Genetic Analysis of the TOR Pathway in Aspergillus nidulans

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We identified five genes encoding components of the TOR signaling pathway within Aspergillus nidulans. Unlike the situation in Saccharomyces cerevisiae, there is only a single Tor kinase, as in plant and animal systems, and mutant phenotypes suggest that the TOR pathway plays only a minor role in regulating nitrogen metabolism.

The TOR signaling pathway is a well-conserved mechanism controlling cell growth in eukaryotes, especially in response to nutrient availability (16, 23). One of the roles of the two Tor kinases (Tor1p and Tor2p) in Saccharomyces cerevisiae is to repress the transcription of genes subject to nitrogen metabolite repression (3, 6). In nitrogen-rich conditions, TOR signaling prevents nuclear accumulation of Gln3p, maintaining it within a cytoplasmic complex with Ure2p (3), preventing activation of a suite of nitrogen utilization genes. The major regulator of nitrogen metabolism in the filamentous fungus Aspergillus nidulans, the GATA factor AreA (19), is a homologue of Gln3p and a second partially redundant transcription factor, Gat1p. Despite detailed molecular and genetic characterization of nitrogen metabolism in A. nidulans (4), a TOR pathway has not been described or implicated in nitrogen signaling. However, one putative component of the pathway has been identified previously (9). We adopted a genetic approach to identify components of this pathway in A. nidulans, selecting mutants resistant to the immunosuppressive and antifungal compound sirolimus (rapamycin) that first revealed the TOR pathway in S. cerevisiae (13). The mutant phenotypes are consistent with the TOR signaling pathway having a minor role in A. nidulans nitrogen signaling.

Sirolimus causes growth inhibition in yeasts and filamentous fungi, including Aspergillus fumigatus (26). In A. nidulans, sirolimus has fungistatic activity, causing severely reduced growth and conidiation (Fig. 1). Mutations in areA do not alter sirolimus sensitivity, whereas S. cerevisiae gln3 gat1 double mutants show partial resistance (3). Unlike for S. cerevisiae, where sirolimus treatment in a nitrogen-rich medium results in rapid expression of genes subject to nitrogen metabolite repression, such as the general amino acid permease gene GAPI and the glutamine synthase gene GLN1 (3, 6), we did not detect induction of AN5678.2 (homologue of S. cerevisiae GAPI), gluA (glutamine synthase), areA, or mepA (ammonium permease) in Northern blot analysis over a 30-min time course (data not shown). This indicates that, unlike in S. cerevisiae, sirolimus does not override nitrogen metabolite signaling in A. nidulans.

In S. cerevisiae, sirolimus toxicity occurs via complex formation with the prolyl-isomerase-like protein FKBP12 (14). Specific mutations in FPR1, encoding FKBP12, TOR1, and genes encoding two downstream components of the TOR pathway, TIP41 (17) and TAP42 (10), result in distinct sirolimus-resistant phenotypes. We used UV irradiation of both haploid (yA2 pabaA1; areA49) and diploid (pabaA1/suA1adE20 yA1 adE20; acrA1; gatA1; pyroA4; acuA303; sB3; nicB8; riboB2) A. nidulans strains to select mutants resistant to sirolimus (200 ng/ml). The sirolimus resistance phenotypes identified in the haploid strains segregated normally in crosses, and the mutants selected were associated with either linkage group (LG) I or LG II. Sirolimus resistance in all six diploid strains tested was associated with LG I, but it proved impossible to obtain sirolimus-resistant haploids from these diploid strains, suggesting that the mutant alleles disrupt an essential function.

To identify the mutations within the sirolimus-resistant strains, we used a combination of bioinformatics, PCR, and sequencing. Assembly and annotation of the public A. nidulans genome sequence proceeded during the course of our work. BLAST searches using protein sequences from other organisms submitted to A. nidulans databases aided identification.

A single Tor kinase gene homologue on LG I which we named torA (accession no. AJ717403) was identified in A. nidulans. Based on reverse transcription (RT)-PCR, the putative protein is 2,385 amino acids long and contains all the domains associated with Tor function (Fig. 2A). Full disruption of Tor activity is likely to be lethal, as has been shown in other fungi, including S. cerevisiae (20) and Fusarium fujikuroi (Sabine Teichert and Bettina Tudzynski, personal communication). PCR amplification and sequencing of the sirolimus-resistant diploid torA3 strain revealed a single point mutation at position 5751 that would result in Pro replacing Ser-1871 within the highly conserved sirolimus-binding domain (8). The dominant nature of the allele indicates that the mutant protein must retain some aspects of its function in the presence of sirolimus. Its apparent lethality in the haploid suggests either multimerization, where the heterodimer retains partial function in the presence of sirolimus, or that the wild-type Tor kinase retains essential sirolimus-tolerant functions affected by this mutation. In S. cerevisiae and mammalian systems, there is evidence for sirolimus-sensitive and -insensitive functions, but Tor multimerization has not been observed (15, 20, 21, 25, 29).

An FPR1 homologue was identified on LG II and named fpra (accession no. AJ717400). The predicted protein se-
The effects of sirolimus on the growth of the wild-type (+/+ +) and torA3 heterozygote (torA3//+) diploids, as well as the wild-type (+), fprA1, jipA10, nmrA1, and jipA10 nmrA1 haploid strains, are shown. Nitrogen source utilization was tested by growing the strains on supplemented minimal medium containing NH4+ , threonine (thr), uric acid (UA), urea, NO3−, or 2-pyrrolidone (pyrr) at 10 mM. Sirolimus (rap) was added at a final concentration of 200 ng/ml to supplemented minimal medium containing 10 mM NH4+ . Nitrogen metabolite derepression was assessed using chlorate (ClO3−) at 500 mM or dl-aspartic acid β-hydroxamate at either 1 mM (DLaa1) or 10 mM (DLaa10) or on minimal medium supplemented with 10 mM NH4+ .

Resulting in a conformation change that prevented sirolimus binding. Finally, a single T→C transition at position 485 in fprA7 altered one of three invariant residues (Phe-100→Ser) required for interaction between FKBP12-sirolimus and Tor kinase (22, 28).

The sirolimus-resistant mutations selected in the haploid strain and located on LG I are within jipA (9). The A. nidulans homologue of S. cerevisiae TIP41. PCR and sequencing of the jipA coding region in two mutants showed that single nucleotide insertions had caused frameshift mutations, truncating the putative protein after 92 (jipA45) and 49 (jipA10) residues (Fig. 2B).

The heterozygous diploid torA3/torA+ showed increased sensitivity to 10 mM dl-aspartic acid β-hydroxamate in the presence of NH4+ , indicating partial derepression of the asparaginase gene ahrA (11, 27). Both fprA− and jipA− showed weak pleiotropic effects on several nitrogen sources and had severely reduced growth on 2-pyrrolidone. The jipA− strains showed marginally reduced growth irrespective of the nitrogen sources tested. The fprA1 and torA3 diploid strain showed enhanced growth on threonine. Interestingly, in S. cerevisiae FKBP12 has a role in aspartokinase feedback regulation of the threonine biosynthetic pathway (2).

A. nidulans does not have an orthologue of yeast URE2 (12). However, a structurally unrelated protein, NmrA, is known to interact with and inhibit AreA (1, 24) and therefore has similar function to Ure2p, which represses Gin3p. In S. cerevisiae, activity of the phosphatase Sit4p, required to release Gin3p from Ure2p, is inhibited by an interaction with Tap42p as part of the TOR network (3). Within the A. nidulans genome, we identified tapA (accession no. AJ717402) and sitA (accession no. AJ717404), homologues of TAP42 and SIT4, respectively, and confirmed their expression and intron structure using RT-PCR. As this arm of the TOR signaling network is conserved in A. nidulans, it was important to determine whether JipA
effected nitrogen signaling through NmrA. We therefore constructed jipA10 nmrA/H9004 double mutants. These were sirolimus resistant, had reduced growth on nitrogen sources equivalent to jipA10 single mutants, were hypersensitive to DL-aspartic acid/H9252-hydroxamate, and were more sensitive to chlorate than either parental strain (Fig. 1). Results of plate tests for nitrite reductase activity were intermediate to those obtained with single mutants (data not shown). Deletion of nmrA results in asparaginase and nitrate reductase derepression (1), but poor growth observed on 2-pyrrolidone suggested that NmrA has a positive effect on the expression of some genes (Fig. 1). One interpretation is that NmrA may remain associated with AreA when it is active. The lack of a clear epistatic relationship suggests that JipA does not act through NmrA. Double mutants containing jipA10 and areA alleles lacking specific domains (N terminus, areA/H90042-389 [5]; C terminus, areA/H9004743-864 [24]; and C-terminal GATA domain and consensus nuclear export signal, areA120 [7, 24]) also failed to give clear epistatic relationships (data not shown), indicating that TOR signaling is not effected specifically through any one of these regions.

Both jipA− and fprA− strains showed their most severe nitrogen utilization phenotypes on 2-pyrrolidone. Northern analysis was used to measure expression levels of the utilization genes lamA and lamB (18). Expression of both genes was significantly lower in jipA10 than the wild type over a 2-h time course after transfer from NH4+/H11001 to 2-pyrrolidone (Fig. 4). However, only lamA expression was significantly reduced in jipA10 and fprA1 strains when induced by β-alanine, which is a good nitrogen source for both strains. This is consistent with the defects in 2-pyrrolidone utilization being due, in part, to reduced transcription of lamA.

We have therefore identified five genes encoding components of the TOR signaling pathway within A. nidulans, namely, torA, fprA, jipA, sitA, and tapA, using a combination of mutant selection, database searching, PCR, and sequencing. The conservation of these components and the presence of only a single Tor kinase make A. nidulans a good model for the situation in animals and plants. There is no clear evidence to suggest TOR signaling acts through AreA. However, the mutant phenotypes suggest that TorA, FprA, and JipA play a minor role in nitrogen metabolism, which in the case of jipA− and fprA− strains results in reduced transcription of at least one gene involved in nitrogen assimilation (lamA).

Nucleotide sequence accession numbers. Sequences from this study were submitted to GenBank and assigned accession no. AJ717400, AJ717401, AJ717402, AJ717403, and AJ717404.
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