Rapamycin-induced Gln3 Dephosphorylation Is Insufficient for Nuclear Localization

Sit4 AND PP2A PHOSPHATASES ARE REGULATED AND FUNCTION DIFFERENTLY*

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Gln3, the major activator of nitrogen catabolite repression (NCR)-sensitive transcription, is often used as an assay of Tor pathway regulation in Saccharomyces cerevisiae. Gln3 is cytoplasmic in cells cultured with repressive nitrogen sources (Gln) and nuclear with derepressive ones (Pro) or after treating Gln-grown cells with the Tor inhibitor, rapamycin (Rap). In Rap-treated or Pro-grown cells, Sit4 is posited to dephosphorylate Gln3, which then dissociates from a Gln3-Ure2 complex and enters the nucleus. However, in contrast with this view, Sit4-dependent Gln3 dephosphorylation is greater in Gln than Pro. Investigating this paradox, we show that PP2A (another Tor pathway phosphatase)-dependent Gln3 dephosphorylation is regulated oppositely to that of Sit4, being greatest in Pro- and least in Gln-grown cells. It thus parallels nuclear Gln3 localization and NCR-sensitive transcription. However, because PP2A is not required for nuclear Gln3 localization in Pro, PP2A-dependent Gln3 dephosphorylation and nuclear localization are likely parallel responses to derepressive nitrogen sources. In contrast, Rap-induced nuclear Gln3 localization absolutely requires all four PP2A components (Pph21/22, Tpd3, Cdc55, and Rts1). In pph21Δ22Δ, tpd3Δ, or cdc55Δ cells, however, Gln3 is dephosphorylated to the same level as in Rap-treated wild-type cells, indicating Rap-induced Gln3 dephosphorylation is insufficient to achieve nuclear localization. Finally, PP2A-dependent Gln3 dephosphorylation parallels conditions where Gln3 is mostly nuclear, while Sit4-dependent and Rap-induced dephosphorylation parallels those where Gln3 is mostly cytoplasmic, suggesting the effects of these phosphatases on Gln3 may occur in different cellular compartments.

Target of Rapamycin (Tor)2 is widely acknowledged as a global regulator whose actions control and integrate an array of cellular processes ranging from transcription to cell division (1–4). One of the principal roles of Tor is participation in the adjustment of cell metabolism and cell biology to intra- and extracellular nutrient fluctuations. Investigation of the Saccharomyces cerevisiae Tor proteins (Tor1 and Tor2) has added much to our understanding of their mammalian homologue (mTor) (1). Central to many of these investigations has been the GATA-binding transcriptional activator, Gln3, well known for its control of nitrogen catabolite repression (NCR)-sensitive transcription (5–9).

Nitrogen Catabolite Repression in S. cerevisiae—NCR is the regulatory mechanism through which S. cerevisiae responds to environmental nitrogen availability and selectively utilizes good nitrogen sources in preference to poorer ones (5–9). It permits cells to rapidly and successfully cope with nutritional changes ranging from starvation after a rain storm to luxurious nitrogen excess when situated in decaying fruit. NCR-sensitive regulation is achieved by controlling the action of Gln3. When cells are growing in the presence of sufficient quantities of a good nitrogen source (e.g. glutamine), Gln3 is restricted to the cytoplasm; an outcome that requires the negative regulator, Ure2. This prevents Gln3 from binding to its target sites in the promoters of NCR-sensitive genes (10–12). Hence the transcription of these genes, whose products are required to transport and catabolize poor nitrogen sources, is repressed. At the opposite end of the nutrient spectrum, when cells are growing in the presence of poor sources (e.g. proline) or there are insufficient quantities of good nitrogen sources available, Gln3 is nuclear and bound to NCR-sensitive promoters (10–12). As a result, NCR-sensitive transcription is greatly derepressed. It is this derepressed transcription of NCR-sensitive genes that permits the cell to scavenge its environment more broadly for alternative poor nitrogen sources until more favorable conditions again prevail. Between these extremes are a variety of additional nitrogen sources, which can be roughly categorized as good (e.g. ammonia, serine, high concentrations of urea) or intermediate (glutamate or aspartate) (13).

Tor Pathway Regulation of Gln3 and NCR-sensitive Transcription—A connection between the Tor pathway (1, 14–25) and NCR-sensitive transcription was established quite unexpectedly by results from several transcriptome analyses in which Tor activity was specifically inhibited by the clinically important drug, rapamycin (15, 17–19). These and other studies laid the groundwork for development of an engaging molecular model explaining how NCR was achieved (Fig. 1A) (16). The seminal findings were four important correlations in which rapamycin treatment of cells provided with a good nitrogen...
source elicited: (i) Gln3 dephosphorylation, (ii) Gln3 nuclear localization, (iii) increased NCR-sensitive transcription, and (iv) a strong Sit4 requirement for all three processes (15–19). According to the model and its subsequent refinements (Fig. 1A), under conditions of nitrogen excess, Tor (now known to be the protein complex TorC1) phosphorylates Tap42 (Tor-associated protein) thereby promoting its association with Sit4 (14–16, 20, 21, 23–25). The Tap42-Sit4 complex, bound to TorC1, is inactive and hence unable to dephosphorylate Gln3 (15, 16, 21, 24, 25). This, in turn, prevents a phosphorylated Gln3-Ure2 complex from dissociating. When Tor kinase activity is inhibited by limiting nutrients or treating cells with rapamycin, the Tap42-Sit4 complex is released from TorC1, thereby becoming active until Tap42 and Sit4 themselves dissociate from one another (25). Active Tap42-Sit4 complex dephosphorylates Gln3, which brings about its dissociation from Ure2, thereby permitting free Gln3 to enter the nucleus and activate NCR-sensitive transcription (15, 16, 22, 23, 25).

For the past several years, we have been investigating Tor regulation of Gln3 by testing predictions that emanate from the above model. Observations and conclusions from experiments in our laboratory and others that have generated the questions we address here are briefly summarized in the next two sections.

Does Rapamycin Treatment Faithfully Mimic a Condition of Limiting Nitrogen?—A key tenant of the Tor model described above is that Tor itself or some molecule preceding it in the regulatory pathway receives the signal of nitrogen excess or limitation. Tor-mediated events then implement the response to that signal. In the case of nitrogen limitation, implementation can be mimicked by treating cells with rapamycin (16, 26). Thus, the signal generated by nitrogen limitation or excess has been thought to enter the Tor regulatory pathway at or above the level of events triggered by rapamycin treatment (Fig. 1A).

We, on the other hand, have been led to an alternative view that rapamycin treatment does not congruently mimic nitrogen limitation and that the dominant nitrogen signal controlling Gln3 enters the Tor regulatory pathway below the level of rapamycin action or enters via another regulatory pathway (9, 27, 28). This view derives from four observations. (i) Rapamycin-induced CIT2 transcription is nitrogen source-dependent. It occurs in ammonia but not proline- or glutamate-grown cells, arguing that the nitrogen signal dictates whether or not rapamycin treatment will increase CIT2 transcription (27, 28). (ii) Gln3 phosphorylation and intracellular localization in nutrient limitation and starvation differ from those generated by rapamycin treatment (29, 30). (iii) Latrunculin, an inhibitor of actin polymerization, inhibits nuclear Gln3-Myc13 localization and NCR-sensitive transcription following transfer of cells from ammonia to proline medium, but not after rapamycin addition (31). (iv) Nuclear localization of Gln3 in proline-grown but not rapamycin-treated cells requires participation of Golgi-to-endosome trafficking proteins (32). Parenthetically, the latter report confirms our earlier conclusion that “movement of Gln3 and Gat1 into and out of the nucleus, like that of Rtg3 another phosphorylated transcription factor reported to be regulated by the Tor1,2 signal transduction pathway, involves the participation of a general protein trafficking pathway” (30).

Sit4 and PP2A Phosphatase Participation in the Tor Regulatory Pathway and Control of Gln3—Another key feature of the Tor model are the prominent roles of type 2A (PP2A) and type 2A-related (Sit4) protein phosphatases (1). Both phosphatases have been previously thought to be regulated similarly with respect to nutrient supply and also to respond similarly to rapamycin treatment (Fig. 1A and B) (14, 20). PP2A and Sit4 consist...
of catalytic subunit(s) (redundant Pph21/22 for PP2A or Sit4), associated with proteins that regulate their specificities and/or activities (14, 20). Phosphorylated Tap42 binds to Pph21/ Pph22 and Sit4, whereas Tpd3 plus Cdc55 or Rts1 bind only to Pph21/22 (14, 20). The PP2A complexes are thought to possess different target specificities (20).

The centrality of Sit4 phosphatase to Tor regulation and hence directly or indirectly to NCR prompted us to analyze its effects on Gln3 first. We found that Sit4 is required for nuclear Gln3-Myc$^{13}$ localization in proline-grown or rapamycin-treated glutamine-grown cells, confirming multiple earlier reports performed with cells growing in complex rich media (33). A paradoxical result, however, emanated from analysis of Sit4-dependent Gln3-Myc$^{13}$ dephosphorylation (33). If Sit4 occupies the position posited in the Tor model (Fig. 1A), one would expect it to be most active under conditions in which Gln3 is most dephosphorylated and nuclear (in proline-grown cells) and least active when Gln3 is most phosphorylated and nuclear (in glutamine-grown cells). Experiments with rapamycin treatment, Msx treatment increased Gln3-Myc$^{13}$ dephosphorylation and intracellular localization in much the same way as we did in our earlier studies of Sit4 (33).

Involvement of PP2A (Pph21/22) in the regulation of NCR-sensitive transcription was discovered only recently by the demonstration that the phosphatase was required for rapamycin treatment or growth with a poor nitrogen source. Moreover, in contrast with rapamycin treatment, Msx treatment increased rather than decreased Gln3-Myc$^{13}$ phosphorylation (34). In sum, the above information about Sit4 did not comfortably fit within the model presented in Fig. 1A. Therefore we turned our attention to the PP2A phosphatase.

**TABLE 1**

| Strain  | Pertinent genotype | Back-ground | Parent | Complete genotype | Primer |
|---------|--------------------|-------------|--------|-------------------|--------|
| TB123   | W.T. Gln3-Myc$^{13}$ | TB          | MATa   | leu2-3,112, rma3-52, rts1, trp1, his4, GAL+, HMLa, GLN3-Myc$^{13}$[KanMX] | None   |
| TB136-2a| sit4Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, GAL+, HMLa, GLN3-Myc$^{13}$[KanMX], sit4:kanMX | None   |
| 03666c  | sit4Δpph21Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, GLN3-Myc$^{13}$[KanMX], sit4:kanMX, pph21::kanMX | None   |
| FV42    | pph21Δ | TB          | Jk9-3da| MATa, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, Gln3-Myc$^{13}$[KanMX], pph21::kanMX | None   |
| FV43    | pph22Δ | TB          | Jk9-3da| MATa, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, Gln3-Myc$^{13}$[KanMX], pph22::kanMX | None   |
| 03643c  | sit4Δpph22Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, GLN3-Myc$^{13}$[KanMX], sit4:kanMX, pph22::kanMX | None   |
| 03655d  | pph21Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], pph22::kanMX | None   |
| 03644a  | pph22Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], pph22::kanMX | None   |
| 03705d  | pph21Δpph22Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], pph22::kanMX | None   |
| FV177   | tep3Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], tep3::NatMX | None   |
| FV206   | cdc55Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], tep3::NatMX | None   |
| FV209   | rts1Δ Gln3-Myc$^{13}$ | TB          | MATa   | sit4Δ, leu2-3,112, rma3-52, rts1, trp1, his4, HMLa, GLN3-Myc$^{13}$[KanMX], rts1::NatMX | None   |
cells. Therefore, we conclude that Sit4-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is not sufficient to elicit its nuclear localization, which is a central tenet of the model in Fig. 1A. (iii) Rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization strongly requires PP2A especially in glutamine-grown cells, whereas similar Gln3-Myc\textsuperscript{13} localization in response to growth with a poor nitrogen source does not.

**MATERIALS AND METHODS**

*Strains and Culture Conditions*—*S. cerevisiae* strains used in this work are listed in Table 1. Growth conditions were identical to those described in Tate et al. (33). Cultures (50 ml) were grown to mid-log phase (\(A_{600 nm} = 0.5\)) in YNB minimal medium containing the indicated nitrogen source at a final concentration of 0.1%. Allantoin was provided at 0.2%. Appropriate supplements (120 \(\mu g/ml\) leucine, 20 \(\mu g/ml\) uracil, 20 \(\mu g/ml\) histidine, tryptophan, and arginine) were added to the medium as necessary to cover auxotrophic requirements. Where indicated, cells were treated with 200 ng/ml rapamycin for 20 min as described earlier (33).

*Western Blot Analyses*—Western blot analyses were performed using a combination of methods described by Cox et al., Tate et al., and Liu et al. (29, 34, 37). Western blot results were recorded on Kodak BioMax XAR film. A wide range of exposures were collected for each sample and gamma settings of the final images uniformly changed to ensure that no minor bands were lost and that the final images reproduced the x-ray films as closely as possible.

*Indirect Immunofluorescence Microscopy*—Cell collection and immunofluorescent staining were performed as previously described (12, 30, 33, 38). Unless indicated otherwise, cells were fixated by addition of 0.55 ml of 1 m potassium phosphate buffer (pH 6.5) and 0.5 ml of 37\% formalin to a 5-ml aliquot of the culture to be assayed. This was followed by incubation for 60–70 min. at 30 °C. Cells were imaged using a Zeiss Axioplan 2 imaging microscope with a \(\times 100\) Plan-Apochromat 1.40 oil objective at room temperature. Images were acquired using a Zeiss Axio camera and AxioVision 3.0 (Zeiss) software, and processed with Adobe Photoshop and Illustrator programs. Gamma settings were altered where necessary to avoid any change or loss in cellular detail relative to what was observed in the microscope; changes were applied uniformly to the image presented.

* Determination of Intracellular Gln3-Myc\textsuperscript{13} Distribution*—More comprehensive descriptions of Gln3-Myc\textsuperscript{13} localization were achieved by manually scoring Gln3-Myc\textsuperscript{13} localization in 200 or more cells in multiple, randomly chosen fields from which each image presented was taken. Scoring was performed exclusively using unaltered primary image files viewed with Zeiss AxioVision 3.0 and 4.6.3 software.

Cells were classified into one of three categories: cytoplasmic (cytoplasmic Gln3-Myc\textsuperscript{13} fluorescence only), nuclear-cytoplasmic (Gln3-Myc\textsuperscript{13} fluorescence appearing in the cytoplasm as well as co-localizing with DAPI-positive material), and nuclear (Gln3-Myc\textsuperscript{13} fluorescence co-localizing only with DAPI-positive material). A representative collection of “standard” images demonstrating the differences in these categories is shown in Fig. 2. The intracellular distribution of Gln3-Myc\textsuperscript{13} in panels 4–6 is clearly different from that in panels 1–3 or 9 and 10. It is for this reason that we use three-category scoring in contrast to scoring Gln3-Myc\textsuperscript{13} localization as cytoplasmic or nuclear. In the first report that control of intracellular Gln3-Myc\textsuperscript{13} localization was associated with a cytoplasmic membranous system, Gln3-Myc\textsuperscript{13} fluorescence was shown to be contained within or attached to tubular structures extending out from the nucleus (30, 31). Because this fluorescence did not co-localize with DAPI-positive material, but was either partially within the nucleus or in its immediate proximity, we classified cells with these characteristics as nuclear-cytoplasmic. Arrows in panels 4–7 point out instances areas where Gln3-Myc\textsuperscript{13} is in proximity of the nucleus, but does not completely co-localize with DAPI-positive material (Fig. 2). Higher resolution examples of this appear in Fig. 11 of Ref. 31, Figs. 7–9 of Ref. 30, and Fig. 4 of Ref. 34.

Scoring precision has been described in detail by Tate et al. (33, 38, 39). However, we emphasize again as earlier (39) that the nuclear-cytoplasmic category is, of necessity, somewhat arbitrary because scoring is not an objective instrument-based measurement. Placing cells in the nuclear-cytoplasmic category is based on subjective visual evaluation of the degree to which co-localization does or does not occur for a cell to be scored as cytoplasmic versus nuclear-cytoplasmic versus nuclear. When the fluorescent signal is not clearly restricted to a single cellular compartment (Fig. 2, panels 1–3 or 8–10), scoring depends upon repeated decisions of whether it is nuclear-cytoplasmic or a category flanking it.

**FIGURE 2.** Cytoplasmic (images 1–3), nuclear-cytoplasmic (images 4–7), and nuclear (images 8–10) localized Gln3-Myc\textsuperscript{13}. Images appear in pairs; Gln3-Myc\textsuperscript{13} and DAPI-positive fluorescence are merged in the upper image of each pair, while Gln3-Myc\textsuperscript{13} fluorescence alone appears in the lower one. Arrows in image 1 indicate tubular structures often observed when Gln3-Myc\textsuperscript{13} localizes in the cytoplasm. Arrows in images 4–7 identify areas where Gln3-Myc\textsuperscript{13} fluorescence occurs in proximity with the nucleus but does not fully co-localize with DAPI-positive material in cells classified as nuclear-cytoplasmic. Note how the categories merge into one another at the boundaries. Images were derived from glutamine (images 1–3), urea (images 4, 6, 7), ammonia (image 5), proline (image 8), and ammonia + Msx (images 9, 10). All images were taken from cultures of wild type, W.T., strain (TB123).
Similar experiments were generally repeated two or more times with similar results. Experiment to experiment variation has been documented in detail (33) and can also be ascertained by comparing similar experimental conditions in this and previous work (33, 38, 39).

RESULTS

PP2A Is Required For Rapamycin-induced Nuclear Gln3-Myc\textsuperscript{13} Localization—Type 2A-related Sit4 phosphatase has been previously shown to participate in multiple downstream effects observed after treating cells with the Tor inhibitor, rapamycin (1, 14, 20, 35). By comparison, considerably less is known about the participation of Type 2A phosphatase PP2A in such effects. Therefore, we assessed the effects of deleting the \textit{PPH21} and/or \textit{PPH22} genes, which encode largely redundant catalytic subunits of PP2A. Neither of the single deletions (\textit{pph21}/H9004 or \textit{pph22}/H9004) exhibited a demonstrable effect on rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells (Fig. 3, C and D). In contrast, deleting both \textit{PPH21} and \textit{PPH22} eliminated the ability of rapamycin to induce nuclear Gln3-Myc\textsuperscript{13} localization (Fig. 3, C and D). The gross phenotype of the \textit{pph21}/H9004 \textit{pph22}/H9004 mutant was similar to that generated by \textit{sit4}/H9004, \textit{sit4}/H9004 \textit{pph21}/H9004, or \textit{sit4}/H9004 \textit{pph22}/H9004 mutations (Fig. 3, A and B). Therefore, both Sit4 and PP2A phosphatases were required for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells.

Gln3-Myc\textsuperscript{13} Phosphorylation Profiles in \textit{sit4} and \textit{pph21} and \textit{pph22} Mutants Are Markedly Different—The similarity of the \textit{sit4} mutant phenotype with respect to Gln3-Myc\textsuperscript{13} localization led us to expect that Gln3-Myc\textsuperscript{13} phosphorylation profiles in the two mutants would also be similar. This, however, was not the case. The Gln3-Myc\textsuperscript{13} phosphorylation profile observed in \textit{sit4} mutant provided with various nitrogen sources was quite different from that observed in \textit{pph21} and \textit{pph22} mutants (compare Fig. 4, A and B). When proline was provided as the sole nitrogen source, Gln3-Myc\textsuperscript{13} phosphorylation in the \textit{pph21} \textit{pph22} mutant was much greater than in \textit{sit4} cells
made the phosphorylation profiles reported in Fig. 4 all the more exceptional and prompted us to determine whether PP2A was uniformly required for nuclear Gln3-Myc13 localization in cells provided with nitrogen sources ranging from rich to poor.

As shown in Fig. 5, Gln3-Myc13 was uniformly cytoplasmic in wild-type cells provided with glutamine (panels A and B). Treating these cultures with rapamycin elicited a limited increase in nuclear Gln3-Myc13 localization, which was Pph21/22-dependent (panels A and B). With asparagine-, ammonia-, or serine-grown cells, Gln3-Myc13 was again restricted to the cytoplasm of untreated wild-type cells. With this group of nitrogen sources, however, there was a greater rapamycin-response in the wild type and it was less Pph21/22-dependent than with glutamine (panels A–D, serine is an especially good example).

When intermediate nitrogen sources, aspartate or glutamate, were provided, Gln3-Myc13 was no longer exclusively cytoplasmic in untreated wild-type cells; it became more nuclear (Fig. 6, panels A and B). The Pph21/22 requirement for this nuclear localization became minimal in untreated cells to nonexistent in those treated with rapamycin. This trend toward greater nuclear Gln3-Myc13 localization and diminishing Pph21,22 requirement reached its maximum with the most derepressive nitrogen sources, proline, or allantoin (Fig. 6, panels C and D). Gln3-Myc13 was now almost completely nuclear in both untreated and rapamycin-treated wild-type cells; localization was now also independent of Pph21,22.

**Overall Gln3-Myc13 Phosphorylation Is NCR-sensitive in the Absence of Pph21/22**—The striking influence a nitrogen source exerted on the Pph21/22 requirement for Gln3-Myc13 localization prompted us to carefully ascertain whether similarly parallel effects could be demonstrated for Gln3-Myc13 phosphorylation. Therefore, we assayed the amount of Pph21/22-dependent Gln3-Myc13 dephosphorylation in cultures provided with a range of nitrogen sources using the Western blot phosphatase shift analysis described in Fig. 4. With this assay, the greater the Pph21/22-dependent Gln3-Myc13 dephosphorylation, the greater the difference between the electrophoretic mobilities of Gln3-Myc13 from wild type versus mutant extracts. Gamma settings were uniformly altered as indicated under "Materials and Methods" to avoid any loss of minor bands.

FIGURE 4. Gln3-Myc13 phosphorylation levels are influenced differently by the nitrogen source provided to wild type (TB123), sit4Δ (TB136 –2a), and pph21Δpph22Δ (03705d) cultures. Western blots of crude extracts from each of the three strains and three growth conditions (glutamine, Gln; proline, Pro; or ammonia, Am. provided as sole nitrogen source) were prepared and Gln3-Myc13 visualized as described under "Materials and Methods." The amounts of phosphatase-dependent Gln3-Myc13 dephosphorylation can be assessed by comparing the mobilities of Gln3-Myc13 in extracts from wild type and similarly cultured pph21Δpph22Δ or sit4Δ cells. The greater the phosphatase-dependent Gln3-Myc13 dephosphorylation, the greater the difference in electrophoretic mobilities of Gln3-Myc13 from wild type versus mutant extracts. Gamma settings were uniformly altered as indicated under "Materials and Methods" to avoid any loss of minor bands.

(compare lanes A and B). These differing amounts of phosphatase-dependent Gln3-Myc13 dephosphorylation can be seen by comparing the mobilities of Gln3-Myc13 in extracts from wild type and similarly cultured pph21Δpph22Δ or sit4Δ cells. The greater the phosphatase-dependent Gln3-Myc13 dephosphorylation, the greater the difference in electrophoretic mobilities of Gln3-Myc13 from wild type versus mutant extracts. Conversely, when glutamine was provided, Gln3-Myc13 phosphorylation in the pph21Δpph22Δ mutant was significantly less than in sit4Δ cells. This suggested the existence of previously undetected differences in the regulation of Sit4 and PP2A.

**Nuclear Gln3-Myc13 Localization Does Not Require Pph21/22 in Cells Provided with Poor Nitrogen Sources**—Early studies demonstrated that PP2A and Sit4 responded similarly to the cell nitrogen supply, their abilities to phosphorylate their targets were low when a rich nitrogen source was provided and higher during nitrogen starvation (14, 20). A parallel correlation was reported for Gln3-Myc13 localization, i.e. it was cytoplasmic when cells were provided with a rich nitrogen source and nuclear during nitrogen starvation (16). This parallelism...
Overall, two clear correlations emerged: (i) Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation and nuclear localization correlated similarly with the derepressiveness of the nitrogen source provided, i.e. both were greatest in cells provided with the most derepressive nitrogen sources (proline or allantoin) and least with those grown with the most repressive nitrogen sources (glutamine or asparagine), and (ii) Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation and the Pph21/22 requirement for nuclear Gln3-Myc\(^{13}\) localization responded oppositely to the nitrogen source provided, i.e. dephosphorylation was greatest with nitrogen sources exhibiting the least Pph21/22 requirement for nuclear Gln3-Myc\(^{13}\) localization (allantoin or proline) and least with nitrogen sources exhibiting the greatest Pph21/22 requirements (glutamine or asparagine). Thus gross Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation on poor nitrogen sources was not a prerequisite for its nuclear localization.

**FIGURE 5.** Effect of high quality nitrogen sources on Gln3-Myc\(^{13}\) localization in wild type (TB123) and pph21\(^{-}\)/H9004 pph22\(^{-}\)/H9004 (03705d) strains. +Rap indicates cultures were treated with rapamycin. Experiment format and data presentation were as in Fig. 3. The nitrogen sources used appear below the histograms and above the microscopic images.

Overall, two clear correlations emerged: (i) Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation and nuclear localization correlated similarly with the derepressiveness of the nitrogen source provided, i.e. both were greatest in cells provided with the most derepressive nitrogen sources (proline or allantoin) and least with those grown with the most repressive nitrogen sources (glutamine or asparagine), and (ii) Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation and the Pph21/22 requirement for nuclear Gln3-Myc\(^{13}\) localization responded oppositely to the nitrogen source provided, i.e. dephosphorylation was greatest with nitrogen sources exhibiting the least Pph21/22 requirement for nuclear Gln3-Myc\(^{13}\) localization (allantoin or proline) and least with nitrogen sources exhibiting the greatest Pph21/22 requirements (glutamine or asparagine). Thus gross Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation on poor nitrogen sources was not a prerequisite for its nuclear localization.

**Pph21/22- and Sit4-dependent Gln3-Myc\(^{13}\) Dephosphorylation Respond Inversely to the Nitrogen Source**—The surprising behavior of Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation and the data observed in Fig. 4 prompted us to carefully compare Pph21/22- and Sit4-dependent Gln3-Myc\(^{13}\) dephosphorylation activities. To this end, we repeated the above phosphorylation measurements with the gel lanes organized such that pph21\(^{-}\)/H9004 mutant extracts were flanked by those from the sit4\(^{-}\)/H11001 strain (Fig. 7, lanes D–F). Pph21/22- and Sit4-dependent Gln3-Myc\(^{13}\) dephosphorylation differed most when either the best (glutamine) or poorest (allantoin or proline) nitrogen sources were provided. In the case of the best nitrogen source, glutamine, Pph21/22-dependent Gln3-Myc\(^{13}\) dephosphorylation was much less than that which was Sit4-dependent. Conversely, in the cases of the poorest nitrogen sources, proline or allantoin, Pph21/22-dependent dephosphorylation was much greater than that which was Sit4-dependent. In other words, Pph21/22- and Sit4-dependent Gln3-Myc\(^{13}\) dephosphorylation responded inversely to the nitrogen source provided.
As noted in Fig. 1B, PP2A exists in three different complexes, one when the catalytic subunits, Pph21 or Pph22, associate with Tap42 and two others when they associate with Tpd3 and a third subunit, Cdc55 or Rts1 (20). Therefore, we queried which of these complexes were required for Gln3-Myc<sup>13</sup> dephosphorylation by first comparing the phenotypes of tpd3Δ and pph21Δpph22Δ mutants. As shown in Fig. 8, the phenotypes of tpd3Δ and pph21Δpph22Δ mutants were indistinguishable with respect to intracellular Gln3-Myc<sup>13</sup> localization. This led to an expectation that tpd3Δ and pph21Δpph22Δ mutants would also exhibit similar Gln3-Myc<sup>13</sup> phosphorylation/dephosphorylation profiles. This expectation was fulfilled almost uniformly throughout the range of nitrogen sources. Gln3-Myc<sup>13</sup> phosphorylation levels in wild-type versus tpd3Δ mutant cells differed least with the best nitrogen sources and most when the poorest sources were provided (Fig. 9, lanes A–C) just as occurred with the pph21Δpph22Δ mutant (Fig. 9, lanes C and D). The similarity of pph21Δpph22Δ and tpd3Δ mutant phenotypes was even more evident when the two mutant phosphorylation profiles were compared directly (Fig. 9, lanes D–F). There was, however, one noticeable difference: Gln3-Myc<sup>13</sup> dephosphorylation was somewhat greater in a glutamine-grown tpd3Δ mutant than in pph21Δpph22Δ cells; again it is most clear when the two mutant phosphorylation profiles are compared directly (Fig. 9, lanes D–F).

Influence of Rapamycin on Gln3-Myc<sup>13</sup> Phosphorylation in pph21Δpph22Δ and tpd3Δ Mutants Provided with Various Nitrogen Sources—Just as occurred in untreated cells, the influence of rapamycin on Gln3-Myc<sup>13</sup> dephosphorylation in pph21Δpph22Δ or tpd3Δ mutant cells depended heavily on the nitrogen source provided to the cells (Fig. 10). Rapamycin did not elicit a demonstrable decrease in Gln3-Myc<sup>13</sup> phosphorylation with the wild type or either mutant when a poor nitrogen source (proline or allantoin) was provided (Fig. 10, A and B, lanes B and C). When a better nitrogen source was provided (ammonia), rapamycin induced Gln3-
Asparagine), rapamycin treatment elicited Gln3-Myc\textsuperscript{13} dephosphorylation in both mutants (Fig. 10, A and B, lanes B and C). In extracts of the \textit{pph21}\textDelta\textit{pph22}\Delta mutant, the amount of the bottom most species markedly increased in the rapamycin-treated samples relative to those that were untreated (black dots). In extracts of the \textit{tdp3}\Delta mutant, the intensity of an upper band decreased while that of a lower band increased (black dots). Most importantly, Gln3-Myc\textsuperscript{13} was dephosphorylated in these glutamine-grown, rapamycin-treated mutants to the same degree as the wild type. This did not occur with any other nitrogen source.

Effects of Nitrogen Source and Rapamycin on Gln3-Myc\textsuperscript{13} Localization and Phosphorylation in \textit{cdc55}\Delta and \textit{rts1}\Delta Mutants—The above data demonstrated four remarkable characteristics of \textit{pph21}/22 and Tpd3. (i) Neither protein was required for nuclear Gln3-Myc\textsuperscript{13} localization in cells provided with poor nitrogen sources. (ii) Both proteins were required for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells. (iii) Neither protein was required, or at best was minimally required, for Gln3-Myc\textsuperscript{13} dephosphorylation when glutamine was provided as nitrogen source, and (iv) both proteins were required for Gln3-Myc\textsuperscript{13} dephosphorylation in cells provided with poor nitrogen sources. The similarity of \textit{pph21}/22 and \textit{tdp3}\Delta mutant phenotypes strongly suggested that \textit{pph21}/22 functioned in association with a \textit{Tpd3-Cdc55} and/or \textit{Tpd3-Rts1} complex to effect rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells and Gln3-Myc\textsuperscript{13} dephosphorylation in proline-grown cells. This prompted us to determine which of the Tpd3 complexes participated in the regulation of Gln3-Myc\textsuperscript{13}.

As shown in Fig. 11, both \textit{Cdc55} and \textit{Rts1} were required for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells, \textit{i.e., cdc55}\Delta and \textit{rts1}\Delta mutant cells exhibited phenotypes similar to those of the \textit{pph21}/22 and \textit{tdp3}\Delta mutants. This indicated that both complexes were required for rapamycin to induce nuclear Gln3-Myc\textsuperscript{13} localization with a good nitrogen source. In contrast, \textit{Cdc55} was not required for nuclear Gln3-Myc\textsuperscript{13} localization with a poor nitrogen source, proline, again characteristics shared with \textit{pph21}/22 and \textit{Tpd3}. There was, however, a modest to minimal requirement of \textit{Rts1} for nuclear Gln3-Myc\textsuperscript{13} localization in proline-grown cells (Fig. 11, C and D).

The requirements of both \textit{Cdc55} and \textit{Rts1} for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization predicted, on the one hand, that these proteins would be required for Gln3-Myc\textsuperscript{13} dephosphorylation in glutamine-grown cells. On the other hand, the absence of \textit{pph21}/22 and \textit{Tpd3} requirements for rapamycin-induced Gln3-Myc\textsuperscript{13} dephosphorylation argued that we might not see \textit{Cdc55} and/or \textit{Rts1} requirements. To settle this question experimentally, we performed experiments similar to those in Fig. 10 with the \textit{cdc55}\Delta and \textit{rts1}\Delta mutants. Remarkably, given the requirement of \textit{Cdc55} for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization, Gln3-Myc\textsuperscript{13} was dephosphorylated identically in extracts from glutamine-grown, rapamycin-treated wild type and \textit{cdc55}\Delta cells (Fig. 12A, lanes B–E, black dots, the Gln3-Myc\textsuperscript{13} mobility profiles were indistinguishable). Therefore, as with \textit{pph21}/22 and \textit{Tpd3}, \textit{Cdc55} was required for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization, but not its dephosphorylation. The \textit{cdc55}\Δ mutant phenotype in proline-grown cells was also nearly identical to those of \textit{pph21}/22 and \textit{tdp3}\Δ mutant strains (compare Figs. 9 and 11); Gln3-Myc\textsuperscript{13} phosphorylation

![FIGURE 7. Pph21/22-dependent Gln3-Myc\textsuperscript{13} dephosphorylation responds inversely to that which is Sit4-dependent with a spectrum of nitrogen sources, ranging from the best (glutamine) to the worst (proline or allantoin). The degree of Pph21/22- or Sit4-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is determined by assessing the difference in phosphorylation levels between W.T. (TB123) and \textit{pph21}/22 (03705d) (lanes A and C versus B) or \textit{sit4}\Delta (TB136–2a) (lanes C versus D) mutant strains, respectively. The effects of nitrogen source on Gln3-Myc\textsuperscript{13} dephosphorylation in the two mutants can also be compared directly (lanes D–F). Experimental procedures were as described in Fig. 4.](image-url)
increased in untreated proline-grown cdc55Δ cells relative to the wild-type strain (Fig. 12A, lanes A and B). Gln3-Myc13 phosphorylation levels were not, however, altered by rapamycin treatment (lanes C and D). If anything, they increased.

For the rts1Δ mutant, there was a difference from the phenotype of the cdc55Δ strain. Unlike the cdc55Δ mutant, Gln3-Myc13 phosphorylation increased in untreated glutamine-grown rts1Δ cells relative to wild type, just as occurred with the pph21Δpph22Δ mutant (Fig. 12B, lanes A and B, and Fig. 10). Recall the most rapidly migrating Gln3-Myc13 species in wild type was noticeably diminished in the pph21Δpph22Δ mutant. Gln3-Myc13 dephosphorylation also increased following rapamycin addition to rts1Δ cells, but only to the untreated wild-type level. It did not reach the level achieved in a rapamycin-treated wild type (Fig. 12B, lanes B–E, black dots). The Gln3-Myc13 phosphorylation/dephosphorylation profile in proline-grown cells was quite similar to that observed with the cdc55Δ mutant. Note here, as in Fig. 12A, that rapamycin treatment of proline-grown wild type and rts1Δ cells increased the level of Gln3-Myc13 phosphorylation (Fig. 12B, lanes B–E).

In sum, both Tpd3-Cdc55 and Tpd3-Rts1 complexes were required for rapamycin-induced nuclear Gln3-Myc13 localization in glutamine-grown cells. The Tpd3-Cdc55 complex was not required for nuclear Gln3-Myc13 localization in untreated proline-grown cells, supporting conclusions reached with the pph21Δpph22Δ and tpd3Δ mutants. The Rts1 requirements, though quite similar, were not identical to those of Cdc55: there were subtle differences in the requirements for both nuclear Gln3-Myc13 localization and dephosphorylation. The Tpd3-Cdc55 complex was not required at all for Gln3-Myc13 dephosphorylation in rapamycin-treated, glutamine-grown cells, or nuclear Gln3-Myc13 localization in proline-grown cells, while the Tpd3-Rts1 complex appeared to be minimally to modestly necessary for both processes.

**DISCUSSION**

Data presented above fill several critical gaps in our understanding of selective nitrogen utilization in *S. cerevisiae*. They also profoundly change our view of the relationship between nitrogen source quality, Gln3 dephosphorylation and the requirements of Sit4 and PP2A for nuclear Gln3 localization. The current mechanism by which the Tor pathway regulates Gln3 localization (Fig. 1A) has been predominantly developed from experiments characterizing Gln3 responses to short duration rapamycin treatment. Using this approach, strong correlations between predicted and observed Gln3 dephosphorylation...
and localization patterns have been reported. The most pivotal of these correlations was that rapamycin induces Sit4-dependent Gln3 dephosphorylation and nuclear localization. Unfortunately, similarly positive correlations between Gln3 dephosphorylation and nuclear localization in response to nitrogen source quality in either wild type or \textit{sit4}/H9004 mutant cells have not been observed.

In the current experiments we have demonstrated, for the first time, a strong correlation between nitrogen source quality, PP2A-dependent Gln3-Myc\textsuperscript{13} dephosphorylation and nuclear localization in response to nitrogen source quality in either wild type or \textit{sit4}Δ mutant cells have not been observed.

In the current experiments we have demonstrated, for the first time, a strong correlation between nitrogen source quality, PP2A-dependent Gln3-Myc\textsuperscript{13} dephosphorylation and nuclear localization. PP2A-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is greatest when cells are provided with repressive nitrogen sources (e.g. proline or allantoin) and Gln3-Myc\textsuperscript{13} is nuclear. It is lowest when cells are provided with repressive nitrogen sources (e.g. glutamine or asparagine) and Gln3-Myc\textsuperscript{13} is cytoplasmic. However, our data additionally support the idea that PP2A-dependent Gln3-Myc\textsuperscript{13} dephosphorylation and nuclear localization are more likely to be two parallel outcomes from the signal of nitrogen source quality than two sequential steps in the nuclear localization pathway. The most compellingly evidence in support of this idea is that PP2A-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is not required for its nuclear localization in cells provided with repressive nitrogen sources.

In our most important experiments with respect to Sit4 and the model in Fig. 1A, we show that Sit4-dependent Gln3-Myc\textsuperscript{13} dephosphorylation in rapamycin-treated cells is insufficient to achieve nuclear localization of this transcription factor. In mutants, respectively, lacking each of three PP2A complex components, rapamycin induced Gln3-Myc\textsuperscript{13} dephosphorylation to the same levels observed in rapamycin-treated wild-type cells. Yet in each case, Gln3-Myc\textsuperscript{13} remained in the cytoplasm. Therefore, rapamycin-induced Gln3-Myc\textsuperscript{13} dephosphorylation, the pivotal event regulating Gln3 localization in the current model (Fig. 1A), no longer appears to be the primary determinant of Gln3 localization.

**Rapamycin Treatment Is Not a Mimic of Nitrogen Catabolite Repression**—Ever since the groundbreaking discoveries linking the Tor pathway to NCR (15–18), rapamycin treatment has been envisioned to inhibit the Tor proteins downstream of the nitrogen availability signal and hence to mechanistically mimic growth with a poor nitrogen source. However, in light of past and now current data, one must seriously question how faithfully rapamycin treatment mimics relief of NCR? As detailed in the introduction, not only is the rapamycin response itself nitrogen source-dependent (27, 28) and its requirements for cytoskeletal and Golgi-to-endosome trafficking proteins different from those of NCR (31, 32), we now show that PP2A phosphatase requirements for rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization in glutamine-grown cells, and growth with poor nitrogen sources are remarkably different. Together these observations suggest that the degree to which rapamycin treatment mimics derepressive growth conditions is limited.

**Pph21/22- and Sit4-dependent Gln3-Myc\textsuperscript{13} Dephosphorylation Respond Inversely to the Quality of the Nitrogen Source**—An intriguing discovery of the present work and one that will potentially have broad impact on Tor regulation is the finding that Pph21/22- and Sit4-dependent Gln3-Myc\textsuperscript{13} dephosphorylation respond oppositely to nitrogen source quality. At one extreme, Pph21/22-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is least with the most repressive nitrogen sources, whereas Sit4-dependent dephosphorylation is greatest. At the other, Pph21/22-dependent Gln3-Myc\textsuperscript{13} dephosphorylation is greatest under derepressive conditions where Sit4-dependent Gln3-Myc\textsuperscript{13} localization is least (Fig. 7). If this overall pattern of regulation occurs more broadly with other targets beyond Gln3, it will alter our view of global regulation by these phosphatases.
when Gln3-Myc\textsuperscript{13} is nuclear, it is no longer available to be influenced by cytoplasmic Sit4-dependent events. Consistent with this suggestion, Gln3-Myc\textsuperscript{13} phosphorylation increases rather than decreases following rapamycin treatment of wild-type cells growing with poor nitrogen sources (Refs. 29, 33, and the present work). Additionally, rapamycin treatment does not elicit Gln3-Myc\textsuperscript{13} dephosphorylation in a proline-grown \textit{pph21\textsuperscript{-}pph22\textsuperscript{-}} mutant and increases it further when \textit{tpd3\textsuperscript{-}}, \textit{cdc55\textsuperscript{-}}, or \textit{rts1\textsuperscript{-}} mutant strains are provided with poor nitrogen sources in which Gln3-Myc\textsuperscript{13} is nuclear. It is improbable that the inability of rapamycin to elicit Gln3-Myc\textsuperscript{13} dephosphorylation in these mutants derives from its requirements for Pph21/22, Tpd3, Cdc55, or Rts1 because the dephosphorylation occurs almost normally when these mutants are growing in glutamine medium.

Alternatively, or in addition, the inverse responses may be indicative of a feedback regulatory loop. Such loops have been repeatedly identified among the components of the Tor and other regulatory pathways (11).

All of these conclusions must, however, be framed within at least two constraints. We, and all others, have assayed “gross” Gln3-Myc\textsuperscript{13} phosphorylation levels. There is ample structural evidence from both Zheng’s laboratory and ours that Gln3 localization depends on multiple structural motifs (40, 41), a conclusion also recently embraced by Puria \textit{et al.} (32). The specific modification of any one of them could easily escape detection. Second, there is no concrete evidence that Pph21/22 itself directly dephosphorylates Gln3-Myc\textsuperscript{13} \textit{in vivo} as opposed to acting through one or more downstream molecules whose intracellular localizations may differ from those of the phosphatases that influence their action.

\textbf{Requirements of Tpd3, Cdc55, and Rts1 for Gln3-Myc\textsuperscript{13} Dephosphorylation and Nuclear Localization—Catalytic Pph21/22 subunits complex with multiple regulatory/specificity subunits. On the one hand, they can form a rapamycin-dissociable complex with Tap42, and on the other, a rapamycin-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{Cdc55 and Rts1 are required for nuclear Gln3-Myc\textsuperscript{13} localization in response to rapamycin treatment but not growth with a poor nitrogen source. The strains used in this experiment were W.T. (TB123), cdc55\textsuperscript{-} (FV206), and rts1\textsuperscript{-} (FV209). Experimental format and data presentation were as in Fig. 5.}
\end{figure}
Pph21/22-dependent Gln3-Myc\textsuperscript{13} Dephosphorylation

(A) W.T. + - - + + 
cdc55\textsuperscript{Δ} - + + + - Rap - - - - -

(B) W.T. + - - + + 
rt51\textsuperscript{Δ} - - + + - Rap - - - - -

Pro
Gln

FIGURE 12. The effects of nitrogen source and loss of Cdc55 and Rts1 on rapamycin-induced Gln3-Myc\textsuperscript{13} dephosphorylation. The wild type was TB123. Experimental procedures and data presentation were as in Fig. 4.

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