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NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin–protein ligase

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Summary

When yeast cells growing on a poor nitrogen source are supplied with NH_4^+ ions, several nitrogen permeases including the general amino acid permease (Gap1p) are rapidly and completely inactivated. This report shows that inactivation by NH_4^+ of the Gap1 permease is accompanied by its degradation. A functional *NPI1* gene product is required for both inactivation and degradation of Gap1p. Molecular analysis of the *NPI1* gene showed that it is identical to *RSP5*. The *RSP5* product is a ubiquitin–protein ligase (E3 enzyme) whose physiological function was, however, unknown. Its C-terminal region is very similar to that of other members of the E6-AP-like family of ubiquitin–protein ligases. Its N-terminal region contains a single C_2 domain that may be a Ca^{2+}-dependent phospholipid interaction motif, followed by several copies of a recently identified domain called WW(P). The Npi1/Rsp5 protein has a homologue both in humans and in mice, the latter being involved in brain development. Stress-induced degradation of the uracil permease (Fur4p), a process in which ubiquitin is probably involved, was also found to require a functional *NPI1/RSP5* product. Chromosomal deletion of *NPI1/RSP5* showed that this gene is essential for cell viability. In the viable *npi1/rsp5* strain, expression of *NPI1/RSP5* is reduced as a result of insertion of a Ty1 element in its 5′ region. Our results show that the Npi1/Rsp5 ubiquitin–protein ligase participates in induced degradation of at least two permeases, Gap1p and Fur4p, and probably also other proteins.

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Introduction

Several of the plasma-membrane permeases for nitrogenous compounds in *Saccharomyces cerevisiae* are tightly regulated according to the nitrogen sources available in the growth medium (Wiame *et al.*, 1985; Grenson, 1992). They include the general amino acid permease (Gap1p) (Jauniaux and Grenson, 1990), proline permease (Put4p) (Jauniaux *et al.*, 1987; Vandenbol *et al.*, 1989), ureidosuccinate and allantoate permease (Dal5p) (Rai *et al.*, 1988) and the inducible γ-aminobutyrate specific permease (Uga4p) (André *et al.*, 1993). The best studied of these nitrogen-regulated permeases is the general amino acid permease encoded by the *GAP1* gene (Wiame *et al.*, 1985; Grenson, 1992). This multi-spanning membrane protein catalyses active transport of apparently all amino acids, many of which are potential nitrogen sources, as well as D-isomers and other toxic analogues. The deduced sequence of Gap1p is similar to that of a family of amino acid permeases identified in several bacteria and fungi (Jauniaux and Grenson, 1990). Gap1 permease is most active in cells grown on a poor nitrogen source, like urea or proline, but it is inactive when cells are provided with a good nitrogen source, such as NH_4^+ ions or glutamine. Adding NH_4^+ ions to cells grown on proline blocks expression of the *GAP1* gene and pre-existing Gap1 permease is rapidly and completely inactivated (Grenson, 1983a). The latter phenomenon, called nitrogen catabolite inactivation, has been genetically dissected (Grenson, 1983a,b; 1992; Wiame *et al.*, 1985). Two genes, *NPI1* and *NPI2* (nitrogen permease inactivator), are essential for the NH_4^+-triggered inactivation of Gap1p; adding NH_4^+ ions to *npi1* or *npi2* mutant cells blocks Gap1p synthesis, but pre-synthesized Gap1 permease remains active (Grenson, 1983a). The *NPI1*- and *NPI2*-dependent inactivation system is also potentially functional when cells are grown on a poor nitrogen source. However, under these conditions, its action appears to be prevented or compensated in a manner that is dependent on the *NPR1* gene product (nitrogen permease reactivator) (Grenson,

1983a,b). Cloning of the *NPR1* gene showed that it encodes a protein having the characteristics of a serine/threonine protein kinase (Vandenbol *et al.*, 1987; 1990). The molecular mechanism underlying nitrogen catabolite inactivation of the Gap1 permease is unknown. Stanbrough and Magasanik (1995) recently showed that the addition of glutamine causes rapid dephosphorylation and inactivation of the Gap1 permease with the same kinetics. However, these experiments were performed in *per1* mutant cells lacking the ammonia inactivation response. The glucose-induced catabolite inactivation of various sugar transporters (Matern and Holzer, 1977; Bisson and Fraenkel, 1984; Ramos and Cirillo, 1989) appears to be similar to nitrogen catabolite inactivation and involves mechanisms which are also largely unknown.

This study shows that the yeast Gap1 permease is degraded during nitrogen catabolite inactivation. This degradation is dependent on the *NPI1* gene. A functional *NPI1* product is also required for induced degradation of the uracil permease, a process which probably involves ubiquitin (Galan *et al.*, 1994). Molecular characterization of the *NPI1* gene shows that it encodes Rsp5p, a protein exhibiting structural and functional properties of a ubiquitin–protein ligase but whose physiological function was unknown (Huibregste *et al.*, 1995). The *NPI1* gene is essential for cell viability, strongly suggesting that the role of the ubiquitin–protein ligase encoded by this gene extends beyond degradation of the Gap1 and Fur4 permeases.

Results

Gap1 permease is degraded during nitrogen catabolite inactivation: role of the Npi1 protein

To investigate the molecular mechanisms underlying nitrogen catabolite inactivation of the general amino acid permease (Gap1p), we started by using site-directed mutagenesis to introduce the 10-amino-acid epitope from the human *c-myc* protein into the amino-terminal region of Gap1p. Strain (*gap1*, *ura3*) was transformed with a centromere-based plasmid containing the epitope-tagged *GAP1* construct. The Ura^+ transformants containing this plasmid grew normally on a minimal medium containing citrulline (a Gap1p-specific substrate) as the sole source of nitrogen and they exhibited a high [^{14}C]-citrulline-uptake activity, indicating that the epitope-tagged Gap1 protein remained functional (data not shown). Total cell extracts were prepared from cells grown on minimal proline medium, resolved on SDS–PAGE and probed with monoclonal anti-*c-myc* antibodies. An immunoreactive signal was detected in cells transformed with the epitope-tagged *GAP1* construct, but not in cells containing the untagged *GAP1* plasmid (Fig. 1, lanes 1 and 2). The immunodetected Gap1 polypeptide had an apparent mass (~50 kDa) smaller than that calculated from the amino acid sequence (65 kDa), which is not unusual for highly hydrophobic proteins. Adding NH_4^+ ions to proline-grown cells caused the citrulline-uptake activity of the epitope-tagged Gap1 permease to decrease rapidly and completely, indicating sensitivity to nitrogen catabolite inactivation (Fig. 2A). In contrast, the pre-existing Gap1 uptake activity in an isogenic *npi1* mutant strain was preserved after adding NH_4^+ ions to the growth medium (Fig. 2A). Hence, as for the wild-type Gap1 permease (Grenson, 1983a), catabolite inactivation of the epitope-tagged permease requires a functional *NPI1* product. Cell samples were withdrawn at different times after addition of NH_4^+ ions to the growth medium and were analysed by Western blotting. The amount of Gap1 polypeptide in wild-type cells gradually declined after the addition of ions and was barely detectable within two hours (Fig. 2B). In contrast, in the *npi1* mutant strain, the level of Gap1 polypeptide was preserved for at least two hours after the addition of NH_4^+ ions (Fig. 2B). Therefore, catabolite inactivation of the Gap1 permease is accompanied by its degradation and this process requires a functional *NPI1* product.

Cloning of the NPI1 *gene*

The *NPI1* gene was cloned by screening a low copy number library, representing the genome of strain Σ1278b, for

Fig. 1. Epitope tagging of the Gap1 permease. Cells of the (*gap1*, *ura3*) strain were transformed with a centromere-based plasmid carrying the *c-myc*-tagged (lane 1; $GAP1^{c\text{-}myc}$) or the wild-type (lane 2) *GAP1* gene. The cells were grown on minimal proline medium and crude extracts were analysed by Western blotting with monoclonal anti-*c-myc* antibodies. An unidentified polypeptide with a relatively high molecular mass was immunodetected in both cell extracts, indicating that they contained similar amounts of protein.

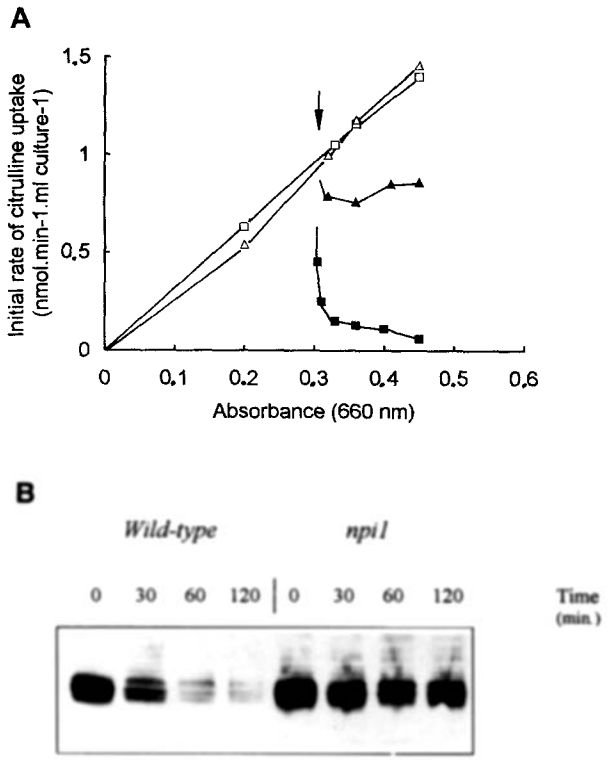

Fig. 2. The Gap1 permease is inactivated and degraded in a *NPI1*-dependent manner upon addition of NH_4^+ ions.
A. Differential plot showing development of Gap1 activity as a function of increased cellular mass in exponentially growing cultures. Cells of the (*gap1*, *NPI1*$^+$, *ura3*) (squares), and (*gap1*, *npi1*, *ura3*) (triangles) strains were transformed with a centromere-based plasmid carrying the *GAP1*$^{c\text{-}myc}$ gene. The cells were grown on minimal proline medium (open symbols); the vertical arrow indicates the addition of 10 mM $(NH_4)_2SO_4$ to part of the cultures (closed symbols). Samples of the growing cultures were withdrawn and assayed for Gap1 activity by measuring the initial rate of [^{14}C]-citrulline incorporation. The assays in the (*gap1*, *NPI1*$^+$, *ura3*) strain (■) were performed 7, 15, 30, 60, 90 and 120 min after addition of NH_4^+ ions; and in the (*gap1*, *npi1*, *ura3*) strain (▲), at 15, 30, 60 and 120 min.
B. Immunoblot analysis of epitope-tagged Gap1 polypeptide. Cell extracts were prepared from culture samples withdrawn at different times after adding NH_4^+ ions to the cultures of the the (*gap1*, *NPI1*$^+$, *ura3*) (wild type) and (*gap1*, *npi1*, *ura3*) (*npi1*) strains expressing the epitope-tagged Gap1 permease.

plasmids complementing an *npi1* mutation (Table 1). The recipient strain (*npr1*, *npi1*, *ura3*) grows very slowly on minimal proline medium containing D-histidine (0.05%) or L-lysine (0.1%). This is due to the *npi1* mutation which restores Gap1 activity in the *npr1* mutant strain, resulting in incorporation of toxic concentrations of L-lysine or D-histidine. In contrast, a strain containing an *NPI1* plasmid should not be poisoned by D-histidine or L-lysine, as Gap1 permease is inactive in an (*npr1*, *NPI1*$^+$) mutant strain. The second selection criterion used in the cloning strategy was the ability of a (*npi1*, *npr1*) double-mutant strain to grow normally on minimal media containing proline (0.1%) or γ-aminobutyrate (0.1%) as sole nitrogen source, while the (*npr1*, *NPI1*$^+$) single-mutant strain grows slowly on these media. The reduction of proline and γ-aminobutyrate transport as a result of an *npr1* mutation is indeed largely suppressed by an additional *npi1* mutation (Table 1). After transformation of the (*npi1*, *npr1*, *ura3*) recipient strain, we recovered one plasmid (YCpJYS2) carrying a 5.6 kb insert and complementing the *npi1* mutation (Table 1; Fig. 3). Plasmid YCpJYS2 was subsequently introduced into strain (*npi1*, *ura3*) and tested for its ability to restore NH_4^+-triggered inactivation of the Gap1 permease. Addition of NH_4^+ ions to cells bearing plasmid YCpJYS2 produced rapid and complete inactivation of Gap1p, whilst pre-existing Gap1 activity was maintained in the recipient strain (Fig. 4). This result provided strong evidence that the cloned DNA fragment contained the *NPI1* gene. The nucleotide sequence of the *npi1*-complementing insert was found to be a sequence of chromosome V. The originally cloned DNA fragment contained at least two complete open reading frames (ORFs) with lengths of 1718 bp (*YER124c*) and 2427 bp (*YER125w*). Sub-cloning experiments indicated that the *NPI1* coding region corresponds to *YER125w* (Fig. 3). To confirm this, a DNA fragment containing *YER125w* was cloned from the *npi1* strain. As expected, this fragment was unable to complement the *npi1* mutation (not shown). The only mutation found by complete sequencing of this clone was the insertion of a yeast transposon (Ty1a) at position −500 upstream from the translation-initiation codon (Fig. 3). This suggested that the region

Table 1. Complementation by the YCpJYS-2 plasmid of the *npi1*-linked phenotypes.

Strain	Plasmid	Growth					Activity of Gap1 permease (nmoles min^{-1} mg prot^{-1})
		Proline + D-histidine	Proline + L-lysine	Proline	GABA	Glutamate	
Wild type	None	−	−	+++	+++	+++	45
ura3 npr1 NPI1$^+$	pFL38	++	++	++	+	+++	5
ura3 npr1 npi1	pFL38	−	−	+++	+++	+++	29
ura3 npr1 npi1	YCpJYS-2	++	++	++	+	+++	4

Cells were grown on minimal medium with the indicated compounds as the sole nitrogen source added at the following concentrations: L-proline, γ-aminobutyrate (GABA), glutamate and L-lysine: 0.1%; D-histidine: 0.05%. The activity of general amino acid permease (Gap1p) was assayed by measurement of the initial rate of [^{14}C]-citrulline uptake in cells grown on minimal proline medium.

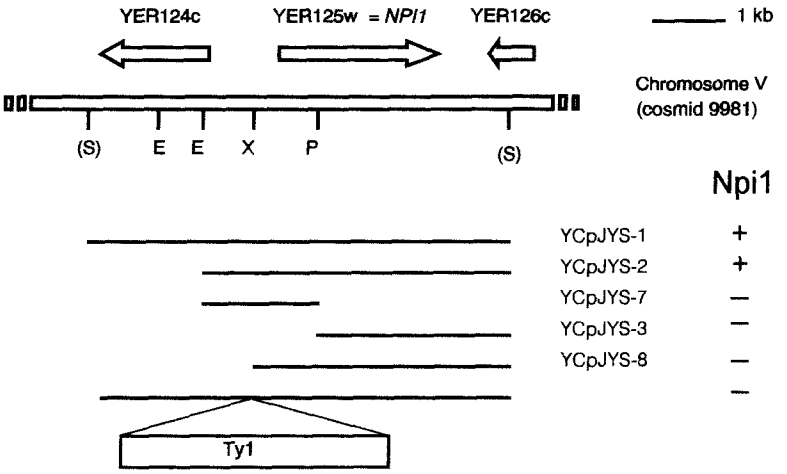

Fig. 3. Chromosomal location and subcloning of the *NPI1* gene. Restriction map of part of cosmid 9981 of chromosome V (Dietrich, F. S. *et al.*, 1994, unpublished; accession number U18916). The thin horizontal lines represent DNA fragments tested for their ability to restore a Npi1$^+$ phenotype to *npi1* cells when carried on a low-copy-number plasmid. Restriction sites: E, *Eco*RI; X, *Xho*I; P, *Pvu*II; S, *Sau*3A.

upstream from this position is essential for *NPI1* function. To confirm this, another subclone of the wild-type DNA fragment was constructed that lacked the region upstream from −470 (Fig. 3, plasmid YCpJYS-8): this subclone was unable to complement the *npi1* mutation (not shown). A Northern blot analysis of total RNAs isolated from the wild-type and *npi1* strains was performed using a *YER125w* internal DNA fragment as a probe. This experiment revealed a single transcript ~2.8 kb in size that did not vary according to the nitrogen source and that was less abundant in the *npi1* strain than in the wild type (Fig. 5). Taken together, these results demonstrate that *YER125w* is the coding region of the *NPI1* gene. They also indicate that a sufficiently high level of *NPI1* expression requiring the region upstream from −470 must be reached to promote efficient inactivation and degradation of the Gap1 permease in response to NH_4^+.

The NPI1 *gene encodes the Rsp5 ubiquitin–protein ligase, a protein conserved from yeast to humans*

The sequence of the *NPI1/YER125w* gene and of the deduced protein (809 amino acids (aa); 91.8 kDa) were reported in databanks in the course of sequencing of chromosome V (Dietrich, F. S. *et al.*, 1994, unpublished; accession numbers: L11119, U18916). The codon usage of *NPI1* is not biased (calculated codon-bias index = 0.17; Bennetzen and Hall, 1982), suggesting that its translated product is not very abundant. Analysis of the hydropathy values of Npi1p did not reveal any predicted transmembrane region. It was also reported in databanks that mutant alleles of this gene were isolated as extragenic suppressors of mutations in Spt3p, a protein that interacts with the TATA-box-binding protein (TBP) (Eisenmann *et al.*, 1992), and the gene was called *RSP5* (F. Winston, unpublished; accession number: 18916). Recently, Huibregste *et al.* (1995) reported that the product of the *NPI1/YER125W/RSP5* gene is in fact a member of the E6-AP-like family of ubiquitin–protein ligases. Proteins of this family share a C-terminal domain of ~300 amino acids which contains a perfectly conserved cysteine residue responsible for thioester-bond formation with ubiquitin. Although Rsp5p was shown to exhibit structural and functional properties of a ubiquitin–protein ligase (Huibregste *et al.*, 1995), the proteins affected by Rsp5p remained unknown. Our observation that *NPI1* and *RSP5* are one and the same gene shows that at least one pathway affected by this ubiquitin–protein ligase is NH_4^+-triggered degradation of the Gap1 permease.

Among members of the E6-AP-like ubiquitin–protein ligase family, the Npi1/Rsp5 protein is closely related to

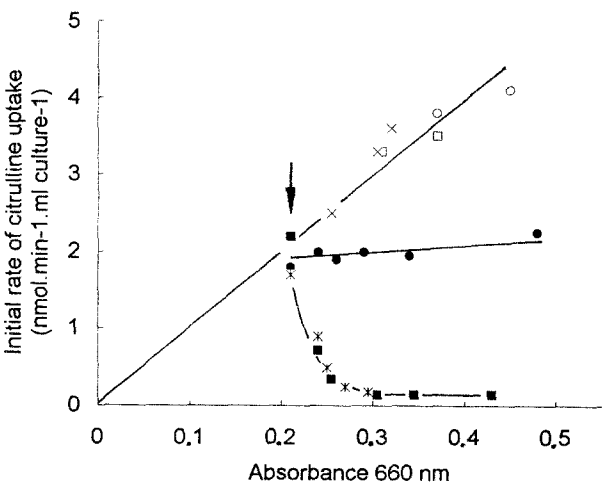

Fig. 4. Ability of YCpJYS2 plasmid to restore nitrogen catabolite inactivation of the Gap1 permease into the *npi1* mutant strain. The [^{14}C]-citrulline uptake activity of Gap1 permease was measured as a function of cell mass in exponentially growing cultures. Cells were grown on minimal proline medium; the vertical arrow indicates addition of 10 mM $(NH_4)_2SO_4$ to part of the cultures. Strains: Σ1278b (wild type) growing with (*) or without (×) NH_4^+ ions; 27038a (*npi1*, *ura3*) bearing plasmid pFL38 and growing with (●) or without (○) NH_4^+ ions; 27038a (*npi1*, *ura3*) bearing plasmid YCpJYS2 and growing with (■) or without (□) NH_4^+ ions.

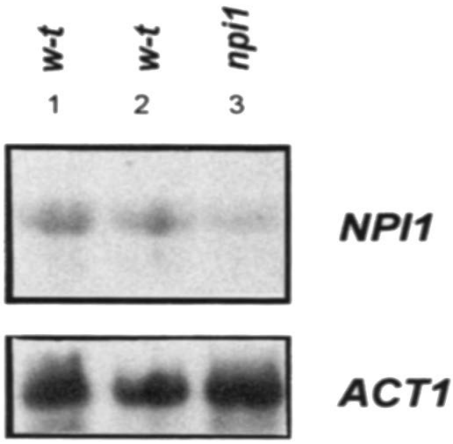

Fig. 5. Northern RNA hybridization analysis of *NPI1* expression. Total RNA was extracted from (*ura3, NPI1*$^+$) (w-t) and (*ura3, npi1*) strains grown on minimal medium containing proline (lanes 1 and 3) or NH_4^+ ions (lane 2) as the sole nitrogen source. The DNA probe was prepared by labelling a 1.9 kb *Acc*I–*Acc*I fragment covering most of the *NPI1* coding region (positions 320–2225 in Fig. 6). The actin RNA (*ACT1*) were used as an internal control.

two mammalian cDNA products, namely mouse NEDD-4 (Kumar *et al.*, 1992) and a human protein referred to here as hORF3 (Nomura, N. *et al.*, 1995, unpublished; accession number: D42055) (Fig. 6). The cellular functions of these two proteins are unknown apart from the fact that NEDD-4 mRNA is predominantly present in the early embryonic brain and its concentration drops during development (Kumar *et al.*, 1992). In addition to the C-terminal domain (42.6% identity in 306 aa overlap), the three proteins share N-terminal sequence similarities. A single C_2 domain is present at the N-terminus of Npi1p and hORF3 (Figs 6 and 7; Bork and Sudol, 1994). The N-terminal region of mouse NEDD-4 was not completely sequenced, thus it is uncertain whether or not this protein also contains a C_2 domain at this position. The C_2 domain was found in many proteins, including some isozymes of protein kinase C (Azzi *et al.*, 1992) and synaptotagmins (p65) (Perin *et al.*, 1990). We also found a single C_2 domain in two incomplete plant cDNA products (Fig. 7). Experiments showed that some C_2 domains mediate interaction with phospholipids (Perin *et al.*, 1990); in some cases, this interaction was shown to be stimulated by Ca^{2+} ions (Clark *et al.*, 1991). Other experiments showed that one of the two C_2 domains of synaptotagmin I is a high-affinity receptor site for clathrin AP-2, indicating that this domain can also play a role in endocytosis (Zhang *et al.*, 1994). In a position C-terminal to the C_2 domain, the yeast Npi1p and the two related mammalian proteins contain three to four copies of a recently reported ~30 aa domain called WW(P) (Figs 6 and 8) (André and Springael, 1994; Bork and Sudol, 1994; Hofmann and Bucher, 1995). The WW(P) domain has also been identified in single or double copies in several proteins of yeast, nematode and vertebrate origin. Among these are dystrophin, the cytoskeletal protein encoded by the Duchenne muscular dystrophy locus, a protein (YAP65) that has been identified for its ability to associate to the Src homology domain 3 (SH3) of the Yes proto-oncogene product (Sudol, 1994), and a human protein related to GTPase activating proteins. The role of the WW(P) domain remains unknown. However, its distribution in cytoskeletal and putative signalling proteins makes it likely that WW(P) is a protein–protein interaction motif.

Npi1p is also involved in degradation of the uracil permease

That *NPI1* encodes the Rsp5 ubiquitin–protein ligase prompted us to test its role in the degradation of yeast uracil permease (Fur4p). The turnover of this permease is strikingly enhanced under stress conditions as compared to that observed in exponentially growing cells. Turnover is rapid when cells approach the stationary-growth phase, when they are starved for various nutrients, and when protein synthesis is inhibited. The degradation of

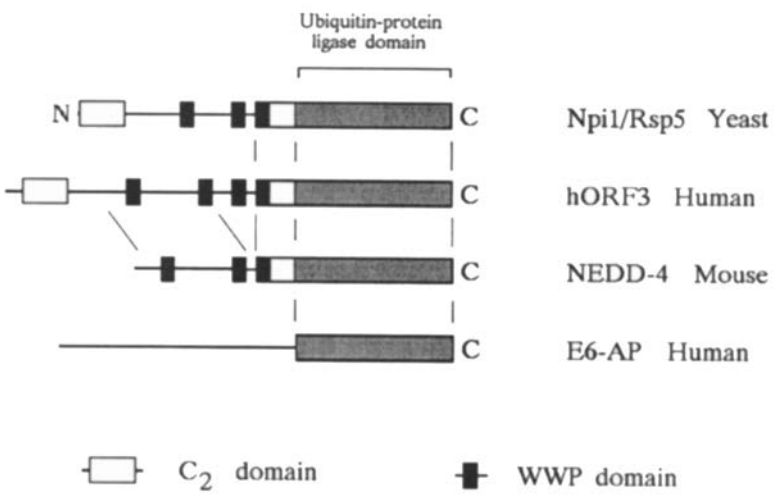

Fig. 6. Diagram of yeast Npi1p and related mammalian proteins. The C_2 domains from yeast Npi1p, human ORF3 and other proteins are aligned in Fig. 7. The WW(P) repeats are aligned in Fig. 8. The proteins related to Npi1p are human ORF3 (Nomura, N. *et al.*, 1995; accession number D42055), mouse NEDD-4 (Kumar *et al.*, 1992; accession number D10714) and human E6-AP (Huibregtse *et al.*, 1993; accession number L07557).

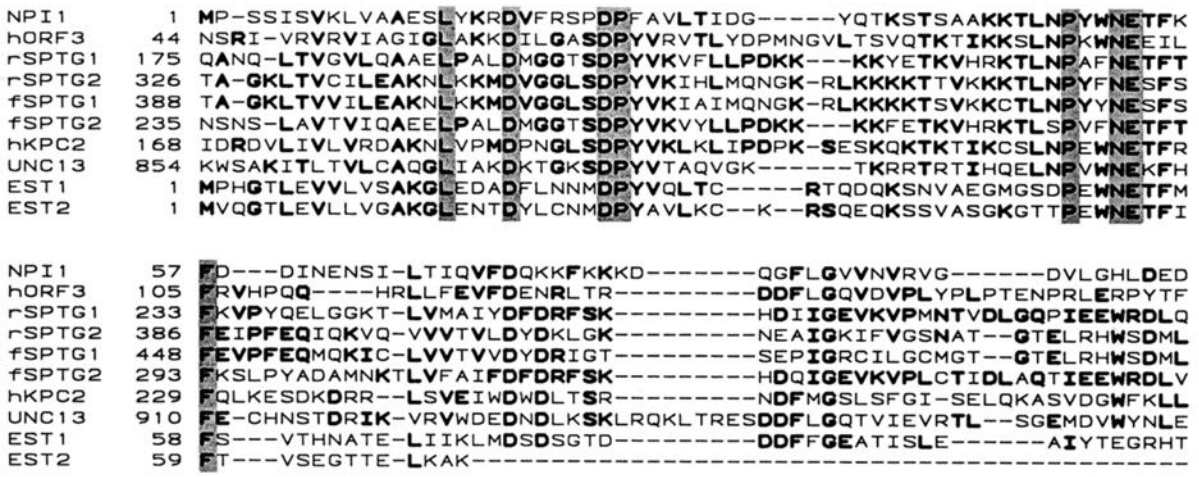

Fig. 7. The yeast Npi1p contains a C_2 domain. The C_2 domain of Npi1p is aligned with the C_2 domain(s) from: hORF3, human ORF3 (Nomura, N. *et al.*, 1995; accession number D42055); rSPTG1 and −2, rat synaptotagmin II (Geppert *et al.*, 1991); fSPTG1 and −2, *Drosophila melanogaster* synaptotagmin (Perin *et al.*, 1991); hKPC2, human protein kinase C, β-II type (Coussens *et al.*, 1986); and UNC13, unc-13 gene product from *Caenorhabditis elegans* (Maruyama and Brenner, 1991). EST1, −2, incomplete cDNA product from rice (Yuzo, M. and Takuji, S. unpublished; accession number D24373); and *Arabidopsis thaliana* (Bardet, C. *et al.*, unpublished; accession number Z17560), respectively. Amino acid identities are indicated in bold. Invariant amino acids are shaded. Amino acid numbers of hORF3 , EST1 and EST2 are uncertain.

Fur4p permease induced under stress conditions appears to be preceded by its endocytosis and to take place within the vacuole (Volland *et al.*, 1994). Uracil permease has a sequence (Arg–Ile–Ala–Leu–Gly–Ser–Leu–Thr–Asp), in an internal hydrophilic loop putatively located at the cytoplasmic side of the membrane, that is very similar to the 'destruction box' required for the ubiquitin-dependent proteolysis of mitotic cyclins (Glotzer *et al.*, 1991). A mutation in this sequence (Arg → Ala) protects Fur4p against degradation, leading to the suggestion that ligation of ubiquitin could be a signal for uracil-permease degradation (Galan *et al.*, 1994). To test the role of Npi1p in stress-induced degradation of the uracil permease, cycloheximide was added to exponentially growing cells which expressed the *FUR4* gene under the control of the inducible *GAL10* promoter. Cycloheximide caused a sharp decrease in uracil uptake ($t_{1/2}$ = 60 min) and a drop in the amount of immunodetected Fur4p permease (Fig. 9). The same experiment performed in parallel in isogenic *npi1* mutant cells showed that these cells were protected against both loss of uracil uptake ($t_{1/2}$ = 130 min) and uracil-permease degradation. Therefore, the product of the *NPI1/RSP5* gene is involved not only in NH_4^+-triggered inactivation and degradation of the Gap1 permease, but also in the degradation of the uracil permease induced by inhibition of protein synthesis. These results are also consistent with Npi1/Rsp5p having a direct role in permease turnover rather than in the regulation of this process according to nitrogen sources or stress conditions.

NPI1 *is an essential gene*

To determine the phenotypic properties of a *npi1* null mutation, we constructed a *npi1*Δ*:LEU2* in which the entire *NPI1* coding sequence was replaced by the yeast *LEU2* gene (see the *Experimental procedures*). The disrupting DNA fragment was used to transform the *leu2/leu2* diploid strain, and several Leu^+ transformants were recovered. Southern analysis and polymerase chain reaction (PCR) amplification of total DNA isolated from several Leu^+ transformants identified clones exhibiting single-copy replacement of *NPI1* by *LEU2*. These diploids were sporulated and subjected to meiotic analysis. For each of the asci analysed, a 2:2 segregation for viability of the spores was observed, and all viable cells were Leu^-. The Npi1/Rsp5 protein is therefore essential for cell viability. These results also suggest that the function of Npi1p might extend beyond degradation of permeases. The fact that the *npi1*

Fig. 8. Sequence alignment of the repeated WW(P) domains from yeast Npi1p, mouse NED$_D$-4 and human ORF3. Amino acid identities are indicated in bold. The numbering of residues in NED$_D$-4 and hORF3 are uncertain.

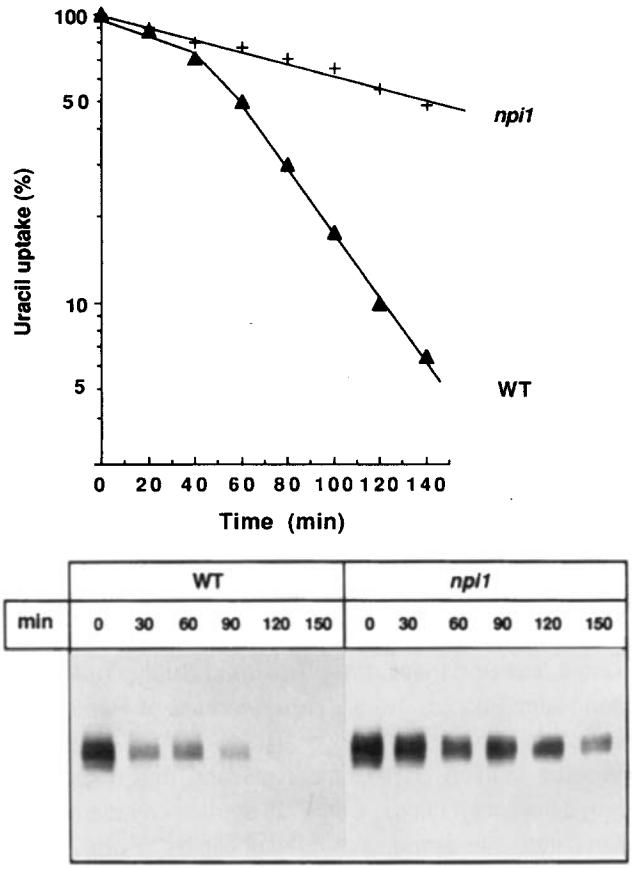

Fig. 9. The Npi1p protein is required for stress-induced degradation of uracil permease. 23344c (*ura3, NPI1*$^+$) and 27038a (*ura3, npi1*) cells transformed with the plasmid p195gF were grown to the same cell density (10^7 cells ml^{-1}). At t = 0, cycloheximide was added to the medium.
A. Uracil uptake was measured at various times.
B. Protein extracts were prepared at the times indicated and analysed for uracil permease by Western immunoblotting.

strain is fully viable suggests that the reduced level of *NPI1* expression exhibited by this mutant is sufficient to allow the function(s) of Npi1/Rsp5p that are essential for cell viability, whilst a higher level of expression appears to be required for induced degradation of the Gap1 and Fur4 permeases.

Discussion

Nitrogen catabolite degradation of the Gap1 permease

Our findings show that nitrogen catabolite inactivation of the Gap1 permease is accompanied by its degradation. The gradual loss of Gap1 uptake activity that occurs after addition of NH_4^+ ions to the growth medium might be a direct consequence of permease degradation. Alternatively, the Gap1 permease could be inactivated and subsequently degraded. The latter interpretation is consistent with the apparent delay between the loss of Gap1 uptake activity and the disappearence of immunoreactive Gap1 polypeptide (Fig. 2). A few examples of permease degradation have been previously described in yeast. For instance, under nitrogen-starvation conditions, turnover of the the maltose permease is much more rapid during catabolism of glucose than ethanol (Lucero *et al.*, 1993). The uracil permease (Fur4p) is degraded under various stress conditions such as the presence of cycloheximide (Volland *et al.*, 1994). Degradation of Fur4p is severely impaired in two mutant strains, *end3* and *end4* (Raths *et al.*, 1993), that are deficient in the internalization step of receptor-mediated endocytosis. Degradation of Fur4p following endocytosis takes place in the vacuole, as Fur4p accumulates in the *pep4* mutant (Volland *et al.*, 1994), a strain that lacks a major vacuolar protease (Woolford *et al.*, 1986). Inositol-mediated inactivation of the inositol permease (Itr1p) is also accompanied by endocytic internalization followed by degradation in the vacuole (Lai *et al.*, 1995). By analogy, it seems likely that NH_4^+-induced degradation of Gap1p is preceded by specific endocytosis of the permease followed by its delivery to intracellular compartments. The probable internalization of Gap1p could be directly responsible for the initial loss of Gap1 uptake activity that occurs when NH_4^+ ions are added to the medium.

Degradation of Gap1 and Fur4 permeases requires normal levels of the Rsp5/Npi1 ubiquitin–protein ligase

The NH_4^+-triggered inactivation and degradation of Gap1p are abolished in the *npi1* mutant strain. Cloning of the *NPI1* gene revealed that it codes for the Rsp5 ubiquitin–protein ligase (E3 enzyme) (Huibregste *et al.*, 1995). Ubiquitin is a 76-amino-acid polypeptide which is covalently conjugated to specific protein substrates. Ubiquitin–conjugate formation requires the combined action of three classes of proteins: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and in some cases additional proteins (E3) referred as to ubiquitin–protein ligases (Jentsch, 1992; Ciechanover, 1994). Only two E3 enzymes have been identified in yeast so far. One is Ubr1p acting through the N-end rule pathway (Bartel *et al.*, 1990). The other is Rsp5p which is similar to human E6-AP and related proteins (Huibregtse *et al.*, 1993; 1995). Scheffner *et al.* (1995) recently showed that formation of a ubiquitin thioester on E6-AP is an intermediate step in E6-AP-mediated ubiquitination. The C-terminal cysteine residue responsible for this ubiquitin thioester formation is conserved in all members of the E6-AP-like family of ubiquitin–protein ligases (Scheffner *et al.*, 1995). The involvment of the Rsp5 ubiquitin–protein ligase in induced degradation of the Gap1 permease could be indirect. For instance, the *npi1* mutant strain could accumulate

proteins preventing NH_4^+-induced degradation of Gap1p. Alternatively, Rsp5p could catalyse direct transfer of ubiquitin to Gap1p, an operation that would be essential for induced degradation of the permease. The latter interpretation is more consistent with the observation that stress-induced degradation of the uracil permease (Fur4p) was also shown to be significantly slowed down in the *npi1* mutant strain. Indeed, consistent with a direct role of ubiquitin in this degradation process, a mutation in a sequence of the Fur4 permease that is very similar to the 'destruction box' required for the ubiquitin-dependent proteolysis of mitotic cyclins (Glotzer *et al.*, 1991) results in protection of Fur4p against degradation (Galan *et al.*, 1994). It is noteworthy that it was recently reported that the Ste6 transporter accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants (Kölling and Hollenberg, 1994).

The major function of ubiquitin conjugation is the targeting of the protein substrate for degradation by the cytosolic proteasome. However, modification of protein targets by ubiquitin has also been implicated in limited proteolysis, post-translational processing, and signalling (Ciechanover, 1994). As was suggested for the yeast Ste6 transport protein (Kölling and Hollenberg, 1994), the putative ubiquitination of Gap1 and Fur4 permeases could direct them into the vacuolar degradation pathway by providing a signal for endocytosis. The role of the vacuole in the degradation of specific ubiquitin-tagged proteins in yeast is supported by the observation that ubiquitin–protein conjugates are found in the vacuole (Simeon *et al.*, 1992). Ubiquitinated protein conjugates were also found in mammalian lysosomes (Laszlo *et al.*, 1990).

Deletion of the chromosomal *NPI1* gene showed that *NPI1* is essential for cell viability, indicating that the function of Npi1p probably extends beyond degradation of Gap1 and Fur4 permeases. The fully viable *npi1* mutant strain exhibited reduced *NPI1* expression as a result of a Ty1 insertion 500 bp upstream from the translation-initiation codon. One interpretation of these data is that Npi1p participates in an essential cellular function that requires a level of Npi1p much lower than for induced degradation of permeases. An alternative possibility is that the major role of Npi1p would be limited to the turnover of permeases (and eventually of other plasma-membrane proteins). For instance, a minimal level of Npi1p such as that present in the *npi1* strain might be sufficient to ensure steady-state turnover of membrane proteins, while simultaneous degradation of an entire subset of permeases, such as those sensitive to NH_4^+ or those sensitive to stress conditions, might require a higher amount of Npi1p no longer available in the viable *npi1* mutant strain. In the complete absence of Npi1p, proteins (including Gap1 and Fur4p) would irreversibly accumulate in the plasma membrane, ultimately leading to cell death. Isolation of a thermosensitive *npi1* mutant strain and identification of other proteins whose turnover is dependent on Npi1p will help in determining why Npi1p is essential for cell viability.

The Npi1/Rsp5 protein is conserved from yeast to humans

The yeast Rsp5/Npi1 protein has at least one homologue in the mouse and in humans. The cellular function of these two proteins is undefined, though the mouse NEDD-4 protein seems to be implicated in the early development of the mouse brain (Kumar *et al.*, 1992). All three proteins contain a C-terminal, ubiquitin–protein-ligase domain similar to E6-AP preceded by three to four copies of a recently reported motif called WW(P). The WW(P) domain has been identified in single or multiple copies in several other proteins of yeast or animal origin, including cytoskeletal or putative signalling proteins. Although the function of the WW(P) domain remains undefined, it seems reasonable to suppose that it mediates interaction with a protein substrate conserved throughout eukaryotes (André and Springael, 1994; Bork and Sudol, 1994; Hofmann and Bucher, 1995). The N-termini of Rsp5/Npi1p and of its human homologue also contain a C_2 domain. Previous studies on this motif showed that it can perform different functions. Some C_2 domains mediate Ca^{2+}-stimulated interaction with phospholipids (Perin *et al.*, 1990; Clark *et al.*, 1991), while one of the two C_2 domains of synaptotagmin I is a high-affinity receptor for clathrin AP-2, indicating a possible role in endocytosis (Zhang *et al.*, 1994). Further experiments will be required to test whether the C_2 domain of Npi1p locates the protein to the plasma or internal membranes, plays a role in Gap1 and Fur4 endocytosis, and/or mediates any other function.

The sequence homology between yeast Rsp5/Npi1p and the two mammalian proteins suggests that all three proteins perform very similar functions. In this respect, it is noteworthy that several cell-surface receptors in higher organisms are subject to ubiquitination (Ciechanover, 1994). Among these, the platelet-derived growth factor (PDGF) receptor β, upon binding of its ligand, is internalized and ultimately delivered to, and degraded in, lysosomes. Ligand binding by the PDGF receptor induces its poly-ubiquitination, while a cytosolic tail-truncated PDGF receptor is resistant to ligand-induced ubiquitination and degradation (Mori *et al.*, 1992). The molecular mechanisms underlying the induced degradation of yeast Gap1 and Fur4 permeases might be similar, at least in part, to this process.

Experimental procedures

Strains, growth conditions and methods

The *S. cerevisiae* strains used in this study are all isogenic

with the wild-type Σ1278b (Béchet *et al.*, 1970) except for the mutations mentioned. These are 23344C (*MATα, ura3*), 23346c (*MATa, ura3*), 27029c (*MATα, ura3, leu2*), 27034b (*MATa, ura3, leu2*), 27038a (*MATa, npi1, ura3*), 25136a (*MATa, npi1, npr1, ura3*), 25696d (*MATa, gap1, ura3*), 25966c (*MATα, gap1, np1, ura3*) and 27039 (*MATα/MATa, ura3/ura3, leu2/leu2, NPI1/npi1Δ (LEU2)*). Cells were grown in a minimal buffered medium (Jacobs *et al.*, 1980) with 3% glucose as the carbon source. Nitrogen sources were added to this medium at the following final concentrations: $(NH_4)_2SO_4$ (10 mM), proline (0.1%), citrulline (0.1%) and γ-aminobutyrate (0.1%). In the Fur4 permease assays, cells were grown in a medium containing 0.7% yeast nitrogen base without amino acids and galactose as a carbon source (Volland *et al.*, 1994). Yeast cells treated with lithium acetate (Ito *et al.*, 1983) were transformed according to Sherman *et al.* (1986). Total RNAs used in the Northern blot experiment were prepared as described in Sherman *et al.* (1986). The *E. coli* strain used was JM109. All procedures for manipulating DNA employed standard methods (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989).

Plasmids

The YCpGAP1 plasmid carries a complete *GAP1* gene (Jauniaux and Grenson, 1990) in the centromere-based vector pFL38 (Bonneaud *et al.*, 1991). The YCpGAP1^{myc} plasmid carries the epitope-tagged version of the *GAP1* gene. The 10-amino-acid epitope derived from human c-*myc* protein (Evan *et al.*, 1985) was inserted between amino acids 62 and 63 of Gap1 (Ljungdahl *et al.*, 1992) by site-directed mutagenesis using the Altered Sites Mutagenesis System (Promega). The *GAP1^{myc}* gene was completely sequenced to verify the accuracy of the construct. The YIpJYS2 plasmid used to isolate the *npi1Δ*::(*LEU2*) deletion strain was constructed by inserting into plasmid pFL34 (Bonneaud *et al.*, 1991) a 2.8 kb DNA fragment containing the *LEU2* gene flanked by the 5′ region (0.6 kb just upstream from the ATG initiation codon) and the 3′ region (0.2 kb just downstream from the stop codon) of the *NPI1* gene. The two latter DNA fragments were obtained by PCR amplification. The high-copy-number plasmid p195gF carrying the *FUR4* gene (Jund *et al.*, 1988) under the control of the *GAL10* promoter has been described (Volland *et al.*,1994).

Cloning of the NPI1 *gene*

S. cerevisiae strain 25136a (*npi1, npr1, ura3*) was transformed with a low-copy-number library representing the total genome of strain Σ1278b (Marini *et al.*, 1994), yielding a total of 76 000 Ura$^+$ transformants. At least two of them presented a Npi1$^+$ Npr1$^-$ phenotype (see the text). DNA was isolated from these clones and used to transform *Escherichia coli*. The recovered plasmids all contained the same 7 kb genomic DNA fragment. A 5.6 kb subclone constructed in the centromere-based vector pFL38 (plasmid YCpJYS2) complemented the phenotypes linked to the *npi1* mutation. The cloned *NPI1* gene was sequenced using a series of oligonucleotide primers deduced from the sequence of chromosome V (accession number U18916).

Construction of the npi1Δ *strain*

The *npi1Δ* mutant strain harbouring a chromosomal deletion of *NPI1* was constructed by the one-step gene-replacement method (Rothstein, 1991). The 3.6 kb DNA fragment carried by plasmid YIpJYS2 was isolated and used to transform the diploid homozygotous (*ura3, leu2*) strain. Twenty Leu$^+$ transformants were analysed by PCR amplification. For this, we used two oligonucleotide primers which hybridize to sequences flanking the *NPI1* DNA regions used to direct integration of the *LEU2* marker. Among the twenty diploid clones tested, two exhibited two amplified DNA fragments with the expected sizes of 3.2 kb and 3.6 kb. A Southern blot experiment using a *NPI1* DNA probe confirmed that these two clones had single-copy replacement of *NPI1* by *LEU2*.

Permease assays

Citrulline is a specific substrate of general amino acid permease. The method of measuring the initial rates of incorporation of 0.1 mM [^{14}C]-citrulline (Amersham) was essentially that of Grenson *et al.* (1966). A 5 ml sample of cells that had reached the state of balanced growth (Wiame *et al.*, 1985) was placed into a pre-warmed, rotating flask containing the radiolabelled amino acid. Samples (1 ml) were removed at 0.5, 1, 1.5 and 2 min, filtered through a nitrocellulose membrane (Millipore, 0.45 μm) and immediately washed three times with 3 ml cold water. The filters were dried under infrared lights and counted for radioactivity. Uracil uptake was assayed as previously described (Volland *et al.*, 1994).

Yeast cell extracts and immunoblotting

Cell extracts were prepared as previously described (Volland *et al.*, 1994). Proteins from 0.2 ml (Fur4p) or 0.8 ml (Gap1p) culture were loaded onto each lane of a 10% SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and treated with monoclonal anti-*c-myc* IgG_1 (9E10, Santa Cruz Biotechnology Inc.) (Gap1 permease) or with a rabbit antiserum against a C-terminal peptide (Fur4 permease) (Volland *et al.*, 1992; 1994). Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse (Gap1p) or anti-rabbit (Fur4p) IgG secondary antibody followed by chemiluminescence (ECL, Amersham).

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