In the yeast *Hansenula polymorpha*, the *YNT1* gene encodes the high affinity nitrate transporter, which is repressed by reduced nitrogen sources such as ammonium or glutamine. Ynt1 protein is degraded in response to glutamine in the growth medium. Ynt1 disappears independently of *YNT1* glutamine repression as shown in strains where *YNT1* repression is abolished. Ynt1-green fluorescent protein chimera and a mutant defective in vacuolar proteinase A (\(A^{lep9}\)) showed that Ynt1 is degraded in the vacuole in response to glutamine. The central hydrophilic domain of Ynt1 contains PEST-like sequences whose deletion blocked Ynt1 down-regulation. Site-directed mutagenesis showed that Lys-253 and Lys-270, located in this sequence, were involved in internalization and subsequent vacuolar degradation of Ynt1. Ynt1-ubiquitin conjugates were induced by glutamate and not nitrate. We conclude that glutamine triggers Ynt1 down-regulation via ubiquitinylation of lysines in the central hydrophilic domain, and proteolysis in the vacuole.

Nitrate is a nitrogen source used by plants, fungi, certain yeasts, and bacteria. In most nitrate assimilation organisms, nitrate induces a set of genes encoding the enzymatic machinery involved in its own assimilation. The nitrate assimilation pathway involves its uptake by a specific transporter, followed by its reduction to nitrite and ammonium by consecutive action of nitrate and nitrite reductases. This pathway demands a high investment of energy per mole of nitrate assimilated because it must be reduced to ammonium to be utilized. As a result, most organisms restrict the use of nitrate to those environments not containing “preferred nitrogen sources” such as ammonium, asparagine, or glutamine (1–5). Furthermore, organisms have evolved molecular mechanisms, termed Nitrogen Catabolite Repression (NCR), by which preferred nitrogen sources repress genes involved in the assimilation of non-preferred nitrogen sources (4–6).

Isolation of genes involved in assimilation of nitrate and construction of the corresponding null mutants makes the yeast *Hansenula polymorpha* a valuable tool for studying different issues related to nitrate assimilation (5). This yeast presents a single high affinity bispecific nitrate/nitrite transporter called Ynt1 that contains 12 putative transmembrane domains (7, 8). *YNT1*, *YNR1* and *YNH1*, encoding, respectively, nitrate and nitrite reductases, are induced by nitrate and repressed by the preferred nitrogen sources ammonium or glutamine (5). Ynt1 has been included in the NNP (nitrate/nitrite porter) family that is involved in high affinity nitrate and/or nitrite transport (9) and is a member of the Major Facilitator Superfamily (10). Ynt1 shows high similarity to NrtA (formerly designated CRNA) and NrtB from *Aspergillus nidulans* (11), NIT10 from *Neurospora crassa* (12), and high affinity nitrate transporters from plants and algae (9). Previous studies have shown that nitrate and nitrite reductases are subjected to posttranslational modifications in response to nitrogen sources (17). The little evidence available suggests the posttranslational down-regulation of the *Nicotiana plumbaginifolia* NRT2.1 in response to ammonium (18). In *Arabidopsis thaliana*, phosphorylation of the nitrate transporter CHL1 at residue Thr-101, stimulated by low nitrogen concentration, switches the transporter from a low to a high affinity form (19). In *H. polymorpha*, preliminary studies indicate that Ynt1 undergoes proteolytic degradation in response to the presence of preferred nitrogen sources in the medium (7).

Ubiquitinylation is an internalization signal for endocytosis and subsequent degradation in the vacuole of most yeast integral plasma membrane proteins (20, 21). In *Saccharomyces cerevisiae*, Gap1 permease that is involved in amino acids transport loses its uptake activity when cells are transferred to ammonium. Inactivation results from polyubiquitinylation followed by vacuolar degradation (22, 23). The ubiquitination pattern of yeast integral plasma membrane proteins ranges from the mult ubiquitinylation of the \(\alpha\)-factor receptor Ste2 (24) and the galactose transporter Gal2 (25) to polyubiquitinylation of Gap1 (23) and uracil permease Fur4 (26). The sequence (D/E)XX(S/T) is considered a potential ubiquitinylation site of yeast plasma membrane proteins (20, 21). Alternative ubiquitinylation sites have been mapped within or near the PEST-like sequences of Fur4, Ste3, and Mal61 (20, 27–29). PEST sequences are hydrophilic stretches of variable length containing Pro, Ser, Asp, or Glu residues that tag certain short-lived proteins (30). Although in some cases degradation of PEST-tagged proteins proceeds via polyubiquitinylation and later proteolysis by the 26 S proteasome (31), PEST-tagged yeast plasma membrane proteins are ubiquitinylated and degraded in the vacuole (21). Phosphorylation sites were mapped either within or very close to PEST-like sequences of Fur4, Ste3, and Mal61 (27–29).

The putative secondary structure for fungal nitrate transporters predicts 12 transmembrane domains with short N- and C-terminal tails oriented to the cytoplasm. A large central hydrophilic domain of \(~90~\) residues facing into the cell connects two sets of six transmembrane domains (8) (Fig. 1A). In all these transporters, Ynt1, NrtA, NrtB, TbNrt2, (32) and NIT10, this domain contains regions resembling PEST sequences, and in the case of NIT10 the PESTFind algorithm (33) defines a proper PEST sequence. In the PEST-like sequences of Ynt1 and fungal nitrate
transporters the numerous Lys, Ser and Thr residues are remarkable, which could constitute ubiquitinylation and phosphorylation sites.

In this work we have studied the effect of preferred nitrogen sources on Ynt1 levels and the role of the central hydrophilic domain in this issue. Ynt1 down-regulation is not a direct consequence of YNT1 gene repression; instead, nitrogen sources trigger Ynt1 ubiquitinylation, in a PEST-like sequence, and sorting to the vacuole for degradation.

EXPERIMENTAL PROCEDURES

Microorganisms and Growth Conditions—The H. polymorpha strains used in this work are listed in supplemental Table S1. All strains are derivatives of NCYC495 leu2 ura3 strain. Yeast cells were grown at 37 °C in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) or synthetic medium containing 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 2% (w/v) glucose, and the nitrogen source indicated in each experiment. For expression of Ynt1 under the control of the methanol-inducible MOX1 promoter in MDP100 strain, cells were grown in synthetic medium with 5 mM ammonium chloride and transferred to synthetic medium with 0.5% (v/v) methanol. To repress the expression of Ynt1 under the control of the methanol-inducible MOX1 promoter in MDP100 strain, cells were grown in synthetic medium with 0.5% (v/v) methanol.

Deletion of the Nitrate Transporter Ynt1—250 mg of cells was resuspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 15 mM EDTA, 15 mM EGTA, 10 mM Na3P2O7, 10 mM NaF plus Complete Mini protease inhibitor mixture (Roche Applied Science), and 2 mM phenylmethylsulfonyl fluoride). 0.5 mm O glass-beads were added to cell suspension for homogenization in a FastPrep homogenizer device (Thermosavant LifeSciences, Hampshire, UK) for 20 s at 6.0 m/s. Supernatant was clarified by two consecutive centrifugations at 820 × g for 1 min. The resulting supernatant was routinely centrifuged at 20,500 × g for 30 min at 4 °C except for immunodetection of Ynt1 ubiquitinylation, for which the supernatant was centrifuged at 20,500 × g for 45 min. In both cases pellets were resuspended in 70 μl of 0.25 mg/ml of Triton X-100 and then mixed with 4× sample buffer (4× 12% (w/v) SDS, 6% (v/v) 2-mercaptoethanol, 30% (w/v) glycerol, 0.05% (w/v) Serva blue G, 150 mM Tris-HCl, pH 7). Finally, samples were heated at 40 °C for 30 min and then subjected to SDS-PAGE. For Ynt1-Ub conjugate form determination experiments, cells were processed as described above except that they were harvested with 10 mM NaCl, and lysis buffer supplemented with freshly prepared 50 mM N-ethylmaleimide (Sigma) to prevent deubiquitinylation. Ynt1 immunoprecipitation was carried out from protein extracts prepared essentially as described above. The membrane-enriched fraction obtained from 250 mg of cells was resuspended in 500 μl of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (w/v) Triton X-100, and 140 mM NaCl) + Complete Mini protease inhibitor mixture (Roche Applied Science) + 2 mM phenylmethylsulfonyl fluoride. Protein extracts were incubated with rotation at 4 °C for 2.5 h with 0.5 μl of anti-Ynt1 anti-serum and 20 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) previously incubated for 3 h at 4 °C with rotation. Immunoprecipitate was washed once with 1 ml of radioimmunoprecipitation assay buffer + protease inhibitors and resuspended in 30 μl of 1× sample buffer.

SDS-PAGE and Ynt1 immunodetection were performed as described in Ref. 7. Cell extract preparation and Ynt1 immunodetection were performed for a minimum of three independent experiments. Films were documented using a Bio-Rad densitometer GS-800.
Nitrate Uptake Assay and Nitrate Reductase Activity Determination—Nitrate uptake was determined by extracellular nitrate depletion as described in Ref. 7 using purified H. polymorpha nitrate reductase enzyme (NECi, Lake Linden, MI). Values are expressed as nmol NO₃⁻ transported/(mg of cell)⁻¹·min⁻¹. Mean values ± S.D. from at least three independent experiments are shown.

Nitrate reductase activity was measured according to Ref. 35 and is expressed as nmol NO₂⁻ produced/(mg protein)⁻¹·min⁻¹. Mean values ± S.D. from at least three independent experiments are shown.

Expression of Ynt1-GFP Fusion and Fluorescence Microscopy—To follow Ynt1 cellular localization, the chromosomal copy of YNT1 was fused to GFP. Plasmid pGYF1 was linearized to transform wild-type (WT) and Ynt1 mutant strains. To follow GFP fluorescence, samples of 3 ml were withdrawn from the culture and imaged under an epifluorescence Leica DMRXA microscope (Leica, Mannheim, Germany) equipped with a Photometric CCD camera SenSys (Photometrics Ltd., Tucson, AZ). To stain vacuolar membranes the culture was concentrated to an A₅₅₀ of 40 and incubated with 10 μM FM4–64 (Molecular Probes Inc., Eugene, OR) for 15 min at 37 °C with shaking before the incubation in nitrate-containing medium.

Quantitative Real-time PCR—Total RNA was extracted using RNeasy Mini (QIAGen, Hilden, Germany) and cDNA produced by RETROscript™ kit (Ambion, Austin, TX) using random primers. Real-time PCR over YNT1 and ACT1 genes (control) was performed using primers Q-YNT1-R and -F and Q-ACT1-F and -R (supplemental Table S3).

RESULTS AND DISCUSSION

Glutamine Causes Posttranslational Down-regulation of Ynt1—Incubation of H. polymorpha cells in a medium containing nitrate as sole nitrogen source brings about the synthesis of Ynt1 concomitantly with the appearance of nitrate uptake (7). When cells are transferred to a medium containing Gln, a marked decrease in nitrate transport and Ynt1 protein levels is observed (Fig. 2, A and B); ammonium produced the same effect as Gln on nitrate uptake and Ynt1 (data not shown). Although nitrate reductase activity also decreases when cells are transferred to a Gln-containing medium (Fig. 2A), this decrease does not account for the drop in nitrate uptake.

To evaluate the role of transcriptional versus posttranscriptional regulation of Ynt1 levels by nitrogen sources, YNT1 gene was expressed under the control of the nitrogen-insensitive promoter of H. polymorpha MOX1 gene. Transcription from P_MOX1 is induced by methanol and repressed by glucose (36). The ynt1-disrupted strain harboring the P_MOX1-YNT1 fusion was incubated in methanol to produce Ynt1. Subsequently, when the cells were transferred to glucose to repress P_MOX1, Ynt1 degraded faster in Gln than in nitrate (Fig. 2C). These results indicate the nitrogen source controls Ynt1 at a level beyond transcription. Further evidence for posttranscriptional regulation of Ynt1 was compiled using H. polymorpha Δure2 mutant, where NCR of nitrate assimilation genes is abolished. 3 A similar S. cerevisiae mutant has been described in which NCR control of nitrate assimilation genes is lost (6). The WT and Δure2 strains were incubated in a medium containing nitrate or nitrate plus ammonium (Fig. 2D). In nitrate alone, Ynt1 protein levels, nitrate transport activity, and YNT1 transcript levels were about the same in WT and Δure2. When ammonium was present and NCR activated in WT, Ynt1 protein, activity, and transcript fell dramatically in 90 min. In contrast, in the Δure2 mutant, where NCR is lost, YNT1 transcript was expressed, but Ynt1 protein levels and transport activity fell to the same degree as WT in 90 min. These results contrast with those shown in Fig. 6B. Thus, it is clear that YNT1 transcript level is not a factor controlling short-term Ynt1 down-regulation.

As can be seen in Fig. 2C and in other figures throughout this work, Ynt1 protein was resolved in SDS-PAGE as a set of bands with slight differences in electrophoretic mobility. We have shown that this pattern is due to Ynt1 phosphorylation (data not shown), which is in agreement with the putative phosphorylation sites present in the central cytosolic domain of the nitrate transporter (Fig. 1A). Although the regulatory significance of Ynt1 phosphorylation is unknown, preliminary evidence indicates that Ynt1 phosphorylation is not involved in Ynt1 down-regulation. We are currently investigating the precise role of Ynt1 phosphorylation.

Ynt1 Degradation in the Vacuole—To study possible cellular redistribution of Ynt1 during down-regulation triggered by Gln, a strain contain-

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FIGURE 3. Gln triggers Ynt1 sorting to vacuole and proteolysis. A, Gln (5 mM) was added to cells bearing the Ynt1-GFP fusion incubated in 10 mM nitrate for 1.5 h. Ynt1-GFP was monitored by fluorescence microscopy over 120 min in Gln. Prior to the Gln incubation, cells were labeled with FM4–64 to stain the vacuolar membrane. B, time course Ynt1 immunoblot of WT (PEP4) and Δpep4 extracts after Gln addition to cells incubated in nitrate.

TABLE 1

| Strains | Nitrate transport | Gln effect on Ynt1 protein level (%) at 60 min |
|---------|------------------|---------------------------------------------|
|         | 0 min | 60 min + Gln |                                            |
|         | mol NO₃/min·mg cell⁻¹ | | |
| WT      | 0.46 ± 0.04 | 0 | 7 ± 3 |
| Δ232–286 | 0.66 ± 0.06 | 0.55 ± 0.06 | 92 ± 7 |
| K243R   | 0.50 ± 0.03 | ND* | 7 ± 2 |
| K253R   | 0.67 ± 0.05 | ND | 17 ± 1 |
| K270R   | 0.58 ± 0.04 | ND | 12 ± 2 |
| K243R K253R | 0.63 ± 0.13 | ND | 26 ± 12 |
| K253R K270R | 0.65 ± 0.03 | ND | 52 ± 10 |
| K243R K253R K270R | 0.62 ± 0.12 | 0.55 ± 0.05 | 62 ± 11 |
| Δpep4   | 0.57 ± 0.05 | 0.28 ± 0.06 | 58 ± 1 |

* ND, not determined.

Deletion of the Central Hydrophilic Domain of Ynt1 Impairs Its Down-regulation—Ynt1 and fungal nitrate transporters present a central hydrophilic domain that contains many Pro, Asp/Glu, and Ser/Thr residues and resembles a PEST sequence (Fig. 1A). To test whether this region is involved in nitrate transporter down-regulation, a DNA fragment encoding 55 residues was deleted from YNT1 to produce the Ynt1Δ232–286 mutant strain. This strain maintained the ability to transport nitrate with high affinity and high rate (Table 1). However, Ynt1Δ232–286 mutant retained a high level of nitrate transport and Ynt1 protein after 60 min of incubation of the cells with Gln, whereas WT did not (Table 1 and Fig. 4). Thus, it is clear that the portion of the central domain deleted in this mutant is involved in down-regulation of Ynt1.

Ubiquitinylation of Ynt1 Protein—One possible cellular mechanism for modifying the central domain of Ynt1 that would lead to its degradation is the ubiquitin system (21). To determine whether ubiquitin conjugates of Ynt1 were present in H. polymorpha cell extracts, denaturing gel electrophoresis and Western blotting were employed (Fig. 5A). When cells were incubated in nitrate for an extended period, a pair of faint higher Mr bands relative to Ynt1 was detected with anti-Ynt1 antiserum by extended development of the immunoblot (Fig. 5A, lane 1). The molecular size of these protein bands corresponds to the conjugation of 1 and 2 ubiquitin molecules to Ynt1 (Fig. 5A, lane 2). These higher Mr bands were not detected when the central domain was deleted (data not shown).

Further analyze for the presence of Ynt1-ubiquitin conjugates, the core of the central domain, residues 236–286, was fused to the C-terminal hydrophilic domain of Ynt1Δ232–286 and called Ynt1Δ232–286+C236–286 (Fig. 1B). In this mutant strain, Ynt1 internalization and degradation are blocked, but formation of ubiquitin conjugates is possible.
Down-regulation of the Nitrate Transporter Ynt1

Ynt1Δ232–286 + Ct236–286 internalization blockage could be due to the absence of Ile-232 and Leu-233 in this mutant; di-Leu motifs in Gap1 and Mal61 impair endocytosis but not ubiquitinylation (28, 40). When Ynt1Δ232–286 + Ct236–286 was incubated in nitrate for 2 h, two intense bands with the same molecular size as in WT were detected (Fig. 5A, lane 3). The putative ubiquitin-Ynt1 conjugate protein amount in the Ynt1Δ232–286 + Ct236–286 strain was higher in cells incubated in glutamine than in nitrate (Fig. 5B). To test whether nitrate triggers Ynt1 ubiquitinylation, the strain Ynt1Δ232–286 + Ct236–286 Δynr1, lacking nitrate reductase and therefore unable to assimilate nitrate, was incubated in nitrate for 2 h and then transferred for 1 h to Gln (Fig. 5D). Cells incubated in nitrate showed much less Ynt1-ubiquitin conjugate than those cells incubated in glutamine. Thus, it appears that Ynt1 becomes modified with ubiquitin during down-regulation as result of nitrate assimilation or the shift to a preferred nitrogen source.

Two further steps in the analysis were taken to confirm that ubiquitin is conjugated to Ynt1 during down-regulation. First, myc-ubiquitin was expressed under the control of the H. polymorpha nitrate reductase gene promoter (41) in the Ynt1Δ232–286 + Ct236–286 strain, which showed an increase of Ynt1-ubiquitin conjugate Mr bands over that expected for Ub (Fig. 5D). Second, Ynt1-ubiquitin conjugate was identified using a monoclonal antibody for ubiquitin after immunoprecipitation of Ynt1Δ232–286 + Ct236–286 with anti-Ynt1 (Fig. 5E). Here the monoclonal anti-ubiquitin appears to detect only the higher Mr Ynt1-ubiquitin conjugate, probably because of epitope display problems in the conjugates. Nevertheless, these results make it clear that ubiquitin
is conjugated to Ynt1 during down-regulation.

**Role of Central Domain Lys Residues in Ynt1 Down-regulation**—The pattern of Ynt1 ubiquitinylation is consistent with a mono- ubiquitinylation of two Lys residues or a di-ubiquitinylation of one Lys residue. To evaluate whether Lys residues in the central hydrophilic domain participate in Ynt1 down-regulation, Lys-243, Lys-253, and Lys-270 were replaced by Arg using site-directed mutagenesis. Arg is a chemically conservative replacement for Lys, but it cannot form ubiquitin conjugates. A set of six strains, three with single Arg replacements for central domain Lys residues, two double replacements, and one triple mutant, were constructed. All these mutants had similar or higher nitrate uptake rates than wild type, but most of the mutants were quite different in response to Gln as a Ynt1 down-regulation signal (Table 1, Fig. 6A). These results made it clear that the K243R mutant did not alter the regulation of Ynt1, whereas all mutants with K253R and K270R replacements had altered down-regulation of Ynt1. Whereas the double mutant K253R/K270R and the triple mutant K243R/K253R/K270R (Ynt1 **ΔK** → **R**) had similar properties, further studies were focused on the triple mutant, which exhibited almost the same resistance to Gln-triggered down-regulation as Ynt1 **ΔK253–286** (Table 1). As expected, in the triple mutant no ubiquitinylation was observed (Fig. 5A, lane 4). This indicates that the central domain Lys residues are responsible for themselves for Ynt1 degradation and that the rest of the residues present in the central domain would act as auxiliaries in the ubiquitinylation process.

In the strain expressing the triple mutant, the transfer to a Gln-containing medium did not trigger the drop in nitrate transport activity observed in WT (Table 1). This indicates that the triple Ynt1 mutant remained in the plasma membrane and was fully active despite the presence of Gln in the medium. When the triple Ynt1 mutant was expressed in the Δure2 strain and incubated in nitrate plus ammonium, nitrate uptake and Ynt1 levels were similar to WT in nitrate (Fig. 6B). These results support the conclusion that at least two of the three Lys in the central domain are necessary for the posttranslational down-regulation of Ynt1. Fluorescence microscopy of a strain with the triple mutant Ynt1 fused to GFP showed that after Gln treatment for 120 min, Ynt1 failed to be internalized from the plasma membrane and consequently was not delivered to the vacuole for degradation (Fig. 6C). This behavior was completely different from that found for Ynt1-GFP (Fig. 3A).

**CONCLUSION**

Ynt1 is degraded in response to preferred nitrogen sources such as ammonium or glutamine. The use of Δure2 mutant strain, lacking nitrogen catabolite repression, and that expressing YNT1 under the control of the nitrogen non-regulable MOX1 promoter provided evidence that the drop in Ynt1 levels is not a consequence of glutamine-YNT1 gene repression (Fig. 2, C and D).

Ynt1-GFP fusion protein and the Δpep4 vacuolar protease mutant strain showed the Ynt1 internalization and sorting to vacuole for degradation (Fig. 3). Deletion of the Ynt1 central hydrophilic domain provided evidence of its involvement in Ynt1 internalization (Fig. 4 and Table 1). Ynt1 is ubiquitinylated in response to nitrate and glutamine (Fig. 5, A and B). However, the lack of nitrate assimilation in a nitrate reductase-deficient mutant (Δymr1) showed that nitrate does not trigger Ynt1 ubiquitinylation but does trigger its assimilation to ammonium or glutamine (Fig. 5C). Site-directed mutagenesis of three Lys residues present in the central hydrophilic domain revealed the participation of Lys-253 and Lys-270 in Ynt1 ubiquitinylation and internalization (Fig. 5A, lane 4 and Fig. 6). Our findings highlight the role played by the central hydrophilic domain of the fungal nitrate transporters in their regulation. Ynt1 internalization blockage in Ynt1 **ΔK** → **R**, Ynt1 **ΔK** → **R** ure2, Ynt1 **ΔK253–286**, and Ynt1 **ΔK232–286** + C236–286 mutants showed that the transporter is not inactivated by glutamine.

The results shown here and previously (14) allow us to conclude that Ynt1 has a key role in regulating nitrate assimilation in yeast. Ynt1 down-regulation by internalization and vacuolar degradation, elicited by intracellular preferred nitrogen sources, modulates the amount of Ynt1 in the plasma membrane, and consequently nitrate uptake. This mechanism enables the cell to regulate the nitrate uptake according to the availability of easily assimilable nitrogen sources. In the short term, Ynt1 down-regulation lowers the influx of nitrate to be assimilated, and in the long term the drop of intracellular nitrate would reduce the nitrate-assimilating gene induction. Such a mechanism may also regulate nitrate transport in other nitrate-assimilating organisms. The central domain of high affinity fungal nitrate transporters is very similar in length and composition to that of Ynt1. Although the occurrence of endocytosis in filamentous fungi has been somewhat controversial (42), current evidence supports an endocytic pathway involving membrane proteins (43).

This work, along with others (18, 19), contributes to understanding how eukaryotic nitrate transporters respond to changes in environmental nitrogen sources. Our results allow us to tackle further studies to understand how glutamine triggers Ynt1 ubiquitinylation.

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