CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins

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SUMMARY

A multivesicular body is a vesicle-filled endosome that targets proteins to the interior of lysosomes. We have identified a conserved eukaryotic protein, human CHMP1, which is strongly implicated in multivesicular body formation. Immunocytochemistry and biochemical fractionation localize CHMP1 to early endosomes and CHMP1 physically interacts with SKD1/VPS4, a highly conserved protein directly linked to multivesicular body sorting in yeast. Similar to the action of a mutant SKD1 protein, overexpression of a fusion derivative of human CHMP1 dilates endosomal compartments and disrupts the normal distribution of several endosomal markers. Genetic studies in Saccharomyces cerevisiae further support a conserved role of CHMP1 in vesicle trafficking. Deletion of CHM1, the budding yeast homolog of CHMP1, results in defective sorting of carboxypeptidases S and Y and produces abnormal, multi-lamellar prevacuolar compartments. This phenotype classifies CHM1 as a member of the class E vacuolar protein sorting genes. Yeast Chm1p belongs to a structurally-related, but rather divergent family of proteins, including Vps24p and Snf7p and three novel proteins, Chm2p, Chm5p and Chm6p, which are all essential for multivesicular body sorting. These observations identify the conserved CHMP/Chmp family as a set of proteins fundamental to understanding multivesicular body sorting in eukaryotic organisms.

Key words: Biological transport, Endosomes, Vacuoles, Carboxypeptidases, Nuclear matrix, Gene silencing

INTRODUCTION

Vesicle trafficking provides communication and transport between membrane-bound compartments involved in biosynthesis, degradation and cell surface signaling. Within this membrane-defined network, endosomes function as intermediate sorting sites that link trafficking between the trans-Golgi, lysosomes and plasma membrane (Gruenberg and Maxfield, 1995). Vesicle budding from endosomes, outward into the cytoplasm, removes cargo for recycling back to the cell surface or Golgi apparatus (Mellman, 1996), prior to maturation of the endosomes and their fusion with lysosomes (Bright et al., 1997; Futter et al., 1996; Mullock et al., 1998; van Deurs et al., 1995). The decision to recycle or degrade an endocytosed receptor protein is determined, in part, by competition between this outward endosomal vesicle budding and inward vesicle budding into the endosome. This latter process results in formation of a vesicle-filled endosome, the multivesicular body (MVB) (Felder et al., 1990; Friend, 1969; Hopkins and Trowbridge, 1983). MVB formation places receptor proteins and integral membrane hydrolases within the endosome (Odorizzi et al., 1998), which enables delivery to the lumen of the lysosome after fusion of endosome-lysosome membranes (Luzio et al., 2000).

Since MVB formation occurs in simple eukaryotes (Hicke et al., 1997; Li et al., 1999; Prescianotto-Baschong and Riezman, 1998; Wurmser and Emr, 1998), budding yeast is a useful model system for delineating this process. Genetic screens in Saccharomyces cerevisiae have identified a set of genes, the class E VPS, required for MVB sorting (Odorizzi et al., 1998; Odorizzi et al., 2000). These genes belong to a much larger group whose mutation results in defective vacuolar protein sorting (vps) (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986). vps mutants aberrantly secrete a soluble vacuolar hydrolase, carboxypeptidase Y (CPY), to the extracellular space (Bryant and Stevens, 1998; Raymond et al., 1992; Robinson et al., 1998; Rothman and Stevens, 1986). Class E vps mutants exhibit an additional, distinctive defect in protein sorting: an integral-membrane hydrolase, carboxypeptidase S (CPS), cannot be targeted to the vacuole lumen, its normal site of action. Instead, CPS remains on the vacuole membrane, a fate shared with endocyotosed alpha-factor receptor in class E vps mutants (Li et al., 1999; Odorizzi et al., 1998). Ultrastructural analysis of class E vps mutants by electron microscopy reveals an enlarged, aberrant endosomal structure comprising stacked membranes, the class E compartment (Raymond et al., 1992; Rieder et al., 1996). In combination, these observations strongly suggest that the primary defect in class E vps mutants is the inability to properly invaginate endosomal membrane to form an MVB (Odorizzi et al., 1998).

CPY missorting screens in budding yeast have identified 13 class E VPS genes (Raymond et al., 1992). Six of these genes have been analyzed molecularly: VPS4, VPS23, VPS24, VPS27, VPS28 and SNF7/VPS32 (Babst et al., 2000; Babst et al., 1997; Babst et al., 1998; Li et al., 1999; Piper et al., 1995; Rieder et al., 1996). The encoded proteins have no reported structural similarity, and only a pair of functional inter-
CHMP1 fusion proteins were constructed using the introduced into p2FLAG to generate p2FLAG-SKD1(EQ). pMBP-SKD1(EQ). In addition, the SKD1(EQ) coding sequence was transferred into pmal-c2 (New England Biolabs) to generate pMBP-SKD1 and full-length human (GenBank AF281063) into the unique MVB formation but, to date, no class E Vps protein has been demonstrated to bind it directly. In addition, the relationship between the identified class E Vps proteins and a postulated coat complex has not been elucidated (Wurms et al., 1999).

Mammals have structural relatives of many or all identified yeast class E Vps proteins (Odorizzi et al., 1998), and functional conservation of two members has been demonstrated. Overexpression of an ATPase-defective human SKD1/VPS4 in mammalian cells results in altered protein sorting and dilated endosomal compartments, structures potentially analogous to the class E compartment observed in vps4 mutant yeast (Bishop and Woodman, 2000; Yoshimori et al., 2000). Similarly, deletion of mouse HRS, which encodes a mammalian structural counterpart to yeast Vps27p (Komada et al., 1997), produces enlarged, transferrin receptor-positive endosomal structures (Komada and Soriano, 1999).

We have identified a mammalian protein, CHMP1, which physically interacts with human SKD1, localizes to early endosomes and can disrupt membrane trafficking when overexpressed in mutant form. CHMP1 is structurally conserved in simple and complex eukaryotes and has led to the identification of a family of proteins (Chmps) required for yeast MVB sorting. Structural similarities within the CHMP/Chmp family suggest cooperative or sequential action of these proteins in a related aspect of MVB formation.

MATERIALS AND METHODS

Plasmid construction

Parental vectors were obtained or constructed as follows. CS2+ was a gift from Dave Turner. βAct was constructed from CS2+ by replacing the cytomegalovirus regulatory region with a 300 nucleotide fragment from the promoter of rat β-actin. p2FLAG is a pcDNA3 derivative that encodes a tandem array of FLAG epitopes within the polylinker and was a gift from Phil Stork (Oregon Health Science University). p316-ADH was constructed by inserting the S. cerevisiae ADH promoter and terminator from pBTM116 (Hollenberg et al., 1995) into pRS316 (Sikorski and Hieter, 1989). pLex-A is a pBTM116 derivative containing the ADE2 gene of S. cerevisiae (Chen et al., 1996).

Two hybrid bait plasmid pLex-CHMP1 was constructed by introducing the full-length human CHMP1 coding sequence (GenBank AF281063) into the unique BamHI site of pLex-A. The full-length human SKD1 coding sequence (GenBank AF159063), obtained from two-hybrid screening with CHMP1, was mutagenized with sense and antisense 5’ CTCAGTCCTTCACTCGACCAGTT-CGATTCCTCGGGG 3’ primers using the QuikChange Site-Directed Mutagenesis kit (Stratagene) to produce SKD1(E228Q). The entire open reading frames of SKD1 and SKD1(EQ) were transferred to pmal-c2 (New England Biolabs) to generate pMBP-SKD1 and pMBP-SKD1(EQ). In addition, the SKD1(EQ) coding sequence was introduced into p2FLAG to generate p2FLAG-SKD1(EQ).

Plasmid expression vectors for Escherichia coli expression of CHMP1 fusion proteins were constructed using the EcoRI/BamHI fragment of CHMP1. This fragment was end-filled and introduced into pmal-c2 (New England Biolabs) or pGEX-kT (Guan and Dixon, 1991) to produce MBP-CHMP1 or GST-CHMP1, respectively. GFP fusion expression vectors were constructed as follows. The CHMP1 termination codon was removed by amplification with a sense primer and antisense primer 5’ GCAATCGAGAACCTTC-CTCAAGGGCGCAACCT 3’. The product was fused to GFP coding sequences at its C-terminus in the vector CS2+ to create CS-CHMP1-GFP. The coding sequence of SKD1(EQ) was fused at its N-terminus to GFP sequences and introduced into βAct (see above) to create βAct-GFP-SKD1(EQ). The plasmid encoding a GFP-CPS fusion was constructed based on the strategy described previously (Odorizzi et al., 1998). Briefly, an expression cassette was constructed that contained the following elements: the ADH promoter from pBTM116 controlling a GFP-CPS fusion, with GFP fused at its C-terminus to full-length CPS coding sequences. This expression cassette was introduced in pRS421, a 2 µm MET15 plasmid (Brachmann et al., 1998) to generate 421-GFP-CPS. 316-ADH-CHM1 was constructed from 316-ADH (see above) by introducing the S. cerevisiae CHM1 open reading frame generated by amplification with 5’ GCAATCGAGACCTTCAACATAATTTGAGCGACAGGGTTTG 3’ and 5’ GGCACACTGAGTAACTAAA-AATGCAATAGCTTACAGTG 3’.

Two-hybrid screen

Yeast strain L40 containing pLex-CHMP1 was used to screen human brain and leukocyte libraries in pAct2 (Clontech). Transformants were plated onto CSM(-His, -Trp, -Leu) (Bio101) containing 1 mM 3-aminotriazole.

Cell culture, immunocytochemistry and antibodies

HEK-293 (human embryonic kidney) or COS7 (African green monkey kidney) cells were cultured on eight-well Nunc SuperCell culture slides (Intemountain Scientific) in Dulbecco’s modified eagle medium with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin (Gibco BRL). Twenty-four hours after plating, cells were directly fixed (see below) or transfected using LipofectAMINE in the presence of Opti-MEM1 (Gibco BRL). Five hours post-transfection, cells were washed, fed using DMEM with serum, and incubated for another 24 hours. Cells were fixed with 4% paraformaldehyde, washed, blocked, permeabilized and exposed to primary and secondary antibodies. Rabbit polyclonal anti-CHM1P1 (1:300) was made by injection of GST-CHMP1 fusion protein mixed with Freund’s incomplete adjuvant into rabbits by Pocono Rabbit Farm & Laboratory Inc. The antibody was subsequently purified over a MBP-CHMP1 Affigel (Bio-Rad) column and eluted with low pH. The CHMP1 antibody does not crossreact with any of the other CHMP proteins we have identified, including closely related CHMP2. Mouse monoclonal antibodies used were specific for transferrin receptor (B3/25.4; 1:100; a gift of Ian Trowbridge, The Salk Institute), lysobisphosphatidic acid (6C4; 1:10; a gift of Jean Gruenberg, University of Geneva), Rab7 (1:300; a gift of Suzanne Pfeffer, Stanford), early endosomal antigen-1 (EEA1; Transduction Laboratories; 1:200), cation-independent mannose-6 phosphate receptor (M6PR; 1:300; a gift of William Brown, Cornell University), LAMP-1 (PharMingen; 1:100), mannosidase II (1:20; a gift of Kelly Moremen, University of Georgia), and γ-adaptin (HAA3; Sigma; 1:50). Secondary antibodies used were anti-rabbit Alexa 488 (Molecular probes; 1:300) and anti-mouse Cy3 (Jackson Immunoresearch Laboratories Inc; 1:300), and included Hoechst 33258 (Molecular Probes) at 1.0 µg/ml. Samples were visualized with a Zeiss Axioplan microscope or by laser scanning confocal microscopy on a BioRad MRC 1000.

Percoll gradient and membrane fractionation

The Percoll gradient analysis was performed with HEK293 cells, as
described previously (Czekay et al., 1997) with some modifications (Press et al., 1998). Membrane fractionation analysis was carried out based on a published method (Wan et al., 1998). HEK293 cells were harvested and washed in PBS. Cells were resuspended in 1.5 ml of 10 mM Tris-HCl pH 7.4, 2.5 mM MgCl$_2$, protease inhibitors) and centrifuged for 1 hour at 100,000 g yielding a high salt supernatant and final pellet. The final pellet was resuspended in 0.1% SDS, protease inhibitors) for gel analysis or resuspended in 100 µl of high salt buffer (10 mM Tris-HCl pH 7.4, 5 mM DTT, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitors) and centrifuged for 1 hour at 100,000 g yielding a high pH supernatant and final pellet. The final pellet was resuspended in 100 µl of RIPA buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl, with protease inhibitors) and centrifuged for 1 hour at 100,000 g yielding a high pH supernatant and final pellet. This pellet was then resuspended in 100 µl of high salt buffer (10 mM Tris-HCl pH 7.4, 750 mM NaCl, with protease inhibitors) and centrifuged for 1 hour at 100,000 g yielding a high salt supernatant and membrane-associated pellet. The pellet was then resuspended in 0.1% SDS, protease inhibitors for gel analysis or resuspended in 100 µl of high salt buffer (10 mM Tris-HCl pH 7.4, 750 mM NaCl, with protease inhibitors) and centrifuged for 1 hour at 4°C in a TLA 45 rotor (Beckman), resulting in a soluble supernatant and membrane-associated pellet. The pellet was then resuspended in 0.1% SDS, protease inhibitors for gel analysis or resuspended in 100 µl of high salt buffer (10 mM Tris-HCl pH 7.4, 2.5 mM MgCl$_2$), Nuclei were pelleted by centrifugation at 5000 g for 10 minutes at 4°C in a Sorvall HB4 rotor. The upper layer consisting of cytoplasmic extract was collected and concentrated to 100 µl in a Centricon 10 column. This fraction was centrifuged at 100,000 g for 1 hour at 4°C in a TLA 45 rotor (Beckman), resulting in a soluble supernatant and membrane-associated pellet. The pellet was then resuspended in 100 µl of RIPA buffer (10 mM Tris-Cl pH 11.0, 100 mM Na$_2$CO$_3$, protease inhibitors) and centrifuged for 1 hour at 100,000 g yielding a high pH supernatant and final pellet. The final pellet was resuspended in 100 µl of RIPA buffer. Equal cell equivalents were resolved by SDS-PAGE and CHMP1 was visualized by western blot analysis.

**Co-immunoprecipitation and pulldown assays**

For co-immunoprecipitations, 293 cells were transfected with LipofectAMINE (Gibco-BRL). Forty-eight hours after transfection the cells were washed twice in ice cold PBS, scraped in PBS, pelleted, resuspended in 0.1 M sodium phosphate, pH 7.8, containing 0.1% NP-40, and lysed by repeated freeze-thaw cycles. Co-immunoprecipitation reactions contained 20 µl anti-FLAG M2 affinity gel (Sigma) and 200 µg cell lysate, and were incubated for 2 hours at 4°C. Following several washes in TENN (20 mM Tris-Cl, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), proteins bound to the resin were resolved by SDS-PAGE and analyzed by western analysis with anti-CHMP1 antibody.

MBP-fusions for in vitro binding assays were purified over amylose resin according to the manufacturer’s instructions (New England Biolabs). The yield and purity of each fusion protein was determined by SDS-PAGE. Based on this analysis, equal amounts of fusion protein and resin were added to an equal volume of binding buffer. CHMP1 and GFP were synthesized using the TNT SP6 coupled reticulocyte lysate system (Promega) in the presence of [35 S]methionine. An aliquot of in vitro translated protein was incubated with amylose resin-bound protein (MBP, MBP-SKD1, or MBP-SKD1(EQ)) in 25 µl NETT (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100) for 2 hours at 4°C. Binding reactions were subsequently washed in NETT, and analyzed by SDS-PAGE and autoradiography.

**Yeast strains**

BY4741 and isogenic deletions of chm2, vps24, snf7, chm6 and vps4 were obtained from the Saccharomyces Deletion Project through Research Genetics. CHM1 and CHM5 were replaced by LEU2 in either BY4741 or the snf7 deletion strain using PCR-mediated gene disruption (Wach, 1996). Homologous recombination was confirmed by 5’ and 3’ PCR amplification.

**Carboxypeptidase S sorting assay**

Yeast cultures expressing GFP-CPS were cultured in CSM(-Met) (Bio101), harvested at mid-log phase, and pulse-labeled with 20 mM FM4-64 (Molecular Probes) at 30°C (Vida and Emr, 1995). Samples were visualized by laser scanning confocal microscopy on a BioRad MRC 1000.

**Fig. 1.** Cytoplasmic CHMP1 colocalizes with endosomes. COS7 cells were double-labeled with antibodies to CHMP1 and the indicated trafficking markers, and visualized by indirect immunofluorescence. Labeled structures are early endosomes, TFR and EEA1; late endosomes, Rab7; lysosomes, LAMP-1; medial and trans-Golgi, mannosidase II (Man II); and trans-Golgi, γ-adaptin (γ-adap). Note that CHMP1 and TFR patterns are most highly related.

**CPY sorting assay**

CPY sorting was measured based on several previously described methods (Gaynor et al., 1994; Klionsky et al., 1988; Vida et al., 1990). Yeast was grown in CSM (-Met) to mid-log phase. Cells were spheroplasted for 30 minutes in 0.5 ml CSM containing 1 M sorbitol and 2 µl zymolyase 100T, washed in 1.2 M sorbitol, and resuspended to 10 OD$_{600}$/ml with 135 µl CSM (-Met) containing 1 M sorbitol, 1 mg/ml BSA, and 0.2 U α-glucuronidase type HP-2S (Sigma) and 2.5 µg/ml BSA. Cells were pulse-labeled for 10 minutes with 100 µCi [35 S]methionine at 30°C. Chase was initiated with the addition of chase solution (to a final concentration of 0.2% yeast extract, 5 mM methionine, 1 mM cysteine). After a 40 minute incubation, cultures were spun at 13,000 g to produce supernatant (extracellular; EC) and pellet (intracellular; IC) fractions. EC was adjusted to 1% SDS, IC was resuspended in 150 µl resuspension buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS) and samples were boiled for 4 minutes. After brief pelleting, supernatants were transferred to fresh tubes containing 1 ml IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20) and immunoprecipitated with 1.75 µg CPY antibody (Molecular Probes, mouse monoclonal 10A5-B5). Antibody was bound with 20 µl 50% Protein A/G beads (Santa Cruz). Complexes were washed with NETT and RIPA (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) and resolved by SDS-PAGE.

**Electron microscopy**

Cells were fixed and stained as described previously (Banta et al., 1988) with the following modifications. Cells were fixed overnight at 4°C in 3% glutaraldehyde. Spheroplasts were produced with 70 units/ml β-glucuronidase type HP-2S (Sigma) and 2.5 mg/ml Zymolase 20T (Seikagaku Corporation) for 20 minutes at 30°C in 1.2 M sorbitol in 0.1 M KPO$_4$, pH 4.0. Subsequent to staining, samples were dehydrated and embedded in Epon 812 resin. Sections were analyzed on a transmission electron microscope (Phillips CM120). Cell counts resulted from inspecting the cytoplasm of cells that were stained sufficiently to visualize the plasma and nuclear membrane. Cells with multi-lamellar cytoplasmic membrane structures were scored as positive.
RESULTS

Mammalian CHMP1 is localized to early endosomes

CHMP1 was identified in a two-hybrid screen for Polycomb-like partners (Stauffer et al., 2001). A CHMP1-specific antibody was generated, affinity-purified and used to define the sites of CHMP1 expression. Western analysis of cell lines and tissues demonstrated that CHMP1 is widely expressed and found in both the cytoplasm and nuclear matrix (Stauffer et al., 2001). We have used immunocytochemistry to investigate the localization of CHMP1 in individual cells and report here its distribution and role within the cytoplasm. CHMP1 distributes to a punctate, asymmetrical pattern centered over the microtubule organizing center (data not shown), a pattern similar to several membrane-bound compartments involved in vesicle trafficking. To more accurately define the compartment to which CHMP1 localizes, cells were double-labeled with antibodies to CHMP1 and JOURNAL OF CELL SCIENCE 114 (13)

Fig. 2. CHMP1 is peripherally associated with the membranes of early and late endosomes. (A) HEK293 cytoplasmic extract was centrifuged at 100,000 g to produce soluble (S100) (lane 1) and membrane-associated (P100) (lane 2) fractions. The membrane pellet was sequentially extracted with 750 mM NaCl (lane 3), 0.1 M Na2CO3 (lane 4), and then the residual was completely solubilized (lane 5). Comparable cell-equivalents from each treatment were resolved by SDS-PAGE and analyzed by western blot for CHMP1. (B) Human 293 cytoplasmic extract was layered on, and centrifuged through, a Percoll gradient. The indicated numbered fractions were collected from the top and analyzed by western blot for CHMP1 and the other early (EEA1) and late (Rab7) endosome, and lysosome (LAMP-1) markers. Note that CHMP1 overlaps well with EEA1, but is also detectable in more dense fractions.

Fig. 3. CHMP1 physically interacts with human SKD1/VPS4 in vitro and in vivo. (A) CHMP1 and a control GFP protein were radiolabeled by in vitro translation and incubated with purified, immobilized MBP, MBP-SKD1 or MBP-SKD1(EQ). Bound protein was eluted, resolved by SDS-PAGE and visualized by fluorography. The input lane represents one-tenth the amount of radiolabeled protein used in each binding reaction. Note that only CHMP1 efficiently binds both wild-type and mutant (EQ) SKD1. (B) COS7 cells were transiently transfected with plasmids that produce unfused- or SKD1(EQ) -fused FLAG epitope, plus (lanes 1-4) or minus (lanes 5,6) a CHMP1 expression vector. Cell extracts were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma), and bound material was analyzed by SDS-PAGE, followed by western blot with CHMP1 antibody. Note that SKD1(EQ) can immunoprecipitate endogenous CHMP1 (lane 6). (C) Transfected COS7 cells overexpressing GFP-SKD1(EQ) were analyzed by immunocytochemistry for changes in the endogenous CHMP1 distribution. Transfected (arrowhead) and untransfected (arrow) cells are indicated. Cells were visualized by fluorescence microscopy.
proteins that mark endosomes (transferrin receptor (TfR); early endosomal antigen (EEA1); and lysobisphosphatidic acid (6C4)), lysosomes (LAMP-1), and regions of the Golgi apparatus (mannosidase II (Man II); γ-adaptin). As shown in Fig. 1, although there is considerable overlap between compartments, the CHMP1 pattern is most highly coincident with the early endosomes defined by the transferrin receptor (Trowbridge et al., 1993).

The membrane-association of CHMP1 was further defined by high speed centrifugation analysis. Cytoplasmic extracts from HEK293 cells was centrifuged at 100,000 g for 1 hour to produce soluble (S100) and membrane pellet (P100) fractions. Western analysis demonstrates that the majority of CHMP1 is soluble, but a reproducible fraction pellets with membranes (Fig. 2A, compare lanes 1,2). Sequential treatment of the membrane fraction with high salt and alkali reveals that the CHMP1 is almost quantitatively released by treatment with high salt (Fig. 2A, compare lanes 3-5). This indicates that CHMP1 is peripherally associated with membranes through ionic interactions, an observation consistent with the charged nature of CHMP1 (see below).

This biochemically defined membrane interaction was used to confirm and extend the immunocytochemical localization of CHMP1. Others have demonstrated that early and late endosomes and lysosomes can be resolved, based on their differing densities, by centrifugation through a Percoll gradient (Czekay et al., 1997; Press et al., 1998). We have used this approach to measure the distribution of CHMP1. As shown in Fig. 2B, the bulk of CHMP1 is found at the very top of the gradient, colocalized with soluble proteins (data not shown), which is consistent with the partial membrane association of Fig. 2A. In addition, measurable levels of CHMP1 enter the gradient and overlap with the early endosome pattern defined by EEA1 (Mu et al., 1995). Interestingly, detectable CHMP1 also co-migrates with Rab7 in late endosome fractions that do not exhibit an EEA1 signal (Fig. 2B, fractions 6-8), indicating that CHMP1 is distributed between both early and late endosomes, consistent with its overlap with the TfR by immunocytochemistry (Fig. 1)

**Human CHMP1 interacts with trafficking protein SKD1**

To gain insight into the function of CHMP1, it was fused to LexA and used as bait in a yeast two-hybrid screen (Hollenberg et al., 1995) of human brain and leukocyte cDNA fusion libraries. Remarkably, 80% of the positives corresponded to full-length human VPS4/SKD1 (Bishop and Woodman, 2000; Scheuring et al., 1999; Yoshimori et al., 2000). Mammalian SKD1 is a close structural relative of an ATPase in yeast, Vps4p, which is required for correct vacuolar protein sorting and belongs to the class E VPS group (see Introduction) (Banta et al., 1988; Raymond et al., 1992; Robinson et al., 1988). Babst and co-workers have demonstrated that Vps4p regulates the membrane association of two other class E VPS gene products, Vps24p and SNF7p/Vps32p, through ATP hydrolysis (Babst et al., 1998).

The interaction between CHMP1 and SKD1 has been confirmed using an in vitro binding assay. For this analysis, binding to wild-type SKD1 and an ATPase-defective mutant derivative SKD1(EQ) (Bishop and Woodman, 2000) was compared. Each was expressed in E. coli as a fusion with maltose-binding protein (MBP) and affinity purified. As shown in Fig. 3A, in vitro translated CHMP1 shows significant binding to MBP-SKD1(EQ) above MBP alone (compare lanes 6,7). Furthermore, introduction of the inactivating E to Q mutation in SKD1 appears to increase CHMP1 binding (Fig. 3A, compare lanes 7,8). Although the increase is small, it is reproducible within each experiment.
The ability of CHMP1 and SKD1 to interact was also measured in cellular extracts. Based on the moderate increased binding activity of the SKD1(EQ) mutant in vitro, this point mutant was used in a co-immunoprecipitation assay. COS7 cells were co-transfected with a GFP-tagged SKD1(EQ), and the subcellular localization of CHMP1 was measured by indirect immunofluorescence. The GFP-SKD1(EQ) fusion protein produces a punctate cytoplasmic pattern, as was observed previously (Bishop and Woodman, 2000), which is always matched accurately by locally increased CHMP1 signal (Fig. 3B). Overall, these in vitro and in vivo assays demonstrate a partnership between CHMP1 and SKD1.

**Mutant CHMP1 and SKD1 produce similar effects on endosomal trafficking**

To further define the properties of CHMP1, its C-terminus was fused to GFP and the subcellular pattern analyzed after transient transfection. CHMP1-GFP localizes to structures in the cytoplasm that are visible by phase-contrast microscopy (Fig. 4A, arrowhead). Similar structures are produced by expression of GFP-SKD1(EQ) (Bishop and Woodman, 2000; Yoshimori et al., 2000), but not by unfused GFP (Fig. 4A). The physical interaction between CHMP1 and SKD1, and the similar appearance of the corresponding, overexpressed GFP-fusions, suggest that each GFP-fusion is altering common aspects of endosomal trafficking, and that the fusion of GFP to CHMP1 changes the properties of the protein. We therefore consider GFP-CHMP1 to be a functionally mutant protein. We have tested this idea by comparing the effects of each fusion protein on the morphology of endosomal structures. As shown in Fig. 4B, in comparison of untransfected (arrows) with transfected cells (arrowheads) shows that each of these GFP-fusion proteins strongly affects the distribution of both early (EEA1) and late (LBPA/M6PR) endosomal markers. In general, the fine punctate distribution produced with each marker is converted to larger diameter structures, indicating dilation and fusion of trafficking compartments. The GFP-signal from these fusion proteins only partially colocalizes with the endosomal patterns, suggesting both direct and indirect effects on membrane flow within several trafficking pathways. This analysis provides further support for functional similarity between CHMP1 and SKD1 and for a CHMP1 role in vesicle trafficking.

CHMP1 is structurally-related to a family of conserved proteins

Comparison of CHMP1 primary structure with the protein sequence database reveals that CHMP1 is a conserved protein, found in *S. cerevisiae* and many other eukaryotic organisms (D.R.S. and S.M.H., unpublished). The yeast homolog of CHMP1, Chm1p, shows structural similarity with five other yeast proteins (Fig. 5A). Each protein in this group is related in size (220 amino acid average) and has a conserved, distinctive charge distribution resulting in average predicted isoelectric points of 10.2 and 4.1 for the N- and C-terminal halves, respectively (Fig. 5A; Babst et al., 1998).

**Fig. 5.** CHMP1 is a conserved member of a structurally related, but divergent, family of yeast and mammalian proteins. (A) The six yeast Chm proteins show related size and predicted isoelectric point (pI) values for their N- and C-terminal halves. Yeast CHM open reading frame assignments are CHM1, YKR035w; CHM2, YKL002w; CHM5, YDR466c; and CHM6, YMR077c. (B) Phylogenetic tree for proteins of the yeast Chm (boxed) and human CHMP families is shown. ClustalX was used to construct the tree (Thompson et al., 1997). D is a measure of sequence divergence. GenBank accession numbers for human proteins are: CHMP1, AF281063; CHMP1.5, AF281064; CHMP2 (BC-2), AF042384; CHMP2.5 (CGI-84), AF151842; CHMP3 (NEDF), AF219226; CHMP4 (HSPC134), AF161483; CHMP5 (CGI-34), AF132968; and CHMP6, AW965590.

The ability of CHMP1 and SKD1 to interact was also measured in cellular extracts. Based on the moderate increased binding activity of the SKD1(EQ) mutant in vitro, this point mutant was used in a co-immunoprecipitation assay. COS7 cells were co-transfected with plasmids encoding FLAG epitope alone or FLAG fused to SKD1(EQ), and the ability of each to co-immunoprecipitate exogenous CHMP1 was compared. Not only are significant levels of exogenous CHMP1 bound by SKD1(EQ) in this assay (Fig. 3B, compare lanes 2,4), but also endogenous CHMP1 is detectably co-immunoprecipitated (Fig. 3B, compare lanes 2,6). These observations strongly suggest that CHMP1 and SKD1(EQ) physically interact in the cell. To gain further support for this
A conserved CHMP family

similarity. Each of the six sub-families includes yeast (boxed) and mammalian structural homologs (Fig. 5B). Comparison of any two proteins in the tree reveals sequence similarity that is distributed throughout the length of the protein, not just limited to a defined motif. Despite the weak sequence similarity exhibited by the most divergent members of this family, the common overall structural features illustrated in Fig. 5A appear to mediate a common function, as demonstrated below.

**The entire yeast CHM family is required for high fidelity carboxypeptidase Y sorting**

Several observations suggest that the yeast CHM family might function in a specific aspect of vacuolar protein sorting. Studies outlined above demonstrate that mammalian CHMP1 localizes to endosomes, physically interacts with SKD1, and can disrupt trafficking in a manner similar to SKD1. In addition, CHMP1 shows structural similarity to Vps24p and Snf7p/Vps32p (Fig. 5A), class E Vps proteins that function with the budding yeast counterpart of SKD1. Therefore, we have used a genetic strategy to test the role in vacuolar protein sorting of CHM1 and three other previously uncharacterized CHM family members, CHM2, CHM5 and CHM6.

The standard assay which identifies vps mutants measures maturation and targeting of a soluble hydrolase, CPY, to the vacuole lumen (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986). We have constructed or obtained deletions of each member of the S. cerevisiae CHM family, including previously characterized VPS24 and SNF7/VPS32 (Babst et al., 1998), and analyzed their ability to sort and process CPY. Cells were collected at mid-log phase, converted to spheroplasts, pulse-chase labeled with [35S]methionine, separated into secreted and spheroplast fractions, and then analyzed for the presence of CPY by immunoprecipitation. As shown in Fig. 6A, each chm deletion strain (and vps4) correctly processes over half of its CPY to an intracellular (I) form of 61 kDa (Fig. 6A). The remaining CPY migrates as an immature, unprocessed form of 69 kDa in both the intracellular (I) and extracellular (E) fractions. By contrast, the parental strain (+) quantitatively processes and targets all CPY. All chm single mutants show a comparable defect in CPY sorting, which is not increased in chm double mutants (Fig. 6A; chm1 snf7; and data not shown). The inability of double mutants to increase the phenotypic severity suggests that CHM gene products function in a common pathway required for high fidelity CPY sorting.

**chm deletions form multi-lamellar class E vps compartments**

To further classify chm mutants and to facilitate identification of the specific process that is disrupted, we analyzed mutant cells ultrastructurally using transmission electron microscopy. The defining morphological feature of vps4, vps24, snf7/vps32 and all other class E vps mutants is a multi-lamellar structure visible by electron microscopy (Odorizzi et al., 1998; Piper et al., 1995; Raymond et al., 1992; Rieder et al., 1996). As shown in Fig. 6B, parental yeast typically exhibits at least one prominent vacuole (v), but completely lack the darkly stained,
multi-layered membrane stacks visible in a chm1 mutant (Fig. 6C,D). Tabulation of the frequency of these aberrant structures reveals that none are visible in sections from 260 cells of the parental strain, whereas 14% to 44% of cell sections from chm deletion strains, including previously characterized vps24 and snf7/vps32, show aberrant multi-lamellar structures in the cytoplasm (Fig. 6E). The frequency of these structures varies somewhat between chm mutants, but their general appearance does not. The relative percentages between the mutant strains should not be emphasized, instead the qualitative difference between the WT strain (0% of the cells containing class E compartments) and each of the mutant strains (>14%) is the significant observation. This suggests that each deletion results in a similar ultrastructural change, the formation of a class E vps prevacuolar compartment.

Each yeast CHM gene is required for sorting to the multivesicular body

Odorizzi and coworkers have demonstrated that missorting of CPS is a hallmark of class E vps mutants (Odorizzi et al., 1998). In their analysis, all known class E vps mutations were unable to sort a GFP-CPS fusion protein to the vacuole lumen. Instead, the GFP signal accumulated on the vacuolar membrane. These studies suggested that class E VPS genes function in sorting proteins (and lipid) to the multivesicular body (see Introduction). To confirm the EM assignment of the CHM family as class E VPS, we tested each chm deletion mutant in this CPS sorting assay. The parental strain (+) shows a GFP-CPS fluorescence that is internal to the vacuolar membranes identified with the vital membrane dye FM4-64 (Fig. 7). By contrast, deletion of each chm gene dramatically changes the GFP pattern. These mutants show a GFP signal that is largely excluded from the vacuole interior, but colocalizes with the FM4-64 membrane signal instead. In addition, intense GFP signals are visible in a perivacuolar location, which presumably corresponds to the class E vps compartment (Fig. 6B-D). The sorting defect observed with GFP-CPS for the chm1 mutant can be efficiently rescued by expression of CHM1 from a CEN plasmid under control of the ADH promoter. CHM2, CHM5 and CHM6 genes at single copy and expressed from their own promoters also rescue their corresponding mutations (T.L.H. and S.M.H., unpublished).

DISCUSSION

Based on conserved structural features and functional similarities, we have identified a CHMP/Chmp family of eukaryotic proteins. Genetic studies in yeast demonstrate the Chmp family functions in a common aspect of vesicle trafficking. Specifically, deletion of each of the six S. cerevisiae CHM genes results in similar effects, disrupting prevacuolar/endosomal structure and producing a missorting of two vacuolar hydrolases. The phenotype shared by these mutants points to a role for each of these proteins in MVB sorting, a role already demonstrated for the two previously characterized members of this yeast CHM family, VPS24 and SNF7/VPS32. Studies with CHMP1 in mammalian cells supports this functional assignment and indicates functional conservation across eukaryotes. CHMP1 is peripherally associated with the membranes of both early and late endosomes and alters endosomal structure when a CHMP1-
fusion derivative is overexpressed. Furthermore, mammalian CHMP1 physically interacts with the mammalian homolog of another yeast protein required for MVB sorting, Vps4p. These observations suggest that CHMP1 and its yeast counterpart have a similar role and protein partner during MVB sorting.

Identification of four new yeast class E vps mutants

The phenotypic consequences of \textit{CHM1}, -2, -5 and -6 deletion categorizes them as class E \textit{VPS} genes. For example, these new deletion mutants show aberrant secretion of unprocessed CPY into the extracellular space, a phenotype common to all \textit{vps} mutants, but most similar to other class E \textit{vps} mutations in correctly targeting the majority of CPY (Raymond et al., 1992). It will be important to determine whether these four \textit{CHM} genes are allelic to previously identified class E \textit{vps} mutations, which have not been characterized at the molecular level, or are novel class E \textit{VPS} genes. The identification of new, non-allelic class E \textit{vps} mutations would not be completely unexpected. The weak CPY missorting phenotype exhibited by class E \textit{vps} mutants probably reduced the efficiency with which some were isolated during the initial \textit{vps} mutant screens (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986).

Structural conservation in MVB formation

With the addition of Chm1p, Chm2p, Chm5p and Chm6p, the yeast Chmp family now totals six structurally related proteins. Since each protein is required for CPS internalization in yeast, each Chmp must play a distinct, non-redundant role in the process. Nevertheless, these proteins show a similar, well-conserved size and charge distribution in the absence of strong primary sequence conservation. How might these proteins function in MVB sorting? One explanation for these conserved features envisions a structural role for Chm proteins on the endosome membrane, where Chm proteins might participate in formation of a membrane coat. Consistent with this hypothesis, CHMP1, Vps24p and Snf7p/Vps32p all show partial membrane association (this study and Babst et al., 1998). This proposed coat would be created by Chm proteins as a membrane-associated lattice (Babst et al., 1998). Within this lattice, the common structural features shared by Chm proteins might allow these proteins to be interchangeable to some extent, which could alter the curvature and/or membrane attachment properties of the lattice. Additional studies directed toward testing this model will determine whether CHMP proteins physically interact within one complex, interact in distinct complexes, or act in a sequential fashion on the endosome membrane.

CHMP1-SKD1/VPS4 interaction

The model proposed above for Chmp action in MVB formation would require restructuring of a membrane lattice to create concave curvature to effect invagination. This restructuring might require changes in protein conformation or protein-protein interaction. The protein previously suggested to carry this function is yeast Vps4p, a class E Vps protein structurally unrelated to the Chmp family (Babst et al., 1997; Babst et al., 1998). Interestingly, we have demonstrated that mammalian CHMP1 is a physical partner of human SKD1 as measured by two-hybrid, co-immunoprecipitation and in vitro binding studies. This is likely to be a fundamental, conserved functional interaction, since yeast Chm1p shows an equally strong two-hybrid signal with human SKD1 (T.L.H. and S.M.H., unpublished). Two hybrid analysis of interactions between SKD1 and the several other members of the mammalian CHMP family identifies a strong interaction with CHMP2, also known as BC-2 (Keese et al., 1999), but no signal above background with CHMP3, CHMP4 or CHMP5 (T.L.H. and S.M.H., unpublished). Analysis of yeast CHM mutants supports this specificity of SKD1 interaction: deletion of yeast \textit{CHM1} or \textit{CHM2}, but not \textit{CHM5} or \textit{CHM6}, results in predominant membrane association of Snf7p (C.R.D. and S.M.H., unpublished). The extent of membrane binding is equivalent to that observed in a \textit{vps4} background (Babst et al., 1998). Based on these observations, it is likely that SKD1/Vps4p is acting directly on CHMP1/Chm1p and CHMP2/Chm2p, but indirectly on other CHMP/Chm proteins to mediate restructuring and/or membrane dissociation. In conclusion, by identifying a family of divergent but functionally related proteins responsible for MVB formation, this study provides compelling groundwork for the analysis of the role of the CHMP proteins in cellular trafficking.

Note added in proof

While our manuscript was in review, \textit{CHM1} (\textit{DID2}) and \textit{CHM2} (\textit{DID4}) were identified in a screen for suppressors of the \textit{doa}1 (deubiquitinating enzyme) mutation. This publication verifies our observation that these proteins are class E Vps factors (Amerik et al., 2000).

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core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. *J. Cell Sci.* **110**, 2027-2040.


