

## NH<sub>4</sub><sup>+</sup>-induced down-regulation of the *Saccharomyces cerevisiae* Gap1p permease involves its ubiquitination with lysine-63-linked chains

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### SUMMARY

Addition of ammonium ions to yeast cells growing on proline as the sole nitrogen source induces internalization of the general amino acid permease Gap1p and its subsequent degradation in the vacuole. An essential step in this down-regulation is Gap1p ubiquitination through a process requiring the Npi1p/Rsp5p ubiquitin ligase. We show in this report that *NPI2*, a second gene required for NH<sub>4</sub><sup>+</sup>-induced down-regulation of Gap1p, codes for the ubiquitin hydrolase Doa4p/Ubp4p/Ssv7p and that NH<sub>4</sub><sup>+</sup>-induced Gap1p ubiquitination is strongly reduced in *npi2* cells. The *npi2* mutation results in substitution of an aromatic amino acid located in a 33-residue sequence shared by some ubiquitin hydrolases of the Ubp family. In this mutant, as in *doa4Δ* cells, the amount of free monomeric ubiquitin is at least four times lower than in wild-type cells. Both ubiquitination and down-regulation of

the permease can be restored in *npi2* cells by over-expression of ubiquitin. In proline-grown wild-type and *npi2/doa4* cells overproducing ubiquitin, Gap1p appears to be mono-ubiquitinated at two lysine acceptor sites. Addition of NH<sub>4</sub><sup>+</sup> triggers rapid poly-ubiquitination of Gap1p, the poly-ubiquitin chains being specifically formed by linkage through the lysine 63 residue of ubiquitin. Gap1p is thus ubiquitinated differently from the proteins targeted by ubiquitination for proteolysis by the proteasome, but in the same manner as the uracil permease, also subject to ubiquitin-dependent endocytosis. When poly-ubiquitination through Lys63 is blocked, the Gap1p permease still undergoes NH<sub>4</sub><sup>+</sup>-induced down-regulation, but to a lesser extent.

Key words: Ubiquitin, Gap1, Permease, Lys63-chain, Yeast

### INTRODUCTION

Degradation of many eukaryotic proteins requires their prior modification by conjugation with ubiquitin (Ub). Ub molecules are transferred to lysine residues of target proteins via an E1-E2-E3 enzyme thioester cascade (Ub-activating enzyme/Ub-conjugating enzyme/Ub-protein ligase) (Ciechanover, 1994). Ubiquitination is known mainly as a signal targeting substrate proteins for recognition and degradation by a multisubunit protease, the 26S proteasome (Pickart, 1997). This has been observed both for soluble proteins and for some integral membrane proteins that undergo endoplasmic-reticulum-associated degradation. Another function of ubiquitination has more recently been evidenced. The ubiquitination of some plasma-membrane proteins signals their internalization via the endocytic pathway and subsequent degradation in the lysosome/vacuole (Hicke, 1997). In the case of several such proteins it has been clearly demonstrated that this degradation is exclusively vacuolar and occurs normally in mutants with defective proteolytic or regulatory proteasome subunits (Galan et al., 1996; Hicke and Riezman, 1996; Egner et al., 1995; Riballo et al., 1995). This notably raises the question of why

the proteasome does not target these ubiquitinated proteins. Recognition of ubiquitinated proteins by the proteasome requires formation of a polyubiquitin chain at least four subunits long. The chain is formed through linkage of the carboxy-terminal Gly of one Ub subunit to the ε-amino group of Lys48 of the previous Ub. This type of chain binds to subunit 5a of the proteasome (Pickart, 1997; Deveraux et al., 1994). In addition to Lys48, Ub carries six other lysine residues, and alternative types of polyubiquitin have been shown to form in vivo in yeast through Lys29 and Lys63 (Arnason and Ellison, 1994). The uracil permease, which undergoes basal and stress-accelerated Ub-dependent endocytosis (Galan et al., 1996), was found to be specifically modified by short Lys63-linked polyubiquitin chains (Galan and Haguenaer-Tsapis, 1997). Formation of these chains is required for maximum-rate internalization of the permease, but mono-ubiquitination at two lysine acceptor sites is sufficient to induce basal-rate permease endocytosis. Another interesting case is that of Ste2p, the G-protein-coupled receptor of α-factor pheromone. This receptor undergoes both basal and ligand-induced Ub-dependent down-regulation (Hicke and Riezman, 1996). It was observed that mono-ubiquitination of a single lysine residue is sufficient to

promote both basal and ligand-induced internalization of a C-terminally truncated form of Ste2p (Terrel et al., 1998). Although ligand binding to full-length Ste2p leads to formation of di- and tri-ubiquitinated Ste2p conjugates, there is evidence that this may mainly reflect addition of single Ub moieties onto multiple receptor lysines rather than formation of a polyubiquitin chain on one lysine (Terrel et al., 1998). In contrast to this situation, the mammalian growth hormone receptor was reported to undergo ligand-induced multi-ubiquitination (Govers et al., 1997) followed by lysosomal degradation (Strous et al., 1996).

This report focuses on the nitrogen-regulated ubiquitination of the general amino acid permease (Gap1p) of *S. cerevisiae*. Gap1p is tightly regulated according to the nitrogen source present in the culture medium (Grenson, 1992; Roberg et al., 1997; Springael and André, 1998). Its activity is maximal in cells using proline or urea as the sole nitrogen source. Upon addition of ammonium ions supporting optimal growth, its synthesis is repressed and pre-synthesized permease is inactivated (Grenson, 1983a,b) through progressive internalization and subsequent degradation in the vacuole (Springael and André, 1998).  $\text{NH}_4^+$ -induced inactivation of Gap1p requires the product of the *NPI1* and *NPI2* genes (Grenson, 1983a,b). *NPI1* codes for Rsp5p, a ubiquitin ligase essential to cell viability and highly conserved from yeast to man (Hein et al., 1995; Huibregtse et al., 1995). A recent analysis has shown that in proline-grown cells, only a small fraction of Gap1p molecules are ubiquitinated. After  $\text{NH}_4^+$  addition, the proportion of ubiquitin-conjugated forms increases, leading to rapid endocytosis and degradation of the permease. Ubiquitination and subsequent degradation of Gap1p are impaired in an *npi1* strain displaying severely reduced amount of Npi1p Ub ligase (Hein et al., 1995; Springael and André, 1998). Npi1p is also required for stress-induced ubiquitination and degradation of the uracil permease (Hein et al., 1995; Galan et al., 1996) and for glucose-induced endocytosis and degradation of the maltose permease (Lucero and Lagunas, 1997). The carboxy-terminal tail of Gap1 plays a critical role in  $\text{NH}_4^+$ -induced down-regulation of the permease (Hein and André, 1997; Springael and André, 1998). This region contains a di-leucine peptide and a glutamate residue located in a putative  $\alpha$ -helix. Mutations within these sequences or in the last eleven amino acids of Gap1 protect the permease against  $\text{NH}_4^+$ -induced down-regulation, but these mutant permeases still bind ubiquitin (Springael and André, 1998).

We here report the molecular characterization of *NPI2*, a second gene required for  $\text{NH}_4^+$  inactivation of Gap1 (Grenson, 1983a,b). We show that this gene codes for the ubiquitin hydrolase Doa4p/Ubp4p/Ssv7p and is required for normal ubiquitination and  $\text{NH}_4^+$ -induced down-regulation of the Gap1 permease. In *npi2* cells, the amount of free monomeric ubiquitin is about 4 times lower than in wild-type cells. Effective ubiquitination and down-regulation can be restored in an *npi2* strain by overproduction of Ub. We provide evidence, based on experiments with *npi2* cells overproducing several mutant Ubs unable to form specific kinds of Ub chains, that Gap1p is ubiquitinated at two acceptor sites and that  $\text{NH}_4^+$  triggers formation of Lys63-linked polyubiquitin chains. We also show that formation of such chains accelerates permease internalization.

## MATERIALS AND METHODS

### Strains, growth conditions, plasmids

Strains 23346c (*MATa, ura3*); 27061b (*MATa, ura3, trp1*); 27002d (*MATa, ura3, npi2*), and 27071b (*MATa, ura3, trp1, npi2*) used in this study are all isogenic with the wild-type  $\Sigma 1278b$  except for the mutations mentioned (Béchet et al., 1970). MHY501 (*MATa his3- $\Delta$ 200 leu2-3, 112 ura3-52 lys2-801 trp1-1*) and its isogenic *doa4 $\Delta$*  derivative (MHY693) are described by Papa and Hochstrasser (1993). Cells were grown in minimal buffered medium (pH 6.1) with 3% glucose as the carbon source (Jacobs et al., 1980). Since this medium apparently chelates copper, experiments requiring copper induction of ubiquitin were carried out on cells grown in YNB medium (Difco) without ammonium. Nitrogen sources were added as indicated at the following final concentrations:  $(\text{NH}_4)_2\text{SO}_4$  (10 mM), proline (0.1%), serine (0.1%), threonine (0.1%). The 2 $\mu$ -based multi-copy plasmid YEp96 contains a synthetic yeast ubiquitin gene under the control of the copper-inducible *CUPI* promoter (Hochstrasser et al., 1991). Plasmids encoding mutant ubiquitins in which Lys29 (UbK29R), Lys48 (UbK48R), Lys63 (UbK63R), or all three of these (UbRRR) were replaced by arginine, are derivatives of YEp96 (Arnason and Ellison, 1994). HA-UbK63R is identical to UbK63R except that it contains an haemagglutinin (HA) tag on the N terminus of Ub (Terrel et al., 1998). Overproduction of Ubs was induced with 0.1 mM  $\text{CuSO}_4$  for two hours. Yeast cells treated with lithium acetate (Ito et al., 1983) were transformed according to the method of Sherman et al. (1986). The *E. coli* strain used was JM109. All procedures for manipulating DNA were standard ones (Ausubel et al., 1995; Sambrook et al., 1997).

### Cloning of the *NPI2* gene

*S. cerevisiae* strain 27002d (*MATa, ura3, npi2*) was transformed with a low-copy-number library representing the whole genome of strain  $\Sigma 1278b$  (Marini et al., 1994). One of the 77000 *Ura3*<sup>+</sup> transformants exhibited an *Npi2*<sup>+</sup> phenotype on selection media (see text). DNA was isolated from this clone and used to transform *E. coli*. The recovered plasmid contained a DNA fragment about 6.5 kb long (YCpJYS-10). A 4 kb subclone constructed in the centromere-based vector pFL38 (YCpJYS-22) suppressed the phenotype linked to the *npi2* mutation. The *npi2* mutant allele was cloned by screening bacterial clones of a library representing the genome of the *npi2* strain with an *NPI2* probe. The *NPI2* and *npi2* genes were sequenced using oligonucleotide primers derived from the sequence of the *YDR069c* gene established by systematic sequencing of chromosome IV (Jacq et al., 1997).

### Permease assays

Gap1p permease activity was determined by measuring incorporation of <sup>14</sup>C-labelled citrulline (20  $\mu$ M) as described by Grenson et al. (1966). All permease activities were measured in cells having reached the state of balanced growth (Wiame et al., 1985). The permease was inactivated by adding prewarmed  $(\text{NH}_4)_2\text{SO}_4$  to the culture at the final concentration of 10 mM.

### Yeast-cell extracts and immunoblotting

Crude extracts were prepared as previously described (Hein et al., 1995). Membrane-enriched preparations were obtained as described elsewhere (Springael and André, 1998). For western blot analysis of Gap1p, solubilized proteins were loaded on a 12% SDS-polyacrylamide gel (11% in some experiments) in a Tricine system (Schägger and von Jagow, 1987). After transfer to nitrocellulose the proteins were probed with rabbit antiserum raised against the N-terminal region of Gap1p (1:20000) or of the plasma-membrane H<sup>+</sup>-ATPase (Pma1) (1:10000) (J. O. De Craene and B. André, unpublished). For anti-ubiquitin immunoblot analysis, cell extracts were prepared as previously described, except that they were boiled after resuspension in sample buffer. Proteins were transferred to

Immobilon-P membranes (Millipore), after which the blots were boiled in water for 15 minutes, before saturation and incubation with polyclonal anti-ubiquitin antibodies (Sigma 1:3000). Primary antibodies were detected with horseradish peroxidase-conjugated (HRP-conjugated) anti-rabbit IgG secondary antibody followed by detection of chemoluminescence (ECL, Amersham).

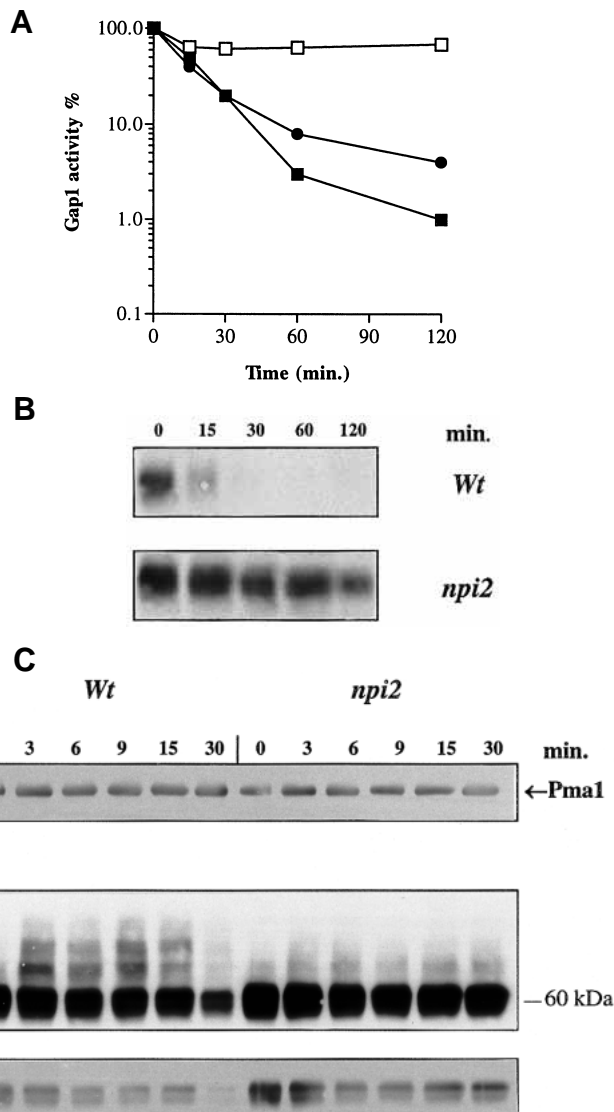
## RESULTS

### The *NPI2* gene product is required for normal ubiquitination and $\text{NH}_4^+$ -induced down-regulation of the Gap1 permease

The product of the *NPI2* gene is required for  $\text{NH}_4^+$ -induced loss of Gap1p permease activity (Grenson, 1983a,b). Recently we have shown that  $\text{NH}_4^+$  added to proline-grown cells triggers internalization and degradation of the permease and that this down-regulation requires conversion of Gap1p to Ub-conjugated forms (Springael and André, 1998). To investigate the role of *NPI2* in down-regulation of Gap1p, we monitored the fate of Gap1p after addition of  $\text{NH}_4^+$  to cultures of wild-type and *npi2* cells growing on proline (Fig. 1A and B).  $\text{NH}_4^+$  induced a loss of permease activity in wild-type cells and a parallel drop in permease immunodetected in total protein extracts. In contrast, *npi2* cells were protected against both loss of permease activity and Gap1p degradation. This resistance of Gap1p to  $\text{NH}_4^+$ -induced down-regulation might be due either to impaired ubiquitination of the permease, as in the *npi1* mutant, or to a defect in a subsequent step such as endocytosis, as in the *act1-1* mutant altered in actin (Springael and André, 1998). To further investigate the role of *NPI2* in  $\text{NH}_4^+$ -induced down-regulation of Gap1p, we tested the efficiency of Gap1p ubiquitination in *npi2* cells. Immunoblotting was performed with membrane-enriched fractions to facilitate detection of ubiquitin-permease conjugates (Fig. 1C). The Gap1p signal detected in proline-grown wild-type cells consists of a doublet around 60 kDa and two minor bands of higher molecular mass. Previous work has shown that the minor upper bands correspond to Ub-conjugated forms of the permease (Springael and André, 1998). In the early minutes after addition of  $\text{NH}_4^+$ , the amount of Ub-conjugated Gap1p increased markedly in wild-type-cells. After 30 minutes, while the control signal corresponding to plasma-membrane  $\text{H}^+$ -ATPase remained stable, the intensity of the Gap1p signal was reduced and ubiquitin-permease conjugates were barely detectable (Fig. 1C). Proline-grown *npi2* cells displayed a more intense Gap1p doublet than did proline-grown wild-type cells (Fig. 1C, lower panel), but this did not lead to higher permease activity. This suggests that the extra-amount of Gap1p could be intracellular or that some elements are limitant for Gap1p activity. The mutant cells, furthermore, displayed a lesser proportion of Ub-conjugated Gap1p forms. After addition of  $\text{NH}_4^+$ , the amount of these forms increased but very slightly and the main Gap1p immunodetected signal remained nearly stable. Taken together, these results show that permease ubiquitination is strongly reduced in *npi2* cells. This ubiquitination defect appears to stabilize Gap1p in proline-grown cells and to protect the permease against  $\text{NH}_4^+$ -induced down-regulation.

### The *NPI2* gene encodes the Doa4/Ubp4/Ssv7 ubiquitin hydrolase

The *npi2* mutant was found to grow very slowly on minimal medium containing L-serine (MSer) or L-threonine (MThr) as



**Fig. 1.** The Gap1p permease is protected against down-regulation in *npi2* cells. Strains 23346c (Wt), 27002d (*npi2*), and 27002d transformed with YCpJYS-22 carrying the cloned *NPI2* gene were grown in minimal medium as described in Materials and Methods.  $(\text{NH}_4)_2\text{SO}_4$  (10 mM) was added to the medium. (A) Gap1p activity was measured by incorporation of  $[^{14}\text{C}]$ -citrulline (20  $\mu\text{M}$ ) before ( $t=0$ ) and at several times after addition of  $(\text{NH}_4)_2\text{SO}_4$ . Wt (■); *npi2* (□), and *npi2* transformed with YCpJYS-22 carrying the cloned *NPI2* gene (●). Results are percentages of initial activities per ml culture. (B) Total protein extracts were prepared at the times indicated and Gap1p was detected by western immunoblotting in extracts prepared from 0.2 ml of culture. (C) Immunoblot of Gap1p from membrane-enriched cell fractions prepared before ( $t=0$ ) and at several times after addition of  $(\text{NH}_4)_2\text{SO}_4$ . The middle and lower panels correspond to two different exposures of the Gap1 immunoblot. The upper panel corresponds to the same immunoblot probed with antibodies against  $\text{H}^+$ -ATPase (Pma1p) as an internal control.

the sole nitrogen source. Although this phenotype is still unexplained, it was used to clone the *NPI2* gene by functional complementation. The recipient strain (*ura3*, *npi2*) was transformed with a low-copy-number plasmid library

representing the genome of strain  $\Sigma$ 1278b. Transformants were screened for growth on M<sub>Ser</sub> medium. A single plasmid carrying a 6.5 kb insert complementing the *npi2* mutation was recovered. Subcloning experiments and phenotypic analysis on M<sub>Ser</sub> and M<sub>Thr</sub> revealed that the complementing gene corresponds to the *YDR069c/DOA4/SSV7* open reading frame encoding the ubiquitin hydrolase Ubp4p (Papa and Hochtrasser, 1993). A plasmid bearing the cloned *NPI2* gene was introduced into the *npi2* strain and tested for its ability to restore NH<sub>4</sub><sup>+</sup>-induced down-regulation of the Gap1p permease. As expected, addition of NH<sub>4</sub><sup>+</sup> ions to this strain triggered down-regulation of Gap1p similar to that observed in the wild-type strain (Fig. 1A). To confirm that *NPI2* is allelic with *DOA4*, we cloned a DNA fragment containing the *YDR069c* ORF of an *npi2* strain and showed that it was unable to complement the *npi2* mutation (data not shown). *DOA4* belongs to a large family of genes encoding de-ubiquitinating enzymes, or Ubps, involved in removing Ub from other proteins (Wilkinson, 1997). Loss of *DOA4* produces several phenotypes including thermosensitivity, a deficient general response to stress, and a deficient DNA replication (Hochtrasser, 1996). In *doa4* cells, proteolysis of all tested substrates of the ubiquitin/proteasome pathway, such as the Mat $\alpha$ 2 repressor (Chen et al., 1993) and the Mat $\alpha$ 2- $\beta$ -galactosidase fusion protein, is impaired (Papa and Hochtrasser, 1993). More recently it was shown that ubiquitination of the Fur4p uracil permease (Galan and Haguenaer-Tsapis, 1997) and Ste2p  $\alpha$ -factor receptor (Terrel et al., 1998) and internalization and degradation of the maltose permease (Lucero and Lagunas, 1997) are impaired in cells with a defective Doa4p. Our data showing that *NPI2* is identical to *DOA4/UBP4* thus further demonstrate that lack of Doa4p Ub hydrolase affects ubiquitination of plasma-membrane proteins.

### The *npi2* mutation is located in a conserved box specific to some Ubp hydrolases

Ub-specific processing proteases (Ubps) (16 in *S. cerevisiae*) vary greatly in length and structural complexity, suggesting functional diversity (Wilkinson, 1997). While the deduced amino-acid sequences of these proteins display little similarity, sequence comparisons reveal two conserved domains, the Cys and His boxes. The Cys domain contains a Cys residue that serves as the active nucleophile of the enzyme. The His domain contains a His residue that contributes to the enzyme active site (Baker et al., 1992). Yeast Ubp-family members further display additional conserved boxes in the region between the Cys and His domains (Wilkinson, 1997). Sequencing of the *npi2* mutant allele revealed a single A→T substitution resulting in replacement of tryptophan 782 by an arginine. This replacement occurs within one of the conserved blocks located between the Cys and His boxes. Although this 14-residue block (EVLGGDNXWYCPKC) is reported to occur in 13 of the 16 yeast Ubp enzymes (Wilkinson, 1997), we found it to be significantly conserved in only 8. We found, moreover, that this block is a part of a larger conserved sequence 33 amino acids long (Fig. 2). This 33-residue motif is also found in the human Tre2 proto-oncogene (Onno et al., 1993), the *Drosophila* Faf protein involved in eye development (Huang et al., 1995), and in several other Ubps present in other organisms. Alignment of these sequences reveals that a glutamate, a leucine, and two

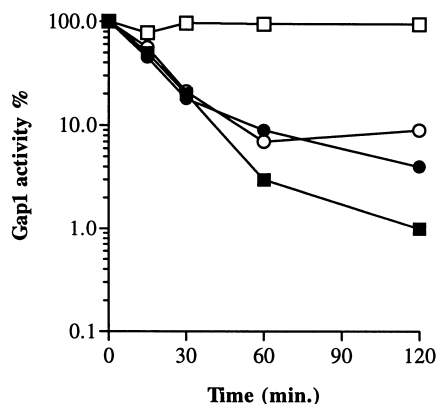
Sc-Ubp4	:	I E D C F R E F T K C E N L E V D E Q Q L C P H C E K R O P S T R
Sc-Ubp5	:	I L D C F R E F T K C E R L C V D E Q Q S C P K L K R Q P S T R
Sc-Ubp7	:	L E D C I N M F T S D E V L S G E N A M D G P R C G P T A S V S T
Sc-Ubp11	:	L E D C I N L F T G D E R L S G D N A M D G P N C R I T D S K S R
Sc-Ubp12	:	L D D C L Q L F S K P E I L G L T D S W Y C P T C K E H R Q A T R
At-Ubp3	:	I T S C L K N F S S T E T L H A E D R F F C D K C C S L Q E A Q R
At-Ubp4	:	I T S C L K N F S S T E T L H A E D R F F C D K C C S L Q E A Q R
Ce-Ubpx	:	I S H C L R W F S E T E T L C G D Q R Y F G E T C S S K Q E A Q R
Ce-Ubpx2	:	V E E A L T A F W Q P E L L D G S N Q Y M C E N C K S K Q D A H R
Dm-Faf	:	L T E S L R E Q Y V K G E L L E G A D A Y H C D K D K R V V T V R
Dm-Ubp64E	:	I E E A L R A F W Q P E T L D G N N Q Y L C E K C R K K C D A H R
Mm-Ubp	:	L R E C I E L F T T M E T L C E H D P W Y C P T C K R H Q Q A T R
Cv-Apo	:	L Q E C I E L F T T W E T L E K E N P W Y C P T C K R H Q L A T R
Hs-Ubp	:	L R D C I E L F T T M E T L C E H D P W Y C P N C K R H Q Q A T R
Hs-Ubpn	:	L R D C I E L F T T M E T L C E H D P W Y C P N C K R H Q Q A T R
Hx-Ubpx	:	L Q D C L R L F S K E E K L T D N N R F Y G S H C R A R R D S L K
Hs-ubpx2	:	L Q E C I E L F T T W E T L E K E N P W Y C P S C K R H Q L A T R
Hs-Tre2	:	L D S C L R A F T S E E L C E S E M Y Y C S K R K T H C L A T R

**Fig. 2.** Sequence alignment of the ELC motifs of ubiquitin hydrolases from *Saccharomyces cerevisiae* (Sc-Ubp4,-5,-7,-11,-12: P32571, P39944, P40453, P50102, P39967, P36026, P39538, P38187), *Arabidopsis thaliana* (At-Ubp3,-4: U76845, U76846), *Caenorhabditis elegans* (Ce-Ubpx,-x2; P34547, Z37812), *Drosophila melanogaster* (Dm-Faf: A49132 and Dm-Ubp64E: X99211), *Mus musculus* (Mm-Ubp: P35123), *Canis vulgaris* (Cv-Apo: Q01988), and *Homo sapiens* (Hs-Ubp, -n,-x, -x2; Q13107, P40818, P51784 and Hs-Tre2; P35125). The site of the *npi2* mutation is indicated by an asterisk. Protein similarity searches were performed with the BLAST network service at the National Center for Biotechnology Information (Altschul et al., 1997). Identification of the ELC motif in yeast Ubps enzymes was based on a PROFILE search (Gribskov et al., 1990).

cysteine residues are invariant in all of them. We therefore propose to name this sequence 'ELC box'. The aromatic residue replaced by arginine in the *npi2* mutant is also highly conserved. Although we cannot exclude an effect of the *npi2* mutation on the stability of the protein, our results are also consistent with an important role of the ELC box in the normal function of Npi2p/Doa4p. In keeping with both hypotheses, the uracil permease was recently found to be identically protected against stress-induced degradation in *doa4* null-mutant cells and in cells with the above-mentioned *npi2* point mutation (data not shown). The role of the ELC box remains to be determined, but its presence in a few members of the Ubp family suggests that these hydrolases might be functionally distinct from the other proteins of the same family.

### Defective down-regulation of Gap1 in *npi2/doa4* mutants is due to a limiting amount of free monomeric ubiquitin

Recent experiments have shown that overproduction of Ub in *doa4*Δ cells can reverse some of the *doa4* phenotypes, notably sensitivity to canavanine or cadmium (Hicke, 1997) and defective ubiquitination of the Fur4p uracil permease (Galan and Haguenaer-Tsapis, 1997) and Ste2p  $\alpha$ -factor receptor (Terrel et al., 1998). It was thus interesting to see whether NH<sub>4</sub><sup>+</sup>-induced ubiquitination and down-regulation of Gap1p are also restored. Wild-type and *npi2* cells were transformed with a plasmid encoding a synthetic ubiquitin gene under the control of the inducible *CUPI* promoter. Overproduction of ubiquitin did not



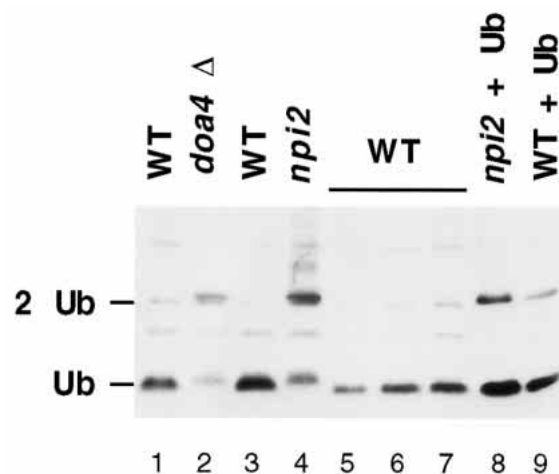
**Fig. 3.** Overproduction of Ub restores  $\text{NH}_4^+$ -induced Gap1p down-regulation in *npi2* cells. Cells of strains 23346c (Wt) and 27002d (*npi2*) transformed with YEp96 (2(*TRP1*) Ub) were grown in YNB medium with proline as a nitrogen source.  $\text{CuSO}_4$  (0.1 mM) was added (or not) to the medium to induce *CUP1*-promoter-driven Ub synthesis and the cells were allowed to grow for another two hours.  $(\text{NH}_4)_2\text{SO}_4$  (10 mM) was then added and Gap1p activity was measured by incorporation of [ $^{14}\text{C}$ ]citrulline (20  $\mu\text{M}$ ) before ( $t=0$ ) and at several times after addition of  $(\text{NH}_4)_2\text{SO}_4$ . Wt cells overexpressing ubiquitin (●) or not (■). *npi2* cells overproducing ubiquitin (○) or not (□).

affect permease down-regulation in wild-type cells, but in *npi2* cells it totally restored  $\text{NH}_4^+$ -induced Gap1p ubiquitination (as detailed below) and down-regulation (Fig. 3). This supports the view that the amount of Ub in *npi2/doa4* cells might be limiting for some cellular processes (Galan and Haguenaer-Tsapis, 1997; Terrel et al., 1998). To investigate this point under our experimental conditions, we used antibodies raised against Ub to probe crude extracts prepared from wild-type, *npi2*, and *doa4* $\Delta$  cells growing exponentially on proline medium. No significant difference was evidenced in the high molecular mass Ub-conjugates present in all cells (not shown). In the low molecular mass ranges, wild-type cells displayed an intense signal corresponding to monomeric Ub and a faint signal likely corresponding to di-Ub (Fig. 4, lane 1). Both the *doa4* $\Delta$  and the *npi2* strains displayed several bands in the region of di-Ub, probably corresponding to Ubs dimers linked to small oligopeptides previously reported to accumulate in *doa4* cells (Papa and Hochtrasser, 1993), and more strikingly a reduced signal for free monomeric Ub (lanes 2 and 4). Serial dilution of the extracts prepared from wild-type cells analyzed on the same gel (lanes 5-7) allowed to estimate that the mutants contain about four times less monomeric Ub than the wild type. Overexpression of Ub from the inducible *CUP1* promoter resulted in at least 20 fold increase in the steady state level of free Ub relative to that in the corresponding untransformed cells (compare lanes 3-4 to lanes 8-9). The level of free Ub in induced transformed *npi2* cells thus exceeds that in untransformed wild-type cells. These data show that defective Gap1p down-regulation in *npi2/doa4* cells is due mainly to the limiting intracellular pool of free monomeric Ub.

#### $\text{NH}_4^+$ induces formation of Lys63-linked polyubiquitin chains

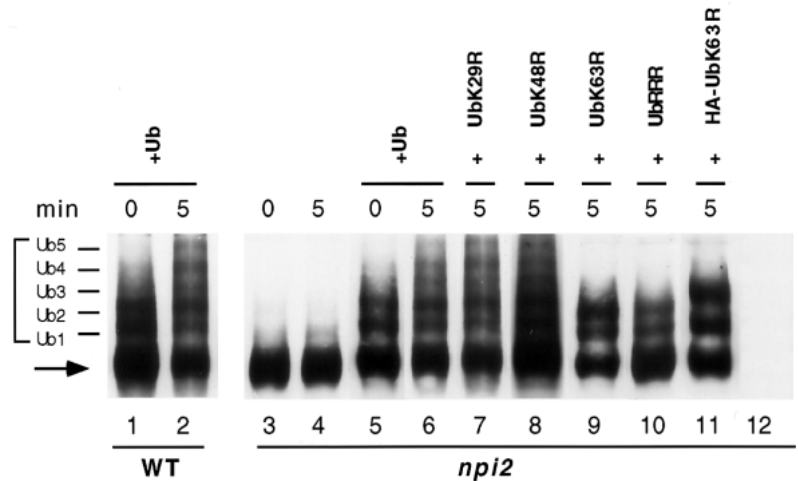
The possibility of restoring normal ubiquitination of at least

some proteins in *doa4* cells by overproduction of Ub was recently used to study the type of ubiquitination undergone by two cell-surface proteins (Galan and Haguenaer-Tsapis, 1997; Terrel et al., 1998). In these studies, various mutant Ubs unable to form certain types of Ub chain were overproduced in the *doa4* cells. We have used here a similar approach to analyze Gap1 ubiquitination before and after addition of  $\text{NH}_4^+$ . Overproduction of ubiquitin in wild-type and *npi2* cells grown on proline medium led to the presence of two intense bands above the major Gap1 signal (Fig. 5, lanes 1 and 5). These upper bands, previously detected as much weaker signals in wild-type cells, correspond to ubiquitinated forms of the protein (see Springael and André, 1998, and Fig. 1C). Accumulation of these ubiquitinated forms did not lead to reduced Gap1p activity (not shown). Addition of  $\text{NH}_4^+$  triggered the very rapid appearance of 2 to 3 additional bands, corresponding to ubiquitin-permease conjugates of higher molecular mass (Fig. 5, lanes 1-2 and 5-6). This modification was accompanied by a decrease in Gap1p activity and a decrease in the amount of Gap1p immunodetected in crude extracts (Fig. 6B). Thus,  $\text{NH}_4^+$  triggers binding of additional Ub molecules to Gap1p. This induced ubiquitination could involve Ub binding to additional lysine residues of the target protein or the formation of Ub chains in which the C-terminal glycine residue of a Ub is linked to the internal lysine residue of a previously bound Ub. We thus overproduced in *npi2* cells a mutant Ub (UbRRR) in which Lys29, Lys48, and Lys63 are replaced by arginine. This Ub variant cannot form multi-Ub chains in vivo (Arnason and Ellison, 1994). When  $\text{NH}_4^+$  was added to *npi2* cells overproducing UbRRR, no bands were formed above the mono- and di-ubiquitinated forms of Gap1p



**Fig. 4.** Mutation or disruption of *DOA4* leads to a decreased monomeric Ub pool. Isogenic MHY501 (Wt, lane 1) and MHY623 (*doa4* $\Delta$ , lane 2) cells, and isogenic 27061b (Wt) and 27071b (*npi2*) cells untransformed (lanes 3-7) or transformed with plasmid YEp96 (lanes 8 and 9) were grown in YNB proline medium and collected during exponential growth. Transformed cells were collected two hours after addition of  $\text{CuSO}_4$  (0.1 mM). Total protein extracts were prepared and Ub was detected by western immunoblotting with polyclonal anti-ubiquitin antibody (Sigma) as described in Materials and Methods. In lanes 1-4, the amount of proteins analyzed corresponded to 0.33 ml of culture per lane ( $A_{600\text{nm}}=1$ ). The amount of proteins analyzed in lanes 5, 6, 7, 8 and 9 corresponded, respectively, to 1/4, 1/2, 3/4, 1/10 and 1/20 of this value. The positions of free Ub and Ub dimers are indicated.

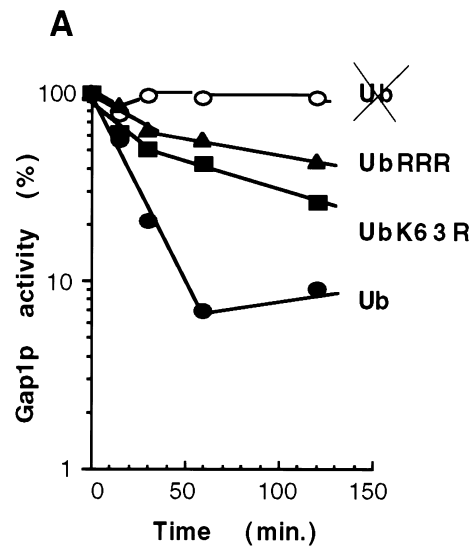
**Fig. 5.** Effect of overproduction of wild-type and mutant Ubs on the ubiquitination of Gap1p. 27061b (Wt) and 27071b (*npi2*) cells either untransformed (lanes 3-4, and 12) or transformed with a plasmid encoding wild-type Ub (lanes 1-2 and 5-6) or mutant Ubs (lanes 7-11) were grown in proline-containing YNB medium. Cells were collected two hours after addition of  $\text{CuSO}_4$  (0.1 mM), then  $(\text{NH}_4)_2\text{SO}_4$  (10 mM) was added and cells were collected 5 minutes later. Membrane-enriched fractions were prepared and Gap1p was detected by western immunoblotting. Arrow represents major Gap1p signal and mono- (Ub1), di- (Ub2), tri- (Ub3), tetra- (Ub4) and penta- (Ub5) ubiquitinated permease are indicated. To check that wild-type and mutant ubiquitins were expressed to the same level, we probed western blots of total extracts with anti-ubiquitin antibody (not shown). Gap1p was undetectable in a membrane-enriched fraction prepared from *npi2* cells grown overnight in the presence of  $(\text{NH}_4)_2\text{SO}_4$  which represses Gap1p expression (lane 12).



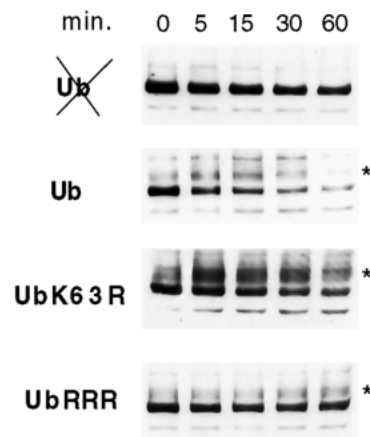
(Fig. 5, lane 10). Although no ubiquitination sites have yet been identified within the Gap1p permease, this result suggests that two residues of Gap1 can act as Ub acceptors. In proline-grown cells, a fraction of the Gap1p molecules would thus be mainly mono-ubiquitinated at one or both acceptor sites;  $\text{NH}_4^+$  addition would induce formation of Ub chains on at least one of these sites. Formation of these polyubiquitin chains is not absolutely essential to  $\text{NH}_4^+$ -induced down-regulation of Gap1, but it does appear necessary to allow its occurrence at an optimal rate: upon addition of  $\text{NH}_4^+$ , *npi2* cells producing UbRRR lost Gap1p activity only half as quickly as cells producing the wild-type Ub (Fig. 6A). Furthermore, immunoblots obtained with crude extracts showed that Gap1p is degraded in *npi2* cells producing the mutant Ub, but more slowly than in the same cells producing wild-type Ub.

To test which lysine residue of Ub is involved in forming polyubiquitin chains on Gap1, we conducted experiments in which a variant Ub singly mutated at either Lys29, Lys48, or Lys63 was produced in *npi2* cells. Overproduction of UbK29R and UbK48R restored the formation of five bands above the major Gap1p signal in response to  $\text{NH}_4^+$  addition. In the presence of UbK63R, only the mono- and di-ubiquitinated forms of Gap1p were evidenced after addition of  $\text{NH}_4^+$  (Fig. 5, lane 9). Both bands exhibited a retarded gel mobility when mutant UbK63R was extended by the addition of HA tag (Fig.

5, lane 11). This clearly indicates that Lys63 is the acceptor residue required for  $\text{NH}_4^+$ -induced ubiquitination of Gap1p. In *npi2* cells overproducing UbK63R, moreover, the loss of permease activity induced by  $\text{NH}_4^+$  was only partial, as in the case of overproduction of UbRRR. We conclude that  $\text{NH}_4^+$



**B**



**Fig. 6.** Mutant ubiquitins UbK63R and UbRRR do not restore normal  $\text{NH}_4^+$ -induced Gap1p down-regulation in *npi2* cells. 27071b (*npi2*) cells either untransformed or transformed with plasmids encoding wild-type Ub, UbRRR, or UbK63R, were grown in proline-containing YNB medium and induced (or not) for two hours with  $\text{CuSO}_4$  (0.1 mM).  $(\text{NH}_4)_2\text{SO}_4$  (10 mM) was then added. (A) Gap1p activity was measured by incorporation of  $[^{14}\text{C}]$ citrulline (20  $\mu\text{M}$ ) before ( $t=0$ ) and at several times after addition of  $(\text{NH}_4)_2\text{SO}_4$ . Results are percentages of initial activities per ml of culture. Strains are: *npi2* ( $\circ$ ), *npi2* + Ub ( $\bullet$ ), *npi2* + UbRRR ( $\blacktriangle$ ), *npi2* + UbK63R ( $\blacksquare$ ). (B) Total protein extracts were prepared at the times indicated after addition of  $(\text{NH}_4)_2\text{SO}_4$ . Gap1p was detected by western immunoblotting in extracts corresponding to 0.2 ml of culture. As shown by probing with anti-ubiquitin, wild-type and mutant Ubs were equally overproduced. Mono- and di-ubiquitinated forms of Gap1p (\*) were clearly evidenced in *npi2* cells overproducing UbK63R or UbRRR (the signals being less intense in the latter case).

induces formation of Lys63-linked polyubiquitin chains on Gap1p, required for rapid and complete  $\text{NH}_4^+$ -induced down-regulation of the permease.

## DISCUSSION

After addition of  $\text{NH}_4^+$  to yeast cells using proline as the sole nitrogen source, Gap1 permease pre-accumulated in the plasma membrane undergoes rapid ubiquitination followed by endocytosis and vacuolar degradation (Springael and André, 1998). Gap1p ubiquitination requires Npi1p/Rsp5p, a ubiquitin protein ligase required for cell viability (Hein et al., 1995; Springael and André, 1998). We here report cloning of *NPI2*, a second gene required for  $\text{NH}_4^+$ -induced down-regulation of the Gap1p permease, and show that it encodes the ubiquitin hydrolase Doa4p. Ubiquitin hydrolases, which cleave Ub from linear or branched conjugates, constitute an unexpectedly large enzyme family, of which 16 members have been identified in *S. cerevisiae* (Wilkinson, 1997; Hochtrasser, 1996). These enzymes share high sequence similarity in two proposed functional domains known as the Cys and His boxes (Baker et al., 1992). These boxes appear to delimit a catalytic core of about 300 residues. Additional blocks of similarity were found between the Cys and His boxes of many isoforms and are assumed to confer specificity or control localization of the enzyme (Wilkinson et al., 1995; Wilkinson, 1997). We show here that the *npi2* mutation impairing Gap1p permease down-regulation results in replacement of a conserved aromatic residue within one of these blocks, which we propose to name the ELC box (Fig. 3). For some of the phenotypes analyzed, the *npi2* mutation appears equivalent to disruption of *DOA4*. We have not determined whether the *npi2* mutation merely reduces the stability of Doa4p or affects a functionally important site, but the critical role of the aromatic residue and the presence of the ELC box in a subset of Ubps (a few in yeast and some in higher eukaryotes, such as the human Tre2 oncogen or the *Drosophila* fat-facets) suggest that these enzymes might share specific functional properties. There is considerable evidence of specific functions among Ub isopeptidases, even though their precise physiological substrates have not yet been identified. For instance, cell fate determination in the *Drosophila* eye is controlled by the fat-facets isopeptidase (Huang et al., 1995), another isopeptidase (Uch-D) is specifically involved in *Drosophila* oogenesis (Zhang et al., 1993), and the yeast Ubp3p appears as an inhibitor of silencing (Moazed and Johnson, 1996).

Strikingly different genetic approaches have led to identification of the *DOA4/SSV7/UBP4* gene: searches for mutants defective in Mat $\alpha$ 2 degradation (do for degradation of  $\alpha$ ) (Hochtrasser and Varshavsky, 1990), isolation of salt-sensitive vacuolar (*ssv*) mutants (Latterich and Watson, 1991), of cells with uncoordinated DNA replication (Singer et al., 1996). Our finding that the *npi2* (nitrogen permease inactivator) mutation is a point mutation in *DOA4* further extends this list. Overproduction of Ub reverses several of the numerous phenotypes conferred by lack of Doa4p, notably sensitivity to cadmium and canavanine (Hicke, 1997) and the inability to ubiquitinate and subsequently degrade certain proteins such as the Ste2p receptor (Terrel et al., 1998), the uracil permease (Galan and Haguenaer-Tsapis, 1997), and as

shown in this paper, Gap1p. These observations suggest that some *doa4* phenotypes are due to decreased Ub levels in *doa4* cells. It has been reported that Ub pools drop dramatically in *doa4* cells entering the stationary phase (Chen and Hochtrasser, 1995) and that free monomeric Ub is no longer detectable in *doa4* cells subjected to prolonged heat stress (Singer et al., 1996). We here extend these observations, showing that the amount of free monomeric Ub is reduced at least 4-fold in *npi2/doa4* cells growing exponentially in minimal medium. Obviously, however, not all Ub-dependent processes are equally affected by lowering of the Ub pools in *doa4* cells. Various proteins of the Ub-proteasome pathway are stabilized to strikingly different degrees (Hochtrasser, 1996). As an extreme example, the Ub pool of *doa4* cells appears sufficient for normal turnover of a membrane-bound protein subject to endoplasmic-reticulum-associated degradation (Galan et al., 1998).

The exact physiological role of Doa4p, still a matter of debate, obviously extends beyond its effect, likely indirect, on the intracellular Ub pool. Doa4p-deficient mutants have been shown to accumulate small Ub-linked peptide fragments (mostly Ub dimers) (Papa and Hochtrasser, 1993, Amerik et al., 1997). A model has been proposed in which Npi2p/Doa4p works in conjunction with the proteasome, cleaving Ub chains still attached to peptide remnants of proteolysis, creating unanchored Ub chain substrates (Amerik et al., 1997). Some *doa4* phenotypes not complemented by overproduction of Ub (Singer et al., 1996), such as uncoordinated replication, might be more directly related to the true function of Doa4p, whatever it may be.

$\text{NH}_4^+$ -induced ubiquitination and down-regulation of Gap1p are thus inhibited in *npi2* cells and both restored by overproduction of Ub. This fact has enabled us to investigate the type of Ub modification involved in these processes. In proline-grown wild-type and *npi2* cells overproducing Ub, a larger fraction of the Gap1p molecules appears to undergo ubiquitination, resulting in accumulation of mono- and di-ubiquitinated species. This modification does not lead to reduced Gap1p activity, suggesting that the permease remains stable at the plasma membrane. Addition of  $\text{NH}_4^+$  to the medium leads within minutes to formation of Ub-permease conjugates bearing up to four or five Ub moieties. The same species are observed, after  $\text{NH}_4^+$  addition, in *npi2* cells overproducing a mutant Ub in which Lys29 or Lys48 have been mutated to arginine. In contrast, *npi2* cells overproducing the triple Ub mutant UbRRR, unable to form Ub chains, or the Ub mutant with an arginine residue instead of Lys63, displayed only di-ubiquitinated Ub-permease conjugates. This strongly suggests that two lysine residues in Gap1p serve as Ub-acceptor sites, and that  $\text{NH}_4^+$  promotes the formation of Lys63-linked polyubiquitin chains. Furthermore, formation of these chains promotes more rapid down-regulation of Gap1p. The uracil permease displays a very similar ubiquitination pattern (Galan and Haguenaer-Tsapis, 1997). Ubiquitination of both transporters appears to involve formation of short Lys63-linked polyubiquitin chains, and in both cases the rate of internalization appears clearly linked to the ability to form these chains. In the case of Gap1p, interestingly, the specific shift from mono- to poly-ubiquitination is triggered by addition of  $\text{NH}_4^+$  to the medium, indicating that polyubiquitination is subject to nitrogen-source regulation. Addition of  $\text{NH}_4^+$  also

generally increases conversion of Gap1p to Ub-conjugated forms (Springael and André, 1998). The mechanisms underlying nitrogen control of Gap1p ubiquitination are unknown. Addition of  $\text{NH}_4^+$  might alter Gap1p phosphorylation, which in turn could render the permease more accessible to mono- and poly-ubiquitination. Regulatory factors responding to  $\text{NH}_4^+$  are likely involved. One such factor could be Npr1p, a protein kinase homologue (Vandenbol et al., 1990) which appears to protect Gap1p and other  $\text{NH}_4^+$ -sensitive permeases from down-regulation in cells growing on proline or urea as the sole nitrogen source (Grenson, 1983b).

Since the Fur4p and Gap1p permeases seem to undergo the same type of ubiquitination, might Lys63-linked polyubiquitin chains be a general feature of plasma-membrane proteins in yeast? A truncated form of the receptor Ste2p, another plasma-membrane protein, is reported to undergo only mono-ubiquitination (Terrel et al., 1998). Perhaps Fur4p and Gap1p ubiquitination on the one hand and Ste2p ubiquitination on the other involve distinct E2/E3 pairs. Ubiquitination of Ste2p is reported to be Ubc4p/Ubc5p-dependent (Hicke and Riezman, 1996), whereas ubiquitination of Fur4p appears normal in *ubc4Δubc5Δ* cells (Galan and Haguenaer-Tsapis, 1997). On the other hand, the Npi1p ubiquitin ligase is required for ubiquitination of both Fur4p and Gap1p (Galan and Haguenaer-Tsapis, 1997; Springael and André, 1998). In yeast, this Ub ligase appears to play a central role in endocytosis of numerous plasma-membrane proteins, or at least of several transporters. It is involved in catabolite inactivation of the maltose transporter (Lucero and Lagunas, 1997) and in  $\text{NH}_4^+$ -induced inactivation of numerous nitrogen-compound permeases in addition to Gap1p (Grenson, 1992). It is tempting to imagine an even broader role for this type of enzyme in ubiquitination of plasma-membrane proteins of eukaryotic cells, since rNedd4, the rat homolog of Npi1p, is involved in the ubiquitination of some epithelial Na channel (ENaC) subunits (Staub et al., 1996, 1997). In addition, it was recently demonstrated that Nedd4 mediates Ub-dependent down-regulation of ENaC in response to increased intracellular  $\text{Na}^+$  (Dinudom et al., 1998).

Gap1p is thus the second example, after the uracil permease, of a yeast plasma-membrane transporter whose rapid internalization requires formation of polyubiquitin chains with linkage through Lys63. It is clear that neither mono-ubiquitination nor the formation of such chains targets proteins for proteasome recognition. The role of these Ub modifications as internalization signals thus remains to be determined. It has been suggested that Ub (chains) might be recognized by a component of the endocytic machinery or might alter target proteins so as to facilitate their binding to such a component (Hicke, 1997). Alternatively, ubiquitination might promote movement of ubiquitinated proteins into membrane regions that actively endocytose, as suggested by the polarized cell-surface staining of Ste6p in mutants displaying reduced ubiquitination (Loayza and Michaelis, 1998). Ubiquitination or lysine-63-mediated polyubiquitination might also contribute to determining lysosomal degradation rather than recycling of internalized plasma-membrane proteins.

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