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Evidence for a modulation of neutral trehalase activity by Ca^{2+} and cAMP signaling pathways in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae neutral trehalase (encoded by *NTH1*) is regulated by cAMP-dependent protein kinase (PKA) and by an endogenous modulator protein. A yeast strain with knockouts of *CMK1* and *CMK2* genes (*cmk1cmk2*) and its isogenic control (*CMK1CMK2*) were used to investigate the role of CaM kinase II in the *in vitro* activation of neutral trehalase during growth on glucose. In the exponential growth phase, *cmk1cmk2* cells exhibited basal trehalase activity and an activation ratio by PKA very similar to that found in *CMK1CMK2* cells. At diauxie, even though both cells presented comparable basal trehalase activities, *cmk1cmk2* cells showed reduced activation by PKA and lower total trehalase activity when compared to *CMK1CMK2* cells. To determine if CaM kinase II regulates *NTH1* expression or is involved in post-translational modulation of neutral trehalase activity, *NTH1* promoter activity was evaluated using an *NTH1-lacZ* reporter gene. Similar ß-galactosidase activities were found for *CMK1CMK2* and *cmk1cmk2* cells, ruling out the role of CaM kinase II in *NTH1* expression. Thus, CaM kinase II should act in concert with PKA on the activation of the cryptic form of neutral trehalase. A model for trehalase regulation by CaM kinase II is proposed whereby the target protein for Ca^{2+}/CaM-dependent kinase II phosphorylation is not the neutral trehalase itself. The possible identity of this target protein with the recently identified trehalase-associated protein YLR270Wp is discussed.

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- Calmodulin
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- YLR270W

Introduction

Neutral trehalase (EC 3.2.1.28), encoded by the *NTH1* gene, has been reported to be post-translationally regulated by two main mechanisms: i) phosphorylation by cAMP-dependent protein kinase (PKA) that converts the non-phosphorylated cryptic trehalase into the phosphorylated active form (1-3), and ii) down-regulation of the phosphorylation step by an unidentified protein (4).

It was postulated that full trehalase activation by PKA requires the previous removal of the endogenous inhibitor protein, which prevents trehalase phosphorylation. This inhibitory protein was reported to be present at

the onset of the transition phase and to be responsible for the observed dependence of neutral trehalase activity on Ca^{2+} ions, a claim reinforced by the demonstration that this modulator protein can bind to a calmodulin-agarose chromatography column (4).

It has been proposed that multifunctional Ca^{2+}/calmodulin protein kinase (CaM kinase II) may act as a general mediator between the Ca^{2+} signal and the Ca^{2+}-dependent physiological response by regulating the phosphorylation of many cellular targets (5). In *Saccharomyces cerevisiae*, the genes *CMK1* and *CMK2* encode the CaM kinase II isozymes with apparent molecular masses of 56 and 50 kDa whose structural and functional properties are closely related, except in terms of autoregulatory activity (6). CMK1p and CMK2p are similar in structure to the mammalian kinase subunit, which is characterized by a segmental organization of its primary structure composed of a serine/threonine protein kinase motif, a CaM regulatory segment on the carboxyl side of the kinase motif, and flanking sequences on the amino or carboxyl terminus which appear to be responsible for the supramolecular association and/or subcellular localization of the enzyme (7).

We report here the involvement of CaM kinase II in trehalase activation by PKA and propose a novel role for this Ca^{2+}/calmodulin-dependent kinase, adding evidence for a synergy between Ca^{2+} and cAMP signaling pathways in trehalase modulation.

Material and Methods

Yeast strains, plasmid and culture conditions

S. cerevisiae YOJ211-9A (*ade2 lys2 his3 trp1 leu2 ura3 CMK1 CMK2*) and its isogenic strain YOJ211-9C (*ade2 lys2 his3 trp1 leu2 ura3 cmk1::TRP1 cmk2::LEU2*), supplied by Dr. Yoshikazu Ohya, University of Tokyo, Tokyo, Japan (6), were grown at 28ºC in YPD medium (1% yeast extract, 2% peptone, 2% glucose) for trehalase activity determinations. The pNL1 plasmid containing a 1.3-kb *Sal*I/*Hin*dIII fragment of the *NTH1* gene spanning 720 bp of the *NTH1* ORF plus 5' upstream promoter joined in frame with *lacZ* (8) was used to transform yeast cells by the lithium acetate method (9). Transformants were selected on YNB dropout medium minus uracil. The transformed cells were grown in YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.01% appropriate auxotrophic requirements) and harvested in the late exponential growth phase for ß-galactosidase activity determinations.

Preparation of cell-free extracts

Cell-free extracts were prepared by shaking 50 mg of cells (dry weight) with glass beads (10). Protein concentration was measured by the method of Stickland (11) using bovine serum albumin as standard.

Enzymatic assays

Basal trehalase activity was assayed in 50 mM maleate buffer, pH 6.0, containing 50 mM trehalose and 100 µg protein from the cell-free extracts in a total volume of 200 µl. After 15 min at 30ºC, the reaction was stopped in a boiling water bath (3 min) and the amount of glucose formed was determined by a modification of the glucose oxidase-peroxidase method (12).

Cryptic trehalase was activated by PKA as described previously (4). Cell-free extract (1 mg) was incubated with an activation mixture containing 1 mM ATP, 10 mM $MgCl_2$, 25 µM cAMP, 25 mM NaF and 2.5 mM theophylline prepared in 50 mM phosphate buffer, pH 7.5, in a final volume of 100 µl. After 15 min at 30ºC, the reaction was stopped by dilution with 400 µl of ice-cold 50 mM maleate buffer, pH 6.0. An aliquot of this mixture corresponding to 100 µg protein was used to quantify trehalase activity as

described above and is referred to as total trehalase activity. Cryptic trehalase activity was calculated by the difference between total trehalase and basal trehalase activities. One unit of trehalase activity catalyzes the release of 1 µmol glucose per minute under the stated conditions.

ß-Galactosidase activity was assayed by measuring p-nitrophenyl-ß-D-galactopyranoside hydrolysis in chloroform/SDS-permeabilized cells and is reported as Miller units (13).

Results and Discussion

In *S. cerevisiae* cells, neutral trehalase activity varies in a predictable way during growth on glucose. Neutral trehalase activity is present at higher levels in cells at the mid-log phase and undergoes a drastic decrease when exponentially growing cells reach diauxie. This variation in neutral trehalase activity was attributed to a modification of the phosphorylation state of the enzyme, since the amount of trehalase polypeptide was not changed (14).

In order to investigate the role played by CaM kinase II in neutral trehalase activation by PKA, basal, cryptic and total trehalase activities were measured in *CMK1CMK2* and *cmk1cmk2* cells grown on glucose and harvested at the exponential growth phase (0.5-2.0 mg dry weight/ml) or at diauxie (2.6-2.7 mg dry weight/ml) (Figure 1).

During exponential growth, cell-free extracts from both strains showed similar cryptic trehalase activities and rates of trehalase activation by PKA until the culture reached a cell density of 2.0 mg dry weight/ml. At the onset of diauxie (2.6 mg dry weight/ml), *CMK1CMK2* cells showed a higher ratio of trehalase activation and a lower basal trehalase activity than *cmk1cmk2* cells although total trehalase activities were similar, i.e., 20.1 ± 1.8 and 17.5 ± 1.9 mU/mg protein, for *CMK1CMK2* and *cmk1cmk2* cells, respectively.

A more evident phenotype for *CMK1CMK2* cells was detected when cultures reached a cell density of 2.7 mg/ml. While *CMK1CMK2* cells showed a higher total trehalase activity (30.4 ± 4.3 mU/mg protein) and a basal activity of 4.9 ± 0.7 mU/mg, the *cmk1cmk2* cells showed values of 15.2 ± 2.0 and 7.0 ± 0.8 mU/mg protein, respectively.

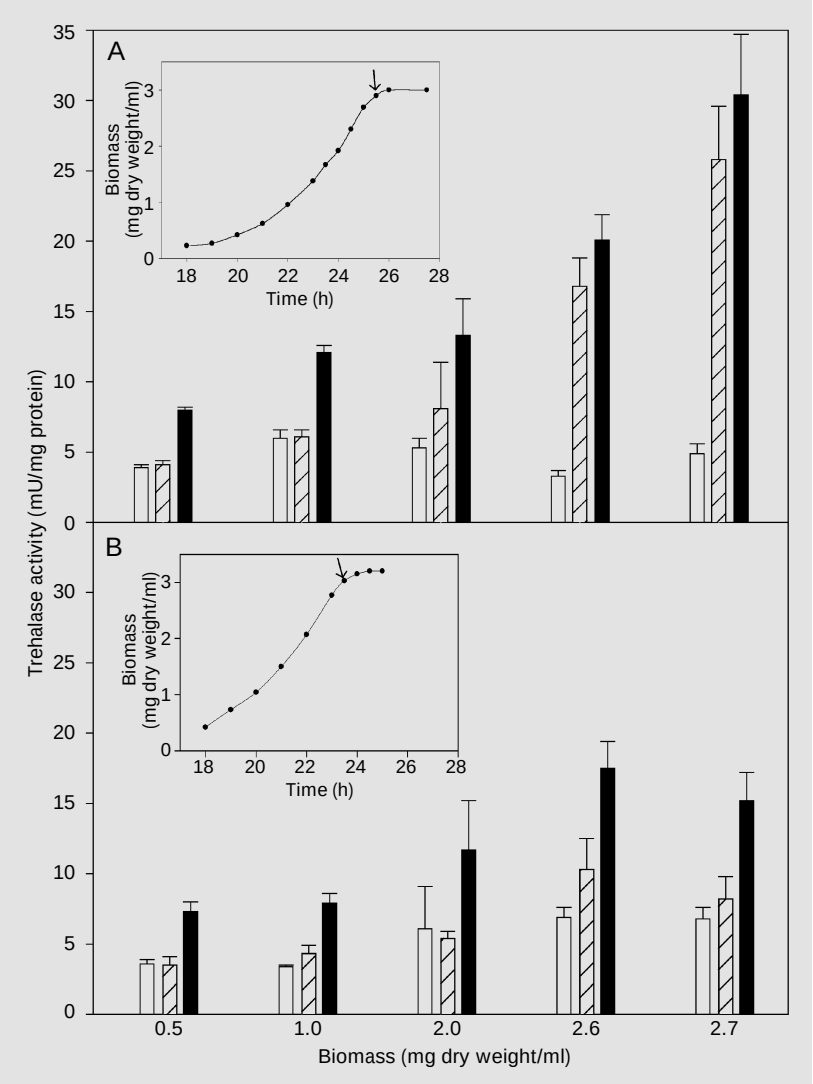

Figure 1. Basal, cryptic and neutral trehalase activities for the YOJ211-9A (*CMK1CMK2*) (A) and YOJ211-9C (*cmk1cmk2*) strains (B). Cells were grown in YPD medium and the growth curves (biomass) are shown in the inset (arrows indicate when glucose was exhausted from the medium). Open bars represent basal trehalase activity; dashed bars, cryptic trehalase activity and filled bars, total trehalase activity (*in vitro* cAMP/ATP activated) determined in cells harvested at the indicated points. Data are reported as means ± SD of three independent experiments.

Actually, total, basal and cryptic activities obtained for the control cells were in agreement with the expected changes in neutral trehalase enzymatic activity during the growth on glucose medium (2,14). However, the data obtained for *cmk1cmk2* cells differed significantly mainly from those at diauxie. Throughout the time considered, cryptic trehalase contributed to approximately 50% of the total trehalase activity in the *cmk1cmk2* cells, even at the onset of the diauxic phase. In *CMK1CMK2* cells, when the total trehalase reached its maximum values at the diauxic phase, the cryptic trehalase represented up to 85% of total activity.

Although the above results point to a misregulation of the trehalase activation mediated by PKA in cells lacking CaM kinase II genes, a possible effect of this kinase on modulation of *NTH1* expression cannot be ruled out.

Neutral trehalase activity is dependent on the combined effect of two factors - the expression level of the *NTH1* gene and the efficiency of cryptic trehalase activation by PKA. *NTH1* expression is driven by stress responsive elements, resulting in the up-regulation of neutral trehalase expression by heat and by other stressors such as H_2O_2, $CuSO_4$, $NaAsO_2$ and cycloheximide or when cells enter diauxie (15).

To determine whether the lack of CaM kinase II activity affects *NTH1* expression, resulting in the observed low total trehalase activity for *cmk1cmk2* cells at diauxie, the expression of *NTH1* was analyzed using a reporter gene. The reporter gene in a multicopy plasmid was constructed by joining in frame a 1.3-kb fragment of the *NTH1* gene, corresponding to 720 bp of the *NTH1* translational initiation site plus 600 bp of its promoter region, to the heterologous *lacZ* gene (8).

Table 1. Effect of *cmk1cmk2* mutations on the expression of the *NTH1-lacZ* reporter gene.

Strain	ß-Galactosidase activity (Miller units)
YOJ211-9A (*CMK1CMK2*)	27.3 ± 3.5
YOJ211-9C (*cmk1cmk2*)	26.9 ± 5.0

Saccharomyces cerevisiae strains YOJ211-9A and YOJ211-9C transformed with pNL1 plasmid containing *NTH1-lacZ* fusion were grown in YNB medium until the late exponential growth phase. ß-Galactosidase activity was determined in permeabilized cells using p-nitrophenyl-ß-D-galactopyranoside as the substrate. Data are reported as means ± SD of three independent experiments.

As shown in Table 1, both *CMK1CMK2* and *cmk1cmk2* cells transformed with the *NTH1-lacZ* fusion showed similar ß-galactosidase activities, indicating that the expression of *NTH1* was not altered by the absence of CaM kinase II genes. These results support the argument that CaM kinase II activity is necessary to achieve full trehalase activation by PKA.

Neutral trehalase can be the target for CaM kinase II phosphorylation, making it susceptible to phosphorylation by PKA. Conversely, an alternative target for phosphorylation could be the endogenous inhibitory protein (4) which down-regulates trehalase by obstructing trehalase activation by PKA.

Evidence favoring the latter as the substrate for CaM kinase II came from the fractionation of yeast extracts by calmodulin-agarose chromatography which showed that the endogenous inhibitory protein, but not trehalase, was able to bind to calmodulin (4). This result, together with those reported here, suggests that CaM kinase II activity is necessary to reach full trehalase activation by PKA.

The identity of the unidentified endogenous inhibitory protein was recently disclosed by an extensive two-hybrid analysis performed on *S. cerevisiae* genes. A 41-kDa protein with unknown function was found to be encoded by the *YLR270W* locus, which interacts with NTH1p (16,17). Although the identity of this protein with trehalase inhibitor (4) has not yet been investigated, we raised the hypothesis that the two proteins

may be the same and renamed them trehalase-associated protein (Tap).

Based on our results, a speculative yet reasonable model for regulation of neutral trehalase by Ca^{2+} and cAMP signaling pathways is proposed (Figure 2). Phosphorylation of cryptic trehalase mediated by PKA is unattainable in the presence of Tap. For cryptic trehalase to become susceptible to activation by PKA, its interaction with Tap has to be dissolved. Phosphorylation by CaM kinase II could induce the dissociation of Tap from cryptic trehalase, thus exposing the sites of cryptic trehalase which are susceptible to phosphorylation by PKA. In this way, the lack of CaM kinase II prevents cells from reaching maximum levels of neutral trehalase activity.

The growth phase where neutral trehalase displayed the highest *in vitro* activation by PKA coincides with the phase of maximum mRNA expression of *CMK1/CMK2* and *YLR270W* genes. A 2.0- and 2.2-fold induction for *CMK1* and *CMK2* mRNA levels at diauxie was described, while the *YLR270W* mRNA expression levels were shown to be enhanced suddenly and transiently 8.3 times (18), a pattern of gene expression similar to that observed for *BCY1* (19).

The pattern of expression of *CMK1*, *CMK2* and *YLR270W* genes during growth supports our results. During the diauxic phase, when Tap and CaM kinase II seem to be expressed, only the *CMK1CMK2* cells can reach the highest levels of trehalase activity. However, at the early exponential phase of growth, when there is no expression of these two proteins, the *cmk1cmk2* cells exhibited the same phenotype as the controls.

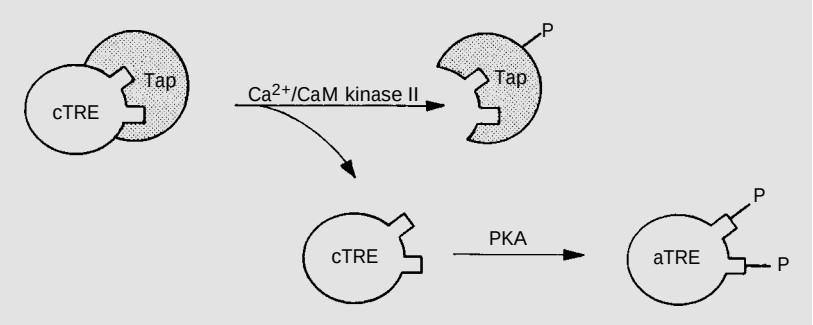

Figure 2. A model for neutral trehalase modulation by Ca^{2+} and cAMP signaling pathways. cTRE = cryptic trehalase, dephosphorylated form; Tap and Tap-P = non-phosphorylated and phosphorylated forms of trehalase-associated protein, respectively; aTRE = active trehalase, phosphorylated form; CaM kinase II = Ca^{2+}/calmodulin-dependent protein kinase II; PKA = cAMP-dependent protein kinase. Here we assumed that both putative sites described in the neutral trehalase sequence (21,22) should be phosphorylated by PKA in order to obtain full trehalase activation.

The data shown here suggest that the major mechanism responsible for the regulation of *S. cerevisiae* neutral trehalase during growth is phosphorylation by different kinases - PKA and CaM kinase II, which might phosphorylate the enzyme itself and Tap, respectively.

The above regulatory mechanism, although detected specifically at diauxie, might also be involved in regulating trehalase under other physiological conditions, such as when yeast cells are subjected to high temperatures. Activation of neutral trehalase during heat stress seems to be regulated by *de novo* protein synthesis and by PKA phosphorylation (15). Therefore, full trehalase activation during heat stress could also be regulated by a combination of cAMP and Ca^{2+} signaling pathways since heat stress evokes Ca^{2+} influx in eukaryotic cells (20).

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