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The Npr1 Kinase Controls Biosynthetic and Endocytic Sorting of the Yeast Gap1 Permease*

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trogen regulation. In cells growing on proline or urea as the sole nitrogen source, newly synthesized Gap1 is delivered to the plasma membrane, where it accumulates. Upon addition of NH_4^+ , a preferential nitrogen source, Gap1 is endocytosed and targeted to the vacuole, where it is degraded. This down-regulation requires ubiquitination of the permease, and this ubiquitination is dependent on the essential Npi1/Rsp5 ubiquitin ligase. In this study, we investigated the role of the Npr1 kinase in the regulation of Gap1 trafficking. We show that Npr1 is required for stabilization of Gap1 at the plasma membrane: when an *npr1^{ts}* mutant growing on proline is shifted to the restrictive temperature, Gap1 down-regulation is triggered, as it is when NH₄⁺ is added to wildtype cells. The fate of newly synthesized Gap1 en route to the plasma membrane is also under Npr1 control: in an $npr1\Delta$ mutant, neosynthesized Gap1 is sorted from the Golgi to the vacuole without passing via the plasma membrane. Similar direct sorting of neosynthesized Gap1 to the vacuole was observed in wild-type cells grown on NH_4^+ . Finally, Gap1 is phosphorylated in NPR1 cells, but this phosphorylation is not strictly dependent on Npr1. Our results show that Npr1 kinase plays a central role in the physiological control of Gap1 trafficking and that this control is exerted not only on Gap1 present at the plasma membrane but also on Gap1 late in the secretory pathway. Npr1 belongs to a subgroup of protein kinases, some of which are reported to exert a positive control on the activity of other permeases. We propose that these kinases also function as regulators of permease trafficking.

Membrane trafficking of the general amino acid permease (Gap1) of Saccharomyces cerevisiae is under ni-

The general amino acid permease (Gap1) of *Saccharomyces cerevisiae* provides an attractive system for genetically dissecting mechanisms that regulate trafficking of a membrane protein according to physiological constraints. In cells growing on proline or urea as the sole nitrogen source, the *GAP1* gene is actively transcribed. Transcription involves the GATA family Gln3 and Nil1/Gat1 transcription factors (1, 2). The newly synthesized Gap1 accumulates at the plasma membrane in an

active and stable form. Secretion of Gap1 to the plasma membrane requires Shr3/Apf1, a nonessential membrane protein of the endoplasmic reticulum (3). *In vitro* studies have shown that Shr3 transiently interacts with Gap1 and several COPII coatomer components to promote packaging of the permease into endoplasmic reticulum-derived COPII transport vesicles (4-6). Sorting of Gap1 from the Golgi to the plasma membrane requires Sec13 (7), a protein originally identified as a component of the COPII vesicle coat (8-11). The Lst4, Lst7, and Lst8 proteins of unknown biochemical function are also involved in sorting Gap1 from the Golgi to the cell surface (12), whereas the last step of Gap1 secretion is dependent on Sec6 (7), a component of the exocyst (13).

Trafficking of Gap1 is subject to nitrogen regulation. When cells are grown on glutamate, newly synthesized Gap1 is sorted directly from the Golgi to the vacuole, instead of to the plasma membrane (7). Pep12, a t-SNARE¹ involved in the fusion of Golgi-derived vesicles with the prevacuolar compartment (14), is required for this direct sorting of Gap1 to the vacuole (7). Gap1 permease at the plasma membrane is also subject to nitrogen regulation: the addition of NH₄⁺ to proline- or ureagrown cells triggers progressive and complete loss of Gap1 activity (15) and repression of GAP1 gene expression (16). The loss of Gap1 activity is the result of Gap1 internalization and subsequent targeting to the vacuole for degradation in a process called down-regulation of Gap1 (17, 18). Like that of many plasma membrane proteins (19, 20), down-regulation of Gap1 requires its conjugation to ubiquitin (18, 21). Npi1/Rsp5, a HECT (homologous to the E6-associated protein Carboxyl Terminus)-type ubiquitin ligase essential to cell viability (17, 22), is required for this ubiquitination (18).

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NPR1 is another gene involved in posttranscriptional control of Gap1. In *npr1* mutants growing on proline or urea, the amount of GAP1 transcripts is unaltered (23), but Gap1 is inactive (24, 25). Other NH₄⁺-sensitive permeases, including the proline permease Put4 (23), the ureidosuccinate and allantoate permease Dal5 (26), and the inducible γ -aminobutyratespecific permease Uga4 (27), are also dependent on Npr1 to be active. Sequencing of the cloned NPR1 gene showed that it encodes a kinase homologue with a serine-rich N terminus (23, 28). Phylogenetic studies have shown that Npr1 belongs to a fungus-specific subfamily of protein kinases (29). Some members of this subfamily (kinases Ptk1/Stk1 and Stk2) are essential determinants of polyamine transport (30); others (kinases Sat4/Hal4 and Hal5) are essential determinants of Trk1-Trk2 potassium transporter activity (31). The molecular mechanisms through which these protein kinases affect the activity of transport proteins remain unclear. Previous studies have

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¹ The abbreviations used are: t-SNARE, target-soluble *N*-ethylmaleimide attachment protein receptor; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; GFP, green fluorescent protein.

	TABLE		1	
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Strain	Genotype	Reference
23344c	MATa ura3	Laboratory collection
21994b	$MAT \alpha \ npr1 \ ura3$	(28)
30788a	$MAT \alpha \ npr1::kanMX2 \ ura3$	This study
27038a	$MAT \alpha npi 1 ura 3$	(15)
30788d	MATα npi1 npr1::kanMX2 ura3	This study
MG2397	MAT a npr1	(61)
33033c	$MAT \alpha \ npi1 \ npr1 \ ura3$	This study
33138c	$MAT \alpha \ npr1 \ pep4$:: $kanMX2 \ ura3$	This study
33144b	MATα pep4::kanMX2 ura3	This study
33144c	MATα npr1::kanMX2 pep4::kanMX2 ura3	This study
$NY13^{a}$	$MAT\alpha$ gal2 $ura3-52$	(62)
$NY279^{a}$	$MAT \alpha \ act 1-1 \ gal2 \ ura3-52$	(62)
$JOD81^a$	MATα gal2 npr1::kanMX2 ura3–52	This study
$JOD121^a$	$MAT\alpha \ act 1-1 \ gal2 \ npr 1 :: kan MX2 \ ura 3-52$	This study
$NY28^{a}$	$MAT\alpha$ gal2 sec4–8 ura3–52	(50)
$JOD233^{a}$	MATα gal2 npr1::kanMX2 sec4–8 ura3–52	This study
33125a	$MAT \alpha \ pep 12 :: kan MX2 \ ura3$	This study
33127c	MATα npr1::kanMX2 pep12::kanMX2 ura3	This study
34288b	MATα GAP1-GFP-kanMX2 ura3	This study
DJOD1	MAT lpha gap1:: $kanMX2 ura3/MAT lpha gap1$:: $kanMX2 ura3$	This study
DJOD2	MATα gap1::kanMX2 npr1::kanMX2 ura3/MATα gap1::kanMX2 npr1::kanMX2 ura3	This study
DJOD3	MATα gap1::kanMX2 npi1 ura3/MATα gap1::kanMX2 npi1 ura3	This study
DJOD4	MATα gap1::kanMX2 npr1::kanMX2 npi1 ura3/MATα gap1::kanMX2 npr1::kanMX2 npi1 ura3	This study
33191b	MATα kanMX2-GAL-GAP1 ura3	This study
33192c	MATα kanMX2-GAL-GAP1 npr1∷kanMX2 ura3	This study
33308c	MATα kanMX2-GAL-GAP1 pep12∷kanMX2 ura3	This study
33307a	MAT α kanMX2-GAL-GAP1 npr1::kanMX2 pep12::kanMX2 ura3	This study
33201b	MAT_{α} kan $MX2$ - GAL - $GAP1$ $npi1$ $ura3$	This study
33191a	MATlpha kan $MX2$ - GAL - $GAP1$ npi1 npr1::kan $MX2$ ura3	This study

^a These strains derive from strain NY13.

shown that Npr1 inactivation in proline-grown cells results in progressive loss of Gap1 activity, a response similar to that observed after the addition of NH_4^+ to wild-type cells (15, 25). Moreover, mutations inhibiting Gap1 down-regulation, which include *npi1* and *gap1^{pgr}*, also suppress the negative effect of the npr1 mutation on Gap1 activity (15, 25, 32). These data are consistent with a role of Npr1 in the control of Gap1 trafficking. Recently, it was shown that Npr1 phosphorylation is regulated by nitrogen through the Tor signaling pathway: nitrogen starvation or growth on proline results in Npr1 dephosphorylation, whereas Npr1 undergoes Tor-dependent phosphorylation in cells grown on NH_4^+ medium or rich medium (33). Under starvation conditions, *i.e.* conditions of high Npr1-supported Gap1 activity (25), Npr1 also promotes Npi1/Rsp5-dependent destabilization of Tat2, a tryptophan permease that is also regulated by nitrogen but inversely compared with Gap1 (34).

We show here that the Npr1 kinase plays a central role in nitrogen-regulated trafficking of the Gap1 permease. Like the Sec13 and Lst proteins, Npr1 is required for transport of neosynthesized Gap1 from the Golgi to the plasma membrane. In $npr1\Delta$ mutants, neosynthesized Gap1 is directly sorted from the Golgi to the vacuole, bypassing the plasma membrane. Npr1 is also required to maintain Gap1 at the plasma membrane because inactivation of Npr1 triggers endocytosis of Gap1, followed by its degradation in the vacuole. A model involving Npr1 in the transport of both neosynthesized and constitutively internalized Gap1 from an internal compartment to the plasma membrane is discussed.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Plasmids—All S. cerevisiae strains (Table I) used in this study are isogenic with $\Sigma 1278b$ (35) or NY13 (36). Cells were grown in minimal buffered medium (37) with 3% glucose as the carbon source, except when indicated otherwise. In steady-state experiments, cells were grown on proline (10 mM) as the sole nitrogen source. In Gap1 neosynthesis experiments, cells were exponentially grown on glutamine (5 mM) and transferred to proline medium to relieve *GAP1* repression. Temperature shifts were performed by filtering cells growing at permissive temperature and resuspending them in

proline medium preheated to the restrictive temperature. Plasmids pGST-NGap1 and FGST05.1, used to produce GST fused to the N terminus of Gap1 (amino acids 1-90) or to the C terminus of Pma1 (amino acids 843-918), respectively, were obtained by PCR amplification of the corresponding gene fragments using total DNA from strain Σ 1278b as template and primers GST1 and GST2 for GAP1 and primers GST3 and GST4 for PMA1 (Table II). The amplified DNA fragments flanked by BamHI (5' end) and XhoI (3' end) restriction sites were cloned into plasmid pGEX-5X-3 (Amersham Pharmacia Biotech). The plasmid constructions were sequenced to verify in-frame fusion to GST and the absence of mutations in the amplified fragments. To construct plasmid YCpCJ025 bearing the Gal1-GAP1-GFP fusion gene, the GAP1 open reading frame was first amplified by PCR using primers 5GAL1-GAP1 and 3GAL1-GAP1 (Table II) and plasmid YCpGAP1 as template. The amplified DNA fragment was then cloned into the BamHI-linearized pRS416-GAL plasmid by recombination in yeast to generate plasmid YCpCJ004. A DNA fragment containing GFP was then amplified by PCR using primers 5GALGAP1GFP and 3GALGAP1GFP2 (Table II) and total DNA of strain 34288b as template. The amplified fragment was then cloned into HindIII-linearized YCpCJ004 by recombination in yeast to generate YCpCJ025. Isolation of the GAL1-GAP1-GFP constructs was performed by comparing the fluorescence of URA3⁺ clones grown on proline glucose or proline galactose media.

Permease Assays—Gap1 permease activity was determined by measuring incorporation of 20 μ M [¹⁴C]citrulline as described by Grenson (38). To avoid competitive inhibition of citrulline transport by glutamine, cells grown on glutamine medium were filtered, washed, and transferred to preheated proline medium just before the transport assay. The permease was inactivated by adding preheated (NH₄)₂SO₄ to the culture (final concentration, 10 mM).

Generation of Antibodies—To raise polyclonal antibodies against the N-terminal region of Gap1 and the C-terminal region of Pma1, the GST-Gap1 and GST-Pma1 fusion proteins were produced in RKB304 bacteria, and their synthesis was induced for 3 h with 1 mM isopropyl-1-thio- β -D-galactopyranoside. The fusion proteins were then purified from lysates by means of agarose beads linked to glutathione (Amersham Pharmacia Biotech). The specificity of the rabbit antisera was tested by comparing the signal pattern obtained in a wild-type versus a gap1 Δ strain or a $pma1\Delta$ strain expressing a plant H⁺-ATPase (39) complementing the $pma1\Delta$ mutation.

Yeast Cell Extracts and Immunoblotting—Crude cell extracts (17) and membrane-enriched preparations (18) were prepared as described previously. In Western blot experiments, the protein concentration was

assessed by densitometry of the Pma1 signal (ImageMaster1D; Amersham Pharmacia Biotech). Equal quantities of protein were loaded on an 8% SDS-polyacrylamide gel in a Tricine system (40). To detect the ubiquitinated forms of Gap1, the extracts were loaded on a 12% gel in Laemmli's system (41). After transfer to a nitrocellulose membrane (Schleicher & Schüll), the proteins were probed with polyclonal antibodies raised against Gap1 (1:10,000) or Pma1 (1:1000). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech) followed by enhanced chemiluminescence (Roche Molecular Biochemicals).

Alkaline Phosphatase Treatment of Protein Extracts—Approximately 10^{8} cells were resuspended in lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) and vortexed for 10 min at 4 °C. The supernatants were transferred to fresh tubes and centrifuged at $1000 \times g$ for 3 min to eliminate cell debris. The supernatant was then centrifuged in fresh tubes at $21,000 \times g$ for 40 min. Pellets were resuspended in 200 μ l of lysis buffer containing 5 M urea, incubated at 0 °C for 30 min, and centrifuged at $21,000 \times g$ for 40 min. The pellets were resuspended in alkaline phosphatase buffer with 0.2% SDS. 20 units of alkaline phosphatase was added to the extracts, which were then incubated for 2 h at 37 °C. Samples were finally subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Genomic Insertions—The pep12 Δ and the npr1 Δ null mutations were constructed by the PCR-based gene deletion method (42). Plasmid pFA6a-kanMX2 served as template to generate DNA fragment npr1::kanMX2 with primers DEL1 and DEL2 and DNA fragment pep12::kanMX2 with primers DEL3 and DEL4 (Table II). Plasmid pFA6a-kanMX6-PGAL1 (43) served to generate the GAL1-GAP1 DNA fragment with primers GAL1 and GAL2. Plasmid pGA5-GFP(S65T)kanMX6 (44) served as template for C-terminal GFP tagging of Gap1 by amplification of a DNA fragment with primers GFP1 and GFP2 (Table II). Strains were transformed with the PCR fragments by the lithium method (45), as described previously (46). Transformants were selected on complete medium containing 200 μ g/ml G418 (Geneticin; Life Technologies, Inc.).

Fluorescence Microscopy—Living cells were fixed on a thin layer of agarose and examined with a Nikon E600 epifluorescence microscope with an HQ-FITC-BP filter cube (Chroma) for blue excitation. Pictures were collected with a SONY DXC-9100P camera and processed with Adobe Photoshop for display.

RESULTS

Role of the Npr1 Kinase in the Phosphorylation of Gap1—The Gap1 permease is most active in cells growing on media containing a poor nitrogen source (proline, urea, low NH_4^+), and the Npr1 kinase is essential to this activity (25). To determine whether Npr1 affects phosphorylation of Gap1, total protein extracts were prepared from the wild-type and *npr1* mutant strains grown on proline medium. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis, and the gels were probed with polyclonal antibodies raised against the N terminus of Gap1 (see "Experimental Procedures") (Fig. 1A). Less Gap1 was detected in the *npr1* mutants than in the wild-type. Furthermore, two distinct bands were clearly detected in the $npr1\Delta$ extract: a minor upper band migrating like the main Gap1 signal detected in the wild-type extract, and a major, faster-migrating lower band. To test whether phosphorylation could account for the slower migration of Gap1 in the wild-type, total protein extracts were treated with alkaline phosphatase (Fig. 1B). This resulted in a decrease in the apparent molecular weight of Gap1. The underphosphorylated form of the permease migrated like the major band observed in the $npr1\Delta$ extract, with or without treatment with alkaline phosphatase (Fig. 1B). Hence, the Npr1 kinase is required for normal accumulation and normal phosphorylation of Gap1. However, Npr1 does not seem to be directly involved in Gap1 phosphorylation: in the $npr1\Delta$ npi1 double mutant, which is also defective in the Npi1/Rsp5 ubiquitin protein ligase, Gap1 is fully active and migrates as a high molecular weight form that is a phosphorylated form of the permease, as judged by the effect of alkaline phosphatase treatment (Fig. 1B). These results show that phosphorylation of Gap1 is not strictly dependent on Npr1.

$\mathrm{TABLE}\ \Pi$ Oligonucleotides used in this study	Primer sequence	5'-GCG GAT CCG CAT GAG TAA TAC TTC T-3' 5'-GCG GAT CCG CAT GAG TAC TTC T-3' 5'-GCG CTC GAG CCT AGT GGT TCA A-3' 5'-CGC CTC GAG GGT TCC CTT TTC GTG TTG AGT AGA-3' 5'-CGC CTC GAG GGT TTC CTT TTC GTG TTG AGT AGA-3' 5'-TAG TAG GGA TTA GTC AGT GGT GTA AGT AGA-3' 5'-TAG TAG GGA TTA GTC AGT GGT GTA CTT ATT AGG GGC CGC CAG TAG GCC AGT AGT GG-3' 5'-CGC CAG AGT AGT GGA GGT CAA TCT ATT TAG GCT TCT ATA GCG GGC CGC CAG TAG GCC ACT AGT GGA TCT G-3' 5'-CGC CAG AGT ATA TCA TCT ATT TAG GGT TCT ATA GCG GGC CGC CAG CTG AAG CTT-3' 5'-CGC CAG AGT ATA TCA TCT ATT AGT GGC AGT AGT GGG CCG GGC CGC CAG TAG GCC ACT AGT GG-3' 5'-CGC CAG AGT ATA TCT CTC GTA CGA AGT GCA AAA CAA GGC GGC CGC CAG CTG AGG CTT 3' 5'-CGC CAG ATT ATT CTT CTC GTA CGA AGT AGT AGT AGT CAA TTT GGA TCT GGA TTT 7-3' 5'-CGC CAA AAA GCC CAT GAA GTT GTA AGT AGT AGT AGT CGA GGC CGC TTA AAC-3' 5'-CAC AAA GCC AAA GTT GTA TTA CGA AGT AGT AGT AGG AGC TGG TGG TGG TGG-3' 5'-CAC AAA GCC AAA GTT GTA ATT CGG AGT GGA GGC GGG TGG TGG TGG-3' 5'-CAC AAA GCC AAG ATG GTA TAG CGA ATT CTG GGT TCG GGG TGG TGG TGG-3' 5'-CAC AAA GCC AAG ATG GTA TAG CGA ATT CTG GGT TGG AGG TGG TGG TGG TGG-3' 5'-CAC AAA GCC AAG ATG GTA TGG GAA TTT CTG GGT TGG AGG TGG TGG TGG TGG-3' 5'-CAC AAA GCC AAG ATG GTA TAG TGG AAT TGG AGG AGG AGG
	Purpose	Gap1-GST fusion Gap1-GST fusion Pma1-GST fusion Pma1-GST fusion NPR1 deletion NPR1 deletion PEP12 deletion GAL1-GAP1 fusion GAL1-GAP1 fusion GAL1-GAP1 fusion Gap1-GFP fusion Gap1-GFP fusion
	Primer	GST1 GST2 GST2 GST3 GST4 GST4 GST4 DEL1 DEL2 DEL3 DEL3 CAL1 GAL1 GAL1 GAL1 GFP1 GFP1



FIG. 1. Reduced accumulation and phosphorylation of Gap1 permease in *npr1* mutant cells. *A*, wild-type (23344c), *npr1-1* (21994b), and *npr1*Δ (33788a) cells were grown on proline as the sole nitrogen source. Total protein extracts were prepared, resolved by electrophoresis, and analyzed by immunoblotting. *B*, wild-type (23344c), *npi1* (27038a), *npr1*Δ (30788a), and *npr1*Δ *npi1*(30788d) cells were grown on proline medium. Gap1 activities (nmol.min⁻¹.mg protein⁻¹) were assayed by measuring uptake of [¹⁴C]citrulline (0.02 mM). Membrane-enriched extracts were prepared and incubated for 2 h at 37 °C in the absence (-) or presence (+) of alkaline phosphatase. The extracts were then resolved by electrophoresis and analyzed by immunoblotting. For the *npr1*Δ mutant, a 10-fold higher amount of protein extracts as loaded to obtain a signal as intense as those for the other strains.

Loss of Npr1 Function Triggers Endocvtosis and Vacuolar Degradation of Gap1—To further investigate the possible role of Npr1 in regulation of Gap1 trafficking, we examined the influence of a thermosensitive *npr1* mutation on Gap1 preaccumulated at the plasma membrane. In the *npr1^{ts}* strain grown at 29 °C on proline medium, Gap1 was as active as in wild-type cells (16.7 *versus* 16.5 nmol.min⁻¹.mg⁻¹ protein, respectively), suggesting a normal level of Gap1 at the plasma membrane. Shifting cells to the restrictive temperature (35 °C) induced progressive and complete loss of Gap1 activity (Fig. 2A). Total cellular extracts were prepared from $npr1^{ts}$ and wild-type cells and resolved by SDS-polyacrylamide gel electrophoresis. The immunoblot shows that loss of Gap1 activity induced by transferring npr1^{ts} cells to 35 °C is accompanied by destabilization of the permease (Fig. 2B). As observed in npr1 mutants grown on proline (Fig. 1A), a small amount of dephosphorylated Gap1 persisted in the $npr1^{ts}$ strain 3 h after the temperature shift (Fig. 2B). The loss of Gap1 activity and the degradation of Gap1 induced by Npr1 inactivation are likely due to endocytosis and vacuolar degradation of the permease, as seen in wild-type cells after the addition of NH_4^+ (17, 18). To confirm the vacuolar location of Gap1 degradation upon Npr1 inactivation, we deleted the PEP4 gene, which is required for enzymatic activation of vacuolar proteases (47), in the $npr1^{ts}$ strain. Shifting $npr1^{ts}$ $pep4\Delta$ cells to 35 °C induced a rapid loss of Gap1 activity (Fig. 2A), but the permease remained stable (Fig. 2B). These results show that the continuous presence of a functional Npr1 protein is required to maintain Gap1 at the plasma membrane: upon loss of the Npr1 function, Gap1 undergoes endocytosis and targeting to the vacuole, where it is degraded.

Ubiquitination of Gap1 Is Essential to Down-regulation Induced by Loss of Npr1 Function—Down-regulation of Gap1 induced by the addition of NH_4^+ to proline-grown cells requires ubiquitination of Gap1; this modification is dependent on Npi1/ Rsp5, a ubiquitin ligase essential to viability (17, 18). To test the role of Npi1/Rsp5 in Gap1 down-regulation induced by the



FIG. 2. Loss of Npr1 function triggers down-regulation of Gap1. A, wild-type (23344c; **D**), $npr1^{ts}$ (MG2397; \triangle), npi1 $npr1^{ts}$ (33033c; \bigtriangledown), and $npr1^{ts}$ $pep4\Delta$ (33138c; **A**) strains were grown on proline medium at 29 °C. At time 0, the cells were shifted to 35 °C. Gap1 activity (nmol.min⁻¹.ml culture⁻¹) was assayed by measuring incorporation of [¹⁴C]citrulline (0.02 mM) before (t = 0) and at various times after the temperature shift. B, immunoblot of Gap1 present in crude extracts prepared from samples of the same cultures collected before (t = 0) and at intervals after the temperature shift.

loss of Npr1 function, we used an $npr1^{ts}$ strain with an additional npi1 mutation. The latter mutation causes a severe reduction of the amount of Npi1/Rsp5, thus preventing ubiquitination of Gap1 without altering cell viability (18). The $npr1^{ts}$ npi1 strain was grown on proline medium at 29 °C and then shifted to 35 °C. Both Gap1 activity (Fig. 2A) and the intensity of the immunodetected Gap1 signal (Fig. 2B) remained as stable as in the wild-type. This shows that Npi1/ Rsp5 plays a major role in Gap1 down-regulation induced by Npr1 inactivation.

To determine whether loss of Npr1 function alters the ubiquitination state of Gap1, we used cell samples from the same cultures to prepare membrane-enriched extracts. In these extracts, the immunodetected Gap1 signal migrates as two major bands (fused into a single band in some experiments) plus one or two minor bands of higher apparent molecular weight (also often fused into a single band) (Fig. 3). These minor upper bands correspond to Gap1 conjugated to one or two molecules of ubiquitin (21). Accordingly, they were visible in the wild-type and in the *npr1^{ts}* mutant grown at 29 °C, but not in the *npi1* or $npr1^{ts}$ npi1 strain (Fig. 3, time 0). In the first minutes after the cells were transferred to the restrictive temperature, the intensity of the minor upper bands markedly increased in the *npr1*^{ts} strain. Furthermore, two additional bands of still higher apparent molecular weight became clearly visible. The latter bands are reminiscent of those corresponding to polyubiquitinated Gap1 forms, which appear in the wild-type after the addition of NH_4^+ (21). No upper bands were visible when the npr1^{ts} npi1 strain was shifted to 35 °C. These results show that loss of Npr1 function induces a drastic increase in the ubiq-

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FIG. 3. Loss of Npr1 function triggers enhanced ubiquitination of Gap1. Wild-type (23344c), $npr1^{ts}$ (MG2397), $npr1^{ts}$ npi1 (33033c), and npi1 (27038a) strains were grown on proline medium at 29 °C. At time 0, the cells were shifted to 35 °C. Cell samples were collected before (t = 0) and at intervals after the temperature shift. Membrane-enriched extracts were prepared, resolved by electrophoresis, and analyzed by immunoblotting.

uitin-conjugated Gap1 fraction. Ubiquitination is essential to Gap1 down-regulation because the permease remains active and stable in the $npr1^{ts}$ npi1 mutant shifted to 35 °C (Fig. 2).

Newly Synthesized Gap1 Is Unstable in the $npr1\Delta$ Mutant— Gap1 regulation according to the nitrogen source concerns not only the permease accumulated at the plasma membrane (17, 18) but also the permease en route to the plasma membrane (7, 12). In cells grown on a poor nitrogen source like urea, neosynthesized Gap1 is transported from the Golgi to the plasma membrane, but in cells grown on glutamate, it is transported from the Golgi directly to the vacuole (7). To test whether Npr1 controls sorting of neosynthesized Gap1, cells were grown on glutamine medium to repress GAP1 gene transcription and then transferred to proline medium to relieve repression. In the wild-type strain, this led to rapid accumulation of newly synthesized Gap1 and a concomitant increase in Gap1 activity (Fig. 4). In $npr1\Delta$ cells shifted from glutamine to proline, Gap1 remained inactive 3 h after transfer (Fig. 4). A Gap1 signal became visible on immunoblots but, as illustrated above for the npr1 strains grown under steady-state conditions on proline (Fig. 1A), it was much less intense than the signal detected in the wild-type, and the corresponding apparent molecular weight was lower. When an $npr1\Delta$ $pep4\Delta$ strain was shifted from glutamine to proline medium, Gap1 accumulation was similar to that observed in the wild-type, but the permease remained inactive (Fig. 4). These results show that in the $npr1\Delta$ mutant, newly synthesized Gap1 does not accumulate at the plasma membrane as it does in the wild-type. Rather, the permease is unstable and is degraded in the vacuole. Note that Gap1 was also detected in the $npr1\Delta$ $pep4\Delta$ strain grown on glutamine medium (Fig. 4). A weak intensity Gap1 signal was also detected in glutamine-grown $npr1\Delta$ cells upon blot overexposure (data not shown). We interpret this effect as follows: the $npr1\Delta$ mutation, which affects the activity of multiple nitrogen permeases, also reduces the uptake of glutamine; this leads to partial relief from repression of GAP1 gene transcription. The resulting low amount of synthesized Gap1 is located mainly in the vacuole (data not shown) and tends to accumulate in the $npr1\Delta$ pep4 Δ deficient in vacuolar proteolysis.

In the npr1 Δ Mutant, Newly Synthesized Gap1 Is Sorted to the Vacuole, Bypassing the Plasma Membrane—The Gap1 permease synthesized in the npr1 Δ strain might either be delivered to the plasma membrane and then immediately endocytosed and targeted to the vacuole for degradation or, as observed when glutamate is available, be transported directly from the Golgi to the vacuole, bypassing the plasma membrane



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FIG. 4. Newly synthesized Gap1 is unstable in the *npr1*Δ mutant. Wild-type (23344c), *npr1*Δ (30788a), *pep4*Δ (33144b), and *npr1*Δ *pep4*Δ (33144c) strains were grown on glutamine medium at 29 °C. The cells were then transferred to proline medium. Top panel, Gap1 activity (nmol.min⁻¹.mg protein⁻¹) was assayed in cells growing on glutamine (*Gln*) and 3 h after cells were transferred to proline medium (*Pro*) by measuring incorporation of [¹⁴C]citrulline (0.02 mM). Bottom panel, immunoblot of Gap1 present in crude extracts prepared from cell samples taken from the same cultures before (*Gln*) and 3 h after transfer to proline medium (*Pro*). To make sure that equal amounts of protein were loaded, Pma1 was also immunodetected.

(7, 12). To explore these two possibilities, we first tested whether an endocytosis deficiency introduced into an $npr1\Delta$ mutant leads to accumulation of Gap1 at the plasma membrane. For this, we deleted the NPR1 gene in the thermosensitive act1-1 mutant defective in endocytosis (48). NH⁺₄-induced endocytosis of Gap1 is defective in this mutant even if cells are grown at 29 °C (18). Hence, cells of the act1-1 mutant and isogenic wild-type strain (NY13) lacking or not lacking the NPR1 gene were first grown on glutamine and then transferred to proline. As expected, the shift to proline led to the development of high Gap1 activity in the wild-type and act1-1 strains but not in the $npr1\Delta$ strain (Fig. 5A). In the $npr1\Delta$ act1-1 double mutant, the activity of Gap1 remained as low as that in the $npr1\Delta$ strain (Fig. 5A). This suggests that Gap1 does not accumulate at the plasma membrane of $npr1\Delta$ cells even if endocytosis is defective. To ascertain that the act1-1 strain grown under these conditions (29 °C, shift for 3 h from glutamine to proline) is indeed defective in endocytosis of Gap1, NH_4^+ was added to proline medium 3 h after the shift (Fig. 5*B*). As expected, Gap1 activity was rapidly lost in the wild-type; in the act1-1 strain, Gap1 was also inactivated, but it was inactivated much more slowly. We conclude that in $npr1\Delta$ cells, newly synthesized Gap1 fails to accumulate at the plasma membrane even if the cells are largely defective in endocytosis. These data are consistent with the view that neosynthesized Gap1 is sorted directly from the secretory pathway to the vacuole, bypassing the plasma membrane.

To further assess the validity of this conclusion, we tested whether blocking exocytosis in the $npr1\Delta$ strain protects the permease against destabilization, as would be expected if Gap1 travels first to the plasma membrane before traveling to the vacuole to be degraded. For this test, we deleted the *NPR1* gene in the *sec4-8* mutant strain and in the isogenic wild-type (NY13) (49). The *SEC4* gene encodes a small GTPase required for fusion of Golgi-derived vesicles with the plasma membrane (50). The cells were first grown on glutamine at 24 °C and then transferred to proline at either 24 °C or 37 °C (Fig. 5*C*). As

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FIG. 5. Destabilization of newly synthesized Gap1 in the npr1 mutant does not require normal endocytosis and exocytosis functions. A, wild-type (NY13), npr1A (JOD81), act1-1 (NY279), and act1-1 npr1 Δ (JOD121) strains were grown on glutamine medium at 29 °C and then transferred to proline medium. Gap1 activity (nmol.min⁻ ¹.mg protein⁻¹) was assayed in cells growing on glutamine (Gln) and 3 h after transfer to proline medium (Pro) by measuring incorporation of [14C]citrulline (0.02 mM). B, 3 h after shifting of the wild-type (NY13; ■) and *act1-1* (NY279; □) strains to proline medium, NH_4^+ was added to the medium (final concentration, 20 mM), and Gap1 activity $(nmol.min^{-1}.mg \text{ protein}^{-1})$ was measured at intervals. C, wildtype (NY13), sec4-8 (NY28), npr1Δ (JOD81), and npr1Δ sec4-8 (JOD233) strains were grown on glutamine medium at 25 °C. The cells were then transferred to proline medium at either 25 °C or 37 °C. Top panel, Gap1 activity (nmol.min⁻¹.mg protein⁻¹) was assayed in cells growing on glutamine (Gln) and 3 h after transfer to proline medium (Pro) at either °C or 37 °C. Bottom panel, immunoblot of Gap1 present in crude extracts prepared from cell samples of the same cultures before (Gln)and 3 h after transfer to proline medium (Pro). To make sure equal amounts of protein were loaded, Pma1 was also immunodetected.



FIG. 6. Newly synthesized Gap1 is stable in an *npr1*Δ *pep12*Δ double mutant. Wild-type (23344c), *npr1*Δ (30788a), *pep12*Δ (33125a), and *npr1*Δ *pep12*Δ (33127c) strains were grown on glutamine medium (Gln) at 29 °C and then transferred to proline medium (Pro). A, Gap1 activity (nmol.min⁻¹.mg protein⁻¹) was assayed in cells growing on glutamine (Gln) and 3 h after transfer to proline medium (Pro) by measuring incorporation of [¹⁴C]citrulline (0.02 mM). B, immunoblot of Gap1 present in crude extracts prepared from cell samples of the same cultures before (Gln) and 3 h after transfer to proline medium (Pro). To make sure that equal amounts of proteins were loaded, Pma1 was also immunodetected.

expected, shifting the wild-type strain to proline at either temperature led to the development of high Gap1 activity and to a high intensity Gap1 signal. It is noteworthy that in the three other strains, *i.e.* sec4-8, $npr1\Delta$, and $npr1\Delta$ sec4-8, a Gap1 signal was already present on glutamine. As suggested above, this is likely due to lower glutamine uptake in these strains than in the wild-type. This effect was in fact more pronounced in NY13-derived strains. Shifting the sec4-8 strain from glutamine to proline at 24 °C led to the development of high Gap1 activity, with a concomitant increase in the immunodetected Gap1 signal (Fig. 5C). Shifting cells from the same culture to proline at 37 °C also led to a strong intensification of the Gap1 signal, but this neosynthesis was not accompanied by an increase in Gap1 activity (Fig. 5C). This confirms that exocytosis of newly synthesized Gap1 requires Sec4. In the $npr1\Delta$ and $npr1\Delta$ sec4-8 mutants, shifting from glutamine (24 °C) to proline (37 °C) did not lead to the high accumulation of Gap1 observed in the $sec4^{ts}$ strain (Fig. 5C). Hence, with regard to Gap1 accumulation, the phenotype of the $npr1\Delta$ mutation is epistatic to that of the sec4-8 mutation, indicating that Npr1 acts before Sec4 in the secretion of Gap1.

Taken together, these data show that nonaccumulation of newly synthesized Gap1 at the plasma membrane in the $npr1\Delta$ strain is not due to rapid endocytosis and vacuolar targeting of Gap1 immediately after its arrival at the cell surface. Rather, newly synthesized Gap1 is likely diverted from the secretory pathway to the vacuole.

Newly Synthesized Gap1 Is Sorted from the Golgi to the Vacuole in the npr1 Δ Mutant—To further test this hypothesis, we investigated whether the effect of the npr1 Δ mutation on Gap1 could be suppressed by blocking transport from the Golgi to the prevacuolar compartment. This transport pathway requires Pep12, a t-SNARE essential to the fusion of Golgi-derived vesicles with the prevacuolar membrane (14). As expected, after the shift from glutamine to proline, Gap1 accumulated to a high level and became highly active in both the wild-type and the pep12 Δ strain, but not in the npr1 Δ strain



FIG. 7. **Gap1-GFP localizes to internal membranes in the** $npr1\Delta$ mutant. A, a haploid wild-type (34288c) expressing Gap1-GFP expressed under the control of the natural GAP1 promoter was grown on proline medium and analyzed by fluorescence (*left panel*) and visible-light (*right panel*) microscopy. B, a wild-type diploid strain (DJOD01) expressing Gap1-GFP under the control of the GAL1 promoter was grown on galactose (200 mM)-proline medium. Glucose (200 mM) was added to repress synthesis of Gap1, and 2 h later, NH₄⁺ (20 mM) was added. Cells were analyzed 60 min after the addition of NH₄⁺ by fluorescence (*left panels*) and visible-light (*right panels*) microscopy. C, wild-type diploid (DJOD01), $npr1\Delta$ (DJOD02), npi1 (DJOD03), and $npr1\Delta$ npi1 (DJOD04) strains expressing Gap1-GFP under the control of the GAL1 promoter were grown on raffinose (50 mM)-proline medium. Cells were analyzed 60 min after the addition of galactose (200 mM) for FM4-64 fluorescence (*right panels*), GFP fluorescence (*middle panels*), and visible-light (*left panels*) microscopy.

(Fig. 6). In the $npr1\Delta$ $pep12\Delta$ double mutant, a high intensity Gap1 signal was immunodetected, and relatively high Gap1 activity was measured, indicating that the $pep12\Delta$ mutation is largely epistatic to the $npr1\Delta$ mutation (Fig. 6). To ascertain that the suppressive effect of $pep12\Delta$ is not due to defective endocytosis of Gap1 in this mutant (51), we compared NH₄⁺-induced endocytosis of Gap1 in the wild-type and the $pep12\Delta$ mutant under similar conditions (29 °C, shift for 3 h from glutamine to proline). Gap1 activity decreased in both strains at a similar rate after the addition of NH₄⁺, indicating that Gap1 internalization is not defective in the $pep12\Delta$ strain. As expected, however, Gap1 degradation was indeed defective in the $pep12\Delta$ strain (data not shown).

Taken together, these data show that Npr1 is essential to accumulation of newly synthesized Gap1 at the plasma membrane. In $npr1\Delta$ cells, Gap1 is sorted from the Golgi to the vacuole (via the late endosome/prevacuolar compartment) without passing via the plasma membrane.

GFP-tagged Gap1 Is Found Mainly in Small, Punctate Structures in the npr1 Mutant—Although Gap1 is inactive in the $npr1\Delta$ mutant, a low level of permease is detectable on immunoblots (Fig. 1A). To assess the subcellular location of this small amount of Gap1, we fused the GFP preceded by a (Gly-Ala)₅ linker to the C terminus of Gap1 (see "Experimental Procedures"). We first examined whether the chimeric protein could localize to the cell surface and function when the fusion gene was expressed under the control of the GAP1 gene's natural promoter. This proved to be the case: in cells grown on proline as the sole nitrogen source, Gap1-GFP was fully active (data not shown) and present at the cell surface (Fig. 7A). We then expressed the GFP-tagged permease under the control of the galactose-inducible GAL1 promoter. Cells transformed with this GAL1-Gap1-GFP construct were first grown on proline-galactose medium, and then glucose was added to the

medium to repress Gap1-GFP synthesis. After 2 h, Gap1-GFP was found mainly at the cell surface. Weak fluorescence was also detected inside the cells, in a compartment identified as the vacuole (Fig. 7B; data not shown). This is likely due to overexpression of the Gap1-GFP protein because this effect was not observed when the protein was expressed under the control of the natural *GAP1* promoter (Fig. 7A). NH_4^+ was then added to the culture medium. After 1 h, the fluorescence was found to have been redistributed mainly to intracellular patches, indicating that the Gap1-GFP fusion protein still responds to NH₄⁺induced regulation. We then monitored the localization of this functional Gap1-GFP protein upon neosynthesis in wild-type and mutant strains (Fig. 7C). The cells were first grown on raffinose-proline medium, conditions under which no fluorescence was detected (data not shown). 1 h after the addition of galactose to the wild-type, as expected, Gap1-GFP accumulated at the cell surface, mainly in the bud (Fig. 7C). In the $npr1\Delta$ mutant, however, Gap1-GFP appeared to be concentrated in punctate structures. These observations confirm the data of uptake assays and immunoblot experiments, *i.e.* that Npr1 is required for accumulation of Gap1 at the plasma membrane. The punctate structures in which Gap1-GFP seems to accumulate in the $npr1\Delta$ strain are reminiscent of the staining pattern of the Golgi or of an endosomal compartment (52). After a longer time interval, the fluorescence was located mainly in the vacuole (data not shown). Finally, in the $npr1\Delta$ strain additionally defective in the Npi1/Rsp5 ubiquitin ligase, neosynthesized Gap1-GFP is retargeted to the plasma membrane (Fig. 7C). This suggests a role of ubiquitin in sorting of Gap1 from the secretory pathway to the vacuole (53).

Neosynthesized Gap1 Is Sorted to the Vacuole in the Presence of NH_4^+ —Loss of Npr1 function and addition of NH_4^+ to the medium both lead to down-regulation of Gap1 present at the plasma membrane. Because loss of Npr1 function also leads to The Journal of Biological Chemistry

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FIG. 8. The presence of NH_4^+ and loss of Npr1 function affect Gap1 activity similarly. Wild-type (33191b), pep12 Δ (33308c), npr1 Δ (33192c), npi1 (33201b), npr1 Δ npi1 (33191a), and npr1 Δ pep12 Δ (33307a) strains in which the GAP1 gene was placed under the control of the GAL1 promoter were grown on galactose as the carbon source and NH₄⁺ or proline as the nitrogen source. Gap1 activity (nmol.min⁻¹.mg protein⁻¹) was measured by incorporation of [¹⁴C]citrulline (0.02 mM) in steady-state growing cells.

direct sorting of newly synthesized Gap1 to the vacuole, we tested whether this missorting also occurs when wild-type cells are grown on NH_4^+ medium. Because the *GAP1* gene is repressed in the presence of NH_4^+ (16), we used yeast strains in which the chromosomal GAP1 gene is expressed under the control of the GAL1 promoter. The results presented in Fig. 8 show that in wild-type cells growing on galactose, Gap1 is inactive if NH_4^+ is the sole nitrogen source. In the pep12 Δ strain, a much higher Gap1 activity was measured. Conversely, Gap1 is inactive in proline-grown $npr1\Delta$ cells but is much more active in the $npr1\Delta$ pep12 Δ double mutant. These results suggest that growth of the wild-type strain in the presence of NH₄⁺ and loss of Npr1 function in proline-grown cells both prevent accumulation of Gap1 at the cell surface. The permease is instead sorted to the vacuole, a process that is Pep12-dependent. The npi1 mutation also restores high Gap1 activity in both the NH₄⁺-grown wild-type and the proline-grown $npr1\Delta$ strain (Fig. 8). This confirms the data from Fig. 7C and suggests that ubiquitin is also involved in direct sorting of Gap1 from the secretory pathway to the vacuole. This result is further investigated in the accompanying article (53).

DISCUSSION

This report shows that the Npr1 kinase plays a central role in nitrogen-regulated trafficking of the yeast Gap1 permease. Upon loss of Npr1 function in proline-grown cells, Gap1 preaccumulated at the plasma membrane is endocytosed and targeted to the vacuole, where it is degraded. This means that inactivation of Npr1 affects Gap1 in the same way as the addition of NH_4^+ to the culture medium (17, 18). Ubiquitination of Gap1 is essential to this down-regulation: inactivation of Npr1 triggers a marked increase in the pool of ubiquitin-conjugated Gap1 and the concomitant appearance of what are probably polyubiquitinated forms of the permease. Failure of Gap1 to undergo ubiquitination, as in cells defective in the Npi1/Rsp5 ubiquitin ligase, protects the permease against down-regulation. Thus, a major role of the Npr1 kinase in proline-grown cells is to prevent the plasma membrane pool of Gap1 from undergoing endocytosis followed by vacuolar degradation, a process requiring Npi1/Rsp5-dependent ubiquitination of the permease.

The Npr1 kinase also affects the fate of newly synthesized Gap1 during secretion to the plasma membrane. By combining $npr1\Delta$ with an *act1-1*, *sec4-8*, $pep12\Delta$, or $pep4\Delta$ mutation, we have shown that in $npr1\Delta$ cells, neosynthesized Gap1 is not

targeted to the cell surface but transported directly from the Golgi to the vacuole, where it is degraded. In the $npr1\Delta$ mutant, direct transport from the Golgi to the vacuole thus becomes the default transport pathway of neosynthesized Gap1. The observation that this transport requires the t-SNARE Pep12 indicates that neosynthesized Gap1 follows the carboxypeptidase Y pathway in the $npr1\Delta$ mutant. If transport between the Golgi and the prevacuolar compartment is blocked because of a $pep12\Delta$ mutation, Gap1 is rerouted to the cell surface. Previous studies have shown that sorting of Gap1 from the Golgi to the plasma membrane requires Sec13 (7), a COPII vesicle coat protein (10), as well as three other proteins of unknown biochemical function, namely, Lst4, Lst7, and Lst8 (12). In cells bearing certain sec13 alleles and in lst mutants, neosynthesized Gap1 is missorted to the vacuole. An additional $pep12\Delta$ mutation leads to rerouting of Gap1 to the plasma membrane. It thus seems that Npr1, Sec13, and the Lst proteins are components of a common mechanism of delivery of Gap1 to the plasma membrane. This mechanism is subject to nitrogen regulation because Gap1 is sorted directly to the vacuole in wildtype cells growing on glutamate instead of urea (7, 12). Using a strain expressing the GAP1 gene under the control of the GAL1 promoter, we have shown that the fate of Gap1 is similar in cells growing on NH₄⁺. Hence, the fate of neosynthesized Gap1 is similarly affected by the presence of NH₄⁺ or glutamate and by the loss of function of Npr1, Sec13, or the Lst proteins: the permease is transported directly from the Golgi to the vacuole via the carboxypeptidase Y pathway.

It is remarkable that Npr1 controls the fate of both Gap1 present in the late secretion pathway and Gap1 present at the plasma membrane. The C-terminal tail of the permease also seems to be important in controlling the fate of Gap1 in both pools. This tail contains a glutamate and a dileucine motif within a predicted α -helix. When these residues are mutated, Gap1 present at the plasma membrane is protected against NH_4^+ -induced down-regulation (32). Deletion of the last 11 amino acids directly following the predicted α -helix also renders Gap1 insensitive to NH₄⁺-induced down-regulation. Interestingly, these C-terminal mutations also restore a high Gap1 activity in the npr1 mutant (32). This suggests that the Cterminal tail of Gap1 is also required for direct sorting of neosynthesized permease from the Golgi to the vacuole. As suggested by the ability of the *npi1* mutation to suppress the $npr1\Delta$ mutation (Fig. 8), ubiquitin also seems to be important in controlling the fate of Gap1 in both pools. This result is confirmed by data presented in the accompanying article (53), in which we show that Npi1/Rsp5-dependent ubiquitination of Gap1 on two distinct lysine residues in its cytosolic N-terminal tail is required for both NH₄⁺-induced down-regulation of Gap1 present at the plasma membrane and sorting of neosynthesized Gap1 from the Golgi to the vacuole. Furthermore, in a study published during the reviewing of this paper, it was reported that it is a polyubiquitination signal on Gap1 that specifies its targeting to the vacuole and that deletion of the last 12 amino acids of Gap1 and a rsp5-1 mutation reduce polyubiquitination of the permease (54). It thus seems that a similar mechanism involving Npr1 kinase, Npi1/Rsp5-dependent mono-ubiquitination and/or polyubiquitination of Gap1, and signals in the Nand C-terminal tails of the permease are involved in the control of both cell surface and internal Gap1. These elements could be part of a sorting mechanism acting at both the plasma membrane and the membrane of an internal compartment such as the late Golgi. Alternatively, they could be part of a single sorting mechanism localized at the membrane of an internal compartment situated at the intersection between the secretory and endocytic pathways. In the latter model, on proline

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medium, Gap1 would constitutively recycle between this compartment and the plasma membrane. Recycling back to the plasma membrane would be dependent on Npr1, Sec13, and the Lst proteins, and the same mechanism would promote transport of neosynthesized Gap1 to the plasma membrane. In the presence of NH₄⁺, Npr1 is inactivated, and ubiquitin-dependent sorting of internal Gap1 to the vacuole would become the default trafficking pathway of Gap1. Experiments are underway to test these models. For instance, we are currently investigating whether the control of Gap1 trafficking also involves factors recently described for their role in recycling (52, 55). We have also tried to locate the Npr1 protein by fusing it to GFP at its C terminus. Although the low expression level of NPR1 made this experiment difficult, the detected fluorescence was found mainly in the cytosol and appeared to be more concentrated in punctate structures reminiscent of the Golgi or of an endosomal compartment.²

The precise molecular mechanisms through which Npr1 promotes targeting of internal Gap1 to the cell surface remain unknown. A simple hypothesis would be that Npr1 directly phosphorylates Gap1 and that this modification is essential to delivery of the permease to the plasma membrane. We show in this study that Gap1 is indeed phosphorylated in wild-type cells growing on proline and that its degree of phosphorylation is reduced in an $npr1\Delta$ mutant. However, the high phosphorylation level of Gap1 in NPR1 cells is more likely the consequence of Gap1 being present at the plasma membrane rather than a direct effect of Npr1 because Gap1 is also highly phosphorylated in the $npr1\Delta$ npi1 double mutant. Several plasma membrane proteins including the uracil (Fur4) and purine-cytosine (Fcy2) permeases were shown to be phosphorylated upon reaching the plasma membrane (56, 57), and preliminary results suggest that the same is true of Gap1.² Hence, although a direct role of Npr1 in limited phosphorylation of Gap1 cannot be ruled out, it seems more likely that phosphorylation of Gap1 in NPR1 cells is a consequence of the permease localizing to the plasma membrane. Npr1 could also act as a regulator of monoubiquitination and/or polyubiquitination of Gap1, i.e. its role could be to protect the permease against this modification when cells grow on a poor nitrogen source.

Our data showing that the absence of Npr1 and the presence of NH₄⁺ affect Gap1 trafficking similarly suggest that Npr1 is inactive in the presence of NH_4^+ . This is consistent with recent data obtained by Schmidt et al. (33) showing that the presence of NH₄⁺ causes phosphorylation of Npr1, whereas nitrogen starvation or addition of rapamycin causes dephosphorylation of Npr1. Interestingly Npr1 appears, under conditions of limited nitrogen supply, to exert opposite effects on the Gap1 and Tat2 permeases, stabilizing the former and destabilizing the latter (33).

Npr1 is a member of an apparently fungus-specific subgroup of protein kinases (29). In this subgroup, three proteins of unknown function (Ydl241c, Ydl025c, and Yor276c) are closely related to Npr1. Whereas deletion of NPR1 causes reduced growth on various nitrogen sources as a result of the inactivation of multiple nitrogen permeases, deletion of the YDL241c, YDL025c, or YOR267c gene has no detectable effect on nitrogen utilization.² Perhaps these kinases control the internal trafficking of other categories of plasma membrane proteins. It was recently reported that disruption of YOR267c partially reduces activation, in response to glucose, of the H⁺-ATPase Pma1 (58). Although this phenotype does not seem to support a role of YOR267c in the control of protein trafficking, it is noteworthy that mutations affecting other proteins involved in trafficking

² J.-O. De Craene, O. Soetens, and B. André, unpublished data.

and turnover of membrane proteins, such as the Npi1/Rsp5 ubiquitin ligase or the Ubc4 ubiquitin-conjugating enzyme (59), also impair glucose activation of Pma1 (60). The Npr subgroup of protein kinases also includes the Hal4 and Hal5 kinases involved in activation of Trk potassium transporters (31) and the Stk1 and Stk2 kinases involved in polyamine transport (30). The mechanisms through which these kinases activate transporter activities remain unknown. We propose that these kinases, like Npr1, function as regulators of permease trafficking.

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