

Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I

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The yeast *Saccharomyces cerevisiae* senses glucose through two transmembrane glucose sensors, Snf3 and Rgt2. Extracellular glucose causes these sensors to generate an intracellular signal that induces expression of *HXT* genes encoding glucose transporters by inhibiting the function of Rgt1, a transcriptional repressor of *HXT* genes. We present the following evidence that suggests that the glucose sensors are coupled to the membrane-associated protein kinase casein kinase I (Yck1). (i) Overexpression of Yck1 leads to constitutive *HXT1* expression; (ii) Yck1 (or its paralogue Yck2) is required for glucose induction of *HXT1* expression; (iii) Yck1 interacts with the Rgt2 glucose sensor; and (iv) attaching the C-terminal cytoplasmic tail of Rgt2 to Yck1 results in a constitutive glucose signal. The likely targets of Yck1 in this signal transduction pathway are Mth1 and Std1, which bind to and regulate function of the Rgt1 transcription factor and bind to the C-terminal cytoplasmic domain of glucose sensors. Potential casein kinase I phosphorylation sites in Mth1 and Std1 are required for normal glucose regulation of *HXT1* expression, and Yck1 catalyzes phosphorylation of Mth1 and Std1 *in vitro*. These results support a model of glucose signaling in which glucose binding to the glucose sensors causes them to activate Yck1 in the cell membrane, which then phosphorylates Mth1 and Std1 bound to the cytoplasmic face of the glucose sensors, triggering their degradation and leading to the derepression of *HXT* gene expression. Our results add nutrient sensing to the growing list of processes in which casein kinase I is involved.

Nutrient sensing is crucial for all cells. How cells sense glucose, the most abundant monosaccharide on earth and the primary carbon and energy source for most cells, is of great interest because defects in glucose sensing can have serious consequences for organisms. One way the yeast *Saccharomyces cerevisiae* senses glucose is through a novel receptor-mediated signaling pathway that begins with two glucose sensors in the cell membrane. Extracellular glucose causes these sensors to generate an intracellular signal that induces expression of several *HXT* genes encoding hexose transporters (1). The glucose signal induces this expression by influencing the function of the Rgt1 transcriptional repressor. In the absence of glucose, Rgt1 is functional and binds to promoters of *HXT* genes and represses their expression (2, 3). Glucose derepresses *HXT* gene expression by inhibiting Rgt1 repressor function. This process requires the SCF^{Grr1} ubiquitin-protein ligase (3, 4), suggesting that it involves protein ubiquitination and degradation. Repression of transcription by Rgt1 also requires the paralogous proteins Mth1 and Std1 (3, 5). Because Mth1 and Std1 interact with both the Rgt1 repressor (5, 6) and the Snf3 and Rgt2 glucose sensors (7, 8), they are good candidates for the components of the signal transduction pathway that are inhibited by the glucose signal. Indeed, it was recently reported that Mth1 is degraded when glucose is added to cells by SCF^{Grr1} (3).

It is not known how Snf3 and Rgt2 generate a signal in response to glucose or how that signal is transduced to Rgt1, Mth1, and Std1. Snf3 and Rgt2 are probably transmembrane receptors for extracellular glucose, because they are similar to

hexose transporters but are unable to transport glucose (9). They differ from bona fide glucose transporters in possessing unusually long C-terminal tails that are predicted to reside in the cytoplasm (10). These tails are sufficient for glucose signaling, because attaching them to a glucose transporter confers on it glucose signaling ability (9), and anchoring the tails by themselves in the cell membrane leads to a constitutive glucose signal (11).

Our current view of this signaling pathway is that the binding of glucose to the glucose sensors induces a conformational change in them that triggers the degradation of Mth1 and Std1. We present evidence that suggests that this process occurs by activation of casein kinase I (Yck), a protein kinase involved in diverse cellular processes that resides in the cell membrane. Our results suggest a model in which glucose-activated Yck catalyzes phosphorylation of Mth1 and Std1 bound to the tails of the glucose sensors, triggering their recognition by the SCF^{Grr1} ubiquitin-protein ligase, which marks them for degradation by the proteasome and leads to inactivation of the Rgt1 repressor and derepression of *HXT* gene expression.

Materials and Methods

Yeast Strains and Growth. *S. cerevisiae* cells were cultivated as described (12). Yeast strains used are YM4128 (*MAT α his3 leu2 lys2 trp1 tyr1 ura3*), YM6107 (*rgt2 Δ snf3 Δ*) (9), YM6212 (*MAT α his3 leu2 met15 ura3 mth1 Δ ::KanMX2 std1 Δ ::KanMX2*), LRB341 (*YCK1 YCK2*), LRB264 (*yck1 Δ*), LRB343 (*yck2 Δ*), LRB362 (*yck1 Δ yck2^{ts}*) (13), L40 (14), and A2085 (15).

Plasmids. DNA fragments used in plasmid constructions were amplified by PCR with the Expand High Fidelity PCR System (Roche Molecular Biochemicals) and cloned by gap repair in yeast (16). Plasmids containing the *HXT1* promoter fused to *lacZ* are pBM2636 and pBM3212 (17). All constructs in pBM2974 (9) were expressed from the *ADHI* promoter (*P_{ADHI}*). Plasmid constructions are as follows: pBM4523, *URA3* in pBM2636 (18) was replaced by *KanMX* from pUG6 (19); pBM4524, GFP from pQBI63 (Wako Pure Chemical, Osaka) and the C-terminal tail of Rgt2 (amino acids 546–end) were fused and cloned into pBM2974; pBM4525, the lipidation signal from Ras2 (GSGGC-CIIS) was attached to Rgt2 just before its stop codon in pBM4524; pBM4526, Val-404 of Rgt2 in pBM3272 (9) was changed to Ile; pBM4527, hemagglutinin (HA) epitope (YPY-DVPDYASL) was attached to the C terminus of Hxt1 in pBM3362 (9); pBM4528, the HA epitope was attached to the C terminus of Rgt2 in pBM3333 (9); pBM4529, the transmembrane domain of Hxt1 (amino acids 1–546) and the Rgt2 tail

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Abbreviation: HA, hemagglutinin.

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(amino acids 579–end) were fused and cloned into pBM2974; pBM4530, Phe-371 of Hxt1 on pBM4529 was changed to Ile; pBM4531, Rgt2 (amino acids 1–578) and Hxt1 (amino acids 547–end) were fused and cloned into pBM2974; pBM4532, Val-404 of Rgt2 on pBM4531 was changed to Ile; pBM4533 and pBM4534, *P_{ADH1}* of pBM4530 or pBM4531 was replaced by the *RGT2* promoter (from –795 nt to –1 nt before ATG), then the *P_{RGT2}*-Hxt1-Rgt2tail or *P_{RGT2}*-Rgt2Δtail fragment was cloned into pRS316 (20); pBM4535, *YCK1* (from ATG to the stop codon) was cloned into pBM2974; pBM4536, 7× His Protein A from pYM10 (21) was fused at the C terminus of Yck1 just before the palmitoylation signal (Cys–Cys) on pBM4535; pBM4537, Lys-98 of Yck1 on pBM4536 was changed to Arg; pBM4538, Yck1 (amino acids 1–536) and Rgt2tail (amino acids 584 to the stop codon) were fused and cloned into pBM2974; pBM4539, Asp-211 of Yck1 on pBM4538 was changed to Ala; pBM4540, *STD1* (–830 nt from ATG to +156 nt after the stop codon) was cloned into pRS313 (20); pBM4541, Ser-129, –132, –135, –136, –137, –140, –141, –144, and –147 of Std1 on pBM4540 were changed to Ala; pBM4542, *MTH1* (–850 nt from ATG to +130 nt after the stop codon) was cloned into pRS313; pBM4561, Ser-118, –121, –125, –126, –129, –130, –133, and –136 of Mth1 on pBM4542 were changed into Thr; pBM4544 and pBM4545, 9xmyc epitope (EQKLISEED) from pYM6 (21) was attached to the C terminus of Std1 on pBM4540 or pBM4541; pBM4560 and pBM4562, 9xmyc was attached to the C terminus of Mth1 on pBM4542 or pBM4561; pBM4546 and pBM4547, *RGT2* or *HXT1* (ATG through the stop codons) was cloned into p415-Cub-PLV (*P_{CYCI}*-Cub-PLV-*T_{CYCI}*, *cen-ARS*, *LEU2*) (gift from I. Stagljar), so that Cub-PLV was fused to the C terminus of Rgt2; pBM4548, Rgt2 “signaling box” (amino acids 665–696) of pBM4546 was replaced by HA epitope; pBM4549, pBM4563, pBM4550, pBM4551, pBM4552, or pBM4553, *MTH1*, *MTH1^{85T}*, *STD1*, *STD1^{95A}*, *YCK1*, or *yck1^{K98R}* (from ATG to the stop codon) was cloned into pNubG-HA-X (*P_{CYCI}*-NubG-HA-*T_{CYCI}*, *cen-ARS*, *TRP1*) (gift from I. Stagljar) so that NubG was fused to the N-terminal of each protein; pBM4554 (pGEX-*STD1*), pBM4555 (pGEX-*STD1^{95A}*), or pBM4556 (pGEX-*MTH1*), *STD1*, *STD1^{95A}*, or *MTH1* (from ATG to the stop codon) was cloned into pGEX-5X-2 (Amersham Pharmacia).

β-Galactosidase (β-Gal) Assays. Yeast cells harboring the indicated plasmids were cultivated overnight in minimal medium containing 2% galactose. The cells were transferred to the same fresh medium containing 2% galactose or 4% glucose. After 5 h of cultivation, β-gal activity of the cell extract was measured with the Yeast β-Galactosidase Assay Kit (Pierce) according to the supplier’s protocol.

Detection of Mth1 and Std1. Yeast cells harboring plasmids were cultivated overnight in rich medium containing 2% galactose. The cells were transferred to the same fresh medium containing 2% galactose or 4% glucose. At the indicated time, cell extracts were prepared as described (22). Western blot analysis was performed with mouse anti-myc antibody 9E10 (Berkeley Antibody, Richmond, CA) and horseradish peroxidase-conjugated rabbit anti-mouse antibodies (Santa Cruz Biotechnology) and visualized with the Supersignal West Pico chemiluminescent substrate (Pierce).

Split-Ubiquitin Method. L40 cells harboring Cub-PLV fusion plasmid and NubG plasmid were cultivated overnight in minimal medium containing 2% galactose or 4% glucose, and β-gal activity of the cells was determined.

Protein Kinase Assays. Each GST-fusion protein expressed in *Escherichia coli* was used still attached to beads and affinity-purified with glutathione-Sepharose 4B (Amersham Pharmacia)

Table 1. *HXT1* expression in strains expressing chimeric sensors

Chimera construct	Mean β-gal activity, U ± SD	
	2% galactose	4% glucose
Low-level expression (single copy, <i>RGT2</i> promoter)		
1 No chimera (pRS316)	64 ± 7	162 ± 2
2 Rgt2 (pBM3272)	70 ± 10	6,830 ± 46
3 Hxt1-tail (pBM4533)	102 ± 22	159 ± 2
4 Rgt2 ^{V404I} (pBM4526)	69 ± 10	92 ± 9
5 Rgt2Δtail (pBM4534)	63 ± 0	137 ± 8
High-level expression (multicopy, <i>ADH1</i> promoter)		
6 No chimera (pRS426)	63 ± 3	180 ± 33
7 Hxt1 (pBM4527)	73 ± 8	226 ± 76
8 Hxt1-tail (pBM4529)	897 ± 181	2,530 ± 558
9 Hxt1 ^{F371I} tail (pBM4530)	428 ± 13	4,150 ± 166
10 Rgt2 (pBM4528)	4,320 ± 15	6,950 ± 687
11 Rgt2Δtail (pBM4531)	238 ± 13	4,220 ± 161
12 Rgt2 ^{V404I} Δtail (pBM4532)	187 ± 49	401 ± 92
13 GFP-tail (pBM4524)	62 ± 1	127 ± 4
14 GFP-tail-CC (pBM4525)	428 ± 74	2,984 ± 356
15 Yck1-tail (pBM4538)	422 ± 50	1,712 ± 34
16 Yck1 ^{D211A} tail (pBM4539)	80 ± 5	123 ± 23
17 Yck1cyt (pBM4536)	25 ± 2	93 ± 7

HXT1-lacZ is expressed from pBM4523 in YM6107 (*Δrgt2 Δsnf3*).

as described in the supplier’s protocol. We purified 7× His-tagged *Yck1* and *Yck1^{K98R}* from yeast A2085 cells harboring pBM4536 or pBM4537 cultivated in yeast extract/peptone/dextrose after the cells were broken with glass beads in buffer A [50 mM Tris-Cl, pH 6.8/100 mM NaCl/0.5% Triton X-100/1 mM DTT/1 mM PMSF/Complete protease inhibitor mixture (Roche Molecular Biochemicals)]. Cell debris was removed by centrifugation, and 7× His-tagged proteins were purified with Ni-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) and then eluted with buffer A containing 250 mM imidazole.

Soluble and membrane protein fractions were prepared from cells cultivated in yeast extract/peptone/dextrose and broken by glass beads in buffer A without Triton X-100. Cell debris was removed by brief centrifugation, and then the cell extract was centrifuged at 10,000 × *g* for 20 min. The supernatant was taken for the soluble protein fraction; the membrane pellet was resuspended in buffer A. Equal amounts of protein were used in the protein kinase assays.

Protein kinase assays were started by mixing 20 μl of purified GST-fusion protein attached to beads, 0.5 μCi of [γ -³²P]ATP (1 Ci = 37 GBq), 100 μM ATP, 10 mM MgCl₂, and protein kinase sample in 50 μl of buffer A. After 30 min incubation at 28°C, beads were washed with buffer A containing 0.5 M NaCl and then washed with buffer A alone. Proteins bound to the beads (GST-fusion proteins) were eluted by boiling and separated by SDS/PAGE. After Coomassie brilliant blue staining, phosphorylated proteins were visualized with a PhosphorImager (Molecular Dynamics).

Results

The Rgt2 Transmembrane Domain Generates the Glucose Signal. Previous reports suggested that the C-terminal tails of the glucose sensors are necessary and sufficient for glucose signaling (9, 11). Indeed, when *RGT2* is expressed at its normal low level, its tail is required for signaling (Table 1, line 5), and Hxt1 carrying the Rgt2 tail causes constitutive *HXT1* expression if it is expressed at high levels (Table 1, line 8) but not if it is expressed at normal levels (Table 1, line 3). We were thus surprised to discover that Rgt2 without its tail can be a functional glucose sensor if it is overexpressed (Table 1, line 11). This seems to be authentic glucose signaling because Hxt1 does not have this

ability (Table 1, line 7), and this signaling is abolished by a *rgt2* mutation (V404I; Table 1, line 12) that affects an amino acid critical for signaling (Table 1, line 4). [We believe this residue is required for glucose binding, because the orthologous residue of Hxt1 (F371) is necessary for its ability to transport glucose (data not shown)]. These results lead us to conclude that the sensor tails are not required for its ability to signal. We imagine that the role of the sensor tails is to recruit Mth1 and Std1 (7, 8) to the vicinity of a protein that modulates their activity and that is activated by the transmembrane domain of the glucose-bound sensors. We suggest that enough Mth1 and Std1 happen to reside near the (overexpressed) tailless sensor for them to be inhibited by the unknown protein that is coupled to the sensors.

We suspected the protein coupled to the glucose sensors is attached to the membrane, because anchoring the tail of the Rgt2 glucose sensor to the membrane, either by giving it a farnesylation signal (Table 1, line 14) or by attaching it to Hxt1 (Table 1, line 8), generates a constitutive glucose signal [but only if it is overexpressed (Table 1, line 3); the higher levels of *HXT1* expression caused by glucose in cells with the Hxt1 carrying the Rgt2 tail or the membrane-tethered Rgt2 tail is probably due to activation by glucose of the *ADH1* promoter (23) that drives their expression]. That this signal is constitutive is supported by the fact that the F371I mutation in *HXT1* that likely impairs its ability to bind glucose does not reduce signaling (Table 1, line 9). We suggest that high levels of the glucose sensor tail attached to the membrane brings enough Mth1 and Std1 near to the unknown protein for it to trigger the event that inhibits their function. These results suggested to us that the transmembrane domain of the glucose sensors activate a membrane protein that initiates inhibition of Mth1 and Std1.

Casein Kinase I Is Required for Glucose Signaling by Rgt2. Glucose stimulates mth1 degradation, a reaction that requires the SCF^{Grr1} ubiquitin protein ligase (3). Because recruitment of substrates by SCF^{Grr1} (e.g., Cln2) requires them to be phosphorylated (24), we further surmised that the unknown protein is a protein kinase that catalyzes phosphorylation of Mth1 and Std1.

Accordingly, we screened for protein kinases that induce *HXT1* expression when overexpressed by using an array of nearly all protein kinase-encoding genes fused to the *GAL1* promoter (25). Because *HXT1* is normally not expressed in cells growing on galactose, each protein kinase could be expressed at high levels on media containing galactose and tested for its ability to induce expression of a *HXT1-lacZ* reporter gene. Thirty strains, each overexpressing a different protein kinase, exhibit detectable *HXT1* expression when growing on galactose (Fig. 1A). The 13 protein kinases that induce the strongest *HXT1* expression are the best candidates for the component of the signal transduction pathway we are seeking, but none of the 12 nonessential protein kinases in this group are required for *HXT1* expression (data not shown), suggesting that the protein kinase we are looking for is either essential or redundant.

This combination of characteristics left casein kinase I (Yck1) (Fig. 1A, lane 6, row E), which is essential for cell viability and is encoded by two genes (*YCK1* and *YCK2*) (26), as the only candidate. Casein kinase I is an attractive candidate, because it is palmitoylated and therefore associates with the cell membrane (27) and because it is also required for the expression of a gene in *Kluyveromyces lactis* encoding a low-affinity glucose transporter (28). We found that *HXT1* expression in a *yck1Δyck2^{ts}* strain is not inducible by glucose, even in cells grown at a temperature semipermissive for *yck2^{ts}* (Fig. 1B). In addition, glucose-triggered degradation of Mth1 and Std1 was not observed in the *yck1Δyck2^{ts}* strain (Fig. 1C). Thus, membrane-bound casein kinase I seems to be a component of the glucose induction pathway upstream of Mth1 and Std1.

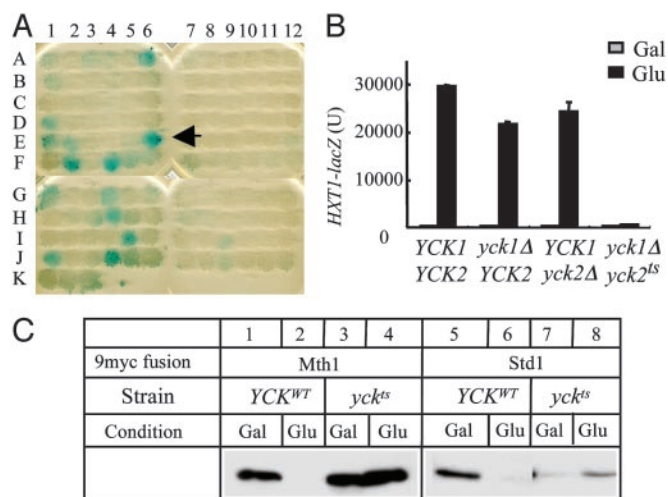


Fig. 1. Casein kinase I (Yck) is involved in the glucose induction pathway. (A) Screening protein kinases for a role in the glucose induction pathway. After being mated with YM4218 harboring *pHXT1-lacZ* (pBM4523), 119 yeast strains expressing GST-protein kinases from the *GAL1* promoter on plasmids (24) were spotted on an X-Gal indicator plate (12) containing 265 mg/liter geneticin (GIBCO) and 2% galactose. The arrow indicates the strain overexpressing *YCK1*. (B) *HXT1-lacZ* expression (from pBM3212) in LB341 (*YCK1YCK2*), LB264 (*yck1ΔYCK2*), LB343 (*YCK1yck2Δ*), or LB362 (*yck1Δyck2^{ts}*) cultivated at 30°C was determined as described in *Materials and Methods*. (C) 9myc-tagged Mth1 or Std1 expressed from their own promoters on a single-copy plasmid (pBM4560 and pBM4544, respectively) in LB341 (*YCK^{WT}*) or LB362 (*yck^{ts}*) cultivated in 2% galactose (Gal) or 4% glucose (Glu) for 30 min at 30°C were detected by Western blotting.

Casein Kinase I Interacts with Rgt2. If Yck is activated by the glucose sensors, it should bind to them. To test for interaction between Rgt2 and Yck1, we used the split-ubiquitin system (14) because it is compatible with membrane proteins. Rgt2 appears to interact with catalytically inactive Yck1 [because of a mutation (K98>R) in the ATP-binding site of Yck1] but not with active Yck1 or Hxt1 (Table 2). Thus, Yck1 appears to interact with Rgt2, and the catalytic activity of Yck1 inhibits this interaction.

A Yck1-Rgt2tail Chimera Can Mimic the Glucose Signal. Tethering the (overexpressed) C-terminal tails of the glucose sensors by themselves to the cell membrane causes constitutive expression of *HXT* genes (ref. 11 and Table 1, line 14). We speculated that they bring Mth1 and Std1 near Yck that happens to be in their vicinity in the plasma membrane. Overexpressing the Rgt2 tail in the cytoplasm is unable to induce *HXT1* expression (Table 1, line 13), probably because Yck is not in the cytoplasm. Likewise, overexpression of Yck1 in the cytoplasm (achieved by deleting the two Cys residues at its C terminus that are required for its palmitoylation) does not lead to induction of *HXT1* expression (Table 1, line 17), probably because it has no means of recruiting Mth1 and Std1. However, attaching the Rgt2 tail to cytoplasmic Yck1 causes constitutive *HXT1* expression (Table 1, line 15), presumably because it is able to recruit its substrates Mth1 and Std1. This *HXT1* expression depends on the catalytic activity of Yck1 (Table 1, line 16).

Potential Casein Kinase I Phosphorylation Sites in Mth1 and Std1 Are Essential for Their Degradation and Derepression of HXT1. If Yck1 is indeed involved in the glucose signaling mechanism, its likely substrates are Mth1 and Std1. Phosphorylation of these proteins could target them to SCF^{Grr1} for their ubiquitination and subsequent degradation, as is the case for other targets of SCF^{Grr1} (24). To search for potential casein kinase I phosphorylation sites in Mth1 and Std1, we aligned them with their

Table 2. Split-ubiquitin system interaction between RGT2 and YCK1

NubG plasmid	Mean β -gal activity, U \pm SD (fold activation over vector alone)			
	Rgt2-Cub-PLV (pBM4546)		Hxt1-Cub-PLV (pBM4547)	
	2% galactose	4% glucose	2% galactose	4% glucose
pNubG-HA-X (vector)	709 \pm 37	31 \pm 1	274 \pm 10	29 \pm 2
NubG-Yck1 (pBM4552)	807 \pm 27 (1.1 X)	31 \pm 5 (1.0 X)	144 \pm 23 (0.5 X)	26 \pm 1 (0.9 X)
NubG-Yck1 ^{K98R} (pBM4553)	3,435 \pm 227 (4.8 X)	88 \pm 14 (2.8 X)	227 \pm 20 (0.8 X)	31 \pm 5 (1.1 X)

Because the expression of Cub-PLV is higher in cells grown on galactose than in those grown on glucose, we calculated the degree of interaction as the difference in expression of strain between strains carrying Cub-PLV fusions and the empty vector (pNubG-HA-X).

orthologues from other *Saccharomyces* yeast species (29) (Fig. 2A). There is a conserved cluster of serine residues in the middle of Mth1 and Std1 (under the solid line in Fig. 2A) that matches the consensus target sequence for casein kinase I (SXXS, bold letters in Fig. 2A) (30). We changed these serines in the conserved cluster and measured the ability of the altered proteins to support repression of *HXT1* expression. Nine serines in Std1 were converted to alanines (Std1^{9SA}), and eight serines in Mth1 were converted to threonines (Mth1^{8ST}) [because their conversion to alanine destroyed function of Mth1 (data not shown)]. Native Mth1 and Std1 mediate 7- to 10-fold repression of *HXT1* expression in the presence of galactose; addition of glucose to cells relieves this repression (Fig. 2B). Mth1^{8ST} and Std1^{9SA} missing their potential casein kinase I phosphorylation sites are functional, given that they promote normal repression of *HXT1* in the presence of galactose, but this repression is not relieved by glucose (Fig. 2B). Furthermore, glucose does not induce degradation of Mth1^{8ST} and Std1^{9SA} (Fig. 2C, lanes 5, 6,

11, and 12) as it does wild-type Mth1 and Std1 (Fig. 2C, lanes 2, 3, 8, and 9). These results are consistent with the idea that glucose stimulates casein kinase I to catalyze phosphorylation of Mth1 and Std1, which leads to their degradation.

The defect in Mth1^{8ST} and Std1^{9SA} regulation is not caused by their inability to bind to the Rgt2 tail, because they interact normally with Rgt2, as detected by the split-ubiquitin method (Table 3). This interaction depends on the Rgt2 signaling box. The degree of this interaction is reduced in the presence of glucose, probably because of glucose-induced degradation of Mth1 and Std1. Indeed, Mth1^{8ST} and Std1^{9SA} exhibit increased interaction with Rgt2 in glucose-grown cells, probably because they are not degraded.

Casein Kinase I Phosphorylates Mth1 and Std1 *in Vitro*. To test whether casein kinase I can phosphorylate Mth1 and Std1, we used Mth1 and Std1 (purified from *E. coli* as GST-fusion proteins) as substrates in a protein phosphorylation assay of Yck1 (fused to 7 \times His-Protein A and affinity-purified from yeast cells). Yck1 catalyzed phosphorylation of Std1 (Fig. 3A, lane 3) and Mth1 (Fig. 3B, lane 3). Inactivation of Yck1 catalytic activity (Yck1-K98R) or removal of its phosphorylation sites from Std1 (Std1^{9SA}) prevented phosphorylation of Std1 (Fig. 3A, lane 4, and Fig. 3B, lane 2), suggesting that the cluster of serines is the major site of phosphorylation of Std1 by Yck1. Changing the equivalent serines of Mth1 (8S>T) did not significantly reduce its phosphorylation *in vitro* catalyzed by Yck1 (data not shown), probably because Mth1 has many more potential Yck1 phosphorylation sites than does Std1. However, the additional serines in Mth1 must not be *in vivo* substrates of Yck1, because the eight serines altered in Mth1^{8ST} are clearly necessary for glucose inhibition of Mth1 function (Fig. 2B and C, compare Mth1 to Mth1^{8ST}). Because inactivation of Yck1-K98R did not reduce Mth1 phosphorylation (perhaps another kinase(s) copurified with Yck1 or contaminated the preparation; data not shown), we tested the Mth1 phosphorylation activity of crude cell lysate fractions. We found that the membrane fraction gave maximal Mth1 phosphorylation activity (Fig. 3C, lanes 3 and 4) and that this activity is substantially reduced in the extract of a *yck1^{ts}* strain (Fig. 3C, lane 4). These results suggest that Mth1 and Std1 are substrates of Yck1.

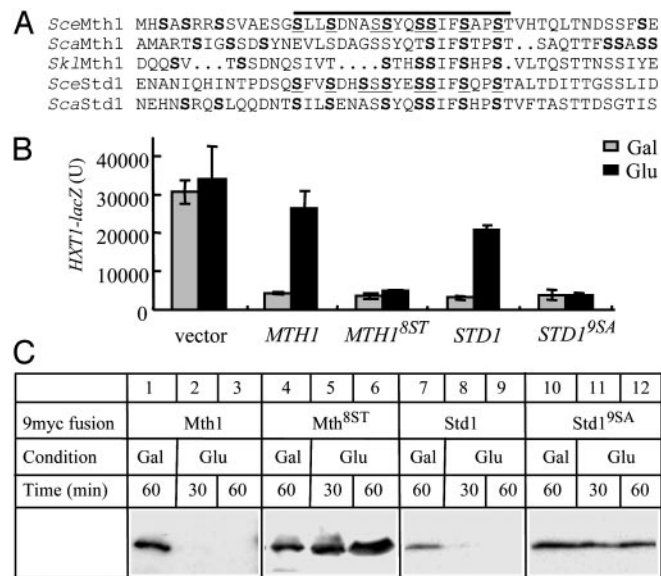


Fig. 2. (A) CLUSTALW alignment of Mth1 and Std1 orthologues from *S. cerevisiae* (*Sce*), *Schwanniomyces castellii* (*Sca*), and *Saccharomyces kluyveri* (*Sk*) (*S. cerevisiae* Mth1 amino acids 104–150 and its orthologous regions are shown) (29). The underlined regions contain consensus Yck phosphorylation sites that are conserved in both Mth1 and Std1 (solid line). (B) *HXT1-lacZ* expression (from pBM3212) in YM6212 (*mth1Δstd1Δ*) with vector (pRS313), *MTH1* (expressed from pBM4542), *MTH1*^{8ST} (pBM4561), *STD1* (pBM4540), or *STD1*^{9SA} (pBM4541) were assayed. Nearly identical results were obtained with the myc-tagged versions of these proteins used for the experiments in C and in Fig. 1C. (C) Mth1 (expressed from pBM4560), Mth1^{8ST} (pBM4562), Std1 (pBM4544), or Std1^{9SA} (pBM4545) in YM6292 cultivated in galactose or glucose for the indicated times were detected by Western blotting.

Discussion

Yck1 As a Component of the Glucose Signaling Pathway. We have described several experimental results that suggest that the Rgt2 glucose sensor (and presumably also the Snf3 glucose sensor) signals glucose availability through casein kinase I (Yck1 and Yck2). First, overexpression of Yck1 generates a constitutive glucose signal that induces *HXT1* expression (Fig. 1A). Second, *YCK1* (or its orthologue *YCK2*) is required for glucose induction of *HXT* expression (Fig. 1B) and the glucose-triggered degradation of Mth1 and Std1 (Fig. 1C). Third, Yck1 interacts with Rgt2 *in vivo* (Table 2). Fourth, a Yck1-Rgt2tail chimera ex-

Table 3. Split-ubiquitin system interaction between RGT2 and MTH1 or STD1

NubG plasmid	Mean β -gal activity, U \pm SD (fold activation over vector alone)			
	Rgt2-Cub-PLV (pBM4546)		Rgt2 Δ box-Cub-PLV (pBM4548)	
	2% galactose	4% glucose	2% galactose	4% glucose
pNubG-HA-X (vector)	663 \pm 76	140 \pm 16	509 \pm 134	217 \pm 7
NubG-Mth1 (pBM4549)	17,300 \pm 568 (26 X)	291 \pm 11 (2.1 X)	402 \pm 30 (0.8 X)	217 \pm 45 (1.0 X)
NubG-Mth1 ^{85T} (pBM4563)	14,700 \pm 452 (22 X)	1,818 \pm 35 (13 X)	536 \pm 28 (1.0 X)	200 \pm 15 (0.9 X)
NubG-Std1 (pBM4550)	2,750 \pm 299 (4.1 X)	202 \pm 4 (1.4 X)	330 \pm 58 (0.7 X)	183 \pm 5 (0.8 X)
NubG-Std1 ^{95A} (pBM4551)	8,650 \pm 2030 (13 X)	883 \pm 196 (6.3 X)	343 \pm 6 (0.7 X)	185 \pm 43 (0.9 X)

pressed in the cytoplasm mimics the glucose signal (Table 1, line 15). Fifth, serine residues in Mth1 and Std1 that match the consensus sequence for phosphorylation by casein kinase I (SXXS) are required for normal regulation of *HXT1* expression by glucose (Fig. 2B) and for degradation of Mth1 and Std1 (Fig. 2C). Finally, Yck1 phosphorylates Mth1 and Std1 *in vitro* (Fig. 3). These results, together with the knowledge that Mth1 and Std1 bind to the C-terminal cytoplasmic tails of the glucose sensors (7, 8), lead us to propose that glucose binding to Rgt2 (and Snf3) activates Yck1 (and Yck2), which then catalyzes phosphorylation of Mth1 and Std1 bound to the tails of the glucose sensors (Fig. 4). Because glucose-induced degradation of Mth1 and Std1 requires the SCF^{Grr1} ubiquitin-protein ligase (ref. 3 and our unpublished observation), their phosphorylation would allow them to be recognized by SCF^{Grr1} for ubiquitination, which targets them for degradation. Indeed, all known proteins that are targets of F-box proteins, like Grr1, must be phosphorylated to interact with this ubiquitin-protein ligase (31).

One well known function of casein kinase I is that it regulates the endocytosis of membrane proteins (13, 32). Although we cannot rule out the possibility that glucose-induced endocytosis of the glucose sensors stimulated by Yck triggers the degradation of Mth1 and Std1, we do not favor this possibility, because internalization of Rgt2 (visualized by using an Rgt2-GFP chimeric protein) is not stimulated by glucose (data not shown), and

glucose signaling is normal in mutants that are defective in endocytosis, such as *end3* or *end4* (our unpublished results).

Signal Generation Mechanism of the Glucose Sensors. The fact that the transmembrane domain of Rgt2 is sufficient for glucose signaling (provided it is expressed at a high level; Table 1, line 11) suggests that the C-terminal tail is not necessary for, but rather enhances, signaling. We believe it does this by bringing Mth1 and Std1 close to Yck. In this view, high levels of the tailless sensor can generate a glucose signal by activating Yck1, which we imagine phosphorylates any Mth1 and Std1 that happens to be in the vicinity. The cytoplasmic Yck1-Rgt2tail chimera mimics a constitutive signal (provided it is expressed at a high level), probably because the tail domain attracts Mth1 and Std1 to the vicinity of Yck1.

Binding of glucose to the glucose sensors could stimulate Yck1 to phosphorylate Mth1 and Std1 simply by stimulating recruitment of Yck1, thereby bringing it into the vicinity of its substrates that are bound to the sensor tail. Alternatively, the glucose-bound sensors could play an active role in stimulating the catalytic activity of Yck1. We currently favor the latter possibility, because glucose is not required for interaction of Yck1 with Rgt2 (Table 2). It is also possible that recruitment of Mth1 and Std1 to the tails of the glucose sensors is stimulated by glucose. We do not favor this idea, because glucose generates a signal through a tailless glucose sensor (Table 1, line 11), suggesting that some event other than recruitment of Mth1 and Std1 is activated by glucose.

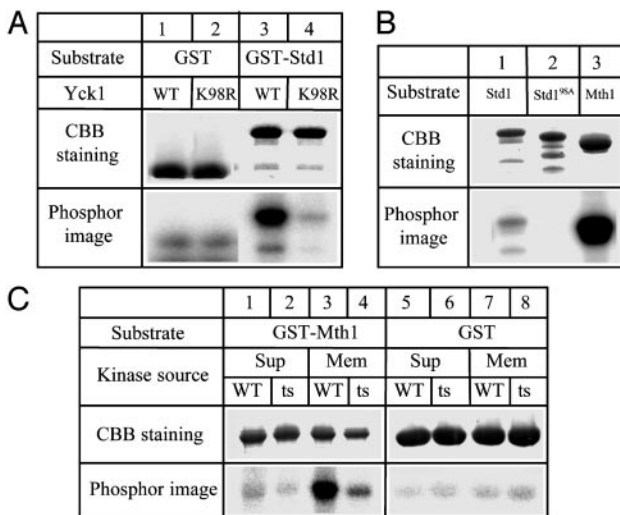


Fig. 3. (A and B) Protein kinase activity of affinity-purified Yck1-7 \times His-ProTA (Yck1-WT) or Yck1^{K98R}-7 \times His-ProTA (Yck1-K98R) toward GST or GST-Std1 was measured. Approximately equal amounts of Yck1 and Yck1-K98R were used in these experiments (determined by an immunoblot with anti-protein A as the probe). (C) The Mth1 phosphorylating activity of crude cell membrane fractions was measured. Soluble protein fraction (Sup) and crude membrane protein fraction (Mem) were prepared from LB341 (WT) and LB362 (ts) as described in *Materials and Methods*. CBB, Coomassie brilliant blue.

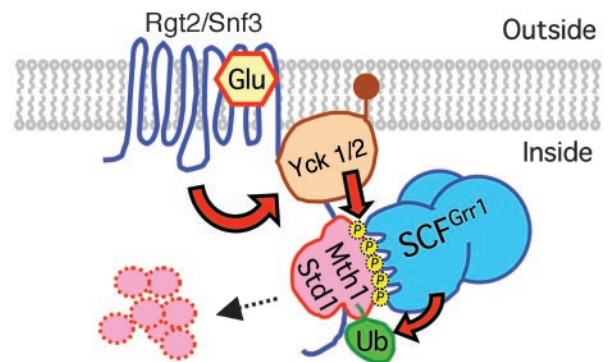


Fig. 4. Current model for transduction of the glucose signal from Snf3 and Rgt2 to Mth1 and Std1. Binding of glucose (Glu) to the transmembrane domain of the glucose sensors (Rgt2/Snf3) is postulated to change their conformation and activate membrane-bound casein kinase I (Yck1/Yck2). Activated casein kinase I catalyzes phosphorylation of Mth1 and Std1, which are bound to the C-terminal tails of the glucose sensors. Phosphorylated Mth1 and Std1 can then be recognized by SCF^{Grr1}, which catalyzes ligation of ubiquitin (Ub) to Mth1/Std1, thereby targeting them to the proteasome. Not shown in this figure is how degradation of Mth1/Std1 leads to the phosphorylation of Rgt1, which prevents it from binding to DNA, leading to derepression of *HXT* gene expression (2, 3).

Std1 seems to be less sensitive to degradation than Mth1. Mth1 cannot be detected 30 min after addition of glucose to cells (Fig. 2C, lane 2), but Std1 protein is still observed after 1 h when the blot is overexposed to film (data not shown). Indeed, Mth1, but not Std1, degradation was observed in a recent study (3). We believe this difference is due to the different regulation of *MTH1* and *STD1* expression. *STD1* expression is induced by glucose [via the Rgt2/Snf3-Rgt1 pathway (33)], which would be expected to counteract Std1 degradation. However, glucose increases *STD1* expression at the same time that it induces its degradation. *MTH1* expression, on the other hand, is repressed by glucose [via the Snf1-Mig1 pathway, (33)], which would be expected to reinforce Mth1 degradation, but glucose reduces *MTH1* expression at the same time that it stimulates its degradation. The relative resistance of Std1 to degradation may account for the different roles in regulation of *HXT* expression that are apparent for Mth1 and Std1 (8).

Do Other Organisms Have Glucose Sensors Similar to Rgt2 and Snf3?

We previously thought that the glucose sensors are yeast-specific, because no glucose transporter-like protein with a long C-terminal tail is apparent in the genome sequences of other organisms. However, our finding that the glucose sensor tail is not required for its function raises the possibility that similar glucose sensors exist in other organisms. Proteins similar to the yeast glucose sensors, but without the long C-terminal tails, have been identified in some fungal species [e.g., *Neurospora crassa* Rco3 (34), *Uromyces fabae* Hxt1 (35), and *Amanita muscaria* MstI (GenBank accession no. CAB06078)]. The mammalian GLUT2 glucose transporter seems to be involved in transducing

a glucose signal that affects gene expression, a function in which its internal cytoplasmic loop has been implicated (36, 37). Also, the mammalian GLUT1 glucose transporter was reported to activate ERK protein kinase by means of its short C-terminal cytoplasmic domain (38).

Casein Kinase I Couples Extracellular Signals to Degradation of Transcriptional Regulators. Casein kinase I is in a family of highly conserved, ubiquitously expressed protein kinases found in all eukaryotic organisms. It has recently become apparent that it regulates diverse processes, including Wnt signaling, circadian rhythms, nuclear import of proteins, and Alzheimer's disease progression (reviewed in ref. 39). Our results add nutrient sensing to the list of processes in which casein kinase I is involved. It seems that casein kinase I regulates Wnt signaling like it regulates glucose signaling in yeast: by triggering the degradation of a protein that regulates transcription (β -catenin) in response to a signal generated by a transmembrane receptor (*frizzled*) via an E3 ubiquitin ligase that includes an F-box protein (SCF ^{β} -TRCP) (40). We expect that casein kinase I will turn up in other signal transduction pathways.

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