Choline is a precursor for the synthesis of phosphatidylcholine through the CDP-choline pathway. *Saccharomyces cerevisiae* expresses a single high affinity choline transporter at the plasma membrane, encoded by the *HNM1* gene. We show that exposing cells to increasing levels of choline results in two different regulatory mechanisms impacting Hnm1 activity. Initial exposure to choline results in a rapid decrease in Hnm1-mediated transport at the level of transporter activity, whereas chronic exposure results in Hnm1 degradation through an endocytic mechanism that depends on the ubiquitin ligase Rsp5 and the casein kinases Yck1/2. We present details of how the choline transporter is a major regulator of phosphatidylcholine homeostasis.

Phosphatidylcholine (PC) is the most abundant cellular phospholipid in eukaryotic cells. PC fulfills an essential role in maintaining membrane permeability barriers while at the same time serving as a substrate for the generation of numerous second messenger signaling molecules (1, 2). Cellular PC homeostasis is controlled by balancing rates of PC synthesis with degradation. Regulatory pathways impinge upon these two processes to coordinate PC level with other cellular processes, such as cell proliferation and vesicular trafficking (3–7). PC synthesis in eukaryotic cells occurs through the CDP-choline and phosphatidylethanolamine (PE) methylation pathways, whereas PC turnover is via multiple lipases.

For PC synthesis through the CDP-choline pathway, choline is activated sequentially to phosphocholine and then CDP-choline, which is condensed with diacylglycerol (DAG), to form PC (Fig. 1). In the yeast *Saccharomyces cerevisiae*, net synthesis of PC through this pathway depends on exogenous choline uptake, a process mediated by a single high affinity choline transporter localized at the plasma membrane encoded by the *HNM1* gene (8–11). The PE methylation pathway catalyzes the addition of three methyl groups to PE to synthesize PC and contributes greatly to total PC synthesis in *S. cerevisiae* when exogenous choline is scarce (12).

Degradation of PC in yeast takes place through the activity of three phospholipases. The phospholipase B Plb1 is localized at the plasma membrane and within the periplasmic space. Plb1 deacylates PC with the glycerophosphocholine produced released extracellularly (13). Nte1, a second phospholipase B, is an integral membrane protein localized at the endoplasmic reticulum and is responsible for intracellular glycerophosphocholine formation. Accumulation of intracellular glycerophosphocholine due to increased Nte1 activity correlates with increased PC synthesis (14). The glycerophosphocholine produced by Nte1 can be further catabolized to choline by the action of the glycerophosphodiesterase Gde1 (15, 16). Intracellular choline can also be generated by the PC phospholipase D Spo14. Spo14 is a soluble enzyme that, upon activation, becomes membrane-bound, localizing at the endosomal compartment and plasma membrane. Spo14 is essential for spore formation and for Sec14-independent vesicular trafficking (17). Choline generated intracellularly by Nte1/Gde1 or by Spo14 is either recycled back into PC through the CDP-choline pathway or excreted out of the cell (9, 17, 18).

The availability of the lipid precursors choline and inositol has a profound effect on yeast phospholipid synthesis. Inositol abundance sustains a high rate of phosphatidylinositol synthesis and allosterically inhibits phosphatidylerine synthase, diverting the common precursor CDP-DAG toward the synthesis of phosphatidylinositol. Simultaneously, a sustained high rate of phosphatidylinositol synthesis drags upon the phosphatidic acid pool via CDP-DAG consumption, leading to transcriptional attenuation of genes encoding enzymes for phospholipid biosynthesis (3, 12, 19). The presence of choline in the growth media enhances the repressive effect of inositol on gene expression, presumably as it affects the same pool of phosphatidic acid via DAG consumption for PC synthesis (3, 12, 20). However, choline on its own lacks such an effect on the transcriptional regulation of phospholipid synthesis genes (20), suggesting the existence of regulatory mechanisms that control
PC metabolism to prevent a skewed phospholipid composition. Indeed, increased turnover of PC through Nte1 takes place when yeast cells grow in inositol-free medium, and this turnover is necessary to prevent transcriptional attenuation triggered by exogenous choline (8).

From these observations a basic question arises. How do yeast cells control PC synthesis when choline is abundant? The ultimate regulation would be limiting the amount of choline channeled into the CDP-choline pathway. Supporting this notion, a pronounced reduction in choline transporter steady state level and consequent choline uptake was observed for yeast cells grown in choline-rich medium independent of inositol content (8, 10, 21). In this paper, we explore the underlying mechanism providing this regulation.

Cells are equipped with several mechanisms to acquire nutrients for growth, coordinating substrate abundance with cellular demand. We show here that upon choline exposure, the sole high affinity choline transporter in S. cerevisiae, Hnm1, is subject to endocytosis and vacuolar degradation. Our data are consistent with the major driver of this event being the choline transporter rate. We go on to determine that Hnm1 endocytosis is required for growth, coordinating substrate abundance with cellular metabolism.

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**MATERIALS AND METHODS**

**Reagents**—[methyl-14C]Choline chloride and [methyl-14C]-methylamine were from American Radiolabeled Chemicals. Antibodies used were anti-mouse horseradish peroxidase (HRP)-linked IgG and anti-rabbit HRP-linked IgG (Cell Signaling), anti-TAP (Open Biosystems), anti-ubiquitin (P4D1, Cell Signaling), anti-GFP (JL-8, Clontech), and anti-Pgk1 (Molecular Probes). Growth media were from Difco. Choline, choline oxidase from Arthrobacter globiformis, horseradish peroxidase, cytochrome, homovanillic acid, and hemicholinium were from Sigma.

**Media and Culture Conditions**—Yeast cells were maintained in YEPD (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) medium or SD (0.67% bacto-yeast nitrogen base without amino acids, 2% dextrose) medium supplemented as required for plasmid maintenance and nutrient auxotrophies. Yeast cells were routinely grown at 25 °C.

**Yeast Strains and Plasmids**—The yeast strains used in this study are shown in Table 1. Single deletion mutants in the BY4741 background were obtained from Euroscarf. When necessary, the genetic KanMX4 marker was replaced with the nourseothricin acetyltransferase gene cassette NatMX4 using plasmid pAG25 or with the Candida albicans URA3 gene (URA3MX cassette) using plasmid pAG60 as described (22). Double mutant strains or single mutant strains carrying a tagged allele were constructed by standard genetic crossovers and sporulation. The HNM1 gene tagged at its 3′-end in frame with the GFP coding sequence was amplified by PCR from genomic DNA of the strain PMY764 using the following primers: 5′-HNM1, CAAATTTGTTAAGTCTCATGAA; 3′-HNM1, TGCTTTTTATGCCTTTTCC. A 5-kb DNA fragment was generated extending from 0.5 kb upstream to 0.5 kb downstream of the HNM1 open reading frame and encompassing the complete insertion encoding GFP plus the HIS3 gene marker. This PCR product was subcloned into a pCR2.1 vector (Invitrogen), and its identity was verified by DNA sequencing. A fragment of 3.2 kb was released using SpeI (352 nt upstream of the HNM1 start codon) and PstI (446 nt downstream of GFP encoding the sequence stop codon) and ligated into the pRS415 low copy plasmid.

**Protein Extraction and Western Blot Analysis**—Logarithmically growing cells from a 5-ml culture (A600 nm, of 0.5) were harvested, washed, and taken up in 200 μl of 50 mM Tris-HCl buffer, pH 8, 20% glycerol-containing Complete protease inhibitor mixture (Roche Applied Science) plus 1 μg/ml pepstatin A. Cells were broken with glass beads using a bead beater for two periods at 4 °C of 1 min intercalated with 1 min on ice. Cell debris was removed by a 3-min centrifugation at 500 × g, and supernatants were saved. Proteins were fractionated by 10% SDS-PAGE, transferred to PVDF membrane, and detected using appropriate primary and HRP-conjugated secondary antibodies followed by enhanced chemiluminescence.

For detection of ubiquitinated Hnm1-GFP adducts, logarithmically growing cells from a 50-ml culture (A600 nm of 0.6) were treated with 10 mM NaCl, 10 mM NaF, and 5 mM N-ethylmaleimide and placed in an ice-water bath. Cells were harvested, washed, and resuspended in 0.5 ml of lysis buffer composed of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 10 mM N-ethylmaleimide, 1 μg/ml pepstatin A, and Complete protease inhibitor mixture (Roche Applied Science). Cells were broken with glass beads using a bead beater at 4 °C for three periods of 1 min intercalated with 3 min on ice. Extracts were recovered by pinching a hole at the bottom of the tube and collection by low speed centrifugation into a new tube. After the addition of 0.5 ml of fresh lysis buffer, the extracts were incubated for 30 min at 4 °C on a spinning wheel. Cell debris was eliminated by centrifugation at 500 × g for 3 min. Supernatants (1 ml) were incubated with 10 μl of anti-GFP monoclonal antibody (JL-8) and 40 μl of a 1:1 slurry of protein A-Sepharose (Amersham Biosciences) in lysis buffer overnight at 4 °C on a spinning wheel. Beads were washed four times with 10 ml of lysis buffer for 15 min on a spinning wheel at 4 °C. Proteins were eluted from the beads with 50 μl of SDS-PAGE loading buffer for 5 min at 95 °C. Proteins were fractionated by 7.5% SDS-PAGE, transferred to PVDF membrane, and probed with anti-ubiquitin monoclonal antibody and HRP-conjugated secondary antibodies followed by enhanced chemiluminescence.
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**TABLE 1**

Yeast strains used in this study

| Strain   | Genotype                          | Source/Laboratory |
|----------|-----------------------------------|-------------------|
| BY4741   | MATA his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | Euroscar          |
| PMY756   | BY4741 HNM1-TAP::HIS3MX6          | Open Biosystems   |
| PMY764   | BY4741 HNM1-GFP::HIS3MX6          | Invitrogen        |
| PMY915   | BY4741 HNM1-TAP::HIS3MX6 chi1Δ::KanMX4 | This study       |
| PMY742   | BY4741 HNM1-TAP::HIS3MX6 pct1Δ::KanMX4 | This study       |
| PMY892   | BY4741 HNM1-TAP::HIS3MX6 chi1Δ::KanMX4 | This study       |
| PMY762   | BY4741 HNM1-GFP::HIS3MX6          | This study        |
| PMY867   | BY4741 HNM1-GFP::HIS3MX6 pctl::KanMX4 | This study       |
| PMY869   | BY4741 HNM1-GFP::HIS3MX6          | This study        |
| PMY888   | BY4741 HNM1-TAP::HIS3MX6 chi1Δ::URAS3MX | This study     |
| PMY908   | BY4741 chi1Δ::KanMX4            | This study        |
| RH448    | MATA leu2 ura3 his4 lys2 bar1    | H. Riezman        |
| RH1597   | MATA leu2 ura3 his4 bar1 end4-i | H. Riezman        |
| SUB62    | MATA his3 leu2 trp1 ura3 (DF5 background) | D. Finley       |
| Y0753    | his3 leu2 lys2 trp1 ura3 (DF5 background) | D. S. Jenkins    |
| LRB341   | MATA his3 leu2 ura3             | L. Robinson       |
| LRB362   | MATA his3 leu2 ura3 yck1-1::URA3 yck2-2a | L. Robinson     |

Microscopy—Yeast cells were grown to mid-log phase at 25 °C in synthetic medium and treated with choline as indicated in the corresponding figure legends. Aliquots (0.5 ml) were withdrawn, and cells were spun down at 500 g for 3 min and resuspended in a small amount of the same medium warmed at 25 °C for visualization. The live cells were mounted on slides and observed using a Zeiss Axiosvert 200 M microscope fitted with a plan-neofluor ×100 oil immersion lens. Images were captured using a Zeiss AxioCam HR using Axiovision version 4.5 software. Staining of vacuolar membranes with FM4-64 or of the cell periphery with calcicfluor white was performed as described (23).

[methyl-14C]Methionine Labeling—Yeast cells were grown in synthetic defined medium containing [methyl-14C]methionine (2500 dpm/nmol) for at least seven generations for steady-state labeling of metabolites derived from the PE methylation pathway. Analyses of radioactivity distribution in aqueous cellular fractions were performed as described previously (9). Most, if not all, of the radioactivity associated with the aqueous fraction was distributed among choline, phosphocholine, glycerophosphocholine, and methionine.

Choline Transport Assay—Cells were harvested, thoroughly washed three times with ice-cold choline-free fresh medium, and resuspended in fresh medium prewarmed at 25 °C. A half-milliliter of 30 μM [14C]choline (~25,000 dpm/nmol) prepared in prewarmed fresh medium was added to 0.5 ml of cell suspension. Uptake was allowed to proceed at 25 °C for 1.5 and 3 min and then stopped by the addition of 5 ml of ice-cold 10 mM choline, 10 mM NaF, 10 mM NaCl. Cells were filtered under vacuum through Whatman GF/C glass fiber filters and rinsed twice with 25 ml of ice-cold Tris-buffered saline containing 2 mM choline. The filters were allowed to dry, and the associated radiolabel was determined by liquid scintillation counting. Under the experimental conditions described above, the amount of choline taken up by cells never exceeded 5% of input.

Choline Mass Assay—Intracellular choline content was estimated enzymatically using choline oxidase coupled to a peroxidase-homovanillic acid detection array. Briefly, cells were grown to mid-log phase and immediately placed on ice. Lipids were extracted using the Folch method (21). The aqueous phase was chromatographed through a column of Dowex AG1-X8 (OH form) developed with water as previously described (21). Choline-containing eluates were concentrated by evaporation under vacuum and resuspended in 10 mM Tris-Cl, pH 8.0, containing 1% (w/v) KCl. To this, 0.2 unit of choline oxidase, 2 units of horseradish peroxidase, and 1 mM homovanillic acid were added. Samples were incubated at 37 °C for 1 h protected from light. Samples were analyzed upon excitation at 315 nm and detected at an emission of 425 nm.

**RESULTS**

**Active Choline Transport Triggers Endocytosis of the Choline Transporter Hnm1**—A functional C-terminal GFP-tagged variant of Hnm1 encoded at the HNM1 locus (Table 2) was used to analyze the stability of Hnm1 protein upon choline exposure by Western blot of protein extracts from cycloheximide-arrested cells. The addition of 200 μM choline substantially reduced the stability of Hnm1-GFP fusion and led to GFP accumulation. The simultaneous addition of 5 mM hemicholinium (a choline analog that blocks choline transport) protected Hnm1 from choline-induced degradation (Fig. 2A) to a level comparable with Hnm1 decay in control cells. Active transport of choline by Hnm1 into the cell appears to be required to decrease Hnm1 level.

We monitored the effect of choline addition on Hnm1-GFP localization by fluorescence microscopy. Intense plasma membrane staining was observed, consistent with the cellular role of Hnm1 as a high affinity transporter for choline (Fig. 2B). After 30 min in the presence of 200 μM choline, a decrease in the intensity at the plasma membrane and the appearance of a few bright spots per cell were evident. At later time points (60 and 90 min), plasma membrane staining was further reduced, whereas internal fluorescence increased. It is known that the GFP fold is relatively resistant to vacuolar degradation. The shift of the fluorescence signal from plasma membrane to round internal compartments is consistent with internalization and vacuolar degradation of Hnm1. Indeed, vacuolar membrane staining with FM4-64 confirmed that Hnm1-GFP was trafficked to the vacuole upon choline exposure (Fig. 2C). Hemicholinium prevented choline-induced delocalization of Hnm1-GFP from the plasma membrane (Fig. 2B), consistent with uptake of choline eliciting a mechanism leading to the internalization and vacuolar degradation of Hnm1.
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Choline uptake rate measurements (Table 3) corroborated these observations. Cells deficient for the Cki1 enzyme took up choline at a low rate under both choline-deficient and -replete conditions. Wild type and cpt1Δ cells exhibited a ~10-fold decrease in the rate of choline uptake in cells grown in the presence of choline compared with those grown in choline-free medium, mirroring their choline-induced change on Hnm1 level and localization. The pct1Δ cells grown in choline-free medium took up choline at 40% of the wild type rate, a drop in transport activity suggested by the reduced level of Hnm1 at the plasma membrane on this mutant strain (Fig. 3, A and B) under the same conditions.

These results taken together indicate that choline-induced Hnm1 degradation is not a response to increased PC synthesis because a block in the CDP-choline pathway did not prevent it. The reduced level of Hnm1 observed in cki1Δ cells grown in choline-free media is consistent with intracellular choline build-up signaling Hnm1 for degradation. A block at the choline kinase not only prevents choline taken up from the medium from being incorporated into PC but also prevents choline generated intracellularly by PC turnover from recycling back into PC (Fig. 1).

In addition to choline, Hnm1 also transports ethanolamine (10, 11). A separate set of enzymes constitutes the CDP-ethanolamine pathway for PE synthesis. We analyzed by Western blot the level of Hnm1-TAP for cells growing in ethanolamine-containing medium (Fig. 3C). Contrary to choline, ethanolamine did not trigger Hnm1 degradation. Similar results were observed for mutant cells deficient in the ethanolamine kinase Eki1, the first enzyme of this pathway. These results highlight the specificity of choline as a metabolite triggering Hnm1 degradation.

Metabolic Labeling of PC Biosynthesis Mutants—We measured the content of choline-containing metabolites in wild type, cki1Δ, pct1Δ, and cki1Δ cells as well as cki1Δ eki1Δ and cki1Δ spo14Δ nte1Δ strains by labeling them at steady state with [14C-methyl]methionine. The radiolabeled methyl group is transferred from S-adenosylmethionine, a downstream metabolite of methionine, to PE during PC synthesis through the PE methylation pathway. PC turnover, in turn, generates choline that is either reused for PC synthesis through the CDP-choline pathway or excreted from the cell (Fig. 1). Strains were grown for 7–8 generations in medium containing radiolabeled methionine to steady state so that metabolite labeling profiles were reflective of metabolite distribution. The absence of Pct1 and Cpt1 elicited the expected metabolic phenotypes: phosphocholine and CDP-choline accumulation for pct1Δ and cki1Δ cells, respectively (Fig. 4A). Surprisingly, different strains carrying a deletion of the Cki1 gene alone or in combination with eki1Δ or spo14Δ nte1Δ inactivated genes did not accumulate an amount of choline comparable with phosphocholine or CDP-choline observed in pct1Δ and cki1Δ cells, respectively. Indeed, the choline level in cki1Δ cells was not different from choline levels measured in wild type cells.

The excretion of choline under blockage of the CDP-choline pathway has been reported previously (25). We assessed excretion of choline in wild type cells and PC synthesis mutants by chasing cells previously labeled to steady state with [methyl-
FIGURE 2. Choline uptake induces choline transporter Hnm1 degradation. A, logarithmically growing wild type cells, expressing a functional Hnm1-GFP variant were treated with 100 μg/ml cycloheximide. After 15 min, choline (0.2 mM) or choline (0.2 mM) plus hemicholinium (5 mM) were added to the cells as indicated. Aliquots were withdrawn from the cultures at the indicated times and processed for immunoblot analysis using antibodies against GFP and against Pgk1. Stationary phase cells, characterized by intense vacuolar and no plasma membrane GFP staining, were included (lane St). B, wild type cells expressing a functional Hnm1-GFP variant were treated or not with choline (0.2 mM) or choline (0.2 mM) plus hemicholinium (5 mM), and at the indicated times, they were observed by fluorescence microscopy. C, wild type cells expressing Hnm1-GFP were exposed to 0.2 mM choline for 2 h. Vacuolar membranes were stained with FM4-64. Cells were observed by fluorescence microscopy. D, wild type cells expressing Hnm1-GFP were exposed to the indicated choline concentrations for 30 min. Cell walls were stained with Calcofluor. Cells were observed by fluorescence microscopy. E, wild type cells were grown to mid-log phase and exposed to different choline concentrations for the indicated times. Choline concentrations are listed on the right and range from 0 to 200 μM. Cells were processed for choline mass determination. Data represent the mean ± S.E. (error bars) of duplicate determination of two independent experiments.
Choline-induced Down-regulation of Hnm1 Involves Endocytosis through a Ubiquitin Ligase Rsp5-mediated Mechanism—

The vacuolar GFP staining observed under choline-rich conditions suggests that Hnm1 plasma membrane level is regulated by endocytic delivery for vacuolar turnover (26–29). We assessed the dependence on endocytosis by looking at Hnm1-GFP trafficking in the endocytic mutant end4-1 strain. END4 encodes a transmembrane actin-binding protein involved in endocytosis, and the product of the allele end4-1 is inactive at 37 °C (30, 31). Upon a shift to the restrictive temperature for the end4-1 allele, the addition of choline did not trigger Hnm1-GFP internalization in the end4-1 strain, whereas in an End4-proficient strain, choline supplementation resulted in Hnm1-GFP trafficking out of the plasma membrane (Fig. 5A).

The ubiquitin ligase Rsp5, among its various functions, regulates the stability of many permeases, targeting them by ubiquitination for endocytosis and vacuolar degradation. The essential character of Rsp5 is associated with the ubiquitin-proteasome-dependent processing of the transcription factor Spt23. Rsp5 is dispensable when yeast cells express a C-terminal truncation of Spt23 (32). We monitored changes in Hnm1-GFP level and localization upon the addition of choline in rsp5/H9004 cells kept alive by the expression of a C-terminally truncated
version of Spt23. Over 3 h of choline exposure, there was a slight decrease in plasma membrane staining and increase in vacuolar lumen staining for rsp5Δ cells, whereas in wild type cells, internal bright spots formed upon choline exposure and plasma membrane localization of Hnm1-GFP declined alongside increasing diffuse vacuolar lumen staining (Fig. 5B). Western blot analysis revealed a strong stabilization of Hnm1-GFP in choline-treated rsp5Δ cells compared with its rapid decrease in wild type cells (Fig. 5C). These results taken together point toward the ubiquitin ligase Rsp5 being responsible for targeting Hnm1 for choline-induced endocytosis and degradation. It is worth highlighting that in rsp5Δ cells, vacuolar membrane staining was evident in all conditions examined (Fig. 5, C and D). It is known that Rsp5 has a role for cargo sorting from late endosomes into multivesicular bodies, and defects in sorting at this step lead to delivery of cargo proteins to the vacuolar membrane (33–35).

We analyzed by Western blotting the ubiquitination of Hnm1 by exposing wild type and rsp5Δ cells expressing Hnm1-GFP to 200 μM choline for 20 min. GFP adducts were immunoprecipitated, separated by SDS-PAGE, and probed for ubiquitin (Fig. 5E). An increased amount of ubiquitination of Hnm1-GFP was evident upon choline exposure for wild type cells, whereas a minor increase was detected for rsp5Δ cells.

We also examined choline transport in the strain defective in casein kinase 1 activity. Inactivation of the casein kinase 1 pair Yck1/Yck2 did not prevent the fast reduction of choline transport upon choline exposure (Fig. 6C). In addition, these results suggest that casein kinase 1 pair Yck1/Yck2 participates in the cascade of events that result in Hnm1 transport from the plasma membrane to the vacuole upon choline exposure.

**FIGURE 5. Rsp5 mediates choline-induced Hnm1 endocytosis and degradation.** A, end4-1 and wild type cells were transformed with a plasmid bearing a GFP-tagged allele of HNM1. Logarithmically growing cells were shifted to 37 °C, and after 15 min, choline (200 μM) was added. At the indicated times, cells were observed by fluorescence microscopy. B and C, rsp5Δ and wild type cells expressing Hnm1-GFP from a plasmid were exposed to 200 μM choline. At the indicated times, cells were processed for fluorescence microscopy (B) and Western blotting (C). D, vacuolar membrane staining with FM4–64 for rsp5Δ cells expressing Hnm1-GFP. E, choline exposure promotes Hnm1 ubiquitination. Yeast cells of the indicated genotypes expressing Hnm1-GFP from a plasmid were exposed or not to 200 μM choline for 20 min. Hnm1-GFP was immunoprecipitated from cell lysates, separated by 7.5% SDS-PAGE, and probed with the indicated antibodies. Molecular weight markers are indicated on the left. DIC, differential interference contrast.
support the notion that a rapid decrease in choline transport occurs independent of targeted endocytosis.

**DISCUSSION**

In this paper, we describe new regulatory mechanisms that control choline uptake. Upon choline exposure, the sole choline transporter in yeast, Hnm1, is endocytosed from the plasma membrane to the vacuole in an Rsp5- and Yck1/2-dependent manner. We also observed a rapid inactivation of Hnm1 transporter activity upon choline exposure that occurred prior to endocytosis.

We show that choline-induced down-regulation of Hnm1 was not a response to high rates of PC synthesis upon choline supplementation. The integrity of the CDP-choline pathway was not necessary for choline to elicit both inactivation and degradation of Hnm1, suggesting that the rate of PC synthesis itself does not regulate choline uptake. Inactivation of the final step in the PC synthesis pathway resulted in an accumulation of CDP-choline and decreased PC synthesis, but the level of Hnm1 at the plasma membrane and its susceptibility to endocytosis were unaffected. Inactivation of the penultimate step resulted in a modest decrease in Hnm1 activity and endocytosis.

The absence of the choline kinase Cki1 decreased choline transport activity. This phenotype arose from the very reduced plasma membrane content of Hnm1. In addition, cki1A cells did not accumulate choline intracellularly but excreted choline into the medium through an Hnm1-independent mechanism. Regulation of intracellular choline concentration seems to be stringent because the choline levels in cki1A cells unexpectedly were not different from those measured for choline kinase-proficient cells. How is Hnm1 targeted for degradation in cki1A if choline levels are not increased? The data suggest that intracellular choline accumulation is not the signal triggering Hnm1 down-regulation. Intracellular choline content did not increase when yeast cells were exposed to 10 μM choline. However, this extracellular choline concentration triggered Hnm1 down-regulation. We show a correlation of concentration around the $K_m$ of Hnm1 for choline transport (0.5 μM), implying that the rate of choline transport can modulate Hnm1 endocytosis. Consistent with the notion that the choline transporter becomes susceptible to degradation upon choline transport is our observation that hemicholinium prevented choline-dependent Hnm1 down-regulation. These observations support a model where Hnm1, when delivering choline into the cytoplasm, acquires a “degradable” conformational state.

Choline has a pivotal role in PC metabolism; it is a precursor for PC synthesis as well as an end product of PC catabolism (Fig. 1). Its dual character contributes to understanding the observation that degradation of Hnm1 is not a response to high rates of PC synthesis through the CDP-choline pathway but merely to choline transport activity. Choline content is the result of a balance between uptake, consumption, generation by PC degradation, and excretion. The existence of an Hnm1-independent mechanism of choline excretion working alongside Hnm1 down-regulation highlights the cellular need to regulate choline content. Unrestricted flow through the CDP-choline pathway would perturb PC metabolism and impact its intercalation with key cellular processes, including vesicle trafficking and regulation of nucleotide levels, because DAG and CTP are both consumed by the CDP-choline pathway (3, 39 – 42).

We show that choline affects Hnm1 endocytosis through the ubiquitin ligase Rsp5. It is highly probable that Rsp5 catalyzes the ubiquitination of Hnm1. First, Hnm1 is resistant to choline-induced degradation in an rsp5Δ mutant strain, consistent with the observed reduction on Hnm1 ubiquitination upon choline exposure. Second, the down-regulation that Hnm1 undergoes...
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upon choline exposure closely resembles the regulation of other yeast transporters by their substrates, for which it has been demonstrated that Rsp5-mediated ubiquitination is necessary to trigger their endocytosis and vacuolar degradation (26, 29).

We also show that casein kinase 1 essential pair Yck1/Yck2 is required for choline-induced Hnm1 endocytosis. This protein kinase is known to be involved in targeting transporters and receptors at the plasma membrane for ubiquitin-mediated endocytosis. Based on the overall conservation of mechanisms and factors involved in the regulated endocytosis of yeast transporters and receptors, we hypothesize that Hnm1 becomes phosphorylated directly or indirectly by Yck1/Yck2, and this event is upstream of Rsp5-mediated ubiquitination of Hnm1.

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