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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 NH₄⁺, 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 (Gap1K⁹K¹⁶) remains fully stable at the plasma membrane after NH₄⁺ 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 Gap1K⁹ and Gap1K¹⁶ mutants, in which a single lysine is mutated, are down-regulated in response to NH₄⁺ 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 Gap1K⁹K¹⁶ is rerouted to and accumulates at the plasma membrane. Finally, Bull and BuΠ2, two proteins interacting with Npi1/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 (Phe⁴ and Ile⁴⁴) (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 NH₄⁺ (a preferential source of nitrogen), Gap1 is internalized by endocytosis and targeted to the vacuole for degradation. Ubiquitination of Gap1 is essential to this NH₄⁺-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 NH₄⁺ 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 NH₄⁺ (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; UbRRR, 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 Gap1<sup>E30K16</sup> 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 Dip1/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 W303-1b (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internal Gap1 (23). Cells were grown in minimal buffered medium (24) with 3% glucose as the carbon source except when mentioned otherwise. In experiments of Gap1 neosynthesis, cells were grown exponentially on glutamine medium and transferred to preheated nitrogen sources available (21). (18) and apparently inactivated by phosphorylation when good protein kinase controlling both cell-surface and internalGap1 (9, 10, 36). These observations raise the interesting possibility that direct sorting of Gap1 from the late secretory pathway to the vacuole involves ubiquitination of the peroxisome—Site-directed mutagenesis of Gap1 was performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene) on plasmid pUC19GAP1 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 <sup>14</sup>C-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 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to the culture (final concentration, 10 mm).

**RESULTS**

**Direct Sorting of Neosynthesized Gap1 to the Vacuole Requires the Npr1/Rsp5 Ubiquitin-Protein Ligase—**In wild-type cells grown on a medium containing urea or proline as sole nitrogen source, newly synthesized Gap1 is sorted from the Golgi to the plasma membrane where it accumulates in an active and stable form. In contrast, in mutant cells lacking the Npr1 kinase, neosynthesized Gap1 is sorted from the Golgi to the vacuole without passing via the plasma membrane (18). This direct sorting of Gap1 to the vacuole also occurs when wild-type cells are grown on a medium containing either glutamate (20) or NH<sub>4</sub><sup>+</sup> (18) as sole nitrogen source, this being consistent with Npr1 being inactive under these favorable nitrogen supply conditions (35).

It was previously reported that loss of Gap1 activity in npr1 mutants is suppressed by the npi1 mutation (17, 18). This mutation results in a markedly reduced level of the HECT-type ubiquitin ligase Npi1/Rsp5, leading to non-ubiquitination of Gap1 (9, 10, 36). These observations raise the interesting possibility that direct sorting of Gap1 from the late secretory pathway to the vacuole involves ubiquitination of the permease. To investigate this possibility, we monitored the fate of newly synthesizedGap1 in wild-type, npr1Δ, npr1Δ, and npi1Δ cells (Fig. 1A). The cells were first grown on glutamine...
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 npr1 Δ 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 npr1 Δ 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...
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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), npi2 (27071b), and npr1Δ npi2 (36065b) strains were grown on glutamine medium at 29 °C. Cells were then transferred to proline medium to which CuSO4 had been added (0.1 mM) 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 mM). 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. To confirm that equal amounts of proteins were loaded, Pma1 was also immunodetected.

W-T  npi2  npr1  npr1 npi2  —  Ub

An activity gradually appeared after galactose addition in the late endosome/pre-vacuolar compartment (37), Gap1 protein Pep12 (required for transport of proteins from the Golgi to the plasma membrane) (36). When NH4+ is added to proline-grown cells (19), Wild-type, npi2, npr1Δ, and npr1Δ npi2 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 npi2 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 npi2 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 Lys48 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 cystolic 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–K63) (Fig. 3A). The mutant strain was transformed with centromere-based plasmids bearing the GAP1, GAP1K9K16, or GAP1K51–K63 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–K63 gene encode fully active Gap1 permeases. The addition of NH3+ 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–K63 gene, indicating that lysine residues at positions 51, 56, 60, and 63 are not important for NH3+-induced down-regulation. In contrast, the permease encoded by the GAP1K9K16 allele remained active and stable after NH3+ 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 GAP1K63 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 NH3+ addition, both permeases were down-regulated, indicating that the lysine residues at positions 9 and 16 are each

targeted to the cell surface rather than to the vacuole. To confirm this result, we placed the GAP1 gene under the control of the galactose inducible GAL1 promoter and monitored the neosynthesis of Gap1 by adding galactose to cells grown on a raffinose/proline medium (Fig. 1B). As expected, this resulted in the progressive increase of Gap1 activity in the wild type, indicating that Gap1 was delivered to the plasma membrane, whereas the permeases remained inactive in the npr1Δ mutant.

In the npr1Δ pep12Δ double mutant lacking the t-SNARE (target soluble N-ethylmaleimide attachment protein receptor) protein Pep12 (required for transport of proteins from the Golgi to the late endosome/pre-vacuolar compartment) (37), Gap1 activity gradually appeared after galactose addition in a manner 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 npi2 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 occurs when NH3+ is added to proline-grown cells (19). Wild-type, npi2, npr1Δ, and npr1Δ npi2 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 npi2 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 npi2 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.
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Fig. 3. The Gap1K9K16 mutant is resistant to NH4+-induced down-regulation. A, topological model of Gap1 and sequence of the N-terminal cytosolic region. The lysine residues replaced by arginine in mutagenesis experiments are underlined. B, strain 30692a (gap1Δ ura3) transformed with plasmid YCpGap1 (C), YCpGap1K9K16 (A), YCpGap1K9 (B), YCpGap1K16 (D), or YCpGap1K51 (E) was grown on proline medium. At time 0, NH4+ (20 mM) was added to the cultures. Gap1 activity (nmol min−1 ml−1) was assayed before and at various times after NH4+ addition by measuring the uptake of [14C]citrulline (0.02 mM). C, immunoblot of Gap1 present in crude extracts of cell samples collected from the same cultures before and various times after the addition of NH4+.

Fig. 4. Both of the ubiquitin acceptor lysines of Gap1 bind Lys63-linked poly-ubiquitin chains. Strain OS21 (gap1Δ npi2 trp1 ura3) transformed with the URA3 plasmid YCpGap1, YCpGap1K9K16, YCpGap1K9, or YCpGap1K16 was grown on proline medium and transferred to proline medium 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. Upper panel, each transformant was additionally transformed with the TRP1 YE906 plasmid carrying the ubiquitin gene under the control of the CUP1 promoter. Lower panel, each transformant was additionally transformed with the TRP1 plasmid carrying the mutant UbK63R ubiquitin gene.

The data presented in Fig. 4 (lower panel) show that upon overexpression of UbK63R instead of normal ubiquitin in cells expressing GAP1K9 or GAP1K16 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 Lys63-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-acclimated at the plasma membrane resistant to NH4+-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 gap1Δ npr1Δ strain with the plasmid-borne GAP1, GAP1K9, GAP1K16, or GAP1K9K16 gene. Cells were grown on a medium containing NH4+ 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 gap1Δ npr1Δ strain expressing the wild-type GAP1 allele. The results were the same when the GAP1K9 or GAP1K16 allele was expressed in the same strain. High Gap1 activity was measured, however, in cells producing the
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 JAA89 (gap1Δ npr1-1) transformed with plasmid YCpGap1K9G (○), YCpGap1K9K16 (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.

Fig. 6. Sorting to the vacuole of Gap1 in an npr1Δ mutant does not require poly-ubiquitin chain formation. The wild-type (27061b) and npr1Δ (36012c) strains and the npr1Δ npi2 (36005b) strain without plasmid or transformed with the plasmid bearing the wild-type (Ub) or mutant (UbRRR) ubiquitin gene were grown on glutamine medium at 29 °C. Cells were then transferred to proline medium containing CuSO4 (0.1 mM). Upper panel, Gap1 activity (nmol min^-1 mg prot^-1) was assayed in cells growing on glutamine and 3 h after transfer to proline medium by measuring the incorporation of [14C]citrulline (0.02 mM). Lower panel, immunoblot of Gap1 present in crude extracts prepared from samples of the same cultures 3 h after transfer to proline medium. To confirm that equal amounts of proteins were loaded, Pma1 was also immunodetected.

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The 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 Gap1K9Δ and Gap1K16Δ 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) which lysines at positions 29, 48, and 63 are replaced by arginine (Fig. 6). 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.

Bu1 and Bu2. 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—Bu1 and Bu2 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 Bu1 proteins (XPXXY) and some or all of the three repeats of the WW(P) domain in Npi1/Rsp5. As Bu1 is not a substrate of Npi1/Rsp5, it has been proposed that the Bu proteins function together with Npi1/Rsp5 in protein ubiquitination (42, 43). To assess the role of Bu1 and Bu2 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. 7, 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+-induced down-regulation showed an increased intensity of the upper bands corresponding to ubiquitin-conjugated forms of Gap1 (Fig. 7C). These upper bands were barely detectable in the bul1Δ bul2Δ double mutant, indicating that ubiquitination of Gap1 is largely defective in this mutant (Fig. 7C). 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 Bu1 and Bu2 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
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 Gap1W10D 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 Gap1W10D 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 that, 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 an npi1, npi2, bul1Δ bul2Δ, or Gap1K16 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 prevacuolar/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 retarget 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 Npi1/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 NH₂⁻-induced down-regulation of cell-surface Gap1 but still allow 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 act at both the plasma membrane and internal membrane level to attach ubiquitin on N-terminal lysines of Gap1, a modification triggering the sorting of the permease to the vacuolar degradation pathway. Second, the early steps of Gap1 endocytosis would not require Npi1/Rsp5-Bul-dependent ubiquitination. Upon reaching an internal compartment lying at the intersection of the endocytic and secretory pathways, internalized Gap1 would either be ubiquitinated or not. In the first case it would be sorted to the vacuole for degradation (this would happen when Npr1 is inactive, i.e. under good nitrogen supply conditions). In the second case it would be recycled to the cell surface (this would occur when Npr1 is active, i.e. under poor nitrogen supply conditions). The same rules would govern the fate of neosynthesized Gap1 reaching this internal compartment, i.e. ubiquitination would lead to sorting to the vacuole (good nitrogen supply conditions), and non-ubiquitination 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 NH₄Cl 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 neo-synthesized Gap1 is missorted to the vacuole in lst4Δ 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 bul1Δ bul2Δ 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 NH₂Cl to urea- or proline grown cells (19). Furthermore, we could not observe hyperaccumulation of mono-ubiquitinated Gap1 in the bul1Δ bul2Δ 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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