Ubiquitin Is Required for Sorting to the Vacuole of the Yeast General Amino Acid Permease, Gap1

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In yeast, ubiquitin plays a central role in proteolysis of a multitude of proteins and serves also as a signal for endocytosis of many plasma membrane proteins. We showed previously that ubiquitination of the general amino acid permease (Gap1) is essential to its endocytosis followed by vacuolar degradation. These processes occur when NH$_4^+$, a preferential source of nitrogen, is added to cells growing on proline or urea, i.e. less favored nitrogen sources. In this study, we show that Gap1 is ubiquitinated on two lysine residues in the cytosolic N terminus (positions 9 and 16). A mutant Gap1 in which both lysines are mutated (Gap1$^{K9K16}$) remains fully stable at the plasma membrane after NH$_4^+$ addition. Furthermore, each of the two lysines harbors a poly-ubiquitin chain in which ubiquitin is linked to the lysine 63 of the preceding ubiquitin. The Gap1$^{K9}$ and Gap1$^{K16}$ mutants, in which a single lysine is mutated, are downregulated in response to NH$_4^+$ although more slowly. In proline-grown cells lacking Npr1, a protein kinase involved in the control of Gap1 trafficking, newly synthesized Gap1 is sorted from the Golgi to the vacuole without passing through the plasma membrane (accompanying article, De Craene, J.-O., Soetens, O., and André, B. (2001) J. Biol. Chem. 276, 43939–43948). We show here that ubiquitination of Gap1 is also required for this direct sorting to the vacuole. In an npr1Δ mutant, newly synthesized Gap1$^{K9K16}$ is rerouted to and accumulates at the plasma membrane. Finally, Bul1 and Bul2, two proteins interacting with Npl1/Rep5, are essential to ubiquitination and down-regulation of cell-surface Gap1, as well as to sorting of neosynthesized Gap1 to the vacuole, as occurs in an npr1Δ mutant. Our results reveal a novel role of ubiquitin in the control of Gap1 trafficking, i.e. direct sorting from the late secretory pathway to the vacuole. This result reinforces the growing evidence that ubiquitin plays an important role not only in internalization of plasma membrane proteins but also in their sorting in the endosomes and/or trans-Golgi.

Ubiquitin is a 76-amino acid protein, which, in all eukaryotes, undergoes conjugation to a multitude of proteins. Although ubiquitination generally serves as a recognition signal for degradation by the proteasome (1, 2), studies in yeast have shown that ubiquitination of plasma membrane proteins results in their endocytosis followed by vacuolar degradation (3). Proteins subject to this mechanism include the G-protein-coupled mating pheromone receptors Ste2 (4) and Ste3 (5) and several transporters: the ABC proteins Ste6 (6) and Pdr5 (7), the uracil permease Fur4 (8), the amino acid permease Gap1 (9, 10), the tryptophan permease Tat2 (11), the galactose permease Gal2 (12), and the zinc transporter Zrt1 (13). Ubiquitination of most of these proteins has been shown to involve the ubiquitin-conjugating enzymes (E2) encoded by the UBC1–4 genes and an HECT-type ubiquitin ligase (E3) encoded by the essential NPI1/RSP5 gene (14). Ubiquitin has been shown to contain an endocytosis signal in the form of two surface patches surrounding two critical residues (Phe$^4$ and Ile$^{44}$) (15). However, the protein components of the endocytosis machinery involved in ubiquitin recognition remain unknown. It also remains undetermined as to whether ubiquitin also plays a role in the late steps of endocytosis and whether plasma membrane proteins undergo successive cycles of ubiquitination-de-ubiquitination during transit to the vacuole.

Here we have investigated the role of ubiquitin in the internal trafficking of the general amino acid permease (Gap1), which is tightly regulated by nitrogen. On proline or urea medium, i.e. conditions of poor nitrogen supply, the Gap1 gene is transcribed to high levels (16), and the synthesized Gap1 permease accumulates at the plasma membrane in an active and stable form (17, 18). Upon the addition of NH$_4^+$ (a preferential source of nitrogen), Gap1 is internalized by endocytosis and targeted to the vacuole for degradation. Ubiquitination of Gap1 is essential to this NH$_4^+$-induced down-regulation (9, 10). In the npi1 mutant, which displays an abnormally low level of the HECT-type ubiquitin ligase Npi1/Rep5, or the npi2 mutant lacking the Npi2/Doa4 de-ubiquitinating enzyme, Gap1 is not ubiquitinated and stays at the plasma membrane after NH$_4^+$ addition (10, 19). Furthermore, as shown for the uracil permease Fur4 (8), Gap1 is poly-ubiquitinated, the ubiquitin moieties being attached to the lysine 63 of the preceding ubiquitin (19) (henceforth called the lysine 63-linked poly-ubiquitin chain). Gap1 poly-ubiquitination is required for down-regulation of the permease at a maximal rate (19). The fate of newly synthesized Gap1 in the late secretory pathway is also under nitrogen control. On proline or urea medium, neosynthesized Gap1 is delivered to the plasma membrane, but in a medium containing glutamate (20) or NH$_4^+$ (18) as the sole nitrogen source, Gap1 is directly sorted from the Golgi to the vacuole without passing via the cell surface. A similar situation has been observed on proline medium with cells lacking Npr1, a

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1 The abbreviations used are: Gap1, general amino acid permease; UbRBR, ubiquitin in which lysine residues 29, 48, and 63 are replaced by arginine; Vps, vacuolar protein sorting; E2, ubiquitin-conjugating enzymes; EGFR, epidermal growth factor receptor; HECT, homologous to E6-AP C-terminus.
protein kinase controlling both cell-surface and internal Gap1 (18) and apparently inactivated by phosphorylation when good nitrogen sources are available (21).

In this paper we show that Gap1 is ubiquitinated on two lysine residues in its extreme N terminus, at positions 9 and 16. Using the Gap1K9K16 variant in which both lysine residues are mutated, we show that ubiquitination of Gap1 is required not only for down-regulation of the protein pre-accumulated at the cell surface but also for direct sorting of the protein from the late secretory pathway to the vacuole, as occurs in an npr1Δ mutant. We further show that ubiquitination and degradation of both cell-surface and internal Gap1 requires Bul1 and Bul2, two proteins interacting with the Npi1/Rsp5 ubiquitin ligase. As this paper was being reviewed, it was reported by others (22) that sorting of Gap1 to the vacuole requires its polyubiquitination and that the specific role of Bul1 and Bul2 is to specify this modification (see “Discussion”).

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Plasmids—All Saccharomyces cerevisiae strains used in this study (see Table I) are isogenic with YEp96 (26). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In steady-state experiments, proline (10 mM) was the sole nitrogen source.

In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine (5 mM) or NH4OH (100 mM) and transferred to proline medium to relieve GAP1 repression. In ubiquitin overexpression experiments, cells were grown on glutamine medium and transferred to preheated YNB (yeast nitrogen base without NH4OH or amino acids; Difeco) medium containing 10 mM proline, 3% glucose, and 0.1 mM CuSO4 to induce expression of ubiquitin. The 2×/H9262 and 2×/H9018 are derived from YEp96 (26). Centromeric plasmid YCPGAP1 (27) is based on the YCPFL38 plasmid (28). Plasmid YCPFL39 was used to complement trp1 auxotrophy (28).

Mutagenesis—Site-directed mutagenesis of GAP1 was performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene) on plasmid YCPGAP1 as recommended by the supplier. The primers used for each construct are described in Table II. Each construct was checked entirely by sequencing.

Permease Assays—Gap1 activity was determined by measuring incorporation of 20 μM 14C-labeled citrulline as described by Grenson (29). To avoid competitive inhibition of citrulline transport by glutamine, cells grown on glutamine medium were filtered, washed, and transferred to preheated proline medium just before the transport assay. The permease was inactivated by adding preheated (NH4)2SO4 to the culture (final concentration, 10 mM).

Yeast Cell Extracts and Immunoblotting—Crude cell extracts (9) and membrane-enriched preparations were prepared as previously described (10). In Western blot experiments, protein concentrations were estimated by densitometry of the Pma1 signal (ImageMaster1D, Amersham Pharmacia Biotech). Equal quantities of protein were loaded on an 8% SDS-polyacrylamide gel in a Tricine system (30). After transfer to a nitrocellulose membrane (Schleicher and Schuell), the proteins were probed with polyclonal antibodies raised against Gap1 (1: 10 000) or Pma1 (1: 1 000). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech) followed by enhanced chemiluminescence (Roche Molecular Biochemicals).

Gene Deletion—The bul1Δ, bul2Δ, and npr1Δ null mutations were constructed by the polymerase chain reaction-based gene deletion method (31). Plasmid pUG6 (32) served as template to generate DNA fragment loxP npi1::KanMX4 loxP with primers DEL1 and DEL2. DNA fragment loxP bul1::KanMX4 loxP with primers D5-bul1 and D3-bul1 (Table II), and DNA fragment loxP bul2::KanMX4 loxP with primers D5-bul2 and D6-bul2 (Table II). Excision of the KanMX4 cassette was performed by transformation of yeast cells with plasmid pSH47 carrying the Cre recombinase gene (32) and inducing Cre expression for 2 h. Cells were transformed by the lithium method (33) as modified by Gietz et al. (34).
medium to repress transcription of the GAP1 gene, then transferred to proline medium to relieve repression. In the wild type, this resulted in the appearance of a high intensity Gap1 signal on immunoblots and of high Gap1 activity in citrulline uptake assays (Fig. 1A). The npi1 strain displayed a similar phenotype. In the npr1Δ npi1 mutant, in keeping with the observation that Gap1 is directly sorted from the secretory pathway to the vacuole (18), no Gap1 activity was measured and only a low quantity of Gap1 was detectable after the cells were shifted to proline medium. The npr1Δ npi1 strain displayed a phenotype similar to that observed with the wild-type and npi1 strains, indicating that in the double mutant, neosynthesized Gap1 is

**Table II**

| Primer | Purpose | Primer sequence |
|--------|---------|-----------------|
| DEL1   | NPR1 deletion | 5’-TAG TAC GGA TTA GTC AGT GGC GTA CCT AGT GGC AAC AAT CGC GGC CGC CAG CTG AAG CTT CGT ACG C-3’ |
| DEL2   | NPR1 deletion | 5’-AGT AGA TTA TGA ACA GGA GGT CAA TCT ATT TAG GCT TCT ATA GCG GCC GCA TAG GCC ACT AGT GGA TCT G-3’ |
| OR5    | K9K16 mutagenesis | 5’-CG TAC GAG AGG AAT AAT CCA GAT AAT CTG AGA CAC AAT GG-3’ |
| OR6    | K9K16 mutagenesis | 5’-CC ATT GTG TCT CAG ATT ATC TGG ATT ATT CCT CTC GTA CG-3’ |
| OR7    | K51K56K60K63 mutagenesis | 5’-GT TCA GGG TCC AGA TGG CAA GAC TTT AGA GAT TCT TTC AGA AGG GTA AGA CCT ATT GAA G-3’ |
| OR8    | K51K56K60K63 mutagenesis | 5’-C TTC AAT AGG TCT TAC CCT TCT GAA AGA ATC TCT AAA GTC TTG CCA TCT GGA CCC TGA AC-3’ |
| OR11   | K9 mutagenesis | 5’-CT TCG TAC GAG AGG AAT AAT CCA GAT AAT C-3’ |
| OR12   | K9 mutagenesis | 5’-G ATT ATC TGG ATT ATT CCT CTC GTA CGA AG-3’ |
| OR13   | K16 mutagenesis | 5’-CCA GAT AAT CTG AGA CAC AAT GGT ATT ACC-3’ |
| OR14   | K16 mutagenesis | 5’-GGT AAT ACC ATT GTG TCT CAG ATT ATC TGG-3’ |
| D5-BUL1| BUL1 deletion | 5’-G AGA CTG TTC GTG TGT GTC AAC AGG TAT ATC GTA CGC TAA GCG GCC GCC AGC TGA AGC TT-3’ |
| D3-BUL1| BUL1 deletion | 5’-A TCT ATA AGA AAA GTA ACG AGA ATT TTT TCT AAT GTT TTT GCG GCC GCA TAG GCC ACT AG-3’ |
| D5-BUL2| BUL2 deletion | 5’-G CAG ATT TGA GAT ATA TTC TGG GGA ACA AAA GAA GTA TTA GCG GCC GCC AGC TGA AGC TT-3’ |
| D3-BUL2| BUL2 deletion | 5’-T ATT TGT AAA ACT GCG AGA TTA CTG TTA GTG TTG TAT GGT GCG GCC GCA TAG GCC ACT AG-3’ |

**Fig. 1.** Sorting to the vacuole of Gap1 neosynthesized in an npr1 mutant involves the Npi1/Rsp5 ubiquitin ligase. A, wild-type (W-T) (23344c), npr1Δ (30788a), npi1 (27038a), and npr1Δ npi1 (30788d) strains were grown on glutamine medium at 29 °C. The cells were then transferred to proline medium. Upper panel, Gap1 activity (nmol.min⁻¹ mg prot⁻¹) was assayed in cells growing on glutamine (Gln) and 3 h after transfer to proline medium (Pro) by measuring the incorporation of [¹⁴C]citrulline (0.02 m). The maximal Gap1 activity in the wild type (100%) corresponded to 20 nmol.min⁻¹ mg prot⁻¹ protein. Lower panel, immunoblot of Gap1 present in crude extracts prepared from cell samples taken from the same cultures before (Gln) and 3 h after transfer to proline medium (Pro). To make sure that equal amounts of protein were loaded, Pma1 was also immunodetected. B, strains GAL1-GAP1 (M, 33191b), GAL1-GAP1 npr1Δ (A, 33192c), GAL1-GAP1 pep12Δ (X, 33308c), GAL1-GAP1 npr1Δ pep12Δ (C, 33307a), GAL1-GAP1 npi1 (7, 33201b), and GAL1-GAP1 npr1Δ npi1 (7, 33191a) were grown on raffinose-proline medium. Galactose (200 mM final concentration) was added to induce GAP1 expression. Gap1 activity (nmol.min⁻¹ mg prot⁻¹) was assayed before and at various times after the addition of galactose by measuring the uptake of [¹⁴C]citrulline (0.02 mM).
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**Fig. 2. Sorting to the vacuole of Gap1 neosynthesized in an npr1Δ mutant requires a normal pool of ubiquitin.** Wild-type (27061b), npr1Δ (36012c), npi2Δ (27071b), and npr1Δ npi2Δ (36005b) strains were grown on glutamine medium at 29 °C. Cells were then transferred to proline medium to which CuSO₄ had been added (0.1 m) to induce ubiquitin overexpression. Upper panel, Gap1 activity (nmol/min/mg prot) was assayed in cells growing on glutamine (Gln) and 3 h after transfer to proline medium (Pro) by measuring incorporation of [¹⁴C]citrulline (0.02 m). Lower panel, immunoblot of Gap1 present in crude extracts prepared from cells samples taken from the same cultures before (Gln) and 3 h after transfer to proline medium. To confirm that equal amounts of proteins were loaded, Pma1 was also immunodetected.

Gap1 activity and npr1Δ show that sorting of Gap1 from the pep12Δ npr1Δ B cells resulted in the endocytosis of Gap1, as shown by staining with a strain overexpressing Gap1. Upper panel, Wild-type 4 strain overexpressing Gap1 was as active as in the wild type, and subsequent down-regulation of Gap1, which normally occur in the progressive increase of Gap1 activity in the wild type, indicating that the lysine residues at positions 9 and 16 are each similar to that in the pep12Δ mutant. This confirms that the pep12Δ mutation largely suppresses the effect of the npr1Δ mutation, even though the activity in the pep12Δ strain is lower than in the wild type (18). The same phenotype was observed in the npr1Δ npi2Δ strain (Fig. 1B). These results confirm those of Fig. 1A and show that sorting of Gap1 from the late secretory pathway to the vacuole requires the ubiquitin ligase Npi1/Rsp5. They also show that when Npi1/Rsp5 is lacking, at least part of the neosynthesized Gap1 is rerouted to the plasma membrane.

**Direct Sorting of Neosynthesized Gap1 to the Vacuole Requires a Normal Pool of Ubiquitin**—To further assess the role of ubiquitin in the direct sorting of Gap1 from the Golgi to the vacuole, we monitored the fate of neosynthesized Gap1 in npr1Δ cells also lacking the Npi2/Doa4 ubiquitin hydrolase. This enzyme facilitates ubiquitin recycling from proteasome-targeted substrates (38). In mutants affected in the Npi2/Doa4 ubiquitin hydrolase, the internal pool of ubiquitin is reduced severalfold (8, 19, 39); this impairs ubiquitination and the subsequent down-regulation of Gap1, which normally occur when NH₄⁺ is added to proline-grown cells (19). Wild-type, npi2Δ, npr1Δ, and npr1Δ npi2Δ strains were grown on glutamine medium and then shifted to proline medium (Fig. 2). As expected, Gap1 remained inactive in the npr1Δ strain, and the quantity of Gap1 detected was much lower than in the wild type. In the npr1Δ npi2Δ strain, Gap1 was as active as in the wild type, and an even higher amount of Gap1 was detected after the cells were shifted to proline medium, indicating that the npi2 mutation results in rerouting of neosynthesized Gap1 to the plasma membrane. In the npr1Δ npi2Δ strain overexpressing ubiquitin, a phenotype similar to that of the npr1Δ strain was observed, confirming that the effect of the npi2 mutation can be overcome by increasing the internal ubiquitin pool. Hence, direct sorting to the vacuole of neosynthesized Gap1 in npr1Δ cells requires a normal pool of ubiquitin, and if this pool is too limiting, Gap1 is rerouted to the plasma membrane.

**Lysine Residues at Positions 9 and 16 Are Essential to Down-regulation of Gap1**—The data presented above show that direct sorting to the vacuole of newly synthesized Gap1, as occurs in the npr1Δ mutant, requires normal levels of both Npi1/Rsp5 ubiquitin ligase and monomeric ubiquitin. These results suggest that ubiquitination of Gap1 could be required for its sorting to the vacuole. To test this hypothesis, experiments were conducted to isolate a mutant form of Gap1 resistant to ubiquitination. Previous work has identified the lysine residues of several permeases to which ubiquitin is attached (11, 13, 14). In the case of the uracil permease, for instance, ubiquitin is covalently linked to two lysine residues in a PEST region at the extreme N terminus (positions 38 and 41) (40). When these residues are mutated, ubiquitination and endocytosis of the permease are impaired. Each residue, furthermore, is subject to poly-ubiquitination, the ubiquitin moieties of the poly-ubiquitin chains being linked via the Lys⁶³ residue of ubiquitin (41). In the case of the tryptophan permease Tat2, a protein homologous in sequence to Gap1, mutation of the five lysine residues present in the 31 N-terminal amino acids preceding the first transmembrane domain were needed to protect the permease against down-regulation induced by rapamycin treatment (11). These observations prompted us to mutagenize lysine residues present in the cytosolic N terminus of Gap1. Two Gap1 mutants were thus constructed in which lysine residues were replaced with arginine, respectively, at positions 9 and 16 (Gap1K⁹K¹⁶) and positions 51, 56, 60, and 63 (Gap1K⁵¹–⁶³) (Fig. 3A). The gap1Δ strain was transformed with centromere-based plasmids bearing the Gap1, Gap1K⁹K¹⁶, or Gap1K⁵¹–⁶³ gene expressed under the natural GAP1 promoter. Citrulline uptake assays performed on transformed cells grown on proline medium revealed that both the Gap1K⁹K¹⁶ and the Gap1K⁵¹–⁶³ gene encode fully active Gap1 permeases. The addition of NH₄⁺ to Gap1 cells resulted in the endocytosis of Gap1, as shown by progressive loss of Gap1 activity (Fig. 3B), followed by degradation of the permease, as shown by progressive disappearance of the Gap1 signal detected on immunoblots (Fig. 3C) (10). A similar situation was observed with cells expressing the Gap1K⁵¹–⁶³ gene, indicating that lysine residues at positions 51, 56, 60, and 63 are not important for NH₄⁺-induced down-regulation. In contrast, the permease encoded by the Gap1K⁹K¹⁶ allele remained active and stable after NH₄⁺ addition (Fig. 3, B and C), demonstrating that the lysine residue(s) at position(s) 9 and/or 16 is/are crucial to down-regulation of Gap1. To determine the contribution of each lysine residue, we constructed alleles Gap1K⁹ and Gap1K¹⁶. In the corresponding proteins, the lysine residue at position 9 or 16, respectively, is replaced with arginine. Experiments showed that both of these Gap1 variants are fully active on proline medium (Fig. 3B). After NH₄⁺ addition, both permeases were down-regulated, indicating that the lysine residues at positions 9 and 16 are each
sufficient to promote \( \text{NH}_4^+ \)-induced down-regulation of Gap1. However, the Gap1\(^{K9K16}\) and Gap1\(^{K16}\) variants were down-regulated significantly more slowly than the native Gap1 protein, indicating that both lysine residues are required to promote maximal-rate down-regulation.

Lysine Residues 9 and 16 of Gap1 Are Acceptor Sites for \text{Lys}^{63}\text{-linked Poly-ubiquitin Chains}—We next examined the ubiquitination state of Gap1, Gap1\(^{K9K16}\), Gap1\(^{K9}\), and Gap1\(^{K16}\). To facilitate detection of ubiquitin-permease conjugates, we performed immunoblotting with membrane-enriched fractions (10). We used cells overexpressing ubiquitin: gap1\(\Delta\) cells expressing the plasmid-borne GAP1, GAP1\(^{K9}\), GAP1\(^{K16}\), or GAP1\(^{K9K16}\) allele were additionally transformed with a high-copy vector bearing the ubiquitin gene under the control of the inducible CUP1 promoter (26). The signal immunodetected in proline-grown cells expressing native Gap1 consisted of a major band at 60 kDa plus minor upper bands at higher molecular masses, corresponding to ubiquitin-conjugated forms of the permease (Fig. 4, upper panel) (10, 19). In keeping with previous observations (10, 19), the amount of ubiquitin-conjugated forms of Gap1 increased in the early minutes after \( \text{NH}_4^+ \) addition. Upper bands were also observed in cells expressing the GAP1\(^{K9}\) or GAP1\(^{K16}\) allele, showing that these Gap1 mutant forms are still ubiquitinated. No upper bands were detected in cells expressing the GAP1\(^{K9K16}\) allele (Fig. 4, upper panel) even if the immunoblots were overexposed (not shown). These data show that Gap1 is ubiquitinated specifically on the lysine residues at positions 9 and 16.

That Gap1 is ubiquitinated on only two acceptor sites was previously deduced from experiments aimed at determining the type of ubiquitin chain linked to Gap1 (19). Upon overexpression in an npi2 mutant of a ubiquitin mutant unable to form any kind of poly-ubiquitin chain, Gap1 forms attached to zero, one, or two ubiquitin moieties were detected (19). A similar pattern was specifically observed with cells overexpressing a ubiquitin variant (Ub\(^{K63R}\)) defective in formation of \text{Lys}^{63}\text{-linked chains}, indicating that poly-ubiquitin chain formation on Gap1 involves linkage through the lysine 63 residue of ubiquitin (19). The data presented in Fig. 4 (lower panel) show that upon overexpression of Ub\(^{K63R}\) instead of normal ubiquitin in cells expressing GAP1\(^{K9}\) or GAP1\(^{K16}\) genes, a single upper band accumulates above the main Gap1 signal. In contrast, two bands accumulated above the main Gap1 signal in cells expressing wild-type GAP1. These results show that both lysine residues (positions 9 and 16) are ubiquitinated with \text{Lys}^{63}\text{-linked chains.}

Ubiquitination of Gap1 Is Required for Direct Sorting from the Late Secretory Pathway to the Vacuole—The data presented in Fig. 3 show that non-ubiquitination of Gap1 at positions 9 and 16 renders the permease pre-accumulated at the plasma membrane resistant to \( \text{NH}_4^+ \)-induced down-regulation. To determine whether these lysine residues of Gap1 are also required for direct sorting of neosynthesized permease from the late secretory pathway to the vacuole, we transformed a \( \text{gap1} \Delta \) strain with the plasmid-borne GAP1, GAP1\(^{K9}\), GAP1\(^{K16}\), or GAP1\(^{K9K16}\) gene. Cells were grown on a medium containing \( \text{NH}_4^+ \) at high concentration (100 mM) so as to repress GAP1 gene expression and were then shifted to proline medium to relieve repression (Fig. 5). As expected, no Gap1 activity was measured in the \( \text{gap1} \Delta \) strain expressing the wild-type GAP1 allele. The results were the same when the GAP1\(^{K9}\) or GAP1\(^{K16}\) allele was expressed in the same strain. High Gap1 activity was measured, however, in cells producing the
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Fig. 5. The Gap1K9K16 mutant neosynthesized in an npr1Δ mutant is rerouted to the cell surface. Strain 30629a (gap1Δ) transformed with plasmid YCpGap1 ( ), and strain JA389 (gap1Δ npr1-1) transformed with plasmid YCpGap1K9 ( ), YCpGap1K16 (X), or YCpGap1K9K16 ( ) were grown on NH4+ (100 mM) medium, and cells were then transferred to proline medium. Gap1 activity (nmol.min⁻¹ mg prot⁻¹) was assayed before and at various times after transfer on proline medium.

Gap1K9K16 variant. Hence the Gap1K9K16 variant, defective in ubiquitination, is rerouted to the plasma membrane upon neosynthesis in an npr1Δ mutant. This result confirms those presented above (Figs. 1 and 2) and shows that ubiquitination of Gap1 is essential to its direct sorting from the late secretory pathway to the vacuole. Ubiquitination of a single lysine residue (position 9 or 16) is apparently sufficient for the sorting of Gap1 to the vacuole, because the Gap1K9 and Gap1K16 mutants both behave like wild-type Gap1.

We next assessed whether poly-ubiquitin chain formation is required for the sorting of Gap1 to the vacuole. For this, we monitored the activity and immunodetected levels of Gap1 neosynthesized in an npr1Δ npi2 strain overexpressing either normal ubiquitin or a ubiquitin mutant (UbRRR) in which the lysines at positions 29, 48, and 63 are replaced by arginine (Fig. 5). This ubiquitin variant is unable to form poly-ubiquitin chains in vivo (26). As illustrated above, overexpression of normal ubiquitin largely suppressed the effect of the npi2 mutation in the npr1Δ strain, i.e. Gap1 remained poorly active and did not accumulate to high levels after the shift of the cells from glutamine to proline. Although a slightly higher activity and level of Gap1 were reproducibly observed when UbRRR instead of normal ubiquitin was overexpressed in the npr1Δ npi2 strain, the effect of the npi2 mutation was also largely overcome by UbRRR expression. This indicates that poly-ubiquitin chain formation is not essential to the direct sorting of Gap1 to the vacuole.

Bul1 and Bul2, Two Additional Factors Required for Ubiquitination and Down-regulation of Cell-surface Gap1. Are Also Required for Direct Sorting of the Permease to the Vacuole—Bul1 and Bul2 are highly similar proteins (51% identity) shown in two-hybrid system, cosedimentation, and immunoprecipitation experiments to interact with the Npi1/Rsp5 ubiquitin ligase (42, 43). There is evidence that this interaction involves a PY-motif in the Bul proteins (XPPXY) and some or all of the three repeats of the WW(P) domain in Npi1/Rsp5. As Bul1 is not a substrate of Npi1/Rsp5, it has been proposed that the Bul proteins function together with Npi1/Rsp5 in protein ubiquitination (42, 43). To assess the role of Bul1 and Bul2 in controlling Gap1 trafficking, bul1Δ and bul2Δ single mutants and a bul1Δ bul2Δ double mutant were isolated. We first analyzed the influence of the bul1Δ and bul2Δ mutations on Gap1 activity and stability after the addition of NH4+ to cells grown on proline medium. In the bul1Δ bul2Δ mutant, Gap1 was totally protected against the NH4+ induced inactivation and degradation observed in the wild-type strain (Fig. 5, A and B). We next assessed the level of Gap1 ubiquitination in this mutant. In keeping with previous observations (10, 19), immunoblots of membrane-enriched extracts of wild-type cells harvested after NH4+ addition showed an increased intensity of the upper bands corresponding to ubiquitin-conjugated forms of Gap1 (Fig. 6C). These upper bands were barely detectable in the bul1Δ bul2Δ double mutant, indicating that ubiquitination of Gap1 is largely defective in this mutant (Fig. 6C). Similar experiments with the bul1Δ and bul2Δ single mutants revealed that Gap1 is still ubiquitinated and largely sensitive to NH4+ induced down-regulation in these strains (not shown). Hence, the Bul1 and Bul2 proteins appear to share a redundant function essential to Gap1 ubiquitination and subsequent down-regulation of cell-surface Gap1. We finally tested whether the direct sorting of neosynthesized Gap1 to the vacuole occurring in an npr1Δ strain also involves the Bul proteins. The npr1Δ strain and an npr1Δ bul1Δ bul2Δ triple mutant were grown on high NH4+ medium and were then shifted to proline medium (Fig. 7D). As expected, no Gap1 activity was detected in the npr1Δ mutant. In contrast, the Gap1 activity of the npr1Δ bul1Δ bul2Δ strain was as high as that of the wild type, showing that neosynthesized Gap1 is rerouted to the plasma membrane in this mutant. This result confirms that a deficiency in Gap1 ubiquitination not only protects Gap1 present at the plasma membrane against NH4+ triggered down-regulation but also prevents Gap1 neosynthesized in an npr1Δ strain from being sorted to the vacuole.

DISCUSSION

In this report we show that ubiquitination of the yeast Gap1 permease takes place on two lysine residues in the extreme N terminus of the protein (positions 9 and 16), a region recently...
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Fig. 7. Bul1 and Bul2 are involved in the down-regulation and direct sorting of Gap1 to the vacuole. A, the wild-type (■) 23344c) and the OS27-1 (■ bul1∆ bul2∆) strain were grown on proline medium. At time 0, NH4+ (20 mM) was added. Gap1 activity (nmol min−1 mg prot−1) was assayed before and at various times after NH4+ addition by measuring the uptake of 14C-citrulline (0.02 mM). B, immunoblot of Gap1 present in crude extracts from cell samples collected from the same cultures before and various times after NH4+ addition. C, the wild-type (W-T) 23344c) and the OS27-1 (bul1∆ bul2∆) strains were grown on proline medium and transferred to proline supplemented with CuSO4 (0.1 mM) 2 h before the addition of NH4+. Cells were collected before and 5 min after NH4+ addition. Membrane-enriched fractions were prepared, and Gap1 was detected by Western immunoblotting. D, the wild type (W-T) 23344c) and strains OS27-1 (■, bul1∆ bul2∆), 30788a (▲, npr1Δ), and JA410 (◆, bul1∆ bul2∆ npr1Δ) were grown on NH4+ (100 mM) medium, and cells were transferred to proline. Gap1 activity (nmol min−1 mg prot−1) was assayed before and at various times after transfer by measuring the uptake of 14C-citrulline (0.02 mM).

shown to be cytosolic (44). Furthermore, each lysine residue carries a chain consisting of at least two ubiquitin moieties linked to each other via the lysine 63 residue of ubiquitin. A similar situation has been described for the uracil permease (Fur4), which is poly-ubiquitinated by Lys63-linked chains on residues 38 and 41 (40). The Gap1K9K16 variant, in which both lysine residues are replaced with arginine, is fully protected against endocytosis and subsequent degradation, which normally occur when NH4+ is added to cells growing on proline or urea as the sole nitrogen source. The Gap1W7 and Gap1K16 variants, in which a single lysine residue is changed, are down-regulated after NH4+ addition. This indicates that ubiquitination of Gap1 on a single lysine residue (at position 9 or 16) is sufficient for effective NH4+-triggered endocytosis and degradation. However, down-regulation of both Gap1W7 and Gap1K16 is slightly slower than wild-type Gap1, indicating that ubiquitination of Gap1 on both lysines is required for maximal rate down-regulation. We have also shown, in addition to the Npi1/Rsp5 ubiquitin ligase, at least one of the two highly similar proteins found to interact with Npi1/Rsp5, i.e. Bul1 or Bul2 (42, 43), is essential to NH4+-induced ubiquitination and down-regulation of Gap1. Taken together, these results suggest that a complex containing Npi1/Rsp5 and at least one of the Bul proteins promotes binding of ubiquitin to lysine residues 9 and 16 of Gap1. Further experiments will be needed to determine whether subsequent poly-ubiquitination of Gap1 via the Lys63 residue of ubiquitin (19) is also mediated by the Npi1/Rsp5-Bul system or whether another ubiquitin ligase enzyme is involved.

Our data further show that ubiquitin plays an essential role in another pathway of Gap1 trafficking, direct sorting of neo-synthesized Gap1 from the late secretory pathway to the vacuole. This direct sorting to the vacuole occurs in cells in which Npr1 is inactive, i.e. in the npr1Δ mutant grown on proline medium and in wild-type cells growing under good nitrogen supply conditions (18, 20). We have shown here that if, under these conditions, ubiquitination of Gap1 is defective (as a result of a npi1, npi2, bul1Δ bul2Δ, or Gap1K9K16 mutation), the permease is rerouted to the plasma membrane. We conclude that in the wild-type strain grown on proline medium, Gap1 is targeted to the plasma membrane, but that loss of the Npr1 function results in sorting of Gap1 to the vacuole (18). This sorting requires ubiquitination of the permease on at least one of the two lysine residues 9 and 16. In the absence of Gap1 ubiquitination in an npr1Δ mutant, Gap1 is rerouted to the plasma membrane. The mechanisms by which the Npr1 kinase positively regulates the sorting of internal Gap1 to the cell surface remain undetermined. Phosphorylation of Gap1 is not strictly dependent on Npr1, suggesting that the effect of Npr1 is indirect (18). The precise role of ubiquitin in the targeting of Gap1 to the vacuole also remains undetermined. Ubiquitin might serve as a signal for packaging Gap1 into vesicles bound for the vacuole, for instance vesicles budding from the late Golgi and/or ones formed by invagination of the prevacuole/late endosome membrane to form a multivesicular body (45). When this ubiquitination is defective, Gap1 could be recycled from this compartment to the plasma membrane.

Our data thus contribute to the growing body of evidence that ubiquitin plays an important role in the sorting of membrane proteins in the endosomal and/or late Golgi system(s) (46). In the same line, an unexpected link between ubiquitin and the endosomal system was recently evidenced by the partial association of the Npi2/Doa4 ubiquitin hydrolase enzyme with the late endosome (47). Furthermore, suppressor mutations bypassing the requirement for the Npi2/Doa4-de-ubiquitinating enzyme appear to affect genes coding for components of the vacuolar protein-sorting (Vps) pathway (47). In another study, a mutant form of the tryptophan permease (Tat2) incapable of binding ubiquitin appeared to be stabilized under
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conditions that normally lead to direct sorting of the permease from the late secretory pathway to the vacuole (11). Remarkably, Npr1 is necessary for destabilization of Tat2, indicating that Tat2 and Gap1 are inversely regulated by Npr1 (35). Also consistent with a role for ubiquitin in sorting to the vacuole is the observation that the class E Vps protein encoded by the VPS23/STP22 gene (48) and its mammalian homologue encoded by the tsg101 tumor susceptibility gene (49) both contain a domain similar to that present in the ubiquitin-conjugating enzymes (E2). This domain, however, lacks the conserved cysteine residue to which ubiquitin is covalently linked in classical E2 enzymes (50, 51). Mutations in the STP22/VPS23 gene were originally isolated for their ability to partially re-target to the cell surface a thermosensitive mutant Ste2 receptor, which, at a restrictive temperature is diverted from the secretory pathway to the vacuole for degradation (48). In mouse tsg101 mutant fibroblasts, the cation-independent mannose-6-phosphate receptor, which normally cycles between the trans-Golgi and endosomal compartments, is re-sorted to the cell surface (49). These trafficking events are reminiscent of what happens to neosynthesized Gap1, rerouted to the plasma membrane when its sorting to the vacuole is prevented by mutations impairing ubiquitination. In the case of the epidermal growth factor receptor (EGFR), ubiquitin is reported to play a specific role in sorting of internalized EGFR to the lysosome (thus preventing its recycling to the cell surface) (52). In the tsg101 mutant, furthermore, activated EGFR is largely recycled back to the cell surface rather than being degraded in the lysosome (49). Finally, it was recently reported that the lowered ubiquitin level of doa4 yeast mutants prevents sorting of the Ste6 ABC transporter from endosomal membrane to the vacuole (53).

Our results also show that the same cis- and trans-acting elements involved in Gap1 ubiquitination control the fate of both cell-surface and internal Gap1. Namely, the Npi2/Rsp5 ubiquitin ligase enzyme, the Bul1 and Bul2 proteins, and the lysine residues at positions 9 and 16 in Gap1 are required for both down-regulation of Gap1 pre-accumulated at the cell surface and sorting to the vacuole of Gap1 present in the late secretory pathway. Npr1 also controls both pools of Gap1 by preventing their sorting to the vacuolar degradation pathway under poor nitrogen supply conditions (18). Finally, mutations affecting sequences in the C-terminal tail of Gap1, including a dileucine motif, restore high Gap1 activity in an npr1 strain; they further prevent NH4+-induced down-regulation of cell-surface Gap1 but still allows binding of ubiquitin to Gap1 (10, 27). We currently envisage several models to account for these observations. First, the same mechanism involving the Npi1/Rsp5-Bul complex and sequences in both cytosolic tails of Gap1 would lead to delivery to the plasma membrane (poor nitrogen supply conditions). Finally, the type of ubiquitination undergone by Gap1 may also crucially influence the fate of the permease. Namely, we previously reported that when cells grow on poor nitrogen sources like proline or urea, a small fraction of the Gap1 is mono-ubiquitinated, but the addition of NH4+ or inactivation of Npr1 specifically induces poly-ubiquitination by Lys63-linked poly-ubiquitin chains (10, 18, 19). Hence, mono-ubiquitination of Gap1 present at the cell surface or late secretory pathway could trigger transport of the permease to an internal sorting compartment. There, either Gap1 would be poly-ubiquitinated, leading to its sorting to the vacuole via the multivesicular body pathway (good nitrogen supply conditions), or de-ubiquitinated, leading to its recycling to the plasma membrane (poor nitrogen supply conditions). Our current experiments aim at testing the validity of these models.

As this paper was being reviewed, it was reported that neosynthesized Gap1 is missorted to the vacuole in lst4A mutant cells and that this missorting requires a normal Bul function (22). These authors further reported that Gap1 is poly-ubiquitinated in urea-grown cells overexpressing ubiquitin. In the bulΔ bul2A strain, furthermore, poly-ubiquitination of Gap1 was found to be defective, and accumulation of mono-ubiquitinated Gap1 forms was instead observed. These data differ from ours because poly-ubiquitination of Gap1 (via Lys63-linked chains) has been observed only upon the addition of NH4+ to urea- or proline grown cells (19). Furthermore, we could not observe hyperaccumulation of mono-ubiquitinated Gap1 in the bulΔ bul2A strain (Fig. 7C). Neither could we show an essential role of poly-ubiquitin in sorting of Gap1 to the vacuole (Fig. 6). This experiment, however, has been carried out in cells defective in the Npi2/Doa4 de-ubiquitinating enzyme; perhaps poly-ubiquitin is required for sorting of Gap1 to the vacuole only if cells contain a normal Npi2/Doa4 enzyme. Finally, the same authors (22) proposed that Bul1 and Bul2 function as E4 enzymes specifying poly-ubiquitination of Gap1. These conclusions imply that lack of poly-ubiquitination of Gap1 would prevent down-regulation of cell surface Gap1, since our data clearly show that a normal Bul function is essential to endocytosis followed by vacuolar degradation of Gap1 (Fig. 7, A and B). Clearly, further experiments are needed to decipher the exact role of mono- versus poly-ubiquitin in control of Gap1 trafficking. These studies will undoubtedly provide a deeper understanding of the role of ubiquitin in mechanisms regulating the internal trafficking of membrane proteins.

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