Bro1 is an endosome-associated protein that functions in the MVB pathway in *Saccharomyces cerevisiae*

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Summary

Multivesicular bodies are late endosomal compartments containing lumenal vesicles that are formed by inward budding of the limiting endosomal membrane. In the yeast *Saccharomyces cerevisiae*, integral membrane proteins are sorted into the lumenal vesicles of multivesicular bodies, and this process requires the class E subset of *VPS* genes. We show that one of the class E *VPS* genes, *BRO1/VPS31*, encodes a cytoplasmic protein that associates with endosomal compartments. The dissociation of Bro1 from endosomes requires another class E Vps protein, Vps4, which is an ATPase that also regulates the endosomal dissociation of ESCRT-III, a complex of four class E Vps proteins (Vps2, Vps20, Vps24 and Snf7/Vps32) that oligomerize at the endosomal membrane. We also show that the endosomal association of Bro1 is specifically dependent on one of the ESCRT-III components, Snf7. Our data suggest that the function of Bro1 in the MVB pathway takes place on endosomal membranes and occurs in concert with or downstream of the function of the ESCRT-III complex.

Key words: Multivesicular, Vesicle, Transport, Vacuole

Introduction

Endosomes function to segregate endocytosed macromolecules destined to be degraded in lysosomal/vacuolar organelles from molecules that are either recycled back to the cell surface or routed toward other intracellular destinations. Endosomes also coordinate the transport of newly synthesized lysosomal/vacuolar hydrolases arriving from the trans-Golgi network. A critical sorting step occurs at multivesicular bodies (MVBs), which are late endosomes that contain lumenal vesicles formed by inward invagination of the limiting endosomal membrane (Hirsch et al., 1968; Sotelo and Porter, 1959). A subset of integral membrane proteins are sorted into the lumenal vesicles of MVBs and are subsequently degraded upon fusion of the limiting MVB membrane with the lysosomal/vacuolar membrane (Futter et al., 1996). In contrast, transmembrane proteins that are excluded from MVB vesicles are either recycled to the plasma membrane or to the Golgi, or, alternatively, are delivered to the limiting membrane of the lysosome/vacuole (Lemmon and Traub, 2000).

Many proteins that are critical to cell growth and development are sorted into MVB vesicles. For example, activated epidermal growth factor receptors in mammalian cells are downregulated through endocytosis and MVB sorting en route to being degraded in the lysosome (Haigler et al., 1979; McKanna et al., 1979). A similar pathway is followed by Ste2, a G protein-coupled pheromone receptor that is downregulated and degraded in the vacuole of the budding yeast *Saccharomyces cerevisiae* (Hicke et al., 1997; Odorizzi et al., 1998). These receptors as well as many other cell-surface proteins that are targeted via the MVB pathway are modified by the attachment of ubiquitin to their cytoplasmic domains. Ubiquitin is a highly conserved 76-amino acid polypeptide that is covalently linked to specific protein substrates by a cascade of ubiquitin-conjugation enzymes. Ubiquitin was originally known for its attachment as a chain of four or more subunits (polyubiquitination) to soluble protein substrates that are targeted for degradation by the proteasome (Weissman, 2001). In contrast, a single ubiquitin or a short chain of less than four ubiquitin subunits is covalently linked to the cytoplasmic domains of cell-surface proteins that are targeted for degradation in the lysosome/vacuole (Hicke, 2001). Prior to proteasomal or vacuolar degradation, ubiquitin is removed from protein substrates by a de-ubiquitinating enzyme, thereby enabling the cell to maintain a constant pool of ubiqiutin (Weissman, 2001).

A direct role for ubiquitin in the MVB pathway has been demonstrated in studies of a yeast vacuolar hydrolase, carboxypeptidase S (CPS). The precursor form of CPS is synthesized as an integral membrane protein that is transported to endosomes directly from the Golgi rather than via endocytosis from the plasma membrane (Cowles et al., 1997). The sorting of CPS into MVB vesicles results in its delivery into the vacuole lumen (Odorizzi et al., 1998), where CPS is proteolytically cleaved from its transmembrane anchor to produce the soluble mature form of the enzyme (Spormann et al., 1992). CPS is mono-ubiquitinated on its cytoplasmic

domain after exiting the Golgi, and mutations in CPS that block this modification result in its mislocalization to the limiting vacuolar membrane (Katzmann et al., 2001). Ubiquitination, therefore, can function as a sorting signal for entry into the MVB pathway.

Ubiquitinated CPS is bound by ESCRT-I (Endosomal Sorting Complex Required for Transport), a cytoplasmic protein complex that transiently associates with endosomal membranes (Katzmann et al., 2001). ESCRT-I is comprised of Vps23, Vps28 and Vps37, all three of which are encoded by class E *VPS* (Vacuolar Protein Sorting) genes, a set of 17 genes that are required for the sorting of both CPS and Ste2 into the MVB pathway (reviewed by Katzmann et al., 2002). Two additional ESCRT complexes consisting of class E Vps proteins have been described recently. Similar to ESCRT-I, both ESCRT-II (Vps22, Vps25 and Vps36) and ESCRT-III (Vps2, Vps20, Vps24 and Snf7/Vps32) transiently associate with endosomes (Babst et al., 2002a; Babst et al., 2002b). Although their precise functions are not yet known, genetic data suggest that ESCRT-II functions downstream of ESCRT-I and initiates the recruitment and assembly of ESCRT-III at the endosomal membrane (Babst et al., 2002b). Another class E Vps protein, Vps4, is an ATPase that catalyzes the dissociation and disassembly of all three ESCRT complexes from endosomal membranes (Babst et al., 2002a; Babst et al., 2002b; Babst et al., 1998).

One of the class E *VPS* genes that has not been characterized as encoding a component of the ESCRT complexes is *VPS31*. The *VPS31* gene is allelic to *BRO1*, which was originally implicated in the protein kinase C/MAP kinase signaling pathway (Nickas and Yaffe, 1996). Recently, mutations in *BRO1* were shown to block the ubiquitin-dependent downregulation of the general amino acid permease, Gap1, from the plasma membrane (Springael et al., 2002). Similarly, mutations in *BRO1* were found to restore amino acid uptake in cells harboring defects in the plasma membrane amino acid sensor complex, presumably by blocking the downregulation and degradation of amino acid permeases (Forsberg et al., 2001). These observations are consistent with a role for *BRO1* in the sorting of cell-surface proteins to the vacuole.

We show that *BRO1* encodes a soluble cytoplasmic protein that associates with endosomes. Similar to the ESCRT complexes, Bro1 accumulates on endosomal membranes in *vps4* mutant cells, suggesting a role for the Vps4 ATPase in regulating the endosomal dissociation of Bro1. Furthermore, biochemical fractionation and fluorescence microscopic studies indicate that Bro1 association with endosomes requires the ESCRT-III component, Snf7. Interestingly, unlike Gap1 (Springael et al., 2002), the ubiquitination of CPS is not blocked by a deletion of the *BRO1* gene, suggesting that ubiquitination of cargo proteins downregulated from the plasma membrane may be subject to different requirements than the ubiquitination of cargo proteins sorted directly from the Golgi.

Materials and Methods

Yeast strains and plasmid constructions

The following yeast strains were used in this study: SEY6210 [*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-Δ801 suc2-Δ9* (Robinson et al., 1988)]; MBY3 [SEY6210; *vps4Δ::TRP1* (Babst et al., 1997)]; GOY65 [SEY6210; *bro1Δ::HIS3* (this study)]; PBY34 [SEY6210; *VPS10-GFP::TRP1* (Burda et al., 2002)]; DBY11 [SEY6210; *VPS10-GFP::TRP1 bro1Δ::HIS3* (this study)]; DBY12 [SEY6210; *VPS10-GFP::TRP1 vps4Δ::HIS3* (this study)]; GOY66 [SEY6210; *bro1Δ::HIS3 vps4Δ::TRP1* (this study)]; BWY102 [SEY6210; *vps24Δ::HIS3* (Babst et al., 1998)]; MBY12 [SEY6210; *vps4Δ::TRP1 vps24Δ::HIS3* (Babst et al., 2002a)]; MBY28 [SEY6210; *vps2Δ::HIS3* (Babst et al., 2002a)]; MBY41 [SEY6210; *vps4Δ::TRP1 vps2Δ::HIS3* (Babst et al., 2002a)]; EEY9 [SEY6210; *snf7Δ::HIS3* (Babst et al., 2002a)]; EEY12 [SEY6210; *vps4Δ::TRP1 snf7Δ::HIS3* (Babst et al., 2002a)]; EEY2-1 [SEY6210; *vps20Δ::HIS3* (Babst et al., 2002a)]; MBY37 [SEY6210; *vps4Δ::TRP1 vps20Δ::HIS3* (Babst et al., 2002a)]; CBY31 [SEY6210; *pep12Δ::HIS3* (Burd et al., 1997)]; GOY54 [SEY6210; *pep12Δ::HIS3 bro1Δ::TRP1* (this study)]; TVY614 [SEY6210; *pep4Δ::LEU2 prb1Δ::HISG prc1Δ::HIS3* (Katzmann et al., 2001)]; MBY52 [SEY6210; *vps4Δ::TRP1 pep4Δ::LEU2 prb1Δ::HISG prc1Δ::HIS3* (Katzmann et al., 2001)]; and GOY55 [SEY6210; *bro1Δ::TRP1 pep4Δ::LEU2 prb1Δ::HISG prc1Δ::HIS3* (this study)]. Media for yeast culture are identical to those previously described (Cowles et al., 1997).

The *HIS3* gene was used to replace the open-reading frame (ORF) of *BRO1* in SEY6210, MBY3 and PBY34 by homologous recombination (Longtine et al., 1998), resulting in GOY65, GOY66 and DBY11, respectively. The same method was used to replace the ORF of *VPS4* in PBY34 with *HIS3*, resulting in DBY12. To construct GOY54 and GOY55, the wild-type *BRO1* gene was amplified by PCR using as template the genomic DNA from SEY6210 together with primers GO104p (containing a *Spe*I restriction site) and GO106p (containing a *Sal*I restriction site), which anneal 500 base pairs upstream and downstream, respectively, of the *BRO1* ORF. The resulting PCR product was ligated as a *Spe*I-*Sal*I fragment into *Spe*I-*Sal*I-digested pRS416 (Sikorski and Hieter, 1989), resulting in plasmid pGO187. The PCR method of gene splicing by overlap extension (gene SOE) (Yon and Fried, 1989) was used to construct a unique *Eco*RI restriction site at the start codon of *BRO1* within pGO187, resulting in plasmid pGO221, which was then digested with *Hin*dIII, blunted with T_4 DNA polymerase, and subsequently digested with *Eco*RI. In parallel, a plasmid consisting of the pBluescriptSK$^-$ vector (Stratagene) containing the wild-type *TRP1* gene from *Saccharomyces cerevisiae* was digested with *Bam*HI, blunted with T_4 DNA polymerase, then digested with *Eco*RI to yield a *Bam*HI$_{blunt}$-*Eco*RI fragment containing the *TRP1* gene that was ligated with the *Hin*dIII$_{blunt}$-*Eco*RI fragment of pGO221, resulting in the replacement of *BRO1* codons 1-770 with *TRP1*. This plasmid served as a PCR template using primers GO104p and GO106p, and the resulting PCR product was transformed into CBY31 and TVY614 to construct GOY54 and GOY55, respectively.

The *HIS3-CPS* fusion was constructed by gene SOE PCR using plasmid templates pRS415 (Sikorski and Hieter, 1989) for *HIS3* and pDP83 (Cowles et al., 1997) for *CPS1*. The resulting PCR product was ligated into pRS416, resulting in plasmid pGO96. SEY6210 cells transformed with pGO96 were mutagenized with ethyl methanesulfonate, resulting in ~25% viability. The mutagenized cells were diluted in rich growth medium and grown at room temperature for 2 hours, then harvested by centrifugation, resuspended in double-distilled water, and spread at a concentration of 5000 cells/petri dish onto 200 petri dishes consisting of agar growth medium that lacks supplemental histidine. The growth medium contained 30 mM 3-amino-1,2,4-triazole, a competitive inhibitor of the His3 enzyme, in order to suppress growth of wild-type cells because of the activity of newly synthesized His3-CPS fusions being transported through the early secretory pathway. After 10 days of incubation at room temperature, surviving colonies were transformed with pGO45 and examined by fluorescence microscopy in order to determine the localization of green fluorescence protein (GFP)-CPS.

The *GFP-BRO1* fusion was constructed by PCR amplification of

BRO1 using pGO187 as a template, together with primers GO98p (which places a unique *Eco*RI restriction site at the start codon of *BRO1*) and GO106p. (Primer sequences are available upon request.) The resulting PCR product was digested with *Eco*RI and *Sal*I, then ligated into *Eco*RI-*Sal*I-digested pGO36, which is identical to the pGOGFP plasmid that was described in Cowles et al. (Cowles et al., 1997), except that pGO36 has a pRS416 vector backbone rather than a pRS426 vector backbone (Sikorski and Hieter, 1989). The resulting *GFP-BRO1*-containing plasmid is pGO249. Plasmids containing the $vps4^{K179A}$ (pMB24) and $vps4^{E233Q}$ (pMB49) alleles were described in Babst et al. (Babst et al., 1997). The plasmid containing *GFP-CPS* (pGO45) was described in Odorizzi et al. (Odorizzi et al., 1998).

Fluorescence microscopy

Cells expressing GFP fusion proteins were pulse-labeled or labeled continuously with FM 4-64 at 30°C as previously described (Vida and Emr, 1995). GFP and FM 4-64 fluorescence as well as Nomarski optics were observed using a Leica DMRXA fluorescence microscope equipped with a Cooke Sensicam digital camera (Applied Scientific Instruments). Images were processed using Slidebook software (Intelligent Imaging Innovations).

Immunoprecipitations and subcellular fractionations

For immunoprecipitation of CPS, 2 sets of 5 A_{600} units of cells grown at 30°C to mid-logarithmic phase in liquid synthetic medium were harvested by centrifugation at 500 ***g*** and resuspended in 1 ml of medium. Fifty microcuries of Tran^{35}S-label (ICN Biochemicals) was added, and cells were shaken for 10 minutes at 30°C. Labeling of newly synthesized proteins was terminated by adding 5 mM methionine, 1 mM cysteine, and 0.2% yeast extract, and cultures were shaken for an additional 0- or 30-minute chase period at 30°C. CPS was subsequently immunoprecipitated using rabbit anti-CPS antiserum (Cowles et al., 1997) and Protein A-sepharose (Pharmacia) from total cell extracts that had been precipitated by the addition of 10% (vol/vol) trichloroacetic acid (TCA) and washed twice with ice-cold acetone. Immunoprecipitates were treated with endoglycosidase H to remove carbohydrate modifications as previously described (Cowles et al., 1997), then resolved by SDS-PAGE and examined by fluorography. For immunoprecipitation of CPY, 5 A_{600} units of cells were labeled with Tran^{35}S-label as described above, then chased for 30 minutes. The cells were then harvested and converted to spheroplasts, then CPY was immunoprecipitated from the intracellular fraction and external medium using rabbit anti-CPY antiserum and Protein A-sepharose as previously described (Darsow et al., 1997). Following SDS-PAGE, the amount of internal and secreted radioactive CPY was quantitated using a Storm 860 phosphorimager (Molecular Dynamics).

For subcellular fractionation, 10 A_{600} units of cells grown at 30°C to mid-logarithmic phase were converted to spheroplasts (Darsow et al., 1997), then harvested by centrifugation at 500 ***g***. Spheroplasts were resuspended gently in 1 ml ice-cold lysis buffer [200 mM sorbitol, 50 mM potassium acetate, 20 mM Hepes, pH 7.2, 2 mM EDTA, supplemented with a protease inhibitor cocktail (Roche)], then subjected to 12 strokes in an ice-cold Dounce tissue homogenizer. Lysates were divided into two 0.5-ml aliquots and centrifuged at 4°C for 15 minutes at 13,000 ***g*** to generate P13 pellets enriched for vacuole, endosome, endoplasmic reticulum and plasma membranes. The 13,000 ***g*** supernatant fractions were centrifuged at 100,000 ***g*** for 1 hour at 4°C in a Beckman TLA100.3 rotor, resulting in S100 supernatant fractions containing soluble proteins and P100 pellet fractions enriched for membranes of the Golgi and small transport vesicles. Protein samples of each fraction were TCA-precipitated and acetone-washed. One-half A_{600} unit-equivalent of each fraction was resolved by SDS-PAGE, transferred to nitrocellulose and examined by western blotting using antibodies against Bro1 (see below), Vps24 (Babst et al., 1998), Snf7 (Babst et al., 1998), Vps4 (Babst et al., 1997), ALP (Molecular Probes) and PGK (Molecular Probes).

For detection of Ub-CPS, denaturing immunoprecipitations using anti-CPS antiserum and Protein A-sepharose were performed as previously described (Katzmann et al., 2001). Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and two A_{600} units of each sample were examined by SDS-PAGE and western blotting using anti-ubiquitin antibodies (Zymed Laboratories). In the wild-type (TVY614), *vps4Δ* (MBY52) and *bro1Δ* (GOY55) strains that were examined, the *PEP4*, *PRB1* and *PRC1* genes encoding vacuolar proteases had been deleted in order to reduce the non-specific cleavage of ubiquitin from CPS after cell lysis.

For sucrose density gradient fractionation, the P13 fraction from *vps4Δ* cells was resuspended in 1 ml of lysis buffer supplemented with 60% sucrose, then loaded beneath 2 ml of 55% and 2 ml of 35% sucrose/lysis buffer solutions. After a 14-hour spin at 200,000 ***g***, 3 fractions were collected: the top 3 ml (F), the remaining 2 ml (NF), and the sediment (P). Each fraction was TCA-precipitated and washed in acetone, and one-half A_{600} unit was examined by SDS-PAGE and western blotting.

Preparation of antiserum against Bro1

A 908-bp DNA fragment of *BRO1* encompassing codons 542-844 was subcloned into pGEX-KG, and the resulting glutathione *S*-transferase fusion protein was inducibly expressed in *Escherichia coli*, purified from bacterial extracts following SDS-PAGE, then used to immunize New Zealand White rabbits as previously described (Cowles et al., 1997).

Results

BRO1/VPS31 is a class E VPS gene required for the MVB pathway

We previously showed that the class E subset of *VPS* genes are required for the MVB pathway by visually screening a collection of mutant yeast strains for defects in the localization of a chimera consisting of the GFP fused to the cytoplasmic domain of CPS (Odorizzi et al., 1998). We also isolated mutations in class E *VPS* genes using a genetic selection for mutant yeast strains that are defective in sorting a His3-CPS fusion protein (Fig. 1). His3 normally functions as a soluble enzyme that catalyzes histidine biosynthesis in the cytoplasm. Sorting of His3-CPS via the MVB pathway results in the degradation of His3 in the vacuole lumen (Fig. 1A); as a result, wild-type (*his3Δ*) cells expressing His3-CPS are unable to survive in the absence of supplemental histidine normally provided in the growth medium (Fig. 1C). However, mutations that block the MVB pathway should mislocalize His3-CPS to the vacuole membrane, providing His3 access to its substrate in the cytoplasm (Fig. 1B). Thus, when we randomly mutagenized cells expressing His3-CPS with ethyl methanesulfonate, then plated the cells onto medium that lacks supplemental histidine, we isolated numerous class E *vps* mutants, including *vps4* (Fig. 1C), *vps25*, *vps28* and *vps36* (data not shown). In addition, several other mutants were isolated that do not overlap with known class E *vps* complementation groups but, nevertheless, exhibit defects in the sorting of GFP-CPS (data not shown). The His3-CPS fusion, therefore, serves as a reliable indicator of mutations that block the MVB pathway. Defective sorting of Ste6, the **a**-factor transporter that is downregulated from the plasma membrane, has also been demonstrated using a hybrid reporter protein in

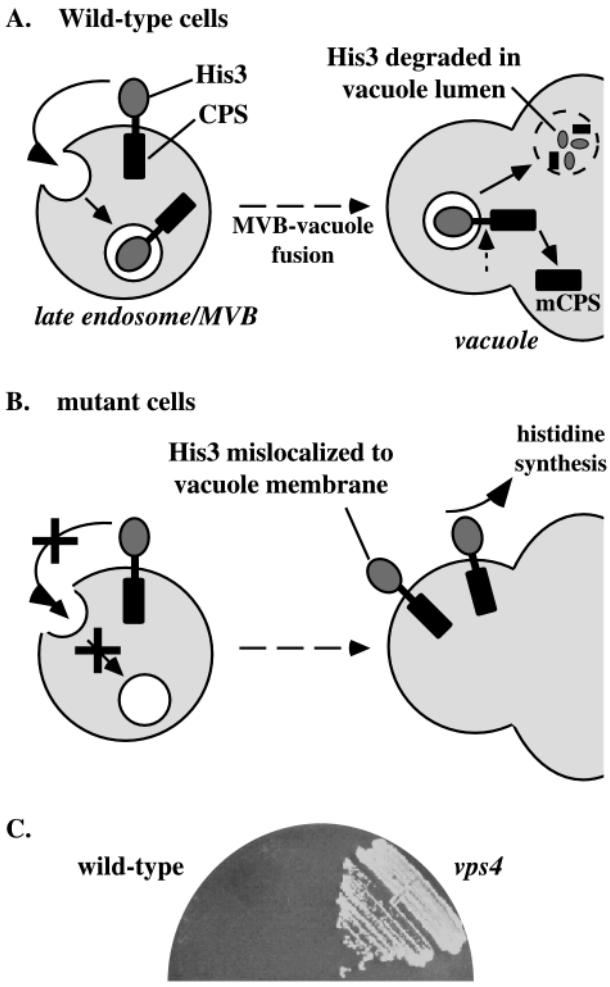

Fig. 1. His3-CPS is a reporter for mutations that block the MVB pathway. Schematic diagram of His3-CPS sorting in wild-type cells (A) and in mutant cells defective in either MVB cargo selection or MVB vesicle formation (B). (C) The growth of wild-type cells and a representative class E *vps* mutant, *vps4*, that have been streaked onto medium lacking supplemental histidine.

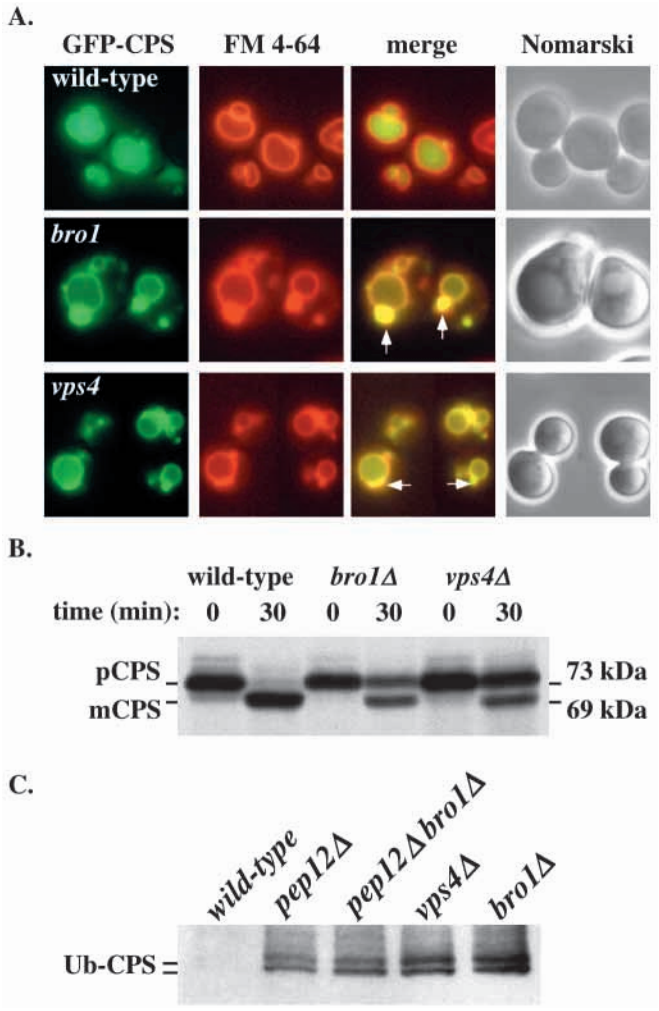

Fig. 2. *BRO1* is required for sorting CPS via the MVB pathway. (A) Fluorescence microscopic localization of GFP-CPS and FM 4-64. Arrows indicate class E compartments. (B) Newly synthesized CPS was immunoprecipitated from lysates of cells that had been pulse-labeled with [^{35}S]methionine/cysteine for 10 minutes then chased in non-radioactive medium for 0 or 30 minutes. Immunoprecipitates were resolved by SDS-PAGE and examined by fluorography. (C) Immunoprecipitates of CPS were resolved by SDS-PAGE, transferred to nitrocellulose, and examined by western blotting using anti-ubiquitin antibodies. Note that CPS is normally differentially modified by the addition of two or three oligosaccharide moieties and is, therefore, observed in C as a doublet (Spormann et al., 1992); however, in B, the immunoprecipitates were treated with endoglycosidase H in order to facilitate the detection of precursor CPS (pCPS) and mature CPS (mCPS).

which His3 was fused to the C-terminal cytoplasmic domain of Ste6 (Losko et al., 2001).

The isolation of class E *vps* mutants using His3-CPS reinforced the idea that class E *VPS* genes have a central role in the MVB pathway. One of the class E *VPS* genes that had not previously been characterized in detail for its role in Golgi-to-vacuole protein sorting is *VPS31*. Analysis of the *VPS31* nucleotide sequence revealed that it is allelic to the *BRO1* gene. *BRO1* stands for *BCK1*-like Resistance to Osmotic shock and was originally identified in a study showing that a *bro1* mutation worsens the viability of cells that are mutant for several components of the protein kinase C/MAP kinase signaling pathway, including the MEK kinase Bck1 (Nickas and Yaffe, 1996). A similar genetic interaction has been found between the *bck1* mutation and other class E *vps* mutations, including *vps24*, *vps28* and *vps36* (M. Nickas, personal communication). These observations may indicate a functional relationship between class E *VPS* genes and signaling through the protein kinase C/MAP kinase pathway. Alternatively, class E *vps* mutations may non-specifically exacerbate the relatively poor viability caused by mutations in MAP kinase components.

Similar to other class E *VPS* genes, *BRO1* is required for the sorting of GFP-CPS via the MVB pathway. In wild-type cells, the delivery of GFP-CPS into the vacuole lumen is in sharp contrast to FM 4-64 (Fig. 2A), a fluorescent lipophilic compound that intercalates into the plasma membrane and is delivered to the vacuole membrane by endocytosis (Vida and Emr, 1995). However, *bro1Δ* cells and other class E *vps* mutants, such as *vps4Δ*, mislocalize GFP-CPS to the vacuole

membrane and to the class E compartment, an aberrant late endosomal structure located adjacent to the vacuole (Fig. 2A).

BRO1 and other class E *VPS* genes are also required for the efficient proteolytic maturation of the native CPS enzyme. The precursor form of CPS (pCPS) is synthesized as a type II integral membrane protein that, upon delivery to the vacuole, is proteolytically cleaved at a site adjacent to its transmembrane domain, resulting in the mature vacuolar form of the enzyme (mCPS) (Spormann et al., 1992). This proteolytic maturation event can be monitored by immunoprecipitation of CPS from cells that have been pulsed-labeled with [^{35}S]methionine/cysteine and chased in non-radioactive medium for 0 or 30 minutes (Cowles et al., 1997). In wild-type cells, pCPS is detected as a 73-kDa polypeptide immediately after synthesis (0-minute chase) and is completely converted to its 69-kDa mature form after a 30-minute chase period (Fig. 2B). In *bro1Δ* and *vps4Δ* mutants, however, ~50% of the pool of newly synthesized CPS fails to be proteolytically matured (Fig. 2B). The partial conversion of CPS to its mature form in class E *vps* mutant cells probably occurs in the class E compartment, as newly synthesized vacuolar hydrolases required for CPS maturation also accumulate at this aberrant structure; the pH-dependent activation of these enzymes is thought to be because of the mislocalization of the vacuolar ATPase at the class E compartment (Piper et al., 1997; Piper et al., 1995).

BRO1 is not required for ubiquitination of CPS

Recently, mutations in *BRO1* were found to impair ubiquitination of the general amino acid permease, Gap1, which undergoes ubiquitin-dependent downregulation from the plasma membrane (Springael et al., 2002). Similar to Gap1, CPS is ubiquitinated on its cytoplasmic domain, which is required for the sorting of CPS via the MVB pathway (Katzmann et al., 2001). Therefore, we investigated whether CPS ubiquitination requires *BRO1* by immunoprecipitating CPS from *bro1* mutant cells, followed by western blotting using anti-ubiquitin antibodies. CPS is ubiquitinated before its arrival at endosomes but is de-ubiquitinated prior to being sorted into MVB vesicles (Katzmann et al., 2001). As a result, ubiquitinated CPS (Ub-CPS) is difficult to detect in wild-type cells, but is easily observed upon deletion of the *PEP12* gene (Katzmann et al., 2001), which encodes a t-SNARE required for the fusion of post-Golgi transport vesicles with endosomes (Becherer et al., 1996). As shown in Fig. 2C, the amount of Ub-CPS observed in *pep12Δ* cells is not diminished if *BRO1* has also been deleted in this strain, indicating that *BRO1* is not required for CPS ubiquitination. Interestingly, Ub-CPS can be detected in class E *vps* mutants, including *bro1Δ* and *vps4Δ* cells, even if the *PEP12* gene has not been deleted (Fig. 2C). A similar stabilization of Ub-CPS has been observed in *vps23Δ* (Katzmann et al., 2001) and *vps27Δ* cells (Shih et al., 2002). The de-ubiquitination of CPS, therefore, appears to require the functions of many class E Vps proteins.

BRO1 is not essential for sorting CPY

In addition to mislocalizing CPS to the vacuole membrane, all class E *vps* mutant cells aberrantly secrete a soluble vacuolar enzyme, carboxypeptidase Y (CPY). Newly synthesized CPY

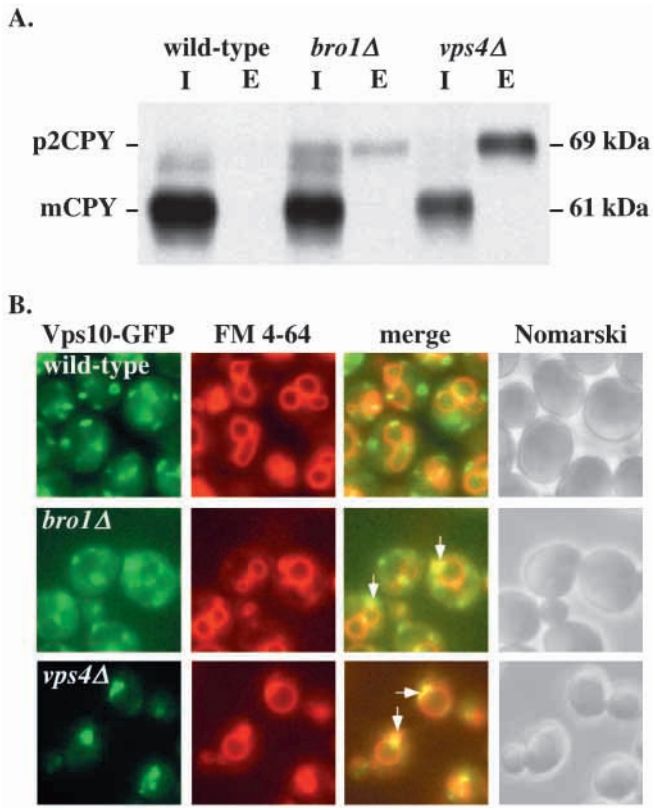

Fig. 3. *BRO1* is not essential for CPY sorting. (A) Newly synthesized CPY was immunoprecipitated from the intracellular fraction (I) and the extracellular medium (E) from cells that had been pulse-labeled with [^{35}S]methionine/cysteine for 10 minutes, chased in non-radioactive medium for 30 minutes, then converted to spheroplasts. Immunoprecipitates were resolved by SDS-PAGE and examined by fluorography. p2CPY, Golgi-modified precursor CPY; mCPY, mature CPY. (B) Fluorescence microscopic localization of Vps10-GFP and FM 4-64. The arrows indicate class E compartments.

is transported from the endoplasmic reticulum to the Golgi, where it receives oligosaccharide modifications, resulting in the 69-kDa p2CPY precursor (Stevens et al., 1982). A vacuolar targeting sequence within the N-terminal pro-peptide region of p2CPY is bound by Vps10, a transmembrane receptor in the late Golgi (Marcusson et al., 1994). The Vps10-p2CPY complex is sorted from the Golgi to endosomes, whereupon p2CPY dissociates from Vps10, and the receptor recycles back to the Golgi, whereas p2CPY is transported further toward the vacuole (Cereghino et al., 1995; Cooper and Stevens, 1996). The pro-peptide region in p2CPY is proteolytically removed upon vacuolar delivery, resulting in mCPY, the mature 61-kDa form of the enzyme (Stevens et al., 1982).

To monitor the requirement for *BRO1* in CPY sorting, wild-type *bro1Δ* and *vps4Δ* cells were pulse-labeled with [^{35}S]methionine/cysteine for 10 minutes, then chased in non-radioactive medium for 30 minutes. Afterward, the cells were converted to spheroplasts, separated into intracellular (I) and extracellular (E) fractions, and CPY was recovered by immunoprecipitation. Virtually all newly synthesized CPY was found intracellularly in its mature form in wild-type cells, reflecting its efficient delivery to the vacuole (Fig. 3A). In contrast, ~50% of CPY was secreted in its p2 form by *vps4Δ*

cells (Fig. 3A) (Babst et al., 1997). Similarly, other class E *vps* mutants have been shown to secrete 30-50% of newly synthesized CPY (Li et al., 1999; Piper et al., 1995; Raymond et al., 1992; Rieder et al., 1996). However, <10% of CPY was secreted to the extracellular medium by *bro1Δ* cells (Fig. 3A). Indeed, an earlier study that compared the sorting defects in class E *vps* mutants showed that *vps31/bro1* mutant cells secrete much less CPY than all of the other class E *vps* mutants that were examined (Raymond et al., 1992). Thus, the *BRO1* gene product appears to have a relatively minor role in CPY sorting compared to other class E *VPS* gene products.

The aberrant secretion of CPY caused by mutations in class E *VPS* genes is thought to be because of a defect in the recycling of the CPY receptor, Vps10, back to the Golgi. Previous indirect immunofluorescence microscopic studies showed that in wild-type cells Vps10 is localized to multiple punctate structures corresponding to Golgi and endosomal compartments. However, upon deletion of *VPS27*, a class E *VPS* gene, Vps10 was observed exclusively at the class E compartment (Piper et al., 1995). Because *bro1Δ* cells secrete very little CPY compared to other class E *vps* mutants, we examined the localization of a Vps10-GFP fusion protein expressed in wild-type *bro1Δ* and *vps4Δ* cells. The Vps10-GFP chimera consists of GFP fused to the C-terminal cytoplasmic domain of Vps10 (Burda et al., 2002). As shown in Fig. 3B, Vps10-GFP was localized to multiple punctate structures in wild-type cells, consistent with its steady-state distribution in Golgi and endosomal compartments (Burda et al., 2002; Piper et al., 1995). In contrast, Vps10-GFP in *vps4Δ* cells colocalized entirely with FM 4-64 at the class E compartment (Fig. 3B). Thus, as observed previously in *vps27Δ* cells (Piper et al., 1995), a deletion of *VPS4* causes a severe defect in the recycling of Vps10 back to the Golgi. In *bro1Δ* cells, however, Vps10-GFP not only colocalized with FM 4-64 at the class E compartment but was also observed on additional punctate structures, the majority of which were not stained with FM 4-64 (Fig. 3B). The localization of Vps10-GFP in *bro1Δ* cells, therefore, is very similar to its localization in wild-type cells. These observations explain the relatively mild CPY sorting defect observed in *bro1* mutant cells (Fig. 3A) (Raymond et al., 1992), and indicate that the recycling of Vps10 back to the Golgi is not strongly dependent on *BRO1*.

BRO1 encodes a soluble cytoplasmic protein that associates with endosomes

BRO1 is predicted to encode an 844-amino acid polypeptide (Bro1) that has a molecular weight of 97.3 kDa. Amino acid sequence alignments predict that Bro1 has a highly conserved N-terminal domain (termed the 'Bro1 domain' by the Pfam protein domain database) (Bateman et al., 2002), a central coiled-coil region and a C-terminal proline-rich domain (Fig. 4B). In order to investigate the intracellular distribution of Bro1, we raised a polyclonal antiserum against its C-terminal 304 amino acids (see Materials and Methods). We then used this antiserum for western blot analysis of yeast cell lysates that were separated by differential centrifugation to yield a 13,000 ***g*** pellet fraction (P13) that contains membranes of the vacuole, endosomes, plasma membrane and endoplasmic reticulum, a 100,000 ***g*** pellet fraction (P100) that contains membranes of the Golgi and small transport vesicles, and a 100,000 ***g*** supernatant fraction (S100) that contains soluble proteins (Marcusson et al., 1994). As shown in Fig. 4A, Bro1 was located predominantly in the S100 fraction of wild-type cells, although a small amount (~5%) was also detected in the P13 and P100 pellets.

We also examined the intracellular localization of Bro1 in vivo using a GFP-Bro1 chimera in which GFP had been fused to the N terminus of Bro1 (Fig. 4B). GFP-Bro1 rescued the sorting of both CPS and CPY when expressed from a low-copy plasmid in *bro1Δ* cells (data not shown), indicating that the fusion protein is fully functional. As shown in Fig. 4B, wild-type cells (in which GFP-Bro1 was expressed in place of endogenous Bro1) exhibited a diffuse cytoplasmic fluorescence which probably corresponds to the distribution of Bro1 in the S100 fraction of cell lysates (Fig. 4A). In addition, a few punctate structures that were positive for GFP fluorescence were also observed (Fig. 4B). These structures probably correspond to endosomal compartments, as they are also stained by FM 4-64 (Fig. 4B, inset). Together, the subcellular fractionation and fluorescence microscopic data indicate that in wild-type cells, Bro1 is predominantly soluble and cytoplasmic, with a portion of its total cellular pool associated with endosomes.

Bro1 accumulates at the class E compartment in the absence of Vps4 ATPase activity

Vps4 is an AAA-type ATPase required for normal endosomal sorting and morphology (Babst et al., 1997). In general, members of the AAA family (ATPases associated with a variety of cellular activities) function as chaperones that disrupt molecular or macromolecular structures, and many AAA proteins assemble into ring-shaped homo-oligomers, which appear to regulate their ATPase activities and mechanism of action (Ogura and Wilkinson, 2001). The ATP-bound form of Vps4 assembles as a homo-oligomer that associates with endosomal compartments, and upon ATP hydrolysis, the Vps4 oligomer disassembles and dissociates from endosomes (Babst et al., 1998).

In addition to regulating its own endosomal localization, Vps4 ATPase activity is required for the endosomal dissociation of several class E Vps proteins, including Vps24 and Snf7/Vps32 (Babst et al., 1998). Vps24 and Snf7 exist in cytoplasmic and membrane-associated pools in wild-type cells (Babst et al., 1998) and are, therefore, distributed among the P13, P100 and S100 fractions (Fig. 4A). In *vps4Δ* cells, however, both Vps24 and Snf7 accumulate at the class E compartment (Babst et al., 1998), resulting in both proteins being found almost entirely in the P13 fraction (Fig. 4A). Similarly, ~50% of the total cellular pool of Bro1 was located in the P13 pellet in *vps4Δ* cells, whereas the remainder was found in the S100 fraction (Fig. 4A). As in the case of Vps24 and Snf7 (Babst et al., 1998), the P13 enrichment of Bro1 appeared to be because of its accumulation at the class E compartment, as GFP-Bro1 was shifted from being primarily cytoplasmic in wild-type cells to being concentrated at the class E compartment in *vps4Δ* cells (Fig. 4B).

To confirm that the P13 pool of Bro1 in *vps4Δ* cells was indeed membrane-associated, this fraction was resuspended in buffer and loaded at the bottom of a sucrose step gradient.

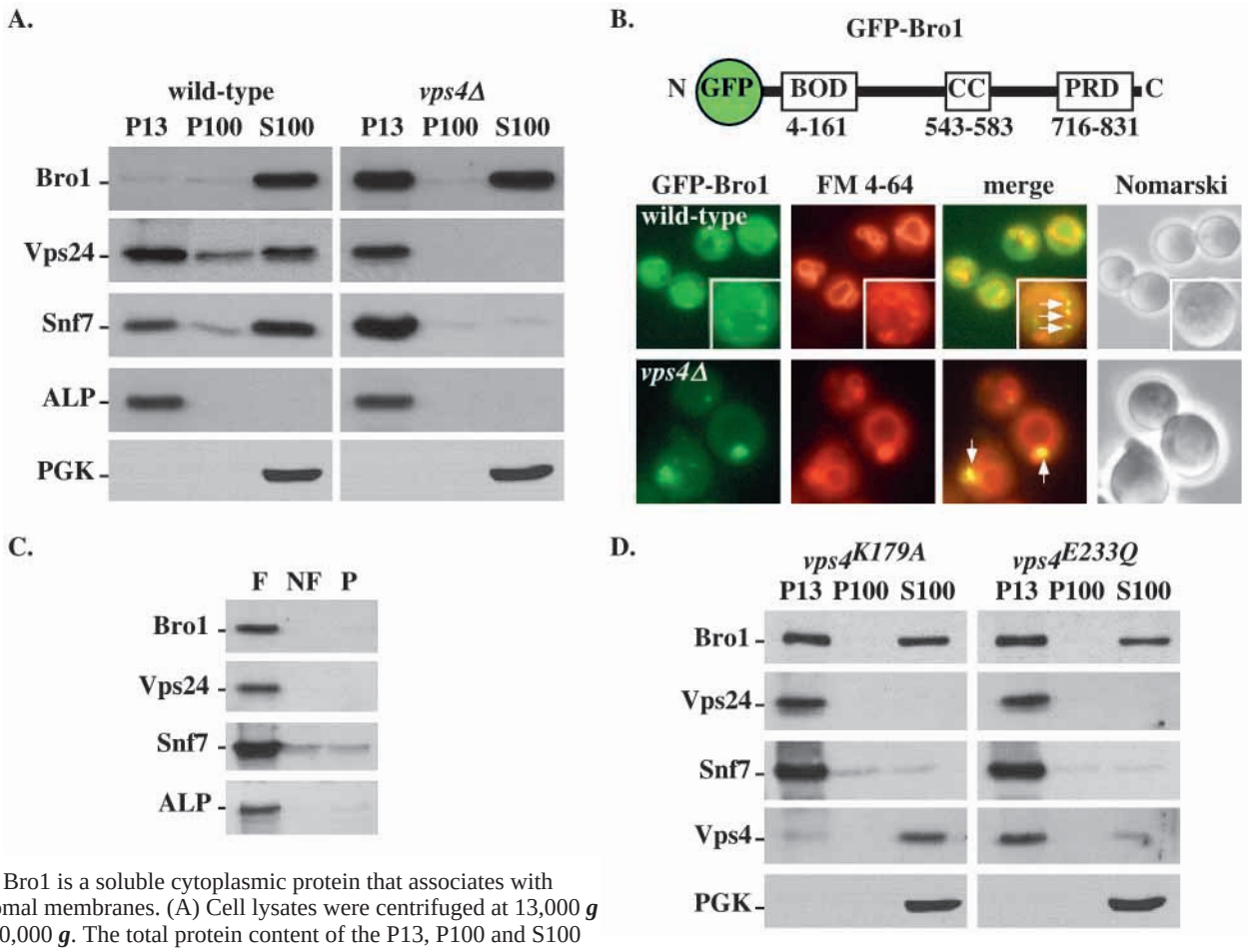

Fig. 4. Bro1 is a soluble cytoplasmic protein that associates with endosomal membranes. (A) Cell lysates were centrifuged at 13,000 ***g*** and 100,000 ***g***. The total protein content of the P13, P100 and S100 fractions was examined by western blotting. Alkaline phosphatase (ALP) is a vacuolar membrane protein, and 3-phosphoglycerate kinase (PGK) is a soluble cytoplasmic enzyme. (B) Fluorescence microscopic localization of GFP-Bro1 and FM 4-64. In the schematic diagram shown above the micrographs, the predicted domains of Bro1 are indicated as BOD ('Bro1 domain'), CC (coiled-coil) and PRD (proline-rich domain). The inset in the top row of panels shows cells subjected to a continuous incubation with FM 4-64 in order to demonstrate the colocalization of GFP-Bro1 with FM 4-64-positive endosomal structures (arrows). Arrows in the bottom set of panels indicate class E compartments. (C) The P13 fraction from *vps4Δ* cells was loaded at the bottom of a sucrose density gradient and centrifuged to equilibrium. Fractions of the floating material (F), the non-floating material (NF) and the pellet (P) were collected and analyzed by western blotting. (D) Subcellular fractionation and western blotting was performed as described in A using *vps4Δ* cells transformed with low-copy plasmids that encode the $vps4^{K179A}$ (pMB24) or $vps4^{E233Q}$ (pMB49) alleles (Babst et al., 1997).

Following centrifugation, the samples were divided into the top fraction containing the membrane-associated floating material (F), the load fraction containing non-floating material (NF), and the pellet fraction (P) that corresponds to large non-membrane-associated material (Babst et al., 1998). Western blot analysis indicated that the P13-associated pool of Bro1, together with Vps24 and Snf7, floated to the top of the gradient along with alkaline phosphatase (ALP), an integral membrane protein of the vacuole (Fig. 4C). Thus, similar to Vps24 and Snf7 (Babst et al., 1998), a membrane-associated pool of Bro1 accumulates at the class E compartment in *vps4Δ* cells.

The accumulation of Vps24 and Snf7 at the class E compartment in *vps4* mutant cells is specifically because of the loss of Vps4 ATPase activity (Babst et al., 1998). Thus, both proteins shift to the P13 fraction in cells expressing mutant forms of Vps4 that are either unable to bind ATP ($Vps4^{K179A}$) or unable to hydrolyze the bound nucleotide ($Vps4^{E233Q}$) (Fig. 4D). Similarly, Bro1 was shifted to the P13 fraction in both $vps4^{KA}$ and $vps4^{EQ}$ mutant cells (Fig. 4D). Therefore, both ATP-binding and ATP-hydrolysis by Vps4 appear to be required for Bro1 to dissociate from endosomes.

The endosomal association of Bro1 requires Snf7

Both Vps24 and Snf7 are components of ESCRT-III, a large, hetero-oligomeric complex that also contains two other class E Vps proteins, Vps2 and Vps20 (Babst et al., 2002a). ESCRT-III associates with endosomal compartments and appears to be comprised of two functionally distinct subcomplexes: a membrane-proximal Vps20-Snf7 subcomplex and a peripheral Vps2-Vps24 subcomplex (Babst et al., 2002a). Vps2 and Vps24 are required for the recruitment of ATP-bound Vps4 and, in turn, ATP hydrolysis by Vps4 catalyzes the dissociation of all four ESCRT-III components from endosomes (Babst et al., 2002a).

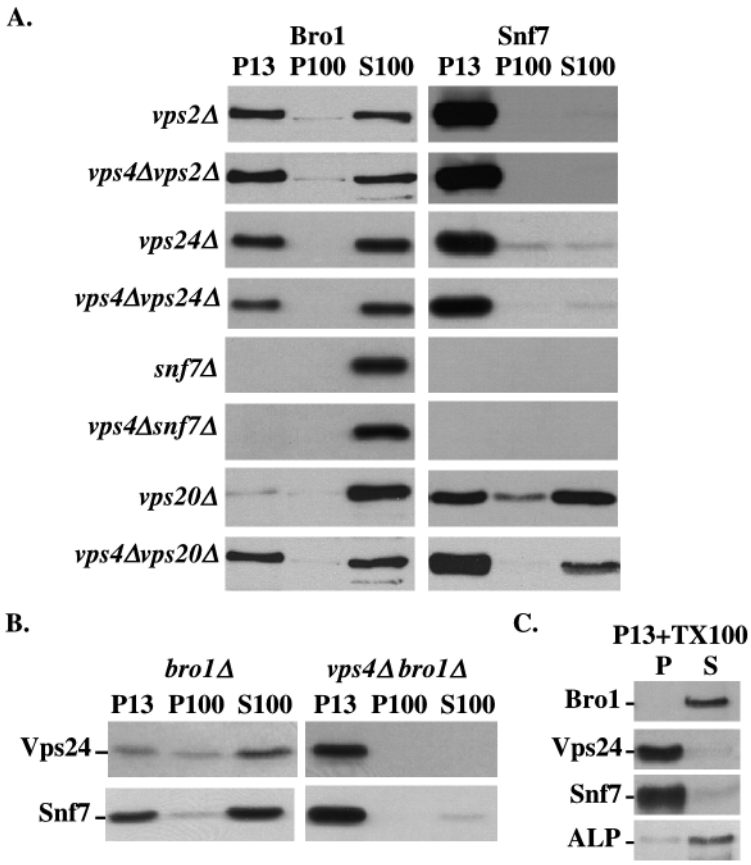

Fig. 5. The subcellular localization of Bro1 requires ESCRT-III components. (A,B) Subcellular fractionation and western blotting was performed as described in the legend to Fig. 4A. (C) The P13 fraction was collected from *vps4Δ* cells, then resuspended in buffer containing 1% Triton X-100 and separated by centrifugation at 100,000 ***g*** for 1 hour into a soluble fraction (S100) and a pellet fraction (P100). The total protein content of each fraction was resolved by SDS-PAGE and examined by western blotting.

Because the data described above suggest that Bro1 also associates with endosomal compartments, we examined whether the distribution of Bro1 is dependent upon the ESCRT-III complex. We found that if either the *VPS2* gene or the *VPS24* gene had been deleted, Bro1 was enriched in the P13 pellet (Fig. 5A), and GFP-Bro1 was concentrated at the class E compartment (Fig. 6). Similarly, Snf7 was shifted almost entirely to the P13 fraction in the absence of either Vps2 or Vps24 (Fig. 5A). This shift in the distributions of Bro1 and Snf7 occurred in *vps2Δ* and *vps24Δ* cells regardless of whether the *VPS4* gene had also been deleted, which is consistent with the Vps2-Vps24 subcomplex being required for the endosomal recruitment of Vps4 (Babst et al., 2002a). Thus, as found previously for ESCRT-III components, Bro1 appears unable to undergo Vps4-dependent dissociation from endosomes in the absence of either Vps2 or Vps24.

Interestingly, we found that if the *SNF7* gene had been deleted either in *VPS4*$^+$ or *vps4Δ* cells, Bro1 remained in the S100 fraction (Fig. 5A) and GFP-Bro1 was mostly cytoplasmic (Fig. 6). However, there was only a modest decrease in the endosome-associated pool of Bro1 upon deletion of the *VPS20* gene in *vps4Δ* cells (Fig. 5A, Fig. 6). Snf7 had similarly been shown to be largely capable of localizing to endosomes independently of Vps20 (Babst et al., 2002a) (Fig. 5A). Thus, the association of Bro1 with endosomal compartments appears to be specifically dependent upon Snf7.

In the absence of Vps4 function, both Bro1 and Snf7 accumulated at the class E compartment and were, therefore, enriched in the P13 fraction (Fig. 4A). As shown in Fig. 5B, however, Snf7 was still found in the P13 fraction in *vps4Δ bro1Δ* cells, and the relatively equal distribution of Snf7 in soluble and membrane-associated fractions of wild-type cells was not altered upon deletion of *BRO1* (compare Fig. 4A with Fig. 5B). Similarly, the distribution of Vps24 in *VPS4*$^+$ and *vps4*Δ cells was not affected if *BRO1* had been deleted (Fig. 5B). To investigate further, we resuspended the P13 fraction from *vps4Δ* cells in buffer containing 1% Triton X-100, then subjected the extract to centrifugation at 100,000 ***g***. Consistent with previous results (Babst et al., 1998), we found that both Snf7 and Vps24 were insoluble upon detergent extraction (Fig. 5C), which is presumably because of their oligomerization into the very high molecular weight ESCRT-III complex (Babst et al., 2002a). In contrast, Bro1 was completely soluble under these conditions (Fig. 5C). Altogether, these observations suggest that Bro1 does not assemble as a core component of the ESCRT-III complex.

Discussion

Protein sorting in the MVB pathway in yeast is dependent upon the class E subset of *VPS* genes (Odorizzi et al., 1998). Our data indicate that *BRO1* is a class E *VPS* gene encoding a soluble cytoplasmic protein that associates with endosomal compartments. Another class E *VPS* gene is *VPS4*, which encodes an AAA-type ATPase (Babst et al., 1997). In the absence of Vps4 ATPase activity, Bro1 accumulates at the class E compartment, an aberrant late endosomal structure, suggesting that the dissociation of Bro1 from endosomes is dependent upon Vps4. Similarly, Vps4 ATPase activity is required for the endosomal dissociation of several other class E Vps proteins, including Vps24 and Snf7 (Babst et al., 1998). Together with Vps2 and Vps20, Vps24 and Snf7 are components of ESCRT-III, a large complex that assembles at endosomal membranes (Babst et al., 2002a). The membrane association/dissociation cycle of Bro1 is dependent upon ESCRT-III, whereas endosomal association and dissociation of ESCRT-III occurs independently of Bro1.

Bro1 is unable to dissociate from endosomes if either *VPS2* or *VPS24* has been deleted, which is consistent with the observation that Vps2 and Vps24 are required for the recruitment of ATP-bound Vps4 to endosomes (Babst et al., 2002a). Vps2 and Vps24 are recruited as a subcomplex to endosomes by Snf7 and Vps20, which may associate directly with endosomal membranes (Babst et al., 2002a). Interestingly, if *SNF7* has been deleted, Bro1 is primarily cytoplasmic with only minimal localization to the class E compartment, even in the absence of Vps4 ATPase activity. In contrast, the

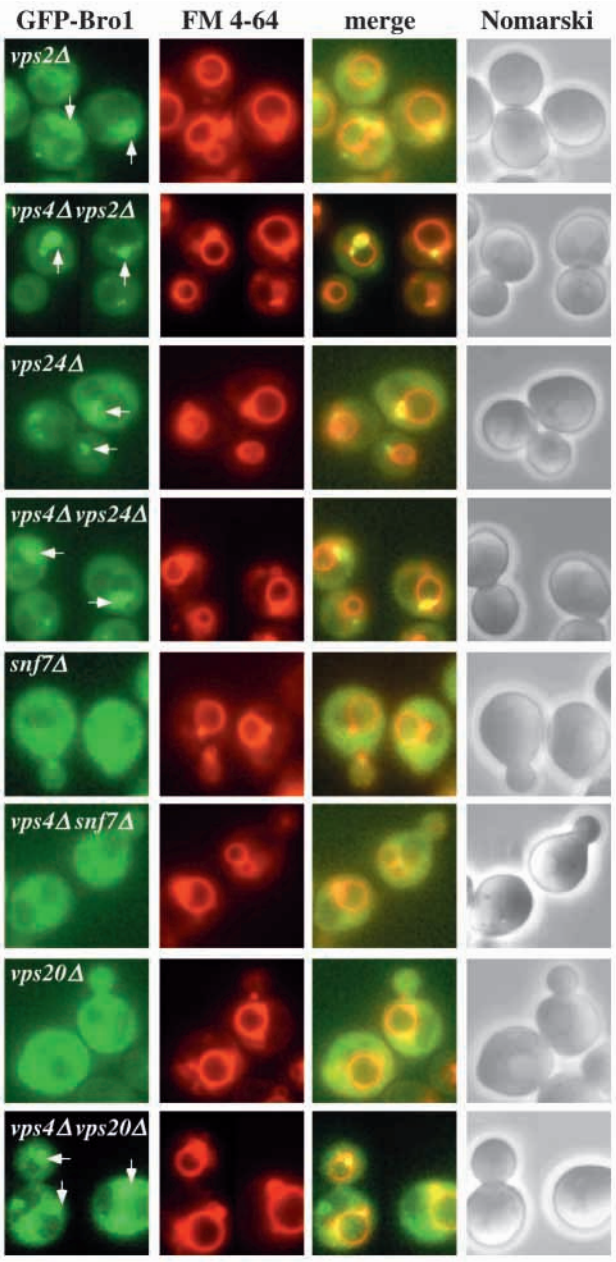

Fig. 6. Localization of GFP-Bro1 in ESCRT-III mutant cells. Fluorescence microscopic localization of GFP-Bro1 and FM 4-64. The arrows indicate GFP-Bro1 localization to class E compartments.

accumulation of Bro1 at the class E compartment is only mildly reduced in *vps4Δ* cells upon deletion of the *VPS20* gene. Similar to Bro1, Snf7 localizes to the class E compartment in *vps4Δ vps20Δ* double-mutant cells (Babst et al., 2002a). Thus, our data suggest that among the ESCRT-III components, Snf7 has a specific role in the endosomal recruitment of Bro1. However, Bro1 is unlikely to form a stable complex with Snf7 at endosomes, as Bro1 is completely solubilized upon detergent extraction of endosomal membranes, whereas Snf7 remains insoluble under these conditions because of its oligomerization into the ESCRT-III complex (Babst et al., 2002a). Furthermore, only ~50% of the cellular pool of Bro1 is trapped at the class E compartment in the absence of Vps4 function, whereas almost 100% of Snf7 (and Vps24) localizes to the class E compartment under these conditions. Altogether, these results suggest that the endosomal association of Bro1 (and possibly its Vps4-dependent dissociation) occurs downstream of ESCRT-III complex assembly. However, it is possible that Bro1 is a peripheral component of ESCRT-III that associates with Snf7-Vps20 less tightly than does Vps2-Vps24. In this scenario, Bro1 could function together with Vps2-Vps24 in the same ESCRT-III complex or, alternatively, either Bro1 or Vps2-Vps24 could bind interchangeably to the membrane-proximal Snf7-Vps20 subcomplex in order to confer distinct ESCRT-III activities.

ESCRT-III functions downstream of two additional ESCRT complexes consisting of class E Vps proteins. ESCRT-I is a ~350-kDa cytoplasmic complex comprised of Vps23, Vps28 and Vps37 (Katzmann et al., 2001). ESCRT-I binds ubiquitinated CPS via the ubiquitin conjugation-like domain of Vps23, suggesting that this complex functions in the recognition of ubiquitinated MVB cargo proteins (Katzmann et al., 2001). ESCRT-II is a ~155-kDa cytoplasmic complex consisting of Vps22, Vps25 and Vps36, and is necessary for the recruitment of ESCRT-III to the endosomal membrane (Babst et al., 2002b). Consistent with these observations, we have found that ESCRT-II is also required for the association of Bro1 with endosomes (G.O., unpublished).

Further evidence that Bro1 functions downstream of other class E Vps proteins is the relatively mild CPY sorting defect observed in *bro1* mutant cells. Although *vps4Δ* and other class E *vps* mutants secrete 30-50% of newly synthesized CPY from the Golgi to the plasma membrane (Li et al., 1999; Piper et al., 1995; Raymond et al., 1992; Rieder et al., 1996), *bro1Δ* cells secrete only ~10% of CPY (Raymond et al., 1992) (this study). The aberrant secretion of CPY by class E *vps* mutants stems from a defect in the recycling of the CPY receptor, Vps10, from endosomes back to the Golgi (Cereghino et al., 1995; Piper et al., 1995). Accordingly, we have found that the normal Golgi/endosomal distribution of a Vps10-GFP fusion observed in wild-type cells is not significantly altered upon deletion of the *BRO1* gene. In contrast, Vps10-GFP is concentrated exclusively at the class E compartment in *vps4Δ* cells, which has also been observed for the native Vps10 distribution in *vps27Δ* cells (Piper et al., 1995). Altogether, these data indicate that the recycling of Vps10 to the Golgi is more dependent upon other class E Vps proteins than it is dependent upon Bro1. One possibility is that Bro1 functions after most of the CPY receptors have recycled out of the endosome, whereas other class E Vps proteins may be required at an earlier stage in endosomal sorting. Alternatively, Bro1 could have a more specific role in sorting MVB cargoes, whereas the activities of other class E Vps proteins could be required in general to maintain normal endosomal function.

The *BRO1* gene was originally identified in a study showing that a *bro1* mutation worsens the viability of *bck1* mutant cells (Nickas and Yaffe, 1996). *BCK1* encodes a MEK kinase that functions in the protein kinase C/MAP kinase pathway (Lee and Levin, 1992). *bck1* mutant cells also exhibit reduced viability upon deletion of other class E *VPS* genes (M. Nickas, personal communication). Although a functional relationship may exist between class E *VPS* genes and signaling through the protein kinase C/MAP kinase pathway, it is also possible that mutations in class E *VPS* genes result in a non-specific

exacerbation of the relatively poor viability caused by mutations in MAP kinase components.

Bro1 is clearly required for the MVB pathway, as a deletion of the *BRO1* gene blocks the sorting of GFP-CPS, which is transported to endosomes from the Golgi, as well as Ste2-GFP, an MVB cargo protein that is endocytosed from the plasma membrane (Odorizzi et al., 1998). Amino acid sequence comparisons indicate that Bro1 has orthologs in a wide range of eukaryotic species, including the mammalian Alix/Aip1 protein that has been implicated in apoptosis (Missotten et al., 1999; Vito et al., 1999), and the Xenopus Xp95 protein, which was identified as a phosphoprotein during meiosis (Che et al., 1999). Bro1 and its putative orthologs have a highly conserved N-terminal ~150-amino acid domain, a central coiled-coil region, and a C-terminal ~150-amino acid proline-rich domain. Interestingly, the *RIM20* gene in yeast, which has been implicated to function in the pH response pathway (Xu and Mitchell, 2001), encodes a protein that is similar to the N-terminal and central regions of Bro1 but which lacks the proline-rich C-terminal domain. However, our observations indicate that Rim20 does not have a role in vacuolar protein sorting, as a deletion of the *RIM20* gene does not cause defects in the sorting of CPS or CPY, and a double deletion of both *RIM20* and *BRO1* does not worsen the sorting defects observed upon deletion of *BRO1* alone (data not shown).

Although the precise function of Bro1 in the MVB pathway is not yet clear, our observations indicate it is not required for the ubiquitination of CPS. CPS ubiquitination is not impaired upon deletion of *BRO1* or other class E *VPS* genes, including *VPS27* (Shih et al., 2002), *VPS23* or *VPS4* (Katzmann et al., 2001). In contrast, mutations in *BRO1* have been found to block ubiquitination of the Gap1 amino acid permease, which undergoes ubiquitin-dependent downregulation from the plasma membrane (Springael et al., 2002). Similarly, deletion of *VPS27* has been shown to block ubiquitination of the uracil permease, Fur4, which is also downregulated from the cell surface (Dupre and Haguenauer-Tsapis, 2001). One possible reason why class E *vps* mutations affect ubiquitination of Gap1 and Fur4 but not CPS could be because of the findings that plasma membrane protein cargoes in yeast are ubiquitinated at the cell surface, whereas CPS is ubiquitinated during its transit from the Golgi to endosomes (reviewed by Katzmann et al., 2002). The ubiquitination of Gap1 and Fur4 requires the ubiquitin ligase, Rsp5 (Hein et al., 1995), the function of which could be sensitive to the membrane trafficking defects that occur upon mutation of class E *VPS* genes. For instance, Rsp5 or other components of the ubiquitination machinery could be mislocalized in class E *vps* mutant cells. In contrast, the ubiquitination of CPS has been proposed to require Tul1, a putative ubiquitin ligase that resides in the Golgi (Reggiori and Pelham, 2002); the function and/or localization of Tul1 may be independent of class E Vps protein activities. Whether Bro1 or other class E Vps proteins have a direct or indirect role in ubiquitination of plasma membrane proteins such as Gap1 and Fur4 awaits further investigation.

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