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Relationship between protein kinase C and derepression of different enzymes

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Abstract **The *PKC1* gene in the yeast *Saccharomyces cerevisiae* encodes for protein kinase C which is known to control a MAP kinase cascade consisting of different kinases: Bck1, Mkk1 and Mkk2, and Mpk1. This cascade affects the cell wall integrity but the phenotype of *pkc1Δ* mutants suggests additional targets that have not yet been identified [Heinisch et al., Mol. Microbiol. 32 (1999) 671–680]. The *pkc1Δ* mutant, as opposed to other mutants in the MAP kinase cascade, displays defects in the control of carbon metabolism. One of them occurs in the derepression of *SUC2* gene after exhaustion of glucose from the medium, suggesting an involvement of Pkc1p in the derepression process that is not shared by the downstream MAP kinase cascade. In this work, we demonstrate that Pkc1p is required for the increase of the activity of enzymatic systems during the derepression process. We observed that Pkc1p is involved in the derepression of invertase and alcohol dehydrogenase activities. On the other hand, it seems not to be necessary for the derepression of the enzymes of the *GAL* system. Our results suggest that Pkc1p is acting through the main glucose repression pathway, since introduction of an additional mutation in the *PKC1* gene in yeast strains already presenting mutations in the *HXKII* or *MIG1* genes does not interfere with the typical derepressed phenotype observed in these single mutants. Moreover, our data indicate that Pkc1p participates in this process through the control of the cellular localization of the Mig1 transcriptional factor.**

Key words: Protein kinase C; *Saccharomyces cerevisiae*; Signal transduction

1. Introduction

The negative effect exerted by glucose on the transcription of different genes in *Saccharomyces cerevisiae* cells is one of the most studied signaling transduction pathways. Catabolic or glucose repression is a term that has been used to define such phenomenon. The principal components of this regulatory system are the transcriptional factor Mig1, the protein kinase Snf1, the protein phosphatase Glc7 and the sugar kinase HxkII [1–3]. Briefly, a complex formed by Mig1p that recruits for this proposal the co-repressor factors Ssn6 and Tup1 exerts the glucose repression. This complex is negatively controlled by Snf1 protein kinase since phosphorylation of Mig1p promotes its translocation from the nucleus to the cytoplasm, relieving the repression when glucose concentration is low [4,5]. For this process is also important the participation of the exportin Msn5p that recognizes the Mig1p phosphorylated form and facilitates its translocation to the cytoplasm [6]. On the other hand, when glucose concentration is high, the Snf1 activity is inhibited through the action of two main upstream regulators: the Glc7 phosphatase and the HxkII sugar kinase. Even though many details of this mechanism are already known, there are many aspects for which scarce information is available. The identity of the kinase that controls the Snf1 activity and the exact way through which the HxkIIp is involved in the control of Snf1 activity are examples of details still unclear.

There are different classes of systems repressed by the presence of high glucose concentrations, but in spite of the existence of a general glucose-repression mechanism not all systems are repressed in the same way. The following class can be cited: proteins involved in the utilization of alternative carbon sources (invertase; enzymes from the so-called galactose and maltose systems); essential proteins for respiration; high-affinity glucose carriers; and proteins involved in different types of stress [3]. These differences are related in many situations to the manner by which the repressor complex interacts with the target genes. Thus, in the case of the *SUC2* gene (that encodes for the invertase), the repressor complex binds to the upstream repressive sequence (URS) at the promoter region. On the other hand, in the case of the *GAL* and *MAL* genes, Mig1p also controls the expression of specific inducers (*GAL4* and *MAL R*). The same situation is observed in the control of respiratory genes where the transcription of Hap4p, an important effector, is also repressed. In its turn, for gluconeogenic genes such as *FBP1* and *PCK1*, there is also the action of the Snf1 complex directly on a specific transcriptional activator (Sip4p) [1].

By working with mutants of PKC MAP kinase signalling pathway, we demonstrated that Pkc1p is specifically involved

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in the glucose depression pathway and enzymes after glucose depletion [7]. Indeed, many other functions have been attributed to Pkc1p that are not shared by the downstream components of the PKC MAP kinase pathway [8,9].

Our data indicates that Pkc1p is necessary for the derepression of enzymatic systems related to the carbon metabolism in yeast. Moreover, we also found evidence for a connection between Pkc1p and the main repression pathway, since the introduction of *pkc1Δ* mutation in strains carrying deletions in *HXKII* or *MIG1* genes does not change the derepressed status exhibited in strains with single mutations in these two genes. Furthermore, the results suggest that Pkc1p would participate in this process, controlling the cellular localization of the Mig1 transcription factor.

2. Material and methods

2.1. Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Yeast cells were grown in medium containing peptone (2%, w/v) and yeast extract (1%, w/v; YP medium), supplemented with glucose (4%, w/v) and 1 M sorbitol as indicated (25 ml, final volume). In all experiments the cells were grown in a rotatory incubator New Brunswick Model G25 (200 rpm) at 28°C until the middle of the exponential phase. At this moment, the cellular suspension was divided in two fractions: 4 and 21 ml samples. Both were harvested and washed by centrifugation (approximately 2000*g*) with 200 mM imidazole buffer (containing 40 mM MgCl2; 400 mM KCl; 1 M sorbitol; pH 7.0). The pellet from the small fraction was used to prepare a cellular extract (control time, repressed cells). The rest of the cells was resuspended in a 25-ml YP medium supplemented with raffinose (2%, w/v), galactose (2%, w/v) or ethanol (2% w/v) plus glucose (0.05% w/v) and 1 M sorbitol (for all situations). Again, samples of 4 ml were taken at given time intervals, immediately harvested and washed by centrifugation (approximately 2000*g*) with the same buffer and used to prepare cellular extracts.

To test the cellular growth in solid media on different carbon sources, yeast cells were grown overnight on YPD plus sorbitol 1 M. 3 μl of cellular suspension with an OD at 600 nm of around 1.5 were inoculated on plates containing YP media supplemented with glucose, galactose, glycerol or ethanol with or without sorbitol 1 M. The plates were incubated at 28°C for 3–4 days.

2.2. Determination of enzyme activities

For determination of invertase and alcohol dehydrogenase activities cell extracts were obtained by adding 500 mg of glass beads (0.5 mm) and 500 μl of 50 mM imidazole buffer without sorbitol to the pellets. The cells were disrupted by vortexing with glass beads for 30 s three times with intervals in ice. Then, 1 ml of the buffer was added plus 2.5 μl 100 mM PMSF and the suspension was centrifuged for 3 min at 13 000 rpm in a microcentrifuge and the supernatant used to measure the enzyme activities. Measurement of specific activity of invertase was performed as described [10] with the modifications [11], except that the assay was carried out at pH 5.1 and 37°C. Alcohol dehydrogenase was measured by using the protocol described in [12].

In the experiments performed to verify the expression of elements of the system GAL, strains containing a *GAL1–lacZ* fusion were grown as described before being transferred to the appropriate derepression medium (see legends of the figures). However, the cell samples were harvested and washed by centrifugation (approximately 2000*g*) in a 0.5 M sodium phosphate buffer (containing 10 mM KCl; 1 mM $MgCl_2$; sorbitol 1 M; pH 7.0). Cell disruption and further sample treatment obeyed the same scheme as the other enzymes. β-galactosidase activity was measured by incubating the extracts with 30 mM ONPG (orto-nitrophenyl-β-D-galactopyranoside). After 30 min the reaction was interrupted by the addition of 1 M Na_2CO_3 and the absorbance read at 420 nm. The enzyme activity was expressed in nmol of *orto*-nitrophenol/min/mg protein. Protein was determined using classical procedures [13].

2.3. RNA isolation and Northern blot analysis

For a shift from growth on glucose to growth on glycerol, yeast cells were grown in 20 ml YPglucose (4%, w/v) plus 1 M sorbitol at 30°C up to the early exponential phase (OD_{600nm} = 0.8–1.2). Then the sample was split in two. 10 ml were washed quickly by centrifugation with 1 M sorbitol and the cellular pellet used to RNA extraction (repressed state). The rest of the suspension was washed quickly by centrifugation with 1 M sorbitol and the pellet resuspended in YP glycerol (3%, w/v) and glucose (0.05%) plus 1 M sorbitol, rapidly mixed and incubated as before. After 2 h, the sample was washed in the same way and the cellular pellet used for RNA extraction (derepressed state).

Isolation of total yeast RNA was performed by using the classical hot acid phenol method. 15 μg of total RNA was separated on 1% (w/v) agarose in 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH, pH 7.5, containing 1% (w/v) formaldehyde. Subsequently, RNA was blotted onto Hybond-N membranes in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and hybridized with gene-specific probes. Probe fragments were radioactively labelled using the rediprime™ II labelling kit (Pharmacia). In all cases equal amounts of RNA were loaded. Gene-specific probes were obtained by PCR. The following primer pairs were used to synthesize *FKS2* and *ACT1* (constitutive endogenous control) fragments, respectively: 5′-ATGTCCTACAACGATCC-3′ (sense) and 5′-GAACCATCTTGATCAGG-3′ (antisense) and 5′-GCTGCTTTGGTTATTGATAAC-3′ (sense) and 5′-GATAGTGGACCACTTTCGTCG-3′ (antisense). The RNA levels were visualized by exposing the membrane to CL-X Posure™ Film from Pierce.

2.4. Subcellular localization of Mig1p by Western blot and by analysis of GFP fluorescence

To study the subcellular localization of Mig1p we used yeast cells transformed with a plasmid containing the *MIG1* gene labeled with a FLAG epitope tag (DYKDDDDK) placed immediately following the initiating methionine. The cells were grown on YPglucose (2%) plus 1 M sorbitol at 30°C up to early exponential phase (repressive state), and then transferred to YPglucose (0.05%) plus 1 M sorbitol for 30 min (depressive state). The detection of the FLAG-tagged Mig1p was performed as described previously [14].

On the other hand, strains containing the transport domain of MIG1 fused to *GFP* gene (GFP states for green fluorescent protein) were grown on YP medium supplemented with 4% glucose until mid-exponential phase. Then 1-ml samples were harvested and washed by

Table 1
Saccharomyces cerevisiae strains used in this study

Strains	Genotype	Source
W303	Mat**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Johan Thevelein
YSH 296	Mat**a** *mig1Δ*::URA 3	Stefan Hohmann
YSH 850	W303 MAT**a** *pkc1Δ*::HIS 3)	Stefan Hohmann
YSH 310	W303 Mat**a** *hxk2Δ*::LEU 2	Stefan Hohmann
LBFM 318	YSH 310 MAT**a** *pkc1Δ*::HIS *hxk2Δ*::URA	This work
LBFM 397	YSH 296 MAT**a** *mig1Δ*::URA 3 *pkc1Δ*::HIS	This work
LBFM 403	W303 Mat**a** *MIG1*(TD):: ::*GFP::lacZ*	This work
LBFM 404	YSH850 Mat**a** *MIG1*(TD)::*GFP::lacZ*	This work
LBFM 410	W303 Mat**a** *pGAL1::lacZ*	This work
LBFM 411	YSH 850 Mat**a** *pGAL1::lacZ*	This work

centrifugation (13 000 rpm in a microcentrifuge) with a sorbitol (1 M) plus 4% glucose (4%, w/v) cold solution. The remaining cell suspension was transferred to YP raffinose (2%, w/v) medium for 1 h. The cells were harvested and washed by centrifugation with a cold solution containing sorbitol (1 M) plus glucose (2%, W/v; repressed cells) or glycerol (3%, w/v; derepressed cells). Finally, 10–15 μl of these cell suspensions were mixed with 10–15 μl of agarose (1%, w/v) at 37°C for microscopy observations. Images were registered by using a Zeitz Laborlux S Microscopic with accessory apparatus for fluorescence (Ploemopak Filter 12) connected to a Sony Progressive 3 CCD. The images were processed with an Axio Vision 3.0 software.

2.5. Reproducibility of results

All experiments of enzyme activities were performed at least three times and the results shown are the mean of independent experiments.

3. Results

3.1. Invertase activity in the pkc1Δ mutants

It has been suggested [7] that the involvement of the Pkc1p in the derepression process be most probably exerted by an alternative pathway and not directly through the control of Snf1 activity. This conclusion was based on the results of the Snf1 activity measured in wild-type and in *pkc1Δ* mutant cells pre-grown on glucose up to the early-exponential phase or up to the mid-exponential phase. In wild-type and in *pkc1Δ* strains the enzyme activity increases when cells are transferred to derepression conditions (i.e. low glucose concentrations or non-fermentable carbon sources). These results suggest that the repressive phenotype observed in *pkc1Δ* strains cannot be related to a defect in the activation of Snf1p.

However, taking into account that Pkc1p is involved in other cellular process, we decided to investigate in more detail the derepression process by using cells presenting deletions in the *MIG1* or *HXK2* genes and in corresponding strains containing an additional mutation in the *PKC1* gene. The derepression pattern observed in the respective single mutants, *mig1Δ* or *hxkIIΔ*, was not modified by the introduction of a deletion mutation at *PKC1* gene in such strains (Fig. 1A–C). However, it should also be observed that in glucose-grown cells the level of the invertase activity in the mutant *mig1Δ* is always lower than in the mutant *hxkIIΔ*. Moreover, when the *mig1Δ* mutant is transferred to a derepressing condition there is a clear and progressive increase of the enzyme activity. On the other hand, in the mutant *hxkIIΔ* the invertase activity does not change when cells are transferred to derepressing conditions.

Together, these and previous results [7] seem to suggest that, although Pkc1p is not involved in the control of the Snf1 activity, it is in some way connected to the modulation of the activity of the main glucose repression pathway.

3.2. Further functional interactions between of Mig1p and Pkc1 p

Mutations in genes encoding proteins of the PKC MAP kinase pathway can only be cultivated in the presence of osmostabilizing agents, such as sorbitol or NaCl, because such mutants undergo cell lysis resulting from a deficiency in cell wall construction [14]. This is particularly the case in *pkc1Δ* mutants, a fact that suggests the involvement of Pkc1p and not the other components of the pathway in the control of other cellular functions. It was demonstrated that under thermal stress, this pathway regulates the transcription of the *FKS2* gene that encodes for a subunit of the glucan synthase

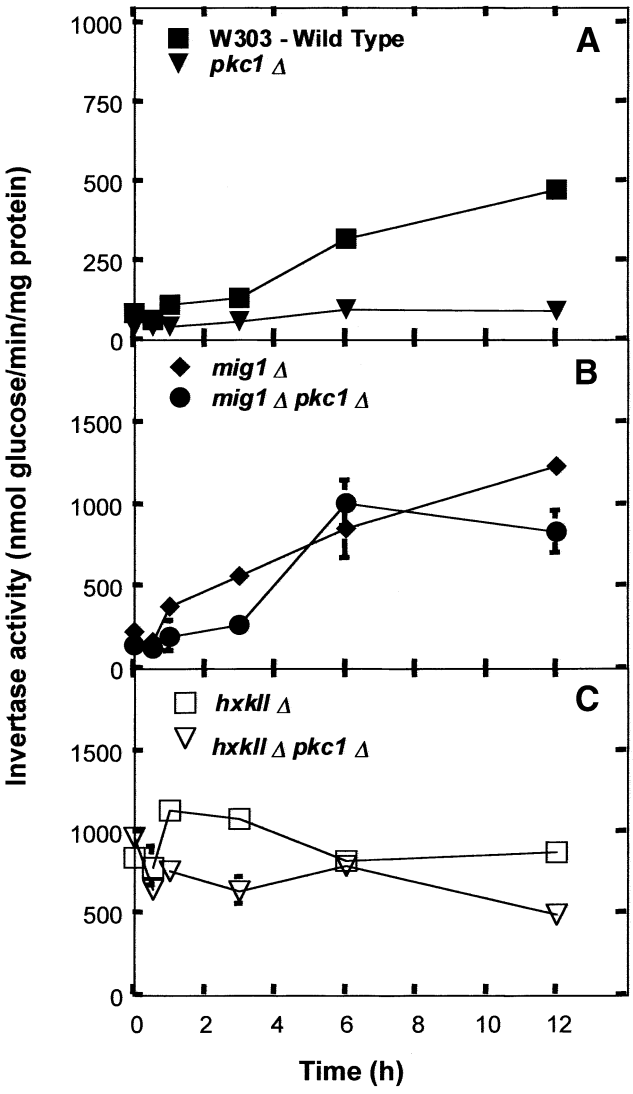

Fig. 1. Invertase activity under repressive and non-repressive growth conditions. The cells were grown on glucose up to OD_{600nm} 1.0–1.5 and then transferred to raffinose. The enzyme activity was measured before and at the indicated times after the transfer of cells to a non-repressive condition. Strains: W303-1A, ■; *pkc1Δ* mutant, ▼; *mig1Δ* mutant, ♦; *mig1Δ pkc1Δ* mutant, ●; *hxk2Δ* mutant, □; *hxk2Δ pkc1Δ* mutant, ▽.

[15,16]. Moreover, it is known that *pkc1Δ* mutants grow poorly on non-fermentable carbon sources [7]. Combining this information with the emerging idea of a possible connection between Pkc1p and the main glucose repression pathway, we decided to investigate the existence of other functional interactions. Crucial to this strategy was the fact that Mig1p seems to be also involved in the control of *FKS2* gene expression [17,18].

The results presented in Fig. 2A,B indicate that inclusion of a *mig1Δ* mutation, but not of *hxk2Δ* mutation, recovered the capacity of *pkc1Δ* mutants to grow without sorbitol even if glucose or galactose are the carbon sources. The results shown in Fig. 3A,B, beyond demonstrating a special difficulty of *pkc1Δ* mutants to grow on non-fermentable carbon sources, also strengthen the idea of a special relationship between Pkc1p and Mig1p.

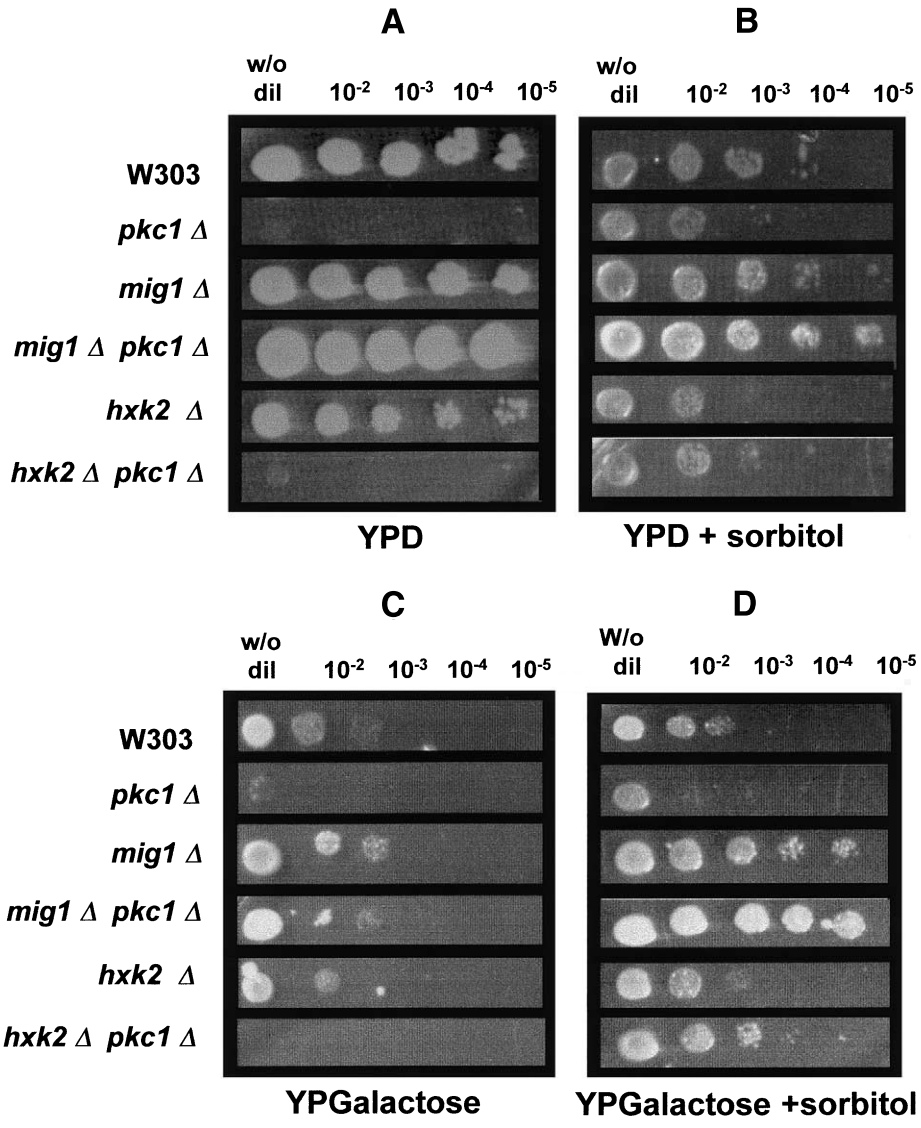

Fig. 2. Growth on agar plates with different carbon sources. The different strains (wild-type W303-1A and p*kc1Δ*, *mig1Δ*, *mig1Δ pkc1Δ*; *hxk2Δ*; *hxk2Δ pkc1Δ* mutants) were grown overnight in YPglucose media plus 1 M sorbitol, harvested and resuspended in 1 M sorbitol. Samples of 3-μl cell suspension with the same cell density were spotted on agar plates containing YPglucose or YPgalactose without (A and C, respectively) or with (B and D, respectively) 1 M sorbitol. The plates were incubated at 28°C for 3–4 days.

Since *FKS2* expression is increased under glucose derepression, we decided to check its expression in strains containing mutations in *PKC1* and/or *MIG1* genes. As can be seen in Fig. 4, the expression of *FKS2* gene in a *bck1Δ* mutant is not affected, suggesting that it is independent of this MAP kinase pathway (Fig. 4, lanes 1 and 2). However, in a *pkc1Δ* mutant its expression is barely detected under both repressive (Fig. 4, lane 3) and derepressive (Fig. 4, lane 4) conditions. In the *mig1Δ* mutant, the expression is clearly present in both cases (Fig. 4, lanes 5 and 6), confirming that its expression is under the control of Mig1p as described previously [17]. Beyond that, in the *pkc1Δ mig1Δ* mutant the level of expression of *FKS2* gene (Fig. 4, lanes 7 and 8) is completely different from that observed in the *pkc1Δ*, being more similar to those observed in *mig1Δ* mutant. Together, these results indicate the existence of a functional relationship between Pkc1p and Mig1p.

3.3. Derepression of other enzyme activities in *pkc1Δ* mutants

With the possible involvement of Pkc1p in the glucose-repression mechanism, we investigated whether other enzymes regulated by glucose repression would be affected by Pkc1p activity. The results presented in Fig. 5 show that Pkc1p is also necessary for derepression of alcohol dehydrogenase activity (Fig. 5A). Moreover, our results also demonstrated that the introduction of a *pkc1Δ* mutation in strains with supplementary mutations in the *MIG1* or *HXK2* genes only interfere with the derepression pattern observed in the *mig1Δ*, but not in the *hxk2Δ* single mutants (Fig. 5B,C). Curiously, the proposed mechanism for the control of *ADH2* gene expression does not seem to involve the main glucose repression pathway. It seems that only Snf1 activity is in some way required for the derepression process [1]. In its turn, the expression of the *GAL1* gene is probably under the control of the main glucose repression pathway. However, the results shown in

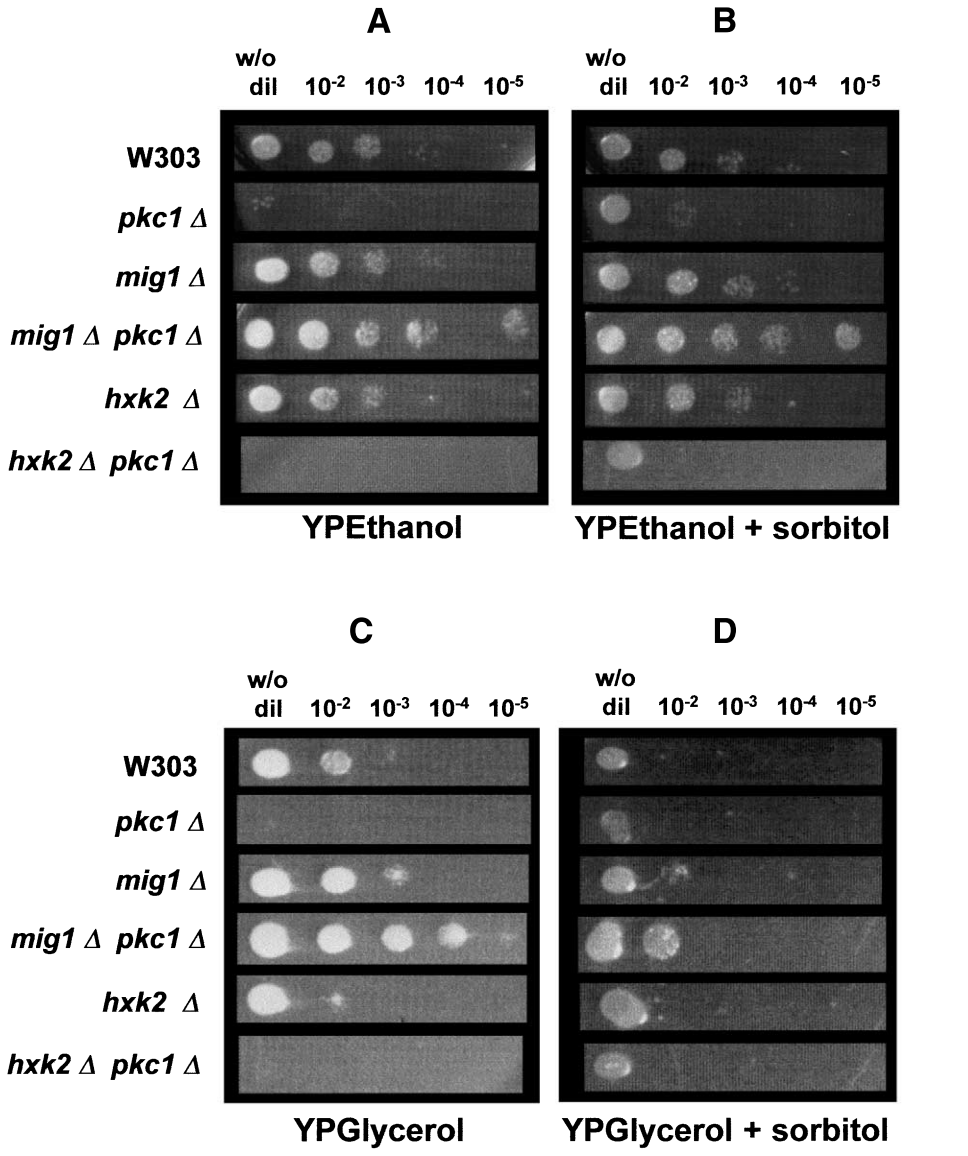

Fig. 3. Cellular growth on agar plates with different carbon sources. The different strains (wild-type W303-1A and p*kc1Δ*, *mig1Δ*, *mig1Δ pkc1Δ*; *hxk2Δ*; *hxk2Δ pkc1Δ* mutants) were grown overnight in YPglucose media plus 1 M sorbitol, harvested and resuspended in 1 M sorbitol. The cell suspensions were diluted to the same cell density and 3-μm aliquots were spotted on agar plates containing YPethanol or YPglycerol without (A and C, respectively) or with (B and D, respectively) 1 M sorbitol. The plates were incubated at 28°C for 3–4 days.

Fig. 6 demonstrated that the expression of *GAL1* gene in wild-type and *pkc1Δ* strains followed the same pattern, indicating that in this system Pkc1p is not required for an appropriated derepression process.

3.4. Possible mechanisms of control of Mig1p

Mig1p is located in the nucleus in conditions of high glucose and conversely when cells are transferred to low glucose, Mig1p becomes phosphorylated and translocates to the cytoplasm [4]. Evidence exists linking Pkc1p in the control of the cellular localization of transcriptional factors in both yeast [9] and animal [19] cells. Therefore, we decided to study the mechanism by which Pkc1p could be involved in the regulation of the cellular localization of Mig1p. In addition, a data base [20] search indicated eight potential protein kinase C phosphorylation sites in Mig1p.

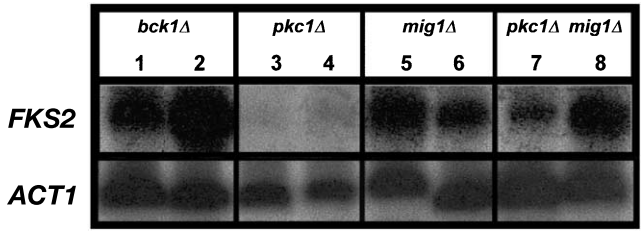

Fig. 4. Northern analysis of *FKS2* mRNA levels in repressive and derepressive conditions. Strains: *bck1Δ* (lanes 1 and 2), *pkc1Δ* (lanes 3 and 4), *mig1Δ* (lanes 5 and 6) and *pkc1Δ mig1Δ* (lanes 7 and 8). They were grown on 4% glucose to mid-exponential phase (lanes 1, 3, 5 and 7) and shifted to a media containing 3% glycerol and 0.05% glucose (lanes 2, 4, 6 and 8). Immediately before and at 2 h post-transfer, samples were taken for Northern blot analysis of the expression of *FKS2*. *ACT1* (actin) expression was used as standard.

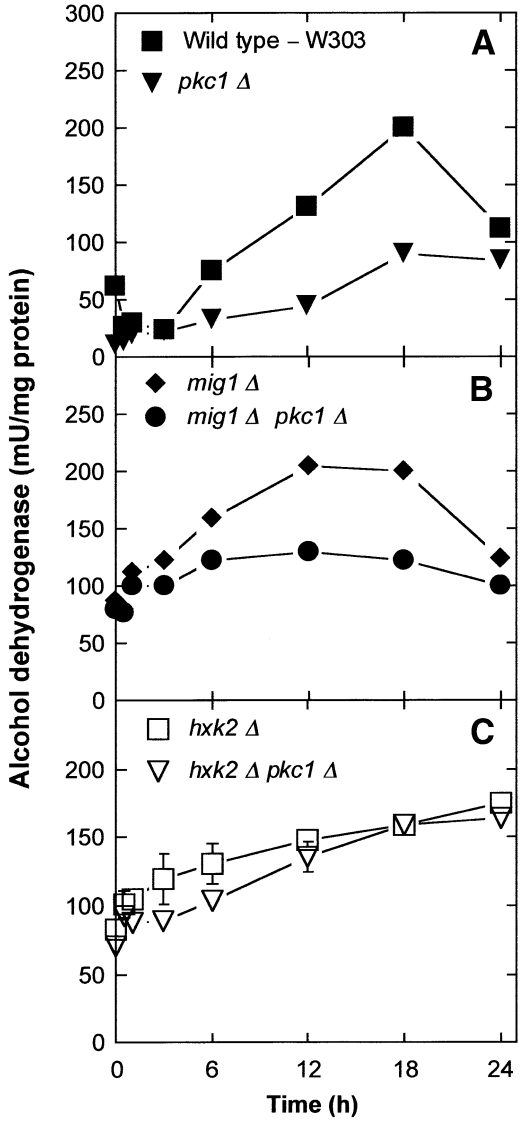

Fig. 5. Alcohol dehydrogenase activity in yeast cells under repressive and non-repressive growth conditions. The cells were grown on 4% glucose up to OD 1.0–1.5 and then transferred to 2% ethanol+0.05% glucose. The enzyme activity was measured before and at indicated times after the transfer of cells to a non-repressive condition. 1 mU is defined as the amount of enzyme that converts 1 mmol of NADH/min in the optimal conditions of measurement. Strains: W303-1A, (■) and *pkc1Δ* mutant (▼) (A); *mig1Δ* (◆) and *mig1Δ pkc1Δ* (●) mutants (B); *hxk2Δ* (□) and *hxk2Δ pkc1Δ* (▽) mutants (C).

The results presented in Fig. 7A show that Mig1p from *pkcΔ* mutant is in the insoluble fraction and most likely nuclear and there appears to be no difference in Mig1p mobility between repressed and derepressed cells. However, the fact that Mig1p is not detected in the lysate supernatant but only in the pellet does not prove that it is being retained in the nucleus. It could be argued that it is just forming, for instance, inclusion bodies and is therefore insoluble.

Therefore, to clarify the situation we introduced in wild-type and *pkc1Δ* strains a plasmid containing the transport domain of Mig1p fused with the GFP gene. As can be observed in Fig. 7B, cells of the wild-type strain grown on glucose (4%, w/v) presented a high and concentrated fluorescence, suggesting a nuclear localization of Mig1p. When these cells were transferred to media containing raffinose (2%, w/v), the intensity of fluorescence became weaker and diffuse throughout the cell, indicating a relocation of Mig1p to the cytoplasm. Conversely, in a *pkc1Δ* strain Mig1p seems to be localized in the nucleus to both repressive and derepressive treatments (Fig. 7B). Thus the results suggest that Pkc1p is required for the translocation of Mig1p from the nucleus to the cytoplasm when yeast cells are moved from glucose repressing to derepressing conditions.

4. Discussion

Pkc1p seems to have cellular functions other than the control of cell wall construction through the PKC MAP kinase pathway. Pkc1p is involved in the control of yeast cell division through the activation of Cdc28 at START [21] and it has been demonstrated that Pkc1p is required for the appropriate localization of some transcription factors to specific stimuli [9]. In addition, previous results from our laboratory suggest the involvement of Pkc1p in the glucose-induced activation of the plasma membrane ATPase and in the derepression mechanism of the glucose-repressible genes [7,8].

Therefore, taking in account the available information on other potential roles played by Pkc1p, we first investigated the invertase activity in cells presenting deletions in *MIG1* or *HXKII* genes combined with *pkc1Δ* mutation. This mutation does not interfere with the normal pattern exhibited by the *mig1Δ* or *hxkIIΔ* single mutants, i.e. increasing when cells are transferred to derepressive condition and/or higher activity, even in the presence of high glucose concentrations (Fig. 1). These results suggest that the probable participation of Pkc1p in the control of *SUC2* gene expression occur in connection with the classical glucose repression pathway.

It is known that Mig1p participates in the control of *FKS2* gene expression in derepressing conditions [17,18]. As shown

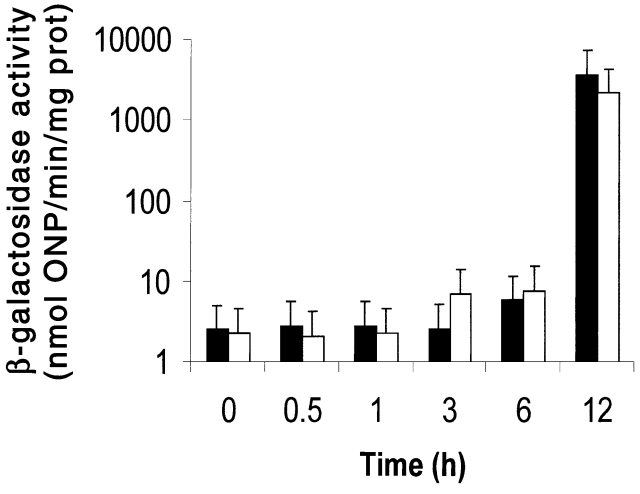

Fig. 6. *GAL1* gene expression in wild-type and *pkc1Δ* mutant cells previously grown on 4% glucose up to OD 1.0–1.5 and then transferred to galactose. Cells were transformed with a plasmid containing the *lacZ* gene from *Escherichia coli* under control of a *GAL1* gene promoter. The gene expression was followed by measuring the β-galactosidase activity before and at indicated times after the transfer of cells to a non-repressive condition.

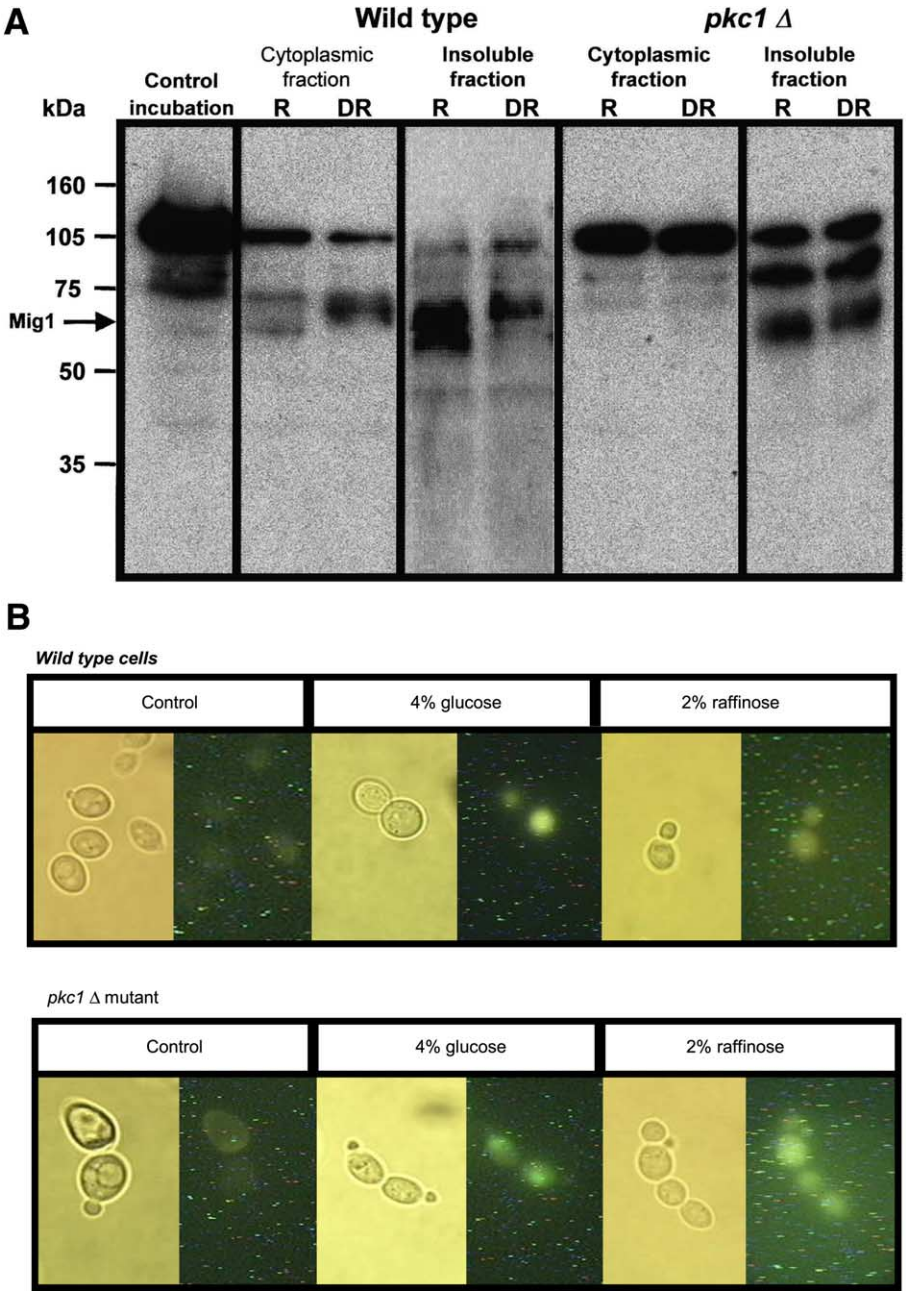

Fig. 7. Subcellular localization of Mig1p in wild-type and *pkc1Δ* mutant cells. A: Yeast cells expressing a FLAG epitope-tagged Mig1p. After growth on media containing 2% glucose, cells were transferred to and kept at 0.05% glucose for 30 min. Western blot from cell lysates was revealed with an anti-FLAG antibody. Control incubation was performed using yeast cells non-expressing the tagged Mig1p. B: Cells were transformed with a plasmid containing the *MIG1* transport domain fused with *GFP* gene. After growth on media containing 4% glucose, cells were transferred to 2% raffinose for 1 h. Images were taken through a Zeitz Laborlux S Microscopic with accessory apparatus for fluorescence (for more details, see Section 2). Control means non-transformed cells (natural fluorescence).

here, only strains presenting combined mutations in the *PKC1* and *MIG1* genes reversed the phenotype exhibited by *pkc1Δ* mutants, indicating that there is a specific and functional interaction between Pkc1p and Mig1p (Figs. 2 and 3). Since a *mig1Δ* mutant displayed a nine-fold increase in *FKS2* gene expression compared with the congenic wild-type strain [17], a plausible hypothesis would be that Mig1p function is controlled (in part) by Pkc1p.

A contradiction emerges upon comparison of invertase activity (Fig. 1) and growth in different carbon sources (Figs. 2 and 3). Care is required in the interpretation of such data. Firstly, there is already strong evidence pointing to the direct involvement of Hxk1lp in the regulation of glucose repression of *SUC2*, as Hxk1lp can be found in the nucleus [22], where it participates in regulatory DNA–protein complexes necessary for glucose repression [23]. Secondly, it was shown that Mig1p but not Hxk1lp is involved in the control of *FKS2* expression [17], suggesting a specific interaction of Pkc1p in the main glucose repression pathway that is likely to be downstream of Snf1p.

Thus, in terms of glucose repression, it is expected that the phenotypes of both *pkc1Δ hxkIlΔ* or *pkc1Δ mig1Δ* mutants would be the same as the one exhibited by the single mutants (*hxkllΔ* or *mig1Δ*). Hxkllp exerts a more prominent role, most probably because it interacts with and inhibits Snf1p [24] and also through its participation in DNA–protein complexes controlling *SUC2* expression [22,23]. This indicates why invertase activity is higher in *hxkIIΔ* cells than in *mig1Δ* cells when grown on glucose. However, in the control of the *FKS2* gene expression (Fig. 4), Pkc1p would exert its effects by regulating the action of the Mig1 transcriptional factor. In this sense, our results offer an explanation for the fact that even in the presence of sorbitol *pkc1Δ* is harder to grow than other strains containing mutations in the genes encoding downstream kinases of the PKC MAP kinase pathway. Indeed, they also corroborate the findings that multiple mechanisms are involved in the control of *FKS2* gene expression, whilst until now none indicated a role for Hxkllp [17].

Moreover, it should be added that Pkc1p also seems to be involved in the control of other genes which encode for key proteins (17) of all the components of the cell wall biosynthesis, i.e. (1-3)-β-glucan (*FKS1* and *GAS1*), (1-6)-β-glucan (*KRE6*), chitin (*CSD2*) and *N*- and *O*-linked mannoproteins (*MNN1*). This is probably the reason why the *pkc1Δ* mutant presents more difficulty to grow in glucose-containing media.

It is known that Pkc1p is required for the translocation of nuclear factors from the nucleus to the cytoplasm [9]. Interestingly, a protein kinase C from animal cells has also been described as contributing to a mechanism involved in the localization of transcription factors [17]. Thus, considering the data ([8], this work) we imagine that Pkc1p could contribute to both the glucose derepression pathway and *FKS2* expression by controlling the cellular localization of Mig1p. This seems to be the case, as in the *pkc1Δ* mutant Mig1p remains in the nucleus even after the cells are transferred from glucose repressing to derepressing conditions (Fig. 7A,B. Nevertheless, the mechanism by which Pkc1p controls the subcellar localization of Mig1p is not clear, but in our opinion there are at least two possibilities. Pkc1p could phosphorylate Mig1p since there are potentially eight protein kinase C phosphorylation sites in Mig1p [20]. This phosphorylation could trigger and/or facilitate the translocation of Mig1p. On the other hand, Pkc1p could be involved in the regulation of translocation factors that are required to export Mig1p from nucleus to the cytoplasm. We are currently investigating these possibilities.

However, the involvement of Pkc1p in the derepression of glucose-controlled systems seems to be still more complex. For instance, the genes that encode enzymes involved in galactose metabolism are glucose repressed, indicating that the main glucose pathway has an important role [25]. According to the current model, in the presence of glucose Mig1p blocks the expression of genes such as *GAL4* and the regulator Gal80p binds to Gal4p, preventing the transcriptional activation of genes that encode enzymes for galactose metabolism. On the other hand, in the presence of galactose, Gal3p impairs the binding of the Gal80p inhibitor to Gal4p, Gal4p can then be phosphorylated and activate *GAL* transcription. In the *pkc1Δ* mutant, and in the presence of an effector molecule, Mig1p may play either a secondary role to Pkc1p and/or a glucose-repression independent mechanism may operate to activate *GAL* transcription.

Moreover, and to add more complexity to the involvement of Pkc1p in the control of glucose-repressed enzymes, the increase in alcohol dehydrogenase activity when yeast cells are transferred from glucose to ethanol seems to be dependent on Pkc1p. Interestingly, Mig1p has not been described as being involved in the control of *ADH2* gene transcription. Indeed, the introduction of a *pkc1Δ* mutation in a *mig1Δ* strain blocked the increase in the alcohol dehydrogenase activity, suggesting an alternative mechanism by which Pkc1p participates in the derepression process. It is known that Adr1p, a transcriptional factor that acts at an upstream activating sequence (UAS) on the *ADH2* and *GUT1* (encoding glycerol kinase, an essential enzyme in glycerol metabolism) promoters, has a positive effect when the glucose levels are low [1–3,26]. Thus, since the *pkc1Δ* mutant grows poorly on ethanol or glycerol carbon sources, it is possible that Pkc1p could regulate the cellular localization of Adr1. However, more experiments are required to clarify this complex mechanism.

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