Deletion of RTS1, Encoding a Regulatory Subunit of Protein Phosphatase 2A, Results in Constitutive Amino Acid Signaling via Increased Stp1p Processing

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The yeast Saccharomyces cerevisiae has developed a complex regulatory network enabling it to control the production of nutrient transporters depending on substrate availability in the environment. Hexose transporters and amino acid transporters are examples of nutrient transporters that are transcriptionally induced by their substrates (14, 24, 44). Amino acids are imported into the cells through amino acid permeases (AAPs) (20). The yeast AAPs belong to the amino acid/polyamine/organocation (APC) superfamily of transporters (28). They exhibit a range of affinities and specificities for all 20 common amino acids and a number of other compounds (42). The presence of extracellular amino acids results in increased transcription of about a third of the AAP genes (14). Amino acids are detected in the environment, 10 kDa of the N terminus are endoproteolytically cleaved off, resulting in relocation of the transcription factors to the nucleus (3). Ssy5p is the endoprotease responsible for processing of Stp1p (1, 2). Signaling and processing are moreover dependent on the F-box protein Grr1p (1, 5, 11, 26), which is part of the E3 ubiquitin ligase SCF (Skp1-Cullin-F-box) complex SCF^{Grr1} (38). Though SCF^{Grr1} normally targets proteins for degradation by the 26S proteasome, activation of Stp1p is not dependent on the 26S proteasome (1). Other factors involved in amino induction include casein kinase I (1). Furthermore, Ptr3p is found to interact with Yfr021wp in a two-hybrid screen (18). The same work reports that yfr021wΔ and ypl100wΔ strains exhibit ptr3Δ-like phenotypes, i.e., they are unable to grow at a high concentration of histidine.

Mutants that exhibit constitutive expression of the target AAP genes independently of the components of the SPS sensor have been identified (15). Here we report a screen likewise aimed at identifying factors down-regulating SPS-mediated signaling, but allowing for factors that, when mutated, cause (constitutive) signaling only if the sensor is present. Using a strain deficient in histidine synthesis, we selected for growth on minimal ammonium medium supplemented with the dipeptide Gly-His. Dipeptides, including Gly-His, are taken up by the permease Ptr2p (39), which is under transcriptional control of...
the amino acid-sensing pathway (4, 8). Subsequent cleavage of internalized Gly-His satisfies the growth requirement for histidine (39). Thus, growth on Gly-His reflects activation of the amino acid-sensing pathway (4, 8). Subsequent cleavage of internalized Gly-His satisfies the growth requirement for histidine (39). Thus, growth on Gly-His reflects activation of the amino acid-sensing pathway (4, 8).

TABLE 1. Strains used in this study

| Strain | Relevant genotype | Reference |
|--------|------------------|-----------|
| M4054  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ | 19        |
| M4605  | MATa SUC2 mal gln2 CUP1 ura3 his3 leu2 gap1Δ | This work |
| M5397  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ | This work |
| M4871  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ sy1Δ | P. S. Nielsen |
| M4723  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ pr3Δ | P. S. Nielsen |
| M4724  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ sy5Δ | P. S. Nielsen |
| M5470  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ grr1Δ | This work |
| M4600  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ sy1Δ | This work |
| M5437  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ pr3Δ | This work |
| M5439  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ sy5Δ | This work |
| M4608  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ stp1Δ stp2Δ stp3Δ | This work |
| M5471  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ grr1Δ rts1Δ | This work |
| M5447  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ STP1-ZZ | 41 |
| M5445  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ sy1Δ STP1-ZZ | 41 |
| M5443  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ pr3Δ STP1-ZZ | 41 |
| M5444  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ sy5Δ STP1-ZZ | 41 |
| M5559  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ grr1Δ STP1-ZZ | This work |
| M5474  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ STP1-ZZ | This work |
| M5501  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ pr3Δ STP1-ZZ | This work |
| M5524  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ sy5Δ STP1-ZZ | This work |
| M5629  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ sy1Δ STP1-ZZ | This work |
| M5525  | MATa SUC2 mal gln2 CUP1 ura3 gap1Δ rts1Δ grr1Δ STP1-ZZ | This work |
**RESULTS**

Isolation of mutants constitutive in amino acid signaling. In order to select mutants with increased activity of the amino acid-sensing pathway, a screen was designed in which a *his3 leu2* derivative of the reference strain M4054 was subjected to transposon mutagenesis. The host strain was transformed with a mixture of yeast genomic fragments representing a mutagenized *LEU2*-based plasmid library (46). Mutants of interest were selected using three different screening criteria. First, we took advantage of the fact that dipeptide uptake (39), as well as branched-chain amino acid uptake (7), is very low in cells grown on minimal medium in the absence of leucine. Dipeptides, including Gly-His, are taken up by the peptide transporter Ptr2p (39), which is under the transcriptional control of the amino acid-sensing pathway (4). Mutants with constitutive signaling were thus selected on SD medium supplemented with the Gly-His dipeptide, which, upon intracellular cleavage, provides the histidine required for growth (39). A subsequent selection was carried out on SD medium supplemented with the amino acids Ile, His, and Val, and the sulfonamide herbicide metsulfuron methyl. This type of compound inhibits synthesis of branched-chain amino acids and therefore prevents growth of cells unable to take up branched-chain amino acids from the medium (53), such as mutants deficient in amino acid signaling (29, 30). Finally, colonies of interest were tested for constitutive activity of the *BAP2* promoter.

Among the 11 mutants thus isolated, three had transposon insertions in the PP2A subunit-encoding gene *RTS1* (34). The remaining transposons were localized in *VPS15, VPS34*, and *VPS16*, coding for proteins involved in vacuolar protein sorting, and *STHI*, coding for the ATPase subunit in the chromatin remodeling complex RSC. Two others had mutations in ORFs with unknown function, *YFR044C* and *YFR045W*. Two mutants were not mapped. In the present work, the relationship between the phenotype and the locus affected by transposon insertion was analyzed in more detail for the *RTS1* gene.

**Deletion of RTS1 results in constitutive activation of BAP2 and AGPI transcription.** Since transposon insertion very often completely inactivates a gene, we wished to analyze the behavior of a strain deleted for *RTS1*. One of the selection criteria used in the original screen was the ability to take up Gly-His on SD medium, thereby promoting growth of a histidine-requiring strain because of intracellular cleavage of the dipeptide. To find out if an *rts1*Δ strain exhibits the same phenotype, we deleted the *RTS1* ORF in a *his3* derivative of the reference strain M4054. Indeed, the lack of *RTS1* enabled growth on the dipeptide as a histidine source (not shown).

In order to be able to analyze the *rts1*Δ mutation in the absence of extracellular amino acids or dipeptides, we also constructed a *HIS3 rts1*Δ strain (M5397). First we wished to confirm the activation of target promoters for the amino acid signaling by testing for growth on YPD supplemented with metsulfuron methyl; the strain grew, as expected (not shown). Then we tested whether this activation was constitutive by yet another criterion, using the dipeptide L-leucyl-L-ethionine. If this dipeptide is taken up, it is cleaved to yield the toxic methionine analogue L-ethionine. Also, uptake of the dipeptide L-leucyl-L-ethionine is mediated up by the peptide transporter Ptr2p (39) and is thus under transcriptional control of the amino acid-sensing pathway (4). Insensitivity to L-leucyl-L-ethionine therefore reflects that the pathway is not activated, while growth inhibition in the absence of an inducing amino acid indicates constitutive activation of the amino acid-sensing pathway. The *rts1*Δ strain M5397 was unable to grow on minimal medium in the presence of L-leucyl-L-ethionine, suggesting constitutive activity of the signaling pathway (not shown).

We also investigated activation of the promoter of the broad-specificity AAP gene *AGPI* using a fusion of the promoter to the *E. coli lacZ* gene. Cells were grown in SD medium and incubated for 40 min with or without 100 μM L-leucine. The experiment was repeated with 5 mM L-citrulline or an equal volume of water. These concentrations have been previously found to fully or almost fully induce the *AGPI* promoter (16, 40). The results show that *AGPI* transcription in *rts1*Δ cells is constitutive, i.e., unaffected by leucine or citrulline addition. In the wild-type control experiment, *AGPI* promoter activation was low in the absence of amino acids and increased strongly in response to either leucine or citrulline (Table 2).

Transcriptional induction of AAP genes was previously shown to involve Stpt1p endoproteolysis (3). We used the Stpt1p-ZZ construct previously described (40), in which the C terminus of Stpt1p was fused to a doublet of the IgG-binding Z domain of the *S. aureus* protein A. This insert was integrated in the wild-type strain and the *rts1*Δ strain, resulting in strains M5447 (40) and M5474, respectively. The fraction of processed Stpt1p in *rts1*Δ cells in the absence of leucine was greater than in wild-type cells (30% versus 13%), whereas addition of leucine led to almost complete (above 90%) Stpt1p processing in both wild-type and *rts1*Δ cells (Table 3 and Fig. 1). Quantification of antibody binding showed that the relative amount of processed Stpt1p in the absence of inducer was more than doubled in *rts1*Δ cells compared to wild-type cells.

**TABLE 2. AGPI promoter activity in wild-type cells and rts1Δ cells in response to leucine and citrulline**

| Inducer   | Mean β-galactosidase activity ± SEM |
|-----------|-------------------------------------|
|           | M4054 (wild type) | M5397 (rts1Δ) |
| L-Leucine | 1.5 ± 0.1 | 10.5 ± 0.4 |
|           | 8.4 ± 1.0 | 9.9 ± 0.9 |
| L-Citrulline | 0.1 ± 0.1 | 11.4 ± 0.6 |
|           | 8.7 ± 1.4 | 13.5 ± 0.4 |

* Activity in Miller units in the absence (−) and 40 minutes after the addition (+) of inducer.

**TABLE 3. Quantification of processed Stpt1-ZZ in wild-type and rts1Δ cells in response to leucine**

| Strain      | Mean amt of processed Stpt1-ZZ ± SEM |
|-------------|--------------------------------------|
|             | Without inducer | With inducer |
| M5447       | 12.8 ± 1.1      | 90.2 ± 0.4    |
| M5474       | 30.4 ± 5        | 92.7 ± 1.6    |

* Samples were taken in the absence and 20 minutes after addition of 100 μM L-leucine. Relative amounts of full-length and processed Stpt1-ZZ were determined by performing the experiment twice.
Remarkably, Stp1p processing to the extent of 30% is sufficient to generate an activity of the AGP1 promoter comparable to that observed in wild-type cells after amino acid addition (compare Table 2 and Table 3). In fact, this is expected from previous comparisons of dose-response relationships using quantification of Stp1p processing versus AGP1 promoter activity as the read-out (40, 41). The sensing of L-leucine exhibits the characteristics of wild-type cells in response to 100 \( \mu \text{M} \) l-leucine, and all were sensitive to metsulfuron methyl on YPD, and all were defective in signaling due to the low, remaining activity of the amino acid signaling pathway in an \( \Delta \) STP1 (40). The most obvious interpretation is that saturation of the promoter activity occurs already at modest levels of activated Stp1p.

**Epistasis relationships.** In order to further investigate the role of RTS1, we constructed strains M4600 (\( \Delta \) ssy1 \( \Delta \) rts1\( \Delta \)), M5437 (\( \Delta \) prr3 \( \Delta \) rts1\( \Delta \)), M5439 (\( \Delta \) ssy5 \( \Delta \) rts1\( \Delta \)), M5470 (\( \Delta \) grr1 \( \Delta \) rts1\( \Delta \)), and M4608 (\( \Delta \) stp1 \( \Delta \) stp2 \( \Delta \) stp3 \( \Delta \) rts1\( \Delta \)). STP3 is homologous to STPI and STP2, and Helge A. Andersen (personal communication) has found that deletion of STP3 further reduces the low, remaining activity of the amino acid signaling pathway in an \( \Delta \) stp1 \( \Delta \) stp2 \( \Delta \) mutant. All of the resulting strains were sensitive to metsulfuron methyl on YPD, and all were insensitive to l-leucyl-l-ethionine (not shown), suggesting that the amino acid-sensing pathway was inactive.

β-Galactosidase assays were performed on extracts from cells in which the E. coli lacZ gene was placed under the control of the AGP1 promoter. β-Galactosidase measurements confirmed that the multiple mutants behaved similarly to the single mutants deficient in signaling, i.e., AGP1 transcription was very low even in the presence of extracellular amino acids (Table 4). Using the Stp1-2Z construct, we also measured relative amounts of processed and full-length Stp1p in each of the strains, in the absence and presence of amino acids. Stp1p was exclusively present as the full-length protein in all single and multiple mutants (Fig. 2). In other words, \( \Delta \) ssy1, \( \Delta \) prr3, \( \Delta \) ssy5, and \( \Delta \) grr1 are epistatic over \( \Delta \) rts1.

**DISCUSSION**

In this work we have attempted to identify potential negative regulators of the amino acid-sensing pathway. For this purpose we have designed a screen allowing selection of mutants with constitutive activity of the pathway. The screen described in this report was performed in the absence of inducer in cells lacking \( \Delta \) GAPI, and we investigated transcription levels of a lacZ reporter gene placed under the control of the amino acid-inducible promoters of the AGP1 and BAP2 genes, which are known targets of the SPS-mediated pathway. The genes that were disrupted by transposon insertion encode proteins that could be involved at any level of the signaling.

The isolated mutants include some in which the transposon insertion disrupted the RTS1 gene. This gene encodes a regulatory subunit of PP2A, which is a major serine/threonine phosphatase involved in several nutrient-induced signaling pathways, in cell growth control and cell division control (9, 35, 45, 56). Protein phosphatase 2A exists in several isoforms and is mostly present in cells as a heterotrimeric complex, consisting of a catalytic (C) subunit, encoded by \( \Delta \) PPH21, \( \Delta \) PPH22, or \( \Delta \) PPH3 (45, 49), a scaffolding subunit (A), encoded by \( \Delta \) TDP3 (55), and a regulatory subunit (B or B*), encoded by CDC55 (22) and RTS1 (47, 48), respectively. The B and B* subunits are believed to regulate the activity of PP2A by determining its cellular location and modifying the substrate specificity of the C subunit (31, 54). CDC55 is required for correct cell cycle checkpoint control (35), and \( \Delta \) cdc55A cells display a cold-sensi-

**TABLE 4. AGP1 promoter activity in cells deleted of genes encoding positive factors in amino acid signaling and/or RTS1 in the absence and presence of inducing amino acids**

| Inducer | M4054 (wild type) | M5397 (\( \Delta \) rts1\( \Delta \)) | M4600 (\( \Delta \) ssy1 \( \Delta \) rts1\( \Delta \)) | M5437 (\( \Delta \) prr3 \( \Delta \) rts1\( \Delta \)) | M5439 (\( \Delta \) ssy5 \( \Delta \) rts1\( \Delta \)) | M5471 (\( \Delta \) grr1 \( \Delta \) rts1\( \Delta \)) | M4608 (\( \Delta \) stp1 \( \Delta \) stp2 \( \Delta \) stp3\( \Delta \)) |
|---------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| L-Leucine (100 \( \mu \text{M} \)) |                  |                   |                   |                   |                   |                   |                   |
| -       | 1.7 ± 0.1        | 10.1 ± 0.2        | 1.1 ± 0.1         | -0.1 ± 0.0        | -0.8 ± 0.0        | 0.1 ± 0.0         |                   |
| +       | 7.2 ± 0.6        | 9.6 ± 0.2         | 1.1 ± 0.0         | -0.1 ± 0.0        | -0.6 ± 0.0        | -0.1 ± 0.0        |                   |
| L-Citrolline (5 mM) |                 |                   |                   |                   |                   |                   |                   |
| -       | 0.1 ± 0.0        | 0.95 ± 0.1        | 0.0 ± 0.0         |                   |                   |                   |                   |
| +       | 11.3 ± 1.2       | 0.83 ± 0.1        | 0.0 ± 0.0         |                   |                   |                   |                   |

* Activity in Miller units in the absence (–) and 40 minutes after the addition (+) of inducer.
tive phenotype but no phenotype at elevated temperatures (22). RTS1 was found in two independent genetic screens, as a multicyclic suppressor of hsp60(Ts) mutant alleles (48), and later as a Rox Three Suppressor (12); rtslΔ cells are thermosensitive and exhibit a typical cdc mutant phenotype (47).

We identified RTS1 as a negative component of the SPS-mediated amino acid-sensing pathway. Deletion of RTS1 results in constitutive transcription of AGPI and BAP2. Moreover, ssyl, ptr3, syy5, and grl1 were found to be epistatic over rtsl. These results indicate that the PP2A is involved in the SPS-mediated pathway and suggest that a dephosphorylation step is required to down-regulate signaling in the absence of extracellular amino acids. Rtslp1 associated with Tpd3p and the C subunit might thus dephosphorylate one of the SPS proteins. Alternatively, it could dephosphorylate the Stp transcription factors, resulting in a conformational change that perhaps limits accessibility of the cleavage site to the protease, thereby impairing Stp1p and Stp2p proteolysis and activation in the absence of amino acids.

While PP2A appears to down-regulate amino acid signaling, a corresponding kinase should be involved in the activation of the pathway. Casein kinase I is a candidate for this activity, since the amino acid-sensing pathway is inactive in temperature-sensitive mutants affected in the casein kinase I genes YCK1 and YCK2, and these strains exhibit loss of Stp1p processing (1). Casein kinase I and PP2A are known to act on the same substrate in Xenopus embryos (17) and we propose that they do so in yeast as well. This hypothesis is substantiated by the finding that the yeast Cdc55p subunit forms a complex with casein kinase I (23). Moreover, casein kinase I has also been reported to be involved in the glucose-sensing pathway (50), which mediates transcriptional induction of hexose transporter (HXT) genes in response to extracellular glucose and which shares many similarities with the amino acid-sensing pathway. Casein kinase I is indeed required for phosphorylation of Mth1, which is then targeted for degradation by SCFGrr1 (36, 50). Thus, glucose induction of the HXT1 promoter is deficient in a yck1Δ yck2(Ts) mutant; in addition, this mutant lacks glucose-induced degradation of Mth1p and Std1p (36).

Mth1p and Std1p interact with the transcriptional repressor Rgt1p in the absence of glucose: the resulting complex binds promoter DNA, thereby repressing transcription from the HXT genes (33, 52). Likewise, Stp1p is phosphorylated in a casein kinase I-dependent way prior to its endoproteolytic activation (1). SCFGrr1 recognizes phosphorylated substrates; this is the case for Mth1p (50) and for the G1 cyclin Cln2p (25). Although Stp1p is phosphorylated by casein kinase I (1), there is no reason to invoke it as a target for SCFGrr1. A possible target for SCFGrr1 is Prt3p, which in fact has been found to be subject to posttranslational modification, exhibiting a slower-migrating band (13) that might be due to ubiquitination. Alternatively, the target for SCFGrr1 could be a protein not yet known to be part of the pathway. Further work is needed to propose which protein is a target for PP2A associated with Rts1p. There may, however, be cross talk between the amino acid-sensing and the target of rapamycin (TOR) pathways, since N. Eckert-Boulet (unpublished data) has observed that treatment of rts1Δ cells with rapamycin enhanced the constitutive signal, also at the level of Stpl-ZZ processing.

Interestingly, it has recently been found that PP2A is involved in regulating the induction of HXT1 by glucose and that Cdc55p is the regulatory subunit involved. The 1A-3-3 proteins Bmh1p and Bmh2p also appear to be involved (51). These observations add to existing similarities between the glucose induction pathway and the amino acid induction pathway. It will be interesting to see whether Bmh1p and Bmh2p also are involved in the SPS-mediated pathway and whether Rts1p interacts with casein kinase I like Cdc55p does.

In summary, we have shown that Rts1p, one of the two known regulatory subunits of PP2A, is a down-regulator of the SPS-mediated amino acid-sensing pathway. This finding illustrates a new role in nutrient-induced signaling for PP2A and further highlights the reuse of components in different signaling pathways.

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