FEBS Letters 581 (2007) 2009–2016

Ras-pathway has a dual role in yeast galactose metabolism

Mario G. Mirisola*, Alessia Gallo, Giacomo De Leo

Dipartimento di Biopatologia e Metodologie Biomediche, Via Divisi, 83, Università degli studi di Palermo, 90133 Palermo, Italy

Received 30 January 2007; revised 2 April 2007; accepted 2 April 2007

Available online 23 April 2007

Edited by Horst Feldmann

Abstract In the yeast *Saccharomyces cerevisiae* the genes involved in galactose metabolism (GAL1,7,10) are transcriptionally activated more than a 1000-fold in the presence of galactose as the sole carbon source in the culture media. In the present work, we monitored the activity of the GAL10 gene promoter in different Ras-cAMP genetic backgrounds. We demonstrate that overexpression of C-terminus of the nucleotide exchange factor Cdc25p stimulates GAL10 transcription in yeast strains carrying the contemporary deletion of both RAS genes. Moreover, the deletion of the chromosomal CDC25 gene provokes impaired growth on galactose based media in yeast strain lacking both RAS genes and adenylate cyclase (whose viability is assured by the presence of the Bcy1-11 allele). Surprisingly, reconstitution of the Ras-pathway inhibits GAL10-promoter activation. Activation of GAL10 gene promoter is indeed possible in the presence of Ras protein but only in strains with chromosomal deletion of adenylate cyclase. These results indicate a dual role of Ras-pathway on galactose metabolism and suggest that Cdc25p has a Ras-independent role in cellular metabolism.

Keywords: Exchange factor; GTP-binding protein; Signal transduction; Galactose metabolism; Leloir pathway; Protein kinase A

1. Introduction

The Leloir pathway is the main enzymatic pathway for the conversion of galactose to glucose in organisms philogenetically distant as *Escherichia coli* and humans [1]. This conversion is mainly realized by three enzymes codified by genes collectively referred to as *GAL* genes in yeast: galactokinase (EC 2.7.1.6, GALK) corresponding to *GAL*1 in yeast, phosphorylates intracellular galactose to galactose-1-phosphate; galactose-1-phosphate uridyltransferase (EC 2.7.7.12 GALT), corresponding to *GAL*7, transfers UMP from UDP-glucose to gal-1P forming UDP-gal, and, finally, UDP galactose 4′-epimerase (EC 5.1.3.2, GALE), whose yeast counterpart is *GAL*10, converts UDP-gal to UDP-glc, via a NAD^+ dependent reaction [2].

*Corresponding author. Fax: +39 091 6554624.
E-mail address: mirisola@unipa.it (M.G. Mirisola).

Abbreviations: PKA, protein kinase A; ATCC, American type culture collection; PAH, phenylalanine hydroxylase; RT-PCR, reverse transcriptase-polymerase chain reaction

Yeast cells respond to the presence of galactose as the sole carbon source with a several 1000-fold increase of the *GAL* genes expression [1–3]. These genes undergo a dual transcriptional regulation. They are repressed in the presence of glucose and activated in response to galactose [4]. Several experiments have demonstrated that activation and repression of the *GAL* genes act via independent pathways [5–7]. The major transcriptional activator of the *GAL* genes is the transcription factor Gal4p [8]. Gal4p is in turn subjected to two levels of regulation. It responds to the presence of intracellular galactose by 4–5-fold increase of its transcriptional level [6,8,9], and by post-translational modification of its interaction with the repressor Gal80p [10].

Many studies, in yeasts, have related the c-AMP-PKA pathway to diauxic shift [11–14]. Genetic manipulations of the Ras-cAMP pathway often result in galactose deficient phenotypes. Yeast strains expressing an hyperactivated *RAS2* allele (*RAS*2G19V) show growth impairment on galactose-based media [15]. Furthermore, in strains with an activated allele of the adenylate cyclase (CYR1T1651I), contemporary deletions of both *RAS* genes or deletion of *RAS*1 combined with the presence of an attenuated *RAS*2 allele result in temperature-sensitive phenotype in galactose [16]. The Ras pathway has been demonstrated to be positively affected by two different nucleotide exchange factors: the essential Cdc25p and the non essential Sdc25p [17,18]. The overexpression of the former provokes growth inhibition in galactose [19] and enhanced transcription of the *GAL*4 gene [20]. Cdc25p has also been demonstrated to be essential for growth in galactose-based media [21]. We investigated the role of *CDC*25 and *RAS2* gene products in galactose metabolism, monitoring the activity of the *GAL*10 gene promoter fused to human phenylalanine hydroxylase cDNA used as a reporter gene. This reporter was expressed in various yeast strains differing for the Cdc25p-Ras-cAMP pathway and the resulting activity of the *GAL*10 promoter measured.

2. Materials and methods

2.1. Media and yeast manipulation

YPD was used as rich medium (2% bacto peptone, 1% yeast extract and 2% glucose). Selective synthetic media were prepared with yeast nitrogen base without aminoacid at 0.67% final concentration, with the appropriate mixture of aminoacids and nucleic acid bases at the concentration indicated by Sherman [22], with glucose at 2% final concentration. Standard media containing carbon source different from glucose were obtained with 2% galactose in place of glucose. Genetic manipulation of yeasts were as described by Mortimer and Schild [23]. Yeasts transformations were carried out as described by Ito [24]. All components of media were from DIFCO (Becton Dickinson, CA, USA).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.04.018

Standard molecular biology methods have been performed as indicated by Sambrook et al. [25].

List of strains		
AAT3B	*a ade2 can1-100 CRI4 his3 leu2-3,112 lys1-1 ras1Δ ras2::URA3 ura3-52*	Mirisola et al. [16]
AAT3BΔ1	*a ade2 can1-100 cdc25::LEU2 CRI4 his3 leu2-3,112 lys1-1 ras1Δ ras2::URA3 ura3-52*	Mirisola et al. [16]
ABH7C	*α ade2 bcy1-11 can1-100 cyr1::HIS3-Δ1 his3 leu2-3, 112 Lys1-1 ras1Δ ras2::URA3 ura3-52*	Mirisola et al. [16]
ABH7CΔ1	*α ade2 bcy1-11 can1-100 cdc25::LEU2 cyr1::HIS3-Δ1 his3 leu2-3, 112 Lys1-1 ras1Δ ras2::URA3 ura3-52* ras1Δ allele with ras1 deletion without any marker was obtained after gene conversion of the Ras1::URA3 allele in the presence of the plasmid Yep13Hras1Δ and selection with 5-FOA as described by Mirisola et al. [16]	This work

WOF6.1 (ras1Δras2Δcyr1::URA3 bcy1-11) strain was obtained transforming the ras1Δras2Δcyr1::HIS3 bcy1-11 (ABE2A, Verrotti et al., 1992) strain with the EcoRI/BamHI fragment of the pWOF6 vector (Fasano O. unpublished).

2.2. PAH reporter construction

The 1.8 kb *Sma*I/*Hpa*I fragment of the American type culture collection (ATCC) clone ATCC 61604, containing the entire coding region of the human phenylalanine hydroxylase (PAH) has been cloned into the unique *Sal*I restriction site of the YEp51 yeast shuttle vector [26], after Klenow treatment to fill it in. The proper orientation of the insert has been verified by restriction analysis and joint ends have been verified by sequencing.

2.3. RNA preparation

The desired yeast strain was grown at 30 °C, on the appropriate selective glucose-based medium, to $OD_{560\,nm} = 0.8/1$. Cells were collected by centrifugation and resuspended in the same medium, except for induced cultures, where galactose replaced glucose at the same concentration, and cultured at 30 °C for additional 24 h. After centrifugation, the pellet, resuspended in acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5), was vortexed for 5′ at 65 °C in the presence of one volume of acid-washed glass beads (SIGMA G-1277, Milano, Italy) and one volume of phenol pH 5. The aqueous phase was extracted twice with chloroform and RNA was finally precipitated with 2.5 V of absolute ethanol, 0.3 M sodium acetate, pH 5.5. After centrifugation, the pellet was resuspended in TE 10:1. The RNA quality was verified evaluating the ribosomal RNA appearance after gel electrophoresis in denaturing conditions. Poly $(A)^+$ RNA was obtained using a commercially available kit (QuickPrep™ Micro mRNA Purification Kit, Amersham, Milano, Italy), according to the manufacturer's instruction.

2.4. RT-PCR

Poly $(A)^+$ (1 μg) or total RNA, obtained as described above from the indicated yeast strain, was subjected to retro transcription in the presence of the RT primer indicated below in the presence of 1 μl of the Improm (Promega, Madison, WI, USA) reverse transcriptase at 42 °C for 1 h, according to the manufacturer's instructions. Different amounts of each cDNA were used for the subsequent PCR. The primers for the each PCR are indicated as For and Rev primers. PCR conditions for hPAH; *GAL4*; *GAL80* were as follow: 94 °C 30″; 51 °C 30″, 72 °C 45″ for two cycles and 94 °C 30″ 60 °C 30″ 72 °C 45″ for additional 28 cycles. PCR conditions for CAP were 94 °C 30″ 51 °C 30″ 72 °C 1′15″ for 30 cycles. PCR conditions for *CDC25* were 94 °C 30″ 58 °C 30″ 72 °C 1′ for 30 cycles. PCR conditions for *TYR1* were 94 °C 30″ 54 °C 30″ 72 °C 1′30″ for 30 cycles.

2.5. Northern blot hybridisation

Total RNA (10 μg) were fractionated in a 1.0% agarose–formaldehyde gel and blotted onto nitrocellulose membrane (Schleicher & Schuell, PROTRAN BA 83, Dassel, Germany), via capillary action as described by Sambrook [25]. Filters were baked at 80 °C for 2 h under vacuum. RNA blots were prehybridized with prehybridization buffer

Primer name:	DNA sequence 5′ → 3′
hPAH-RT	gagggcactgcaaaggattcc
hPAH probe For	aaacctgacccacattgaatctagacc
hPAH probe Rev	tgccccatgttttcttttcttcctc
GAL4-RT	ccctgtagtgattccaaacg
GAL4-For	gcggggtttttcagtatctacga
GAL4-Rev	gssgcaagcctgaaagatga
GAL80-For	tccttgccgaccagcgtatac
GAL80-Rev	gcatgcacgaaaaagggaaat
GAL80-RT	gcatgcacgaaaaagggaaat
CAP-For	cgcggtcgacatatgcctgactctaagtacaca
CAP-Rev	cgcggatccaagcttacacatgaaaaaaaccttt
CAP-RT	cgcggatccaagcttacacatgaaaaaaaccttt
Cdc25-RT	ttgcgattttagtcctcttgctg
Cdc25-For	tggcgggacaaaagaggcactgat
Cdc25-Rev	cgtcacggatcgcaacagctctcta
TYR1-RT	gcctaatattataggaaatcagcatt
TYR1-For	gaaggaaagaggacagcatatcca
TYR1-Rev	gcctaatattataggaaatcagcatt