Ubiquitination of the yeast Gap1 permease at the plasma membrane triggers its endocytosis followed by targeting to the vacuolar lumen for degradation. We previously identified Bro1 as a protein essential to this down-regulation. In this study, we show that Bro1 is essential neither to ubiquitination nor to the early steps of Gap1 endocytosis. Bro1 rather intervenes at a late step of the multivesicular body (MVB) pathway, after the core components of the endosome-associated ESCRT-III protein complex and before or in conjunction with Doa4, the ubiquitin hydrolase mediating protein deubiquitination prior to their incorporation into MVB vesicles. Bro1 markedly differs from other class E vacuolar protein sorting factors involved in MVB sorting as lack of Bro1 leads to recycling of the internalized permease back to the plasma membrane by passing through the Golgi. This recycling seems to be accompanied by deubiquitination of the permease and unexpectedly requires a normal endosomato-vacuole transport function.

The machineries responsible for endocytosis and degradation of plasma membrane proteins are crucial to normal regulation of cell surface-associated functions such as transport, signaling, and adhesion (1, 2). The proteins are first internalized from the cell surface into small endocytic vesicles, a step that generally requires prior ubiquitination of cargo proteins at the plasma membrane. The endocytosed proteins then move through early and late endosomes to finally reach the lysosomal/vacuolar lumen where they are degraded. The last steps of this endocytic pathway, from endosome to vacuolar lumen, are shared with a subset of biosynthetic membrane proteins derived from the late secretory pathway (3). Namely, the delivery of both endocytic and biosynthetic membrane proteins to the lysosomal/vacuolar lumen requires their prior sorting into the multivesicular body (MVB) pathway (4, 5). MVBs arise from endosomes, as the protein-carrying endosomal membrane invaginates and buds into the endosome, forming internal vesicles. The proteins sorted to these MVB vesicles are ultimately delivered to the lumen of the lysosome/vacuole when the MVB fuses with this organelle. Besides its role in the early steps of endocytosis, ubiquitin is also important for MVB sorting, as entry of several proteins into MVB vesicles depends on their prior ubiquitination (6–8). In yeast mutants defective in the class E Vps factors, proteins normally targeted to the vacuolar lumen accumulate in an aberrant late endosome, referred to as the class E compartment, as well as at the limiting membrane of the vacuole (5, 9). Several of these Vps factors are organized in three distinct endosome-associated protein complexes. The ESCRT-I complex participates in recognition of ubiquitinated proteins that are targeted into the MVB pathway (6). ESCRT-II and -III function downstream from ESCRT-I, sorting proteins into MVB internal vesicles (10, 11). The MVB pathway involves several other factors as follows: the Vps27-Hse1 complex to recruit the ESCRT-I complex and to recognize ubiquitinated MVB cargo proteins (12, 13); the AAA-type ATPase Vps4 to dissociate the ESCRT complexes from the endosomal membrane; and the ubiquitin hydrolase Doa4, recruited by ESCRT-III, to deubiquitinate proteins prior to their sorting into MVB vesicles (6, 14, 15). The MVB sorting machinery appears well conserved, because most of the yeast class E Vps proteins have mammalian homologues whose functions are probably similar to those of their yeast counterparts (16).

We use the yeast general amino acid permease Gap1 as a model to gain further insight into the process of regulated trafficking of membrane proteins. Gap1 is suitable for this purpose, as its membrane trafficking is tightly controlled by the nature of the nitrogen source. Under conditions of poor nitrogen supply (proline or urea), Gap1 is abundantly synthesized and accumulates in a highly active and stable form at the plasma membrane. Upon addition of a preferential nitrogen source like ammonium (NH₄⁺), the GAP1 gene is repressed, and the permease present at the cell surface is rapidly poly-ubiquitinated on two lysine residues in the N-terminal tail (Lys-9 and Lys-16). This ubiquitination triggers Gap1 endocytosis followed by sorting to the vacuole/lysosome lumen for degradation (17–22). Ubiquitination of Gap1 at the cell surface is dependent on the ubiquitin ligase Rsp5/Npi1, the Bul proteins, and the Doa4/Npi2 ubiquitin hydrolase, the latter being required to maintain a normal level of free ubiquitin (19, 20, 22, 23). The nitrogen source controls Gap1 trafficking also in the late secretory pathway (24). Although newly synthesized Gap1 is sorted from the Golgi to the plasma membrane in proline- or urea-grown cells, it is directed to the vacuole, bypassing the cell surface, when glutamate (24), ammonium (22), or other amino acids (25) are present. The same direct sorting of Gap1 to the vacuole occurs on proline or urea medium in cells lacking a functional Npr1 (26), a protein kinase inhibited under favored nitrogen supply (17, 27, 28). Like endocytosis, this direct vac-
Bro1 Function in MVB Sorting

Down-regulation of Gap1 also requires the NPI3 gene; in npi3 mutant cells, the permease stays mainly at the plasma membrane after ammonium addition. The cells also show an apparent defect in Gap1 ubiquitination (30). The npi3 mutant was initially isolated together with npi1 and npi2 strains in a screen for mutations restoring high Gap1 activity in the npr1 mutant (17). Accordingly, Npi3 is also required for direct sorting of Gap1 to the vacuole that occurs in the absence of Npr1, indicating that the Npi3 function is not limited to Gap1 endocytosis (30). Cloning of NPI3 revealed that it is identical to BRO1, a gene originally described to interact genetically with components of the Pkc1-mitogen-activated protein kinase pathway (31). BRO1/NPI3 has also been identified as AS6; the as6 mutation restores amino acid uptake in cells defective in a signaling pathway that induces transcription of several amino acid permease genes (32). Bro1/Npi3/As6 has also been isolated in two separate screens for factors required for vacuolar protein sorting (5, 33, 34) and was named Vps31. For simplicity, the protein will hereafter be referred to as Bro1. In a recent study, Bro1 was shown to be a soluble class E Vps factor associating with endosomes and required for the MVB sorting of the vacuolar hydrolase carboxypeptidase S (CPS) (35). Bro1 is conserved throughout the eukaryotic kingdom, and its homologues include mouse AIP1/Alix, a cytosolic protein involved in apoptosis but whose function has remained unclear (36, 37).

Here we report that Bro1 is not essential for ubiquitination or for endocytosis of Gap1. Instead, the protein intervenes at a late step of MVB sorting, likely after the ESCRT-III core complex (31). In contrast to other class E Vps factors of the late step of MVB sorting, likely after the ESCRT-III core complex (36, 37). Bro1 functions as a soluble class E Vps factor in a recent study, Bro1 was shown to be a soluble class E Vps factor associating with endosomes and required for the MVB sorting of the vacuolar hydrolase carboxypeptidase S (CPS) (35).

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The Saccharomyces cerevisiae strains used in this study (Table I) are isogenic with Σ1278b (38). The gene deletions and excision of the KanMX4 cassette were performed as described elsewhere (22). The primer sequences are available upon request. Cells were transformed as described previously (22). The plasmids used in this study are listed in Table I. Plasmid pJOD10 is identical to pCB252 (26). Cells were grown in minimal buffered medium (pH 6.1) (39) with 3% glucose as the carbon source except where indicated otherwise. Nitrogen sources were added as indicated to the following final concentrations: (NH4)2SO4, 10 mM; proline, 0.1%.

Permease Assays—Cells carrying the gap1Δ mutation were transformed with the YCPGap1 or YCPGap1ΔN343 plasmid. Gap1 permease activity was determined by measuring the initial rate of incorporation of 14C-labeled citrulline (20 μM), a specific substrate of the permease (40). Gap1 was inactivated by adding (NH4)2SO4 (10 mM) to the culture. The permease activity was determined in each strain at least three times.

Yeast Cell Extracts and Immunoblotting—Cells bearing the gap1Δ mutation were transformed with plasmid YCPGap1 or YCPGap1ΔN343. Crude extracts (19) and membrane-enriched preparations (20) were obtained as described previously. Gap1 immunoblots were performed as described (22). Extract preparation and Gap1 immunoblotting were performed a minimum of three times for each strain.

Fluorescence Microscopy—Cells transformed with the pJOD10 plasmid were grown in minimal buffered medium (pH 6.1) with 3% galactose and 0.3% glucose as carbon sources and 0.1% proline as the nitrogen source. Glucose was added to the final concentration of 3.5% 2 h before starting the experiment in order to stop Gap1-GFP synthesis. Gap1-GFP internalization was induced by adding (NH4)2SO4 (10 mM) to the culture. To visualize vacuoles, cells were incubated in medium containing 40 μM FM4-64 (Molecular Probes, Eugene, OR), shaken for 15 min at 30 °C, transferred to fresh medium, and chased for ~1 h. Cells were viewed with a Nikon Eclipse E600 microscope equipped with appropriate fluorescence light filter sets. Images were captured with a Nikon Dxm1200 digital camera and processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA). All the Gap1 location experiments were performed at least three times.

RESULTS

Gap1 Is Sorted into the MVB Pathway—To date, a number of yeast proteins have been shown to reach the vacuolar lumen via the MVB pathway (e.g. CPS, Ste2, and Fur4) (5, 41). The sorting of these proteins into intra-endosomal vesicles necessitates the interplay of several class E Vps factors, many of which are organized in complexes (6, 10, 11). In class E vps mutants, membrane proteins normally transiting through the MVB pathway mainly accumulate in an aberrant late endosome referred to as the class E compartment and/or are missorted to the vacuole limiting membrane. We thus sought to determine the fate of Gap1 after ammonium addition to various mutants lacking one of the following class E Vps factors: Vps23, Vps36, Snf7/Vps32 (components of the ESCRT-I, -II, and -III complexes, respectively), or Vps4 (the AAA-type ATPase required for ESCRT complex dissociation from the endosomal membrane) (6, 10, 11).

The presence of Gap1 at the cell surface is reflected by permease activity that is easily monitored by 14C-labeled citrulline uptake assay. In accordance with previous studies (19, 20), we observed high Gap1 activity in proline-grown wild type cells; this activity decreases rapidly after addition of 20 mM NH4+ (Fig. 1A). In end3A cells defective in endocytosis (42), however, the permease activity remained substantially high (Fig. 1A), as shown previously with thermosensitive act1 mutant cells (20). The internalization process was also studied by using a green fluorescent protein-tagged version of the permease (Gap1-GFP). The Gap1-GFP protein is correctly directed to the cell surface where it is functional and largely sensitive to NH4+-triggered down-regulation. Internalization of the chimeric protein is somewhat delayed, however, compared with native Gap1, so that 2 h after NH4+ addition some permease...

### Table I

| Strain | Genotype | Ref. |
|--------|----------|-----|
| EK008  | gap1Δ ura3 | Laboratory collection |
| EN121  | gap1Δ end3Δ ura3 | This study |
| EN063  | gap1Δ vps33Δ ura3 | This study |
| EN057  | gap1Δ npi2Δ ura3 | This study |
| EN054  | gap1Δ snf7Δ ura3 | This study |
| EN050  | gap1Δ vps4Δ ura3 | This study |
| EN076  | gap1Δ bro1Δ ura3 | This study |
| CJ013  | gap1Δ vps6Δ ura3 | Jacob and Andrèa |
| 36216b | gap1Δ bro1Δ vps6Δ ura3 | This study |
| 23344c | ura3 | Laboratory collection |
| 27092a | bro1Δ ura3 | 30 |
| 36222d | gap1Δ bro1Δ end3Δ ura3 | This study |
| EN123  | gap1Δ pep12Δ ura3 | This study |
| EN124  | gap1Δ bro1Δ pep12Δ ura3 | This study |
| EN080  | gap1Δ bro1Δ vps23Δ ura3 | This study |
| EN082  | gap1Δ bro1Δ snf7Δ ura3 | This study |
| EN064  | vam3Δ ura3 | This study |
| EN083  | bro1Δ vam3Δ ura3 | This study |
| 36210a | bro1Δ pep4Δ ura3 | This study |
| 36233b | gap1Δ vam3Δ ura3 | This study |
| 36234a | gap1Δ bro1Δ vam3Δ ura3 | This study |

### Plasmid

| YCpFL38 | CEN-ARS URA3 | 77 |
| YCpGap1 | YCpFL38 GAP1 | 22 |
| YCpGap1D192 | YCpFL38 GAP1ΔN343 | 22 |
| p416GAL1 | CEN-ARS URA3 | 78 |
| pJOD10 | p416GAL1 GAP1-GFP | 26 |

* C. Jacob and B. Andre, unpublished data.
FIG. 1. **Gap1 is sorted into the MVB pathway.** A, Gap1 activity was determined before \((t = 0\) min) and at several times after addition of \((\text{NH}_4)_2\text{SO}_4\) (10 mM) to cultures of proline-grown wild type (Wt) and mutant cells by measuring the incorporation of \[^{14}\text{C}\]citrulline (20 \(\mu\)M): wild type (Wt) (\(\square\), EK808), end3Δ (\(\square\), EN121), vps23Δ (\(\bullet\), EN054), vps36Δ (\(\Delta\), EN057), snf7Δ (\(\circ\), EN059), and vps4Δ (\(\sigma\), EN050). Results are percentages of initial activities per ml of culture. All cells were transformed with the YCpGap1 plasmid. B, localization of Gap1-GFP in the wild type and in the various class E vps mutants indicated in A. Cells were transformed with plasmid pJOD10 and visualized before \((t = 0\) min) and at several times after \(\text{NH}_4^+\) addition. Cells were labeled with FM4-64 to stain the vacuolar membrane and endosomal compartments \((t = 120\) min). C, Western analysis of Gap1 in total protein extracts prepared before \((t = 0\) min) and at several times after addition of \(\text{NH}_4^+\). Strains were as in A.
activity is still measured. In proline-grown wild type cells (t = 0 min), Gap1-GFP was present mainly at the plasma membrane. Addition of NH₄⁺ induced progressive removal of the permease from the cell surface and its targeting to the vacuolar lumen (t = 120 min) (Fig. 1B). Immunodetection of Gap1 in crude extracts indeed showed degradation of the permease in response to NH₄⁺ (Fig. 1C) (20).

Addition of NH₄⁺ resulted in a significant loss of Gap1 activity also in the various class E vpsΔ cells (Fig. 1A), but the internalized permease did not reach the lumen of the vacuole. Instead, NH₄⁺ addition led to a progressive shift of Gap1-GFP into the various vpsΔ cells from the cell surface to compartments next to the vacuole, with weak fluorescence on the vacuolar limiting membrane, as indicated by FM4-64 staining (Fig. 1B). The structures adjacent to the vacuole likely represent the class E compartment (43), and the internalized Gap1 thus appears to accumulate mainly on the membrane limiting this compartment instead of being sorted to the vacuolar lumen. Accordingly, immunoblots showed that the Gap1 protein was not degraded in the various vpsΔ mutants (Fig. 1C). In all vpsΔ cells, addition of NH₄⁺ also induced the appearance of several additional bands above the main Gap1 signal. These were sustained during the whole experiment. As discussed below, these upper bands correspond to ubiquitin-conjugated forms of Gap1.

Hence, Gap1 is normally internalized but not targeted to the vacuolar lumen in mutants lacking components of the ESCRT complexes. Instead, the permease accumulates in a limited number of punctate structures likely corresponding to the class E compartment, whereas a fraction of the protein also reaches the limiting vacuolar membrane.

**Gap1 Is Internalized but Recycled Back to the Cell Surface in bro1Δ Cells**—Bro1 was recently described as a class E Vps factor associated with endosomal membranes and required for MVB sorting of CPS (35). Interestingly, we independently identified Bro1 as a factor essential to normal ubiquitination and subsequent down-regulation of Gap1 and other permeases including the uracil permease Fur4 (30). After ammonium addition to bro1Δ mutant cells, Gap1 remains highly active and is not degraded (Fig. 2, A and C) (30). Consistently, most of theGap1-GFP remained at the cell surface even 2 h after NH₄⁺ addition (Fig. 2B). However, some fluorescence was clearly found also on the vacuole limiting membrane, as indicated by staining with the lipophilic dye FM4-64. This suggests that in bro1Δ cells a fraction of the Gap1 is internalized but that its sorting to the vacuolar lumen is impaired. This phenotype is markedly different from that displayed by the above-mentioned class E Vps-factor mutants, where Gap1 is internalized and accumulates largely in the class E compartment, whereas only a fraction of the permease reaches the limiting membrane of the vacuole.

That Gap1 resides largely at the plasma membrane in bro1Δ cells after NH₄⁺ addition suggests that Bro1 might play a role in some early steps of endocytosis (e.g. in Gap1 ubiquitination) in addition to MVB sorting. Alternatively, Gap1 might be efficiently internalized in bro1Δ cells but not correctly sorted to the vacuole. As a consequence, the permease would largely recycle back to the cell surface, a smaller fraction being missorted to the limiting vacuolar membrane (Fig. 2B). To test the latter hypothesis, we isolated bro1Δ strains containing additional mutations that impair transport from the endosomes to the trans-Golgi network (TGN), a step required for recycling of Chs3 (chitin synthase III) and Snc1 (v-SNARE) from the endocytic pathway back to the plasma membrane (44, 45). Endosome-to-Golgi transport depends on several factors, such as the Golgi-associated GTPase Ypt6, the VFT complex (Vps51-Vps52-Vps53-Vps54), and the late Golgi/endosomal SNARE proteins Tlg1 and Tlg2 (44, 46–49). Interestingly, deletion of the TLG1 gene alone is lethal in our wild type background (21275b), as reported for the W303 strain (50), but the same deletion is viable in the npi3/brα1 mutant, although growth is reduced. Addition of NH₄⁺ to bro1Δ ypt6Δ cells caused rapid loss of Gap1 activity, similar to that displayed by the wild type and ypt6Δ strains, but this internalization was not accompanied by degradation of the permease (Fig. 2, A and C). Also consistent with efficient Gap1 internalization in bro1Δ ypt6Δ cells is the NH₄⁺-triggered decline in the intensity of Gap1-GFP fluorescence present at the cell surface. Similar results were obtained with bro1Δ mutants carrying vps52Δ or tlg1Δ deletions, but the tlg2Δ mutation did not alter the phenotype of bro1Δ cells (data not shown). NH₄⁺-induced Gap1 internalization, but not degradation, may thus be restored if the bro1Δ strain is additionally defective in factors involved in endosome-to-Golgi transport. This observation is entirely consistent with Gap1 being internalized but rapidly recycled back to the cell surface in bro1Δ cells. The data also show that, as in the bro1Δ single mutant, at least part of the Gap1-GFP internalized in the bro1Δ ypt6Δ strain reaches the vacuole limiting membrane. This strain also displayed fragmentation of the vacuole, a characteristic of the ypt6Δ cells (data not shown) (46). Finally, as down-regulation of Gap1 is normal in the ypt6Δ mutant, it seems unlikely that the process involves a passage of the permease through the Golgi (51).

These data thus show efficient Gap1 internalization and a defect in Gap1 MVB sorting in the bro1 mutant. Yet in contrast to what happens in the class E mutants described above, most of the internalized permease apparently recycles back to the plasma membrane via the TGN, and only a part of the protein reaches the vacuole limiting membrane.

**Bro1 Is Not Essential to Gap1 Ubiquitination**—Addition of NH₄⁺ triggers ubiquitination of Gap1, and this modification is crucial to endocytosis of the permease (20, 22). This ubiquitination is readily detected on immunoblots of membrane-enriched cell extracts as several additional bands appeared above the main Gap1 signal shortly after addition of NH₄⁺ to proline-grown cells (Fig. 3A) (20). These bands are not detected in cells expressing the altered Gap1K9K16 permease, where arginine replaces both ubiquitin-acceptor lysines (22).

As shown above, Gap1 is efficiently internalized in bro1Δ mutants that are additionally defective in recycling (Fig. 2). Furthermore, this internalization requires ubiquitination of the permease, because the mutant Gap1K9K16 permease remained active after addition of ammonium to the bro1Δ ypt6Δ strain (Fig. 2A). This was confirmed with a GFP-tagged version of Gap1K9K16 (data not shown). These results show that Bro1 is essential neither to ubiquitination nor to internalization of the Gap1 permease. We did, however, reproducibly observe a reduced level of ubiquitinated Gap1 in the bro1Δ mutant (30); the upper bands corresponding to the ubiquitin-conjugated forms of Gap1 were markedly less intense in the bro1Δ strain than in the wild type, their detection requiring immunoblot overexposure (Fig. 3A).

One possible interpretation of these results is that Bro1 is involved in ubiquitin fixation to Gap1 at the plasma membrane but that the ubiquitination defect observed in bro1Δ cells is not sufficient to impair the early steps of Gap1 endocytosis. To test this hypothesis, we monitored Gap1 ubiquitination in an end3Δ strain defective in endocytosis. As expected, addition of NH₄⁺ to these cells resulted in the rapid appearance of additional bands above the main Gap1 signal (Fig. 3B). That these upper bands...
correspond to ubiquitin-conjugated forms of Gap1 is shown by their total absence in end3Δ/H9004 cells expressing the Gap1 K9K16 mutant. In the bro1Δ end3Δ double mutant, the intensity of the upper bands was not significantly different from that observed with the end3Δ single mutant. This result indicates that Bro1 is not essential per se to Gap1 ubiquitination at the plasma membrane. It also shows that the lesser ubiquitination of Gap1 in bro1Δ cells is apparent only if the endocytosis function is normal. This suggests that Gap1 could be partially deubiquitinated after its endocytosis in bro1Δ cells.

Gap1 Reaches the Pep12-containing Endosome before Returning Back to the Plasma Membrane in bro1Δ Cells—We next sought to determine more precisely at which step of the endocytic pathway Gap1 is recycled back to the plasma membrane in the bro1Δ mutant. For this, we monitored the fate of the permease in bro1Δ cells additionally defective in distinct steps of endocytosis. We first combined the bro1Δ mutation with a deletion of the PEP12 gene. In mutants defective in this t-SNARE protein, the endocytic pathway is impaired in fusion of early endosome-derived vesicles with the late endosome/prevacuolar compartment, but protein recycling from the early endosome to the plasma membrane appears not to have interference (44, 52, 53). Pep12 is also required for TGN- and vacuole-to-late endosome/prevacuolar compartment transport steps (52, 53).

As expected, Gap1 was inactivated upon addition of NH₄⁺ to the pep12Δ single mutant, almost as efficiently as in the wild type, but the internalized permease was not degraded (Fig. 4, A...
Bro1 Function in MVB Sorting

Bro1 is not essential for Gap1 ubiquitination. Western analysis of Gap1 in membrane-enriched protein extracts prepared from cells growing on proline and harvested before (t = 0 min) and at several times after addition of NH₄⁺. A, strains: wild type (Wt) (23344c), bro1Δ (27092a); B, strains: wild type (Wt) (EK008) and bro1Δ end3Δ (36222d) transformed with YCpGap1 and end3Δ (EN121) transformed with either YCpGap1 or YCpGap1K30K16.

and C). Moreover, NH₄⁺ addition caused the appearance of additional bands above the main Gap1 signal that were maintained in the mutant throughout the experiment. These bands correspond to ubiquitin-conjugated forms of Gap1 and were not detected while using the Gap1K30K16 form of the permease (data not shown). Analysis of Gap1-GFP in pep12Δ cells showed an accumulation of internalized permease in numerous small intracellular dots, without any fluorescence in the vacuolar lumen (Fig. 4B). Thus, NH₄⁺ triggers Gap1 ubiquitination and internalization in pep12Δ cells, but the permease is blocked in small vesicular structures instead of reaching the vacuolar lumen. Furthermore, a large fraction of the permease seems to remain in a ubiquitinated form. A very similar fate of Gap1 was observed in bro1Δ pep12Δ double mutant cells (Fig. 4) showing that the pep12Δ mutation is epistatic over the bro1Δ defect. This indicates that Gap1 has to reach the Pep12-containing compartment before it can be recycled back to the cell surface in the bro1Δ mutant. The fact that Gap1 is efficiently internalized and ubiquitinated in bro1Δ pep12Δ cells further confirms that Bro1 is not essential to these processes. As shown previously, however, ubiquitinated forms of Gap1 were hardly detected in bro1Δ single mutant cells (Fig. 4C). The fact that these forms were readily detected in the bro1Δ pep12Δ cells is consistent with the view that Gap1 internalized in bro1Δ cells undergoes deubiquitination at a step downstream from Pep12, i.e. later in the endocytic pathway or during recycling.

Bro1 Functions at a Late Step of the MVB Sorting Pathway—Bro1 thus functions downstream from Pep12 (Fig. 4), and its absence leads to partial relocation of internalized Gap1 to the peripheral vacuolar membrane (Fig. 2). Hence, Bro1 is involved in MVB sorting of Gap1, a conclusion consistent with the recent characterization of Bro1 as a class E Vps factor required for MVB sorting of CPS (35). Yet Bro1 clearly differs from the class E Vps factors evaluated in this work (Vps23, Vps36, Snf7, and Vps4), because a Bro1 deficiency leads to recycling of internalized Gap1 back to the cell surface. To pinpoint more precisely the step at which Bro1 intervenes in MVB sorting, we constructed double mutant strains combining deletions of the BRO1 and VPS23 (ESCRT-I) or SNF7/VPS32 (ESCRT-III) genes, and we monitored the fate of Gap1 in these cells before and after NH₄⁺ addition. On proline medium, all strains exhibited high Gap1 activity, Gap1-GFP being located at the cell surface. After addition of NH₄⁺, both double mutant strains displayed a phenotype similar to that of the two vpsΔ single mutants, i.e. Gap1 was internalized but not degraded and Gap1-GFP was entrapped mostly in internal structures (one to three per cell) next to the vacuole, a fraction of the permease being present at the vacuole limiting membrane (Fig. 5). Structures adjacent to the vacuole were also stained with the lipophilic dye FM4-64 and most likely represent the class E compartment (43). Finally, in both the bro1Δ vpsΔ double mutants as well as in the vpsΔ single mutants, several bands appeared above the main Gap1 signal that were sustained throughout the experiment. These bands reflect ubiquitination of Gap1 and were not detected when the Gap1K30K16 form of the permease was used (data not shown). Similar results were obtained with the bro1Δ vpsΔ strain (data not shown). As observed previously, no ubiquitinated forms of Gap1 were detected in bro1Δ single mutant cells.

Taken together, these results clearly show that the various class E vpsΔ mutations are epistatic over the bro1Δ defect, i.e. Bro1 apparently acts after the Vps23 and Snf7 factors, at a late step of the MVB sorting pathway. Moreover, our data confirm that Bro1 is not dispensable to either Gap1 ubiquitination or Gap1 endocytosis, because the permease is efficiently internalized and accumulates in ubiquitinated forms in the bro1Δ vpsΔ double mutant cells. Yet ubiquitinated Gap1 is barely detected in bro1Δ single mutant cells (Fig. 5C). This suggests that the deubiquitination of Gap1, proposed to occur after internalization in bro1 mutant cells, takes place after the ESCRT-I–III-dependent steps.

Gap1 Recycling in bro1Δ Cells Requires a Normal Vam3 Function—The above analyses of epistasis relationships are thus consistent with Bro1 acting after the main components of the ESCRT-III complex. On the other hand, recent data suggest that Bro1 might interact with at least one component of ESCRT-III; in a systematic study of protein complexes, Bro1 was found to copurify with Snf7 and Vps4 (54). Furthermore, association of Bro1 with endosomes depends on Snf7. As the association of ESCRT-III with endosomes is not Bro1-dependent, however, Bro1 is proposed not to assemble as a core component of ESCRT-III (35). These results, together with our observations, suggest that Bro1 might intervene just after the main ESCRT-III components in order to mediate correct sorting of Gap1 into MVB vesicles. In bro1Δ mutant cells, Gap1 would largely recycle from the endosomal membrane back to the cell surface, whereas a smaller fraction would reach the vacuole limiting membrane. The deubiquitination of Gap1 proposed to occur in the bro1Δ strain might thus be catalyzed by Dof4, because the association of this deubiquitinating enzyme with endosomes also depends on ESCRT-III components (6, 15).

Should this model be true, Gap1 recycling in bro1Δ cells should not depend on the last step of the endocytic pathway, i.e. endosome-to-vacuole transport. To test this, we determined the fate of Gap1 in bro1Δ cells additionally lacking the VAM3 gene. A defect in this vacuolar t-SNARE protein impairs the fusion of various transport intermediates with the vacuole (55, 56). Upon addition of NH₄⁺ to vam3Δ single mutant cells, Gap1 was, as expected, internalized as in the wild type (Fig. 6). Gap1 activity was rapidly lost (Fig. 6A), and Gap1-GFP shifted from the cell surface into numerous internal punctate structures (Fig. 6B). These structures, often stained also by FM4-64, likely correspond to endosomes unable to fuse with the vacuole. In keeping with the Vam3 function in vacuolar homotypic fusion,
the cells lacked large, distinct vacuoles (55, 56). Also as expected, the Gap1 permease internalized in the \textit{vam3}/H9004 strain was not degraded (Fig. 6). Yet in contrast to various mutants impaired in earlier steps of endocytosis (\textit{pep12}/H9004, \textit{vps23}/H9004, and \textit{snf7}/H9004; Figs. 1, 4, and 5), Gap1 did not accumulate in a ubiquitinated form in \textit{vam3}/H9004 cells (Fig. 6). It has been proposed that proteins following the MVB pathway are deubiquitinated by the ubiquitin hydrolase Doa4 prior to their incorporation into MVB vesicles (6, 15). Most likely then, Gap1 also undergoes this deubiquitination.

Yet in the \textit{bro1}/H9004\textit{vam3}/H9004 double mutant, although Gap1 degradation was also impaired, the permease unexpectedly appeared to remain ubiquitinated; higher molecular weight forms were readily detected in this strain, in contrast to the \textit{vam3}/H9004 single mutant (Fig. 6C). These upper bands were not visible in extracts of the same cells expressing the Gap1 K9K16 mutant permease (not shown). It thus seems that \textit{bro1}/H9004\textit{vam3}/H9004 cells are impaired in the deubiquitination of Gap1 that probably occurs in \textit{vam3}/H9004 cells when the permease is sorted into the MVB pathway. This suggests that Bro1 functions before or in conjunction with Doa4 and that a defect in Bro1 prevents the Gap1 deubiquitination that normally occurs at the endosomal membrane.

Remarkably, NH$_4^+$ addition to the \textit{bro1}/H9004\textit{vam3}/H9004 double mutant also caused a clear loss of Gap1 activity (Fig. 6A). Gap internalization was confirmed by the observed Gap1-GFP shift from the cell surface to multiple internal structures after NH$_4^+$ addition, exactly as in \textit{vam3}/H9004 single mutant cells (Fig. 6B). Hence, the \textit{vam3}/H9004 mutation impairs recycling of Gap1 in the \textit{bro1}/H9004 mutant. A similar result was obtained with \textit{bro1}/H9004\textit{vam7}/H9004 cells (data not shown). Vam7 is another t-SNARE protein required for the same step as Vam3 in transport to the vacuole (57, 58). In contrast, Gap1 remained largely stable at the cell surface after NH$_4^+$ addition to a \textit{bro1}/H9004\textit{pep4}/H9004 strain defective in vacuolar proteolysis (59, 60) (Fig. 6A). These data clearly show that recycling of Gap1 in the \textit{bro1}/H9004 mutant is dependent on the function of Vam3, a t-SNARE protein required for protein transport to the vacuole.

**DISCUSSION**

Vacuolar lumen targeting of several yeast plasma membrane proteins, e.g. the mating factor receptors Ste2 and Ste3 and the
uracil permease Fur4, depends on their prior sorting into the MVB pathway. In class E vps mutant cells, these proteins accumulate in the class E compartment (5, 41, 61, 62). Here we report similar findings regarding the Gap1 permease, the down-regulation of which is triggered by addition of ammonium to the medium. Ubiquitination has been shown essential for MVB sorting of a number of proteins (6, 7). In class E vps mutants, a large fraction of Gap1 is found in ubiquitin-conjugated forms (Figs. 1C and 5C), as is the case with CPS (6). Although this question has not been directly addressed in our study, it seems most likely that ubiquitin fixation to Gap1 is required for MVB sorting of the permease.

We further report that Bro1 intervenes in MVB sorting of the Gap1 permease, and this is consistent with the recent identification of Bro1 as a class E Vps factor required for MVB sorting of CPS (35). The fate of Gap1 is markedly different, however, in mutants lacking Bro1 than in mutants lacking one of the other class E Vps factors. Two hours after NH$_4^+$ addition
to the bro1Δ mutant, most of the permease is still present at the cell surface, a part being found in the peripheral vacuolar membrane, without clear Gap1-GFP staining of punctate structures close to the vacuole. Moreover, in contrast to the classical E vps mutants, bro1Δ cells display less ubiquitin-conjugated Gap1 than the wild type. Although these observations are consistent with a dual role of Bro1 at both early (internalization) and late (MVB sorting) steps of endocytosis, we could show that Gap1 is efficiently ubiquitinated and internalized in bro1Δ cells after NH4+ addition. However, the internalized permease is largely recycled back to the plasma membrane, and a fraction is missorted to the vacuole limiting membrane. Recycling of Gap1 to the cell surface requires a normal endosome-to-Golgi transport function (Ypt6, Vps52, and Tlg1) (Fig. 2) indicating that it involves a passage of the permease through the TGN. Like Gap1, the uracil permease Fur4 also might undergo recycling, as Fur4 remains highly active in the bro1Δ mutant under conditions where it is normally down-regulated (30). This contrasts substantially with the fate of internalized Ste2 in the bro1Δ vps31 mutant, as the Ste2 receptor was reported to accumulate in the class E compartment in this strain (5). These results suggest that recycling of Ste2 could be less efficient and/or that retrieval of permeases from the endocytic pathway might be an active and selective process.

Although we first assumed Gap1 to be recycled from the class E compartment/endosome in the bro1Δ mutant, we unexpectedly observed that this recycling requires the vacuolar t-SNARE Vam3 (Fig. 6) and also Vam7, both of which function in endosome-to-vacuole traffic (55–58). This raises the interesting possibility that Gap1 recycling in the bro1Δ mutant might occur only after the permease has reached the limiting membrane of the vacuole. The Gap1-GFP detected at this location in bro1Δ cells might thus be the permease in transit to the cell surface. Alternatively, the effects of the vam mutations might be indirect, i.e. the mutations might somehow impair Gap1 retrieval from the endosome (Fig. 7). Yet the function of this compartment appears normal in vam3Δ cells, as transport and recycling of Vps10 between the Golgi and the late endosome remain unaffected (55). Also, despite their abnormalities, the small vacuoles of vam3Δ cells are competent in vacuole fusion and contain normal composition of vacuolar markers (56, 58). To date, recycling of yeast plasma membrane proteins has been
Lack of Bro1 does not impair ubiquitination of Gap1 at the plasma membrane (Fig. 6). Yet in bro1Δ single mutant cells, in which Gap1 recycles, ubiquitin-conjugated forms are hardly detected. But if Gap1 recycling in bro1Δ cells is prevented by impairing the endocytic pathway (in end3, pep12, and class E vps), the permease is readily detected in ubiquitin-conjugated forms. These observations suggest that recycling of Gap1 in bro1 mutant cells is accompanied by deubiquitination of the permease. Where does this deubiquitination take place and what is the deubiquitinating enzyme involved? Previous studies have shown that a late step of the MVB pathway involves

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5 E. Nikko, A.-M. Marini, and B. André, unpublished observations.
The ubiquitinated Gap1 would then reach the limiting membrane of the vacuole in a Vam3-dependent manner, and only after this step, Gap1 would recycle, and this recycling would be accompanied or preceded by deubiquitination catalyzed by another deubiquitinating enzyme.

The remarkable differences in the fate of Gap1 between various class E vps mutants and bro1Δ cells indicate that Bro1 ensures a particular role in the MVB pathway. Also in keeping with this view, we observed different FM4-64 staining in bro1Δ versus vps23Δ or snf7Δ cells (Fig. 2B and 4B), suggesting that the class E compartment is less prominent in bro1Δ cells. Furthermore, recurring of the CPY-sorting receptor Vps10 from the late endosome back to the Golgi is unaffected in bro1Δ/vps23Δ cells (9, 35) but impaired in mutants defective in other class E vps factors. Also noteworthy is the classification of Bro1 as a class F vps factor on the basis of vacuolar morphology (34). Class F vps mutants often have a large vacuole that is surrounded by small vacuole-like structures (9). Yet the exact molecular function of Bro1 in the MVB pathway remains to be discovered. Our genetic analysis shows that pep12Δ, vps23Δ, and snf7Δ mutations are all epistatic over the bro1 defect, suggesting that Bro1 acts at a late step of the MVB sorting pathway. This is in agreement with a recent study (54) reporting that Bro1 copurifies both with Snf7 and Vps4. It is also consistent with the Snf7-dependent association of Bro1 with the endosomal membrane (35). Finally, as discussed above, our data are compatible with Bro1 acting before or in conjunction with Doa4 in the MVB pathway. A likely mammalian counterpart for Bro1 is mouse AIP1/Alix (30), a protein recently shown to interact with the Snf7-dependent association of Bro1 with the endosomal membrane (36). Furthermore, overexpression of the C-terminal part of Alix leads to cytoplasmic vacuolization (73). Current knowledge of Bro1-AIP1/Alix proteins allows us only to speculate as to their precise function and to propose that they may intervene in protein trafficking by participating, directly or indirectly, in membrane deformation processes, e.g., those involved in the invagination of the late endosomal membrane to form MVB vesicles.

Acknowledgments—We are grateful to Sandra Lecomte, Nasiha MRabet, and Patrice Godard for their excellent technical support. We thank members of the laboratory and Rosine Haguenauer-Tsapis for fruitful discussions.

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