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Nitrogen-regulated Ubiquitination of the Gap1 Permease of *Saccharomyces cerevisiae*

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Addition of ammonium ions to yeast cells growing on proline as the sole nitrogen source induces rapid inactivation and degradation of the general amino acid permease Gap1 through a process requiring the Npi1/Rsp5 ubiquitin (Ub) ligase. In this study, we show that NH_4^+ induces endocytosis of Gap1, which is then delivered into the vacuole where it is degraded. This down-regulation is accompanied by increased conversion of Gap1 to ubiquitinated forms. Ubiquitination and subsequent degradation of Gap1 are impaired in the *npi1* strain. In this mutant, the amount of Npi1/Rsp5 Ub ligase is reduced >10-fold compared with wild-type cells. The C-terminal tail of Gap1 contains sequences, including a di-leucine motif, which are required for NH_4^+-induced internalization and degradation of the permease. We show here that mutant Gap1 permeases affected in these sequences still bind Ub. Furthermore, we provide evidence that only a small fraction of Gap1 is modified by Ub after addition of NH_4^+ to mutants defective in endocytosis.

INTRODUCTION

In eukaryotic cells, the degradation of many proteins requires their initial modification by conjugation to a 76-amino acid peptide called ubiquitin (Ub). In general, the covalent bond between Ub and lysine residues marks the substrate proteins for degradation by the 26S proteasome (reviewed by Hochstrasser, 1996). In the yeast *Saccharomyces cerevisiae*, involvement of the Ub pathway has been demonstrated in the degradation of several soluble proteins such as the Matα2 repressor (Chen *et al.*, 1993), the Gcn4 transcriptional activator (Kornitzer *et al.*, 1994), the fructose 1,6-bisphosphatase (Schork *et al.*, 1995), and several cyclins (Deshaies *et al.*, 1995; Seufert *et al.*, 1995; Yaglom *et al.*, 1995). There is now growing evidence, both in higher and lower eukaryotes, that Ub may also be used as a signal for endocytosis of some cell surface proteins and their subsequent degradation in the lysosome and vacuole (Hochstrasser, 1996). For instance, the yeast α-peptide transporter Ste6 is stabilized and accumulates in a ubiquitinated form in *end4* cells defective in the internalization step of endocytosis (Kölling and Hollenberg, 1994). Evidence supporting a role of Ub in turnover of cell surface proteins was further provided by Hicke and Riezman (1996) in the case of ligand-induced endocytosis of the Ste2 pheromone receptor. In a C-terminally truncated form of Ste2 still competent for ligand-induced endocytosis, substitution of a single lysine residue for arginine within a DAKSS sequence was shown to impair both ligand-induced ubiquitination and endocytosis of the receptor (Hicke and Riezman, 1996). Other recent experiments in yeast suggest that Ub is used as a signal for endocytosis of the uracil permease Fur4 (Galan *et al.*, 1996), the galactose permease Gal2 (Horak and Wolff, 1997), the pheromone receptor Ste3 (Roth and Davis, 1996), and the multidrug resistance protein Pdr5 (Egner and Kuchler, 1996). The mechanism by which Ub promotes endocytosis is still unknown. Whether binding of Ub to a cell surface protein constitutes a sufficient signal for endocytosis also remains undetermined. It has been suggested that Ub might be recognized by a component of the endocytosis machinery or might promote movement of ubiquitinated proteins into membrane regions that actively endocytose (Hicke and Riezman, 1996).

This report focuses on the regulation of turnover of the general amino acid permease (Gap1) in *S. cerevisiae*. The synthesis, the activity, and more recently the

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sorting of this permease in the late secretory pathway have been shown to be regulated according to the nitrogen source used by the cells (Wiame *et al.*, 1985; Grenson, 1992; Roberg *et al.*, 1997). Upon addition of NH_4^+ to cells grown on a less-favored nitrogen source such as proline, synthesis of the Gap1 permease is strongly reduced, and presynthesized permease is completely inactivated and degraded (Grenson, 1983a; Hein *et al.*, 1995). The products of the *NPI1* and *NPI2* genes are required for both inactivation and degradation of Gap1. We have previously shown that *NPI1* is an essential gene encoding the Ub–protein ligase Rsp5 (Huibregtse *et al.*, 1995); this suggests that the Ub pathway is involved in the turnover regulation of Gap1 (Hein *et al.*, 1995). In keeping with a role of Npi1/Rsp5 in permease turnover, this enzyme is required for basal and stress-induced ubiquitination and degradation of the uracil permease Fur4 (Galan *et al.*, 1996). Several mutations affecting the C-terminal hydrophilic tail render the Gap1 permease resistant to NH_4^+-triggered inactivation and degradation (Hein and André, 1997); one is a di-leucine→di-alanine substitution ($Gap1^{LL\rightarrow AA}$). In higher eukaryotic cells, the di-leucine motif has been shown to act as a signal for internalization of several cell surface proteins (Shin *et al.*, 1991; Letourneur and Klausner, 1992; Aiken *et al.*, 1994; Haft *et al.*, 1994; Dittrich *et al.*, 1996). A role of di-leucine in targeting membrane proteins to endosome and lysosome has also been reported (Sandoval and Bakke, 1994). The di-leucine of Gap1 is located in a region predicted to adopt a stable α-helical conformation. This putative helix also contains a glutamate residue which, when replaced by a lysine, leads to resistance of Gap1 to NH_4^+-induced inactivation and degradation. Finally, a Gap1 permease lacking the last 11 amino acids directly following the putative α-helix also remains active and stable after addition of NH_4^+ to the medium (Hein and André, 1997).

The present work shows that a small fraction of Gap1 is ubiquitinated in cells grown on proline medium. Addition of NH_4^+ increases the conversion of Gap1 to Ub-conjugated forms. This conversion is followed by rapid internalization of the permease and subsequent degradation in the vacuole. In *npi1* mutant cells, in which the level of Npi1/Rsp5 Ub ligase is much reduced, Gap1 ubiquitination is impaired, and the permease remains stable on the plasma membrane. Finally, mutations affecting the C-terminal tail of Gap1 impair NH_4^+-induced endocytosis and degradation of Gap1, but the mutant permeases are still ubiquitinated.

MATERIALS AND METHODS

Strains, Growth Conditions, and Plasmids

S. cerevisiae strains isogenic with the wild-type Σ1278b (Béchet *et al.*, 1970) are 24346c (*MAT**a**, ura3*), 27061b (*MAT**a**, ura3, trp1*), 27038a (*MAT**a**, ura3, npi1*) (Grenson, 1983a), JOD0097 (*MAT**a**, ura3, gap1::kanMX2*), and RTY1 (*MAT**a**, ura3, trp1, pep4::kanMX4*). Strains nonisogenic with Σ1278b are NY279 (*MAT**a**, ura3-52, act1-1*) and its isogenic parental strain NY13 (*MAT**a**, ura3-52*) (Shortle *et al.*, 1984; Goud *et al.*, 1988). Cells were grown in minimal buffered medium (pH 6.1) with 3% glucose as the carbon source (Jacobs *et al.*, 1980). Nitrogen sources were added as indicated at the following final concentrations: $(NH_4)_2SO_4$, 10 mM; proline, 0.1%. The YEpJYS-2 plasmid contains the *NPI1* gene of the YCpJYS-1 plasmid (Hein *et al.*, 1995), inserted into the 2μ-based multicopy vector pFL44 (Bonneaud *et al.*, 1991). The YCpGAP1 plasmid contains the *GAP1* gene (Jauniaux and Grenson, 1990) in the centromere-based vector pFL38 (Bonneaud *et al.*, 1991). $YCpGap1^{pgr}$, $YCpGAP1^{LL\rightarrow AA}$, and YCpGAP1Δ2 are modified versions of plasmid YCpGAP1, encoding altered Gap1 permeases (respective alterations: $E_{582}\rightarrow K_{582}$, L_{575} $L_{576}\rightarrow A_{575}$ A_{576}, and truncation of the last 11 amino acids; Hein and André, 1997). The 2μ-based multicopy plasmid YEp96 contains a synthetic yeast Ub gene under the control of the copper-inducible *CUP1* promoter; YEp105 is identical to YEp96 except that it encodes a c-Myc-tagged version of Ub (Hochstrasser *et al.*, 1991). Yeast cells treated with lithium acetate (Ito *et al.*, 1983) were transformed according to the method of Sherman *et al.* (1986). The *Escherichia coli* strain used was JM109. All procedures for manipulating DNA used standard methods (Ausubel *et al.*, 1995; Sambrook *et al.*, 1997).

Permease Assays

Gap1 permease activity was determined by measuring incorporation of ^{14}C-labeled citrulline as described by Grenson (1966). All permease activities were measured in cells that had reached the state of balanced growth (Wiame *et al.*, 1985). The permease was inactivated by adding prewarmed $(NH_4)_2SO_4$ to the culture at a final concentration of 10 mM.

Yeast Cell Extracts and Immunoblotting

Crude cell extracts were prepared as previously described (Hein *et al.*, 1995). For membrane-enriched preparations, ~10^8 yeast cells were filtered (Millipore, Bedford, MA; 0.45 μm), washed with cold water plus NaN_3 (10 mM), and resuspended in 0.2 ml lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA) containing the following proteinase inhibitors: 100 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 mM NaN_3, 50 mM *N*-ethyl maleimide. An equal volume of glass beads (0.45 μm) was added, and the cells were lysed at 4°C in a 2.0-ml Eppendorf (Madison, WI) tube by vortex mixing for 2 min. The extracts were diluted with 2 volumes of lysis buffer, transferred to new tubes, and centrifuged for 3 min at 3000 rpm. The membrane-enriched fraction was obtained from the supernatant as described elsewhere (Galan *et al.*, 1996). For Western blot analysis, 10 μl of solubilized proteins were loaded on a 12% SDS-polyacrylamide gel in a Tricine system (Schägger and von Jagow, 1987). For the experiment showing the shift of the ubiquitinated form of Gap1 upon expression of Ub-myc, the extracts were loaded on a 12% gel in Laemmli's system (Laemmli, 1970). After transfer to nitrocellulose the proteins were probed with rabbit antiserum raised against the N-terminal region of GAP1 (1:20,000) or against the mouse protein Nedd4 (1:1000) (Kumar *et al.*, 1997). Anti-Gap1 antibodies where shown elsewhere to be specific of Gap1 protein (De craene and André, unpublished data). Primary antibodies were detected with horseradish peroxidase–conjugated anti-rabbit-IgG secondary antibody followed by enhanced chemoluminescence (Amersham, Arlington Heights, IL). All Western blots were quantitated with a densitometer to measure protein amounts.

RESULTS

Ammonium-induced Endocytosis and Vacuolar Degradation of the Gap1 Permease

Addition of NH_4^+ to wild-type cells growing on proline as the sole nitrogen source induces loss of Gap1 permease activity and degradation of the permease (Grenson, 1983a; Hein *et al.*, 1995). As previously reported for other yeast cell surface proteins (Schandel and Jenness, 1994; Volland *et al.*, 1994; Egner *et al.*, 1995; Riballo *et al.*, 1995; Hicke and Riezman, 1996; Roth and Davis, 1996; Horak and Wolf, 1997), this degradation could take place in the vacuole after internalization of the permease. NH_4^+-induced loss of measurable Gap1 activity could thus reflect progressive removal of Gap1 from the plasma membrane. Alternatively, Gap1 could be inactivated before its internalization. To further elucidate the mechanisms underlying nitrogen-regulated turnover of the Gap1 permease, we assayed the activity of Gap1 in a strain carrying a thermosensitive mutation, *act1-1*, in the actin gene (Shortle *et al.*, 1984). This mutant is defective in the internalization step of receptor-mediated endocytosis of the α-factor (Kübler and Riezman, 1993). It is also defective in endocytosis of the uracil permease (Fur4), observed after inhibition of protein synthesis by cycloheximide (Galan *et al.*, 1996). The *act1-1* mutation has a strong effect at 37°C, a temperature at which Gap1 is partially inactivated, so we performed the experiment at 29°C, at which temperature *act1-1* cells still exhibit defective endocytosis, although the effect is less pronounced than at 37°C (Kübler and Riezman, 1993; Galan *et al.*, 1996). The results show that NH_4^+-induced inactivation of Gap1 is severely impaired in the *act1-1* mutant compared with the wild type (Figure 1A). An internalization step thus seems required for complete NH_4^+-triggered loss of Gap1 activity. In other words, the so-called nitrogen catabolite inactivation of Gap1 (Grenson, 1983a) is likely the result of progressive internalization of the permease by endocytosis.

To determine whether degradation of Gap1 after internalization takes place within the vacuole, we examined Gap1 in yeast cells with a defective vacuolar proteinase A (*pep4* mutant). This proteinase is required for maturation of several vacuolar proteases (Ammerer *et al.*, 1986; Woolford *et al.*, 1986). In the *pep4*Δ strain, Gap1 is inactivated by NH_4^+ as efficiently as in the isogenic wild type (Figure 2A). The amount of Gap1 protein in crude cellular extracts was analyzed using antibodies raised against the N-terminal region of the permease. The unique signal immunodected in wild-type cells corresponds to the Gap1 protein, as no signal is visible in a *gap1*Δ strain (our unpublished observations). Addition of NH_4^+ to cells growing on proline as the sole nitrogen source led to rapid degradation of the Gap1 protein. In the *pep4*Δ mutant, however, Gap1 was strongly protected against NH_4^+-induced degradation (Figure 2B). These results indicate that after internalization, Gap1 is targeted for vacuolar proteolytic breakdown. The phenomenon including internalization and subsequent

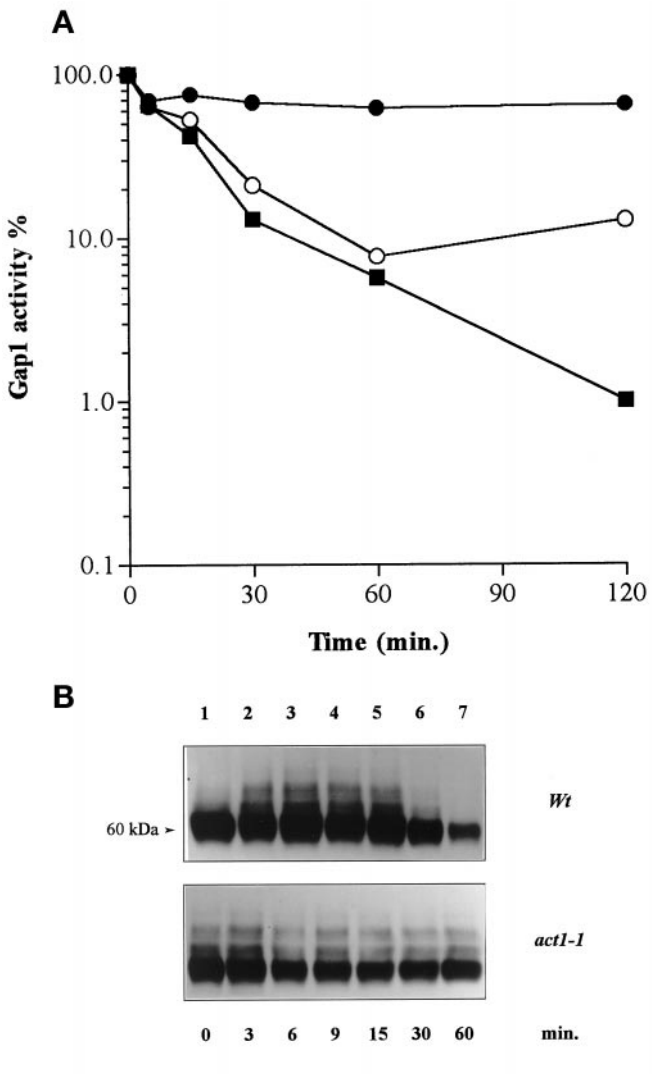

Figure 1. Ammonium-induced down-regulation of Gap1 is impaired in *act1-1* mutant cells. (A) Cells were grown on proline medium, and Gap1 activity was assayed by measuring incorporation of [^{14}C]citrulline (0.1 mM) before (t = 0) and at various times after addition of $(NH_4^+)_2SO_4$ (10 mM) in strains NY13 (wild type; ○), NY279 (*act1-1*; ●), and 24346c (wild type derived from Σ1278b; ■). The Gap1 activities were calculated in nanomoles per minute per milliliter to avoid dilution effect due to NH_4^+-triggered arrest of Gap1 synthesis. In proline-grown cells, initial Gap1 activity is lower in *act1-1* cells than in isogenic wild-type cells (1.3 vs. 4.2 $nmol \cdot min^{-1} \cdot ml^{-1}$). (B) Immunoblot of Gap1 from membrane-enriched cell fractions prepared before (t = 0) and at several times after addition of $(NH_4)_2SO_4$. Note that the disapearance of Gap1 signal in the NY13 wild-type strain is slower compared with the wild type derived from Σ1278b.

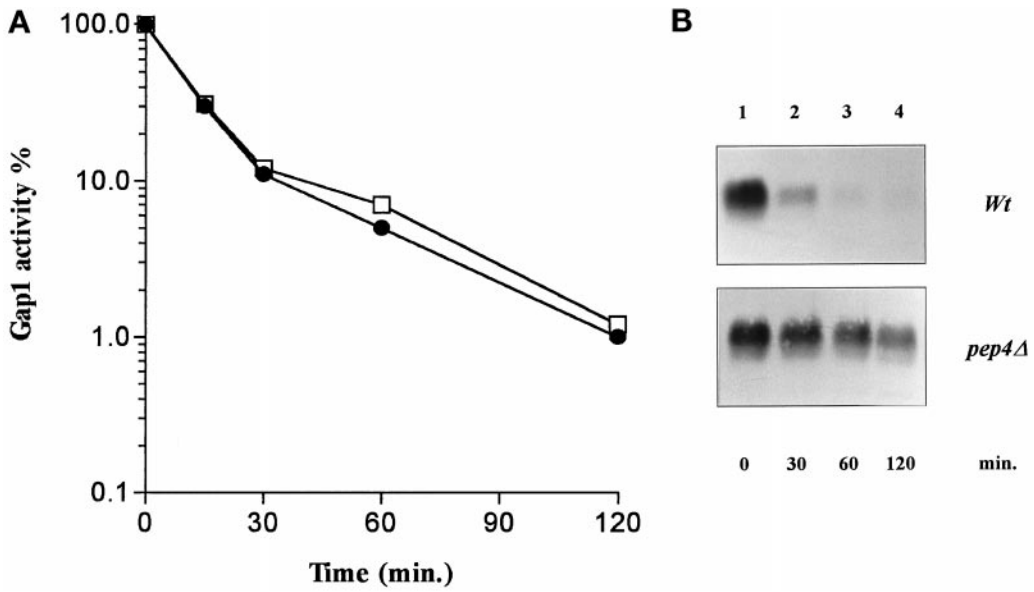

Figure 2. Ammonium-induced degradation of Gap1 is dependent on vacuolar proteases. (A) Cells were grown on proline medium, and Gap1 activity was measured by incorporation of [^{14}C]citrulline (0.1 mM) before (t = 0) and at several times after addition of $(NH_4^+)_2SO_4$ (10 mM) in strains RTY1 (*pep4*Δ; ●) and 27061b (wild type; □). The Gap1 activities were calculated in nanomoles per minute per milliliter. (B) Immunoblot of Gap1 in crude extracts prepared before (t = 0) and at several times after addition of $(NH_4)_2SO_4$.

degradation of preaccumulated Gap1 will be henceforth referred to as "down-regulation" of the permease.

A High Amount of Npi1/Rsp5 Ub Ligase Is Required for Ammonium-induced Endocytosis and Degradation of Gap1 Permease

In *npi1* mutant cells, the Gap1 permease is known to remain active (i.e., plasma membrane located) and stable after addition of NH_4^+ (Grenson, 1983a; Hein *et al.*, 1995). In the mutant used here, a Ty1 transposon is inserted 500 bp upstream from the translation initiation codon of the *NPI1/RSP5* gene. This Ty1 insertion results in a reduced *NPI1* transcript level, raising the possibility that a fairly large amount of Npi1 is required for NH_4^+-induced down-regulation of Gap1 (Hein *et al.*, 1995). This has now been confirmed using polyclonal antibodies raised against Nedd4, the mouse homologue of Npi1 (Kumar *et al.*, 1992, 1997). In Western blot experiments performed with crude extract of wild-type cells, these antibodies detected two polypeptides, one at ~80 kDa and one at ~90 kDa (Figure 3, lane 1). The apparent mass of the upper band is consistent with the predicted molecular mass of Npi1 (91.8 kDa). The amount of this upper band is reduced >10-fold in the *npi1* strain (Figure 3, lane 2), but it reaches a normal level in *npi1* cells transformed with a high-copy number plasmid bearing a complete *NPI1* gene demonstrating that the 90-kDa signal does correspond with the Npi1 Ub ligase (Figure 3, lane 3). *npi1* cells transformed with the *NPI1*-bearing plasmid also show restored sensitivity of Gap1 to NH_4^+ regu-

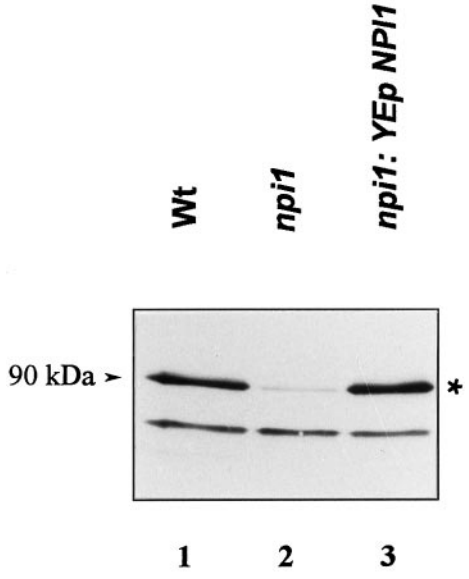

Figure 3. The amount of Npi1 Ub ligase is reduced in *npi1* cells. Crude extracts were prepared from cells grown on proline medium, resolved by electrophoresis, and blotted onto a nitrocellulose membrane. The blot was probed with polyclonal Nedd4 antibodies (Kumar *et al.* 1997). The strains used were 24346c (wild type; lane 1), 27038a (*npi1*; lane 2), and 27038a (*npi1*) transformed with plasmid YEpJYS-2 carrying the *NPI1* gene (lane 3). The signal corresponding to Ub ligase Npi1/Rsp5 is marked with an asterisk.

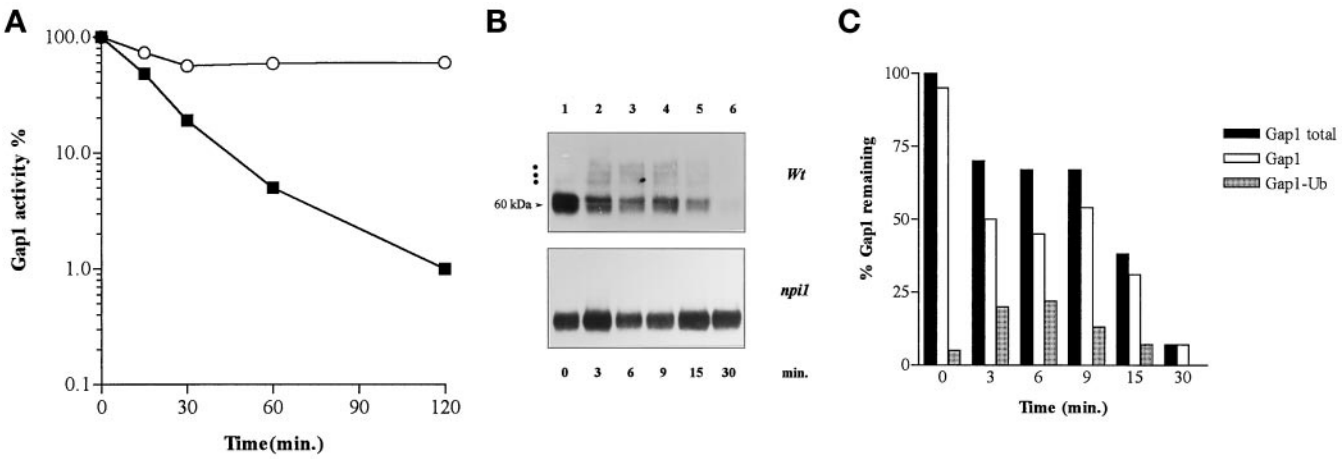

Figure 4. Ubiquitination of Gap1 is impaired in *npi1* cells. (A) Cells were grown on proline medium, and Gap1 activity was measured by incorporation of [^{14}C]citrulline (0.1 mM) before (t = 0) and at several times after addition of $(NH_4)_2SO_4$ (10 mM) in strains 24346c (wild type; ■) and 27038a (*npi1*; ○). The Gap1 activities were expressed in nanomoles per minute per milliliter. (B) Immunoblot of Gap1 from membrane-enriched cell fractions prepared before (t = 0) and at several times after addition of $(NH_4)_2SO_4$. The major Gap1 signal is composed of two bands at ~60 kDa, and the positions of ubiquitinated forms are indicated with dots. (C) Quantitation of the Gap1 signal in immunoblot shown in the upper part of B (wild-type cells). The immunoblot was scanned with a densitometer to measure the amount of total and ubiquitinated Gap1 present at each time point. Each value is expressed as percentage of the Gap1 found in all forms at t = 0 min.

lation (Hein *et al.*, 1995). Thus, the reduced amount of Npi1 present in *npi1* cells, although sufficient to ensure cell viability, is limiting for NH_4^+-induced down-regulation of the permease. Also consistent with this conclusion is the observation that when Gap1 was assayed in *npi1* cells transformed with either a low- or a high-copy number plasmid bearing the promoter-truncated *npi1* gene, only multiple copies of the *npi1* gene were able to restore NH_4^+-induced loss of Gap1 activity (our unpublished results).

Ammonium Induces Npi1-dependent Ubiquitination of Gap1 Permease

The fact that efficient NH_4^+-induced down-regulation of Gap1 requires the presence of Npi1 Ub ligase in relatively high amounts indicates that Ub must be involved in this regulatory process. As previously suggested for several other yeast cell surface proteins (Egner and Kuchler, 1996; Galan *et al.*, 1996; Hicke and Riezman, 1996; Roth and Davis, 1996), conjugation of Ub to Gap1 might constitute a signal required for endocytosis of the permease. Ubiquitination of Gap1 was tested by Western blotting analysis of membrane-enriched extracts. These preparations contained >90% of the plasma membrane H^+-ATPase Pma1 immunodetectable in crude extracts (our unpublished observations) and possibly membrane of other compartments. The Gap1 signal detected in the proline-grown wild-type strain consists of two intense bands at ~60 kDa, which we shall call the major Gap1 signal, and at least one minor band at ~70 kDa, which is visible if exposure is long enough (Figure 4B, lane 1). A second minor band just above the first and additional bands of still higher molecular weight were also detected upon still longer exposure (our unpublished observations). The difference of ~10 kDa between the major Gap1 signal and the first minor band is about as one would expect if a Ub molecule (~9 kDa) is linked to the permease. In accordance with this prediction, there appear no minor bands in the lanes corresponding to *npi1* mutant cells. After addition of NH_4^+ to wild-type cells, in parallel with a decrease in the intensity of the major Gap1 signal, a ladder consisting of the first two minor bands plus a third one becomes clearly visible (Figure 4B). Quantitation of the immunoblot signals indicates that the upper minor bands represent up to 25% of the total Gap1 signal (Figure 4C). The three minor bands most probably correspond with Ub-conjugated forms of the permease, as they are barely detectable in extracts of *npi1* cells. To test this assumption, we prepared membrane-enriched fractions from the wild-type strain overexpressing either normal Ub or an epitope-tagged form of Ub (Ub-myc). Because Ub-myc is larger than Ub, proteins binding Ub-myc instead of Ub are retarded in a gel mobility assay (Hochstrasser *et al.*, 1991; Galan *et al.*, 1996; Roth and Davis, 1996). We carried out a Western blot experiment with modifying electrophoresis conditions to improve signal detection. The results are shown in Figure 5. The Gap1 signal obtained with proline-grown cells overexpressing normal Ub consists of a major band, resolved as a doublet upon longer migration, and two additional minor bands of higher molecular weight (Figure 5, lane 1). Extracts of cells har-

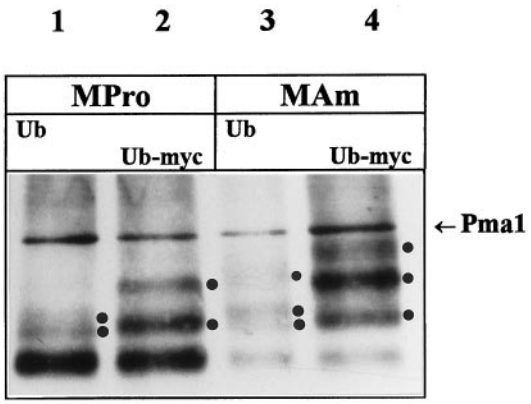

Figure 5. Effect of Ub and Ub-myc expression on the ubiquitination profile of Gap1. Cells of strain 27061b (wild type) bearing plasmid YEp96 (Ub; lanes 1 and 3) or YEp105 (Ub-myc; lanes 2 and 4) were grown in the presence of $CuSO_4$ to induce synthesis of Ub and Ub-myc from the *CUP1* promoter. Two hours after induction, membrane-enriched cell fractions were prepared before (lanes 1 and 2) and 5 min after addition of ammonium (lanes 3 and 4) and subjected to Western analysis with anti-Gap1 antibodies. The positions of the ubiquitinated forms of Gap1 are indicated with dots, and the band marked with an arrow corresponds to the H^+-ATPase Pma1 used as an internal control.

vested 5 min after NH_4^+ addition display a less-intense major Gap1 signal and a third additional minor band of higher molecular weight (Figure 5, lane 3). These results are approximately similar to those obtained without overexpression of Ub (Figure 4). In contrast, overexpression of Ub-myc does alter the migration pattern of the minor bands, whether the cells are grown on proline or ammonium ions; these minor bands are more intense, and some are shifted to a higher molecular weight (Figure 5, lanes 2 and 4). Specifically, in both proline- and NH_4^+-grown cells, the second minor band is clearly retarded, indicating that this signal corresponds to a ubiquitinated form of the permease (Figure 5, lane 2 vs. lane 1 and lane 4 vs. lane 3). The third minor band appearing after NH_4^+ addition also shifts to higher molecular weight upon expression of Ub-myc. Although the first minor band, directly above the major Gap1 signal, is retarded only slightly if at all upon expression of Ub-myc, it does most likely correspond to a ubiquitinated form of Gap1, because it is not detected in *npi1* cells, and the corresponding molecular weight is as expected for a monoubiquitinated form of Gap1. Taken together, these results show that minor bands just above the major Gap1 signal must indeed correspond with Ub-conjugated forms of the Gap1 permease. The fact that the intensity of the Ub-conjugated forms increases upon expression of Ub-myc suggests that the presence of the myc epitope on Ub leads to stabilization of at least some ubiquitinated forms of the Gap1 permease. A similar stabilization effect has been observed for Ub-conjugated forms of the uracil permease (Haguenauer-Tsapis and Galan, personal communication).

Ubiquitination of Gap1 Mutants Resistant to NH_4^+-induced Internalization and Degradation

Mutations affecting the C terminus of Gap1 have been shown to protect the permease against NH_4^+-induced inactivation (Hein and André, 1997). Two such mutations, $Gap1^{LL\rightarrow AA}$ and $Gap1^{pgr}$, are located in a region predicted to adopt a stable α-helix conformation, whereas another (Gap1Δ2) results in a truncated permease lacking the last 11 C-terminal amino acids directly following this putative helix (Figure 6). The resistance of these mutant permeases to NH_4^+-induced down-regulation (Figure 7, A and B) might be due to a defect in ubiquitination, but alternatively, the permeases might be ubiquitinated but not down-regulated. To test whether these C-terminal mutations affect ubiquitination of Gap1, we prepared membrane-enriched extracts of *gap1*Δ cells expressing either wild-type Gap1 or the mutant $Gap1^{pgr}$, $Gap1^{LL\rightarrow AA}$, or

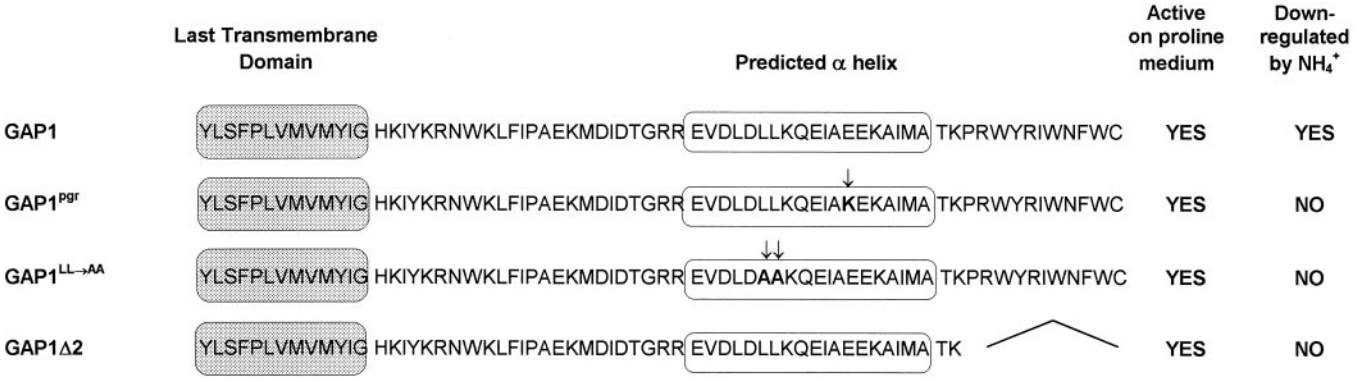

Figure 6. Sequence of the C-terminal region (residue 533–601) of the Gap1 permease and mutant derivatives. Arrows indicate the positions of the amino acid substitutions (shown in bold). The deleted region is indicated by joined lines.

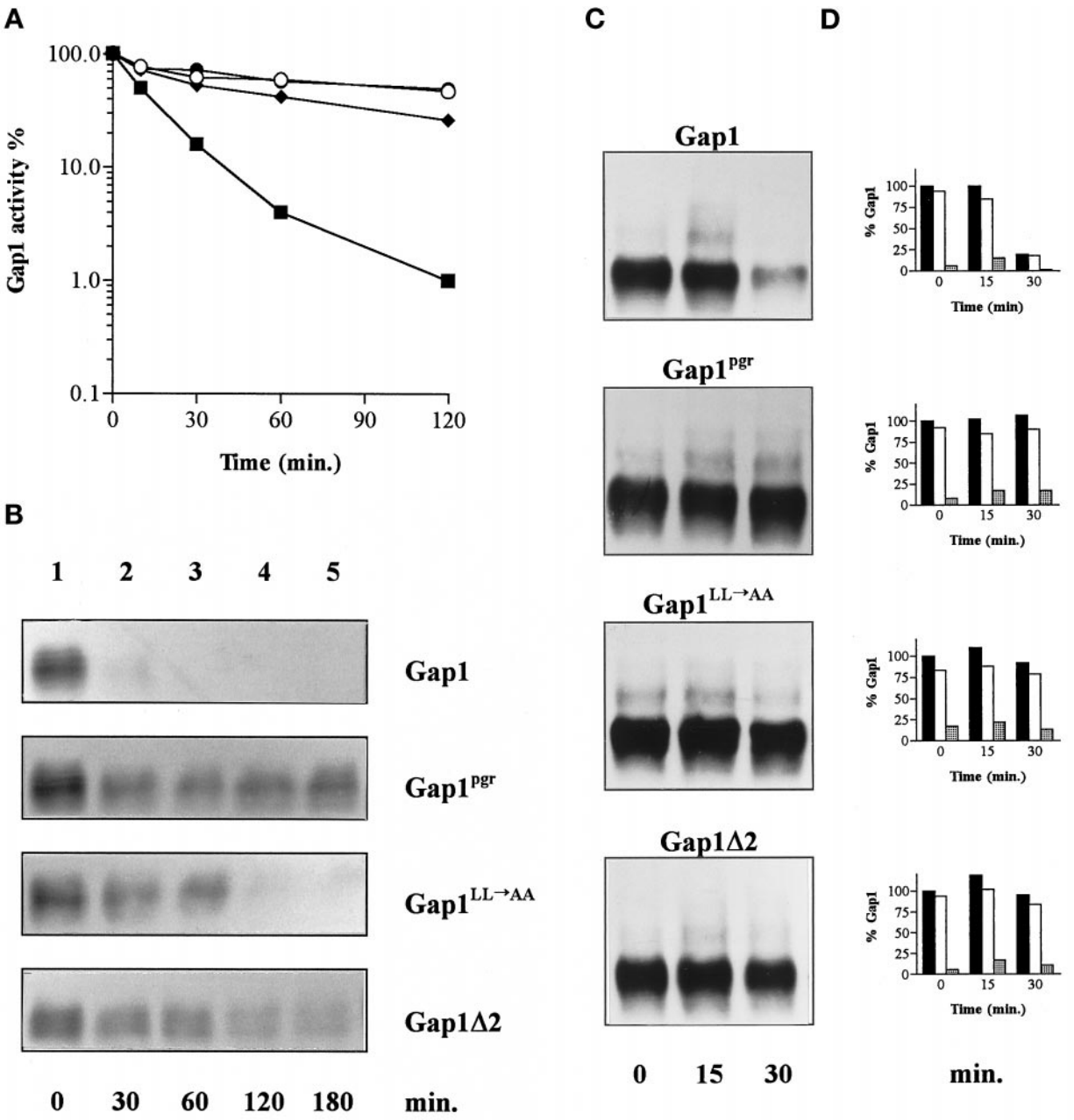

Figure 7. Ubiquitination of C-terminal Gap1 mutants defective in down-regulation. (A) Cells were grown on proline medium, and Gap1 activity was measured by incorporation of [^{14}C]citrulline before (t = 0) and at several times after addition of $(NH_4)_2SO_4$ (10 mM) in strain JOD0097 (*gap1*Δ) expressing the wild-type Gap1 permease (■) or one of the three mutant permeases, Gap1^{pgr} (○), Gap1$^{LL\rightarrow AA}$ (♦), or Gap1Δ2 (●). The Gap1 activities were expressed in nanomoles per minute per milliliter. (B) Immunoblot of Gap1 from crude extracts prepared before (t = 0) and several times after $(NH_4)_2SO_4$ addition. (C) Immunoblot of Gap1 from membrane-enriched fractions of cells prepared before (t = 0) and 15 and 30 min after addition of $(NH_4)_2SO_4$. (D) Quantitation of blots shown in C. Measure of the amount of Gap1 present at each time point. Each value is expressed as a percentage of the total Gap1 found in all forms at (t = 0). Closed bars, all forms of Gap1; open bars, unconjugated forms of Gap1; stippled bars, ubiquitinated forms of Gap1.

Gap1Δ2. We then compared these extracts by Western analysis (Figure 7C). In cells expressing wild-type Gap1, addition of NH_4^+ led to an intensification of the minor bands corresponding to ubiquitinated forms of the permease rapidly followed by a decrease of the major Gap1 signal. In contrast, in cells expressing the mutant Gap1^{pgr} permease, the major Gap1 signal remained stable after addition of NH_4^+. Yet these cells

did display bands corresponding to ubiquitinated forms of the permease; these bands are already visible in proline-grown cells, and addition of NH_4^+ increased their intensity. Thus, NH_4^+ enhances ubiquitination of Gap1^{pgr}, but this modification does not lead to permease down-regulation. Perhaps the Gap1^{pgr} permease is less efficiently ubiquitinated than the wild-type permease and is thus protected against degradation. This, however, seems unlikely, because quantitation of variously exposed blots revealed that the relative level of ubiquitinated permease detected 15 min after NH_4^+ addition is approximately similar for the wild-type and Gap1^{pgr} permeases (Figure 7D). The Gap1$^{LL\rightarrow AA}$ and Gap1Δ2 mutant permeases are also significantly protected against NH_4^+ down-regulation; after NH_4^+ addition, both are destabilized at a much lower rate than wild-type Gap1, and yet minor bands corresponding to ubiquitinated forms of the Gap1$^{LL\rightarrow AA}$ and Gap1Δ2 permeases appear clearly, indicating that the reduced sensitivity of the two proteins to NH_4^+-induced down-regulation is not due to complete failure to be ubiquitinated.

The fact that Gap1^{pgr} remains largely active after NH_4^+ addition indicates that it remains plasma membrane located. It is noteworthy, however, that addition of NH_4^+ does not lead to complete conversion of the Gap1^{pgr} permease to ubiquitinated forms (Figure 7C and our unpublished results). This contrasts with the behavior of α-factor receptor Ste2 observed in *end4* cells defective in the internalization step of endocytosis. In this latter case, ligand binding leads to nearly complete conversion of Ste2 to ubiquitinated forms (Hicke and Riezman, 1996). To further investigate this question, we examined the ubiquitination of wild-type Gap1 in *act1-1* cells defective in endocytosis (Figure 1B). When the *act1* cells were grown on proline medium, the minor bands corresponding to ubiquitinated forms of the permease were more intense than the corresponding bands of the proline-grown isogenic wild type. After addition of NH_4^+, the intensity of these minor bands did not increase significantly. In contrast, addition of NH_4^+ to wild-type cells led to strong intensification of the minor bands detectable in extracts from proline-grown cells. Thus, in contrast to the situation reported for the Ste2 receptor, the Gap1 permease does not seem to be completely converted to Ub-conjugated forms in a mutant defective in endocytosis.

DISCUSSION

In this article, we show that ammonium-triggered degradation of the Gap1 permease depends on vacuolar proteases and is preceded by internalization of the protein by endocytosis. In *act1-1* cells defective in the internalization step of endocytosis, the Gap1 permease remains largely active (i.e., plasma membrane located) and stable after addition of NH_4^+, indicating that nitrogen catabolite inactivation of Gap1 (Grenson, 1983a) most likely results from removal of the permease from the plasma membrane. What we have called Gap1 down-regulation, i.e., the internalization and subsequent degradation of Gap1, thus seems to occur via the same pathway as for other cell surface proteins such as the uracil (Fur4), maltose (Mal61), and galactose (Gal2) permeases, the pheromone receptors Ste2 and Ste3, and the multidrug resistance protein Pdr5 (Singer and Riezman, 1990; Davis *et al.*, 1993; Volland *et al.*, 1994; Egner and Kuchler, 1995; Riballo *et al.*, 1995; Horak and Wolf, 1997). In the Gap1 system, however, down-regulation is induced by adding a preferred nitrogen source to the medium, suggesting that regulatory factors responding to nitrogen must be specifically involved.

Our results show that ubiquitination of the Gap1 permease is required for its down-regulation. Ubiquitinated forms of Gap1 were detected on Western blots as several minor bands migrating to positions just above the major Gap1 signal. At least three minor bands were detected in most experiments; they likely correspond to mono-, di-, and tri-ubiquitinated forms of the permease. In proline-grown cells, these bands represent only a small fraction (~5%) of the immunodetected Gap1 signal, but they markedly rise in proportion a few minutes after addition of NH_4^+. Both basal and NH_4^+-stimulated ubiquitination are severely impaired in *npi1* mutant cells, in which the level of immunodetected Npi1/Rsp5, a Ub ligase essential to cell viability (Hein *et al.*, 1995; Huibregtse *et al.*, 1995), is reduced at least 10-fold compared with the wild type. This reduced level of Npi1/Rsp5 is due to a Ty element inserted 500 bp upstream from the initiation codon of the *NPI1* gene (Hein *et al.*, 1995). The reduced level of Npi1/Rsp5 in *npi1* mutant cells is thus sufficient to ensure cell viability but limiting for ubiquitination of Gap1. It is likely that Npi1/Rsp5 is directly involved in ubiquitination of Gap1. In keeping with a relatively high level of Npi1/Rsp5 protein being needed for NH_4^+-induced permease down-regulation, Gap1 is a relatively abundant protein, and Npi1/Rsp5 is involved in NH_4^+-induced inactivation of other, probably numerous, nitrogen-sensitive permeases, including the proline (Put4) and allantoate/ureidosuccinate (Dal5) permeases (Grenson, 1983a). Furthermore, the Npi1/Rsp5 Ub ligase is also involved in turnover control of nitrogen-insensitive permeases such as the uracil (Fur4) (Hein *et al.*, 1995; Galan *et al.*, 1996) and maltose (Mal61) permeases (Lucero and Lagunas, 1997).

The mechanism by which Ub binding induces down-regulation of Gap1 remains unknown. As suggested for several other cell surface proteins, Ub molecules attached to Gap1 might provide a signal triggering endocytosis of the permease. This model finds

support in the fact that ubiquitinated forms of Gap1 accumulate to some extent in *act1-1* cells defective in endocytosis, and that Gap1 remains plasma membrane located after addition of NH_4^+ to *npi1* cells defective in ubiquitination. It is also possible that ubiquitination would also be involved in another step of down-regulation, such as vacuolar sorting of internalized permease. For instance, deubiquitination of internalized permeases might lead to their recycling back to the plasma membrane, whereas maintenance of their ubiquitinated state might target them to vacuolar breakdown. Although recycling of protein after internalization is generally considered as the default pathway in higher eukaryotic cells (Mayor *et al.*, 1993), no such pathway has been documented in yeast. Yet in the *sec18* mutant strain recently shown to be impaired in forward progression of molecules from the plasma membrane to the vacuole (Hicke *et al.*, 1997), the maltose permease remains active under conditions that normally induce its internalization and vacuolar degradation. This raises the possibility that at least some cell surface proteins might undergo recycling after endocytosis (Riballo *et al.*, 1995). Clearly, elucidating the exact role of Ub in down-regulation of Gap1 requires further investigation.

The $Gap1^{LL\rightarrow AA}$ and Gap1Δ2 permeases carrying mutations in the C-terminal tail of the permease (Figure 6) are significantly protected against NH_4^+-induced degradation, but this protection is not due to complete failure of the proteins to be ubiquitinated. These mutant Gap1 proteins might be less efficiently ubiquitinated and thus partially protected against down-regulation. Another possibility is that these C-terminal mutations alter down-regulation at a step downstream from ubiquitination. Another mutant, $Gap1^{pgr}$, carries a glutamate-to-lysine substitution within the C-terminal tail (Figure 6). This mutant permease is strongly protected against NH_4^+-triggered degradation, but it is apparently nevertheless converted to ubiquitinated forms in a manner similar to that observed for the wild-type permease. This would mean that, in addition to Ub binding, sequences located within the C-terminal tail of the permease are required for normal down-regulation. The exact role of the glutamate residue substituted in the $Gap1^{pgr}$ mutant is unknown. It is located within an EEKAI sequence reminiscent of the DAKSS sequence. The latter is present in the cytosolic tail of the Ste2 receptor and is essential to ubiquitination and endocytosis of a truncated form of the receptor (Hicke and Riezman, 1996). Although the EEKAI sequence of Gap1 clearly differs from the DAKSS sequence of Ste2, mutagenesis experiments on the DAKSS motif have shown that EAKSS and DAKAS promote efficient internalization, whereas AAKSS does not (Rohrer *et al.*, 1993). Replacing the lysine residue (DARSS) impairs both ubiquitination and endocytosis of the truncated form of Ste2 (Hicke and Riezman, 1996). That the E→R substitution in the EEKAI sequence of Gap1 markedly impairs down-regulation without apparently affecting ubiquitination suggests that this sequence plays a role in a subsequent step of the down-regulation pathway.

Our data also show that ubiquitinated forms of Gap1 are more abundant in the endocytosis-defective *act1-1* strain, but that addition of NH_4^+ to these cells does not cause a rise in the level of ubiquitinated permease, the latter forms representing a relatively constant fraction of the immunodetected Gap1 protein. The situation of the $Gap1^{pgr}$ mutant permease is quite similar; added NH_4^+ triggers conversion of the permease to ubiquitinated forms, but only a fraction of the permease undergoes this modification. These results were unexpected in the light of the finding that Ste2 receptor is nearly completely converted to Ub-conjugated forms upon addition of α-factor to mutant cells defective in endocytosis (Hicke and Riezman, 1996). Perhaps the effects of the *act1-1* and $gap1^{pgr}$ mutations are only partial, and residual down-regulation is sufficient to prevent accumulation of ubiquitinated forms of Gap1. However, should this interpretation be true, addition of NH_4^+ would lead to progressive loss of the preexisting Gap1 activity, an effect that has not been observed. Alternatively, a specific defect in endocytosis due to $gap1^{pgr}$ and *act1-1* mutations could prevent further ubiquitination beyond a certain amount of permease. For instance, some limiting components of the ubiquitination machinery (such as the Npi1/Rsp5 Ub ligase itself) might be sequestrated in endocytosis-defective cells. Another explanation for the failure to accumulate Ub conjugated forms in endocytosis mutants is that deubiquitinating enzyme may impose a steady-state level of modification.

One particularity of the Gap1 system, compared with other cell surface proteins that undergo ubiquitination, is the ubiquitination-enhancing effect of added NH_4^+. This suggests that nitrogen-sensitive regulatory factors are involved in this process. One such factor is likely Npr1. Previous work has shown that Gap1 is inactive in an *npr1* mutant grown on proline medium, and that this loss of activity requires Npi1/Rsp5 and the integrity of the C-terminal region of the permease (Grenson, 1983b; Hein and André, 1997). Npr1 thus seems to protect Gap1 against down-regulation in cells grown on a poor nitrogen source. This observation, together with the data of this study, suggests the following model for the regulation of Gap1 turnover according to the nitrogen source. In cells growing on proline medium, Gap1 is abundant and highly active. Only a small fraction of the permease is ubiquitinated. The presence of ubiquitinated Gap1 forms might reflect basal turnover of the permease. Under these growth conditions, the role of Npr1 might be to limit the conversion of the Gap1 proteins into

ubiquitinated forms. Addition of NH_4^+ might either enhance the efficiency of the ubiquitination reaction itself or counter the putative protective action of Npr1 against ubiquitination. The result would be rapid ubiquitination of all preaccumulated Gap1 molecules followed by their down-regulation. Molecular analysis has shown that the *NPR1* encodes a kinase homologue (Vandenbol *et al.*, 1990), suggesting that phosphorylation could be involved in protecting Gap1 against down-regulation.

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