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Sugar induced cell death in yeast is dependent on the rate of sugar phosphorylation as determined by *Arabidopsis thaliana* hexokinase

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Abstract

Sugars like glucose and fructose induce death of yeast cells within a few hours, in the absence of additional nutrients to support growth, while cells incubated in water remain viable for weeks. This sugar-induced cell death (SICD) by glucose and fructose required glucose or fructose phosphorylation since yeast cells deficient in hexose phosphorylation did not die. However, when hexose phosphorylation is restored by complementation with *Arabidopsis thaliana* hexokinase, the cells died. The affinity of *A. thaliana* hexokinase is about 400 times higher for glucose than for fructose, therefore, *A. thaliana* hexokinase was further utilized to study the role of hexose phosphorylation in SICD. The rate of SICD of hexokinase-deficient yeast cells expressing *A. thaliana* hexokinase was significantly slower in fructose than in glucose, indicating that SICD is determined by the rate of hexose phosphorylation. The significance of hexose phosphorylation and its role in SICD is discussed.

Keywords: sugar induced cell death (SICD); *Saccharomyces cerevisiae*; hexose phosphorylation; glucose; fructose

Abbreviations: SICD, sugar-induced cell death

Introduction

Yeast cells grown to the stationary phase accumulate large amounts of reserve carbohydrates such as glycogen and trehalose (Fraenkel, 1982) and are able to survive for long periods under starvation conditions (Granot and Snyder, 1991, 1993). Upon transfer to growth media containing sugar, nitrogen source and additional minor nutrients, the cells resume growth. Surprisingly, when yeast cells are incubated in the presence of only a sugar, in the absence of additional nutrients to support growth, the cells lose viability within a few hours (Granot and Snyder, 1991, 1993), a phenomenon we term Sugar-Induced Cell Death (SICD). Incubation for more than 3 weeks either in water or in all the nutrients except for a sugar does not cause death (Granot and Snyder, 1991). It has been suggested that, unlike other nutrients, sugar signals growth, and because the medium does not contain sufficient nutrients to support growth the cells rapidly lose viability (Granot and Snyder, 1991, 1993). Indeed, short exposure (min) of stationary-phase yeast cells to fermentable or nonfermentable sugars without additional nutrients to support growth induced early growth events such as vacuole dispersal, which precede budding, culminating in advanced budding upon transfer to rich media (Granot and Snyder, 1991, 1993).

The nature of the growth signal and SICD were analyzed by means of glucose analogs. L-Glucose, a glucose analog which does not enter the cell, does not induce growth events or SICD (Granot and Snyder, 1991). 6-Deoxyglucose, a glucose analog which enters the cell but is not phosphorylated and is not metabolized, signals growth but not SICD, suggesting that SICD is not a direct outcome of the growth signal, but that it may require glucose phosphorylation. The hypothesis that hexose phosphorylation is necessary for SICD is supported by results obtained with yeast mutants bearing mutations in hexose-phosphorylating genes. The yeast *Saccharomyces cerevisiae* possesses three genes which code for enzymes capable of phosphorylating hexoses. Two enzymes, Hexokinase 1, coded by *HXK1* and Hexokinase 2, coded by *HXK2* phosphorylate either glucose or fructose, while Glucokinase 1, coded by *GLK1*, phosphorylates only glucose (Fraenkel, 1982). Hence, *hxk1 hxk2* double-mutant cells are unable to phosphorylate fructose but are able to phosphorylate glucose by *GLK1*. Such double-mutant cells undergo SICD when incubated with glucose, but remain viable when incubated with fructose, indicating that SICD in fructose requires fructose phosphorylation (Granot and Snyder, 1993).

The involvement of hexose transport in SICD was also studied. Glucose and fructose are transported into yeast cells by high-affinity and by low-affinity transport (for review see Bisson *et al*, 1993). The high-affinity transport whose K_m is 2 mM for glucose and 5 mM for fructose is mediated by several nonessential transporters and sugar receptors like *SNF3* (Bisson *et al*, 1987; Coons *et al*, 1995; Ozcan *et al*, 1996; Yang and Bisson, 1996). Whether the low-affinity transport (K_m is 10–20 mM for glucose and 20–50 mM for fructose) is mediated by transporters or by passive diffusion is as yet unclear (Bisson *et al*, 1993; Lagunas, 1993; Coons *et al*, 1995). However, cells with null allele of *SNF3* lack the high-affinity hexose transport and neither grow on supplemented media containing 5.5 mM glucose (Bisson *et al*, 1987; Neigeborn *et al*, 1986) nor undergo SICD upon incubation in 5.5 mM glucose (Granot and Snyder, 1993)

but grow in supplemented media containing 110 mM glucose (Bisson *et al*, 1987; Neigeborn *et al*, 1986) and undergo SICD in 110 mM glucose (Granot and Snyder, 1993). Therefore, SICD may occur independently of high-affinity hexose transport.

In the present study, we used the well defined *A. thaliana* hexokinase cDNA, expressed in triple-mutant hexose-kinase deficient yeast cells (Dai *et al*, 1995) to test directly the importance of glucose phosphorylation for SICD and to analyze the dependence of SICD on the rate of hexose phosphorylation.

Results

SICD depends on glucose phosphorylation

Glucose phosphorylation had previously been indirectly shown to be necessary for SICD, by using a glucose analog, 6-deoxyglucose, that enters the cell but is not phosphorylated. No SICD is oberved upon incubation in 6-deoxyglucose, suggesting that SICD may require glucose phosphorylation (Granot and Snyder, 1991). To prove the necessity of glucose phosphorylation for SICD, a triple-mutant yeast strain, DFY632, defective in glucose phosphorylation because of mutations in glucokinase and the two hexokinase genes (Walsh *et al*, 1991) was used. The mutant cells are unable to grow on glucose as carbon source. However, when the cells are transformed with pHK1, a plasmid containing *A. thaliana* hexokinase cDNA under a constitutive yeast promoter, the cells regain their ability to grow on glucose (Dai *et al*, 1995), indicating that *A. thaliana* hexokinase phosphorylates glucose efficiently. When DFY632 cells transformed with pHK1 were incubated in 110 mM glucose, in the absence of additional nutrients to support growth, the cells started to die within a few hours (Figure 1), while cells transformed with pFL61, the vector plasmid without the hexokinase gene, remained viable. These results directly indicate that glucose phosphorylation is necessary for SICD.

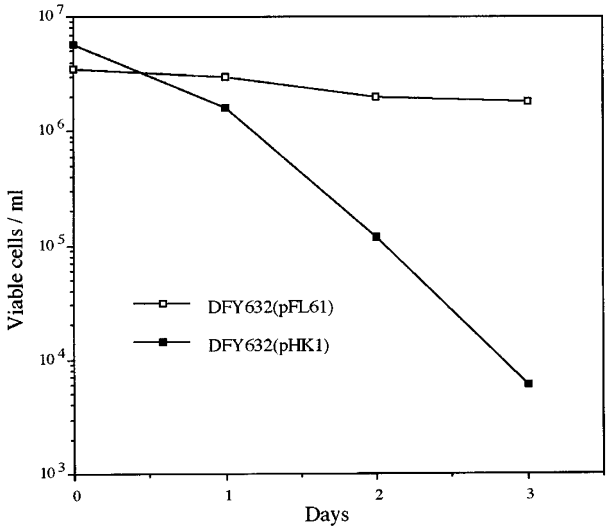

Figure 1 Effect of glucose phosphorylation on cell viability in 110 mM glucose. DFY632 cells transformed with either pFL61 or with pHK1 were grown on −URA+galactose to stationary phase. The cells were washed thoroughly in water, resuspended in 110 mM glucose without additional nutrients and incubated at 37°C. Viability of the cells was followed by dilution and plating on YEPG plates.

A. thaliana hexokinase allows growth in various concentrations of glucose and fructose

The affinity of *A. thaliana* hexokinase is much higher for glucose ($K_{m(glucose)}$=44 μM than for fructose ($K_{m(fructose)}$=17 mM, Dai *et al*, 1995). Hence, it might be possible to determine the role of hexose phosphorylation rate in SICD by comparison of SICD of DFY632 cells expressing *A. thaliana* hexokinase in various concentrations of glucose or fructose. If the rate of hexose phosphorylation determines the rate of SICD, it is expected that SICD at concentrations below fructose K_m and above glucose K_m will be slower in fructose than in glucose. To facilitate such experiments, we examined the proliferation of DFY632 cells transformed with pHK1 at various glucose and fructose concentration, as an indication of hexose uptake and *in vivo* phosphorylation by *A. thaliana* hexokinase. As shown in Figure 2, DFY632 cells transformed with pHK1 failed to grow on 1.1 mM hexose but do grow on 2.75, 5.5, 11 and 110 mM glucose or fructose. However, the growth rates at 2.75 and 5.5 mM were different for glucose and fructose. At 2.75 mM, i.e., near the high-affinity glucose-uptake K_m, proliferation rate in glucose was slightly above that in fructose. At 5.5 mM, that is around the high-affinity fructose uptake and double the high-affinity glucose uptake, proliferation rate in glucose was still faster than that in fructose. At 110 mM, the proliferation rate in glucose was reduced compared with that in 11 mM glucose. This reduction is probably due to a substrate inhibition of glucose uptake, previously described for *hxk2* yeast mutants, which occurs at glucose concentrations above 20 mM (McClellan and Bisson, 1988). Nonetheless, at 11 mM, which is above glucose and fructose high-affinity uptake K_m, the proliferation rates in glucose and fructose were similar.

The rate of SICD mediated by *A. thaliana* hexokinase is different in glucose and in fructose

To determine the role of the hexose phosphorylation rate on SICD, stationary phase DFY632 cells transformed with *A. thaliana* hexokinase were incubated in 2.75, 5.5, 11 and 110 mM of either glucose or fructose, without additional nutrients to support growth, and SICD was monitored. At 2.75, 5.5 and 11 mM, SICD in fructose was slower than in glucose while at 110 mM, SICD rates in both sugars were similar (Figure 3). The time for 50% of the cells to die, $t_{1/2}$ of SICD, was calculated for 5.5, 11 and 110 mM (Figure 4). At 110 mM, $t_{1/2}$ of SICD of either glucose or fructose was similar, around 17 h. At 11 mM, $t_{1/2}$ of SICD in fructose was 35 h as compared with 20 h in glucose, and at 5.5 mM $t_{1/2}$ of SICD in fructose had further increased to 69 h as compared with 23 h in glucose. Since 11 and 5.5 mM are above the phosphorylation K_m of *A. thaliana* hexokinase for glucose and below its K_m for fructose, these results suggest that the rate of SICD is determined by the rate of hexose phosphorylation.

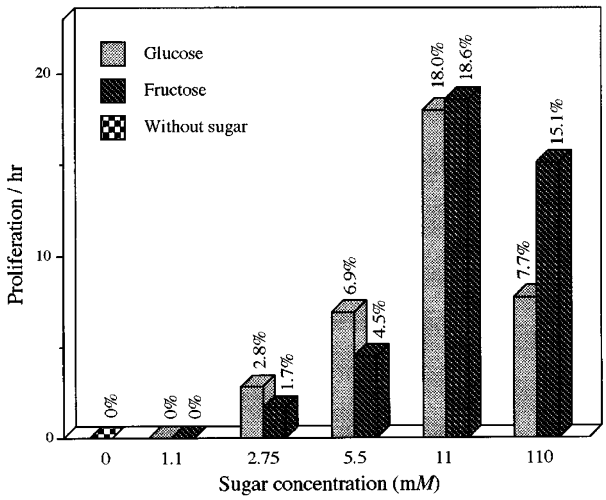

Figure 2 Proliferation rate in various hexose concentrations. DFY632 cells transformed with pHK1 were grown to the stationary phase on −URA+galactose. Cells from the same stationary culture were washed thoroughly and resuspended at approximately 1×10^7 cells/ml in −URA liquid medium containing 1.1, 2.75, 5.5, 11 or 110 mM of either glucose or fructose, and incubated at 30°C. At set intervals, samples were taken, diluted and plated on YEPG plates to follow proliferation. Proliferation rate at the logarithmic phase was calculated for each hexose concentration as the increased proportion of cells per hour.

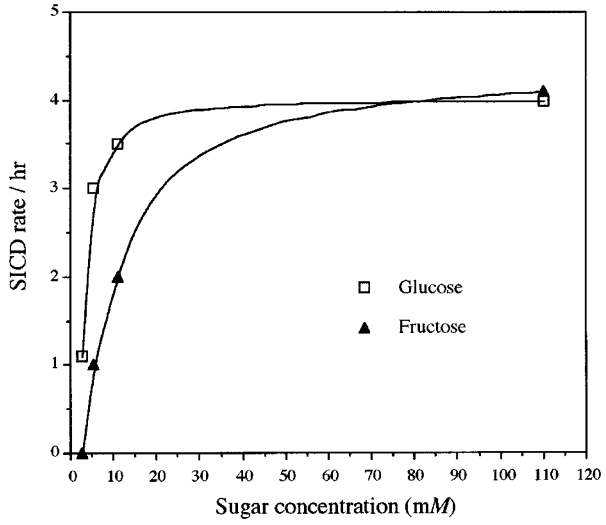

Figure 3 SICD rate at various hexose concentrations. DFY632 cells transformed with pHK1 were grown to the stationary phase on −URA+galactose. The cells were washed thoroughly in water and resuspended in 2.75, 5.5, 11 or 110 mM of glucose or fructose, without additional nutrients, to a final concentration of about 1×10^7 cells/ml. In addition, cells were resuspended in water as a control. The cells were incubated at 37°C and their viability was monitored by dilution and plating on YEPG plates. The rate of SICD was calculated for each hexose concentration as the proportion of dying cells per hour.

Discussion

Exposure of yeast cells to sugar such as glucose or fructose, without sufficient of the nutrients necessary for growth,

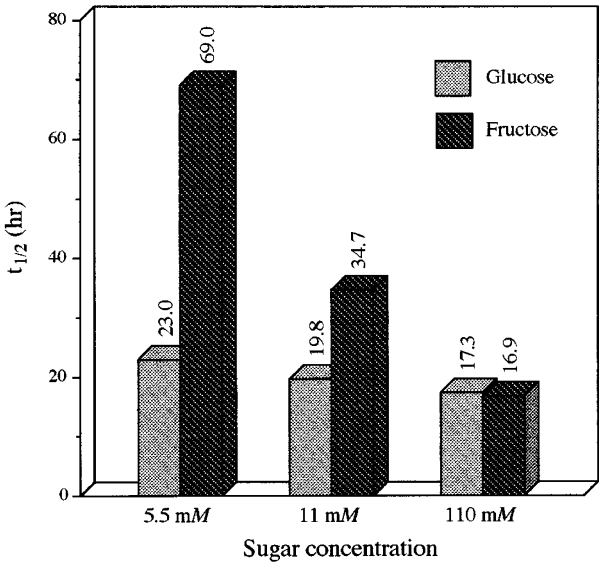

Figure 4 $t_{1/2}$ of SICD as in Figure 3. Once SICD rate for each glucose and fructose concentration was calculated, $t_{1/2}$ for each culture was determined as the time taken for 50% of the cells to die.

causes sugar induced cell death (SICD), while exposure to all the nutrients except for a sugar has no effect (Granot and Snyder, 1991, 1993). A mutant strain, DFY632, which is unable to phosphorylate glucose or fructose, clearly demonstrated that SICD requires glucose phosphorylation since DFY632 cells remained viable in 110 mM glucose but undergo SICD when expressing *A. thaliana* hexokinase cDNA (Figure 1). Theoretically, one could argue that *A. thaliana* hexokinase is required for hexose uptake which in turn causes SICD. Nonetheless, several observations indicate that *A. thaliana* hexokinase is not necessary for glucose uptake at 110 mM glucose. First, although it is still controversial whether hexokinases are required for high-affinity glucose transport whose K_m is 2 mM, it is established that hexokinases are dispensable for glucose uptake at higher concentrations (Bisson and Fraenkel, 1984; Smits *et al*, 1996). Accordingly, by using *SNF3* null mutants which lack high affinity glucose uptake we previously showed that SICD takes place at 110 mM glucose independently of high-affinity hexose uptake (Granot and Snyder, 1993). Second, *hxk2* deficient mutant cells exhibit substrate inhibition of glucose uptake at glucose concentrations above 20 mM (McClellan and Bisson, 1988). *A. thaliana* hexokinase did not complement this substrate inhibition as demonstrated by decreased proliferation rate at 110 mM glucose (Figure 2). The inability to complement the substrate inhibition eliminates the possibility that *A. thaliana* hexokinase is involved with glucose uptake at 110 mM glucose. Therefore, *A. thaliana* hexokinase caused SICD through its role in hexose phosphorylation rather than through a potential role in hexose uptake, confirming that glucose phosphorylation is essential for SICD.

To elucidate the dependence of SICD on the rate of hexose phosphorylation we took advantage of the differing affinities of *A. thaliana* hexokinase for glucose and fructose,

and compared the SICD rate in glucose with that in fructose at various concentrations. The SICD rate was the same in both glucose and fructose at 110 mM but was significantly slowed at 11 mM in fructose compared with glucose and further reduced in fructose at 5.5 mM: both concentrations are below *A. thaliana* hexokinase K_m for fructose and above its K_m for glucose. Nonetheless, the proliferation rates of the cells at 11 mM were similar in glucose and fructose (Figure 2), suggesting that the proliferation rate is determined by hexose uptake rather than by hexose phosphorylation and that the SICD rate is determined by the rate of hexose phosphorylation.

Similar conclusion can be drawn from comparison of proliferation rate and SICD rate at different concentrations of glucose. Although the proliferation rate in 110 mM glucose was significantly reduced compared with 11 mM glucose, most probably because of substrate inhibition of glucose uptake at 110 mM glucose, SICD in 110 mM glucose was slightly faster than in 11 mM glucose, suggesting that the rate of glucose phosphorylation rather than glucose uptake determines the rate of SICD.

When yeast cells are exposed to glucose or fructose, hexose phosphorylation is necessary for SICD; however, the subsequent molecular and biochemical processes which lead to cell death are not known. Likewise, it is not known whether the presence of phosphorylated hexoses *per se* is sufficient for SICD or whether the phosphorylation reaction is also necessary. Since SICD occurs in the presence of nonfermentable sugars and carbon sources such as acetate and ethanol (Granot and Snyder, 1993), in which phosphorylated hexoses are produced via gluconeogenesis without hexose phosphorylation (Fraenkel, 1982), it is possible that the phosphorylation reaction *per se* is not required and that the presence of phosphorylated hexoses is sufficient for SICD. Accordingly, it is conceivable that subsequent metabolism of the phosphorylated hexoses is required for SICD. Recently we have isolated yeast mutants which survive in glucose (Granot, unpublished results) suggesting that SICD in yeast is at least partially genetically controlled. Further analysis of these mutants and their complementing genes may uncover the nature of SICD.

Materials and Methods

Yeast strains and media

The yeast, *Saccharomyces cerevisiae*, strain used was DFY632 – *MAT**a**, ura3-52*, *hxk1::LEU2*, *hxk2::LEU2*, *glk1::LEU2*, *lys1-1*, *leu2-1* (S288C background, congenic with strains published in Walsh *et al*, 1991). Yeast cells were grown on YEPG liquid media, consisting of 1% Yeast Extract (Difco), 2% Bacto Peptone (Difco) and 110 mM (2%) galactose (Sherman *et al*, 1986). Selective media for uracil auxothrophic growth, −URA+sugar, contained 0.5% ammonium Sulfate, 0.17% Yeast Nitrogen Base without amino acids (Difco), 0.2% Casamino Acids (Difco), 0.004% Adenine (Sigma), 0.008% Tryptophan (Sigma) and 110 mM of either galactose, glucose or fructose.

Glucose or fructose liquid media contain 2.75, 5.5, 11 or 110 mM of either glucose of fructose in double-distilled water, sterilized by filtration.

Yeast transformation and plasmids

pFL61 is a yeast shuttle vector containing *URA3* gene as a selective marker and the constitutive phosphoglycerate kinase (*PGK*) promoter and terminator (Minet *et al*, 1992). pHK1 – contains *A. thaliana* hexokinase cDNA in pFL61 (Dai *et al*, 1995) under *PGK* promoter. Yeast transformations were carried out by growing DFY632 cells in YEPG liquid media to mid-logarithmic phase, treating the cells with lithium acetate according to Ito *et al* (1983) and selecting for transformants on −URA+galactose plates.

Viability tests

Yeast cells transformed with either pFL61 or with pHK1 were grown in 50 ml of −URA selective medium containing 110 mM galactose, since DFY632 cells transformed with pFL61 are unable to grow on glucose. The cells were grown to stationary phase for at least 1 day after they reached approximately $0.5-1.0\times10^8$ cells/ml. The cells were pelleted at 4000 x *g*, washed three times with 40 ml of water and resuspended in a small volume of water. Cells from the same stationary culture were then transferred to 5 ml of −URA medium containing 2.75, 5.5, 11 or 110 mM of either glucose or fructose to a final concentration of about 1×10^7 cells/ml and incubated in a roller drum at 37°C (Viability experiments were done at 37°C because SICD occurs faster at 37°C than at 30°C). Samples were taken at approximately 24 h intervals, diluted and plated on YEPG plates to determine the percentage of viable cells at each time. The SICD rate (proportion of dying cells/h) was calculated for each hexose concentration with the equation $dx/dt=(\text{SICD rate})\cdot x$ in which x represents number of viable cells and dx stands for the decrease in the number of viable cells between two time points − dt. Once SICD rate was calculated, $t_{1/2}$ was determined for each culture as the time at which 50% of the cells were dead.

Proliferation rate assay

DFY632 cells transformed with pHK1 were grown in −URA+galactose to the stationary phase. The cells were washed three times with 40 ml of water and resuspended in a small volume of water. Cells from the same stationary culture were transferred to 5 ml of −URA media containing 1.1, 2.75, 5.5, 11 or 110 mM of either glucose or fructose to approximately the final concentration of 1×10^7 cells/ml, and incubated at 30°C. Proliferation was measured by taking samples about every 5 h, diluting and plating on YEPG plates on which newborn cells that did not inherit the plasmid would also grow and be counted. Proliferation rate was calculated as the increased proportion of cells per hour by the equation $dx/dt=(\text{Proliferation rate})\cdot x$ in which x represents number of cells and dx stands for the increase in the number of cells between two time points − dt.

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