We have characterized the regulation of spermidine transport in yeast and identified some of the genes involved in its control. Disruption of the SPE2 gene encoding S-adenosylmethionine decarboxylase, which catalyzes an essential step in polyamine biosynthesis, upregulated the initial velocity of spermidine uptake in wild-type cells as well as in the polyamine transport-deficient pcp1 mutants. Exogenous spermidine rapidly inactivated spermidine transport with a half-life of 10–15 min via a process that did not require de novo protein synthesis but was accelerated by cycloheximide addition. Conversely, reactivation of spermidine influx upon polyamine deprivation required active protein synthesis. The stability of polyamine carrier activity was increased 2-fold in polyamine-depleted spe2 deletion mutants, indicating that endogenous polyamines also contribute to the down-regulation of spermidine transport. Ligand-mediated repression of spermidine transport was delayed in end3 and end4 mutants that are deficient in the initial steps of the endocytic pathway, and spermidine uptake activity was increased 4- to 5-fold in end3 mutants relative to parental cells, although the stability of the transport system was similar in both strains. Disruption of the NPR1 gene, which encodes a putative Ser/Thr protein kinase essential for the reactivation of several nitrogen permeases, resulted in a 3-fold decrease in spermidine transport in NH4+-rich media but did not prevent its down-regulation by spermidine. The defect in spermidine transport was more pronounced in NH4+-than proline-grown npr1 cells, suggesting that NPR1 protects against nitrogen catabolite repression of polyamine transport activity. These results suggest that (a) the polyamine carrier is an unstable protein subject to down-regulation by spermidine via a process involving ligand inactivation followed by endocytosis and that (b) NPR1 expression fully prevents nitrogen catabolite repression of polyamine transport, unlike the role predicted for that gene by the inactivation/activation model proposed for other nitrogen permeases.

Putrescine and the polyamines spermidine and spermine are ubiquitous molecules required for macromolecular biosynthesis (1, 2), for posttranslational maturation of the essential protein eukaryotic initiation factor-5A (3), and for the control of various ion channel activities (4). Intracellular polyamine pools are actively regulated by de novo synthesis, degradation, excretion, and import from extracellular sources. Polyamine-specific carriers are widely distributed in prokaryotes and eukaryotes (5–7) and can replenish polyamine pools upon inhibition of the biosynthetic enzymes (8–10). Mammalian polyamine transport activity is also acutely controlled by cell cycle events (11, 12) and hormonal stimulation (5, 9, 10, 13, 14). Polyamine transport is a saturable, carrier-mediated and energy-dependent process (5, 15, 16). Although its physiological properties have been extensively studied, the molecular characteristics of the diamine and polyamine carrier proteins have only been elucidated in prokaryotes (7, 17–19).

In the yeast *Saccharomyces cerevisiae*, exogenous polyamines are internalized by specific, saturable plasma membrane uptake system(s) (20–24). Recently, we (22) and others (24) have identified the putative Ser/Thr protein kinase that, as a major determinant of high affinity polyamine transport in yeast. The closely homologous *PTK1* (STK1) gene, which directs a lower affinity, low capacity polyamine transport system, has also been cloned and characterized (22, 23). However, the exact role of these kinases in the regulation of polyamine transport as well as the molecular identity of the putrescine and polyamine carrier(s) in yeast have not yet been determined.

In mammalian cells, polyamine transport activity is tightly regulated by negative feedback mechanisms that depend on intracellular polyamine levels. A marked induction of polyamine transport activity is triggered by polyamine depletion, such as that caused by inhibition of ornithine decarboxylase or S-adenosylmethionine decarboxylase (AdoMetDC) (5, 6, 8–10, 25–28), and rapidly repressed by the addition of exogenous polyamines (9, 26, 29–31). The expression of ornithine decarboxylase antizyme, which is translationally regulated by polyamine levels (32), plays an important role in the acute down-regulation of mammalian polyamine uptake (29–31). However, an exhaustive search in the *Saccharomyces* Genome Data Base has disclosed no antizyme-like gene in yeast, and little is known on the regulation of diamine and polyamine transport in lower eukaryotes. A Ca2+-sensitive gene product has been reported to negatively regulate putrescine transport in Neuros-

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* This work was supported by Grant MT-12551 from the Medical Research Council of Canada (to R. P., M. A., and D. R.), and by a Student’ship from the Fonds Concerté d’Aide à la Recherche/Fonds de la Recherche en Santé du Québec (to I. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ This paper is available on line at http://www.jbc.org

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 4, Issue of January 23, pp. 2109–2117, 1998

ADDED: This article has an accompanying advertisement.

THE Spermidine Transport System Is Regulated by Ligand Inactivation, Endocytosis, and by the Npr1p Ser/Thr Protein Kinase in *Saccharomyces cerevisiae*†

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(Received for publication, September 9, 1997, and in revised form, November 15, 1997)
pura crassa, although its molecular identity is as yet unknown (33).

More recently, we reported that spermidine transport in yeast is subject to negative regulation by substrate availability (22). The aim of the present study was to gain insight into the components and mechanisms responsible for the regulation of polyamine transport in yeast. By using various mutant strains, we now provide evidence that spermidine transport is mediated by an unstable carrier protein that is down-regulated by intracellular polyamines of both endo- or exogenous origins via a mechanism involving ligand-mediated inactivation and endocytosis. In addition, we show that the NPR1 gene, which encodes a Ser/Thr protein kinase structurally related to Ptk1p and Ptk2p, and is required for the derepression of various nitrogen permeases upon transfer to poor nitrogen sources (34, 35), plays an important role in the expression of high-affinity spermidine uptake activity under conditions (e.g., high [NH\textsubscript{3}]) that were previously thought to down-regulate Npr1p activity.

MATERIALS AND METHODS

Strains, Media, and Plasmids—The yeast strains used in this study are listed in Table I and were routinely grown in YPD medium (1% yeast extract, 2% peptone, 2% \( \text{g/l} \) glucose). The minimal medium used to study the influence of nitrogen sources on polyamine transport was made of 2% \( \text{g/l} \) glucose and 0.17% amino acid-free yeast nitrogen base (Difco) without (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and supplemented with the appropriate essential amino acids. This medium was supplemented with either 10 mM \( \text{(NH}_4\text{)}_2\text{SO}_4 \) (minimal \( \text{NH}_4 \) conditions) or \( \text{NH}_2\text{-glucose} \) (minimal \( \text{glucose} \) conditions) to contain 2 mM unlabeled L-citrulline. At predetermined times, the reaction was stopped by adding 1 ml of ice-cold stop buffer (citrate/glucose buffer containing 20 mM MGBG, 5% (w/v) trichloroacetic acid and stored at \(-20^\circ\text{C}\) until chromatographic analysis. After thawing, cell lysates were sonicated in an ultrasonic water bath for 3 cycles of 45 s in an ice-water slurry and microcentrifuged for 5 min at room temperature. Supernatants were filtered through 0.45-\( \mu \text{m} \) filters. Spermidine and Citrulline Uptake Assays—Terminal methylene-\( ^3\text{H} \)Spermidine trihydrochloride (2.65 \( \times \) 10\textsuperscript{4} Ci/mol) was obtained from Amersham Corp. Spermidine uptake assays were performed as previously described (22), using 10 \( \mu \text{M} \) [\( ^3\text{H} \)]Spermidine (50 Ci/mol) as substrate, unless otherwise indicated. Analysis of spermidine uptake characteristics was carried out according to Michaelis-Menten kinetics as described (22). Citrulline transport was measured by incubating 100 \( \mu \text{l} \) yeast cell suspension (5 \( \times \) 10\textsuperscript{7} cells/ml) in citrate/glucose buffer (22) containing 20 mM \( \text{L-}^\text{3H} \)citrulline (57.8 Ci/mol) was purchased from Amersham Corp. Spermidine uptake assays were performed as described previously (22), using 10 \( \mu \text{M} \) [\( ^3\text{H} \)]spermidine (50 Ci/mol) as substrate, unless otherwise indicated. Isolation of polyamine transport-deficient yeast mutants—Previous characterization of polyamine transport-deficient yeast mutants from this laboratory (22) and other laboratories (23, 24) share mutations in the PTK2 gene as a common genetic defect. Such mutants are most likely regulatory mutants, since PTK2 encodes a putative Ser/Thr protein kinase of unknown function and is devoid of transmembrane domains (22, 24). To generate polyamine transport mutants of a different genotype, wild-type DBY747 cells were chemically mutagenized and selected for their ability to grow in the presence of 20 mM MGBG, a toxic polyamine analog (22, 40). One isolate from this selection (DRY405) exhibited a marked defect in spermidine transport (Fig. 1A) as well as a complete lack of specific putrescine uptake (data not shown). Kinetic analysis showed that the deficiency in spermidine uptake activity resulted from a \( \approx 50\% \) decrease in \( V_{\text{max}} \) with no change in the \( K_m \) for transport (Fig. 1B), consistent with a structural mutation in the polyamine permease gene. Moreover, spermidine transport deficiency and growth sensitivity to MGBG could not be restored in DRY405 cells after transformation with the cloned PTK2 and NPR1 genes that are both required for normal polyamine transport (cf. below) (22). Taken together, these observations suggest that DRY405 cells might express a dysfunctional poly-

| Strain | Genotype | Ref. |
|--------|----------|-----|
| DBY747MATA leu2 his3 ura3 trp1 | 70 |
| DBY747spe2 \( \Delta \) | spe2\( \Delta \)MATA leu2 his3 ura3 trp1 \( \Delta \)spe2-5::LEU2 | This study |
| DRY405MATA leu2 his3 ura3 trp1 pcp1 | \( \Delta \)spe2-5::LEU2 pcp1 |
| DRY405spe2 \( \Delta \) | spe2\( \Delta \)MATA leu2 his3 ura3 trp1 \( \Delta \)spe2-5::LEU2 pcp1 |
| RH144-3D | MATA leu2 his3 ura3 bar1-1 ts end3 |
| RH266-1D | MATA leu2 his3 ura3 bar1-1 ts end4 |
| RH268-1C | MATA leu2 his3 ura3 bar1-1 ts end4 |
| 33436c | MATA ura3-1 |
| 21994b | MATA ura3-1 npI-1 |
| 27038a | MATA ura3-1 npI-1 |
| El457 | MATA ade1 leu2-3,112 trp1-289 |
| Stock laboratory |

2 I. Gamache, M. Kaoouas, K. Torossian, D. Ramotar, M. Audette, and R. Poulin, unpublished results.
and the initial velocity of \(^3\text{H}\) spermidine uptake was then determined for 1 min at increasing substrate concentrations. Data represent the means ± S.D. for triplicate determinations from representative experiments.

To assess the relative contribution of preincubation with extracellular spermidine to down-regulate spermidine uptake activity, and specific \(^3\text{H}\) spermidine uptake was then determined. A mutant was found to be recessive, as diploids produced in various genetic backgrounds did not influence spermidine uptake activity. These data suggest that influx of exogenous spermidine by low levels of accumulated spermidine. Putrescine addition had no effect on spermidine transport velocity following incubation in polyamine-free medium suggesting that endogenous polyamines negatively regulate spermidine transport. On the other hand, suppression of polyamine biosynthesis slightly enhanced the rate of spermidine accumulation in \(pcp1\) mutants grown in the presence of spermidine (data not shown), although the initial velocity of uptake remained unaffected (Table II). Moreover, both the initial uptake velocity and rate of spermidine accumulation were markedly enhanced in \(pcp1spe2\Delta\) double mutants grown in polyamine-free medium. Thus, the \(pcp1\) mutation still allows the polyamine-regulated expression of a functional permease with a strongly impaired affinity.

An elevated initial velocity of spermidine transport was generally correlated with a lower spermidine content, either after preincubation in polyamine-free medium or disruption of the AdoMetDC gene (Table II). The latter phenomenon also likely accounts for the decrease in putrescine levels accompanying accumulation of exogenous spermidine. Moreover, intracellular spermidine levels in cells with wild-type polyamine transport activity (\(PCP1\)) grown in the presence of spermidine reached a similar value whether endogenous biosynthesis was functional (\(SPE2\)) or not (\(spe2\)), suggesting that the size of the spermidine pool is adjusted by a common mechanism when an extracellular source of the polyamine is available. However, incubation with exogenous spermidine failed to significantly enlarge \((p > 0.05)\) spermidine content in \(pcp1spe2\Delta\) cells, despite a detectable reduction in putrescine content. In fact, although the size and composition of the putrescine and polyamine pools were not affected by the \(pcp1\) mutation, steady-state spermidine accumulation was 3-6-fold lower in these mutants upon \(SPE2\) inactivation, consistent with a chronic repression of a defective spermidine uptake system by low levels of accumulated spermidine. Putrescine addition had no effect on spermidine transport velocity (data not shown), indicating that fluctuations in putrescine content resulting from the different incubation conditions and genetic backgrounds did not influence spermidine uptake activity. These data suggest that influx of exogenous spermidine strongly down-regulates spermidine transport, whereas endogenous levels of polyamines, but not putrescine, also exert a significant, but much less stringent negative control of spermidine transport. Moreover, marked down-regulation of polyamine uptake activity by exogenous spermidine was accompanied with only minor (e.g. in \(pcp1spe2\Delta\) cells) or even without

| Strain                  | Spermidine Intracellular content | Initial | Velocity of Spermidine uptake |
|-------------------------|----------------------------------|---------|-------------------------------|
|                         | Putrescine | Spermidine | Spermine | pmol/min/10\(^7\) cells |
| Wild-type (\(PCP1\))    | 0        | 42.2 ± 5.5 | 216 ± 60 | 16.3 ± 1.2 | 119 ± 9 |
| \(PCP1\)               | 100      | 3.6 ± 0.7  | 527 ± 30 | 15.9 ± 0.4 | 30 ± 5  |
| \(pcp1\)               | 0        | 108 ± 13  | 21 ± 0.5 | <1           | 224 ± 4 |
| \(PCP1\)               | 100      | 9.0 ± 2.7 | 596 ± 69 | <1           | 38 ± 2  |
| \(pcp1\)               | 0        | 154 ± 28  | 3.1 ± 0.1 | <1           | 165 ± 6 |
| \(PCP1\)               | 100      | 13.2 ± 0.9 | 528 ± 27 | <1           | 21 ± 1  |
| \(pcp1\)               | 0        | 141 ± 19  | 2.0 ± 0.2 | <1           | 49 ± 4  |
| \(PCP1\)               | 100      | 127 ± 17  | 177 ± 21 | <1           | 10 ± 1  |
| \(pcp1\)               | 0        | 43.5 ± 5.8 | 233 ± 30 | 14.5 ± 1.7  | 65 ± 1  |
| \(PCP1\)               | 100      | 12.6 ± 0.7 | 728 ± 16 | 15.3 ± 1.1  | 12 ± 1  |
| \(pcp1\)               | 0        | 101.2 ± 4.5 | 1.5 ± 0.2 | <1           | 93 ± 1  |
| \(PCP1\)               | 100      | 66.3 ± 4.9 | 88.5 ± 3.1 | <1           | 13 ± 1  |

**TABLE II**

Effect of exogenous spermidine on intracellular putrescine and polyamine contents and initial velocity of spermidine uptake in wild-type DBY747 strain as well as in the \(pcp1\) (\(PCP1\)) and \(pcp1\) (\(PCP1\)) mutants. Cells were grown to mid-exponential stage in YPD medium and the initial velocity of \(^3\text{H}\) spermidine uptake was then determined for 1 min at increasing substrate concentrations.
detectable changes (e.g. in pcp1SPE2 cells) in total spermidine content, suggesting that only a minor fraction of the total spermidine pool is required for feedback transport inhibition.

The latter assumption was further confirmed by determining the concentrations of spermidine required to repress polyamine uptake activity (Fig. 2). Following overnight preincubation with increasing concentrations of spermidine, subsequent spermidine uptake activity was inactivated with an IC_{50} = 2.4 ± 0.7 μM, i.e. a value close to the K_{m} of spermidine for transport in S. cerevisiae (4 to 5 μM) (20, 22). This observation is again consistent with a close connection between substrate internalization and the mechanism of transport inactivation triggered by spermidine.

**Down-regulation of Spermidine Transport Does Not Require de Novo Protein Synthesis**—Substrate-mediated down-regulation of spermidine transport could either result from decreased synthesis, increased turnover, or posttranslational inactivation of the polyamine permease. Thus, the effect of exogenous spermidine on the half-life (t_{0.5}) of polyamine uptake activity was examined in wild-type cells in the presence of 10 μg/ml cycloheximide (CHX). Spermidine (100 μM) was added to cells preincubated under polyamine-free conditions to fully derepress spermidine transport, and the rate of decay of spermidine transport activity was then determined. As shown in Fig. 3A, spermidine triggered an immediate and rapid loss of spermidine uptake activity, with a t_{0.5} ~10–15 min, until a new equilibrium value was reached after 1 h. After CHX addition, spermidine uptake activity decayed much more slowly, with a ~35% decrease in velocity observed after 3 h. Furthermore, protein biosynthesis inhibition enhanced the extent of substrate-induced inactivation of spermidine transport without affecting the t_{0.5} of the decay process. Down-regulation of spermidine uptake was not prevented when 5 mM NaN_{3} was added prior to spermidine addition (data not shown), demonstrating that ligand-mediated inactivation of spermidine transport did not directly depend on metabolic energy.

The time course of derepression of spermidine transport was examined by preincubating wild-type cells in the presence of spermidine and then transferring cultures to spermidine-free conditions in the presence or absence of CHX. Spermidine uptake activity increased in a time-dependent manner following transfer to polyamine-free medium, with a 2.5-fold increase observed after 3 h, and a significant derepression already detectable 30 min after the onset of polyamine deprivation (Fig. 3B). Derepression of spermidine uptake absolutely depended on de novo protein biosynthesis since it was completely abolished by CHX. Moreover, a continuous supply of exogenous spermidine was required to fully destabilize the transport system, as shown by the slower rate of decay of spermidine uptake activity noted upon transfer to spermidine-free conditions in the presence of CHX.

**Intracellular Polyamine Depletion Stabilizes the Spermidine Transport System**—Since endogenous polyamine pools also exert a down-regulatory effect on polyamine transport, we determined the decay of spermidine uptake activity after CHX addition in spe2Δ mutants preincubated in polyamine-free medium. As shown in Fig. 4, the spermidine transport system was more stable in the spe2Δ mutants than in the parental strain. Thus, the increase in spermidine transport activity observed upon depletion of endogenous polyamines is clearly associated with a decreased rate of its inactivation, in a manner similar to deprivation of exogenous polyamines.

**Polyamine-induced Down-regulation of Spermidine Transport Partly Depends on Endocytosis**—Several plasma membrane transporters in yeast are cleared from the cell surface by endocytic internalization (43–48). The END3 (49) and END4 genes (50, 51) encode proteins that are involved in actin cytoskeleton organization, and their expression is required at early steps of the endocytic pathway for the internalization of several membrane proteins (52). To assess whether endocytosis is involved in the inactivation and/or degradation of the poly-
amine carrier, we analyzed the stability of this transporter in end3 and end4 mutants that are thermosensitive for growth (52). Whereas the end3 mutants also exhibit a defect in endocytosis at the permissive temperature (25 °C), the genetic lesion of end4 cells is more stringently temperature-dependent (52).

At the permissive temperature, spermidine transport under fully derepressing (i.e. polyamine-free) conditions was 4- to 5-fold higher in end3 cells than in the parental strain (Fig. 5). As expected, no significant difference in spermidine uptake was observed between end4 mutants and wild-type cells at 25 °C, and spermidine transport velocity in wild-type cells was considerably lower than at 30 °C, consistent with a requirement for metabolic energy for the uptake process. To assess the effect of impaired endocytosis on the turnover of the polyamine uptake system, we then determined the t₀.5 of spermidine transport inactivation in end3 and end4 mutants grown at 25 °C under fully derepressing conditions and then transferred to a non-permissive temperature (37 °C) in the presence of CHX. Fig. 6A shows that following deprivation of exogenous polyamines, the initial rate of decay of spermidine uptake activity was similar in end3 mutants and in the parental strain (t₀.5 = 60 ± 12 and 55 ± 9 min, respectively) but was notably accelerated in end4 mutants (t₀.5 = 21 ± 6 min). Thus, the increased spermidine uptake activity observed in end4 mutants is clearly not associated with a lower rate of inactivation of the polyamine transport system, and in fact, the defect in endocytosis present in the end4 mutants decreases the stability of this carrier.

It has been shown that substrate-mediated inactivation and degradation of yeast plasma membrane carriers such as the copper transporter Ctr1p (53) occur via a mechanism that does not require prior endocytic internalization. To determine whether spermidine can inactivate the polyamine carrier system independently from endocytosis, we first deprived end3 and end4 mutants and wild-type cells from exogenous polyamines at 25 °C, then transferred cultures for 10 min into spermidine-containing medium at the non-permissive temperature to initiate the inactivation process, and finally transferred cells to polyamine-free medium in the presence of CHX while continuously monitoring the time course of spermidine transport velocity. Spermidine rapidly triggered the decay of spermidine uptake activity in the parental strain, and spermidine removal slowed down but did not prevent the time-dependent inactivation of spermidine transport (Fig. 6B). Moreover, although the initial rate of inactivation of spermidine transport was significantly lower in the end3 and end4 mutants immediately after spermidine addition, a steady decay of sper-
Polyamine Transport Regulation in Yeast

An unexpected finding from the above experiments was that the lack of NPR1 expression in a NH₄⁺-rich medium (i.e. 38 mM (NH₄)₂SO₄) strongly depressed spermidine transport activity, although under the same conditions, Gap1p activity is reduced to almost undetectable levels in an NPR1-independent manner (34, 35, 54). We indeed verified that Gap1p activity (as measured by the rate of [¹⁴C]citrulline transport) was dramatically up-regulated upon transfer from NH₄⁺ to proline as sole nitrogen sources for growth (data not shown). We next determined the effect of NCR on spermidine uptake activity in wild-type and npr1 cells preincubated in polyamine-free minimal medium containing either NH₄⁺ or proline as sole nitrogen sources. Spermidine transport in wild-type cells was clearly not repressed by growth on NH₄⁺ and was in fact reproducibly higher than that measured in proline-grown cells (Fig. 8). Interestingly, the differential effect of the npr1 mutation on spermidine uptake was much more pronounced in NH₄⁺ than proline-grown cells. These results indicate that the spermidine transport system is resistant to NCR and that NPR1 expression might in fact protect this transporter against an NH₄⁺-dependent inactivating mechanism.

One such inactivating mechanism could obviously be the action of the ubiquitin-protein ligase Npl1p, which targets several yeast membrane transporters for degradation through the endocytosis-vacuolar pathway (34, 57, 59–61). Although NPI1 is an essential gene (57), partly Npl1p-deficient mutants exhibit reduced rates of endocytosis and ubiquitination of target proteins such as the uracil permease Fur4p (60) and a pleiotropic up-regulation of amino acid permease activity under NH₄⁺-repressing conditions (34, 61). Thus, if spermidine transport is under NH₄⁺-induced repression by Npl1p in a manner similar to Gap1p, polyamine uptake activity should be up-regulated in npi1 mutants. However, there was no difference in spermidine transport between wild-type and npi1 cells under
Amines affect polyamine permease stability in a qualitatively permease. However, the fact that intra- and extracellular poly-site or lies in a different, extracellular domain of the polyamine stabilization by spermidine is identical to the substrate binding possibility that the regulatory site responsible for carrier de-

Mixing with the endogenous pool. This interpretation would

That the underlying mechanism involves a rapid and direct regulation exerted by exogenous spermidine might indicate

Acids and free nucleotides, a factor that could reduce their free

Port system has been described in yeast (63). Furthermore, due

To their mixed polycationic/aliphatic character, intracellular

Compartmentalization has yet to be investigated in

Like for mammalian polyamine transport (5, 6, 8–10, 25–31), high affinity spermidine uptake activity in yeast is clearly under negative control by polyamines of both endogenous and exogenous origins. However, extracellular spermidine quantitatively exerts a more stringent feedback inhibition on its own high affinity transport than the endogenous pool. Likewise, deprivation of exogenous polyamines had a disproportionately large stimulatory effect on spermidine transport as compared with polyamine depletion due to a genetic block in endogenous synthesis, as evidenced in pcp1::spe2Δ double mutants. The weaker feedback inhibition of spermidine transport exerted by endogenous polyamines could be due to sequestration of de novo synthesized polyamines in the vacuole, as well as to binding to macromolecular polyanions. Although subcellular polyamine compartmentalization has yet to be investigated in S. cerevisiae, it has been estimated that at least 25% of total cellular spermidine is confined to the vacuole in N. crassa (62), and a vacuolar, H+–ATPase-dependent active polyamine trans-

Some features of the rapid repression elicited by spermidine on polyamine transport are reminiscent of the ligand inactiva-
tion mechanism recently proposed for the down-regulation of the inositol permease Itr1p (45) and the copper transporter Ctr1p by their substrate (53). The rate of degradation of these carriers is dramatically enhanced upon exposure to their substrate via a feedback mechanism that likely involves direct ligand binding to an allosteric site on the exo- or endofacial side of the carrier (45, 53). However, spermidine-mediated down-regulation of the polyamine permease exhibits features that are intermediary between the ligand inactivation mechanisms respectively described for the Itr1p and Ctr1p carriers. Endocytosis has been shown to form an integral part of the inactivation/degradation mechanism elicited by inositol binding to Itr1p, since end3Δ and end4Δ mutants are spared from ligand-mediated inactivation of Itr1p after removal of inositol from the medium (45). On the other hand, Ctr1p undergoes copper-induced degradation while still inserted in the plasma membrane and does not require endocytic internalization for its down-regulation (53). In contrast, we have observed that spermidine-mediated inactivation of polyamine transport is only delayed by defective endocytosis, suggesting that spermidine triggers the destabilization process independently from internalization of the polyamine permease and that ligand inactivation might in fact accelerate the endocytic degradation of the transporter. Moreover, deficient endocytosis did not decrease the basal rate of turnover of the polyamine uptake system, the t0.5 of spermidine uptake activity being even shorter in end4Δ mutants. A role for endocytosis in the regulation of polyamine transport is nonetheless suggested by the fact that spermidine uptake was strongly up-regulated in end3Δ mutants grown at the permissive temperature. The marked instability of the yeast polyamine uptake system would predict that a high rate of insertion and endocytic removal of carrier molecules into and from the plasma membrane is required to maintain adequate transport activity. Since the t0.5 of the transport system was not affected by the end3Δ mutation, increased spermidine uptake activity in end3Δ cells could result from defective recycling of functional polyamine permease molecules at the endocytic internalization step, resulting in a steady-state increase in the number of active spermidine carriers in the plasma membrane.

Our results are thus consistent with a model where spermi-
dine controls the rate of degradation of the polyamine permease via a posttranslational, ligand inactivation mechanism that destabilizes the carrier. The polyamine permease appears to turn over rapidly, and up-regulation of spermidine transport clearly depends on de novo protein biosynthesis, either because derepression reflects an increase in the number of polyamine permease molecules or because a spermidine-repressed, essential reactivator is induced following polyamine deprivation. Upon its binding to an allosteric site, spermidine accelerates the rate of endocytic internalization of functional transporters, apparently without an involvement of the Npf1p ubiquitin-protein ligase that controls the endocytic degradation of several

DISCUSSION

Like for mammalian polyamine transport (5, 6, 8–10, 25–31), high affinity spermidine uptake activity in yeast is clearly under negative control by polyamines of both endogenous and exogenous origins. However, extracellular spermidine quantitatively exerts a more stringent feedback inhibition on its own high affinity transport than the endogenous pool. Likewise, deprivation of exogenous polyamines had a disproportionately large stimulatory effect on spermidine transport as compared with polyamine depletion due to a genetic block in endogenous synthesis, as evidenced in pcp1::spe2Δ double mutants. The weaker feedback inhibition of spermidine transport exerted by endogenous polyamines could be due to sequestration of de novo synthesized polyamines in the vacuole, as well as to binding to macromolecular polyanions. Although subcellular polyamine compartmentalization has yet to be investigated in S. cerevisiae, it has been estimated that at least 25% of total cellular spermidine is confined to the vacuole in N. crassa (62), and a vacuolar, H+–ATPase-dependent active polyamine transport system has been described in yeast (63). Furthermore, due to their mixed polycationic/aliphatic character, intracellular polyamines may largely exist as complexes bound to nucleic acids and free nucleotides, a factor that could reduce their free fraction to less than 10% (64). Thus, the more efficient down-regulation exerted by exogenous spermidine might indicate that the underlying mechanism involves a rapid and direct allosteric interaction with the polyamine permease, without mixing with the endogenous pool. This interpretation would account for the closely similar values of the $K_m$ of spermidine for high affinity uptake and the $IC_{50}$ of exogenous spermidine for transport repression. The present data cannot rule out the possibility that the regulatory site responsible for carrier de-

Stabilization by spermidine is identical to the substrate binding site or lies in a different, extracellular domain of the polyamine permease. However, the fact that intra- and extracellular poly-

Amines affect polyamine permease stability in a qualitatively similar manner suggests that the allosteric site for ligand in-

In mammalian cells, polyamine depletion up-regulates poly-

Some features of the rapid repression elicited by spermidine on polyamine transport are reminiscent of the ligand inactiva-
tion mechanism recently proposed for the down-regulation of the inositol permease Itr1p (45) and the copper transporter Ctr1p by their substrate (53). The rate of degradation of these carriers is dramatically enhanced upon exposure to their substrate via a feedback mechanism that likely involves direct ligand binding to an allosteric site on the exo- or endofacial side of the carrier (45, 53). However, spermidine-mediated down-regulation of the polyamine permease exhibits features that are intermediary between the ligand inactivation mechanisms respectively described for the Itr1p and Ctr1p carriers. Endocytosis has been shown to form an integral part of the inactivation/degradation mechanism elicited by inositol binding to Itr1p, since end3Δ and end4Δ mutants are spared from ligand-mediated inactivation of Itr1p after removal of inositol from the medium (45). On the other hand, Ctr1p undergoes copper-induced degradation while still inserted in the plasma membrane and does not require endocytic internalization for its down-regulation (53). In contrast, we have observed that spermidine-mediated inactivation of polyamine transport is only delayed by defective endocytosis, suggesting that spermidine triggers the destabilization process independently from internalization of the polyamine permease and that ligand inactivation might in fact accelerate the endocytic degradation of the transporter. Moreover, deficient endocytosis did not decrease the basal rate of turnover of the polyamine uptake system, the t0.5 of spermidine uptake activity being even shorter in end4Δ mutants. A role for endocytosis in the regulation of polyamine transport is nonetheless suggested by the fact that spermidine uptake was strongly up-regulated in end3Δ mutants grown at the permissive temperature. The marked instability of the yeast polyamine uptake system would predict that a high rate of insertion and endocytic removal of carrier molecules into and from the plasma membrane is required to maintain adequate transport activity. Since the t0.5 of the transport system was not affected by the end3Δ mutation, increased spermidine uptake activity in end3Δ cells could result from defective recycling of functional polyamine permease molecules at the endocytic internalization step, resulting in a steady-state increase in the number of active spermidine carriers in the plasma membrane.

Our results are thus consistent with a model where spermi-
dine controls the rate of degradation of the polyamine permease via a posttranslational, ligand inactivation mechanism that destabilizes the carrier. The polyamine permease appears to turn over rapidly, and up-regulation of spermidine transport clearly depends on de novo protein biosynthesis, either because derepression reflects an increase in the number of polyamine permease molecules or because a spermidine-repressed, essential reactivator is induced following polyamine deprivation. Upon its binding to an allosteric site, spermidine accelerates the rate of endocytic internalization of functional transporters, apparently without an involvement of the Npf1p ubiquitin-protein ligase that controls the endocytic degradation of several
yeast transporters (57, 60). The observed kinetics of repression/derepression of polyamine transport is consistent with a predominantly posttranslational control, as is the case for the regulation of yeast ornithine decarboxylase by spermidine (20, 43). Without appropriate molecular tools, we cannot currently discriminate between the possibilities that ligand inactivation of the polyamine permease involves its proteolytic degradation, conformational changes that irreversibly inhibit its activity as a result of allosoteric interaction, and/or spermidine-regulated posttranslational modifications.

In connection with the latter hypothesis, we have explored the possibility that polyamine transport might be regulated by the NPR1-dependent inactivation/reactivation system governing the posttranslational control of several nitrogen permeases (34, 35). We have indeed found that NPR1 expression promotes spermidine transport, but its inactivation does not abolish high affinity spermidine uptake nor prevent its ligand-induced down-regulation, as noted for ptk2::lacZ disruption mutants (22). However, inactivation of both PTK1 and PTK2 was sufficient to fully suppress high affinity spermidine transport and feedback transport repression (22). Thus, although Npr1p, Ptk1p, and Ptk2p all encode putative, homologous Ser/Thr protein kinases, Npr1p action clearly requires functional PTK1 and/or PTK2 genes. Surprisingly, spermidine transport was strongly depressed in npr1 mutants under conditions (i.e. high [NH₄⁺]) where Npr1p activity has been predicted to be down-regulated by NCR (34, 35). In fact, the npr1 mutation does not further decrease the already low activity of the Gap1p or Put4p permeases in NH₄⁺-rich media, although it up-regulates their expression at the mRNA level (34, 38, 54, 65, 66). Moreover, our data clearly demonstrate that the spermidine transport system is not subject to NCR, and if anything, NH₄⁺ would rather be a weak inducer of spermidine transport.

Obviously, the model of inactivation/reactivation of nitrogen permeases by NCR as originally proposed (34, 35) is not compatible with the observed mechanism of regulation of polyamine transport. In that model, NPR1-regulated permeases such as Gap1p are constitutively degraded via a ubiquitin-dependent pathway involving the NPI1 and NPR1 genes, and rich nitrogen sources such as NH₄⁺ and l-glutamine down-regulate Npr1p expression at a posttranscriptional level (34, 35, 38). This level of regulation by nitrogen catabolites was suggested to involve the increased turnover of Npr1p, which, like Ptk1p and Ptk2p (22), has strong PEST sequences (67) that might signal its rapid degradation. However, no report is yet available on the regulation of Npr1p protein levels nor catalytic activity by NCR. Our data show that NH₄⁺ certainly does not impair NPR1 expression as pertaining to spermidine transport regulation. If Npr1p activity per se is not down-regulated by NH₄⁺, reactivation of nitrogen permeases by NPR1 should thus involve some downstream, NCR-antagonized target of Npr1p. The simplest case would be that NPR1-dependent permeases require phosphorylation for their activity and/or stability and that NCR involves the posttranslational activation of a phosphatase directed to phosphorylated, active permeases. In support of this hypothesis, l-glutamine down-regulates Gap1p through its rapid dephosphorylation, a modification that inactivates the permease and promotes its degradation (68). There are precedents for a role of Ser/Thr protein phosphatases in the control of membrane transport in yeast. For instance, protein phosphatase 2B (or calcineurin) causes the switch of the Trk1p K⁺ transporter from a low to a high affinity mode of K⁺ transport upon Na⁺ stress (69).

Although spermidine transport is regulated by Npr1p, it is clearly resistant to NCR and is not inactivated via an NPI1-catalyzed pathway. The Mep1p permease responsible for low affinity, high capacity NH₄⁺ uptake is another NPR1-dependent, NPR1-independent nitrogen permease but is nonetheless subject to NCR (55, 56). Thus, there is no obligatory link between NPR1, NCR, and the NPR1-dependent pathway of ubiquitination/degradation in the control of nitrogen permease activity. That Npr1p deficiency had a substantially larger effect on spermidine transport in NH₄⁺- than proline-based medium suggests that the putative kinase could in fact protect the polyamine transport system against NCR. Why would Npr1p expression confer resistance of spermidine transport to NCR but not for other Npr1p-dependent permeases? One possibility is that Npr1p regulates the expression and/or activity of intermediary factors specifically targeting the spermidine uptake system such as Ptk1p and Ptk2p, and that NPR1 function requires intact PTK1 and PTK2 genes. We have indeed found that ptk2::lacZ disruption mutants exhibit phenotypic responses similar to npr1 cells in response to nitrogen sources.² In fact, any of the Ser/Thr protein kinases known to affect spermidine transport could either activate the polyamine permease through phosphorylation, transcriptional induction, and/or inactivation of a putative permease phosphatase. The ongoing molecular identification of the PCP1 gene should help to elucidate the unique features of spermidine transport regulation in yeast.

Acknowledgments—We acknowledge the expert assistance of Sylvie Pilote for high pressure liquid chromatography polyamine analysis and of Guy Chouinard in the construction of the pYES-PTK2 expression vector and in complementation analysis of the pop1 mutants. We are grateful to Dr. Celia W. Tabor for the generous gift of the SPE2 gene knock-out construct and to Dr. Howard Reisman for providing the end3 and end4 mutant strains. We are grateful to Dr. Brice Legendre for providing the npi1 and npr1 mutants and the pMV33 plasmid as well as for helpful discussions during the preparation of this manuscript.

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