Distinct Phosphatase Requirements and GATA Factor Responses to Nitrogen Catabolite Repression and Rapamycin Treatment in \textit{Saccharomyces cerevisiae}*

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In yeast, rapamycin (Rap)-inhibited TorC1, and the phosphatases it regulates (Sit4 and PP2A) are components of a conserved pathway regulating the response of eukaryotic cells to nutrient availability. TorC1 and intracellular nitrogen levels regulate the localization of Gln3 and Gat1, the activators of nitrogen catabolite repression (NCR)-sensitive genes whose products are required to utilize poor nitrogen sources. In nitrogen excess, Gln3 and Gat1 are cytoplasmic, and NCR-sensitive transcription is repressed. During nitrogen limitation or Rap treatment, Gln3 and Gat1 are nuclear, and transcription is derepressed. We previously demonstrated that the Sit4 and Pph21/22-Tpd3-Cdc55/Rts1 requirements for nuclear Gln3 localization differ. We now show that Sit4 and Pph21/22-Tpd3-Cdc55/Rts1 requirements for NCR-sensitive and Rap-induced nuclear Gat1 localization markedly differ from those of Gln3. Our data suggest that Gln3 and Gat1 localizations are controlled by two different regulatory pathways. Gln3 localization predominantly responds to intracellular nitrogen levels, as reflected by its stronger NCR-sensitivity, weaker response to Rap treatment, and strong response to methionine sulfoximine (Mxs, a glutamine synthetase inhibitor). In contrast, Gat1 localization predominantly responds to TorC1 regulation as reflected by its weaker NCR sensitivity, stronger response to Rap, and immunity to the effects of Mxs. Nuclear Gln3 localization in proline-grown (nitrogen limited) cells exhibits no requirement for Pph21/22-Tpd3-Cdc55, whereas nuclear Gat1 localization under these conditions is absolutely dependent on Pph21/22-Tpd3/Cdc55. Furthermore, the extent to which Pph21/22-Tpd3-Cdc55 is required for the TorC1 pathway (Rap) to induce nuclear Gat1 localization is regulated in parallel with Pph21/22-Tpd3-Cdc55-dependent Gln3 dephosphorylation and NCR-sensitive transcription, being highest in limiting nitrogen and lowest when nitrogen is in excess.

One consequence of motif, gene, or genome duplication is the subsequent existence of multiple proteins with the same or overlapping functions. In some cases the duplicated genes evolve, acquiring easily distinguishable new functions or regulatory profiles (1–4). In others, the differences are more subtle, and a more careful study is required before they become apparent. This is the case with the \textit{Saccharomyces cerevisiae} GATA family transcription factors, Gln3 and Gat1/Nil1.

Both Gln3 and Gat1 are responsible for nitrogen catabolite repression (NCR)-sensitive expression of genes encoding the transport and enzyme proteins needed to scavenge poor nitrogen sources (e.g. proline) when more readily usable ones are limiting or unavailable (5–8). Gln3 and Gat1 binding sites in NCR-sensitive promoters contain the core sequence GATAA respectively, by Ure2 (15, 16). A possible mechanism to explain this regulation first derived from the \textit{in vitro} demonstration of a Ure2-Gln3 complex, whose existence was subsequently confirmed by others (17–21). Similar attempts to isolate a Ure2-Gat1 complex, however, have not been successful (18).

A milestone in the study of GATA factor regulation occurred with the discoveries that (i) NCR-sensitive transcription aberrantly increases after the addition of the TorC1 serine/threonine kinase inhibitor, rapamycin, to cultures growing under the repressive conditions of excess nitrogen (18, 19, 22, 23) and (ii) NCR-sensitive and rapamycin-induced gene expression positively correlates with intracellular Gln3 and Gat1 localization (18, 19, 24, 25). Gln3 and Gat1 are cytoplasmic, and NCR-sensitive gene expression is repressed in wild type yeast extract/peptone/dextrose- or glutamine-grown cells, whereas after treating these cells with rapamycin or culturing them in nitrogen-limited or starvation conditions, Gln3 and Gat1 become nuclear, and NCR-sensitive transcription is derepressed.

Work from many laboratories has established Tor1/2 in \textit{S. cerevisiae} and mTor in mammals as highly conserved global regulators coordinating cell division with nutrient supplies and a wide variety of metabolically related processes ranging from...
protein synthesis to autophagy and protein degradation; expression of hundreds of genes increase or decrease more than 2-fold in rapamycin-treated cells (19, 22, 23, 26–29). Although many biochemical details of the regulatory pathway remain to be elucidated, the broad outlines of Tor regulation are well known, or models of them have been proposed (e.g. Refs. 18 and 30–36).

The present work focuses on the downstream serine/threonine phosphatases of the pathway Sit4 and PP2A (see the summary pathway in Fig. 1 of Ref. 47). The latter can exist as a heterotrimer, consisting of a catalytic subunit (Pph21 or Pph22), a scaffold protein (Tpd3), and a regulatory subunit (Cdc55 or Rts1) (30, 37); heterotrimeric Pph21/22-Tpd3-Cdc55/Rts1 is immune to the effects of rapamycin treatment (33). Both Sit4 and each PP2A catalytic subunit can also associate with the phosphorylated form of Tor-associated protein, Tap42; TorC1 phosphorylates Tap42 under conditions of nitrogen excess but not starvation (31, 33). In nitrogen excess, the Tap42-phosphatase complexes are bound to TorC1 and are inactive. During nitrogen starvation or inhibition of TorC1 by rapamycin, the Tap42-phosphatase complexes are released from TorC1 and thereby become transiently active until they in turn dissociate into their inactive components, Tap42 and Sit4 or Pph21/22 phosphatase catalytic subunits (34, 38, 39).

The mechanistic connections between rapamycin treatment, Gln3 localization, and NCR-sensitive transcription were initially extended and strengthened by the observations that: (i) rapamycin-induced nuclear Gln3-Myc13 localization and NCR-sensitive transcription required Sit4 (18); (ii) rapamycin treatment led to dissociation of the Gln3-Ure2 complex (18, 19); (iii) methionine sulfoximine (Msx), a glutamine synthetase inhibitor, elicited nuclear Gln3-Myc13 localization and increased NCR-sensitive transcription leading to the conclusion that “Tor-controlled transcription activators Gln3, Rtg1, and Rtg3 (Rtg1/3 activate retrograde gene expression) are regulated in parallel with Pph21/22-Tpd3-Cdc55-dependent (NCR-sensitive) control of Pph21/22-Tpd3-Cdc55 under these conditions. Furthermore, the extent to which Pph21/22-Tpd3-Cdc55 is required for the TorC1 pathway (rapamycin treatment) to induce nuclear Gln3 localization is regulated in parallel with Pph21/22-Tpd3-Cdc55-dependent Gln3 dephosphorylation and NCR-sensitive transcription; it is highest in limiting nitrogen and lowest when nitrogen is in excess. Together these data permit us to draw a consistent picture of intracellular GATA factor localization in response to the nature of the nitrogen source (NCR-sensitive regulation) that differs from the response to TorC1 pathway (rapamycin-induced) regulation. They also suggest that nitrogen source-dependent (NCR-sensitive) control of Pph21/22-Tpd3-Cdc55 regulates the ability of Gat1 localization to respond to rapamycin.

MATERIALS AND METHODS

Strains and Culture Conditions—S. cerevisiae strains used in this work are listed in Table 1 and are isogenic to wild type TB50. Their construction was described earlier (47). Growth conditions are identical to those previously reported (46, 47). Cells were cultured to mid-log phase (A600 nm = 0.5) in 50 ml of YNB (yeast nitrogen base without amino acids or nitrogen)
minimal medium containing the indicated nitrogen sources at a final concentration of 0.1% unless indicated otherwise. Supplements were added to the medium to cover auxotrophic requirements as described earlier (45, 47). The rapamycin concentration was 200 ng/ml. Strains were transformed with CEN-based plasmid pRS416-Gat1-GFP or pRS416-Gln3-GFP whose construction and detailed validation for normal regulation have appeared (42, 52). Only freshly prepared transformants were assayed.

Western Blot Analyses—Western blot analyses were performed using previously described methods except for the concentration of acrylamide used for electrophoresis (7%) (47). Results were recorded on Kodak BioMax XAR film using a wide range of exposures to ensure that all minor species were detected. Films were then digitally photographed, and the images were assembled for publication; gamma settings (Adobe Photoshop) of the photographic images were not changed.

**Scoring Intracellular Gat1-GFP Localization**—A comprehensive description of intracellular Gat1-GFP localization and movement cannot be adequately achieved from one or two subjectively selected images of microscopic fields containing 4–8 cells. Therefore, we manually scored all of the cells in the two images collected from each culture sample. Scoring was performed using primary, unaltered .zvi file images and Zeiss AxioVision 4.6.3 software. Cells were classified into one of three categories: (i) cytoplasmic (cytoplasmic Gat1-GFP fluorescence only; examples in Fig. 1B, panels A and B), (ii) nuclear-cytoplasmic (Gat1-GFP fluorescence in the cytoplasm and additionally Gat1-GFP fluorescence in a clearly delimited structure similar to one in which Gat1-Myc13 (indirect immunofluorescence (44)) or Gat1-GFP (fluorescence) and DAPI-staining co-localize (Fig. 1C); examples in Fig. 1B, panels C and D, white arrows NC), or (iii) nuclear (Gat1-GFP fluorescence exclusively restricted to a clearly delimited structure similar to one in which Gat1-Myc13 (indirect immunofluorescence (44)) or Gat1-GFP (fluorescence) and DAPI-staining co-localize (Fig. 1C); examples in Fig. 1B, panels E and F, white arrows N). DAPI staining throughout the time course of the experiments was not possible because DAPI does not uniformly stain the nuclei of live cells in the absence of ethanol treatment, and its fluorescent signal fades within 5–10 min. This has been seen before (54). Identifying nuclear Gat1-GFP, however, is straightforward as shown by the pairs of images in Fig. 1C, panels H–M. Criteria applied in the scoring of Gat1-GFP localization are the same as those described in detail earlier for Gln3 (46, 47). The three scoring categories described above are color-coded as red, yellow, and green bars, respectively, in subsequent histograms (Fig. 1, A and D).

Scoring data obtained from two images of each culture sample (Fig. 1A) were averaged, and these averages are the values presented in all of the figures except Fig. 1A, in which data from the individual images are presented. The average number of cells scored per sample was 91. This number is lower than we

**TABLE 1**

*S. cerevisiae* strains used in this work

| Strain | Pertinent genotype | Complete genotype |
|--------|--------------------|-------------------|
| FV063  | Wild type          | MATa, Leu2-3,112,  |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| 03827c | sit1Δ              | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| 03839c | sit1Δpph21Δ        | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| 03879c | pph21Δpph22Δ       | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| FV210  | tpd3Δ              | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| FV207  | cdc55Δ             | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| FV208  | rts1Δ              | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
| FV226  | cdc55Δrts1Δ        | MATa, Leu2-3,112, |
|        |                    | ura3-52, trp1,    |
|        |                    | HIS3, GAT1-MYC13[ ] |
normally use for indirect fluorescence measurements (200 cells) because live cells cannot be concentrated without artifactually altering intracellular Gat1-GFP distribution. Data from three independent samples are presented. Following recent recommendations (55), the three actual data points are presented per condition rather than an average of them with an error bar; this permits direct assessment of the raw data reproducibility. Transformant-to-transformant and day-to-day pre-

FIGURE 1. Time course, scoring, and precision of methods used to assay rapamycin-induced nuclear Gat1-GFP localization. Panel A, five samples (S-1 to S-5) were taken before rapamycin addition with two images collected from each sample as described under “Materials and Methods.” Thereafter, rapamycin was added to the glutamine-grown wild type (FV063) culture, and sampling (10 samples, S-6 to S-15) was continued. Electronic acquisition of two images per sample was, respectively, completed at the times indicated on the abscissa. Spacing between results from the pairs of images in the histogram was increased to indicate this. For example, the 3.0- and 4.0-min time points were derived from the same culture sample. The histograms are color-coded as described under “Materials and Methods.” Panel B, examples of cells scored by the criteria outlined under “Materials and Methods.” Images A and B, cytoplasmic; images C and D, nuclear cytoplasmic (NC); images E and F, nuclear (N). Panel C, co-localization of Gat1-GFP and DAPI-positive signals in three pairs of images (H and I, J and K, and L and M) is shown. Panel D, day-to-day, transformant-to-transformant precision of the scoring method is shown. Each bar of the histogram derives from cells scored in the images (2 each) of three samples derived from three independent transformants; error bars indicate the S.D. of these data, n = 9 samples. The absence of error bars indicates the S.D. was 0.
Phosphatase Requirements for NCR and Rapamycin Responses

cision of scoring can be assessed by the data in Fig. 1D. Each bar of the histogram was derived from the images (2 each) of three samples derived from three independent transformants; error bars indicate the S.D. of these data, n = 9 samples. Reproducibility of the data can be further assessed by comparing data in like conditions and strains in control experiments that appear throughout the work. Pairs of representative microscopic images taken from those quantitated in the histograms are also presented above the histograms for each condition. All experiments were performed multiple times with similar results.

RESULTS

Time Course of Gat1 Localization in Response to Rapamycin—Confidently establishing protein phosphatase requirements associated with control of intracellular Gat1 localization in response to rapamycin treatment or the environmental nitrogen supply required high quality semiquantitative assays. To this end, we refined Gat1-GFP fluorescence assays to maximize their reliability and reproducibility. The final method involved repeatedly sampling growing experimental cultures and rapidly collecting two images from each sample. The two images were completed usually within 1 min of one another. As shown by the raw data in Fig. 1A, we obtained good reproducibility in the pairs of images and could straightforwardly follow movement of Gat1-GFP from the cytoplasm to the nucleus after rapamycin treatment. The first clear evidence of movement occurred in this strain by 6–7 min with Gat1-GFP stably residing in the nucleus by 19 min. Complete time courses (as in Fig. 1A) were performed for all strains, and conditions were tested (15–22 samples per culture). Due to limitations of space, however, we have presented Gat1-GFP localization data from only three samples taken before rapamycin treatment and another three samples taken after the localization of Gat1-GFP, which in response to rapamycin addition, had stabilized (samples S-11 to S-13 in Fig. 1A).

Phosphatase Catalytic Subunit Requirements for Rapamycin-induced Nuclear Gat1-GFP Localization—To determine the phosphatase requirements for rapamycin-induced Gat1-GFP localization, wild type and phosphatase mutant cells were grown in minimal glucose medium and treated with rapamycin. Gat1-GFP strongly responded to rapamycin treatment with nuclear localization being observed in nearly all wild type cells (Fig. 2, A and B). This response, however, possessed only modest requirements for Sit4 and PP2A as evidenced by the fraction of sit4Δ and pph22Δ/pph22Δ cells exhibiting nuclear-cytoplasmic and nuclear Gat1-GFP localization compared with those in which Gat1-GFP was restricted to the cytoplasm (Fig. 2, A and B).

To assay the potential contributions of individual PP2A catalytic subunits to Gat1-GFP localization, we eliminated the effects of Sit4 by performing the experiments in a sit4Δ background. There was a modest decrease in the number of cells in which Gat1-GFP was exclusively nuclear relative to the Gat1-GFP distribution observed in the sit4Δ (compare Fig. 2, A and B with C and D). Comparing the individual responses of the mutants, the requirement for Pph21 appeared to be slightly greater than that for Pph22. However, the degree of change while reproducible was insufficient to convincingly conclude that Pph21 and Pph22 possess partially non-overlapping functions. This question is more clearly addressed later.

It is important to emphasize that although participation of Sit4 and Pph21/22 in rapamycin-induced nuclear Gat1-GFP localization could be confidently demonstrated, the requirements were far from complete. It is possible that the two phosphatases possess additive functions in support of the rapamycin response, but neither of them was dispensable.

Phosphatase Regulatory Subunit Requirements for Rapamycin-induced Nuclear Gat1-GFP Localization—Early studies of PP2A subunit composition and function demonstrated this phosphatase is controlled by three regulatory complexes; one in which Pph21/22 interacts with Tap42 and two others consisting of a Tpd3 subunit complexed with either Cdc55 or Rts1 (31, 33). Therefore, we assessed the relative contributions of each regulatory subunit to PP2A activity required for rapamycin-induced nuclear Gat1-GFP localization. Because the Tpd3 subunit is common to both Tpd3-Cdc55 and Tpd3-Rts1 complexes but has been reported not to be associated with the Pph21/22-Tap42 complex (33), we assayed a tpd3Δ to indirectly ascertain the extent to which the Pph21/22-Tap42 complex performed overlapping functions with the Tpd3 complexes. The more direct approach of using a tap42Δ or temperature-sensitive tap42 mutant could not be performed because (i) the deletion is inviable, and (ii) the previously reported sensitivity of Gln3 and Gat1 localization to stress, including that associated with inactivating a temperature sensitive Tap42 mutant protein (53, 56), precluded unambiguous interpretation of data generated by heat inactivation of Tap42. The rapamycin response of Gat1-GFP localization in a tpd3Δ was very similar to that observed in a pph21Δ/pph22Δ (Fig. 3, A and B). This indicated that the Pph21/22-Tap42 complex could not significantly substitute for functions provided by the Pph21/22-Tpd3-Cdc55 and Pph21/22-Tpd3-Rts1 complexes. Additionally, rapamycin-induced nuclear Gat1-GFP localization exhibited a substantially greater requirement for Cdc55 than for Rts1 (Fig. 3, C and D). Loss of Cdc55 increased the fraction of cells in which Gat1-GFP was cytoplasmic or nuclear-cytoplasmic relative to wild type, whereas only a slight negative effect, if any, was observed in the rts1Δ (Fig. 3, C and D). There appeared to be a very slight additive effect observed in the cdc55Δ/rts1Δ double mutant, but it was insufficient to be used as corroborating evidence in support of a significant Rts1 contribution to the overall results.

Pph21/22-Tpd3-Cdc55/Rts1 Requirements for the Rapamycin Response Are Drastically Influenced by the Nitrogen Supply—The PP2A requirement for rapamycin-induced nuclear Gat1-GFP localization reported above was significantly weaker than the absolute requirement observed earlier for Gln3-Myc13 in glutamine-grown cells (compare Fig. 3, C and D, W.T. Gln + Rap, versus pph21/22/pph22Δ Gln + Rap in Ref. 47 with the analogous experiments in Figs. 2, A and B, or 3, A and B, of this work). Therefore, we extended our analysis assayng rapamycin-induced Gat1-GFP localization in nitrogen limited, proline-grown cells where there is no demonstrable Pph21/22 requirement for nuclear Gln3-Myc13 localization (47). In sharp contrast with Gln3-Myc13, rapamycin-induced nuclear Gat1-
GFP localization was completely abolished in pph21Δpph22Δ proline-grown cells (Fig. 4, A and B). The Sit4 requirement was similar to that observed in glutamine medium, being quite limited compared with that for Pph21/22.

The relative requirements of the individual Pph21 and Pph22 catalytic subunits for rapamycin-induced nuclear Gat1-GFP localization also increased somewhat in proline-grown cells (Fig. 4, C and D). The increase was modest, however, relative to that seen for the pph21Δpph22Δ double mutant. As occurred in glutamine-grown cells, there was a moderately greater requirement for Pph21 than for Pph22.

The above observations concerning the PP2A catalytic subunit requirements were substantiated by those observed in the regulatory subunit mutants. There was an absolute requirement for Tpd3 in rapamycin-treated proline–grown cells, a phenotype identical to that observed in the pph21Δpph22Δ (Fig. 5, A and B). Again, Pph21/22-Tap42 could not replace functions provided by the Pph21/22-Tpd3-Cdc55 and/or Pph21/22-Tpd3-Rts1 complexes. The phenotypes of the cdc55Δ and rts1Δ mutants were largely unchanged from those seen in glutamine-grown cells (Fig. 3, C and D), with nuclear Gat1-GFP localization exhibiting a greater requirement for Cdc55 than Rts1. However, in rapamycin-treated proline-grown cells, the two regulatory subunits could both be clearly shown to function (Fig. 5, C and D). In contrast with the situation in glutamine-grown cells, rapamycin-induced nuclear Gat1-GFP localization

FIGURE 2. Requirements of the catalytic subunits of Sit4 and Pph21/22-Tpd3-Cdc55/Rts1 protein phosphatases for intracellular Gat1-GFP localization in untreated and rapamycin-treated (+Rap), glutamine-grown wild type (FV063), sit4Δ (FV066), pph21Δpph22Δ (03879c), sit4Δpph21Δ (03827c), and sit4Δpph22Δ (03839c) mutant cells. Microscopic images in panels A and C derived from images used to determine intracellular Gat1-GFP localization in the histograms of panels B and D. Times that appear on the abscissa are the averages of the two times at which the pairs of images from each sample were completed after the addition of rapamycin to the culture.
was almost abolished in the proline-grown \textit{cdc55} and \textit{rts1} double mutant, which possessed a phenotype similar to that of the \textit{tpd3} (Fig. 5, C and D).

\textbf{Rapamycin-induced and Nitrogen Supply-dependent Gat1-GFP Localization Are Regulated Differently}—Untreated samples in the above series of experiments permitted us to evaluate the NCR sensitivity of Gat1-GFP localization. Gat1-GFP was nuclear-cytoplasmic in about 20% of glutamine-grown wild type cells (Figs. 2, A and B, 3, A and B, and 6, A and B), and this value increased to about 50% when proline replaced glutamine as the nitrogen source (Figs. 4, A and B, 5, A and B, and 6, K and L). In other words, Gat1-GFP localization was NCR-sensitive, but much less so than Gln3 (compare Figs. 4, A and B, 5, A and B, and 6, K and L, W.T. Pro, in this work with Fig. 6, C and D, W.T. Pro, in Ref. 47). Most surprisingly, however, this nuclear-cytoplasmic Gat1-GFP localization was uniformly and completely abolished in \textit{pph21pph22}, \textit{tpd3}, \textit{cdc55}, and \textit{cdc55rts1} mutants irrespective of the nitrogen source (Figs. 4, A and B and 5, A–D). It partially remained only in the \textit{rts1}, which again possessed the weakest phenotype of all the mutants tested.

These results demonstrated an absolute requirement of Pph21/22-Tpd3-Cdc55 and Sit4 activity for nuclear Gat1-GFP localization in both repressive and derepressive nitrogen sources. They additionally indicated an overriding influence of the nitrogen supply on rapamycin-induced nuclear Gat1-GFP localization when cells harbored defects in Pph21/22-Tpd3-Cdc55 activity. More strikingly, the rapamycin effect was just opposite that expected for NCR-sensitive regulation, \textit{i.e.} the mutant phenotype was strongest in proline medium and weakest in glutamine-grown cells.
To develop a more comprehensive understanding of the control nitrogen supply exerted on Gat1 regulation, we followed intracellular Gat1-GFP localization in untreated and rapamycin-treated wild type and \textit{pph21}\textsubscript{H9004} \textit{pph22}\textsubscript{H9004} cells growing in a battery of nitrogen sources ranging from repressive to derepressive (Fig. 6, A to L). When untreated wild type cells were provided with repressive nitrogen sources (e.g. glutamine or 0.4% serine), Gat1-GFP was, as expected, cytoplasmic in a large majority of cells (Fig. 6, A–D). In 20% or less of the glutamine-grown cells, Gat1-GFP was observed to be nuclear-cytoplasmic (Fig. 6, A and B). Gat1-GFP was nuclear-cytoplasmic in even fewer 0.4% serine-grown cells (Fig. 6, C and D). Under these growth conditions rapamycin treatment resulted in Gat1-GFP being nuclear in most wild type cells and, more importantly, nuclear-cytoplasmic or nuclear in about 40 – 60% of the \textit{pph21}\textsubscript{pph22}\textsubscript{Δ} cells (Fig. 6, A–D). When a less repressive nitrogen source (e.g. ammonia or 0.1% serine) was used, the fraction of wild type cells in which Gat1-GFP was nuclear-cytoplasmic increased relative to the previous experiment (Fig. 6, E–H), the expected outcome of an NCR-sensitive process. At the same time, the response to rapamycin began to diminish in the \textit{pph21}\textsubscript{pph22}\textsubscript{Δ}, although it was still detected. In other words, the two responses move in opposite directions. Finally, full derepression, such as seen in glutamate or proline media, resulted in the highest levels of Gat1-GFP in untreated wild type cells, but the \textit{pph21}\textsubscript{pph22}\textsubscript{Δ} became totally unresponsive to rapamycin (Fig. 6, I–L).
Confirming Differences in Gln3 and Gat1 Localization Using Identical Assays—The above experiments semiquantitatively analyzed the responses of Gat1-GFP in wild type and Tor pathway phosphatase mutants to rapamycin and growth in repressive versus derepressive medium. These responses differed strikingly from those observed in previous, equally semiquantitative parallel studies of Gln3-Myc13 (46, 47). Although unlikely, it was conceivable that the differing Gln3 and Gat1 responses might derive from the assay methods that were used; Gat1-GFP localization was assayed in live cells throughout this paper, whereas Gln3-Myc13 localization in our previous publications was assayed by indirect immunofluorescence in fixed cells. To evaluate this possibility, we assayed Gat1 and Gln3 responses using identical assays. Unfortunately, it is not possible to quantitatively determine the intracellular distribution of Gln3 using a GFP assay because it is not currently possible to distinguish nuclear-cytoplasmic from nuclear Gln3-GFP. This is due to the low concentration of Gln3 in the cell when the gene is expressed from its native promoter, which results in an extremely low signal to noise ratio. On the other hand, we were fortunate in that all but one of the most important differences in the regulation of Gln3 and Gat1 localization were qualitative, i.e. Gln3 is almost completely cytoplasmic under conditions where Gat1 is almost completely nuclear and vice versa. Therefore, we focused on situations where the differences were greatest and transformed two aliquots of a wild type, sit4Δ, or pph21Δpph22Δ culture with pRS416-Gat1-GFP or pRS416-Gln3-GFP (42,

**FIGURE 5. Requirements of the Pph21/22-Tpd3-Cdc55/Rts1 regulatory subunits for intracellular Gat1-GFP localization in untreated and rapamycin-treated, proline-grown wild type (FV063), pph21Δpph22Δ (03879c), tpd3Δ (FV210), cdc55Δ (FV207), rts1Δ (FV208), and cdc55Δrts1Δ (FV226) mutant cells.** The experimental format and presentation of data are the same as in Fig. 2.
Gat1-Myc13 mobility was similarly unaltered in a sit4Δ mutant (Fig. 8). In a glutamine-grown, rapamycin-treated sit4Δ mutant, the opposite situation was observed, i.e. Gln3 was cytoplasmic, whereas Gat1 was nuclear—cytoplasmic or nuclear (Fig. 7). In a proline-grown, untreated, or rapamycin-treated pph21Δpph22Δ mutant, Gln3 was again highly nuclear, whereas Gat1-GFP was cytoplasmic (Fig. 7). In short, under all of these conditions Gln3 and Gat1 were in different cellular compartments. The only distinction we were unable to test was the relative Gln3 and Gat1 rapamycin and NCR sensitivities in wild type cells because these two distinctions are quantitative, not qualitative.

Effect of Phosphatase Gene Deletions on the Electrophoretic Mobility of Gat1-Myc13—Experiments described above clearly demonstrated Pph21/22-Tpd3-Cdc55 participation in the regulation of intracellular Gat1-GFP localization. If this participation was direct, one might expect to find changes in Gat1 phosphorylation levels as initially reported (Ref. 18 and data not shown). However, past attempts to detect changes in the electrophoretic mobility of Gat1-Myc13 either in response to rapamycin treatment or in vitro treatment of cell extracts with calf intestinal alkaline phosphatase have yielded only negative results (44). The above localization requirements identified new conditions under which to search for a change in Gat1-Myc13 electrophoretic mobility.

The electrophoretic mobility of Gln3-Myc13 increases in untreated sit4Δ or pph21Δpph22Δ cells relative to wild type and does so in a phosphatase-specific, nitrogen source-dependent manner (46, 47). Following this lead, we examined Gat1-Myc13 mobility in crude extracts derived from untreated and rapamycin-treated wild type, sit4Δ, and pph21Δpph22Δ cells. Treating glutamine- or proline-grown wild type cells with rapamycin did not detectably alter the electrophoretic mobility of Gat1-Myc13 (Fig. 8, A and B, respectively, lanes 1, 4, and 5). Gat1-Myc13 mobility was similarly unaltered in a sit4Δ irrespective of (i) the nitrogen source provided (Fig. 8, D and E, respectively, lane 2) and (ii) whether or not cells were treated with rapamycin (Fig. 8, D and E, lane 3). In contrast, nitrogen source-dependent changes in Gat1-Myc13 electrophoretic mobility were observed when extracts from a proline-grown pph21Δpph22Δ were compared with those from wild type cells. Although difficult to visualize in a single exposure, when multiple gels and image exposures were analyzed, one could identify three doublets migrating with markedly different electrophoretic mobilities in extracts of the pph21Δpph22Δ mutant (Fig. 8A, lanes 2 and 3, and overexposures and Fig. 8C). Two changes were detected in Gat1-Myc13 mobilities when pph21Δpph22Δ mutant and wild type extracts were compared. In the first case a species present at low concentrations in wild type cells (Fig. 8A, upper panel, lanes 1–4, upper species of the bottom doublet, lower most black dot) increased in the mutant. In the second case a slower migrating doublet appeared in the mutant that was not present in the wild type extract (Fig. 8, upper and lower panels, lanes 2 and 3, upper most black dot). Even when the blot was vastly overexposed, this upper doublet was not observed in proline-grown wild type cells (Fig. 8A, lower panel, lanes 2 and 3, black dot). It was also absent in glutamine-grown wild type and pph21Δpph22Δ cells (Fig. 8B, lanes 2 and 3). Finally, the electrophoretic profile observed in proline-grown pph21Δpph22Δ cells was not affected by rapamycin addition (Fig. 8A, lanes 2 and 3). Therefore, the appearance or increased levels of these Gat1-Myc13 species paralleled NCR sensitivity and were Pph21/22-dependent. These results are the same as those reported for Gln3-Myc13 in the pph21Δpph22Δ mutant (47). Unfortunately, using multiple conditions and procedures, we were unable to determine whether the slower mobility of the Gat1-Myc13 species observed in Fig. 8 derived from increased phosphorylation. This was largely due to protein degradation during extraction and phosphatase treatment of extracts from proline-grown mutant cells.

DISCUSSION

Assembling the present data with our earlier work has permitted us to construct a more comprehensive view of Gln3 and Gat1 responses and phosphatase requirements to nitrogen availability versus rapamycin treatment. Responses to each of these conditions are GATA factor-specific and possess unique requirements for the Tor pathway phosphatases Sit4 and Pph21/22-Tpd3-Cdc55 (Fig. 9). Together, our data support the idea that Gln3 and Gat1 localizations are each separately controlled by two independent pathways receiving distinct nitrogen supply-related input signals. Although both GATA factors share overlapping responses to the nitrogen environment, each possesses unique primary and secondary responses exhibiting different phosphatase requirements.

Differential Sensitivity of GATA Factor Localization to NCR and Rapamycin Treatment—NCR sensitivity of nuclear Gln3-Myc13 localization is far more clear-cut than that of Gat1-GFP. Gln3-Myc13 is nuclear or nuclear-cytoplasmic in none of the wild type cells cultured with a repressive nitrogen source such as glutamine (Fig. 3, A and B, W.T. Gln, of Ref. 47). Under similar conditions, Gat1-GFP is nuclear-cytoplasmic in 20–30% of the cells (Fig. 2, A and B). These responses correlate well with nuclear Gln3 localization being much more negatively regulated under repressive growth conditions by Ure2 than is Gat1 (compare Fig. 6, A and B, W.T. Gln versus ure2Δ Gln, with Fig. 6, C and D, W.T. Gln versus ure2Δ Gln, of Ref. 48). At the other end of the spectrum, Gln3-Myc13 is localized to the nuclei of nearly all wild type cells cultured with a derepressive nitrogen source, such as proline, with the remainder exhibiting a nuclear-cytoplasmic distribution. Few if any are cytoplasmic (Fig. 6, C and D, W.T. Pro, of Ref. 47). In contrast, Gat1-GFP is exclusively nuclear in few wild type proline-grown cells, is nuclear-cytoplasmic in about half of the cells, and cytoplasmic in the remainder. In short, Gln3-Myc13 localization is more NCR-responsive than that of Gat1-GFP (Fig. 9A).

Conversely, Gln3 and Gat1 sensitivities to rapamycin inhibition of TorC1 are just the opposite. Rapamycin treatment of wild type cells cultured with a repressive nitrogen source elicits
nuclear localization of Gat1-GFP in nearly all cells, with Gat1-GFP being nuclear-cytoplasmic in the remaining few (Fig. 2, A and B). Gln3-Myc<sup>C13</sup>, in contrast, remains cytoplasmic in about 30–40% of rapamycin-treated glutamine-grown wild type cells (Fig. 3, A–D, W.T. Gln + Rap, of Ref. 47). Gln3-Myc<sup>C13</sup> is nuclear-cytoplasmic and nuclear in the remaining cells, with the nuclear-cytoplasmic category usually being somewhat predominant. In short, Gat1-GFP localization is more rapamycin-responsive than that of Gln3-Myc<sup>C13</sup> (Fig. 9A). If we assume a single linear regulatory pathway with the nitrogen supply signal entering at the upper end of it and controlling TorC1 activity, as previously suggested (18, 35), then both Gln3 and Gat1 localization would be expected to respond similarly to rapamycin treatment and growth in proline medium with rapamycin treatment generating the strongest effects. These outcomes are not observed experimentally.

**Phosphatase-specific Requirements for NCR-sensitive and Rapamycin-induced GATA Factor Localization**—Although both Gln3-Myc<sup>C13</sup> and Gat1-GFP do to different degrees relocate to the nucleus under derepressive growth conditions or rapamycin treatment under repressive growth conditions, the protein phosphatase requirements for these re-localizations are different and specific to both the perturbation imposed and the GATA factor monitored (Fig. 9A). In the strains we used (TB50 genetic background), there was an absolute and shared requirement of Sit4 for nuclear Gln3-Myc<sup>C13</sup> and Gat1-GFP localization in response to derepressive growth conditions, i.e. in proline-grown cells (Figs. 9A, 4, A and B, of this work and Fig. 6D, W.T. None, versus sit4Δ none, of Ref. 46). There was additionally a GATA factor-specific requirement; nuclear Gat1-GFP localization under these conditions possessed an absolute requirement for Pph21/22 and Tpd3 (Fig. 5, A and B), whereas there was no such requirement for nuclear localization of Gat1-GFP in untreated and rapamycin-treated wild type (FV063) and pph21Δpph22Δ (03879c) mutant cells. The nitrogen source provided is indicated above each pair of panels. Concentration of these nitrogen sources was 0.1% unless indicated otherwise.
nuclear Gln3-Myc\textsuperscript{13} localization (Fig. 6, C and D, W.T. Pro, versus \textit{pph21Δpph22Δ} Pro, of Ref. 47) (Fig. 9A).

Analogous shared and GATA factor-specific phosphatase requirements also exist for their rapamycin-induced nuclear localization in repressive media as well (Fig. 9A). Both GATA factors exhibit shared requirements for Pph21/22-Tpd3-Cdc55 and Sit4. However, the strength of these requirements is vastly different and GATA factor-specific. In nitrogen-rich medium, the phosphatase requirements are far stronger for Gln3-Myc\textsuperscript{13} than for Gat1-GFP (for Sit4, compare Fig. 2, A and B, W.T. Gln+Rap, versus \textit{sit4Δ} Gln+Rap, and Fig. 7, \textit{sit4Δ} Gln + Rap, of this work with Fig. 3, A and B, W.T. Gln + Rap, versus \textit{sit4Δ} Gln + Rap, of Ref. 47. For Pph21/22, compare Fig. 2, A and B, W.T. Gln + Rap, versus \textit{pph21Δpph22Δ} Gln + Rap, of this work with Fig. 3, C and D, W.T. Gln + Rap, versus \textit{pph21Δpph22Δ} Gln + Rap, of Ref. 47). Rapamycin-induced nuclear Gln3-Myc\textsuperscript{13} localization is absolutely Pph21/22-Tpd3- and Sit4-dependent in these strains and conditions, whereas for Gat1-GFP their requirement is much weaker. Furthermore, nuclear Gln3-Myc\textsuperscript{13} localization requires both PP2A regulatory subunits, Cdc55 and Rts1 (Fig. 11, A–D of Ref. 47), whereas nuclear Gat1-GFP localization only partially requires Cdc55 (Fig. 3, C and D). In nitrogen limiting (proline) medium, nuclear Gln3 localization exhibits no Pph21/22 requirement irrespective of whether cells are treated with rapamycin (Fig. 7 of this work and Fig. 6, C and D, of Ref. 47). In contrast, nuclear Gat1 localization in untreated, proline-grown cells as well as in response to rapamycin treatment exhibits absolute requirements for Pph21/22, Tpd3, and Cdc55 (Figs. 5 and 7).

The above associations can be instructively structured in another way (Fig. 9A). Nuclear Gln3-Myc\textsuperscript{13} localization, whether in response to
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FIGURE 7. Gln3-GFP and Gat1-GFP localizations respond oppositely under multiple conditions. Two aliquots of a wild type (FV063), sit4Δ (FV066), or pph21Δpph22Δ (03879c) culture were transformed with pRS416-Gat1-GFP and pRS416-Gln3-GFP (42, 52). Transformants were grown under identical conditions and inoculated as soon as possible into YNB-serine (Ser), glutamine (−Gln), or −proline (−Pro) medium. At an A600nm of 0.45–0.5, the respective cultures were sampled 6 times, and then where indicated, rapamycin (200 ng/ml) or methionine sulfoximine (2 mM Sigma) was added. The treated cultures were sampled for an additional 30–35 min. All images were derived from samples in which GATA factor localization had become stable as described under “Materials and Methods.”

FIGURE 8. Electrophoretic mobility of Gat1-Myc13 in wild type (FV063), pph21Δpph22Δ (03879c), and sit4Δ (FV066) in untreated and rapamycin-treated (+Rap) cells. The nitrogen source provided in each of the cultures is indicated to the right of the images. The bottom images in panels A and B are overexposures of the upper images. They are presented to permit determination of whether minor low mobility bands existed in lanes 1, 4, and 5 relative to those in lanes 2 and 3. Black dots between lanes 2 and 3 of panel A highlight species that increase in the pph21Δpph22Δ mutant extract relative to wild type. The gel depicted in C was electrophoresed for a longer time than the one in A. The left image of C is a digital photograph similar to those in A, B, and D, and E. The right image of C is a scan of the x-ray film photographed in the left panel.
Tpd3-Cdc55/Rts1 requirement derives from the product to which the nitrogen source is degraded. This is unlikely, however, because the strong Pph21/22 requirement observed when cells are grown in a more derepressive 0.1% serine medium significantly decreases as the concentration of serine is increased to a more repressive level of 0.4% (Fig. 6, C, D, G, and H). Because only serine was used in the experiment, the degradation product does not change from the derepressive to the repressive condition; it is ammonia in both cases. Together, these observations argue that Pph21/22-Tpd3-Cdc55/Rts1 and Sit4 perform quite different functions in the regulation of GATA factor localization.

The dichotomy of Sit4 and Pph21/22-Tpd3-Cdc55/Rts1 phosphatase regulation imposed on Gln3 and Gat1 localization is not the first time striking nitrogen supply-dependent differences have been observed in downstream components of the Tor signal transduction pathway. Two additional examples have been reported.

In the first example, Pph21/22-Tpd3-Cdc55/Rts1-dependent Gln3-Myc13 dephosphorylation (increased electrophoretic mobility) is NCR-sensitive, being greatest in derepressive conditions (proline medium) and least in the repressive conditions that occur in glutamine medium (e.g. Fig. 7, W.T. versus pph21Δ/ρph22Δ, in Ref. 47). It is important to note that a similar Pph21/22-dependent increase in the electrophoretic mobility of Gat1-Myc13 occurs in proline but not glutamine medium (Fig. 8A). However, the correlation cannot be extended further because the source of changes in Gat1-Myc13 mobility remains unknown. In contrast, Sit4-dependent Gln3-Myc13 phosphorylation responds in the opposite manner, being least under derepressive conditions (proline medium) and greatest under repressive conditions (Fig. 7, W.T. versus sit4Δ, in Ref. 47).

In the second example, we demonstrated that the ability of rapamycin treatment to elicit Rtg1/3-dependent transcription of genes, encoding the early tricarboxylic acid cycle enzymes in cells growing in high glucose medium, was completely dependent upon the nitrogen source provided (40, 41). Rapamycin-induced transcription occurred at high levels in ammonia-grown cells but not detectably in glutamate- or
proline-grown cells (40, 41). The nitrogen effect was downstream of that elicited by rapamycin.

**GATA Factor-specific Responses to Methionine Sulfoximine Inhibition of Glutamine Synthetase**—The final set of observations pointing to the distinct regulation of Gln3 and Gat1 localization derive from experiments with Msx (43, 44, 57). Treating cells with Msx results in rapid and complete nuclear Gln3-Myc13 and Gln3-GFP localization (Ref. 43 and Fig. 7). In contrast, Gat1-Myc13 and Gat1-GFP are unaffected and almost completely cytoplasmic (Ref. 44 and Fig. 7). If Gln3 and Gat1 localizations were regulated exclusively by a linear regulatory pathway in which the nitrogen signal is sensed by TorC1 complex which then generates a response to it, Gat1 and Gln3 should have responded similarly to Msx.

**TorC1 Pathway Phosphatase Regulatory Subunits Regulate GATA Factor Localization**—Increasing lines of evidence have accumulated supporting the idea that Gat1 and Gln3 localization and NCR-sensitive transcription responds to multiple cellular signals operating through different regulatory pathways. However, one of the most important characteristics of Pph21/22 regulation of GATA factor localization are the regulatory subunits that function along with them, *i.e.* the Tpd3-Cdc55 and Tpd3-Rts1 complexes. To date, phosphorylated Tap42 has been demonstrated to be the regulatory subunit that associates with Pph21/22 and Sit4 catalytic subunits to implement a TorC1-regulated response to the cell nutrient environment (18, 31, 33, 34). In contrast, the Pph21/22-Tpd3-Cdc55/ Rts1 complexes have been envisioned to function downstream of the proteins dephosphorylated by Pph21/22-Tap42 and Sit4-Tap42 (34) or as a means of antagonizing these activities (33). This raises the pivotal question of the precise function(s) of Pph21/22 in the regulation of GATA factor localization. Do the Pph21/22-Tpd3 complexes themselves dephosphorylate Gln3 and/or Gat1, or are they performing less obvious functions? In this regard we should reemphasize several correlations. Nuclear Gat1 localization in proline-grown (limiting nitrogen) cells is absolutely dependent on Pph21/22-Tpd3-Cdc55. Yet Gln3, whose regulation is more sensitive to the nature of the nitrogen source than to rapamycin-treatment, is absolutely independent of Pph21/22-Tpd3-Cdc55 under these conditions. Furthermore, the extent to which Pph21/22-Tpd3-Cdc55 is required for the TorC1 pathway (rapamycin treatment) to induce nuclear Gat1 localization is regulated in parallel with Pph21/22-Tpd3-Cdc55-dependent Gln3 dephosphorylation and NCR-sensitive transcription; it is highest in limiting nitrogen and lowest when nitrogen is in excess. Finally, the only circumstance in which we could demonstrate a Pph21/22-Tpd3-Cdc55/Rts1 requirement with respect to Gln3 localization was its response to rapamycin in glutamine-grown cells. These correlations are consistent with the idea that nitrogen source-dependent (NCR-sensitive) control of Pph21/22-Tpd3-Cdc55 regulates the ability of Gat1 localization to respond to rapamycin (this work). This is also true for Gln3 in excess nitrogen (47). By this reasoning, Pph21/22-Tpd3-Cdc55 may be involved not only in implementing the TorC1 response to the nitrogen supply but perhaps also in transmitting the signal of intracellular nitrogen levels to TorC1. Work now in progress seeks to investigate the interplay of these regulatory pathways and the roles each regulatory protein plays in it.

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