The cells expressing GFP or DsRed epitope-tagged proteins were fixed using 3.7% formaldehyde with PBS, 2.0% paraformaldehyde, or –20 °C methanol. The cells were permeabilized in either 0.1% saponin with PBS or 0.2% Triton X-100, 1.0% bovine serum albumin and stained for either Lamp2 (1:200; HAB1461 or GL2A7 Developmental Hybridoma, Iowa City, IA), EGFR (1:200; NeoMarkers, Fremont CA), p230 (BD Bioscience, San Jose, CA), or mannose-6-phosphate receptor (1:1000, a gift from Dr. Peter Lobel). For detection of FLAG-tagged proteins, mouse anti-FLAG (Sigma) was used at a dilution of 1:750. The secondary antibodies included Alexa 488-conjugated goat anti-mouse IgG (1:750), Alexa 594-conjugated goat anti-rabbit IgG (1:5000) (Jackson ImmunoResearch), Alexa 647-conjugated goat anti-mouse IgG (1:750), Alexa 488-conjugated goat anti-rabbit IgG (1:750), and Alexa 594-conjugated goat anti-rabbit IgG (1:750) (Molecular Probes, Eugene, OR) diluted in 0.1% saponin with PBS and bovine serum albumin. The cells were visualized using an epifluorescence microscope (Olympus Inc., Melville, NY) with a 100× oil immersion objective. The images were acquired using MetaFire analysis software for confocal microscopy, the images were collected at single wavelengths on an Olympus FVX confocal fluorescence microscope with a 60× Planapo objective (1.4 numerical aperture oil) using Fluoview 2.0.39 software.

**Affinity Purification**—Transfected cells were washed and scraped into PBS with 1× protease inhibitor cocktail and 1.0 mM phenylmethylsulfonyl fluoride and pelleted at 1,000 × g for 5 min. The cells were solubilized using 1.0% Triton X-100 with PBS plus 1× protease inhibitor cocktail plus 1.0 mM phenylmethylsulfonyl fluoride at 4 °C. The supernatants were obtained, and each supernatant was added to 100 μl of anti-FLAG (M2) agarose beads at 4 °C overnight. The beads were pelleted and washed 10 times with PBS. The beads were eluted three times with 1.0 mg/ml FLAG peptide and then eluted twice with 0.1 M glycine, pH 2.5. For Western analysis, the samples were run on 10% SDS-PAGE, transferred to nitrocellulose, and probed for proteins of interest.

**Mass Spectrometric Analysis of Proteins**—For mass spectrometry analysis, the samples were separated on 10% SDS-PAGE and stained by Coomassie Blue. Bands of interest were excised and analyzed by the University of Utah Mass Spectrometry Core Facility. Each excised protein gel band was transferred to a 1.5-ml tube and destained. In-gel digestion was then performed by adding 100 μl ammonium bicarbon-
ate containing modified trypsin (Promega, Madison, WI) at a 1:50 enzyme-substrate ratio and incubated at 37 °C overnight. Tryptic peptides were extracted twice in 100 µl extraction buffer (0.1% trifluoroacetic acid, 5% acetonitrile, pH 2.5). Two extracted peptide solutions were combined and dried in a Speed-Vac device. Analysis was carried out using a Simadzu HPLC system interfaced to a Finnigan LCQ Deca ion trap mass spectrometer (Finnigan MAT, San Jose, CA) with electrospray ionization. Each tryptic peptide sample was reconstituted in 10 µl of 5% acetonitrile, 0.1% formic acid and was injected onto an HPLC column (Thermo Hypersil-Keystone, Bellefonte, PA; 100 × 0.2 mm, 5-µm particle size). A 53-min gradient of 10–80% solvent B (solvent A was 5% acetonitrile, 0.1% formic acid; solvent B was 80% acetonitrile, 0.1% formic acid) was selected for separation of peptides. The spectra were acquired in automated triple play mode for recording of mass and mass spectrometry/mass spectrometry data. The scan range for mass spectrometry mode was set at m/z 400–1800. Automated analysis of peptide tandem mass spectra was performed with SEQUEST/MASCOT computer algorithms for protein identification.

RNA Interference—siRNA pools matching selected regions of LIP5 and CHMP5 cDNA sequences and a random sequence pool were purchased from Dharmacon Research (Lafayette, CO). Individual RNA oligonucleotide sequences are listed in Table I. siRNA for TSG101 was as described (23). Transfections were performed on HeLa cells or 293T plated at 50% confluence using OligofectAMINE reagent (Invitrogen) with siRNA pools at a final concentration of 100 nM. Twenty-four hours post-transfection, the cells were trypsinized and plated onto 100-mm plates. Forty-eight hours later the cells were either processed for immunofluorescence microscopy or solubilized in 1.0% Triton, PBS, protease inhibitor cocktail with phenylmethylsulfonyl fluoride followed by SDS-PAGE and Western analysis probing for either LIP5, CHMP5, TSG101, or EGFR.

Viral Replication Assays—Human 293T cells in 6-well plates were transfected twice at 24-h intervals with 1.33 µg of siRNA using Lipofectamine 2000 (Invitrogen) as described previously (23). The cells were cotransfected with an HIV-1 vector system comprising the following plasmids: 0.38 µg of pCMVΔR8.2 (24), 0.12 µg of pRSV-Rev (25), 0.12 µg of pMD.G (26), and 0.38 µg of pWPTS-nlsLacZ (www.tronolab.unige.ch) during the second transfection. Supernatants containing virions were harvested 48 h after the second transfection, and the cells were harvested at the same time for Western blot analyses. To measure titers, virus particles were diluted 1:100 in Dulbecco’s minimal essential medium containing 10% fetal calf serum, 1-glutamine, and 20 µg/ml DEAE-dextran, and infectivity was assayed by transducing human HeLa-M cells with the diluted vector in 96-well plates. After 24 h, the cells were fixed with 0.5% glutaraldehyde, washed twice with PBS, and stained with an X-gal staining solution containing 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM MgCl₂, and 0.4 mg/ml X-gal. For Western analysis the following primary antibodies were used: rabbit anti-HIV CA antibody from Hans-Georg Krausslich (Heidelberg, Germany; at 1:4000), rabbit anti-HIV MA from Didier Trono (Geneva, Switzerland; at 1:40,000), and murine monoclonal anti-TGSI014A10 from Genetex, Inc. (San Antonio, TX; at 1:1000).

RESULTS

Characterization and Subcellular Distribution of LIP5—To examine the role of LIP5 in membrane trafficking in mammalian cells, we generated a polyclonal antibody directed against a bacterially expressed LIP5. The specificity of the LIP5 antibody was examined by SDS-PAGE and Western analysis. The antisera detected recombinant LIP5 protein, and this interaction could be blocked by preincubation of anti-LIP5 antisem with recombinant LIP5 (Fig. 1A). LIP5 has a predicted molecular mass of 34 kDa, but the recombinant protein showed an apparent molecular mass of 39 kDa on SDS-PAGE. The yeast LIP5 homologue, Vta1p, also migrates more slowly than an apparent molecular mass of 39 kDa on SDS-PAGE. The molecular mass of 34 kDa, but the recombinant protein showed a slightly higher molecular mass shown) was cytosolic. LIP5-GFP has a predicted molecular mass of 61 kDa but showed a slightly higher molecular mass when analyzed by SDS-PAGE. Overexpression of LIP5-GFP or LIP5-FLAG did not affect membrane trafficking in the endocytic or secretory pathways, as determined by localization of organelle specific markers including Alexa 594-Tf, Alexa 594-
Lamp2 (Fig. 3 showed no alteration in the distribution of Alexa 594-Tf or transfected with LIP5-GFP. A, 24 h post-transfection cells were either incubated with 5 μg/ml Alexa 594-Tf or Alexa 594-EGF for 30 min at 37 °C and imaged live using an epifluorescence microscope or cells were fixed, permeabilized, and incubated with antibodies to p230 (a trans-Golgi marker) or Lamp2 (a late endosome/lysosomal marker) followed by Alexa 594-conjugated goat anti-mouse IgG and then imaged. B, cells expressing LIP5-GFP were incubated with Alexa 594-conjugated dextran (molecular weight 10,000) at 37 °C for 60 min. The cells were washed extensively and imaged for LIP5-GFP and Alexa 594-dextran localization.

EGF, p230 (trans-Golgi marker), and Lamp2 (late endosome/lysosomal marker) (Fig. 2A). Similar results were observed for Tf receptor and EGFR (data not shown). The distribution of a fluid phase marker, Texas Red dextran, which is found in endosomes and lysosomes, was unaffected upon overexpression of GFP-LIP5 (Fig. 2B).

Decreased expression of LIP5, however, revealed a role for LIP5 in MVB trafficking. Decreased expression of LIP5 was accomplished by transfecting HeLa cells with siRNA pools directed against LIP5. No loss of LIP5 protein was seen in cells transfected with a random siRNA pool (Fig. 3A) or siRNA directed against Lamin (data not shown). The average reduction in LIP5, as determined by Western analysis, was ~85% (n > 10). The cells transfected with siRNA against LIP5 showed no alteration in the distribution of Alexa 594-Tf or Lamp2 (Fig. 3B). The yeast homologue of LIP5, Vta1p, plays a role in MVB formation, and deletion of VTA1 results in a marked decrease in the degradation of internalized plasma membrane proteins (17, 18). To test whether the loss of LIP5 affects the degradation of plasma membrane proteins in mammalian cells, we examined the effect of LIP5 silencing on the internalization and degradation of EGFR. In control cells, either untransfected cells or cells transfected with a random siRNA pool, the addition of EGF resulted in the rapid degradation of EGFR (Fig. 3C). In contrast, the addition of EGF to LIP5 siRNA-treated cells led to decreased degradation with a 2-fold change in EGFR half-life. The addition of nonspecific RNA interference oligonucleotides had no effect on the rate of EGFR degradation.

A change in EGFR degradation could reflect a decrease in proteolysis or a decrease in internalization of receptor from the cell surface. To distinguish between these possibilities, we examined cells for EGFR distribution by immunofluorescence. EGFR is found predominantly on the cell surface (Fig. 3D). The addition of EGF to control cells or cells transfected with a random siRNA pool resulted in the disappearance of cell surface EGFR and a marked reduction in immunofluorescence throughout the cell. In cells depleted of LIP5, the addition of EGF resulted in the disappearance of EGFR from the cell surface and the accumulation of EGFR in large intracellular vesicles. The increased accumulation of EGFR was observed in 53% (n = 37/70) of the cells silenced for LIP5. Incubation with EGF did not affect transferrin receptor or Lamp2 distribution in LIP5 silenced cells (data not shown). These results demonstrate that LIP5 is involved in the sorting and down-regulation of the EGFR. In the absence of LIP5, the degradation of EGFR is reduced, and EGFR accumulates in intracellular vesicles.

LIP5 Interacts with CHMP5—To determine whether LIP5 interacts with other proteins, we expressed GFP-LIP5-FLAG in Cos-7 cells, solubilized cells with detergent, and affinity-purified LIP5 using anti-FLAG beads followed by elution with FLAG peptide. SDS-PAGE/Coomassie analysis of the eluate revealed two bands, overexpressed GFP-LIP5-FLAG, and a second protein of ~27 kDa (Fig. 4A), which was identified by mass spectrometry as Hscp177/CHMP5 (n = 3). This protein belongs to a family of small coiled-coil proteins (21). The S. cerevisiae homologue of CHMP5 is Vps60p, a class E Vps protein whose precise role in MVB formation has not been defined (4). We generated a rabbit polyclonal antibody to bacterially expressed CHMP5. The antibody detected two prominent bands in bacterial lysates expressing GST-CHMP5, but a 27-kDa band was dramatically diminished when the antibody was preabsorbed with purified recombinant CHMP5 (Fig. 4B). Based on these data and silencing data discussed below, we conclude that the 27-kDa protein is CHMP5. Subcellular fractionation followed by Western analysis showed CHMP5 to be primarily cytosolic (Fig. 4C). The CHMP5 antibody did not work for immunofluorescence or immunoprecipitation. To examine CHMP5 localization in vivo, we generated a CHMP5 protein with either a carboxyl-terminal GFP or DsRed. CHMP5-DsRed or CHMP5-GFP was found on vesicles near the nucleus (Fig. 4D). Coexpression of CHMP5-DsRed and LIP5-FLAG resulted in LIP5-GFP, a cytosolic protein, now associating with the CHMP5-DsRed vesicles.

There are 10 mammalian CHMP proteins that exhibit a series of common features including a predicted coiled-coil motif. It is therefore possible that other CHMP proteins interact with LIP5 through conserved sequence or structural elements. To test this possibility, we generated FLAG-tagged CHMP2A, CHMP4B, and CHMP5 constructs, transfected the constructs into Cos-7 cells, purified the FLAG-tagged CHMP proteins using FLAG affinity resin, and examined the eluates for copurified LIP5. Purification of CHMP5-FLAG resulted in the co-purification of endogenous LIP5 (Fig. 4E). Purification of either CHMP2A-FLAG or CHMP4B-FLAG did not result in the copurification of endogenous LIP5. These data demonstrate that the
association of LIP5 and CHMP5 is specific.

CHMP5 Overexpression/Silencing Alters Trafficking in the Endocytic Pathway—Studies have shown that expression of other CHMP proteins with a large amino- or carboxyl-terminal epitope affected the endocytic apparatus (22, 27–30). Expression of CHMP5-DsRed (data not shown) or CHMP5-GFP altered the distribution of early as well as late endocytic markers including Alexa 594-Tf and EGFR (Fig. 5A). In contrast, LIP5-GFP overexpression did not alter early or late endocytic trafficking (Fig. 2). Overexpression of CHMP5-DsRed or CHMP5-GFP resulted in an altered localization of CHMP5; CHMP5-DsRed (data not shown) or CHMP5-GFP (Fig. 5A) was now found to be associated with membranes. In contrast, endogenous CHMP5, as determined by Western analysis (Fig. 4C), or overexpressed CHMP5 with a smaller carboxyl-terminal FLAG epitope was cytosolic (data not shown). The CHMP-DsRed or GFP constructs are suggested to act as dominant negatives (22, 27–28). To characterize the role of CHMP5 in endocytic trafficking more precisely, we compared the effect of CHMP5-GFP/DsRed overexpression to depletion of CHMP5 by siRNA. Cells transfected with siRNA pools specific for CHMP5 showed an 80% decrease (n > 8) in CHMP5 protein as determined by Western analysis (Fig. 5B). No reduction in CHMP5 protein was seen in cells transfected with either random or LIP5-specific siRNA. Depletion of CHMP5 by siRNA did not alter the distribution of Alexa 594-Tf or Lamp2 (Fig. 5C). CHMP5 depletion did, however, affect EGFR trafficking (Fig. 5D). When CHMP5 protein levels were decreased, degradation of the EGFR was reduced, with a half-life of 16 min compared with 8 min in cells transfected with random oligonucleotide pools (Figs. 3D and 5E). Simultaneous depletion of both CHMP5 and LIP5 did not lead to further changes in the rate of degradation of EGFR, and the morphology of the vesicles in which EGFR accumulated was similar to LIP5 silencing alone (data not shown). After the addition of EGF to CHMP5-GFP-expressing cells, the EGFR was found in enlarged perinuclear vesicles associated with CHMP5-GFP (Fig. 5A). These vesicles were much larger than the EGFR vesicles seen in EGF-treated cells that were depleted of CHMP5, suggesting that overexpression of a large epitope-tagged CHMP5 gives rise to a different phenotype compared with CHMP5 depletion. A small but reproducible morphologic difference in EGFR localization was observed in cells depleted of LIP5 compared with cells depleted of CHMP5; EGFR appeared to accumulate in enlarged perinuclear vesicles in cells depleted of LIP5, whereas in cells depleted of CHMP5 EGFR accumulated in smaller structures that showed a broader cell distribution (Fig. 3E versus Fig. 5D).

Reduction in LIP5 Protein Levels Alters HIV Budding—In addition to the sorting and degradation of internalized membrane proteins, depletion or alteration of a number of MVB proteins in mammalian cells reduces HIV-1 budding efficiency (11, 31–33). To determine whether LIP5 or CHMP5 also play a role in viral budding, we measured the release of virus-like particles and infectious HIV-1 particles from cells transfected with siRNA oligonucleotides designed to deplete LIP5, CHMP5, or TSG101 (positive control) (Fig. 6). An siRNA oligonucleotide

![Fig. 3. Depletion of LIP5 reduces EGFR degradation.](image-url)
encoding the TSG101 inverted sequence was also used as a negative control (23).

All three target proteins were effectively depleted, as determined by Western blotting of cellular extracts, and the intracellular Gag expression levels did not change significantly upon depletion of any of the target proteins. As expected, TSG101 depletion, the apparent mammalian orthologue of Vta1p, plays a role in mammalian MVB formation. First, EGFR down-regulation is dependent upon a functional MVB sorting pathway. We demonstrate that reducing the levels of LIP5 by siRNA decreases the rate of degradation of EGFR and that EGFR accumulates in a mammalian class E compartment. We also demonstrate that the reduction in EGFR degradation in LIP5 depleted cells is not due to alterations in EGFR localization or EGFR-EGF internalization. Second, reduction in LIP5 levels also reduces the production of infectious HIV-1 particles. Other mammalian proteins involved in MVB formation have been shown to be important in viral budding (11, 31–33). Reduction in LIP5 levels resulted in a 70% reduction in HIV-1 viral vector release and infectivity. Surprisingly, depletion of LIP5 levels resulted in a 70% reduction in HIV-1 viral vector release and infectivity (23/24). Although the change in infectivity varied significantly between different experiments (n = 6), the levels were invariably increased by CHMP5 depletion. We therefore conclude that the presence of CHMP5 in the cell actually inhibits viral vector release and infectivity. This is the first example in which depletion of a protein implicated in MVB formation increased viral particle release.

**DISCUSSION**

The *S. cerevisiae* homologue of LIP5, Vta1p, is a protein involved in MVB formation (17, 18). At least 17 cytosolic "class E" proteins are required for MVB sorting in *S. cerevisiae*, and many of these proteins are grouped based on complementation analyses or the complexes in which they are found (4, 34). These complexes are called ESCRT I, II, and III with the nomenclature corresponding to the order in which they act in MVB formation (4). To date, all of the proteins required for MVB formation in yeast have mammalian orthologues (4, 10), and functional studies show their involvement in MVB formation (9, 22, 23, 35–37). Here, we provide two lines of evidence that LIP5, the apparent mammalian orthologue of Vta1p, plays a role in mammalian MVB formation. First, EGFR down-regulation is dependent upon a functional MVB sorting pathway. We demonstrate that reducing the levels of LIP5 by siRNA decreases the rate of degradation of EGFR and that EGFR accumulates in a mammalian class E compartment. We also demonstrate that the reduction in EGFR degradation in LIP5 depleted cells is not due to alterations in EGFR localization or EGFR-EGF internalization. Second, reduction in LIP5 levels also reduces the production of infectious HIV-1 particles. Other mammalian proteins involved in MVB formation have been shown to be important in viral budding (11, 31–33). Reduction in LIP5 levels resulted in a 70% reduction in HIV-1 viral particle release.

Overexpression of LIP5 with a large epitope did not alter the endocytic apparatus as assessed by the localization of endogenous markers (Alexa 594-Tf, Alexa 594 EGF, p230 trans-Golgi...
marker, and Lamp2) or the uptake of a fluid phase marker, whereas overexpression of other proteins with large epitope tags that are involved in MVB formation does alter the endocytic apparatus (22, 27, 28, 30). Our studies show that LIP5 interacts with Hspc177/CHMP5. The \textit{S. cerevisiae} homologue of CHMP5 is Vps60p/Mos10, a class E VPS protein involved in MVB sorting (21). Previously, we demonstrated that the yeast homologues of these two proteins, Vta1p and Vps60p, were involved in MVB sorting and also interacted physically (18). Silencing of either LIP5 or CHMP5 protein demonstrated that both proteins play a role in the degradation of the EGFR. The effect of silencing these proteins on EGFR half-life is similar to that seen when Hrs (mVps27) or mVPS37A, proteins that act early in MVB sorting, is silenced (36, 38). Silencing both LIP5 and CHMP5 simultaneously did not result in an additive effect on EGFR degradation.

CHMP5 is a member of a large family of small coiled-coil proteins (21). Ten CHMP proteins have been identified in mammals, and six have been identified in yeast (4, 10). These proteins have a conserved domain organization but show only modest sequence similarity. Expression of CHMPs fused to large epitope tags in normal endocytic structures. These structures are endosomes that contain both early and lysosomal markers. Hybrid organelles have been seen in other cases, for example when cells are treated with the phosphatidylinositoll 3-kinase inhibitor wortmannin (39). However, hybrid organelles are not typically seen when MVB proteins are silenced. We have considered two explanations for these hybrid organelles. One possibility is that the large epitope on an individual overexpressed CHMP protein results in the amplification of weak nonspecific interactions between different CHMPs and their targets. These interactions lead to aberrant sorting/fusion of vesicles. A second possibility is that CHMP proteins containing large epitope fusions can enter into membrane-bound ESCRT sorting complexes but then become trapped in aberrant assemblies that cannot be efficiently recycled and therefore effectively deplete the cell of multiple ESCRT (and perhaps other) sorting factors. Regardless of which

![Figure 5](image-url)
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act sequentially. The yeast data suggest that Vta1p interacts with Vps20p, a member of the ESCRT III complex. Deletion of VPS60 results in the prolonged retention of Vta1p on the class E compartment, indicating that Vps60p is required for the recycling of Vta1p (18). Based on these results, LIP5, the mammalian equivalent of Vta1p, may recruit CHMP5. The presence of CHMP5 may affect the property of the MVB, resulting in larger EGF-R vesicles, which leads to a change in viral budding properties. The exact effect of LIP5 on MVB formation is currently unknown.

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