Interaction of Knr4 protein, a protein involved in cell wall synthesis, with tyrosine tRNA synthetase encoded by TYS1 in *Saccharomyces cerevisiae*

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Abstract

The Knr4 protein, known to be involved in the regulation of cell wall assembly in *Saccharomyces cerevisiae*, strongly interacts with the tyrosine tRNA synthetase protein encoded by TYS1 as demonstrated by the genetic two-hybrid system and a biochemical pull-down experiment using GST–Tys1p fusion. Data reported here raise the possibility that this physical interaction between these proteins is required for dityrosine formation during the sporulation process. In addition, it is shown that the efficiency of spores formation was drastically reduced in diploid cells homozygous for the disruption of KNR4 or for a temperature-sensitive mutation of TYS1, although this effect could be independent of their protein interaction. Altogether, these data provide novel functions of Knr4p and Tys1p to those that were known before. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Cell wall; Sporulation; Two-hybrid screen; Dityrosine; Aminoacyl tRNA synthetase

1. Introduction

The cell wall of *Saccharomyces cerevisiae*, in spite of its apparent rigidity, is a highly dynamic structure. Its general biological function includes mechanical protection, determination of cell shape and modulation of selective uptake of molecules. Moreover, the cell wall structure can adapt to different physiological states (conjugation, sporulation, stationary phase, etc.) and morphological changes, i.e. pseudohyphal and agar-invasive growth. Such a plasticity requires the coordinated regulation of the expression of genes involved in the biosynthesis of cell wall components, namely β-glucans and mannoproteins, and in their assembly at the cell surface [1,2]. Although spore wall assembly has received less attention, it has been shown that regulatory molecular mechanisms comparable to those identified in the formation of vegetative cell walls occur for the formation of wall ascospores during the sporulation process [3–5].

Screening for genes that might be involved in the assembly of cell wall structural components, we isolated KNR4 as a suppressor of Calcofluor White hypersensitivity of several cell wall mutants [6]. It was reported that the loss of KNR4 function results in a 50% reduction of the level of both β-1,3-glucan and β-glucan synthase activity and in a high cell wall permeability to the 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-gal chromophore) [7]. Deletion of KNR4 resulted in a hypersensitivity of the cells to Calcofluor White, whereas overexpression caused a strong reduction in mRNA levels of CHS1, CHS2 and CHS3 encoding chitin synthases and delayed smoos formation upon exposure to α-factor [6]. While Knr4p affects expression of genes encoding chitin synthases, this effect must be indirect, likely through the interaction with other regulatory proteins since Knr4p does not appear to be a nuclear protein and it has been localized to the presumptive buds site in unbudded cells and at the incipient bud site during bud emergence [6]. We therefore devised a two-hybrid screen using Knr4p as a bait
and identified several partners, among which the tyrosine tRNA synthetase encoded by TYS1 turned out to be a frequently isolated product.

2. Materials and methods

2.1. Yeast, bacteria strains and plasmid construction

Yeast strains used in this study are listed in Table 1. Strains of *Escherichia coli* DH5α were used for plasmid amplification and BL21 for heterologous expression of yeasts Tys1p and Knr4p. Plasmid pGEX-TYS1 containing a glutathione S-transferase (GST)-Tys1 fusion was obtained by PCR amplification of a 1.2-kbp *TYS1* fragment using the primers 5′-AGATCTTCTCTTGAGGACC-GGTTG-3′ and 5′-CAGCTCTCTGTCTGCA-CGCGGTG-3′ (underlined are the *Bam*HI and *Nco*I restriction sites) and cloning of this fragment digested by *Bam*HI and *Nco*I into plasmid pGEX-3x. Plasmid pGAL10-TYS1 consists of the *TYS1* open reading frame (ORF) amplified with primers 5′-GGATCCTGGATCTATTCAAAAGAAAAGTT-3′ and 5′-GAGCTCTTTAAGCTATTCTCCTGATGTTG-3′ and 5′-GAGCTCTTTAAGCTATTCTCCTGATGTTG-3′ inserted into the *TYS1* ORF from genomic DNA of the strain AR27 with the high fidelity Vent polymerase from New England Biolabs. The PCR product was cut by *Xba*I and *Hind*III restriction enzymes, and subcloned in the vector pMALC2 from New England Biolabs, in frame with the maltose-binding protein (MBP) encoding gene. The fusion protein was expressed in *E. coli*, purified from a 12% SDS-polyacrylamide gel and used to immunize rabbits (Eurogentec Sa, Belgium). Specificity of the antibody was further rechecked for Ade+ and His+ phenotypes in the deletion strain (AR27) and a *knr4*Δ strain (HM10), and optimal dilution was found between 1/2000 and 1/40,000.

2.2. Production of anti-Knr4p antibodies

The oligonucleotides KNR4 start *Xba*I (ACTAGTATG-GATCTTCTCTGAGGACC-GGTTG-3′) and KNR4 end *Hind*III (AAGCTTTAAAGCTATTCTCCTGATGTTG-3′) were used to amplify the KNR4 ORF from genomic DNA of the strain AR27 with the high fidelity Vent polymerase from New England Biolabs. The PCR product was cut by *Xba*I and *Hind*III restriction enzymes, and subcloned in the vector pODB80 from New England Biolabs, in frame with the maltose-binding protein (MBP) encoding gene. The fusion protein was expressed in *E. coli*, purified from a 12% SDS–polyacrylamide gel and used to immunize rabbits (Eurogentec Sa, Belgium). Specificity of the serum was checked by testing it against protein extracts from a control strain (AR27) and a *knr4*Δ strain (HM10), and optimal dilution was found between 1/2000 and 1/40,000.

2.3. Culture media

Yeast media contained either 2% (w/v) bactopeptone, 1% (w/v) yeast extract and 2% (w/v) glucose (YEPOD) or 0.17% (w/v) yeast nitrogen base without amino acids and ammonium, 0.5% (w/v) ammonium sulfate and 2% (w/v) glucose (SC), supplemented with auxotrophic requirements. For solid media, agar (Difco) was added at 2% (w/v) final concentration. Unless specified, yeast cells were cultured at 30° C and growth was followed by measurement of OD600 (one unit OD600 corresponded to about 0.25 mg cell dry mass or 2×107 cells ml⁻¹).

2.4. The two-hybrid screening

The two-hybrid host strain pJ67-4A [9] was first transformed with pODB-KNR4, which expresses full-length Knr4p fused to GAL4-binding domain and which was constructed as follows: the *KNR4* ORF was amplified without termination codon using primers KNR4-sens: 5′-GGATCCTGGATCTATTCAAAAGAAAAGTT-3′ and KNR4-rev: 5′-ATCTGAGAAGCTATATTCTCTGCTGATGTTG-3′ and cloned in *Bam*HI and *Pst*I sites (underlined) of the plasmid pODB80 [10]. The transformed cells were grown overnight in SC minus Trp medium. Yeast cells were diluted in fresh YEPD media to OD600 = 0.25, grown until OD600 = 0.6 and then collected by centrifugation. They were transformed with a library of fusion between the GAL4 activation domain (GAD) and yeast genomic fragments in all three reading frames [9], and plated on SC minus Trp and Leu. After 3 days of growth at 30° C, the plates were replicated on SD minus Ade and His+ media. The plasmids that conferred sustained growth of transformed cells on these media were sent for partial sequencing (Genome Express, France). Complete sequences were retrieved using SGD and YPD data bases.

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Table 1

List of yeast strains used in this work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJ69-4A</td>
<td>MATa trp1::kanΔ leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1::HIS3 GAL2::ADE2 met2::GAL7-lacZ</td>
<td>[9]</td>
</tr>
<tr>
<td>AR27</td>
<td>MATa ura3-52</td>
<td>[6]</td>
</tr>
<tr>
<td>AR27-2N</td>
<td>MATa MATa ura3-52/ura3-52</td>
<td>This study</td>
</tr>
<tr>
<td>SS28</td>
<td>MATa MATa ura3-52/ura3-52</td>
<td>[19]</td>
</tr>
<tr>
<td>ts2</td>
<td>MATa ade2-101 his3A 200 tyr1 ura3-52 tys1Δ</td>
<td>[19]</td>
</tr>
<tr>
<td>HM10</td>
<td>AR27 knr4::KANΔ</td>
<td>[6]</td>
</tr>
<tr>
<td>HM20</td>
<td>AR27 KNR4::kanΔ</td>
<td>[6]</td>
</tr>
<tr>
<td>HM21</td>
<td>AR27-2N knr4::KANΔ TYS1::kanΔ</td>
<td>This study</td>
</tr>
<tr>
<td>AD1</td>
<td>AR27 TYS1::kanΔ</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.5. Protein-binding (pull-down) assays

Crude extracts containing overexpressed GST-Tys1p or MBP-Knr4p were prepared from E. coli BL21 transformed with pGEX-TYS1 or pMBP-KNR4. A 1.5 ml of transformed bacteria were grown in LB media with ampicillin to an OD 600 = 0.6 before addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested after 4 h of induction by centrifugation. The pellet was resuspended in 13.5 ml of STE buffer, containing 50 mg ml⁻¹ of lysozyme and subjected to cell lysis as described by Frangioni and Neel [11]. Cellular extracts (100 μl) containing GST or GST-Tys1 were mixed with glutathione-Sepharose beads (50% v/v in phosphate-buffered saline (PBS) buffer), and incubated with agitation at 4°C for 1 h. Then, the extracts containing MBP-Knr4p were added to both samples and incubation was continued overnight at 4°C. The resin was washed six to eight times with ice-cold PBS buffer. Bound proteins were eluted with elution buffer (HEPES 75 mM, pH 7.4 containing NaCl 150 mM and EDTA 1 mM) and incubated with agitation at 4°C for 1 h. Determination of dityrosine content in the spore wall was carried out as described by Briza et al. [13].

2.6. Sporulation conditions and assay of dityrosine content

Diploid strains were grown on solid YEPD or SC media with supplements, and then spread on solid 1% K-acetate media, supplemented with adenine (20 μg ml⁻¹ for strains bearing Ade⁻ auxotrophy). For the cells transformed with pYEDP-TYS1, galactose (2%) was used in the place of glucose in presporulation media, and galactose (2%) was added in the K-acetate media. Sporulation was checked after 2–3 days of incubation under optical microscopy.

3. Results and discussion

3.1. Isolation of Tys1p as a protein interacting with Knr4p

The KNR4 ORF fused in frame to the binding domain of GAL4 inserted in pJ67-4A strain was used as a bait to search for protein partners. After the transformation of this strain with genomic libraries made in pGAD plasmids, namely pGAD-C2, pGAD-C3 and pGAD-C1 [9], 10 clones that exhibited an efficient growth on SD media without adenine and histidine were recovered, and the plasmids were isolated and sequenced. Three of them contained a TYS1 DNA fragment encoding the C-terminal part (started from the amino acid at position 197) of the tyrosine tRNA synthase. Four others contained the CIN5/HAL6 gene which encodes a transcription factor that belongs to the basic leucine zipper protein family [14], and whose overexpression increases salt tolerance of the cells [15]. The YMR262 which encoded a protein of yet unknown function was also recovered three times. Quantification of these protein–protein interactions was then assessed by measurement of the β-galactosidase activity from GAL7-lacZ (Table 2). The interaction of Knr4p with Tys1p was by far the strongest, showing a β-galactosidase activity that was even four times higher than that obtained with the Snf1p-Snf4p interaction, commonly used as a positive control. On the other hand, β-galactosidase levels resulting from Cin5p-Knr4p and Ymr262p-Knr4p interactions were, respectively, 2.5-fold and close to the background activity obtained with empty vectors (Table 2). These low β-galactosidase activities appeared somehow contradictory with a sustained growth of the transformed strains on SC minus Ade and SC minus His (not shown). Since the Tys1p showed the strongest interaction with Knr4p, we further analyzed this interaction and looked for its physiological meaning.

Table 2
Growth on SC minus Ade and His and β-galactosidase activity in transformed cells

<table>
<thead>
<tr>
<th>pJ67-4A strain transformed with</th>
<th>Growth on SC minus Ade</th>
<th>SC minus His</th>
<th>β-Galactosidase activity* (nmol ONPG hydrolyzed min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pODB80-KNR4+pGAD</td>
<td>–</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>pODB80-KNR4+pGAD-TYS1</td>
<td>+</td>
<td>+</td>
<td>189</td>
</tr>
<tr>
<td>pODB80+pGAD-TYS1</td>
<td>–</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>pODB80-KNR4+pGAD-CIN5</td>
<td>+</td>
<td>+*</td>
<td>24</td>
</tr>
<tr>
<td>pODB80+pGAD-CIN5</td>
<td>–</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>pODB80-KNR4+pGAD-YMR262</td>
<td>+</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>pODB80+pGAD-YMR262</td>
<td>–</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>pSNF11 (BD)+pGAD-SNF4</td>
<td>+</td>
<td>+</td>
<td>42</td>
</tr>
</tbody>
</table>

*Values of β-galactosidase activity are from a typical experiment that was repeated two times with similar value (within 10% of deviation).
A direct physical interaction between Knr4 and Tys1 proteins was demonstrated by a protein-binding ‘pull-down’ in vitro experiment using GST–Tys1 fusion that was fixed on glutathione-Sepharose beads and incubated with MBP–Knr4 fusion. The interaction was identified using antibodies raised against this latter protein fusion. The results are shown in Fig. 1. No detectable MBP–Knr4 was retained on GST, whereas an intense band on the Western blot was revealed in the elution fraction from the resin carrying the GST–Tys1 fusion with the anti-MBP–Knr4 antibodies. Similar results were obtained using protein extracts from AR27 wild-type strain, as a source of KNR4 protein, instead of MBP-Knr4 from *E. coli* lysates (data not shown). These results indicate that Knr4p can directly bind to Tys1p.

### 3.2. Dityrosine levels in the sporulating cultures of the strains with changed amounts of *KNR4* and *TYS1* proteins

The finding of interaction between Knr4p and a aminoacyl tRNA synthetase was at first glance unexpected. Therefore, we sought for a cellular process in which these two proteins might be involved together. The main and essential function of tyrosine tRNA synthetase is in protein synthesis, which does not seem to be affected in *knr4* mutant strains. Therefore, it is unlikely that the function of Tys1p, which is an abundant aminoacyl tRNA synthetase in protein synthesis, is somehow affected by Knr4p during vegetative growth. On the other hand, it was shown in a genome-wide analysis of the sporulation process that *TYS1* was the single aminoacyl tRNA synthetase encoding gene whose transcription was strongly activated at the middle stage of the sporulation, i.e. starting at around 7 h after the induction process [16]. Interestingly, this kinetic profile of *TYS1* activation almost coincided with that of *KNR4* and was identical to that of *DIT1* and *DIT2* which encode two key enzymes in the formation of the dityrosine, a major component of the outermost layer of the spores wall [17,18]. In view of the fact that Knr4p is involved in the cell wall assembly, the co-induction of these genes at the end of meiosis raised the possibility

![Fig. 1. Physical interaction between Knr4p and Tys1p in a pull-down assay. Resin-bound GST and GST–Tys1p fusion were incubated with MBP-Knr4 as indicated in Section 2. After being washed, bound proteins were eluted with the elution buffer (HEPES 75 mM, pH 7.4, NaCl 150 mM, EDTA 1 mM) and a fraction (20 μl) of each sample was electrophoresed on SDS-12.5% polyacrylamide gels. (A) The bound MBP-Knr4p was visualized by Western blotting with anti-KNR4 polyclonal antibodies, and (B) eluted GSTp and GST–Tys1p by Coomassie blue coloration.](image)
that Knr4p and Tys1p might participate in spore wall formation during sporulation. There was, however, a drawback to estimate this requirement due to the fact that loss of TYS1 function is lethal [19]. Therefore, only diploid strains heterozygous for TYS1 deletion could be used to assess our hypothesis. Nonetheless, it can be seen in Table 3 that the total dityrosine levels in spores from diploid strains heterozygous for KNR4 and TYS1 were already reduced by 25–40%, while the sporulation efficiency of these mutants was equal to that of their isogenic wild-types. These data are thus in favor of an implication of these two proteins in the formation of the dityrosine layer. Since Tys1p has the ability to bind L-tyrosine, this protein might function in an initial step of dityrosine formation, for example as carrier of L-tyrosine to channel this amino acid towards dityrosine formation. Alternatively, Tys1p might be part of the enzymatic mechanisms involved in the incorporation of soluble tyrosine-containing precursors into the spore wall [18–20]. This potential function does not however completely justify the need for interaction between Knr4p and Tys1p. However, evidence has been given for a role of Knr4p in chitin deposition [7]. Hence, a physical Knr4p–Tys1p interaction would be one of the molecular elements responsible for coupling chitosan and dityrosine formation during prospore wall formation. However, the fine demonstration of this hypothesis appears to be a difficult task due to the fact that diploid cells homozygous for the disruption of TYS1 are inviable.

3.3. The KNR4 and TYS1 proteins appear to be necessary for efficient sporulation process

While spores from diploid cells homozygous for KNR4 deletion contained very low levels of dityrosine, the sporulation efficiency of such a mutant strain was also strongly reduced (Table 3), such as that the actual dityrosine content expressed per spores from this mutant was comparable to that in spores from diploid cells heterozygous for TYS1 or KNR4 deletion (Table 2). The 3–4-fold reduction of spore formation was reproducibly observed in two different genetic backgrounds homozygous for disruption of KNR4. Since the inability of cells to produce chitosan or dityrosine layers has no effect on sporulation efficiency [21], this result indicates that Knr4p may participate in another step in the sporulation process. We investigated whether TYS1 might also affect sporulation efficiency using a temperature-sensitive mutant of this gene isolated by Hopper’s group [19]. We created isogenic diploids in both the control and the tysIΔ mutant using the HO plasmid and carried out sporulation at 24°C. As for the knr4Δ/knr4Δ homzygous diploid, the sporulation efficiency of the dysIΔ/tysIΔ diploid was notably decreased (to less than 5%) as compared to the control diploid strain, although the overall efficiency of spores formation of this latter strain was below 20%. The sporulation defect of the dysIΔ/tysIΔ diploid could be restored after transformation with a plasmid bearing TYS1 under the control of GAL10 promoter, when the transformed diploids had been cultured in a galactose medium prior to sporulation on K-acetate media. Strangely enough, most of the asci obtained in these conditions contained only two spores (not shown). One possible reason is that overexpression of TYS1 gene is toxic for the cells (Robert Martin, IBMGC, Strasbourg, personal communication).

These data indicate that both KNR4 and TYS1 are required for efficient sporulation. There is however no evidence that this additional function requires interaction between the two proteins. Moreover, it could be that the reduced spore formation of the diploid cells homozygous for KNR4 deletion or dysIΔ mutation originated from their defects during vegetative growth of the diploid cells. In the case of TYS1, this can be due to a reduced translational activity that might alter the overall protein synthesis, whereas the impairment of cell assembly due to the loss of KNR4 function might affect proper signalling in response to environmental stimuli. In favor of this idea are our data that overexpression of KNR4 suppresses cell wall defects caused by mutations in several genes [6], including the recently identified CWH43 gene which encodes a transmembrane protein implicated in environmental sensing upstream of the BCK2 branch of the PKC1 integrity pathway [22].

To summarize, this genetic screen allowed us to identify two partners of Knr4p that are involved in at least two distinct functions, namely Tys1p in protein synthesis and Cin5p/Hal6p in transcriptional regulation genes in response to salt tolerance. A large-scale two-hybrid analysis identified Bas1p, a transcriptional factor, as another partner of Knr4p [23]. More recently, we isolated two other proteins implicated more directly in cell wall assembly that strongly interact with Knr4p (H. Martin-Yken et al., in preparation). Thus, Knr4p is clearly a regulatory protein, which participates through its multi-partner interactions, in different cellular processes, including cell wall assembly and transcription, and thus playing an important role in vegetative growth and during sporulation.

Acknowledgements

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