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Understanding the mechanism of glucose-induced relief of Rgt1-mediated repression in yeast☆

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ABSTRACT

The yeast Rgt1 repressor inhibits transcription of the glucose transporter (*HXT*) genes in the absence of glucose. It does so by recruiting the general corepressor complex Ssn6-Tup1 and the *HXT* corepressor Mth1. In the presence of glucose, Rgt1 is phosphorylated by the cAMP-activated protein kinase A (PKA) and dissociates from the *HXT* promoters, resulting in expression of *HXT* genes. In this study, using Rgt1 chimeras that bind DNA constitutively, we investigate how glucose regulates Rgt1 function. Our results show that the DNA-bound Rgt1 constructs repress expression of the *HXT1* gene in conjunction with Ssn6-Tup1 and Mth1, and that this repression is lifted when they dissociate from Ssn6-Tup1 in high glucose conditions. Mth1 mediates the interaction between the Rgt1 constructs and Ssn6-Tup1, and glucose-induced downregulation of Mth1 enables PKA to phosphorylate the Rgt1 constructs. This phosphorylation induces dissociation of Ssn6-Tup1 from the DNA-bound Rgt1 constructs, resulting in derepression of *HXT* gene expression. Therefore, Rgt1 removal from DNA occurs in response to glucose but is not necessary for glucose induction of *HXT* gene expression, suggesting that glucose regulates Rgt1 function by primarily modulating the Rgt1 interaction with Ssn6-Tup1.

1. Introduction

The yeast Rgt1 repressor is a DNA-binding transcription factor that regulates expression of glucose responsive genes, including genes encoding a family of glucose transporters (HXTs) [1,2]. Rgt1 represses expression of *HXT* genes in the absence of glucose by recruiting the general corepressor Ssn6-Tup1 complex, which in turn recruits global corepressors, such as chromatin and nucleosome remodelers, or directly interacts with the RNA transcription machinery [1,3–5]. Ssn6-Tup1 also functions by masking the activation domain of a DNA-binding repressor and thereby preventing recruitment of the coactivators necessary for transcriptional activation [6]. Thus, Ssn6-Tup1 may act differently on different repressors, but an efficient recruitment of Ssn6-Tup1 by gene specific repressors may be critical for establishing repression.

Rgt1-dependent, Ssn6-Tup1-mediated repression occurs in conjunction with the paralogous proteins Mth1 and Std1. Rgt1 does not bind DNA, which thereby causes constitutive expression of *HXT* genes, in cells lacking Mth1 and Std1 [7–9]. Mth1 and Std1 directly interact with Rgt1, enabling Rgt1 to recruit Ssn6-Tup1 to the *HXT* promoters in the absence of glucose, but are degraded by the proteasome in the presence of high levels of glucose, implicating Mth1 and Std1 as Rgt1 regulators [10–14]. However, evidence also indicates that deletion of the *STD1* gene alone has little effect on the regulation of *HXT* gene expression [8,9,15]. Glucose stimulates proteasomal degradation of Std1 but also induces expression of *STD1* gene expression, suggesting attenuation of Std1 degradation by feedback regulation of Std1 expression. By contrast, glucose stimulates Mth1 degradation [14–17] but at the same time represses expression of the *MTH1* gene [9,15]. Therefore, Mth1 degradation is reinforced by glucose repression of *MTH1* gene expression, ensuring rapid removal of Mth1 from cells when glucose becomes available so as to enables prompt induction of *HXT* gene expression. Hence, glucose likely modulates Rgt1 function by mainly regulating Mth1 levels [18].

Rgt1 is phosphorylated and dissociated from the *HXT* promoters in cells grown in high glucose [3,19]. Rgt1 is a phosphoprotein; it is phosphorylated at a basal level in the absence of glucose, but hyperphosphorylated by PKA in high levels of glucose [20–23]. Rgt1 is

Abbreviations: ChIP, chromatin immunoprecipitation; ECL, enhanced chemiluminescence; IP, immunoprecipitation; PKA, protein kinase A.

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Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Source
BY4741	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ*	[37]
FM557	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 rgt1::kanMX*	[37]
YM6545	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 RGT2–1*	[15]
JKY98	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 rgt1::kanMX pHXT1::NAT*	This study
KFY35	*Matα his3Δ1 leu2Δ0 ura3Δ0 met15Δ mth1::kanMX*	[37]
KFY56	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ SSN6-TAP-HIS3MX6*	[38]

phosphorylated at four serine residues within its amino-terminal region, but this does not occur until Mth1 is degraded [24]. The PKA phosphorylation of Rgt1 inhibits its interaction with Ssn6-Tup1 and this phosphorylation is inhibited by Mth1, suggesting that Mth1 mediates the interaction between Rgt1 and Ssn6-Tup1 by inhibiting Rgt1 phosphorylation [25]. Interestingly, a recent work shows that Rgt1 bound to the *HXT1* promoter does not inhibit glucose induction of *HXT1* gene expression in cells lacking Ssn6 or Tup1, raising a possibility that glucose-induced Rgt1 removal from DNA may be not the primary cause of glucose induction of *HXT* gene expression [25]. The relief of Ssn6-Tup1-mediated repression comes about through the destruction or inactivation of the individual repressors, resulting in dissociation of the repressors from Ssn6-Tup1 and/or DNA [4]. Based on these observations, we have hypothesized that dissociation of Rgt1 from DNA occurs in response to glucose, but is not required for glucose induction of *HXT* gene expression, and that Rgt1 dissociation from Ssn6-Tup1 may be sufficient to lift Rgt1-mediated repression.

The goal of this study is to provide direct evidence to support this hypothesis. To do so, we examined glucose regulation of LexA-Rgt1 and GFP-Rgt1 fusions that bind DNA constitutively and found that the Rgt1 constructs repress *HXT1* gene expression in conjunction with Mth1 and Ssn6-Tup1 in the absence of glucose, and that this repression is lifted when they are phosphorylated and dissociated from Ssn6-Tup1 in the presence of glucose. We observed, however, that the Rgt1 constructs lacking PKA phosphorylation sites did not dissociate from Ssn6-Tup1 and thereby repress expression of the *HXT1* gene constitutively. Our results suggest that glucose induction of *HXT* gene expression results primarily from the disruption of the Rgt1-Ssn6-Tup1 interaction, rather than from Rgt1 removal from DNA.

2. Materials and methods

2.1. Yeast strains and plasmids

Yeast strains used in this study are listed in Table 1. Except where indicated, yeast strains were grown in YP (2% bacto-peptone, 1% yeast extract) and SC (synthetic yeast nitrogen base media containing 0.17% yeast nitrogen base and 0.5% ammonium sulfate) supplemented with the appropriate amino acids and carbon sources.

2.2. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [3]. Yeast strains were grown till mid-log phase ($O.D_{600nm}$ = 1.2–1.5) and incubated with formaldehyde (1% final concentration) at room temperature for 15 to 20 min. The cross-linking reaction was quenched by adding glycine to a final concentration of 125 mM for 5 min. The cells were disrupted by vortexing with acid-washed glass beads in ice cold ChIP lysis buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate) containing protease and phosphatase inhibitors. The lysate was sonicated (ultrasonic cell disruptor with a microtip) five times with 10 s pulse. The genomic DNA fragments were immunoprecipitated with anti-HA, LexA, GFP or Ssn6 antibody (Santa Cruz) conjugated with agarose beads. After washing the immunoprecipitated beads, DNA was eluted from both immunoprecipitated and 1/100 input samples. The immunoprecipitated DNA was PCR-amplified using primer pairs directed against the *HXT1* promoter. As a negative control, primer sets were designed to amplify the actin gene promoter region. DNA-binding of Rgt1 was determined by running the PCR products of linear range in 1.5% agarose gel and visualizing by ethidium bromide staining.

2.3. Western blot and immunoprecipitation (IP) analysis

For Western blot analysis, yeast cells ($O.D_{600}$ = 1.5) were collected by centrifugation at 3000 rpm in a table-top centrifuge for 5 min. The cell pellets were resuspended in 100 μl of SDS-buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and boiled for 5 min. After the lysates were cleared by centrifugation at 12,000 rpm for 10 min, soluble proteins were resolved by SDS–PAGE and transferred to PVDF membrane (Millipore). The membranes were incubated with appropriate antibodies (anti-HA, anti-LexA, anti -GFP and anti-TAP antibodies, Santa Cruz) in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and proteins were detected by the enhanced chemiluminescence (ECL) system. For IP, yeast cells were disrupted by vortexing with acid-washed glass beads in ice cold NP40 buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0) containing protease and phosphatase inhibitors. The cell lysates were incubated with appropriate antibodies at 4 °C for 3 h and further incubated with protein A/G-conjugated agarose beads at 4 °C for 1 h. The precipitated agarose beads were washed three times with ice cold NP40 buffer containing protease and phosphatase inhibitor cocktails (Sigma P8215 and Sigma P0044, respectively) and boiled in 50 μl of SDS–PAGE buffer. The resulting proteins were analyzed by Western blot using appropriate antibodies.

2.4. β-Galactosidase assay

To assay β-galactosidase activity with yeast cells expressing the *HXT1-LacZ* reporter, the yeast cells were grown to mid-log phase and the assay was performed as described previously [14]. Results were given in Miller Units [$(1000 \times O.D_{420nm})/(T \times V \times O.D_{600nm})$, where T was the incubation time in minutes, and V is the volume of cells in milliliters]. The reported enzyme activities were averages of results from triplicates of three different transformants.

2.5. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted by RNeasy mini kit (Qiagen) following manufacturer's protocol and 2 μg of total RNA was converted to cDNA by qScript cDNA supermix (Quanta Biosciences). cDNA was analyzed by qRT-PCR using SsoFast Evagreen reagent (Bio-Rad) in CFX96 Real-time thermal cycler (Bio-Rad). *ACT1* was used as an internal control to normalize expression of *HXT1* gene. All of the shown quantification data were the averages of three independent experiments with error bars representing standard deviations (S.D.).