Snf1 Promotes Phosphorylation of the α Subunit of Eukaryotic Translation Initiation Factor 2 by Activating Gcn2 and Inhibiting Phosphatases Glc7 and Sit4[▽]

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Snf1 is the ortholog of mammalian AMP-activated kinase and is responsible for activation of glucose-repressed genes at low glucose levels in budding yeast. We show that Snf1 promotes the formation of phosphorylated α subunit of eukaryotic translation initiation factor 2 (eIF2 α -P), a regulator of general and gene-specific translation, by stimulating the function of eIF2 α kinase Gcn2 during histidine starvation of glucose-grown cells. Thus, eliminating Snf1 or mutating its activation loop lowers Gcn2 kinase activity, reducing the autophosphorylation of Thr-882 in the Gcn2 activation loop, and decreases eIF2 α -P levels in starved cells. Consistently, eliminating Reg1, a negative regulator of Snf1, provokes Snf1-dependent hyperphosphorylation of both Thr-882 and eIF2 α . Interestingly, Snf1 also promotes eIF2 α phosphorylation in the nonpreferred carbon source galactose, but this occurs by inhibition of protein phosphatase 1 α (PP1 α ; Glc7) and the PP2A-like enzyme Sit4, rather than activation of Gcn2. Both Glc7 and Sit4 physically interact with eIF2 α in cell extracts, supporting their direct roles as eIF2 α phosphatases. Our results show that Snf1 modulates the level of eIF2 α phosphorylation by different mechanisms, depending on the kind of nutrient deprivation existing in cells.

Budding yeast cells reprogram gene expression in response to amino acid starvation by a two-pronged regulatory mechanism centered on the protein kinase Gcn2. Gcn2 has low activity in nonstarved cells and is activated by uncharged tRNAs that accumulate during starvation. Gcn2 has a single known substrate, Ser51 in the α subunit of eukaryotic translation initiation factor 2 (eIF2 α). eIF2 delivers methionyl-tRNA^{Met} in a ternary complex (TC) with GTP to the 40S ribosomal subunit, and eIF2 α phosphorylation inhibits TC formation and subsequent steps in the initiation pathway. Although phosphorylated eIF2 inhibits general protein synthesis, it induces the translation of *GCN4* mRNA, encoding a transcriptional activator of amino acid biosynthetic enzymes. Four short open reading frames (uORFs) in the *GCN4* mRNA leader prevent scanning ribosomes from reaching the *GCN4* start codon in nonstarved cells when TC is abundant. The reduction in TC levels provoked by eIF2 α phosphorylation in starved cells allows ribosomes that have translated uORF1 and resumed scanning to bypass the more inhibitory uORFs 3 and 4 and reinitiate downstream at *GCN4* instead. Hence, Gcn2 induces Gcn4 and its target amino acid biosynthetic genes to replenish amino acid pools while reducing general protein synthesis and amino acid consumption (reviewed in reference 10). This dual response to amino acid starvation also operates in mammalian cells, involving Gcn2 and transcription factors Atf4 (8, 28) and Atf5 (35).

Phosphorylation of the activation loop in the Gcn2 kinase domain (KD) is required for its activation. Thr-882 and Thr-

887 are the only sites of autophosphorylation *in vitro*, and mutating them to Ala impairs (T882A) or abolishes (T887A) Gcn2 function (23). We previously isolated substitutions in the Gcn2 KD, R794G and F842L, that constitutively activate Gcn2 in the absence of amino acid starvation or the tRNA binding domain in Gcn2, which mediates activation of Gcn2 by uncharged tRNA (22). Kinase activation by these *GCN2^c* mutations is associated with hyperphosphorylation of T882 (T882-P); however, it has not been demonstrated that physiological activation of wild-type (WT) Gcn2 in amino acid-starved yeast cells involves increased T882 phosphorylation.

Another layer of nutrient control over Gcn2 function involves the phosphorylation of a negative regulatory residue N terminal to the KD, Ser577, which is dependent on the Tor kinases. Yeast Tor1 and Tor2 regulate metabolic processes to coordinate cell growth with nutrient availability, and their inhibition by mutation or rapamycin provokes cellular responses characteristic of nutrient starvation (reviewed in reference 4). Tor kinases promote Gcn2 latency in nonstarved cells by enhancing Ser577 phosphorylation (S577-P), at least partly by inhibiting type 2A protein phosphatases that dephosphorylate S577-P (2). Thus, Tor proteins stimulate general protein synthesis in yeast in part by preventing eIF2 α phosphorylation. As expected, rapamycin elicits dephosphorylation of S577-P by inhibiting Tor function; however, histidine starvation and other stress conditions that increase eIF2 α phosphorylation do not reduce S577-P levels (2). Presumably, the inhibitory effect of S577-P can be overridden when uncharged tRNA accumulates to high levels during amino acid starvation to elicit Gcn2 activation (6).

Budding yeast Snf1, the founding member of the Snf1/AMP-activated protein kinase (AMPK) family of protein kinases, is another key regulator of gene expression by nutrients.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
H1515	<i>MATa ura3-52 trp1-Δ63 leu2-3 leu2-112</i>	3
H1642	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 GCN4-lacZ::TRP1</i>	3
H1894	<i>MATa ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63</i>	T. E. Dever, unpublished
H1895	<i>MATa ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63 GCN4-lacZ::TRP1</i>	T. E. Dever, unpublished
HQY305	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 reg1Δ GCN4-lacZ::TRP1</i>	This study
HQY343	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ GCN4-lacZ::TRP1</i>	This study
HQY344	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ gcn2Δ GCN4-lacZ::TRP1</i>	This study
HQY347	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ reg1Δ GCN4-lacZ::TRP1</i>	This study
HQY356	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ</i>	This study
HQY357	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ gcn2Δ</i>	This study
HQY358	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 snf1Δ::hisG::URA3::hisG</i>	This study
HQY1313	<i>MATa ura3-52 trp1-63 leu2-3 leu2-112 GLC7::13myc::kanMX</i>	This study
JC782-24D	<i>MATa leu2 ura3-52 lys2-1 glc7-1</i>	29
JC782-26C	<i>MATa leu2 ura3-52 lys2-1 glc7-1</i>	J. Cannon, unpublished
CY1194	<i>MATa leu2 ura3-52 lys2-1 trp1-Δ63 glc7-1 snf1Δ::hisG</i>	This study
CY1297	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 sit4Δ::KanMX GCN4-lacZ::TRP1</i>	This study
CY1298	<i>MATa ura3-52 leu2-3 leu2-112 trp1-Δ63 sit4Δ::KanMX snf1Δ GCN4-lacZ::TRP1</i>	This study
CY1299	<i>MATa leu2 ura3-52 lys2-1 trp1-Δ63 glc7-1 snf1Δ::hisG sit4Δ::KanMX</i>	This study
CY1323	<i>MATa leu2 ura3-52 lys2-1 trp1-Δ63 GLC7 snf1Δ::hisG</i>	This study

Its best-understood function is to orchestrate the transcriptional response to glucose limitation, involving induction of genes that regulate the metabolism of alternative carbon sources, gluconeogenesis, or respiration during growth in non-preferred carbon sources. Snf1 kinase activity is stimulated at low glucose levels by upstream kinases that phosphorylate activation loop residue Thr-210, overcoming the inhibitory effect of the Reg1-Glc7 protein phosphatase 1 (PP1) complex that dephosphorylates T210-P under glucose-replete conditions (9). T210 phosphorylation and Snf1 activity are also stimulated by other environmental stresses, including high salinity and alkaline pH (16), and by inactivation of Tor kinases (9, 19). Snf1 also mediates resistance to heat shock (12), toxic cations (21), and hydroxyurea (5) in a manner that does not require increased T210-P or elevated kinase activity, indicating that Snf1 basal activity is sufficient for its function under certain stresses.

In this report, we document the existence of cross talk between Snf1, Gcn2, and two distinct eIF2 α phosphatases in coupling eIF2 α -P formation to amino acid and glucose availability. We found that Snf1 kinase activity is stimulated in histidine-starved cells, where it activates Gcn2. In contrast, Snf1 promotes eIF2 α -P formation in the nonpreferred carbon source galactose by inhibiting dephosphorylation of eIF2 α -P by protein phosphatases Glc7 and Sit4. Thus, we can now state that two major nutrient-signaling kinases that respond to the availability of different nutrients, Snf1 (carbon) and Tor (nitrogen), make critical contributions to setting the level of eIF2 α -P, a key regulator of translation initiation in nutrient-limited cells.

MATERIALS AND METHODS

Strains and plasmids. All of the yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Strain HQY305 was constructed by transforming H1642 with a *reg1 Δ ::hisG::URA3::hisG* fragment, and deletion of *REG1* in the resulting transformant was confirmed by PCR analysis, after which selection for growth on 5-fluoroorotic acid (5-FOA) was used to evict the *URA3* marker. Similarly, HQY343, HQY344, HQY347, HQY356, and HQY357 were constructed from H1642, H1895, HQY305, H1515, and H1894, respectively, by transformation with the *snf1 Δ ::hisG::URA3::hisG* fragment from pHQ1160, confirming the deletion of *SNF1* by PCR analysis, and selecting a Ura⁻ segregant on

5-FOA medium. HQY1313 was constructed from H1515 by transformation with the *GLC7::13myc::kanMX* fragment, and the addition of myc₁₃ coding sequences at the 3' end of the *GLC7* ORF was confirmed by colony PCR.

CY1194 was generated by crossing JC782-26C with HQY358, identifying a *glc7-1 snf1 Δ ::hisG* ascospore clone by its Ura⁺ phenotype and defective accumulation of glycogen (29), followed by selection on 5-FOA medium to evict the *URA3* marker. Strain CY1323 was generated by transforming CY1194 with plasmid pCB227, linearized by digestion with BglII to direct integration to *glc7-1*; this was followed by selection of Ura⁻ clones on 5-FOA medium. The presence of *GLC7* in CY1323 was confirmed by staining for glycogen. Strains CY1297, CY1298, and CY1299 were constructed by replacing *SIT4* with *sit4 Δ ::kanMX4* in

TABLE 2. Plasmids used in this study

Plasmid	Relevant description	Source or reference
pAH15	2 μ m <i>LEU2 GCN2</i>	11
p235	<i>GCN4^c</i>	17
p238	<i>GCN4^c</i> no uORFs	17
p630	2 μ m <i>URA3 GCN2</i>	30
p722	<i>CEN6 URA3 GCN2</i>	30
p2106 (pB11)	<i>CEN6 URA3 gcn2-T882A T887A</i>	23
p2326 (pB34)	2 μ m <i>URA3 gcn2-K628R</i>	23
pB61	2 μ m <i>URA3 gcn2-T882A T887A</i>	23
pB107	<i>CEN6 URA3 GCN2</i>	6
pB115	<i>CEN6 URA3 gcn2-K628R</i>	6
pHQ817	<i>CEN6 LEU2 REG1</i>	This study
pHQ1160	<i>snf1Δ::hisG::URA3::hisG</i>	This study
pHQ1162	<i>CEN6 URA3 SNF1</i>	This study
pHQ1163	<i>CEN6 LEU2 SNF1</i>	This study
pHQ1774	2 μ m <i>URA3 GLC7-13myc</i>	This study
pHQ1873	<i>CEN6 URA3 snf1-K84R</i>	This study
pHQ1874	<i>CEN6 URA3 snf1-T210A</i>	This study
pDH101	<i>FL-GCN2 CEN6 URA3</i>	2
pCB149	<i>FL-GCN2-S577A CEN6 URA3</i>	2
pCB227	<i>GLC7</i> in pRS306	This study
pMO18	2 μ m <i>LEU2 pADH1::3HA-SNF1</i>	19
pMO19	2 μ m <i>LEU2 pADH1::3HA-snf1-T210A</i>	19
pSK134	2 μ m <i>LEU2</i>	19
pSNF1-K84R	<i>snf1-K84R-3HA</i> in pRS316	16
CB1770	2 μ m <i>URA3 SIT4</i>	K. Arndt
pSL2667	2 μ m <i>URA3 pADH-SIT4-HA</i>	G. Sprague
p28.1	<i>CEN6 URA3 GLC7</i>	29
p29.1	2 μ m <i>URA3 GLC7</i>	29

H1642, HQY343, and CY1194, respectively, followed by PCR verification of the replacements.

Plasmids pB11, pB34, pB61, pB107, pB115, pDH101, and pCB149 were described previously (2, 6, 23). Plasmid pHQ817 was made by inserting a 3.68-kb EcoRI-SalI fragment generated by PCR and containing 340 bp of 5' noncoding sequences, the ORF, and 300 bp of 3' noncoding sequences of *REG1* between the EcoRI and SalI sites of YCplac111. To construct pHQ1160, a 590-bp XbaI-BglII fragment containing the *SNF1* 5' noncoding region and a 500-bp BglII-XbaI fragment containing the *SNF1* 3' noncoding region were generated by PCR and inserted at the XbaI site of pUC18 to produce pHQ1157, after which a 3.8-kb BamHI fragment containing *hisG::URA3::hisG* from pHQ221 was inserted at the BglII site of pHQ1157. Plasmid pHQ221 was constructed by ligating the 3.8-kb BamHI-BglII fragment containing *hisG::URA3::hisG* from pNKY51 (1) with XbaI-digested pUC18 after using the Klenow fragment to blunt end both fragments. pHQ1162 and pHQ1163 were constructed by inserting a 3-kb XbaI fragment generated by PCR, containing 590 bp of 5' noncoding sequences, the ORF, and 500 bp of 3' noncoding sequences of *SNF1*, into the XbaI sites of YCplac33 and YCplac111, respectively. pHQ1873 (*snf1-K84R*) and pHQ1874 (*snf1-T210A*) were constructed as follows. (i) PCR was employed with yeast genomic DNA as the template and primers 5' CTC TCG ATC TTG CAG GCT ATG and 5' CAGA TCT AGA CTT GGG ATT GTT TAG CGT C 3' to amplify a *SNF1* fragment containing 475 bp of the promoter and the first 44 codons after digestion with XhoI and XbaI (the latter is underlined in the reverse primer). (ii) Plasmids pSNF1-K84R and pMO19 were used as templates with primers 5' CAAG ACT AGT TTA GCG GAT GGT GCA CAT ATC 3' and 5' CTT AAT CAA CAT GTA CGC GTC 3' to produce 1,030-bp *SNF1* fragments containing codons 45 to 382 after digestion with SpeI and MluI (the latter is underlined in the reverse primer). (iii) The digested PCR fragments from steps i and ii were ligated with pHQ1162 digested with XhoI and MluI. pHQ1774 was constructed as follows. (i) The HindIII-EcoRV fragment containing *GLC7* obtained from p29.1 was inserted into YEplac195. (ii) An EcoRV-BglII *GLC7::l3myc* fragment was amplified by PCR from the chromosomal DNA of HQY1313 and used to replace the cognate fragment in the plasmid obtained in step i. pCB227 was constructed by inserting the HindIII-XhoI fragment from p28.1 into pRS306 digested with SalI and HindIII.

Immunoprecipitations and immunoblot assays. For detection of phosphorylated Gcn2 and eIF2 α , whole-cell extracts (WCEs) were prepared from yeast cultures grown to mid-logarithmic phase in SD medium with minimal supplements (24) as previously described (2), except that the breaking buffer contained 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.0), 100 mM NaCl, 50 mM NaF, 40 mM β -glycerophosphate, 1 mM dithiothreitol (DTT), 5 mM phenylmethylsulfonyl fluoride (PMSF), EDTA-free protease inhibitor cocktail (Roche), and 1 μ g/ml each leupeptin, aprotinin, and pepstatin. For analysis of eIF2 α phosphorylation, aliquots of WCE were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis using antibodies specific for phosphorylated Ser51 in eIF2 α (BIOSOURCE International). The blots were stripped and reprobed with polyclonal antibodies against eIF2 α as described previously (2, 6). For analysis of Thr882 or Ser577 phosphorylation, aliquots of 4 to 8 mg of WCE were incubated with 30 μ l anti-FLAG protein A-agarose beads (Sigma) at 4°C overnight. The immunoprecipitates were washed four times with 1 ml of breaking buffer, resolved by 4 to 12% SDS-PAGE, and subjected to immunoblot analysis using antibodies against Thr882-P (22) or Ser577-P (6). The blots were stripped and reprobed with polyclonal antibodies against Gcn2 (serum HL2523) (23). The immunocomplexes were visualized using anti-rabbit antibodies conjugated with horseradish peroxidase and SuperSignal West Dura (Thermo Scientific) or the enhanced chemiluminescence system (Amersham). NIH Image 1.63 software was used for quantification of Western signals. The Thr882 phospho-specific antibodies are not very efficient, and in some cases, the Thr882-P signal observed for the loading with the lesser amount of FL-Gcn2 was outside the linear range. For consistency, therefore, we always quantified the Thr882-P signals for the loadings with the greater amounts of FL-Gcn2 and ensured that the FL-Gcn2 signals exhibit a dose response between the two loadings.

For coimmunoprecipitation of Gcn2 with hemagglutinin (HA)-Snf1 or of eIF2 α with Sit4-HA or Glc7-myc, WCE preparation and immunoprecipitation were done essentially as described in reference 27, with extraction buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 10% glycerol, 1 mM PMSF, and complete protease inhibitor cocktail (Roche).

For analysis of Snf1 T210 phosphorylation, WCEs were prepared and subjected to immunoblot analysis with antibodies against AMPK T172-P (Cell Signaling Technology) as described previously (18).

Immunocomplex kinase assays. HA-Snf1 was immunoprecipitated from WCEs (2 mg) prepared in the extraction buffer described above (27), using 30 μ l

of anti-HA agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. After immunoprecipitation, beads were washed three times with 1 ml of extraction buffer and two times with 1 ml kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100). Immunocomplexes bound to the beads were divided in half and subjected to Western analysis or kinase assays. The latter were conducted with 20 μ l kinase buffer plus 250 μ M ATP and 10 μ Ci [γ -³²P]ATP at 30°C for 20 min and stopped by adding the same volume of 2 $\times$ SDS sample buffer. The reactions were resolved by SDS-PAGE and analyzed by phosphorimaging.

RESULTS

Elimination of Snf1 reduces eIF2 α phosphorylation in histidine-starved cells. In view of previous reports indicating regulatory roles for Snf1 basal kinase activity at high glucose levels (6, 15, 24), we were intrigued to find that deletion of *SNF1* confers sensitivity to 3-aminotriazole (3AT), an inhibitor of the histidine biosynthetic enzyme His3, in glucose-containing medium (Fig. 1A). Plasmid-borne *SNF1* complemented the 3AT^S phenotype of the *snf1* Δ mutant (Fig. 1B), confirming that it results from the absence of Snf1. Sensitivity to 3AT (3AT^S) is a hallmark of Gcn[−] mutants, evident for the *SNF1* *gcn2* Δ mutant strain in Fig. 1A. To examine whether the 3AT^S phenotype of *snf1* Δ mutant cells results from impaired translational induction of *GCN4*, we asked whether it could be suppressed by dominant alleles of *GCN4* lacking the two most inhibitory uORFs (uORFs 3 and 4) or all four uORFs in the *GCN4* mRNA leader (*GCN4*^c alleles). Translation of *GCN4* mRNA from these alleles is constitutively derepressed and independent of Gcn2 (17). Both *GCN4*^c alleles only partially suppressed the 3AT^S phenotype of the *snf1* Δ mutant (Fig. 1B). As Gcn2 overexpression also is known to derepress *GCN4* (30), we tested whether overexpressing Gcn2 from a low-copy-number (lc) or high-copy-number (hc) plasmid would suppress the 3AT^S phenotype of *snf1* Δ mutant cells. However, this was not the case (Fig. 1B), even though Gcn2 was overexpressed ~5-fold in the hc *GCN2* transformants (data not shown). Finally, we found that *snf1* Δ does not lower the expression of a *GCN4-lacZ* construct in 3AT-treated cells; in fact, *snf1* Δ partially derepresses this reporter under nonstarvation conditions (Table 3). Similar results were reported recently by Arndt and colleagues (25). Hence, it appears that the 3AT^S phenotype of *snf1* Δ does not involve reduced translation of *GCN4*. Consistent with this, Kuo and colleagues reported that eliminating Snf1 decreases the transcription of *HIS3*, whose product is targeted by 3AT, by impairing the transcriptional coactivator Gcn5 (14).

While investigating the 3AT^S phenotype of *snf1* Δ mutant cells, we also explored whether the kinase function of Gcn2 is compromised in *snf1* Δ mutant cells by measuring the induction of Ser51 phosphorylation on eIF2 α (eIF2 α -P) by 3AT treatment. Indeed, the results showed clearly that *snf1* Δ reduces the abundance of eIF2 α -P relative to the total amount of eIF2 α (Fig. 1C). This phenotype is complemented by *SNF1* but is not suppressed by hc *GCN2* (Fig. 1D). As discussed below, the decreased level of eIF2 α -P in *snf1* Δ mutant cells might not reduce *GCN4* translation because eliminating Snf1 derepresses *GCN4* independently of high-level eIF2 α phosphorylation. Because eIF2 α -P is an important negative effector of general translation, in addition to its importance in *GCN4* control, we

FIG. 1. Elimination of Snf1 reduces eIF2 α -P in histidine-starved cells. (A) Strains H1642 (WT), H1895 (*gcn2* Δ), HQY343 (*snf1* Δ) and HQY344 (*snf1* Δ *gcn2* Δ) were grown in liquid SC, and serial dilutions were spotted onto synthetic complete (SC) or SC plates with 3AT and incubated at 30°C for 2 or 4 days. (B) Transformants of HQY343 (*Gcn2* *snf1* Δ) with the empty vector, p235 (*Gcn4*^c, lacking uORFs 3 and 4), p238 (*Gcn4*^c, no uORFs), pHQ1162 (l.c. *SNF1*), p722 (l.c. *GCN2*), or p630 (h.c. *GCN2*) were replica printed to 3AT medium as for panel A. These strains exhibited equal growth on SC plates without 3AT (not shown). (C) Strains H1895 (*gcn2* Δ), H1642 (WT), and HQY343 (*snf1* Δ) were grown in SC-His, diluted in SC-His to an optical density at 600 nm of 0.2, cultured for 6 h (–3AT) or 5 h prior to treatment with 10 mM 3AT for 1 h (+3AT). WCEs were subjected to Western analysis with antibodies specific for eIF2 α -P or total eIF2 α . (D) Strain H1642 (WT) and transformants of *snf1* Δ mutant strain HQY343 with the empty vector, p722 (l.c. *GCN2*), p630 (h.c. *GCN2*), or pHQ1162 (l.c. *SNF1*) were analyzed as for panel C. The ratio of eIF2 α -P normalized to eIF2 α in the presence or absence of 3AT is shown at the bottom.

set out to elucidate the role of Snf1 in promoting eIF2 α phosphorylation.

We first examined the possibility that *snf1* Δ decreases Gcn2 kinase activity by increasing the phosphorylation of Ser577 in Gcn2, a modification required for Gcn2 latency. Thus, we asked whether replacing Ser577 with Ala would suppress the defect in eIF2 α phosphorylation in *snf1* Δ mutant cells, as *S577A* constitutively activates Gcn2 (6). As expected, *Gcn2*^c-*S577A* cells have constitutively induced levels of eIF2 α -P; however, this phenotype was largely reversed by *snf1* Δ (Fig. 2A). Furthermore, as described below, *snf1* Δ does not increase Ser577-P in a FLAG-tagged form of Gcn2 (e.g., Fig. 2B, lanes 1 to 6). Hence, *snf1* Δ reduces eIF2 α -P accumulation in starved cells without altering the Ser577-P content of Gcn2.

Snf1 promotes activation of Gcn2 during histidine starvation. The decrease in eIF2 α -P observed in *snf1* Δ mutant cells

could result from decreased Gcn2 kinase activity or increased eIF2 α phosphatase activity. To distinguish between these possibilities, we assayed the phosphorylation level of Thr-882 in the Gcn2 activation loop as an indicator of Gcn2 activation. Similar to other kinases, Thr882 phosphorylation stimulates a conformational change in the KD that overcomes a nonproductive alignment of catalytic residues in the active site (7, 20). However, it was not known whether T882-P increases on the activation of WT Gcn2 in starved cells. If so, we could determine whether *snf1* Δ reduces T882-P and, hence, Gcn2 activity under these conditions.

Accordingly, we used antibodies specific for T882-P to probe FLAG-tagged Gcn2 (FL-Gcn2) immunoprecipitated from yeast. These antibodies were shown to react with a constitutively activated form of the Gcn2 KD but not with catalytically inactive or unphosphorylatable forms of the mutant KD harboring the K628R and T882A substitutions, respectively (22). The T882-P signal, normalized for the amount of FL-Gcn2 (or for the Ser577-P signal measured using the appropriate phosphospecific antibodies), increased >3-fold in response to 3AT (Fig. 2B, cf. lanes 1 and 2 and lanes 7 and 8; also data not shown). Moreover, the T882-P/FL-Gcn2 ratio showed an obvious increase for activated FL-Gcn2^c-S577A under nonstarvation conditions (Fig. 2B, lanes 5 and 6 versus 1 and 2). Thus, T882-P increases with activation of Gcn2 elicited by histidine starvation or the absence Ser-577-P.

Importantly, the T882-P content of FL-Gcn2 was significantly reduced by *snf1* Δ in the presence of 3AT and possibly also in its absence (Fig. 2B, cf. lanes 3 and 4 versus 1 and 2 and lanes 9 and 10 versus 7 and 8). This strongly suggests that Snf1 is a positive effector of Gcn2 activity and the reduction of eIF2 α -P during starvation of *snf1* Δ mutant cells results at least

TABLE 3. *snf1* Δ does not reduce *Gcn4-lacZ* expression in response to histidine starvation and partially derepresses *Gcn4-lacZ* under nonstarvation conditions in glucose medium^a

Strain	<i>Gcn4-lacZ</i> expression (U)		
	R (SD)	DR (SD + 3AT)	DR/R
WT	6 $\pm$ 0.1	91 $\pm$ 6.0	15
<i>gcn2</i> Δ mutant	2 $\pm$ 0.1	5 $\pm$ 0.1	2.5
<i>snf1</i> Δ mutant	18 $\pm$ 0.5	95 $\pm$ 8.1	5.3

^a Strains H1642 (WT), H1895 (*gcn2* Δ), and HQY343 (*snf1* Δ) harboring an integrated *Gcn4-lacZ* reporter were cultured to early logarithmic phase in SD medium (repressing [R] conditions) and treated with 40 mM 3AT for 4 h (derepressing [DR] conditions). β -Galactosidase activities (in units of nanomoles of *o*-nitrophenyl- β -D-galactopyranoside cleaved per minute per microgram of protein) were measured in WCEs. Means and standard errors were calculated from results obtained with three or more independent transformants.

FIG. 2. Elimination of Snf1 reduces T882-P in the Gcn2 activation loop. (A) Lanes 2 to 9, transformants of *snf1Δ gcn2Δ* mutant strain HQY344 with an *lc* plasmid containing *GCN2-FL* (pDH101) or *FL-GCN2-S577A* (pCB149) and either *SNF1* (pHQ1163) or the empty vector; lane 1, H1895. Cultures grown in SD with minimal supplements were divided, and growth continued in the absence (–) or presence of 40 mM 3AT (+) for 90 min. WCEs were subjected to Western analysis with antibodies against total eIF2α or phospho-specific antibodies against eIF2α-P. Western signals were quantified, and the eIF2α-P to eIF2α ratios were plotted. (B) Transformants of H1895 (*gcn2Δ*) or HQY344 (*gcn2Δ snf1Δ*) with plasmids containing *FL-GCN2* (pDH101) or *FL-GCN2-S577A* (pCB149) were cultured as for panel A, and FL-Gcn2 was immunoprecipitated with anti-Flag M2 agarose affinity gel and subjected to Western analysis with antibodies against T882-P or Ser577-P in Gcn2 or total Gcn2 (FL-Gcn2). Aliquots with 8 and 6 mg of protein were immunoprecipitated and analyzed in adjacent lanes. Western signals were quantified, and the T882-P to FL-Gcn2 ratios were plotted. Means and standard errors were calculated from three independent experiments.

partly from decreased Gcn2 function. As mentioned above, the level of S577-P in FL-Gcn2 does not vary significantly between WT and *snf1Δ* mutant cells in the presence or absence of 3AT (Fig. 2B, middle blot), thus ruling out S577 dephosphorylation as a mechanism of Gcn2 activation by Snf1.

Reg1 negatively regulates eIF2α phosphorylation in response to histidine starvation via Snf1. At high glucose concentrations, Snf1 activity is repressed by the PP1α Glc7 in complex with its regulatory subunit Reg1 (15, 16) such that Snf1 is activated in *reg1Δ* cells at high glucose levels. We asked, therefore, if activation of Snf1 by *reg1Δ* evokes greater Gcn2 activation and eIF2α phosphorylation during histidine starvation. Indeed, *reg1Δ* cells exhibit higher eIF2α-P levels than WT cells upon 3AT treatment (Fig. 3A, lanes 14 and 15 versus 10 and 11), mirrored by a similar increase in the T882-P content of FL-Gcn2 (Fig. 3B, lanes 13 and 14 versus 9 and 10). Importantly, deletion of *SNF1* suppressed these effects of *reg1Δ*, yielding the reduced levels of eIF2α-P and T882-P in the *reg1Δ snf1Δ* double mutant characteristic of the *snf1Δ* single mutant (Fig. 3A and B, right panels). These data strongly support our conclusion that Snf1 stimulates Gcn2 kinase activity and attendant eIF2α phosphorylation under histidine starvation conditions and indicate that Reg1 constrains this Snf1 function in glucose medium. As expected, the S577-P/FL-Gcn2 ratio did not vary significantly among the WT, *reg1Δ* mutant, and *snf1Δ* mutant strains in these experiments (Fig. 3A and B).

Surprisingly, *reg1Δ* provoked a decrease, rather than an increase, in eIF2α-P under nonstarvation conditions (Fig. 3A, left panel), even though it did not reduce the level of T882-P (Fig. 3B,

left panel). Moreover, the decrease in eIF2α-P conferred by *reg1Δ* was reversed by the deletion of *SNF1* (Fig. 3A, left panel). Both the decreased eIF2α-P under nonstarvation conditions and the increased eIF2α-P upon 3AT treatment in *reg1Δ* cells were complemented by plasmid-borne *REG1* (Fig. 3C). The fact that *reg1Δ* reduces eIF2α-P without decreasing T882-P (Gcn2 activity) implies that activating Snf1 in nonstarved *reg1Δ* mutant cells increases the dephosphorylation of eIF2α-P by a protein phosphatase. The relevant phosphatase might be activated by 3AT in *reg1Δ* mutant cells as well, but its effect on eIF2α-P accumulation could be masked by Snf1-mediated activation of Gcn2.

Snf1 kinase activity is induced during histidine starvation. The fact that the increased levels of T882-P and eIF2α-P produced by *reg1Δ* in histidine-starved cells depend on *SNF1*, combined with the known function of Reg1 in negatively regulating Snf1, suggested that activation of Snf1 by the phosphorylation of T210 in the activation loop is required for its ability to stimulate Gcn2. To test this prediction, we examined the ability of *snf1-T210A* and catalytically inactive *snf1-K84R* to complement *snf1Δ* mutant cells for impaired eIF2α-P induction by 3AT. Both *snf1* alleles were indistinguishable from the empty vector (Fig. 4A). Furthermore, in the *snf1Δ reg1Δ* background, plasmid-borne *SNF1*, but not *snf1-T210A*, increased both eIF2α-P and T882-P levels above those in transformants with the empty vector under starvation conditions (Fig. 3D, upper and lower panels). These results imply that the activated level of Snf1 function requiring T210-P is necessary for stimulating Gcn2 activity in histidine-starved cells.

To test this conclusion further, we asked whether 3AT

FIG. 3. Reg1 negatively regulates Snf1 to stimulate Gcn2 phosphorylation on T882 and eIF2 α phosphorylation. (A and B) Transformants of strains H1642 (WT), HQY343 (*snf1 Δ*), HQY305 (*reg1 Δ*), and HQY347 (*snf1 Δ reg1 Δ*) harboring pDH101 (*FL-GCN2*) were cultured in the absence or presence of 3AT, and the eIF2 α -P/eIF2 α (A) and T882-P/FL-Gcn2 (B) ratios were determined as for Fig. 2A and B. For analysis of eIF2 α phosphorylation (A), aliquots of WCE with 10 and 20 μ g of protein were loaded for each strain. For analysis of Gcn2 phosphorylation (B), 4 and 6 mg of protein were immunoprecipitated for each strain. (C) Transformants of H1642 (WT) or HQY305 (*reg1 Δ*) bearing the empty vector or pHQ817 (*REG1*) were cultured and analyzed as for panel A. Ten and 20 μ g of protein were loaded for each strain. (D) Transformants of HQY347 (*snf1 Δ reg1 Δ*) with pDH101 (*FL-GCN2*) and pMO18 (HA-Snf1), pMO19 (HA-Snf1-T210A), or the vector pSK134 (V) were analyzed as for panels A and B; *gcn2 Δ* , strain H1895. In the upper panels, 10 and 20 μ g of protein were loaded for each strain. For detection of Gcn2 phosphorylation (lower panels), 4 and 6 mg of protein were immunoprecipitated for each strain. For panels A, B, and D, means and standard errors were calculated from the results of three or more independent experiments.

increases the phosphorylation of T210 in Snf1 by using phosphospecific antibodies (19, 26). Consistent with previous findings (16), the T210-P content of immunoprecipitated HA-tagged Snf1 was increased by *reg1 Δ* and undetectable in the strain expressing HA-Snf1-T210A (Fig. 4B, lanes 7 to 10 versus 1 and 2). Treatment with 3AT consistently provoked a moderate increase in the T210-P content of HA-Snf1 (cf. lanes 3 and 4 and lanes 5 and 6), suggesting that Snf1 function is stimulated by histidine starvation.

Detection of complexes containing Snf1 and Gcn2 *in vivo*. Having found that Snf1 kinase activity is required for its role in activating Gcn2, we wondered whether it might function directly by phosphorylating Gcn2. If so, we would expect to find that Gcn2 and Snf1 are physically associated *in vivo*. Indeed, Gcn2 coimmunoprecipitated specifically with HA-Snf1, being absent in immunocomplexes from cells expressing untagged Snf1 (Fig. 5A, top blot, lanes 3 and 11). Similarly, Snf1-HA coimmunoprecipitated specifically with Gcn2, being absent in immunocomplexes from *gcn2 Δ* mutant cells expressing Snf1-HA (Fig. 5A, bottom blot, lanes 3, 7, and 11). We were

unable to determine whether association of Gcn2 with Snf1 is induced by histidine starvation because Snf1 is activated during cell harvesting by the *g* stress of centrifugation or nutrient limitation in the dense cell pellet (32). Moreover, Gcn2 is activated in extracts from nonstarved cells (31). Hence, it is currently impossible to prepare native cell extracts for coimmunoprecipitation experiments in which Snf1 and Gcn2 are at basal levels of activation. Thus, whereas activated Snf1 and Gcn2 can clearly associate (Fig. 5A), it is not known if the nonactivated forms of these proteins can do so.

Having detected complexes containing native Gcn2 and Snf1, we explored whether phosphorylation of T882 in the Gcn2 activation loop is mediated by Snf1. Although T882 is autophosphorylated by Gcn2 *in vitro* (23), it was not known whether autophosphorylation is the primary source of T882-P *in vivo*. To address this, we examined the effect of inactivating Gcn2 by the K628R substitution in the active site on the level of T882-P in the mutant protein. If T882 is phosphorylated by Snf1 *in vivo*, then inactivation of Gcn2 should not eliminate T882-P. Indeed, the equivalent inactivating substitution in Snf1

FIG. 4. Snf1 kinase activity is induced in histidine-starved cells and is required to promote eIF2 α phosphorylation. (A) Cells were grown in SD with minimal supplements and treated with 40 mM 3AT for 90 min. WCEs were prepared, and eIF2 α -P was determined as described in the legend to Fig. 2A. Strains are transformants of HQY357 (*snf1Δ gcn2Δ*) with plasmids pHQ1162 (*SNF1*), YCplac33 (V), pHQ1873 (*snf1-K84R*), and pHQ1874 (*snf1-T210A*). Ten and 20 μ g of protein were loaded for each strain. Means and standard errors were calculated from three or more independent experiments. (B) Transformants of HQY343 (*snf1Δ*) with pMO19 (*HA-SNF1-T210A*) or pMO18 (*HA-SNF1*) and HQY347 (*snf1Δ reg1Δ*) with pMO18 (*HA-SNF1 reg1Δ*) were cultured in SD medium and left untreated or treated with 40 mM 3AT for 1 h. WCEs were subjected to Western analysis with antibodies specific for mammalian AMPK phosphorylated on T172.

(K84R) does not diminish T210-P because this residue is phosphorylated by upstream kinases (13, 16). At odds with this possibility for Gcn2, the K628R substitution eliminates T882-P in cells starved with 3AT (Fig. 5B). This makes it unlikely that Snf1 activates Gcn2 directly by T882 phosphorylation.

It was possible that Snf1 phosphorylates Gcn2 on a residue outside the activation loop to stimulate kinase activity, indirectly increasing the T882-P content. To examine this possibility, we coimmunoprecipitated catalytically inactive gcn2-K628R with either functional HA-Snf1 or nonfunctional HA-snf1-T210A and carried out immunocomplex kinase assays with radiolabeled ATP. We observed no labeling of gcn2-K628R in complexes with functional or nonfunctional Snf1-HA (Fig. 5C, lanes 1 and 2 versus 3 and 4). Consistent with this, a glutathione *S*-transferase-Snf1 protein purified from yeast, which was functional for autophosphorylation, did not produce any detectable phosphorylation of purified, catalytically inactive gcn2-K628R (data not shown). Finally, we tested the possibility that association with active Snf1 might increase the ability of Gcn2 to autophosphorylate its activation loop. Indeed, we consistently observed an increase in the labeling of

WT Gcn2, by a factor of 2.3 ± 0.19 , in immunocomplexes with HA-Snf1 versus HA-snf1-T210A (Fig. 5C, lanes 5 and 6 versus 7 and 8; data not shown). These last results suggest an indirect mechanism of Gcn2 activation by Snf1, which is considered further below.

Snf1 inhibits an eIF2 α phosphatase in galactose medium. Because Snf1 is activated in nonpreferred carbon sources and eIF2 α phosphorylation increases at low glucose concentrations (33), we went on to investigate whether Snf1 regulates Gcn2 activity during growth in galactose. When WT and *snf1Δ* mutant strains were shifted from glucose to galactose medium, eIF2 α -P declined in both strains within 20 min (Fig. 6A); however, whereas eIF2 α -P began to recover in WT cells after 4 h, eIF2 α -P remained at low levels in the *snf1Δ* mutant strain throughout the experiment. Thus, Snf1 promotes eIF2 α phosphorylation after cells are shifted from glucose to galactose medium.

We asked next if the reduced level of eIF2 α -P in WT and *snf1Δ* mutant cells evoked by shifting to galactose is associated with reduced Gcn2 activity. In contrast to the reduced level of eIF2 α -P seen in WT cells 90 min after a shift to galactose (Fig. 6A), T882-P in FLAG-Gcn2 was not diminished under these conditions (Fig. 6B, lanes 5 and 6 and lanes 1 and 2). Furthermore, the even larger reduction in eIF2 α -P seen in *snf1Δ* mutant cells after 90 min on galactose (Fig. 6A) is associated with an ~ 1.5 -fold increase in T882-P (Fig. 6B, lanes 7 and 8 versus 3 and 4). Hence, the reduced level of eIF2 α -P seen upon a shift to galactose does not result from lower Gcn2 activity. This implies that a higher level of eIF2 α -P phosphatase activity exists in galactose versus glucose medium and in *snf1Δ* mutant versus WT cells on galactose medium.

Interestingly, the increased T882-P observed in *snf1Δ* mutant cells after 90 min in galactose is associated with an ~ 2 -fold decrease in Ser577 phosphorylation compared to the levels observed in WT or *snf1Δ* mutant cells on glucose (Fig. 6B, lanes 7 and 8 versus 3 to 6). These data suggest that, in galactose medium, Snf1 promotes S577-P and thereby reduces Gcn2 activity. However, the fact that *snf1Δ* mutant cells exhibit lower levels of eIF2 α -P despite increased Gcn2 activity (T882-P level) implies that the inhibitory effect of Snf1 on eIF2 α -P phosphatase activity (deduced from Fig. 6B) exceeds Snf1's inhibitory effect on Gcn2 in galactose medium.

Reg1 reduces eIF2 α phosphorylation in galactose medium by inhibiting Snf1. Having obtained evidence that Snf1 inhibits an eIF2 α -P phosphatase (eIF2 α -PPase) in galactose medium, we wondered whether eliminating Reg1 would elevate eIF2 α -P by activation of Snf1 and attendant downregulation of eIF2 α -PPase activity. Consistent with this idea, *reg1Δ* evoked higher levels of eIF2 α -P in WT cells on galactose (Fig. 6C, upper panels, lanes 5 and 6 versus 1 and 2), similar to those above for histidine starvation. Furthermore, *snf1Δ* suppressed this phenotype in the *snf1Δ reg1Δ* double mutant and reduced eIF2 α -P to the low level observed in *snf1Δ REG1* mutant cells (Fig. 6C, lanes 7 and 8 versus 3 to 6). Thus, *reg1Δ* provokes a Snf1-dependent increase in eIF2 α -P in galactose. Importantly, *reg1Δ* produced only a modest increase in T882-P relative to that measured in WT cells on galactose, comparable to that seen in *snf1Δ* mutant cells (Fig. 6C, lower panels). Thus, an ~ 5 -fold higher level of eIF2 α -P in *reg1Δ* versus *snf1Δ* mutant cells occurs with no significant change in T882-P. This implies that,

FIG. 5. Functional and physical interactions of Snf1 with Gcn2 under conditions of histidine starvation. (A) Transformants of HQY344 (*gcn2Δ snf1Δ*) harboring pSNF1-316 (*SNF1-HA*) and either pAH15 (*GCN2*) (lanes 1 to 4) or the empty vector YEplac181 (lanes 5 to 8) or transformants containing pHQ1162 (*SNF1*) and pAH15 (lanes 9 to 12) were cultured as described in the legend to Fig. 2 in the presence of 3AT for 90 min, and WCEs were immunoprecipitated (IP) with anti-HA agarose and subjected to Western blot (WB) analysis using antibodies against Gcn2 or Gcd6 (top two panels). Gcd6 is a subunit of eIF2B, which presumably does not interact with Snf1, analyzed as a loading control. (Bottom of panel) WCEs were immunoprecipitated with antibodies against Gcn2 and subjected to Western analysis using anti-HA antibodies. In, P, and S correspond to the input, pellet, and supernatant, respectively. For inputs, 1% and 0.2% of the samples were loaded; for supernatants, 1% of the samples was loaded. (B) Transformants of HQY357 (*gcn2Δ snf1Δ*) harboring plasmids containing WT *GCN2* (pB107), *gcn2-K628R* (pB115), or *gcn2-T882A*, *T887A* (pB11) and either WT *HA-SNF1-HA* (pMO18) or *HA-SNF1-T210A* (pMO19) (*SNF1-TA*) were cultured with 40 mM 3AT and analyzed as described in the legend to Fig. 2B. Four and 6 mg of protein were immunoprecipitated for each strain. Means and standard errors were calculated from three independent experiments. (C) Cells were grown in SD medium and treated with 40 mM 3AT for 90 min. WCEs were prepared, HA-Snf1 was immunoprecipitated, and one-half of the immunocomplexes were subjected to Western analysis with anti-Gcn2 and anti-HA antibodies (bottom two panels); the other half was used for *in vitro* kinase assays with [γ - 32 P]ATP, which were resolved by SDS-PAGE and subjected to autoradiography (top two panels). The strains used were transformants of HQY357 (*snf1Δ gcn2Δ*) with plasmids pMO18 (*HA-SNF1*) and p2326 (pB34) (*gcn2-K628R*) (lanes 1 and 2), pMO19 (*HA-SNF1-T210A*) and p2326 (pB34) (lanes 3 and 4), pMO18 and p630 (*GCN2*) (lane 5 and 6), pMO19 and p630 (lanes 7 and 8), and pMO18 and the empty vector YEplac195 (lanes 9 and 10). One-half and 1 mg of protein were immunoprecipitated for each strain.

on galactose, an eIF2 α -PPase is activated when Snf1 function is enhanced by eliminating Reg1.

Protein phosphatase Glc7 reduces eIF2 α phosphorylation in glucose and galactose medium. Previous data suggested that the essential PP1 α Glc7 acts in opposition to Gcn2 in regulating levels of eIF2 α phosphorylation on glucose medium. Consistent with this, we found that the hypomorphic *glc7-1* allele confers increased eIF2 α -P on *GCN2* mutant cells in glucose (Fig. 7A, lanes 2 and 3 versus 10 and 11). We concluded above that Snf1 inhibits an eIF2 α -PPase on galactose medium. If Glc7 is an eIF2 α -PPase inhibited by Snf1 under these conditions, then *glc7-1* should dampen the reduction in eIF2 α -P that normally occurs in *snf1Δ* mutant cells on galactose. The results in Fig. 7A support this prediction, as the eIF2 α -P/eIF2 α ratio is higher in the *glc7-1 snf1Δ* double mutant than in the *GLC7 snf1Δ* mutant strain (cf. lanes 8 and 9 versus 16 and 17). Moreover, *glc7-1* does not increase Gcn2 activity on galactose (or glucose) medium, as *glc7-1* and *GLC7* mutant cells exhibit no significant differences in the T882-P content of FL-Gcn2

(Fig. 7B, lanes 1 to 8 versus 9 to 15). Thus, the moderately higher levels of T882-P on galactose versus glucose, and in *snf1Δ* versus *SNF1* cells, described above for *GLC7* cells, also occur in *glc7-1* mutant cells. The fact that *glc7-1* elevates eIF2 α -P in *snf1Δ* mutant cells without activating Gcn2 supports our conclusion that the eIF2 α -PPase function of Glc7 is inhibited by Snf1 on galactose medium.

In the experiment just described, we observed that *snf1Δ* still evokes a significant decrease in eIF2 α -P on galactose in the *glc7-1* background (Fig. 7A, lanes 8 and 9 versus 6 and 7). This could be explained by noting that *glc7-1* does not completely inactivate Glc7, so that releasing its residual activity from inhibition by Snf1 (in *snf1Δ* mutant cells) should lead to a further reduction in eIF2 α -P. However, an alternative possibility is that Snf1 inhibition of a second eIF2 α -PPase contributes to the increased eIF2 α -P conferred by *snf1Δ* in the *glc7-1* background. Indeed, we obtained evidence previously that the type 2A-related phosphatase Sit4 contributes to the dephosphorylation of eIF2 α on glucose, as *sit4Δ* mutant cells have elevated

FIG. 6. Snf1 stimulates eIF2 α phosphorylation in galactose-grown cells without activating Gcn2. (A) Strains H1642 (WT) and HQY343 (*snf1Δ*) were grown in minimally supplemented SD medium, harvested, washed, and cultured for the indicated times in the same medium containing galactose in the place of glucose. WCEs were analyzed as described in the legend to Fig. 2A. (B and C). Effects of *snf1Δ* and *reg1Δ* on eIF2 α -P and phosphorylation of T882 and S577 in FL-Gcn2 immunoprecipitated from cells cultured in galactose. (B) Transformants described in Fig. 2B were cultured for 90 min in galactose as for panel A and analyzed as described in the legend to Fig. 2B. (C) Strains described in Fig. 3A were cultured as for panel B and analyzed as described in the legend to Fig. 3A and B. For detection of eIF2 α phosphorylation, 10 and 20 μ g of protein were loaded for each strain. For detection of Gcn2 phosphorylation, 4 and 6 mg of WCEs were immunoprecipitated for each strain. Means and standard errors were calculated from three or more independent experiments.

eIF2 α -P levels under amino acid-replete conditions during growth in glucose medium (2). We confirmed this finding here (data not shown) and also observed that *sit4Δ* evokes increased eIF2 α -P in *SNF1* mutant cells on galactose (Fig. 7D, lanes 6 and 7 versus 2 and 3). However, *sit4Δ* does not significantly alter T882-P (or S577-P) levels in glucose or galactose medium (Fig. 7C and data not shown), indicating that Sit4 does not regulate Gcn2 activity. Thus, Sit4, in addition to Glc7, contributes to the dephosphorylation of eIF2 α -P in galactose-grown cells.

Importantly, in the *sit4Δ* background, deletion of *SNF1* still confers a strong reduction in eIF2 α -P (Fig. 7D, lanes 8 and 9 versus 6 and 7), comparable to the effect of *snf1Δ* on *SIT4* mutant cells. This suggests that Snf1 promotes eIF2 α -P in galactose primarily by inhibiting the eIF2 α -PPase activity of Glc7. If Sit4 and Glc7 together make up the bulk of the eIF2 α -PPase activity downregulated by Snf1, then deletion of *SNF1* in the *sit4Δ glc7-1* background should evoke little decrease in eIF2 α -P. This prediction was fulfilled by the results in lanes 14 to 17 of Fig. 7D. Hence, Glc7 and Sit4 are both downregulated by Snf1, and Glc7 is responsible for the majority of the eIF2 α -P dephosphorylation in galactose.

Finally, having found that Sit4 and Glc7 both promote the dephosphorylation of eIF2 α , we sought to demonstrate that eIF2 α associates with Sit4 and Glc7 in cell extracts. Indeed, we found that eIF2 α specifically coimmunoprecipitates with epitope-tagged forms of Sit4 and Glc7 in both glucose and galactose media (Fig. 7E and F). These findings strongly support the idea that Sit4 and Glc7 function directly as eIF2 α phosphatases *in vivo*.

DISCUSSION

Snf1 activates Gcn2 to promote eIF2 α phosphorylation in histidine-starved cells on glucose. We have shown here that histidine starvation elicits Gcn2 activation via increased phosphorylation of T882 in the activation loop and that eliminating Snf1 or impairing its activation by upstream kinases (with the T210A substitution) reduces both T882-P in Gcn2 and phosphorylated eIF2 α in 3AT-treated cells. In addition, histidine starvation evokes a moderate increase in T210-P levels in Snf1, and deletion of *REG1* confers elevated Gcn2-Thr882-P and eIF2 α -P in 3AT-treated cells in a manner dependent on Snf1. Thus, Snf1 is acti-

FIG. 7. Evidence that Snf1 antagonizes the eIF2 α phosphatase activities of Glc7 and Sit4. (A to D) Strains of the indicated genotype were cultured in galactose for 20 min as described in the legend to Fig. 6A and analyzed for eIF2 α -P, T882-P, and S577-P as described in the legend to Fig. 2A and B. (A) Transformants of *glc7-1 snf1Δ* mutant strain CY1194 harboring plasmids containing *SNF1* (pHQ1163) and *GLC7* (p28.1) (WT), *GLC7* and the empty vector (*snf1Δ*), *SNF1* and the empty vector (*glc7-1*), or two empty vectors (*glc7-1 snf1Δ*). (B) The *glc7-1 SNF1* and *glc7-1 snf1Δ* mutant strains are transformants of CY1194 with pDH101 (*FL-GCN2*) and either plasmid pHQ1163 (*SNF1*) or the empty vector (*snf1Δ*); *GLC7 SNF1* strains are cognate transformants of CY1323 (*GLC7 snf1Δ*) containing the same plasmids. (C) The WT and *snf1Δ* strains are H1642 and HQY343, respectively, transformed with pDH101 (*FL-GCN2*). The *sit4Δ SNF1* and *sit4Δ snf1Δ* mutant strains are CY1297 and CY1298 transformed with pDH101. (D) The strains employed are transformants of CY1194 harboring plasmids containing *SNF1* (pHQ1163) and the empty vector (*glc7-1 snf1Δ*), two empty vectors (*glc7-1 snf1Δ*), *GLC7* (p28.1) and pHQ1163 (*GLC7 SNF1*), or p28.1 and the empty vector (*GLC7 snf1Δ*). The *sit4Δ* mutant strains are transformants of CY1299 with two empty vectors (*glc7-1 sit4Δ snf1Δ*), with *SNF1* (pHQ1163) and the empty vector (*glc7-1 sit4Δ*), with p28.1 (*GLC7*) and pHQ1163 (*sit4Δ*), or with p28.1 and the empty vector (*sit4Δ snf1Δ*). For detection of eIF2 α phosphorylation (A and D), 10 and 20 μ g of protein were loaded for each sample. For detection of Gcn2 phosphorylation (B and C), 4 and 6 mg of protein were immunoprecipitated for each strain. Means and standard errors were calculated from three or more independent experiments. (E and F). Cells were grown in SD medium, and one-half of each culture was used for preparation of WCEs. The other half was washed with SD containing galactose in the place of glucose and cultured in the same galactose medium for 20 min and used for the preparation of WCEs. Sit4-HA (E) or Glc7-MYC (F) was immunoprecipitated from the WCEs with anti-HA or anti-myc agarose (Santa Cruz Biotechnology), respectively, resolved by SDS-PAGE, and subjected to Western analysis using antibodies against eIF2 α . Strains: E, *sit4Δ* (CY1297) transformed with *SIT4* (CB1770) or *SIT4-HA* (pSL2667); F, H1515 transformed with either untagged *GLC7* (p29.1) or *GLC7-MYC* (pHQ1774). In, P, and S correspond to the input, pellet, and supernatant, respectively. For inputs, 1% and 0.2% of the samples were loaded; 1% of the supernatants was loaded.

vated by histidine starvation and stimulates Gcn2 activity in glucose-grown cells (model in Fig. 8A). As T882-P and eIF2 α -P are still induced by 3AT in *snf1Δ* mutant cells, albeit to lower levels than in WT cells, there is also a Snf1-independent component of Gcn2 activation evoked by uncharged tRNA.

Our finding that Snf1 and Gcn2 are associated with one another in cell extracts is consistent with a direct role for Snf1 in stimulating Gcn2 activity. However, T882-P is undetectable in catalytically inactive gcn2-K628R immunoprecipitated from yeast, implying that T882-P formation *in vivo* depends primarily on autophosphorylation by Gcn2. Consistent with this, when examining the fraction of Gcn2 that specifically coimmunopre-

cipitates with Snf1, we found no Snf1-dependent phosphorylation of catalytically inactive gcn2-K628R. Interestingly, however, we observed a higher level of phosphorylation of active Gcn2 in such assays containing WT Snf1 versus those containing the *snf1*-T210A mutant.

One explanation for these last findings could be that the Snf1 KD interacts with the Gcn2 KD to mediate allosteric activation of Gcn2 autokinase activity or to block the access of a coimmunoprecipitating protein phosphatase that dephosphorylates the Gcn2 activation loop. Snf1 catalytic activity would be required for these putative regulatory functions involving physical interactions between the KDs of Snf1 and Gcn2, as the Snf1-

FIG. 8. Postulated roles of Snf1 in regulating eIF2 α -P levels by distinct mechanisms in cells limited for histidine or glucose. (A) In cells replete with glucose but limited for histidine, uncharged tRNA^{His} accumulates and activates Gcn2, increasing the phosphorylation of T882 in the Gcn2 activation loop and evoking increased eIF2 α phosphorylation (bold arrows). Snf1's contribution to Gcn2 activation is indicated by thinner arrows. High-level T882-P and eIF2 α -P formation depends on the phosphorylation of T210 in the Snf1 activation loop, which is negatively regulated by the Glc7/Reg1 PP1 complex. Snf1 might also promote eIF2 α -P formation by inhibiting Sit4 or the Glc7 complex with eIF2 α -PPase activity, whose regulatory/targeting subunit (X) is unknown. (B) In amino acid-replete, glucose-limited cells growing on galactose, uncharged tRNA is at low levels (depicted by gray shading) and Gcn2 activity/T882-P is at the basal level. Snf1 does not stimulate Gcn2 function; in fact, it seems to inhibit Gcn2 by promoting S577-P formation (not depicted here; see text for details). Hence, Snf1 promotes eIF2 α -P by inhibiting the eIF2 α -P phosphatase activities of Glc7/X and Sit4.

T210A substitution reduces Gcn2 activation and lowers eIF2 α -P but does not reduce Snf1 binding to Gcn2 in extracts (Fig. 5C). Another possibility is that Snf1 phosphorylates Gcn2 in a manner that enhances Gcn2 autokinase activity, but the hypothetical Snf1-phosphorylated residue is labile *in vitro* and, hence, was undetectable in the gcn2-K628R of Fig. 5C. Additional work is required to elucidate the mechanism of Gcn2 activation by Snf1 in histidine-starved cells.

Although Snf1 stimulates Gcn2 function in histidine-starved cells on glucose, it might also negatively regulate an eIF2 α -PPase to increase eIF2 α -P under these conditions (Fig. 8A). Our previous results indicated that Glc7 (29) and Sit4 (2) both downregulate eIF2 α -P in glucose, and we demonstrated here that this occurs without reducing Gcn2 activity (T882-P level). This provides evidence that both enzymes function directly as eIF2 α phosphatases, rather than dephosphorylating the Gcn2 activation loop, which is supported by our finding that eIF2 α is physically associated with Glc7 and Sit4 in extracts. Thus, if Snf1 inhibited one of these enzymes during histidine starvation, it would contribute to the Snf1-dependent increase in eIF2 α -P under that condition (Fig. 8A). Although it appears that Snf1 stimulates, rather than inhibits, an eIF2 α -PPase in the absence of histidine starvation on glucose medium, as *reg1* Δ reduces eIF2 α -P without changing the activation level of Gcn2 (T882-P) (Fig. 3A and B), it is possible that the situation is reversed during histidine starvation.

Snf1 negatively regulates eIF2 α phosphatases Glc7 and Sit4 in galactose medium. We found that Snf1 is also required to maintain normal levels of eIF2 α phosphorylation in cells shifted to galactose. Interestingly, this Snf1 function does not involve stimulation of Gcn2 activity, as T882-P levels are somewhat higher in *snf1* Δ mutant cells on galactose. Hence, Snf1 must promote eIF2 α -P by downregulating eIF2 α -PPase activ-

ity on galactose (Fig. 8B). This function of Snf1 is also negatively regulated by Glc7/Reg1, as *reg1* Δ provokes a strong increase in eIF2 α -P on galactose (without elevating T882-P) that is abolished in the *snf1* Δ *reg1* Δ double mutant.

We found that mutations impairing Glc7 or Sit4 elevate eIF2 α -P in galactose, showing that both enzymes contribute to eIF2 α dephosphorylation and, hence, are candidates for inhibition by Snf1 in this carbon source. If Sit4 was the sole eIF2 α -PPase downregulated by Snf1 in galactose, then *sit4* Δ should abolish the effect of removing Snf1 on eIF2 α -P levels. This was not the case, however, as *sit4* Δ *snf1* Δ mutant cells exhibited markedly lower levels of eIF2 α -P compared to *sit4* Δ single mutants on galactose. This finding suggests that the eIF2 α -PPase activity of Glc7 is also downregulated by Snf1 on galactose, which is supported by our finding that *glc7-1* blunts the effect of *snf1* Δ in lowering eIF2 α -P. The residual reduction in eIF2 α -P levels produced by *snf1* Δ in *glc7-1* mutant cells likely results from inhibition of Sit4 by Snf1 because *snf1* Δ evokes no reduction in eIF2 α -P in *glc7-1* *sit4* Δ mutant cells, where both phosphatases are impaired. Together, our results indicate that Snf1 inhibits the eIF2 α -PPase activities of both Sit4 and Glc7 in galactose medium and that Glc7 is responsible for most of the eIF2 α -P dephosphorylation seen under these conditions (Fig. 8B). It is unknown what regulatory subunit Glc7 might utilize for its eIF2 α -PPase activity (Fig. 8B). It is unlikely to be Reg1, considering that the increases in eIF2 α -P produced by *reg1* Δ in histidine-starved cells, or after a transfer to galactose, are fully suppressed by *snf1* Δ , indicating that Reg1 acts upstream of Snf1 in regulating eIF2 α -P.

Can we rationalize the fact that Snf1 employs distinct mechanisms to promote eIF2 α phosphorylation in histidine-starved versus galactose-grown cells, activating Gcn2 in the former and downregulating eIF2 α -PPases in the latter? The fact that Snf1 stimulates Gcn2 activity only in histidine-starved cells might be explained by noting that the principal activating ligand for Gcn2, uncharged tRNA, accumulates to high levels under these conditions (34), but probably not after the cells are shifted to galactose. Hence, the Snf1 stimulation of Gcn2 might be observed only when uncharged tRNA accumulates and Gcn2 can be activated by this important stimulatory ligand (Fig. 8A). On the other hand, the ability of Snf1 to effectively inhibit an eIF2 α -PPase, as it does in galactose, might require the elevated Snf1 kinase activity that prevails only in the absence of glucose.

Besides its principal role in helping yeast cells adapt to glucose limitation and use alternative carbon sources, Snf1 has been implicated in responses to environmental stress (9). Our results extend Snf1's purview to include a key translational response to amino acid starvation. By stimulating eIF2 α phosphorylation, Snf1 contributes to the reduction in general protein synthesis evoked by eIF2 α -P, a common response to starvation and stress that conserves amino acids and facilitates the reprogramming of gene expression. Snf1's role in promoting eIF2 α -P accumulation in galactose could be viewed as a means of reducing the energy consumption in protein synthesis during growth on a nonpreferred carbon source.

The other important consequence of eIF2 α phosphorylation is induction of *GCN4* translation and attendant transcriptional activation of amino acid biosynthetic enzymes by Gcn4. However, Arndt and colleagues reported (and our findings confirm) that eliminating Snf1 function does not reduce *GCN4* transla-

tion in amino acid-starved cells. In fact, the translation of a *GCN4-lacZ* reporter is partially derepressed, as is that of numerous Gcn4 target genes, in cells lacking Snf1 function on glucose medium without amino acid limitation, conditions under which eIF2 α -P is at basal levels (25). Thus, it seems likely that inactivation of Snf1 alters a step in translation initiation in a way that reduces the inhibitory effects of the *GCN4* uORFs and partially derepresses *GCN4* translation independently of high-level eIF2 α -P. As noted above, the fact that *snf1 Δ* leads to increased Gcn4 synthesis but confers a 3AT^s phenotype is explained by the fact that Snf1 also stimulates the ability of Gcn4 to activate the transcription of *HIS3* (14).

Together, the available data indicate that Snf1 interfaces with translational and transcriptional responses to amino acid starvation at multiple levels in budding yeast. In cells starved for histidine in minimal glucose medium, Snf1 kinase function is needed for the high-level activation of Gcn2 and eIF2 α -P accumulation that downregulates general translation initiation under starvation conditions. In WT cells, this function of Snf1 is expected to also enhance the translational induction of *GCN4*. However, detecting the absence of this function in *snf1* mutants might be confounded by the fact that Snf1 inactivation derepresses *GCN4* translation by an unknown mechanism. In histidine-starved cells, Snf1 not only is required for high-level eIF2 α phosphorylation by Gcn2 but also promotes transcriptional activation by Gcn4. In galactose medium, Snf1 maintains the appropriate level of eIF2 α -P by inhibiting the eIF2 α -PPase activities of Glc7 and Sit4. The phosphorylation levels of other substrates for these protein phosphatases are also likely enhanced by the activated form of Snf1 present in galactose and other nonpreferred carbon sources.

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