Regulation of the Nucleocytoplasmic Distribution of Snf1-Gal83 Protein Kinase

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Snf1 protein kinase containing the β subunit Gal83 is localized in the cytoplasm during growth of Saccharomyces cerevisiae cells in abundant glucose and accumulates in the nucleus in response to glucose limitation. Nuclear localization of Snf1-Gal83 requires activation of the Snf1 catalytic subunit and depends on Gal83, but in the snf1Δ mutant, Gal83 exhibits glucose-regulated nuclear accumulation. We show here that the N terminus of Gal83, which is divergent from those of the other β subunits, is necessary and sufficient for Snf1-independent, glucose-regulated localization. We identify a leucine-rich nuclear export signal in the N terminus and show that export depends on the Crm1 export receptor. We present evidence that catalytically inactive Snf1 promotes the cytoplasmic retention of Gal83 in glucose-grown cells through its interaction with the C terminus of Gal83; cytoplasmic localization of inactive Snf1-Gal83 maintains accessibility to the Snf1-activating kinases. Finally, we characterize the effects of glucose phosphorylation on localization. These studies define roles for Snf1 and Gal83 in determining the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase.

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of 1 and then exposed to different conditions. In all cases, cells were collected by brief centrifugation, resuspended in residual medium, and placed on a microscope slide. Cells were grown in the indicated medium to an optical density at 600 nm representative of the population. At least 500 cells were examined in each case, and the selected images were representative of the population.

### Results

N terms of Gal83 suffices for Snf1-independent, glucose-regulated nuclear localization. The sequence of Gal83 N-terminal to the conserved glycogen-binding domain (29) is divergent from that of the other β subunits. To examine the role of this divergent sequence in determining the localization of Gal83, we expressed residues 1 to 153, fused to GFP, from the Gal83 promoter on a centromeric plasmid (Fig. 1). The resulting protein, designated Gal83(1-153)-GFP, does not contain the C-terminal sequences that interact with Snf1 and Snf4 (14) and does not provide β-subunit function. Gal83(1-153)-GFP was expressed at higher levels than Gal83-GFP, as judged from fluorescence and immunoblot analyses (data not shown). In wild-type cells, Gal83(1-153)-GFP was cytoplasmic during growth in glucose and relocalized to the nucleus when cells were shifted to limiting glucose or glycerol plus ethanol (Fig. 2A and data not shown). Similar results were observed with gal83Δ cells, and Gal83(1-153)-GFP also accumulated in the nucleus when cells were grown in glycerol plus ethanol (data not shown). These patterns are the same as those exhibited by full-length Gal83-GFP (Fig. 2A) (9, 27). In accord with the absence of Snf1- and Snf4-interacting sequences, Gal83(1-153)-GFP showed the same localization patterns in snf1Δ,
snf1Δ, and sak1Δ mutant cells as in the wild type during growth in glucose and in response to glucose limitation (Fig. 2B and data not shown). Thus, the N-terminal 153 residues of Gal83 are sufficient for glucose-regulated localization. These findings are consistent with previous results that Gal83(1-90)-GFP exhibits partially regulated localization (27).

**Cytoplasmic retention of the C terminus of Gal83 by inactive Snf1 in glucose-grown cells.** To determine if the N terminus is necessary for regulated localization, we expressed Gal83(154-417)-GFP from the native promoter (Fig. 1). In glucose-grown wild-type or gal83Δ cells, Gal83(154-417)-GFP was present in the cytoplasm but also accumulated in the nucleus (Fig. 2C and data not shown), whereas Gal83-GFP was nuclear excluded (Fig. 2A). When cells were shifted to limiting glucose, Gal83(154-417)-GFP became more strongly nuclear (Fig. 2C). Similar results were observed with Gal83(244-417)-GFP, although it was not well expressed (data not shown).

Gal83(154-417)-GFP and Gal83(244-417)-GFP conferred growth on raffinose in sip1Δ sip2Δ gal83Δ cells (data not shown), indicating that they both provide at least minimal β-subunit function, consistent with the presence of Snf1- and Snf4-interacting regions C-terminal to the glycogen-binding domain (residues 161 to 243). Deletion analysis mapped the Snf1-interacting sequence (designated the KIS domain) between residues 198 and 343, which is now known to include part of the glycogen-binding domain, and mapped the Snf4-interacting sequence (ASC domain) distal to residue 343 (14).

Previous evidence indicated that kinase-dead Snf1 inhibits the nuclear localization of Gal83-GFP (9), suggesting that, in glucose-grown cells, the presence of inactive Snf1 inhibits nuclear localization. To test this idea, we first examined snf1Δ mutant cells. Gal83(154-417)-GFP was strongly nuclear localized under high- and low-glucose conditions (Fig. 2C and data not shown). We next examined reg1Δ mutant cells, in which Snf1 is constitutively active; the Reg1-Glc7 protein phosphatase 1 is required for dephosphorylation and inactivation of Snf1 in glucose-grown cells (12, 21). In reg1Δ cells, Gal83(154-417)-GFP was nuclear in high glucose (Fig. 2C), whereas Gal83-GFP and Gal83(1-153)-GFP were not (reference 27 and data not shown). These findings indicate that the presence of catalytically inactive Snf1 was responsible for the partial cytoplasmic retention of Gal83(154-417)-GFP in glucose-grown wild-type cells. When Snf1 was active or absent, Gal83(154-417)-GFP was constitutively nuclear. Thus, inactive Snf1 contributes to the cytoplasmic retention of Gal83 in glucose-grown cells through its interaction with C-terminal sequences of Gal83, and the N-terminal sequence of Gal83 is necessary for the Snf1-independent mechanism that regulates the localization of Gal83 in response to glucose signals.

**N-terminal leucine-rich nuclear export signal is required for glucose-dependent nuclear exclusion of Gal83.** We next took advantage of conveniently located NheI sites to remove residues 19 to 97 from Gal83-GFP (Fig. 1). In snf1Δ and reg1Δ mutant cells, Gal83(Δ19-97)-GFP showed nuclear accumulation in both high and low glucose (Fig. 3A and data not shown). In wild-type cells, the mutant protein was not excluded from the nucleus; it was, in some cases, enriched in the nucleus during growth in high glucose, and it became strongly nuclear in response to glucose limitation (Fig. 3A). These findings indicate that sequences residing within the deleted region are required for the Snf1-independent, glucose-dependent nuclear exclusion of Gal83.

The N terminus of Gal83 contains the sequence LAYTFSQMNV (residues 39 to 48), which matches the leucine-rich NES consensus ΦX_{α}ΦX_{α}XΦ (Φ = L, I, V, F, M) (7, 17, 28). We introduced mutations altering the four critical residues in Gal83-GFP to alanines (L39A, F43A, M46A, V48A) and refer to the quadruply mutant protein as Gal83mutNES-GFP (Fig. 1). In wild-type, snf1Δ, and reg1Δ cells, Gal83mutNES-GFP showed the same patterns of localization as Gal83(Gal83(154-417)-GFP) (data not shown). The alteration S38A, L39A also caused the same pattern, whereas S38A alone had no effect (wild-type cells were examined; data not shown). The truncated Gal83(1-153)mutNES-GFP showed similar nuclear accumulation in both high and low glucose even in wild-type cells.
tions altering the basic residues of either or both clusters to alanines had no effect on the localization of Gal83(1-153)-GFP; alteration of the serines also had no effect (data not shown). Nuclear localization of Gal83(1-153)-GFP may be conferred through an import signal that we have not recognized or through interactions with another protein containing an NLS.

The full-length Gal83 has additional potential NLSs. We altered one of these, KKGR at position 155, to AAGG. This alteration alone, or in combination with alteration of both N-terminal basic clusters, did not impair nuclear localization of Gal83-GFP (data not shown). However, KKGR affected the localization of Gal83(1-243)-GFP (Fig. 1) which, as expected, does not provide an NLS. Thus, this sequence appears to be recognized as an NLS in the context of this truncated protein, although we cannot rule out more complicated models. The localization patterns observed for Gal83(1-153), Gal83(1-243), and Gal83 and their mutant derivatives would then simply reflect the different balance between NLS and NES elements.

2-Deoxyglucose inhibits Snf1 activity but not nuclear accumulation of Gal83(1-153)-GFP. Previous evidence suggested that phosphorylation of glucose is necessary and sufficient for the glucose-dependent nuclear exclusion of Gal83-GFP, and glucose-6-phosphate was proposed as a candidate signal (27). As it is now evident that inhibition of Snf1 activity affects Gal83-GFP localization, we have revisited this issue. We first examined the localization of Gal83(1-153)-GFP in hsk1Δ hsk2Δ gk1Δ cells, which lack hexose phosphorylating activity; localization of Gal83-GFP was normal in hsk1Δ hsk2Δ gk1Δ cells but defective in the triple mutant (27). Cultures were grown on glucose-6-phosphate, restored glucose-regulated localization (data not shown).

Identification of NLSs. We next examined the N terminus of Gal83 for basic nuclear localization signals (NLSs). The only clustered basic residues are KH at position 28 and RHK at position 61; the latter is also a potential protein kinase A site (RHKSS) and resembles the Snf1 consensus site (3). Mutations altering the basic residues of either or both clusters to alanines had no effect on the localization of Gal83(1-153)-GFP; alteration of the serines also had no effect (data not shown). Nuclear localization of Gal83(1-153)-GFP may be conferred through an import signal that we have not recognized or through interactions with another protein containing an NLS.

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153)-GFP remained nuclear after 10 min and 1 h (Fig. 5A and data not shown), whereas Gal83-GFP rapidly relocated to the cytoplasm, as reported previously (27) (Fig. 5B). In snf1Δ and reg1Δ cells, however, Gal83-GFP remained nuclear (Fig. 5B). Thus, the effect of 2-deoxyglucose on the localization of Gal83 is mediated by Snf1.

We next assayed Snf1 catalytic activity. Glucose-grown wild-type cells were shifted to limiting glucose for 10 min and then adjusted to 2% glucose, 0.02% 2-deoxyglucose, or 0.02% 6-deoxyglucose for 10 min. Extracts were prepared, and Snf1 was partially purified and assayed by phosphorylation of the SAMS peptide. The addition of glucose or 2-deoxyglucose inhibited Snf1 activity, whereas 6-deoxyglucose did not (Fig. 5C); no inhibition was observed for hok1Δ hok2Δ glk1Δ cells (data not shown). The inhibition of Snf1 may be indirect, and it has been noted that intracellular phosphorylation of 2-deoxyglucose depletes cellular ATP (30).

In cells lacking phosphoglucose isomerase, the addition of glucose inhibits Snf1 activity but not nuclear localization of Gal83(1-153)-GFP. We examined the localization of Gal83(1-153)-GFP and assayed Snf1 activity in the pgi1Δ mutant, which lacks phosphoglucose isomerase and cannot isomerize glucose-6-phosphate to fructose-6-phosphate (1). Gal83(1-153)-GFP was nuclear excluded in cells grown on 2% fructose plus 0.1% glucose and translocated to the nucleus upon a shift to medium with no added carbon source. The addition of 2% glucose did not (Fig. 6A). In contrast, Gal83-GFP relocated to the cytoplasm upon addition of either fructose or glucose (Fig. 6B). Consistent with these results, Snf1 was activated when cells were shifted from fructose to carbon source-deprived medium, and the addition of 2% glucose or 0.02% 2-deoxyglucose inhibited Snf1 (Fig. 6C). These findings provide evidence that glucose-6-phosphate is not the signal responsible for the glucose-dependent nuclear exclusion of Gal83(1-153)-GFP.

We note that the addition of glucose-6-phosphate (2 mM) to assays of Snf1 prepared from carbon source-deprived pgi1Δ cells had no inhibitory effect on Snf1 activity (data not shown),...
confirming that glucose-6-phosphate does not directly inhibit Snf1 protein kinase (30). Our experiment excludes the possibility that glucose-6-phosphate was metabolized by phosphoglucone isomerase present in the preparation.

**Relationship between Snf1 activity and localization.** These findings indicate that the localization of Gal83, and thus that of Snf1-Gal83 protein kinase, is determined both by signals impinging directly on Gal83 and by the activation status of Snf1. During growth on abundant glucose, Snf1-Gal83 is largely inactive and cytoplasmic, whereas during growth on nonfermentable carbon sources or in response to acute carbon stress, Snf1-Gal83 is active and nuclear enriched (9, 10, 15, 27). We considered the possibility that, during growth on carbon sources that are neither very preferred or nonpreferred, the existence of multiple control mechanisms could result in the cytoplasmic localization of active Snf1-Gal83. The localization of Gal83-GFP in cells growing on some carbon sources is highly strain dependent; however, Gal83-GFP was cytoplasmic in both W303 and an S288C-related strain during growth on sucrose (10, 27). The snf1 mutation was identified by its sucrose-nonfermenting phenotype, suggesting that this cytoplasmic Snf1-Gal83 was active.

To explore this further, we assayed Snf1 catalytic activity in sucrose-grown W303 cells. Cultures were grown to mid-log phase in synthetic complete medium containing 2% sucrose, an aliquot was taken for microscopic examination, and cells were collected for assays of Snf1 activity. Gal83-GFP was cytoplasmic, as was Gal83(1-153)-GFP; however, Snf1 activity was not significantly higher than that of cells grown in 2% glucose (0.63 ± 0.07 and 0.56 ± 0.13 nmol/min/mg, respectively). In control cultures shifted from glucose to sucrose for 10 min or grown on 2% glycerol plus 3% ethanol, Snf1 was activated (fivefold-higher activity) and Gal83(1-153)-GFP accumulated in the nucleus. The utilization of sucrose requires the Snf1-dependent expression of the SUC2 gene encoding invertase, a stable secreted enzyme that hydrolyzes sucrose; however, after adaptation, minimal Snf1 activity is apparently required for continued growth. Consistent with these findings, the snf1Δ mutant exhibits a severe growth defect on sucrose only under anaerobic (fermentative) conditions or in the presence of the respiratory inhibitor antimycin A. Although cytoplasmic Snf1 proved to be largely inactive in this case, it remains possible that other conditions result in cytoplasmic localization of active Snf1.

**DISCUSSION**

We have examined here the roles of the Snf1 and Gal83 subunits in regulating the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase in response to glucose signals. We showed that the N terminus of Gal83, which is divergent from those of the other two β subunits, is necessary and sufficient for the Snf1-independent regulation of nuclear localization. Gal83(1-153)-GFP was cytoplasmic and excluded from the nucleus in high glucose conditions and translocated to the nucleus in response to glucose limitation. Localization of Gal83(1-153)-GFP was not affected by snf1Δ, reg1Δ, or sak1Δ mutations, indicating that it is controlled independently of Snf1.

We identified a leucine-rich NES in the N terminus (LXFXMXV, residues 39 to 48) that is required for the Snf1-independent nuclear exclusion of Gal83 in glucose-grown cells. Mutation of the NES resulted in nuclear accumulation during growth in high glucose for Gal83(1-153)mutNES-GFP in wild-type cells and for Gal83mutNES-GFP in reg1Δ or snf1Δ mutant cells. Mutation of the gene encoding the Crn1 export receptor also resulted in nuclear accumulation of Gal83(1-153)-GFP in high glucose. A leucine-rich NES, VXVXVXL, is conserved at a similar position in the N terminus (residues 44 to 53) of the β subunit Sip2, which is excluded from the nucleus (27). Gal83 also contains other possible NESs, notably LX3LX5VXL (residues 350 to 359), which is conserved in Sip2 and AMP-activated protein kinase β1 and β2; however, its functional role remains uncertain as Gal83(154-417)-GFP showed strong nuclear accumulation in the absence of Snf1. Although Gal83 includes various potential NLSs, including one at position 155 that affects the nuclear localization of Gal83(1-243)-GFP, we did not identify an NLS responsible for the import of Gal83(1-153)-GFP. This region may contain a nonclassical NLS, or import may depend on another unidentified protein with an NLS.

We also present evidence that the C terminus of Gal83 is involved in the regulation of localization through its interaction with Snf1; previous studies showed that activation of Snf1 is required for nuclear accumulation of Snf1-Gal83 (9). During growth in glucose, Gal83(154-417)-GFP was partially cytoplasmic in wild-type cells but strongly nuclear in reg1Δ and snf1Δ cells. Similar patterns were observed with full-length Gal83 proteins lacking the NES. These findings indicate that inactive Snf1 contributes to the cytoplasmic retention of Gal83 in glucose-grown cells through its interaction with C-terminal sequences of Gal83.

Further studies are required to understand the mechanism by which Snf1 activity regulates localization. There are multiple possibilities; for example, a phosphorylation event could inhibit cytoplasmic retention by releasing interaction with an anchoring protein, or phosphorylation could be required for nuclear import of Snf1-Gal83 by unmasking an NLS or affecting interaction with an import factor. Gal83 is phosphorylated by Snf1 in vitro (32), but immunoblot analysis showed no differential modification in response to glucose availability in vivo (K. Hedbacker, unpublished results). The dependence of nuclear accumulation on the activation of Snf1 may be physiologically important because none of the Snf1-activating kinases is located in the nucleus (2, 9, 15); hence, the retention of inactive Snf1 in the cytoplasm maintains accessibility to the activating kinases.

Finally, we present evidence that the signal responsible for the glucose-dependent nuclear exclusion of Gal83(1-153)-GFP requires glucose phosphorylation but is not glucose-6-phosphate. While glucose had no effect in cells lacking hexose kinases, the addition of 2-deoxyglucose to glucose-limited wild-type cells, or the addition of glucose to pgi1Δ mutant cells lacking phosphoglucose isomerase, rapidly inhibited Snf1 activity but did not promote the cytoplasmic localization of Gal83(1-153)-GFP. Thus, previous evidence suggesting glucose-6-phosphate as a candidate signal regulating Gal83 localization (27) can be accounted for by inhibitory effects on Snf1 activity. These findings further suggest that Snf1 activity and localization of Gal83(1-153) are regulated by different signals;
a caveat is that depletion of ATP may limit the phosphorylation of Snf1 by its activating kinases.

The signaling mechanisms that control localization of Snf1-Gal83 remain unclear. Nuclear accumulation of Snf1-Gal83 is defective in a sak1Δ mutant (9); one explanation is that Sak1 is the major kinase responsible for activating Snf1, but substantial activation of Snf1 by a heterologous kinase did not suppress this defect (12). We found that Sak1 is not required for nuclear localization of Gal83(1-153)-GFP, consistent with previous evidence that it is not required for localization of Gal83-GFP in the absence of Snf1. Protein kinase A regulates the localization of the Sip1 β subunit (10) but not that of Gal83-GFP (10, 27) or Gal83(1-153)-GFP (Hedbacker, unpublished).

We have examined the localization of Gal83(1-153)-GFP and Gal83-GFP in an array of mutants lacking protein kinases that have been implicated in nutrient responses but did not identify another kinase that affects localization (Hedbacker, unpublished); functional redundancy may have precluded detection of a defect. We also mutated several potential CK2 sites in Gal83-GFP because CK2 catalytic subunits copurify with Sak1 (6); however, the substitution of alanine for serines 12 and 17, aspartates 94, 95, and 96, or serines 100 through 104 had no effect (Hedbacker, unpublished).

These studies show that regulation of the nucleocytoplasmic distribution of Snf1-Gal83 is complex, involving multiple regulatory mechanisms operating on Snf1 and Gal83. Under the growth conditions that we have examined, Snf1 activity correlated with the localization of Snf1-Gal83. Under other conditions, in particular those encountered during growth in natural environments, these multiple control mechanisms may serve to fine-tune the nucleocytoplasmic distribution of active Snf1-Gal83 and thus modulate the expression of subsets of its target genes.

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