Eukaryotic Cell

Reg1 Protein Regulates Phosphorylation of All Three Snf1 Isoforms but Preferentially Associates with the Gal83 Isoform

Yuxun Zhang, Rhonda R. McCartney, Dakshayini G. Chandrashekarappa, Simmanjeet Mangat and Martin C. Schmidt

Eukaryotic Cell **2011, 10(12):1628. DOI: 10.1128/EC.05176-11.**
Published Ahead of Print 14 October 2011.

EUKARYOTIC CELL, Dec. 2011, p. 1628–1636 Vol. 10, No. 12
1535-9778/11/$12.00 doi:10.1128/EC.05176-11

Reg1 Protein Regulates Phosphorylation of All Three Snf1 Isoforms but Preferentially Associates with the Gal83 Isoform▽

Yuxun Zhang, Rhonda R. McCartney, Dakshayini G. Chandrashekarappa, Simmanjeet Mangat, and Martin C. Schmidt*

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received 11 July 2011/Accepted 5 October 2011

The phosphorylation status of the Snf1 activation loop threonine is determined by changes in the rate of its dephosphorylation, catalyzed by the yeast PP1 phosphatase Glc7 in complex with the Reg1 protein. Previous studies have shown that Reg1 can associate with both Snf1 and Glc7, suggesting substrate binding as a mechanism for Reg1-mediated targeting of Glc7. In this study, the association of Reg1 with the three Snf1 isoforms was measured by two-hybrid analysis and coimmunoprecipitation. We found that Reg1 association with Snf1 occurred almost exclusively with the Gal83 isoform of the Snf1 complex. Nonetheless, Reg1 plays an important role in determining the phosphorylation status of all three Snf1 isoforms. We found that the rate of dephosphorylation for isoforms of Snf1 did not correlate with the amount of associated Reg1 protein. Functional chimeric β subunits containing residues from Gal83 and Sip2 were used to map the residues needed to promote Reg1 association with the N-terminal 150 residues of Gal83. The Gal83 isoform of Snf1 is the only isoform capable of nuclear localization. A Gal83-Sip2 chimera containing the first 150 residues of Gal83 was able to associate with the Reg1 protein but did not localize to the nucleus. Therefore, nuclear localization is not required for Reg1 association. Taken together, these data indicate that the ability of Reg1 to promote the dephosphorylation of Snf1 is not directly related to the strength of its association with the Snf1 complex.

The Snf1 kinase of *Saccharomyces cerevisiae* is a member of the AMP-activated protein kinase (AMPK) family that is conserved among all eukaryotic organisms. The kinase activity of AMPKs is regulated in response to nutrient and energy availability. In mammalian cells, low energy is sensed by an increase in AMP, which is a direct activator of AMPKs (21, 38). In yeast cells, the Snf1 kinase is activated under conditions of glucose limitation (8). The active form of the Snf1/AMPK enzyme is a heterotrimer with one α, one β, and one γ subunit (13). The α subunit contains the kinase domain, and its catalytic activity requires phosphorylation of the kinase activation loop (32, 43a). The mammalian γ subunit contains four potential binding sites for adenosine molecules. Binding of AMP causes a 2-fold allosteric activation of the kinase activity (38, 45). Even more importantly, binding of AMP protects the kinase activation loop from dephosphorylation, which can account for a 1,000-fold increase in kinase activity (38, 45). Yeast Snf1 is also regulated at the level of its dephosphorylation (36), although the role played by AMP is uncertain.

Yeast and mammalian AMPK enzymes exist as distinct isoforms defined by the association of distinct subunits. Mammals carry two α subunit genes, two β subunit genes, and three γ subunit genes, allowing for the formation of 12 distinct isoforms of mammalian AMPK. Yeast cells express a single α subunit (Snf1), a single γ subunit (Snf4), and three different β subunits (Gal83, Sip1, and Sip2). Thus, yeast cells express three isoforms of AMPK that differ only in the identity of the β subunit. The three β subunits are not completely redundant with one another. Cells expressing Gal83 or Sip2 as their only β subunit are Snf^+, whereas cells expressing Sip1 as the only β subunit display a modest Snf^- phenotype and are clearly defective for aerobic growth (40, 53). Furthermore, the β subunits control the subcellular localization of the Snf1 complex (50) and may contribute to substrate specification (40). Studies with fusions to green fluorescent protein (GFP) have shown that the Snf1 isoform containing Gal83 is the only isoform to show significant nuclear localization (22, 50) following glucose limitation. This finding would lead one to believe that the Gal83 isoform is largely responsible for the phosphorylation of nuclear substrates such as the transcription factor Mig1. However, cells expressing Sip2 as the only β subunit are perfectly capable of regulating genes under the control of Mig1, despite the fact that the Sip2 isoform of the Snf1 kinase is excluded from the nucleus (53). Thus, nuclear targets of the Snf1 kinase must cycle through the cytoplasm, or Snf1 isoforms other than Gal83 must have at least some access to the nuclear compartment.

Activation of the AMPK enzyme requires phosphorylation of the kinase activation loop, a mechanism common to many protein kinases (1). The key regulated step controlling the phosphorylation of AMPK in both yeast and mammals is the dephosphorylation reaction (36, 38, 45). In mammals, the identity of the AMPK phosphatase has been proposed to be the PP2C phosphatase. Evidence supporting this comes from the observations that purified human PP2Cα can dephosphorylate AMPK *in vitro* (10) and that reduced expression of PP2Cα by use of small interfering RNA (siRNA) blunts the effect of tumor necrosis factor alpha (TNF-α) on AMPK signaling (44). However, recent studies have also shown that the PP1 phosphatase in complex with the regulatory protein R6 is the pre-

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, 531 Bridgeside Point II, 450 Technology Drive, Pittsburgh, PA 15219. Phone: (412) 648-9243. Fax: (412) 624-1401. E-mail: mcs2@pitt.edu.

▽ Published ahead of print on 14 October 2011.

TABLE 1. *S. cerevisiae* strains used for this study

Strain	Genotype
MSY920	*MAT***a** *ura3-52 leu2Δ0 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3 snf1Δ10*
MSY990	*MATα ura3-52 leu2Δ1 trp1Δ63 his3Δ200 reg1Δ::HIS3*
PJ69-4A	*MAT***a** *ura3-52 leu2-3,112 trp1-901 his3Δ200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*
MSY1127	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sip1Δ::KAN sip2Δ::KAN gal83Δ::KAN*
MSY1038	*MATα ura3-52 leu2 his3Δ200 trp1Δ63 SNF1-3HA reg1Δ::HIS3 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3*
MSY1141	*MAT***a** *ura3 leu2 trp1-901 his3 gal4Δ gal80Δ LYS2::GAL1-HIS3 met2::GAL7-lacZ sip1Δ::KAN sip2Δ::KAN gal83Δ::KAN*
MSY557	*MATα ura3-52 leu2Δ1 trp1Δ63 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3*

dominant AMPK phosphatase in mouse MIN6 β cells (18). The idea that a mammalian PP1 phosphatase is an important regulator of mammalian AMPK is intriguing, since it has long been known that the yeast PP1 phosphatase Glc7 is the primary phosphatase responsible for inactivating Snf1 (48). Members of the PP1 phosphatase family function in association with regulatory subunits that target the PP1 phosphatase to distinct subcellular localizations and specify substrate selection (42). In yeast, the regulatory subunit that targets Glc7 to Snf1 and other proteins in the glucose repression pathway is Reg1 (49). Mutations in the *REG1* gene lead to hyperactivation and hyperphosphorylation of the Snf1 kinase. In a recent study, the altered glycogen metabolism found in *reg1* mutants was identified as an important determinant of Snf1 activity (37). In mammalian cells, the Snf1 homolog is regulated by adenyl nucleotide binding (20). The nucleotide binding sites found in the mammalian enzyme are conserved in Snf4, the yeast γ subunit, although ligand-mediated regulation of Snf1 has yet to be demonstrated. Further studies will be needed to determine whether the deletion of *REG1* acts via glycogen metabolism, changes in adenyl nucleotide levels, PP1 substrate selection, or some combination of the above.

The *REG1* gene was first identified in genetic screens for mutations that relieved glucose repression of the invertase (16, 54) and *GAL7* (31) genes. Subsequent studies showed that Reg1 bound to the yeast PP1 phosphatase Glc7 and that these proteins functioned together in glucose repression (49). Since that time, it has become clear that the PP1 phosphatases act in concert with a wide array of PP1-interacting proteins that function to activate, inhibit, localize, or select substrates for the catalytic subunit (5). The exact mechanism by which the Reg1 protein exerts its influence over Glc7 is not currently known. However, reports that the Reg1 protein interacted with Snf1 in a two-hybrid system (29) and was an abundant component in purified preparations of Snf1 kinase (14) suggested that Reg1 might function by directly recruiting the Glc7 phosphatase to its substrate, the Snf1 kinase. However, not all the data support this model. The same point mutations in the Reg1 protein that disrupt binding to Glc7 also disrupt binding to Snf1, suggesting that these proteins may compete for binding to the same surface of Reg1 (46). If Glc7 and Snf1 compete for binding to the same site on Reg1, it is hard to imagine how Reg1 could target Glc7 to Snf1. Also, there is no mass spectrometry or two-hybrid evidence that Reg1 interacts directly with other substrates of the Glc7-Reg1 complex, such as the Mig1 or Hxk2 protein. In addition, a recent report showed that the change in glycogen metabolism that is present in *reg1* mutants may play an important role in the regulation of Snf1 kinase (37), suggesting that the direct interaction of Reg1 and Snf1 proteins may not be as significant as previously thought. In this study, we examined the ability of the Reg1 protein to interact with the three different isoforms of Snf1. Surprisingly, we found that a single isoform accounts for essentially all the interaction with Reg1 yet the phosphorylation of all three isoforms is regulated by Reg1. These data suggest that binding of Reg1 to the Snf1 complex is not required for its ability to direct Glc7 to the Snf1 kinase complex.

MATERIALS AND METHODS

Yeast strains, and media. The *Saccharomyces cerevisiae* strains used in this study are all derived from S288C and are described in Table 1. Strains with multiple gene deletions were produced by genetic crosses and sporulation. MSY1141 was derived from a cross of PJ69-4a (25) and MSY1127 (Table 1). Growth of cells utilized standard media at 30°C (35) with either 2% or 0.05% glucose (g/100 ml), as indicated. Raffinose medium contained 2% raffinose, 0.01% glucose (g/100 ml), and 1 μg/ml antimycin A. Transformation of yeast strains was done by the lithium acetate method (19).

Plasmid construction. Plasmid pACT-Reg1, expressing the Gal4 activation domain fused to Reg1 amino acids 443 to 1014, with five copies of the V5 epitope at the C terminus, was made by cloning the 2,039-nucleotide (nt) NcoI-BamHI fragment from pReg1-5V5 (46) into the NcoI and BamHI sites of pACT2 (27). Plasmid pACT-Reg1-1014, expressing the Gal4 activation domain fused to amino acids 1 to 1014 of Reg1, with five copies of the V5 epitope at the C terminus, was made by amplifying the DNA encoding the N terminus of Reg1 with a PCR primer that created an in-frame NcoI site at the initiating ATG codon. The PCR product encoding the N-terminal 434 amino acids of Reg1 was then cloned into the NcoI site of pACT-Reg1. Plasmids expressing hemagglutinin (HA)-tagged Snf1 (32) and Flag-tagged β subunits (30) have been described previously. Chimeras between the triple-Flag-tagged Gal83 and triple-Flag-tagged Sip2 open reading frames were created using QuikChange II (Agilent Technologies) mutagenesis and subcloning. Junctions and sequences of the chimeras are shown in Table 2. DNA encoding the yeast codon-optimized enhanced green fluorescent protein (yEGFP) was PCR amplified using plasmid pKT127

TABLE 2. Sip2 and Gal83 chimeras

Plasmid	N-terminal aa	C-terminal aa	Junction sequence	Epitope tag
pG3S2-5	Gal83 1-3	Sip2 7-415	MAG-3-Flag-hpaqkkqt	3-Flag tag
pG3S2-150	Gal83 1-147	Sip2 150-415	GFQQQQEQ-easggpse	3-Flag tag
pG3S2-244	Gal83 1-246	Sip2 248-415	NYMEVSAP-eknptnek	3-Flag tag
pS2G3-5	Sip2 1-4	Gal83 6-417	MGTT-3-Flag-penkdasm	3-Flag tag
pS2G3-150	Sip2 1-147	Gal83 147-417	EEGQQQIR-s-qqqgtveg	3-Flag tag
pS2G3-244	Sip2 1-245	Gal83 244-417	NFVNYIEV-sappdwgn	3-Flag tag
pG3S2-150U	Gal83 1-147	Sip2 150-415	GFQQQQEQ-easggpse	None

(41) as the template and was added to the C termini of the open reading frames for Gal83, Sip2, and the Gal83-Sip2-150 fusion protein. Constructs were confirmed by DNA sequencing. Each protein was expressed from its own promoter (the Gal83 promoter in the case of the Gal83-Sip2-150 chimera), using plasmid pRS316 as the vector (43).

Two-hybrid analysis. Two-hybrid interactions were assessed by growth on synthetic complete medium containing 2% (g/100 ml) glucose and lacking histidine, using strains bearing the *GAL1-HIS3* reporter integrated at the *LYS2* locus. The positive-control plasmids used in these studies encoded the herpes simplex virus capsid protein 22a (11). The entire open reading frame for the Snf1 protein was expressed as a fusion to the Gal4 DNA binding domain in pGBT9 (4). The Reg1 protein (amino acids 443 to 1014) was expressed as a fusion to the Gal4 activation domain in the plasmid pACT2 (Clontech). The Snf4 protein was expressed as a fusion to the Gal4 activation domain by using plasmid pNI12 (17).

Western blotting and immunoprecipitation. Snf1-HA was detected with a 1:2,000 dilution of HA probe (Santa Cruz), Reg1-5V5 was detected with a 1:1,000 dilution of anti-V5 (Invitrogen), and β subunits tagged with a triple-Flag epitope were detected with a 1:1,000 dilution of mouse monoclonal anti-Flag (Sigma). Actin was detected with a mouse monoclonal antibody (Abcam) used at a 1:1,000 dilution. Goat anti-mouse IgG DyLight 800 (Thermo) diluted 1:5,000 was used as the secondary antibody. Blots were processed by using a Snap identification system (Millipore) and scanned by using an Odyssey scanner (Li-Cor). Integrated intensity values for triplicate bands were quantified by using Odyssey scanning software. To detect Snf1 activation loop (Thr210) phosphorylation, protein extracts were prepared using the NaOH cell lysis method (26). Protein extracts (800 μg) were immunoprecipitated in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors, using 20 μl HA probe agarose conjugate (Santa Cruz). Bound proteins were eluted in SDS sample buffer and resolved in SDS gels. Blots were treated with Odyssey blocking buffer (Li-Cor). For detection of phosphorylated Snf1, either phospho-AMPKα (Thr172) antibody (Cell Signaling) (diluted 1:1,000) or a rabbit polyclonal antibody directed against phosphorylated Snf1 T210 (diluted 1:500) was used. Goat anti-rabbit IRDye 800CW (Li-Cor) (1:5,000 dilution) was used as a secondary antibody. Immunoprecipitation of Reg1-V5 and Snf1-HA was detected by preparing glass bead protein extracts in RIPA buffer supplemented with protease and phosphatase inhibitors. Extracts (500 μg) were incubated with 20 μl HA probe agarose conjugate (Santa Cruz) and washed extensively, and bound protein was eluted in SDS sample buffer.

Fluorescence microscopy. Cells lacking all three β subunit genes (MSY557) were transformed with the β subunit-yEGFP plasmids and grown to mid-log phase in medium containing a mixture of glycerol and ethanol as the carbon source. Cells were collected by centrifugation and suspended in buffer containing 10 mM potassium phosphate, pH 6, 2 mM magnesium sulfate, 25 mM ammonium sulfate, and 2% (vol/vol) glycerol. Cells were washed once in the same buffer and immediately examined by microscopy. Images were collected with a Nikon Ti inverted microscope using a 100× 1.4-numerical-aperture (NA) Plan Apochromat objective and a Photometrics HQ2 camera. Images were processed using NIS Elements software. Images of at least 100 cells of each sample were collected, and representative images are shown.

RESULTS

All three Snf1 isoforms show glucose-regulated phosphorylation. Activation of the Snf1 kinase requires phosphorylation of the activation loop at threonine 210 (T210). In this study, we asked whether all three isoforms of the Snf1 kinase exhibit the same degree of glucose-mediated regulation of activation loop phosphorylation. Cells expressing a single β subunit, and therefore a single isoform of Snf1, were grown in high glucose (H) or shifted to low glucose (L) for 2 h. The Snf1 kinase was harvested by immunoprecipitation, and its phosphorylation status at T210 was assessed by quantitative Western blotting using antibodies that detect total Snf1 and the phosphorylated form of Snf1 (Fig. 1). While the Gal83 and Sip2 isoforms exhibited a more pronounced increase in phosphorylation status, all three isoforms exhibited statistically significant ($P < 0.01$) increases in phosphorylation following the shift to low glucose.

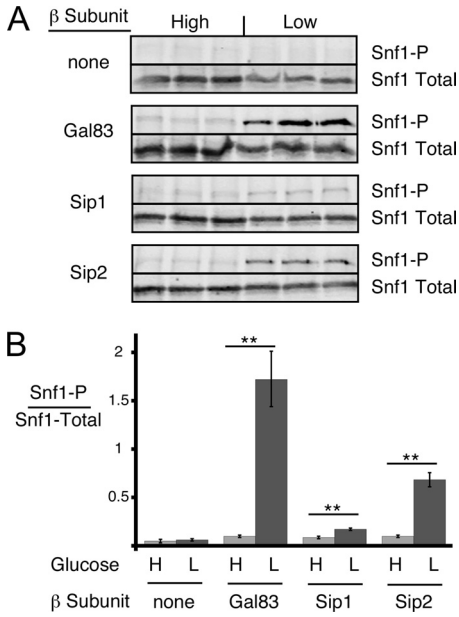

FIG. 1. Glucose-regulated phosphorylation of Snf1 isoforms. Protein extracts were prepared from cells grown in high (H) and low (L) glucose. Cells expressed either no Snf1 β subunit or the single β subunit shown. Snf1 tagged with the HA epitope was collected using anti-HA beads. Proteins were eluted and detected with antibodies directed against phosphorylated Snf1 (anti-PT210) or total Snf1 (anti-HA). Assays were repeated in triplicate. Western blots are shown (A), and the mean values for phosphorylated Snf1 divided by total Snf1 were also plotted, with error bars representing 1 standard error (B). Each isoform showed statistically significantly increased phosphorylation in low glucose (**), with a P value of <0.01.

Reg1-Snf1 interaction detected in the two-hybrid system. In order to better characterize the interaction of the Reg1 protein with the Snf1 kinase, the yeast two-hybrid system was used (4). For a positive-control pair, we used amino acids 307 to 610 of the herpes simplex virus capsid protein 22a fused to the Gal4 DNA binding domain in pGBT9 and the Gal4 activation domain in pGAD424 (11). This viral protein interacts strongly with itself and also provides excellent negative controls, since capsid protein 22a does not show a two-hybrid interaction with any of the yeast proteins used in this study. Two-hybrid plasmids were introduced into the two-hybrid reporter strain PJ69-4a (25). The *GAL1-HIS3* reporter was used to detect an interaction as growth on medium in the absence of added histidine (Fig. 2A). As expected from earlier results (11, 17, 29), we detected a strong interaction between 22a and 22a, between Snf1 and Reg1, and between Snf1 and Snf4 but not between 22a and Snf1. The Snf1-Reg1 interaction was detected using two-hybrid constructs expressing the entire Reg1 protein (residues 1 to 1014) or a smaller construct containing Reg1 residues 443 to 1014. Subsequent studies used the smaller construct (Reg1 residues 443 to 1014) because it was consistently expressed at a higher level (not shown).

Two-hybrid interaction of Reg1 and Snf1 requires the Snf1 β subunits. To further analyze the Reg1-Snf1 interaction, we generated a new two-hybrid reporter strain that lacked the genes for all three Snf1 β subunits. This strain, MSY1141 (Table 1), allowed us to determine whether an intact Snf1

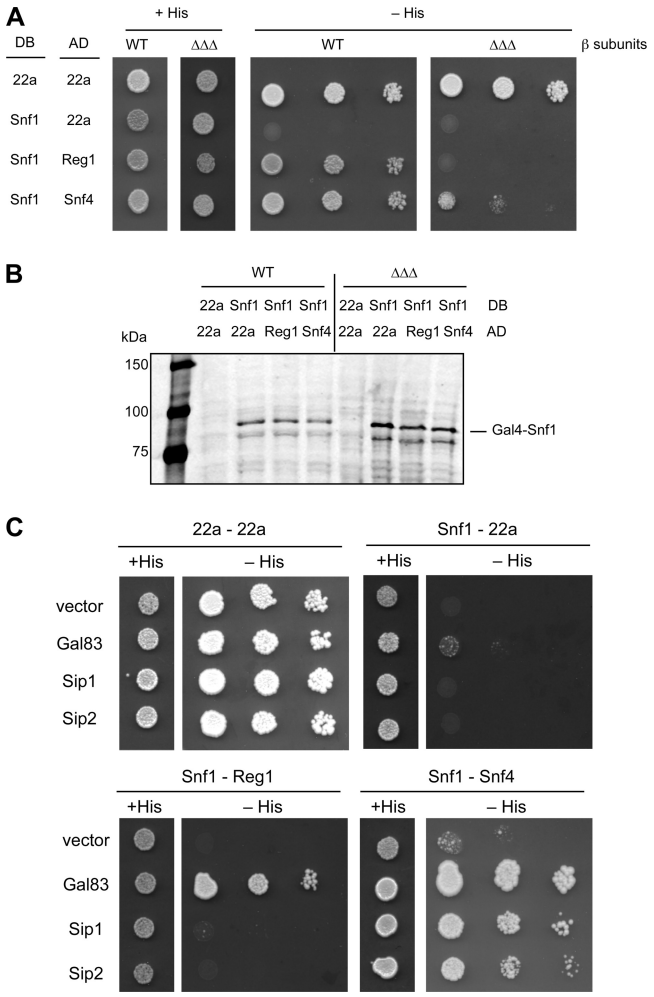

FIG. 2. β subunit requirements for Snf1 two-hybrid interactions. The herpes simplex virus capsid protein 22a forms homodimers and was used as a positive control when paired with itself (11). 22a was used as a negative control when paired with Snf1. Two-hybrid interactions were assessed in yeast strains with the *GAL1-HIS3* reporter gene on media supplemented with (+ His) or lacking (− His) histidine. (A) The requirement for intact Snf1 heterotrimers was assessed by comparing two-hybrid interactions in strains containing (WT) or lacking (ΔΔΔ) all three genes for the β subunits. (B) Western blot showing expression of the Gal4-Snf1 fusion protein in strains with (WT) and without (ΔΔΔ) β subunits. Proteins expressed as fusions with the Gal4 DNA binding domain (DB) or Gal4 activation domain (AD) are indicated above each lane. (C) The β subunit requirement for the Snf1-Reg1 and Snf1-Snf4 interactions was determined by transforming a strain lacking all three β subunit genes with a low-copy-number plasmid expressing a single β subunit or with empty vector, as shown.

heterotrimer was needed for interaction of Snf1 with Reg1 and whether all three isoforms of Snf1 interacted with Reg1. The same two-hybrid pairs were introduced into the triple mutant β subunit deletion reporter strain, and interactions were assessed by growth in the absence of histidine (Fig. 2A). The herpesvirus capsid protein 22a showed a strong self-interaction regardless of whether the β subunits were present (WT) or absent (ΔΔΔ). In contrast, the absence of the β subunits caused a sharp reduction in growth for the Snf1-Reg1 pair and the Snf1-Snf4 pair. The Gal4-Snf1 fusion protein was expressed at similar levels in cells with and without β subunits (Fig. 2B). The finding that the Snf1-Reg1 and Snf1-Snf4 interactions required the Snf1 β subunits was not unexpected. Structural studies of the Snf1 heterotrimer (3) demonstrated the important role of the β subunit in forming the interaction interface between the α (Snf1) and γ (Snf4) subunits, and purification of Snf1 from cells lacking the β subunits showed no association of the γ subunit (15). Furthermore, coimmunoprecipitation studies from our lab had previously noted that Reg1 did not associate with Snf1 in the absence of a β subunit (30). Thus, the two-hybrid interactions in our reporter strain lacking the three β subunits were able to confirm interaction results from independent studies.

The Gal83 isoform of Snf1 shows the strongest interaction with Reg1 in the two-hybrid system. We next sought to determine whether Reg1 interacted with all three isoforms of Snf1. The two-hybrid reporter strain lacking all three genes for the Snf1 β subunits was transformed with low-copy-number plasmids that expressed a single β subunit. Each two-hybrid pair was analyzed for interaction with each of the β subunits or with no β subunit (transformed with empty vector). The herpesvirus capsid 22a self-interaction was independent of the β subunits (Fig. 2C). Similarly, Snf1 and 22a failed to interact, regardless of whether a β subunit was present or not. Interestingly, the Snf1-Reg1 interaction was reconstituted only when the Gal83 protein was present. Neither Sip1 nor Sip2 was able to support Snf1-Reg1 interaction in the two-hybrid system, as measured by growth on medium lacking histidine. Using the more sensitive *lacZ* reporter system, Gal83 promoted the strongest interaction between Snf1 and Reg1; however, Sip2 did provide weak but detectable *lacZ* activity above the background (not shown). The stronger interaction provided by Gal83 was not a product of β subunit expression levels, since overexpression of the Sip2 protein from a high-copy-number plasmid failed to reconstitute the Snf1 interaction with Reg1 on medium lacking histidine (not shown). In contrast to the case with the Snf1-Reg1 pair, any one of the β subunits was able to reconstitute the interaction between Snf1 and Snf4. Thus, all three of the β subunits were expressed, and all three were able to support the two-hybrid reconstitution of the Gal4 transcription factor in the cell nucleus. The ability of Snf1 and Reg1 to interact in the two-hybrid system was independent of Snf1 kinase activity, since a kinase-dead subunit, Snf1-K84R (lysine 84 changed to arginine), was able to interact with Reg1 in a Gal83-dependent manner (data not shown).

The Gal83 isoform of Snf1 shows the strongest interaction with Reg1 in a coimmunoprecipitation assay. Our two-hybrid study suggested that Reg1 interacted preferentially with one of the three Snf1 isoforms. We were concerned that the interaction in the two-hybrid system might be affected by a protein's ability to localize to the nucleus. Indeed, earlier studies from the Carlson lab had shown that the Gal83 isoform of Snf1 was the only one to manifest significant nuclear localization (22). Therefore, we sought an independent assay for Reg1-Snf1 interaction that did not depend on nuclear localization. We expressed epitope-tagged Snf1 from its endogenous chromosomal locus and Reg1 from a low-copy-number plasmid in cells that lacked the genes for Reg1 and all three β subunits. A second low-copy-number plasmid was introduced that ex-

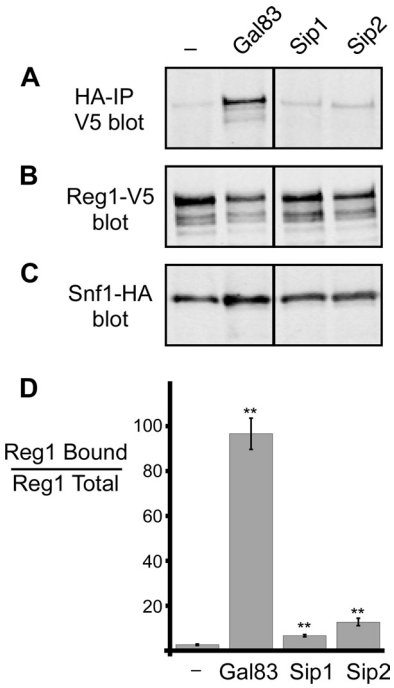

FIG. 3. Coimmunoprecipitation of Reg1 with the three Snf1 isoforms. Protein extracts were prepared from cells expressing Reg1 protein tagged with the V5 epitope and Snf1 tagged with the HA epitope. Cells expressed a single or no β subunit, as indicated. (A) Snf1 and associated proteins were collected with HA beads, and bound Reg1 was detected in an anti-V5 Western blot. (B and C) Total Reg1 and Snf1 expression was monitored by Western blotting. All samples were analyzed in triplicate, and representative blots are shown. (D) The mean amount of Reg1 bound relative to total Reg1 was plotted. Error bars indicate 1 standard error. Statistical analysis using the Student *t* test detected significant associations of Reg1 with all three isoforms of Snf1 compared to association in the absence of a β subunit ($P < 0.01$).

FIG. 4. Reg1 promotes dephosphorylation of the Gal83 and Sip2 isoforms of Snf1. Three independent transformants of cells, with and without Reg1 and expressing either Gal83 or Sip2 as the only β subunit, were grown in high-glucose medium. Extracts were prepared, and the level of Snf1 phosphorylation was determined by quantitative Western blotting. Representative blots are shown (top), and the mean values are plotted below, with 1 standard error indicated. Differences between samples with and without Reg1 were statistically significant (*, $P < 0.05$; **, $P < 0.01$).

pressed a single β subunit or no β subunit. Extracts were prepared in triplicate from cells shifted to low glucose, since these conditions gave the strongest Reg1-Snf1 interaction (46). Snf1 was collected by immunoprecipitation, and the abundance of associated Reg1 was determined by quantitative Western blotting (Fig. 3). The association of Reg1 with the Gal83 isoform was readily apparent. The Sip1 and Sip2 isoforms did interact weakly with Reg1; this was more evident in the statistical analysis of the Western signals than in the visual examination of the Western blot. However, the interaction between Reg1 and the Gal83 isoform was 15- and 8-fold stronger than those observed with Sip1 and Sip2, respectively. Thus, the large majority of the Reg1 interaction with the Snf1 complex is mediated by a single isoform.

Reg1 regulation of phosphorylation of Snf1 isoforms does not correlate with the strength of association. Since Reg1 association with the Snf1 complex is mediated largely by the Gal83 isoform, we next sought to determine whether the ability of Reg1 protein to associate with Snf1 was a requirement for its ability to regulate the phosphorylation status of Snf1. We decided to compare the Gal83 and Sip2 isoforms because they showed distinct differences in Reg1 association (Fig. 2 and 3), while at the same time, these two β subunits are very similar in size (417 and 415 amino acids, respectively) and primary sequence (48% identical in a global alignment). Cells expressing HA-tagged Snf1 but lacking the genes for all three β subunits were transformed with low-copy-number plasmids that expressed either Gal83 or Sip2. In addition, these cells either lacked or expressed the Reg1 protein. Three independent transformants for each combination were grown in high-glucose medium, and the Snf1 protein was analyzed by quantitative Western blotting (Fig. 4). A representative blot is shown, and the mean value for the relative phosphorylation of Snf1 T210 is plotted. Deletion of the *REG1* gene caused a statistically significant increase in Snf1 phosphorylation in cells expressing either the Gal83 or Sip2 isoform. Therefore, the ability of Reg1 to promote Snf1 dephosphorylation is not directly related to the strength of its association with the Snf1 complex.

Reg1 association with Snf1 does not affect the kinetics of dephosphorylation. Addition of glucose to glucose-starved cells leads to rapid dephosphorylation of the Snf1 complex (36), within 1 min. We hypothesized that this rapid dephosphorylation of Snf1 might require the preassociation of Reg1 with the Snf1 complex. To test this hypothesis, we examined the kinetics of Snf1 dephosphorylation in cells that expressed a single isoform of the Snf1 complex. Cells expressing the Gal83 isoform would have more Reg1 preassociated with the Snf1 complex than cells expressing only the Sip2 isoform. These cells were grown in high glucose and then shifted to low glucose for 30 min to allow for phosphorylation of the Snf1 activation loop. Aliquots from high- and low-glucose cultures were removed for analysis. Glucose was then added, and cells were harvested 1, 3, and 5 min after glucose addition. Analysis of total and phosphorylated Snf1 by quantitative Western blotting showed that both the Gal83 and Sip2 isoforms had dephosphorylated Snf1 to its basal level 1 min after addition of glucose (Fig. 5). Therefore, we did not detect any kinetic differ-

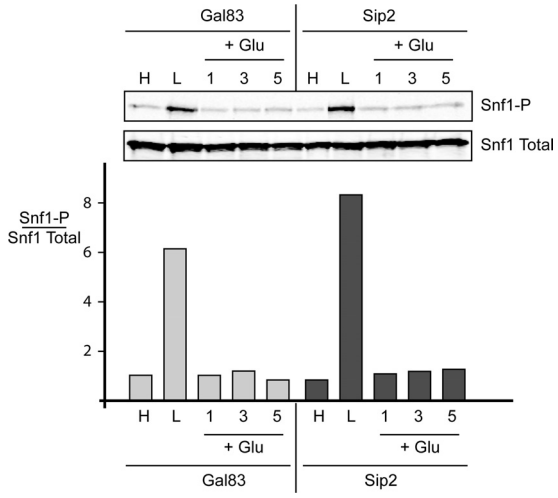

FIG. 5. Snf1 isoforms show similar rates of dephosphorylation. Cells expressing a single β subunit, either Gal83 or Sip2, were grown in high glucose (H) and then shifted to low glucose (L) for 30 min. Glucose was then added back to the cultures and aliquots removed 1, 3, and 5 min after addition of glucose. Samples were analyzed for total Snf1 and for phosphorylated Snf1 by Western blotting. The ratio of phosphorylated Snf1 to total Snf1 is plotted below.

ence in the dephosphorylation of Snf1 isoforms that differed in their association with Reg1.

Gal83 residues 1 to 150 mediate Reg1 association. In order to identify the region of Gal83 that mediates interaction with the Reg1 protein, we constructed a series of chimeric proteins by combining the open reading frames of Gal83 and Sip2. All chimeras were tagged with 3 copies of the Flag epitope near the N terminus. Positions of the fusions are detailed in Table 2. Plasmids expressing no β subunit, Gal83, Sip2, or chimeras were introduced into the two-hybrid reporter strain lacking all three gene for the β subunits. Two-hybrid interaction between Snf1 and Reg1 was assessed by growth in the absence of histidine (Fig. 6A). Consistent with earlier results, cells lacking any β subunit (vector) and cells expressing only Sip2 were unable to support the two-hybrid interaction of Snf1 and Reg1. Gal83 and chimeras that contained the first 150 residues of Gal83 were able to promote the Snf1-Reg1 interaction. The tagged wild type as well as all of the chimeric proteins was expressed and detected by Western blotting of the N-terminal Flag epitope (Fig. 6B). Quantitative Western blotting showed that all proteins were expressed at levels comparable to (within a 2-fold difference) that observed with Gal83. Therefore, the first 150 residues of the Gal83 protein are both necessary and sufficient for the interaction between Snf1 and Reg1.

N-terminal modification does not affect Snf1-Reg1 interaction. In these studies, we used β subunits that contained N-terminal Flag epitopes (Table 2). Since the region of Gal83 required for the Snf1-Reg1 interaction included the N terminus, we were concerned that the presence of the N-terminal tags affected the Snf1-Reg1 interactions. Therefore, we tested the ability of untagged Sip1, Sip2, and Gal83 to promote interaction of Snf1 and Reg1. Low-copy-number plasmids expressing untagged genomic clones of the three β subunit genes were introduced into the two-hybrid reporter strain lacking all three β subunit genes. Only Gal83 was able to promote an interaction (Fig. 7A). Thus, the presence or absence of the

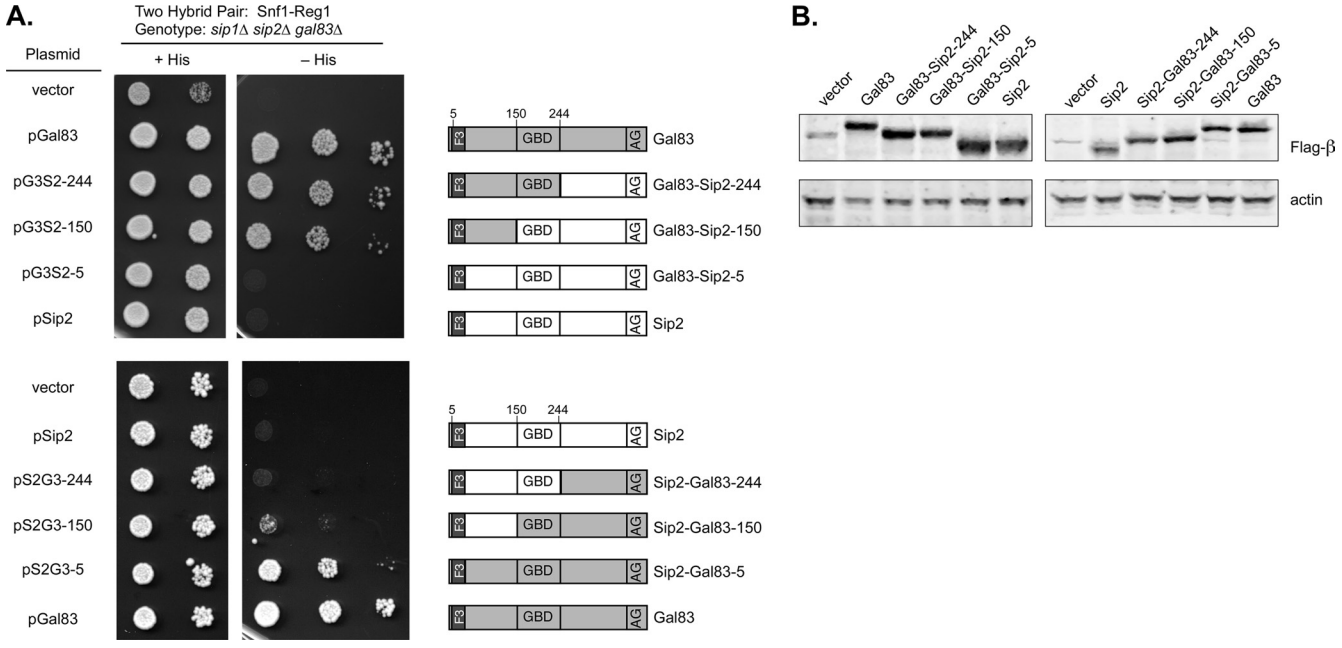

FIG. 6. Gal83 residues necessary for Reg1 interaction map to the N terminus. (A) Two-hybrid analysis of the Reg1-Snf1 interaction was conducted with a strain lacking all three β subunit genes. Wild-type Gal83, wild-type Sip2, or chimeras (plasmids are described in Table 2) were introduced on low-copy-number plasmids, and the Snf1-Reg1 interaction was assessed using medium lacking histidine. Maps of the β subunit chimeras are shown to the right, with Gal83 residues shaded and Sip2 residues in white. All constructs had 3 copies of the Flag epitope (F3) at the N terminus. The positions of the glycogen-binding domain (GBD) and the alpha-gamma interaction domain (AG) are shown. (B) Western blot of triple-Flag-tagged β subunits Gal83 and Sip2 and their chimeras (Flag-β). A control blot of actin is shown below.

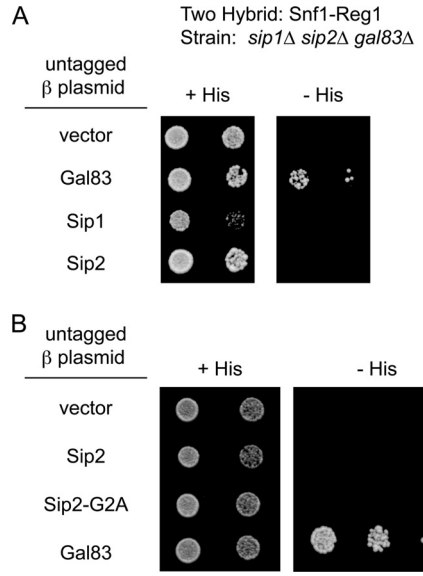

FIG. 7. Interaction of Snf1 and Reg1 is independent of β subunit N-terminal modifications. Two-hybrid analysis of the Reg1-Snf1 interaction was conducted with a strain lacking all three β subunit genes. The Snf1-Reg1 interaction was assessed using medium lacking histidine. (A) Low-copy-number plasmids encoding no β subunit (vector) or untagged wild-type Gal83, Sip1, or Sip2 were introduced. (B) Low-copy-number plasmids encoding no β subunit or untagged Sip2, Sip2-G2A, or Gal83 were introduced.

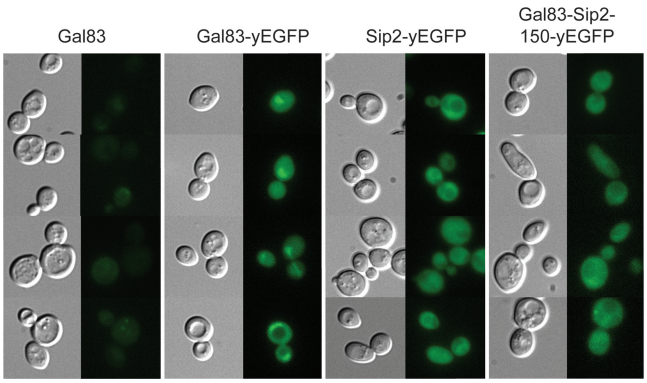

FIG. 8. Subcellular localization of β subunits. A yeast strain lacking all three β subunit genes was transformed with low-copy-number plasmids expressing either Gal83, Gal83-yEGFP, Sip2-yEGFP, or Gal83-Sip2-150-yEGFP, as indicated on the left. DIC and GFP fluorescence images were collected. Representative cells are shown.

Flag tag had no effect on the ability of the individual β subunits to promote the interaction of Snf1 and Reg1.

Earlier studies have reported that the Sip1 and Sip2 proteins are myristoylated at their N termini (24, 28). While neither study showed any direct physical evidence for myristoylated Sip1 or Sip2, both studies showed phenotypic changes when the potential for myristoylation was blocked by changing the glycine residue at position 2 into alanine. The Gal83 protein lacks a glycine at position 2 and cannot be myristoylated. Since the ability to promote Snf1-Reg1 interaction was inversely correlated with the potential for myristoylation, we tested whether the myristoylation of Sip2 affected its ability to promote the interaction of Snf1 and Reg1. Low-copy-number plasmids expressing untagged Gal83, Sip2, or the Sip2-G2A mutant were introduced into the two-hybrid strain and tested for the ability to promote interaction of Snf1 and Reg1 (Fig. 7B). Neither the Sip2 protein nor the Sip2-G2A protein was able to promote the interaction of Snf1 and Reg1 in the two-hybrid assay. Therefore, the ability of Sip2 to be myristoylated did not affect the interaction of Reg1 with Snf1.

Nuclear localization is not required for Snf1-Reg1 interaction. The three β subunits of the Snf1 kinase confer distinct differences in the subcellular localization of the Snf1 complex (50). The Gal83 protein is the only β subunit that shows enriched nuclear localization in response to glucose limitation. However, the control of the nuclear-cytoplasmic distribution of the Gal83 isoform of the Snf1 kinase complex is not simple, as it depends on both the activation state of the Snf1 kinase and the identity of the Snf1-activating kinase (23). Studies using fragments of the Gal83 protein fused to GFP have identified a nuclear export signal (NES) at residues 39 to 48 whose function is dependent on the Crm1 export receptor (22). The identification of the Gal83 nuclear localization signal (NLS) has been more difficult. A potential NLS at residues 155 to 158 affects the nuclear-cytoplasmic distribution of one Gal83 fragment; however, a smaller fragment lacking this sequence also shows nuclear localization (22). Thus, it is not certain whether the NLS responsible for Gal83 nuclear localization is present in Gal83 itself or in a distinct Gal83-associated protein. Since Gal83 is the only β subunit to show nuclear localization and the N terminus of Gal83 (residues 1 to 90) is sufficient to promote nuclear localization (50), we considered the possibility that nuclear localization was required to promote Snf1-Reg1 interaction. To test this idea, we made fusions of selected β subunits to yeast codon-optimized GFP (41). These proteins were expressed from low-copy-number plasmids via their endogenous promoters, and their localization was assessed by fluorescence microscopy. Cells were grown in medium containing a mixture of glycerol and ethanol as the carbon source (conditions that promote nuclear localization of the Gal83 protein) (22). We collected images by differential interference contrast (DIC) microscopy and fluorescence microscopy (Fig. 8). The Gal83 protein showed clear nuclear localization. In contrast, the Sip2 protein failed to show any enriched localization to the nucleus. This pattern of localization for the Gal83 and Sip2 proteins has been reported in earlier studies (50). We next tested the localization of the Gal83-Sip2-150 chimera. This β subunit chimera is a functional β subunit and contains the minimal fragment of Gal83 (residues 1 to 150) needed to promote the two-hybrid interaction of Snf1 and Reg1. Examination of the GFP fluorescence showed no evidence of nuclear enrichment for the Gal83-Sip2-150-yEGFP protein. Western blot analysis of the proteins showed equivalent expression of all yEGFP constructs used in this experiment (not shown). Therefore, the ability of a β subunit to promote the interaction of Snf1 and Reg1 is not linked to its ability to localize to the cell nucleus.

DISCUSSION

The mechanism by which the yeast PP1 phosphatase Glc7 is targeted to different substrates is a subject of great interest.

Glc7 functions in many important and diverse pathways, including kinetochore function, endocytosis, bud site selection, and glucose repression (7). Numerous Glc7 regulatory subunits have been identified, yet the mechanism by which they direct Glc7 to different substrates is less clear. In this study, we examined the mechanism by which a PP1 regulatory protein directs the PP1 phosphatase to a specific substrate. Several mechanisms have been proposed by which PP1 regulatory subunits direct the PP1 phosphatase to specific substrates. These include control of subcellular localization, changes in regulatory subunit abundance and/or modification, allostery, and direct substrate binding (9). Some of these mechanisms have been documented for Glc7, the PP1 enzyme of yeast. For instance, the subcellular localization of Glc7 is controlled by the bound regulatory subunits. The regulatory subunit Gip1 directs PP1 to the bud neck (47), while the regulatory subunit Sds22 directs PP1 to the cell nucleus (33). However, control of localization is not likely to be the regulatory mechanism directing Reg1-Glc7 to the Snf1 complex, since Reg1 is predominantly cytoplasmic (12), while the substrates of interest, the different isoforms of Snf1, show distinct localization patterns (50). Similarly, Reg1 abundance does not change appreciably in response to the carbon source (46, 49), so Reg1 abundance is not likely to be the determining mechanism. Posttranslational modification of Reg1 is a possible mechanism controlling substrate targeting. The Reg1 protein is heavily phosphorylated (6, 39, 52), and it remains a distinct possibility that one or more of its phosphorylation sites play an important role in its ability to direct Glc7 to specific targets. The control of Glc7 through allostery cannot be ruled out, although there is no evidence to support the idea that the binding of Reg1 to Glc7 changes PP1 catalytic activity.

Results from numerous studies support a direct substrate interaction mechanism to explain the ability of Reg1 to direct Glc7 to the Snf1 protein. Like most PP1 regulatory subunits, the Reg1 protein binds to the PP1 catalytic subunit via an RVXF motif that is present in roughly the middle of the Reg1 protein. Mutations in this sequence abrogate Reg1's ability to bind PP1 (2, 12) and to promote inactivation of Snf1 (46). More importantly, the Reg1 protein can also bind to the substrate Snf1, as detected in two-hybrid interaction studies and coimmunoprecipitation experiments (39, 46). Indeed, Reg1 is one of the major components of the affinity-purified Snf1 complex (14). However, not all of the experimental data support a direct substrate interaction mechanism for Reg1. We recently reported that point mutations in the RVXF motif of Reg1 interfered with its ability to bind both Glc7 and Snf1. Since Reg1 binding to Glc7 and Snf1 requires the same amino acids, we infer that Snf1 and Glc7 binding is likely to be mutually exclusive. As such, a direct substrate binding mechanism becomes unlikely. Second, other substrates of the Glc7-Reg1 phosphatase, such as Mig1 (36) and Hxk2 (2), show no evidence for direct binding to Reg1. Thus, some other mechanism for directing Glc7 to substrates in the glucose repression pathway is likely to be operative.

In this study, we examined the ability of the Reg1 protein to interact with and direct the dephosphorylation of the three different isoforms of Snf1. We found that the Reg1 protein promoted the dephosphorylation of all three isoforms. However, the vast majority of Reg1 associated with only the Gal83 isoform of Snf1. Therefore, the ability of Reg1 to promote Snf1 dephosphorylation is not directly related to its ability to interact with the Snf1 complex. In light of the data reported here and earlier (2, 36, 46), we propose that the direct substrate binding mechanism is not the means by which Reg1 directs Glc7 to substrates in the glucose repression pathway.

If substrate binding is not required for Reg1 to promote Snf1 dephosphorylation, what is the mechanism by which Reg1 directs Glc7 to proteins in the glucose repression pathway? Structural studies have noted that the PP1 active site lies at the intersection of three surface grooves, and substrate selectivity may involve extended interactions within and beyond one or more of the surface grooves (5). Regulatory subunits may specify substrates by blocking one or more of these surface grooves while enhancing substrate docking in another groove. Structural studies of PP1 bound to spinophilin, a neuronal PP1 regulatory subunit, showed that spinophilin blocks one substrate binding groove, thereby restricting PP1 specificity (34). Such a mechanism might also operate with the Reg1 protein. Spinophilin and Reg1 show structural similarity. Both are relatively large proteins (817 and 1,014 amino acids, respectively), both have a centrally located RVXF motif that mediates binding to PP1, and both are known or predicted to be unstructured proteins (34, 51). The data presented here suggest that whatever mechanism of substrate selection is used by Reg1, the ability to bind stably to substrates is not likely to be involved. The ability of Reg1 to promote dephosphorylation of the Snf1 isoforms does not directly correlate with its ability to bind the different isoforms. Furthermore, Reg1 directs Glc7 to dephosphorylate other substrates without direct binding (2, 36). Further studies of Reg1 interaction with Glc7 will be needed to determine whether the Reg1 protein specifies PP1 substrate selectivity by blocking or enhancing substrate interaction with the PP1 surface grooves.

ACKNOWLEDGMENTS

We thank Simon Watkins and Greg Gibson at the University of Pittsburgh Center for Biologic Imaging for image collection and processing.

This work was supported by grant GM46443 from the National Institutes of Health.

REFERENCES

1. **Adams, J. A.** 2003. Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? Biochemistry **42:**601–607.
2. **Alms, G. R., et al.** 1999. Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in Saccharomyces cerevisiae: characterizing the effects of a phosphatase subunit on the yeast proteome. EMBO J. **18:**4157–4168.
3. **Amodeo, G. A., et al.** 2007. Crystal structure of the heterotrimer core of Saccharomyces cerevisiae AMPK homologue SNF1. Nature **449:**492–495.
4. **Bartel, P. L., and S. Fields.** 1995. Analyzing protein-protein interactions using two-hybrid system. Methods Enzymol. **254:**241–263.
5. **Bollen, M., et al.** 2010. The extended PP1 toolkit: designed to create specificity. Trends Biochem. Sci. **35:**450–458.
6. **Breitkreutz, A., et al.** 2010. A global protein kinase and phosphatase interaction network in yeast. Science **328:**1043–1046.
7. **Cannon, J. F.** 2010. Function of protein phosphatase-1, Glc7, in Saccharomyces cerevisiae. Adv. Appl. Microbiol. **73:**27–59.
8. **Carlson, M.** 1999. Glucose repression in yeast. Curr. Opin. Microbiol. **2:**202–207.
9. **Cohen, P. T.** 2002. Protein phosphatase 1—targeted in many directions. J. Cell Sci. **115:**241–256.
10. **Davies, S. P., et al.** 1995. 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett. **377:**421–425.

11. **Desai, P., and S. Person.** 1996. Molecular interactions between the HSV-1 capsid proteins as measured by the yeast two-hybrid system. Virology **220:** 516–521.
12. **Dombek, K. M., et al.** 1999. Functional analysis of the yeast Glc7-binding protein Reg1 identifies a protein phosphatase type 1-binding motif as essential for repression of ADH2 expression. Mol. Cell. Biol. **19:**6029–6040.
13. **Dyck, J. R. B., et al.** 1996. Regulation of 5′-AMP-activated protein kinase activity by the noncatalytic beta and gamma subunits. J. Biol. Chem. **271:** 17798–17803.
14. **Elbing, K., et al.** 2006. Purification and characterization of the three Snf1-activating kinases of Saccharomyces cerevisiae. Biochem. J. **393:**797–805.
15. **Elbing, K., et al.** 2006. Subunits of the Snf1 kinase heterotrimer show interdependence for association and activity. J. Biol. Chem. **281:**26170–26180.
16. **Entian, K. D., and F. K. Zimmermann.** 1980. Glycolytic enzymes and intermediates in carbon catabolite repression mutants of Saccharomyces cerevisiae. Mol. Gen. Genet. **177:**345–350.
17. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
18. **Garcia-Haro, L., et al.** 2010. The PP1-R6 protein phosphatase holoenzyme is involved in the glucose-induced dephosphorylation and inactivation of AMP-activated protein kinase, a key regulator of insulin secretion, in MIN6 beta cells. FASEB J. **24:**5080–5091.
19. **Gietz, R. D., et al.** 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast **11:**355–360.
20. **Hardie, D. G.** 2011. Sensing of energy and nutrients by AMP-activated protein kinase. Am. J. Clin. Nutr. **93:**891S–896S.
21. **Hardie, D. G., et al.** 1999. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. Biochem. J. **338:**717–722.
22. **Hedbacker, K., and M. Carlson.** 2006. Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. Eukaryot. Cell **5:**1950–1956.
23. **Hedbacker, K., et al.** 2004. Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. Mol. Cell. Biol. **24:**8255–8263.
24. **Hedbacker, K., et al.** 2004. Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. Mol. Cell. Biol. **24:**1836–1843.
25. **James, P., et al.** 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:**1425–1436.
26. **Kushnirov, V. V.** 2000. Rapid and reliable protein extraction from yeast. Yeast **16:**857–860.
27. **Li, L., et al.** 1994. Specific association between the human DNA repair proteins XPA and ERCC1. Proc. Natl. Acad. Sci. U. S. A. **91:**5012–5016.
28. **Lin, S. S., et al.** 2003. Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in Saccharomyces cerevisiae by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. J. Biol. Chem. **278:**13390–13397.
29. **Ludin, K., et al.** 1998. Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. **95:**6245–6250.
30. **Mangat, S., et al.** 2010. Differential roles of the glycogen-binding domains of beta subunits in regulation of the Snf1 kinase complex. Eukaryot. Cell **9:**173–183.
31. **Matsumoto, K., et al.** 1983. Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in Saccharomyces cerevisiae. J. Bacteriol. **153:**1405–1414.
32. **McCartney, R. R., and M. C. Schmidt.** 2001. Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J. Biol. Chem. **276:**36460–36466.
33. **Peggie, M. W., et al.** 2002. Essential functions of Sds22p in chromosome stability and nuclear localization of PP1. J. Cell Sci. **115:**195–206.
34. **Ragusa, M. J., et al.** 2010. Spinophilin directs protein phosphatase 1 specificity by blocking substrate binding sites. Nat. Struct. Mol. Biol. **17:**459–464.
35. **Rose, M. D., et al. (ed.).** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36. **Rubenstein, E. M., et al.** 2008. Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. J. Biol. Chem. **283:**222–230.
37. **Ruiz, A., et al.** 2011. Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. Proc. Natl. Acad. Sci. U. S. A. **108:**6349–6354.
38. **Sanders, M. J., et al.** 2007. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem. J. **403:**139–148.
39. **Sanz, P., et al.** 2000. Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol. Cell. Biol. **20:**1321–1328.
40. **Schmidt, M. C., and R. R. McCartney.** 2000. Beta-subunits of Snf1 kinase are required for kinase function and substrate definition. EMBO J. **19:**4936–4943.
41. **Sheff, M. A., and K. S. Thorn.** 2004. Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. Yeast **21:**661–670.
42. **Shi, Y.** 2009. Serine/threonine phosphatases: mechanism through structure. Cell **139:**468–484.
43. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122:**19–27.

43a. **Stein, S. C., et al.** 2000. The regulation of AMP-activated protein kinase by phosphorylation. Biochem. J. **345:**437–443.

44. **Steinberg, G. R., et al.** 2006. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab. **4:**465–474.
45. **Suter, M., et al.** 2006. Dissecting the role of 5′-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J. Biol. Chem. **281:**32207–32216.
46. **Tabba, S., et al.** 2010. PP1 phosphatase-binding motif in Reg1 protein of Saccharomyces cerevisiae is required for interaction with both the PP1 phosphatase Glc7 and the Snf1 protein kinase. Cell. Signal. **22:**1013–1021.
47. **Tachikawa, H., et al.** 2001. A Gip1p-Glc7p phosphatase complex regulates septin organization and spore wall formation. J. Cell Biol. **155:**797–808.
48. **Tu, J., and M. Carlson.** 1994. The GLC7 type 1 protein phosphatase is required for glucose repression in Saccharomyces cerevisiae. Mol. Cell. Biol. **14:**6789–6796.
49. **Tu, J., and M. Carlson.** 1995. REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. EMBO J. **14:** 5939–5946.
50. **Vincent, O., et al.** 2001. Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes Dev. **15:**1104–1114.
51. **Ward, J. J., et al.** 2004. Prediction and functional analysis of native disorder in proteins from the three kingdoms of life. J. Mol. Biol. **337:**635–645.
52. **Wu, R., et al.** 2011. A large-scale method to measure absolute protein phosphorylation stoichiometries. Nat. Methods **8:**677–683.
53. **Zhang, J., et al.** 2010. The beta-subunits of the Snf1 kinase in Saccharomyces cerevisiae, Gal83 and Sip2, but not Sip1, are redundant in glucose derepression and regulation of sterol biosynthesis. Mol. Microbiol. **77:**371–383.
54. **Zimmermann, F. K., and I. Scheel.** 1977. Mutants of Saccharomyces cerevisiae resistant to carbon catabolite repression. Mol. Gen. Genet. **154:**75–82.