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Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*

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How eukaryotic cells sense availability of glucose, their preferred carbon and energy source, is an important, unsolved problem. Bakers' yeast (*Saccharomyces cerevisiae*) uses two glucose transporter homologs, Snf3 and Rgt2, as glucose sensors that generate a signal for induction of expression of genes encoding hexose transporters (*HXT* genes). We present evidence that these proteins generate an intracellular glucose signal without transporting glucose. The Snf3 and Rgt2 glucose sensors contain unusually long C-terminal tails that are predicted to be in the cytoplasm. These tails appear to be the signaling domains of Snf3 and Rgt2 because they are necessary for glucose signaling by Snf3 and Rgt2, and transplantation of the C-terminal tail of Snf3 onto the Hxt1 and Hxt2 glucose transporters converts them into glucose sensors that can generate a signal for glucose-induced *HXT* gene expression. These results support the idea that yeast senses glucose using two modified glucose transporters that serve as glucose receptors.

Keywords: glucose/glucose transporter/Rgt2/Snf3/yeast

Introduction

Glucose, the preferred carbon and energy source for most eukaryotic cells, has significant and varied effects on cell function. Consequently, maintaining glucose homeostasis is of great importance to many organisms. How cells perceive and respond to glucose is an important, unanswered question. A signal transduction pathway responsible for glucose-induced gene expression in baker's yeast (*Saccharomyces cerevisiae*) has come into focus recently. Transcription of several of the 20 genes that encode hexose transporters or highly related proteins (*HXT* genes) (Bisson *et al*., 1993; Kruckeberg, 1996; Boles and Hollenberg, 1997) is induced by glucose. Expression of *HXT1* is induced only in response to high concentrations of glucose; transcription of *HXT2* and *HXT4* is induced only by low levels of glucose (Özcan and Johnston, 1995, 1996; Schulte and Ciriacy, 1995). Glucose induction of the *HXT* genes is mediated by a repression mechanism involving the zinc-finger-containing protein Rgt1: in the absence of glucose, Rgt1 binds to the *HXT* promoters and represses their expression; glucose inactivates Rgt1 repressor function, leading to derepression of *HXT* expression (Marshall-Carlson *et al*., 1991; Erickson and Johnston, 1994; Özcan and Johnston, 1995; Özcan *et al*., 1996a). Inhibition of Rgt1 by glucose requires Grr1, which may be part of a ubiquitin-conjugating enzyme complex (Flick and Johnston, 1991; Barrall *et al*., 1994; Vallier *et al*., 1994; Li and Johnston, 1997).

The glucose signal that triggers inhibition of Rgt1 function appears to be generated by Snf3 and Rgt2 (Özcan *et al*., 1996b), which are similar to glucose transporters and are members of a large family of 20 known or predicted glucose transporters in yeast. Most of these Hxt proteins are very similar to each other, sharing between 50 and 100% amino acid sequence identity. Snf3 and Rgt2 are the most divergent family members, being only ~25% similar to their relatives (Bisson *et al*., 1993; Kruckeberg, 1996; Boles and Hollenberg, 1997). A distinguishing characteristic of Snf3 and Rgt2 is their unusually long C-terminal extensions (>200 amino acids) that are predicted to reside in the cytoplasm (Marshall-Carlson *et al*., 1990). All other known or predicted hexose transporters (from any organism) have C-terminal cytoplasmic tails of only ~50 amino acids.

Work in several laboratories suggested that Snf3 plays a regulatory rather than a metabolic role in glucose transport (Marshall-Carlson *et al*., 1990, 1991; Bisson *et al*., 1993; Ko *et al*., 1993; Özcan and Johnston, 1995; Liang and Gaber, 1996; Coons *et al*., 1995, 1997). We have presented evidence that Snf3 and Rgt2 are sensors of extracellular glucose that are involved in generation of an intracellular glucose signal that triggers the induction of *HXT* gene expression (Özcan *et al*., 1996b). The key result that led to this idea is that a dominant mutation in *RGT2* and *SNF3* (Marshall-Carlson *et al.*, 1990) causes constitutive induction of *HXT* gene expression, even in the absence of the inducer glucose (Özcan *et al*., 1996b). We believe this mutation converts the glucose sensors into their glucose-bound form. This result led us to conclude that glucose sensing and signaling is a receptor-mediated process which is independent of glucose metabolism. Here we provide further evidence that glucose signaling is not the result of glucose transport and that the C-termini of Rgt2 and Snf3 are the glucose signaling domains of these glucose sensors.

Results

Snf3 and Rgt2 have separate but overlapping functions

Previous results indicated that Snf3 and Rgt2 probably sense different levels of glucose (Özcan *et al*., 1996b); Snf3 seems to function as a sensor of low levels of glucose because it is required for low glucose-induced expression

Table I. Rgt2p and Snf3p have distinct, but overlapping functions in glucose signaling

	Genotype	Construct	Mean β-galactosidase activity (U) ± SD			
			HXT1::lacZ		*HXT2::lacZ*	
			A Gly	B 4% Glu	C Gly	D Gly + 0.1% Glu
1	WT	vector	1.1 ± 0.15	353 ± 32	14 ± 0.5	348 ± 30
2		*CEN–SNF3*	1.4 ± 0.2	364 ± 22	21 ± 4	335 ± 18
3		*CEN–RGT2*	1.2 ± 0.3	394 ± 80	19 ± 7	314 ± 40
4		*ADH–SNF3*	30 ± 7	329 ± 21	274 ± 23	327 ± 57
5		*ADH–RGT2*	37 ± 9	396 ± 74	298 ± 46	308 ± 26
6	*rgt2*Δ	vector	0.6 ± 0.14	68 ± 7	19 ± 4	397 ± 83
7		*CEN–SNF3*	0.9 ± 0.2	72 ± 8		
8		*CEN–RGT2*	1.6 ± 0.1	403 ± 69		
9	*snf3*Δ	vector	0.7 ± 0.09	227 ± 30	10 ± 1.8	24 ± 2
10		*CEN–SNF3*			21 ± 3	332 ± 77
11		*CEN–RGT2*			11 ± 1.5	27 ± 4
12	*rgt2*Δ *snf3*Δ	vector	0.42 ± 0.2	0.88 ± 0.2	6 ± 1.4	14 ± 3
13		*ADH–HXT1*	0.7 ± 0.2	0.7 ± 0.1	4 ± 0.9	12 ± 1

Abbreviations: Gly, 5% glycerol + 0.5% galactose; Gly + 0.1% glu, 5% glycerol + 0.1% glucose; 4% Glu, 4% glucose. Vector = pRS316; *CEN–SNF3* = pBM3111; *CEN–RGT2* = pBM3272; *ADH–SNF3* = pBM3135; *ADH–RGT2* = pBM3333; *ADH–HXT1* = pBM3362. The data shown in lanes 6C/D and 9A/B are from Özcan *et al*. (1996b).

of *HXT2* (Table I, compare lines 9D and 10D) but not high glucose-induced expression of *HXT1* (line 9B). Rgt2 appears to sense high concentrations of glucose because it is required for full induction of *HXT1* expression by high levels of glucose (Table I, compare lines 6B–8B) but not for induction of *HXT2* by low levels of glucose (line 6D). To support the idea that Snf3 and Rgt2 have different affinities for glucose, we tested whether the *SNF3* or the *RGT2* gene in single copy is sufficient to complement *rgt2* or *snf3* mutants, respectively. The decrease in high glucose-induced *HXT1* expression caused by *rgt2* mutations cannot be restored by *SNF3* (line 7B), nor can the defect in *HXT2* induction by low levels of glucose in a *snf3* mutant be restored by *RGT2* (line 11D). Thus, *RGT2* and *SNF3* have separate, non-redundant functions.

Induction of *HXT1* expression by high concentrations of glucose is completely abolished in the *snf3 rgt2* double mutant (Table I, line 12B). Consequently, the *snf3 rgt2* double mutant grows poorly on high concentrations of glucose (Figure 3; see below). This is in contrast to *snf3* mutants, which exhibit no reduction of high glucose-induced *HXT1* expression (line 9B), and *rgt2* mutants, which have only ~5- to 6-fold reduction in *HXT1* transcription at high concentrations of glucose (line 6B) (Özcan *et al*., 1996b). Thus, Snf3 appears to contribute to induction of *HXT1* transcription by high levels of glucose. We believe that these results reflect the different relative affinities of these sensors for glucose: Rgt2 is probably a sensor of high levels of glucose (a low-affinity receptor); Snf3 is probably a sensor of low levels of glucose (a high-affinity receptor; see Discussion).

Snf3 and Rgt2 are limiting components of the glucose signaling pathway

Previous data obtained with dominant *SNF3-1* and *RGT2-1* mutants suggested that these two proteins are the limiting components of the glucose signaling pathway (Özcan *et al*., 1996b). This is supported by the observation that overexpression of either *SNF3* or *RGT2* causes constitutive expression of *HXT1* and *HXT2* (i.e. even in the absence of glucose) (Table I, lines 4A and 4C, and lines 5A and 5C). We believe this is because the glucose receptors, like all receptors, are in equilibrium between the unliganded and ligand-bound form. Higher levels of the receptors necessarily increase the amount of receptor in the ligand-bound form, leading to constitutive signaling. Note that *HXT1* expression in the absence of glucose is only partially constitutive (lines 4A and 5A); this is because induction of *HXT1* expression at high levels of glucose requires a second, Rgt2-independent pathway (Özcan *et al*., 1996b).

Glucose transport is neither necessary nor sufficient for signaling

To test whether Snf3 and Rgt2 can transport glucose, we expressed them in a strain unable to grow on glucose because it is deleted for seven *HXT* genes (*hxt1*Δ–*hxt7*Δ, called the *hxt* null mutant) (Reifenberger *et al*., 1995, 1997) (Figure 1). Expression in this strain of any one of the seven *HXT* genes restores growth on glucose (Reifenberger *et al*., 1995). We overexpressed in this strain *SNF3*, *RGT2* and *HXT1* from the *ADH1* promoter on a multicopy plasmid and assayed growth on glucose media. Overexpression of *HXT1* fully restored growth of the *hxt* null strain. By contrast, neither *SNF3* nor *RGT2*, when overexpressed, were able to restore growth of the *hxt* null mutant (Figure 1). While we cannot be certain that increased levels of Rgt2 and Snf3 are expressed and reach the membranes in these cells, the fact that expression of *SNF3* and *RGT2* from the *ADH1* promoter leads to constitutive *HXT* gene expression (Table I, lines 4A, 4C, 5A and 5C) suggests that this is the case. Thus, even though they are similar to glucose transporters, Snf3 and Rgt2 appear unable to transport sufficient amounts of glucose to correct the growth defect of the *hxt* null mutant.

To test if *bona fide* glucose transporters can provide for glucose signaling, we expressed the *HXT1* and *HXT2* genes from the *ADH1* promoter on a multicopy plasmid in *snf3* and *rgt2* mutant cells and tested for restoration of *HXT* gene expression (Table II). Both of these plasmids express functional glucose transporters because they

enable a mutant defective in glucose transport to grow on glucose (Figure 1, and data not shown), but neither is able to restore the glucose signaling defect of *snf3* or *rgt2* mutants, indicating that the *HXT* induction defect in these mutants is not due simply to impaired glucose transport.

The C-terminal tails of Snf3 and Rgt2 are necessary for glucose signaling

An unusual feature of Snf3 and Rgt2 that distinguishes them from all other known glucose transporters is their long C-terminal tails, which are predicted to reside in the cytoplasm. The sequences of the Snf3 and Rgt2 tails are dissimilar, except for a stretch of 25 amino acids, 16 of which are identical among the repeats. Snf3 has two of these sequences; Rgt2 has only one (Figure 2). Deletion of the Rgt2 C-terminal tail (*RGT2Δ2*) abolishes its ability to sense high levels of glucose and induce *HXT1* expression (Table III, line 3B), and deletion of the Snf3 tail (*SNF3Δ2*) abolishes its ability to sense low levels of glucose and induce *HXT2* expression (line 7D). Furthermore, the dominant mutations *SNF3-1* and *RGT2-1* (Arg231 and Arg229, respectively, changed to lysine), which cause constitutive (glucose-independent) expression of the *HXT* genes (Table III, lines 10A and C, and 12A and C; see also Özcan *et al*., 1996b), do not manifest their effect when the C-terminal tails of Snf3 and Rgt2 are deleted (lines 11A and C, and 13A and C). Thus, the C-terminal tails of both Snf3 and Rgt2 are essential for glucose signaling.

The 25 amino acid repeats seem to be the functional units of the C-terminal tails of Snf3 and Rgt2 because an Rgt2 protein that retains its 25 amino acid repeat but is missing all distal sequences (*RGT2Δ1*) is still partially functional, mediating 2-fold induction of *HXT1* expression (Table III, compare lines 1B, 2B and 4B). Similarly, Snf3 retaining one of its two repeats (*SNF3Δ1*) is partially functional, providing ~3-fold induction of *HXT2* expression (compare lines 5D, 6D and 8D). However, overexpression of the Snf3 or Rgt2 tails by themselves does not restore the glucose signaling defect of *snf3* or *rgt2* mutants (Table IV, lines 3B, 4B, 7D and 8D). Thus, the C-terminal

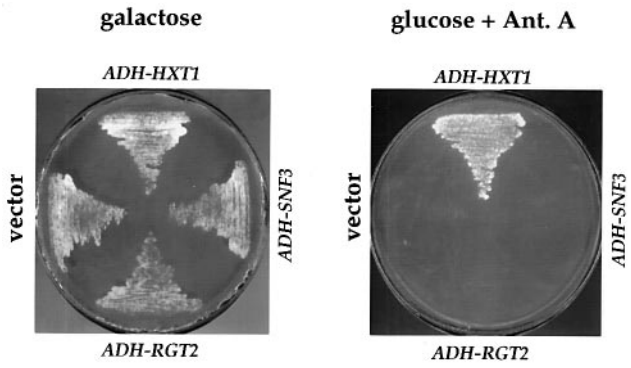
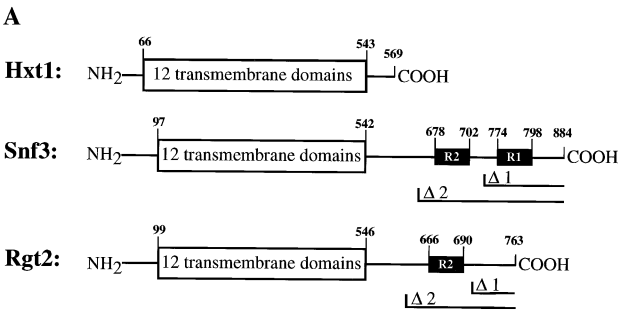


Fig. 1. Analysis of glucose transport activity of overexpressed *SNF3*, *RGT2* and *HXT1* genes in *hxt1Δ–hxt7Δ* (*hxt*) strain. The *hxt* mutant transformed with the ADH1 vector alone, *ADH1–SNF3*, *ADH1–RGT2* and *ADH1–HXT1* was scored for growth on YNB medium containing either 2% galactose or 2% glucose with antimycin A (1 μg/ml). The cells were grown first on YNB medium lacking uracil with 2% galactose as carbon source and then replica plated on YNB medium containing 2% glucose with antimycin A.



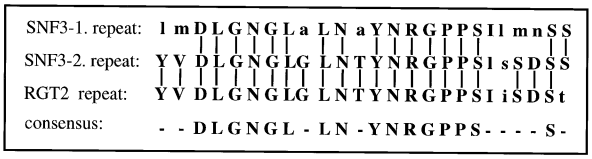


Fig. 2. (**A**) Schematic representation of the Snf3, Rgt2 and Hxt1 protein structure. The repeats of the C-terminal tails of Snf3 and Rgt2 are indicated by boxes. In addition, the C-terminal tail deletions from Table III are shown. The numbers indicate the amino acid position. (**B**) Alignment of the repeated sequences in the C-terminal tail of Snf3 and Rgt2. Snf3 has two repeats [amino acids 678–702 (2. repeat) and 774 to 798 (1. repeat)], Rgt2 has only one (666–690). The amino acids that are identical within the repeats are indicated.

Table II. The glucose signaling defect of *rgt2*Δ and *snf3*Δ mutants is not simply caused by impaired transport[a]

Genotype		Plasmid	Mean β-galactosidase activity (U) ± SD			
			HXT1::lacZ		*HXT2::lacZ*	
			A Gly	B 4% Glu	C Gly	D Gly + 0.1% Glu
1	WT	vector	1.1 ± 0.15	353 ± 32	14 ± 0.5	348 ± 30
2		*ADH–HXT1*	1.3 ± 0.2	328 ± 31	20 ± 4	340 ± 16
3		*ADH–HXT2*	0.8 ± 0.16	336 ± 51	18 ± 4	334 ± 59
4	*rgt2*Δ	vector	0.66 ± 0.14	68 ± 7		
5		*ADH–HXT1*	0.5 ± 0.1	75 ± 11		
6		*ADH–HXT2*	0.6 ± 0.12	61 ± 4		
7	*snf3*Δ	vector			10 ± 1.8	24 ± 2
8		*ADH–HXT1*			11 ± 2	29 ± 4
9		*ADH–HXT2*			9 ± 1	37 ± 6

[a]Abbreviations: see Table I; vector = pRS426; *ADH–HXT1* = pBM3362; *ADH–HXT2* = pBM3138.

Table III. The C-terminal tail of Rgt2p and Snf3p is essential for glucose signaling[a]

	Genotype	Plasmid	Mean β-galactosidase activity (U) ± SD			
			HXT1::lacZ		*HXT2::lacZ*	
			A Gly	B 4% Glu	C Gly	D Gly + 0.1% Glu
1	*rgt2*Δ	vector	0.6 ± 0.14	68 ± 7		
2		*CEN–RGT2*	1.6 ± 0.1	403 ± 69 (5.9×)		
3		*RGT2Δ2*	1.0 ± 0.3	71 ± 10 (1.0×)		
4		*RGT2Δ1*	1.2 ± 0.2	134 ± 24 (2.0×)		
5	*snf3*Δ	vector			10 ± 1.8	24 ± 2
6		*CEN–SNF3*			21 ± 3	332 ± 77 (13.8×)
7		*SNF3Δ2*			11 ± 2	21 ± 4 (0.88×)
8		*SNF3Δ1*			13 ± 2	69 ± 15 (2.9×)
9	WT	vector	1.1 ± 0.15	353 ± 32	14 ± 0.5	348 ± 30
10		*RGT2-1*	33 ± 4	384 ± 13	272 ± 36	367 ± 74
11		*RGT2-1ΔT*	1.3 ± 0.3	360 ± 56	13 ± 3	336 ± 38
12		*SNF3-1*	29 ± 5	349 ± 37	301 ± 31	327 ± 57
13		*SNF3-1ΔT*	0.9 ± 0.1	355 ± 48	16 ± 3	359 ± 32

[a]Abbreviations: see Table I; vector = pRS316; *CEN–RGT2* = pBM3272; *RGT2Δ1* = pBM3312; *RGT2Δ2* = pBM3279; *CEN–SNF3* = pBM3111; *SNF3Δ1* = pBM3319; *SNF3Δ2* = pBM3363; *RGT2-1* = pBM3270; *RGT2-1ΔT* = pBM3277; *SNF3-1* = pBM3259; *SNF3-1ΔT* = pBM3335.

Table IV. Overexpression of the C-terminal tail of Rgt2p and Snf3p is not sufficient for glucose signaling[a]

	Genotype	Plasmid	Mean β-galactosidase activity (U) ± SD			
			HXT1::lacZ		*HXT2::lacZ*	
			A Gly	B 4% Glu	C Gly	D Gly + 0.1% Glu
1	*rgt2*Δ	vector	0.6 ± 0.14	68 ± 7		
2		*CEN–RGT2*	1.6 ± 0.1	403 ± 69		
3		*RGT2-T*	0.6 ± 0.04	64 ± 5		
4		*SNF3-T*	0.5 ± 0.07	67 ± 11		
5	*snf3*Δ	vector			10 ± 1.8	24 ± 2
6		*CEN–SNF3*			21 ± 3	332 ± 77
7		*RGT2-T*			13 ± 3	28 ± 7
8		*SNF3-T*			12 ± 1	22 ± 3

[a]Abbreviations: see Table I; vector = pRS426; *CEN–RGT2* = pBM3272; *CEN–SNF3* = pBM3111; *SNF3-T* = pBM3578; *RGT2-T* = pBM3576.

tails of Rgt2 and Snf3 are necessary, but are not by themselves sufficient, for signaling.

Attachment of the Snf3 C-terminus to Hxt1 and Hxt2 converts these glucose transporters into glucose sensors

To test whether the C-terminal tail of Snf3 allows glucose signaling when attached to other glucose transporters, we attached it to the Hxt1 and Hxt2. Indeed, both *HXT1–SNF3* and *HXT2–SNF3* chimeras partially complement the signaling defect of *snf3* and *rgt2* mutants (Table V, lines 3B, 4B, 8D and 9D). Since neither wild-type *HXT1* nor *HXT2* could repair the signaling defect of *snf3* and *rgt2* mutants (even when overexpressed) (Table II), we conclude that the C-terminal tail of Snf3 confers upon these glucose transporters the ability to signal glucose availability. However, these chimeric proteins do not signal as well as Snf3 or Rgt2, indicating that other residues of Snf3 and Rgt2 (probably in the transmembrane domain) are important for optimal function of the glucose sensors.

Overexpression of the *HXT1–SNF3* chimera from the *ADH1* promoter in the *snf3* and *rgt2* mutant strain also causes a low level of constitutive *HXT* expression (i.e. in the absence of glucose) (Table V, lines 5A and 10C). This is similar to results obtained when *SNF3* and *RGT2* were overexpressed (Table I), and further supports the view that the concentration of glucose sensors is the limiting factor for signaling.

Snf3 and Rgt2 are required for glucose repression of GAL1 and SUC2 expression

The *snf3 rgt2* double mutant is severely defective in induction of *HXT* expression (like *grr1* mutants), and therefore grows poorly on glucose-containing media. Overexpression of *HXT1* (from the *ADH1* promoter on a multicopy plasmid) in the *snf3 rgt2* double mutant restores growth of this mutant on glucose (Figure 3), suggesting that its poor growth on glucose is due to a defect in glucose transport. Overexpression of *HXT1* does not, however, repair the glucose induction defect in *HXT* expression (Table I, lines 13B and D), supporting the idea that the glucose induction signal is generated independently of glucose metabolism.

Because the *snf3 rgt2* double mutant has severely reduced glucose transport, we expected it to be defective in glucose repression of *GAL1* and *SUC2* expression (like

Table V. Attachment of Snf3p C-terminus to Hxt1p and Hxt2p converts them into glucose sensors[a]

Genotype		Plasmid	Mean β-galactosidase activity (U) ± SD			
			HXT1::lacZ		*HXT2::lacZ*	
			A Gly	B 4% Glu	C Gly	D Gly + 0.1% Glu
1	*rgt2*Δ	vector	0.6 ± 0.14	68 ± 7		
2		*CEN–RGT2*	1.6 ± 0.1	403 ± 69 (5.9×)		
3		*pHXT1-HXT1/SNF3*	0.8 ± 0.06	182 ± 41 (2.7×)		
4		*pHXT2-HXT2/SNF3*	0.7 ± 0.14	153 ± 37 (2.3×)		
5		*pADH-HXT1/SNF3*	5 ± 0.8	263 ± 20 (3.9×)		
6	*snf3*Δ	vector			10 ± 1.8	24 ± 2
7		*CEN–SNF3*			21 ± 3	322 ± 77 (13×)
8		*pHXT1-HXT1/SNF3*			9.2 ± 1.2	89 ± 11 (3.7×)
9		*pHXT2-HXT2/SNF3*			11 ± 1.5	117 ± 21 (4.9×)
10		*pADH-HXT1/SNF3*			56 ± 6	94 ± 11 (3.9×)

[a]Abbreviations: see Table I; vector = pRS316; *CEN–RGT2* = pBM3272; *CEN–SNF3* = pBM3111; *pHXT1-HXT1/SNF3* = pBM3436; *pHXT2-HXT2/SNF3* = pBM3654; *pADH-HXT1/SNF3* = pBM3273.

grr1 mutants) (Flick and Johnston, 1991; Özcan *et al*., 1994). Indeed, *GAL1* and *SUC2* expression is not repressed by 4% glucose in the *snf3 rgt2* double mutant, in contrast to *snf3* or *rgt2* single mutants (Table VI). The *snf3 rgt2* double mutant displays a 3-fold decrease in *SUC2* expression at low concentrations of glucose because low levels of glucose induce *SUC2* expression, and this requires *SNF3* (Özcan *et al*., 1997).

Discussion

Snf3 and Rgt2 appear to serve as sensors of glucose that generate an intracellular glucose signal for induction of *HXT* gene expression in yeast. Even though both proteins are very similar to glucose transporters, they apparently do not transport glucose, since they do not enable a mutant deficient in glucose transport to grow on glucose (Figure 1). There are two possible explanations for this observation; Snf3 and Rgt2 could bind glucose without being able to translocate it across the plasma membrane, or they could transport glucose, but with a capacity insufficient to allow growth of the *hxt* null mutant on glucose. Since both proteins should be present in cells at relatively high levels in our experiment (they were expressed from the strong *ADH1* promoter on a multicopy plasmid, and this causes constitutive glucose signaling), we favor the idea that Snf3 and Rgt2 do not translocate glucose, but are instead glucose receptors.

Conversely, transport of glucose does not appear to be sufficient for generation of the glucose signal because overexpression of the *bona fide* glucose transporters *HXT1* or *HXT2* does not restore the glucose signaling defect of *snf3* or *rgt2* mutants. This indicates that the signaling defect of these mutants is not due simply to impaired glucose transport. The facts that Snf3 and Rgt2 do not seem to transport glucose, and that known glucose transporters are unable to signal glucose availability bolsters our confidence in the idea that Snf3 and Rgt2 are glucose receptors that generate an intracellular signal for induction of gene expression.

The *snf3 rgt2* double mutant is completely defective in glucose induction of *HXT* expression, in contrast to the single mutants (Table I). As a consequence, this double

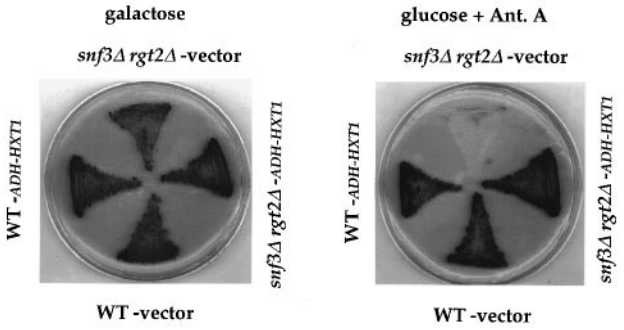


Fig. 3. The growth defect of the *snf3 rgt2* double mutant on 2% glucose with antimycin A (1 μg/ml) is complemented by overexpression of the *HXT1* gene. Cells were grown first on YNB–2% galactose plates and then transferred (replica plated) to YNB–2% glucose plates with antimycin A.

Table VI. The *snf3*Δ *rgt2*Δ double mutant is defective in glucose repression[a]

Genotype	Mean β-galactosidase activity (U) ± SD			
	GAL1::lacZ		*SUC2::lacZ*	
	Glu/gal	Gal	Glu	Gly + 0.1% Glu
WT	<0.1	128 ± 19	<0.1	75 ± 12
*snf3*Δ *rgt2*Δ	33 ± 7	137 ± 26	16 ± 3	26 ± 5
*rgt2*Δ	<0.1	112 ± 15	<0.1	59 ± 11

[a]Abbreviations: see Table I; vector = pRS316; *GAL1::lacZ* = pBM690; *SUC2::lacZ* = pBM3082.

mutant is defective in glucose transport and grows poorly on glucose-containing media. Because of its glucose transport defect, the *snf3 rgt2* mutant is also defective in glucose repression of *GAL1* and *SUC2* expression (like *grr1* mutants) (Table VI). Even though the growth defect of the *snf3 rgt2* double mutant on glucose is corrected by overexpression of *HXT1*, the defect in glucose induction of *HXT* expression is not, lending further support to the idea that the signal for glucose induction is generated independently of glucose metabolism.

Induction of *HXT1* expression by high levels of glucose is reduced ~5-fold in an *rgt2* mutant, but is unaffected by

a *snf3* mutation. Induction of *HXT2* expression by low levels of glucose is abolished in a *snf3* mutant, but unaffected by an *rgt2* mutation. This suggests that the two proteins have different affinities for glucose; Rgt2 appears to be a sensor of high levels of glucose (a low-affinity receptor) and Snf3 appears to be a sensor of low levels of glucose (a high-affinity receptor). Snf3 also contributes to high glucose induction of *HXT1* transcription because the *snf3 rgt2* double mutant has a more severe defect in high glucose-induced *HXT1* expression than the *rgt2* single mutant (Table I, line 12). A function for Snf3 at high concentrations of glucose was also suggested by the observation that it is required for repression of *HXT6* and *HXT7* expression at high levels of glucose (Liang and Gaber, 1996). This is expected behavior for a high-affinity receptor, since it should bind ligand at both low and high concentrations. *SNF3* expression is repressed by glucose (Celenza *et al*., 1988; Bisson *et al*., 1993; Özcan and Johnston, 1995), but the basal level of *SNF3* expression apparently is sufficient to provide enough Snf3 under glucose repression conditions for it to sense glucose and generate the intracellular signal.

Rgt2, presumably being a low-affinity glucose receptor, is expected to sense only high levels of glucose. Indeed, *rgt2* mutations have no effect on low glucose-induced expression of *HXT2* (Table I; also see Özcan *et al*., 1996b). Recent results of Jiang *et al*. (1997) support the idea that Rgt2 functions as a high glucose sensor; Rgt2, but not Snf3, is required for normal inactivation of the maltose permease caused by high levels of glucose. In addition, the dominant *RGT2-1* mutation causes constitutive degradation of maltose permease (even in the absence of glucose) (Jiang *et al*., 1997).

The long C-terminal extensions of Snf3 and Rgt2, which are predicted to reside in the cytoplasm, distinguish these proteins from the yeast Hxt proteins and glucose transporters of many other organisms. The sequences of these tails are similar only in a 25 amino acid repeat (Figure 2). Our results suggest that these repeats are important for Snf3 and Rgt2 signaling function. At least one of the two repeats in the Snf3 tail is required for induction of *HXT2* expression by low levels of glucose (Table III), consistent with previous observations that at least one of the two repeats in Snf3 is necessary for growth of cells on raffinose (Marshall-Carlson *et al*., 1990; Bisson *et al*., 1993). The repeated sequences in the C-terminal tails of Snf3 and Rgt2 contain putative phosphorylation sites for casein kinase II (Figure 2, Bisson *et al*., 1993), and conserved glycine residues that might constitute a nucleotide-binding domain ($DXGXGX_{15–50}GXG$; Saraste *et al*., 1990).

The C-terminal tail of Snf3 is able to generate an intracellular glucose signal when attached to either Hxt1 or Hxt2. This suggests that Snf3 and Rgt2 consist of two domains: a 12 transmembrane domain responsible for binding glucose and a C-terminal domain in the cytoplasm that transmits the glucose signal to the next intracellular component of the signal transduction pathway. However, because attachment of the Snf3 tail to Hxt1 or Hxt2 results in only partial signaling, other residues in the transmembrane domain of Snf3 and Rgt2 must be important for optimal function of the glucose sensors.

The protein(s) that receives the glucose signal from Snf3 or Rgt2, presumably by interacting with their C-terminal tails, has not been identified. Hxk2, the main glucose-phosphorylating enzyme in yeast, seems a likely candidate because it carries out the first step of glucose metabolism and is partially required for glucose induction of *HXT* expression (Bisson *et al*., 1993; Özcan and Johnston, 1995). Yang and Bisson (1996) identified *SKS1*, which encodes a putative serine/threonine protein kinase, as another candidate. However, we found that deletion of *SKS1* has no effect on glucose-induced *HXT* expression (unpublished result).

Recently, a glucose transporter from the yeast *Kluyveromyces lactis* was identified (*RAG4*, DDBJ/EMBL/GenBank accession No. Y14849). It is >60% identical to Snf3 and Rgt2 from *S.cerevisiae*. Interestingly, the *K.lactis* Rag4 protein also has a long C-terminal tail of ~251 amino acids that contains one copy of the same repeated sequence found in the C-terminal tails of Snf3 and Rgt2. This finding is consistent with the idea that the 25 amino acid C-terminal repeat plays an important role in signaling by Snf3 and Rgt2. It is possible that Rag4 also functions as a glucose sensor in the yeast *K.lactis*, and might regulate the high glucose-induced expression of the *K.lactis* hexose transporter *RAG1*.

Other transporters of small molecules also function as sensors. Sensing of certain sugars by bacteria is mediated by sugar transporters (Postma *et al*., 1993; Saier *et al*., 1996). However, this cannot be viewed as a receptor-mediated event, because signal generation is coupled to transport and metabolism (phosphorylation) of the sugar. The glucose transporter Rco3 of *Neurospora crassa* may function as a nutrient sensor; like Snf3 and Rgt2, it is required for expression of glucose transporter activity, glucose regulation of gene expression and glucose repression (Madi *et al*., 1997). Mep2 appears to play a regulatory role in pseudohypal growth in addition to its function as a high-affinity transporter of ammonium ions (Lorenz and Heitman, 1996). Thus, sensing and generation of an intracellular signal by transporters in response to nutrients may be a general phenomenon. It seems possible that similar glucose receptors may be found in mammalian cells, possibly in the insulin-producing cells of the pancreas, which must sense the level of available glucose and respond appropriately.

The ability of cells to sense nutrients and respond by altering gene expression is familiar, but doing this by a receptor-mediated process is unusual. Paradigms for how small molecules affect gene expression are provided by the *lac* operon of *Escherichia coli*, where an intracellular metabolite of the nutrient lactose (allolactose) induces gene expression by binding to and inhibiting function of the Lac repressor (Schlax *et al*., 1995), the *GAL* genes of *S.cerevisiae*, where galactose is thought to bind to the Gal3 protein to effect induction of gene expression (Zenke *et al*., 1996), and steroid hormones in mammalian cells, which bind to intracellular receptors and modify their ability to affect transcription (Yamamoto, 1995). In contrast, glucose sensing and signaling by yeast seems to be a receptor-mediated process, more akin to peptide hormone signaling in mammalian cells. The chemotactic response of bacteria, in which certain nutrients are sensed using cell surface receptors and cause altered cell movement,

provides one of the few other examples of receptor-mediated nutrient sensing (Parkinson and Kofoid, 1992).

Materials and methods

Strains and plasmids

Growth conditions and media have been described previously (Özcan and Johnston, 1995). The strains used in this study are YM4127 (wild-type), YM4718 (*rgt2::HIS3*) and YM4714 (*snf3::hisG*). The complete genotype of these strains is described in Özcan *et al*. (1996b). To construct the *rgt2 snf3* double mutant (YM6107), the complete coding region of *RGT2* was disrupted in the *snf3* mutant YM4714 with a PCR product of *GFP–HIS3*, as described by Niedenthal *et al*. (1996). The *hxt* null strain (*htx1*Δ–*hxt7*Δ, RE700A) was constructed and generously provided by Reifenberger *et al*. (1995).

The plasmid pBM3111, containing the *SNF3* gene on a CEN vector, was constructed by subcloning the *Sal*I–*Pst*I fragment of pBL8 (Marshall-Carlson *et al*., 1990) containing *SNF3* into Ycplac33 (Gietz and Sugino, 1988). The *RGT2–CEN* construct (pBM3272) was obtained by PCR amplification of the *RGT2* gene as an *Xba*I–*Xho*I fragment using the primers OM1018: TGCTCTAGATCCCTTTTTCCTGAAACC (*Xba*I site at –795 relative to the ATG) and OM1019: CCGCTCGAGGTTG-ACCCATTTTGTATTCC (*Xho*I site starts 213 bp downstream of the stop codon). The plasmids pBM3135 and pBM3333, which express the *SNF3* and *RGT2* genes, respectively, from the *ADH1* promoter in the multicopy plasmid pRS426, were created by PCR amplification. The *SNF3* coding region was amplified as a 2.4 kb *Hin*dIII–*Bam*HI fragment with the oligonucleotides OM958: GGCAAGCTTCCATGGACCCTAA-TAGTAACAGTTCTAG (*Hin*dIII site starts at the ATG and mutates the natural *Bam*HI site) and OM957: CGCGGATCCCCGCTTAATTAATA-CATCG (*Bam*HI site starts at the stop codon). For the amplification of the *RGT2* coding region as an *Eco*RI–*Bam*HI fragment, the primers OM1124: CCGGAATTCATGAACGATAGCCAAAACTG (*Eco*RI site starts at +1) and OM1125: CGCGGATCCTTATTGGGGGGAAGTGT-ATTG (*Bam*HI site starts at the stop codon) were used. To create the *ADH–HXT1* plasmid (pBM3362), the *Eco*RV–*Dra*I fragment of *HXT1* was subcloned into pRS426 containing the *ADH1* promoter as a 0.8 kb *Hin*dIII–*Sal*I fragment (pBM2974). The *ADH–HXT2* plasmid (pBM3138) was created by subcloning the *HXT2* coding region as a *Bgl*II–*Bam*HI fragment into pBM2974. The *HXT2* gene was amplified by PCR using the oligonucleotides OM1028: GGCAGATCTATGTCTGAATTCGCTA-CTAGC (*Bgl*II site starts at the ATG) and OM944: CGCGGATCCCTTA-TTCCTCGGAAACTC (*Bam*HI site starts at the stop codon). The *RGT2*Δ*1* plasmid (pBM3312) that is deleted for all sequences downstream of the *RGT2* repeat was constructed by PCR amplification of *RGT2* with the oligonucleotides OM1034: CGCGGATCCATGGCGCCCTTGAAA-CTTTC (*Bam*HI site starts at –1200) and OM1126: CCGGAATTCTTAC-CTCATAGAACTCATCAGTAG (*Eco*RI site starts at +2086), followed by subcloning of the *Eco*RI–*Bam*HI fragment into pRS316 (Sikorski and Hieter, 1989). The plasmid containing *RGT2*Δ*2* (pBM3279), which is deleted for the C-terminal tail of *RGT2*, was created by digesting pBM3272 with *Sal*I (cuts within the *RGT2* gene and in the multicloning site of pRS316) followed by religation. The *SNF3* deletions *SNF3*Δ*1* (pBM3319) and *SNF3*Δ*2* (pBM3363) are the *Eco*RI–*Sal*I fragments of the previously described plasmids snf3Δ7-lacZ and snf3Δ10-lacZ (Marshall-Carlson *et al*., 1990, but without the *lacZ* gene), respectively, subcloned into pRS316. Plasmid pBM3259 (*SNF3-1*) has been described previously (Özcan *et al*., 1996b). The plasmid containing the dominant *RGT2-1* mutation in pRS316 (pBM3270) was created using the same oligonucleotides as described for pBM3272 (OM1018/OM1019). Deletion of the C-terminal tails of *RGT2-1* and *SNF3-1* was accomplished by cutting pBM3270 with *Sal*I (truncates the tail) and religating to yield pBM3277 (*RGT2-1*Δ*T*), and cutting pBM3333 with *Eco*RV (cuts within the *SNF3* tail) and religating to yield pBM3335 (*SNF3-1*Δ*T*). Plasmids pBM3576 and pBM3578, which contain the C-terminal tails of *RGT2* and *SNF3*, respectively, fused to the *ADH1* promoter, were obtained by cloning PCR products into pBM3531 (*ADH1*-HA-pRS424). The oligonucleotides used to amplify the *RGT2* tail were OM1029: CGCGG-ATCCGGGGATTGACTTTGGAAG (*Bam*HI site starts at +1644) and OM1030: CGGCTCGAGTTATTGGGGGGAAGTGTATTG (*Xho*I site starts at the stop codon). The *SNF3* tail was amplified using oligo-nucleotides OM1333 (CGCGGATTCAAACGAAGGGTTTGACAT-TAG; *Bam*HI site starts at +1629) and OM957 (*Bam*HI site starts downstream of the stop codon).

The *HXT–SNF3* chimeras were constructed as follows: pBM3273 (*HXT1–SNF3* tail) was made by amplifying the region of *HXT1* encoding its transmembrane region by PCR using oligonucleotides OM1027 (GGC-AAGCTTATGAATTCAACTCCCGATC) and OM1025 (AATGTCAAA-CCCTTCGTTTCGAAGAAAAAGACGTAAAAGTAAG) with pBM-2648 as template. The region of *SNF3* encoding the C-terminal tail was amplified using oligonucleotides OM1092 (ACTTTTACGTCTTTTTCT-TCGAAACGAAGGGTTTGACATTAG; includes sequences from the 3′ end of the *HXT*21 PCR product for priming in the third PCR described below) and OM957 with pBM3111 as template. The gel-purified products of these two PCRs were combined in a third reaction with OM1027 (primes at the 5′ end of *HXT1*) and OM957 (primes at the 3′ end of *SNF3*) to yield a chimeric product fusing *HXT1* nucleotide 1536 to *SNF3* nucleotide 1627 (relative to the ATG of each gene). The product was cleaved with *Hin*dIII and *Bam*HI (sites incorporated at the end of primers OM1027 and OM957) and inserted between the *Hin*dIII and *Bam*HI sites of pBM2974 [pRS426 (Christianson *et al*., 1992) containing the *ADH1* promoter]. The *ADH1* promoter was swapped for the *HXT1* promoter by replacing the *Hpa*I–*Xba*I fragment of pBM3435 [the 4.1 kb *Hin*dIII fragment containing ~1200 bp of upstream promoter sequence and the entire *HXT1* coding sequence inserted into pRS316 (Sikorski and Hieter, 1989)] with the 1.8 kb *Hpa*I–*Xba*I fragment of pBM3273 containing the *HXTI–SNF3* fusion. pBM3454 (*HXT2–SNF3* tail) was made by amplifying the region of *HXT2* encoding its transmembrane region by PCR using oligonucleotides OM1028 and OM1026 (AATGT-CAAACCCTTCGTTTCAAAGAAAAACACGTAGAAGAATG) with pBM2649 as template. The region of *SNF3* encoding the C-terminal tail was amplified using oligonucleotides OM1092 (includes sequences from the 3′ end of the *HXT2* PCR product for priming in the third PCR described below) plus OM957 with pBM3111 as template. The gel-purified products of these two PCRs were combined in a third reaction with OM1028 (primes at the 5′ end of *HXT2*) and OM957 (primes at the 3′ end of *SNF3*) to generate the chimeric product fusing *HXT2* nucleotide 1510 to *SNF3* nucleotide 1627 (relative to the ATG of each gene). The product of the reaction was cleaved with *Hin*dIII (cuts within *HXT2*) and inserted in the correct orientation in pBM2649 (a 2 μm plasmid containing *HXT2*; contributes the *HXT2* 2883 bp *Hin*dIII fragment containing the promoter and immediate 1203 nucleotides of coding sequence to the final plasmid).

The *Gal1::lacZ* reporter (pBM690) contains the *Eco*RI–*Bam*HI fragment of the *GAL1* promoter from pBM252 (Johnston and Davis, 1984) fused to *lacZ* in the vector Ycp50. The construction of the *SUC2::lacZ* plasmid (pBM3082) was described by Özcan *et al*. (1997).

β-galactosidase (β-Gal) assays

β-galactosidase activity was assayed in permeabilized cells grown to mid-log phase as described previously (Yocum *et al*., 1984). Activities are reported in Miller units. The mean activities are the averages of four to six assays of at least four independent transformants. Cells were pre-grown on yeast nitrogen base (YNB) containing 5% glycerol plus 0.5% galactose lacking the appropriate amino acids, and transferred to YNB medium containing 4% glucose, 5% glycerol plus 0.5% galactose, or 5% glycerol plus 0.1% glucose, and incubated overnight before β-galactosidase activity was assayed.

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