The SNF1/AMP-activated protein kinases (AMPKs) function in energy regulation in eukaryotic cells. SNF1/AMPKs are αβγ heterotrimeric complexes that are activated by phosphorylation of the activation loop Thr on the catalytic subunit. Kinases that activate SNF1/AMPK have been identified, but the protein phosphatases responsible for dephosphorylation of the activation loop are less well defined. For *Saccharomyces cerevisiae* SNF1/AMPK, Reg1-Glc7 protein phosphatase 1 and Sit4 type 2A-related phosphatase contribute to glucose-regulated dephosphorylation, whereas Reg1-Glc7 PP1 plays the major role.

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elevated during growth on glucose (15). These findings indicated that both Reg1-Glc7 and Sit4 function in glucose-regulated dephosphorylation of Thr-210 and do so independently of the intact SNF1 heterotrimer. Evidence for physical interaction of Reg1 and Sit4 with Snf1 (4, 5, 11) supported the idea that these phosphatases directly dephosphorylate Thr-210.

For mammalian AMPK, the relevant phosphatases for the activation loop in vivo are not yet firmly established. Type 2A and 2Ca protein phosphatases dephosphorylate the activation loop Thr in vitro (16, 17); however, studies of mammalian cells implicated Ppm1E, and probably Ppm1F (18), and PPI-R6, a member of the PPI family (19). The S. cerevisiae homolog of Ppm1E and Ppm1F is Ptc1, which is named for its identity as a PP2C family member. We have here used the yeast genetic system to address the role of Ptc1 in regulating activation-loop phosphorylation of SNF1/AMPK.

S. cerevisiae encodes a family of seven PP2C catalytic subunits, Ptc1 through Ptc7; the catalytic subunits of the PP2C family are not usually associated with regulatory subunits. Ptc1 is the best characterized, and it is structurally and functionally distinct from the others, although it may share some functions (for review, see ref. 20). Ptc1 is implicated in MAPK pathways such as the high-osmolarity glycerol and cell wall integrity pathways, the TOR (target of rapamycin) pathway, cation homeostasis, inheritance of cellular organelles, and other functions (20). Analysis of the ptc1Δ mutant using DNA microarrays showed no significant effects on expression of glucose-regulated genes (21, 22). Ptc1 binds to the adaptor protein Nbp2, which mediates its association with multiple protein kinases, but SNF1 was not identified as an interaction partner (23). We here examine the effects of the ptc1Δ mutation and Ptc1 overexpression, in combination with reg1Δ and/or sit4Δ mutations, on Thr-210 phosphorylation of wild-type and mutant forms of SNF1.

**EXPERIMENTAL PROCEDURES**

**Strains**—S. cerevisiae strains had the W303 (ade2 can1 ura3 leu2 his3 trpl) genetic background. The alleles SNF1–8myc::TRP1, snf1Δ::LEU2, snf4Δ::kanMX4, sit4Δ::nat1, reg1Δ::HIS3 and glc3Δ::kanMX4 were used previously (11, 24). The SSIΔ-v1 allele, which is present in many common strain backgrounds, is essential for viability of sit4Δ mutants (25) and does not affect phosphorylation of Thr-210 (11). ptc1Δ::nat1 and ptc1Δ::hphMX4 were constructed by replacing the 0.7-kb SnaBI/BamHI fragment, and ptc2Δ::TRP1 by replacing the 0.9-kb Xbal/NruI fragment. ptc3Δ::LEU2 (26) and ptc6Δ::kanMX4 (27) have been described. ptc4Δ::URA3, ptc5Δ::HIS3, ptc7Δ::hphMX4, and glc3Δ::URA3 were constructed using marker swap plasmids (28) to replace kanMX4 in the cognate alleles (Open Biosystems). Standard methods for genetic analysis and transformation were used.

**Plasmids**—Snf1–8myc and Snf1(1–309)-8myc were expressed from the SNF1 promoter on centromeric plasmids pYLI199, pYLI41, and pYLI414 (15, 24). pXX7 and pYLI200 are derivatives of pYLI411 and pYLI199 in vectors pRS316 (29) and YCp50 (30), respectively. Mutated versions of Snf1, expressed from the native promoter on a centromeric plasmid, have been described (31); catalytic activity of Snf1EA toward a synthetic peptide substrate was reduced 5-fold relative to WT (32). Ptc1 and Ptc1D58N coding sequences were amplified from YEp195-PTC1 (33) and YEp195-Ptc1(D58N) (34) by PCR and used to transform yeast for gap repair of the centromeric vector pMK547 (35); the recovered plasmids, pAR12 and pAR32, express 3xHA-Ptc1 and 3xHA-Ptc1D58N, respectively, from the ADH1 promoter and terminator. pAR48 was generated by gap repair of centromeric vector pRS314 and expressed 3xHA-Ptc1 from its own promoter and terminator. Recovered plasmids were sequenced.

**Growth of Cultures**—Cells were grown to mid-log phase in selective synthetic complete medium (SC) containing 2% (high) glucose, and an aliquot of the culture was harvested by rapid filtration to preserve the phosphorylation state of Thr-210 and was frozen in liquid nitrogen. Another aliquot was collected by rapid filtration, resuspended in SC containing 0.05% (low) glucose for 10 min, collected by filtration, and frozen.

**Immunoblot Analysis**—For analysis of Snf1 phosphorylation, whole cell extracts were prepared (11), and proteins (10 μg) were separated by SDS-PAGE on 7.5% polyacrylamide and analyzed by immunoblotting with anti-Thr(P)-172-AMPK (Cell Signaling Technologies). Membranes were incubated in 0.2 M glycine, pH 2, for 10 min and reprobed with anti-Myc (9E10, Santa Cruz Biotechnology) or anti-polyhistidine (Sigma; Snf1 has a stretch of His residues) to detect Snf1 polypeptides. ECL Plus (GE Healthcare) was used for visualization. Intensity of bands was quantified on appropriate exposures using ImageJ software (36), and phosphorylated Thr-210 was normalized to WT.

**RESULTS**

**Ptc1 Is Dispensable for Snf1 Thr-210 Dephosphorylation in Cells Containing Reg1-Glc7 and Sit4**—To determine whether glucose regulation of the phosphorylation state of Snf1-Thr210 depends on Ptc1, we first examined Myc-tagged Snf1, expressed from a centromeric plasmid in ptc1Δ snfΔ and ptc1Δ snfΔ glcΔ cells. The glcΔ mutation deletes the gene encoding glycogen branching enzyme, thereby avoiding the possibility of inappropriate glycogen synthesis, which results in elevated Snf1 phosphorylation (11). Cells were grown to mid-log phase on a high (2%) concentration of glucose, and an aliquot was shifted to low (0.05%) glucose for 10 min. Cell extracts were prepared, and proteins were analyzed by immunoblotting with antibodies that recognize phospho-Thr-210 and Myc. No defect in Thr-210 dephosphorylation was observed in cells grown on high glucose, and phosphorylation increased appropriately in response to glucose limitation (Fig. 1A). Glucose replenishment of glucose-depleted ptc1Δ cultures resulted in rapid dephosphorylation of Thr-210 (Fig. 1B). Mutants lacking all seven Ptc family members showed normal glucose-regulated dephosphorylation of both Snf1 and the truncated Snf1 kinase domain, Snf1(1–309)-myc (Fig. 1C).

**Ptc1 Affects the Phosphorylation State of Thr-210 in sit4Δ Cells**—To assess the possibility that a role of Ptc1 was masked by the presence of other phosphatases that dephosphorylate Thr-210, we first constructed mutants lacking Sit4 and Ptc1. We examined sit4Δ ptc1Δ glcΔ mutants to avoid the indirect
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FIGURE 1. Snf1 Thr-210 phosphorylation in mutants lacking Ptc1 and other PP2C phosphatases. Cells of the indicated relevant genotype were collected after growth on 2% (H, high) glucose or after a shift to 0.05% (L, low) glucose for 10 min. For B, aliquots of cultures shifted to low glucose were replenished with 2% glucose for 2.5 or 5 min, as indicated. Protein extracts were separated by SDS-PAGE and subjected to immunoblot analysis with anti-Thr(P)-172-AMPK to detect phosphorylated Thr-210. C, cells carrying mutations ptc1Δ through ptc7Δ expressed Snf1 from the genomic locus and Snf1(1–309)-myc from pYL411. All lanes are from the same blot. Arrows mark the positions of Snf1 and Snf1(1–309).

FIGURE 2. Snf1 Thr-210 phosphorylation in sit4Δ ptc1Δ cells. Cells of the indicated genotype were grown, extracts were prepared, and Snf1 Thr-210 phosphorylation was assayed as in Fig. 1. A, cells expressed Snf1 from the genomic locus. Three independent sit4Δ ptc1Δ glc3Δ strains are shown. B, four independent transformants expressed Snf1-myc from centromeric plasmid pYL200. C, quantification of results shown in panel A and obtained on other blots. Intensity of the bands was quantified, and phosphorylated Thr-210 was normalized to Snf1 protein. Values for high glucose (dark bars) and low glucose (open bars) were plotted as a percentage of the value obtained on the same blot for control glc3Δ samples on low glucose (n = 6, except n = 11 for sit4Δ ptc1Δ glc3Δ strains). D, quantification of results, relative to control snf1Δ glc3Δ samples, shown in B. E, cells expressed Snf1(1–309)-myc from pYL411. Transformants of two independent sit4Δ ptc1Δ glc3Δ snf1Δ strains were assayed on the same blot. H, high; L, low.

Ptc1 Is Required for Viability of reg1Δ Cells and for Thr-210 Dephosphorylation of Mutant SNF1—To assess the role of Ptc1 in the absence of Reg1-Glc7, we attempted to construct reg1Δ ptc1Δ mutant cells, but they were inviable. This finding, together with the inviability of reg1Δ sit4Δ cells (11), indicates that the three phosphatases have overlapping function(s) that are essential for cell viability.

The snf1Δ mutation suppressed lethality, and reg1Δ ptc1Δ snf1Δ cells expressing Snf1(1–309), which has reduced catalytic activity (15), remained viable. These cells manifested no defect in Thr-210 dephosphorylation (Fig. 3A). However, Thr-210 phosphorylation in the absence of Reg1-Glc7, we attempted to construct reg1Δ ptc1Δ mutant cells, but they were inviable. This finding, together with the inviability of reg1Δ sit4Δ cells (11), indicates that the three phosphatases have overlapping function(s) that are essential for cell viability.

Effects of inappropriate glycogen accumulation caused by sit4Δ, which results in elevated Snf1 phosphorylation (11); all strains used in subsequent experiments carried glc3Δ, which will henceforth be omitted from the relevant genotypes. During growth on high glucose, Thr-210 phosphorylation was elevated to a small extent relative to Snf1 protein levels, and Thr-210 phosphorylation increased somewhat less robustly in response to glucose limitation, perhaps because cells growing on high glucose are already somewhat preadapted for growth on low glucose. Similar results were obtained in sit4Δ ptc1Δ snf1Δ cells expressing Snf1-myc from a centromeric plasmid (Fig. 2B), although one of the four transformants showed somewhat greater phosphorylation than the others. Quantification of these results is shown in Fig. 2, C and D. An elevation of Thr-210 phosphorylation during growth on glucose was easily evident in sit4Δ ptc1Δ snf1Δ cells expressing Snf1(1–309)-myc (Fig. 2E); in this case, phosphorylation increased robustly in response to glucose limitation, perhaps reflecting the fact that Snf1(1–309) has little activity (15), and thus, cells on high glucose are not pre-adapted for growth on low glucose. These results support a modest role for Ptc1 in regulating the phosphorylation state of Snf1 Thr-210 in sit4Δ cells. Nonetheless, Thr-210 phosphorylation was largely glucose-regulated in cells lacking bothSit4 and Ptc1 but containing Reg1-Glc7.
we expressed three mutant Snf1 proteins that have reduced phosphorylation (Fig. 3). In cells lacking only Reg1-Glc7 or Ptcl, all three exhibited glucose-regulated Thr-210 phosphorylation in wild-type cells (31, 32). In cells expressing each mutant Snf1 protein, as in Fig. 2C, for the transforms expressing Snf1R99A, R102A shown on the left blot of A. Values for high (H) glucose (dark bars) and low (L) glucose (open bars) are shown as percentage of the value obtained for the snf1Δ glc3Δ sample on low glucose. C, quantification of phosphorylated Thr-210, normalized to Snf1 protein, for the transformants expressing each mutant Snf1 protein. Values are shown as percentage of the value obtained for the same transformant on low glucose and are averages for the two transformants shown. may be more readily accessible to Sit4 in the context of the truncated kinase domain, which does not associate with β or Snf4/γ subunits (15), than in the context of the intact SNF1 heterotrimer.

To address the possibility that Ptc1 is required for dephosphorylation of the SNF1 heterotrimer, we expressed three mutant Snf1 proteins that have reduced catalytic activity in reg1Δ ptc1Δ snf1Δ cells. Snf1R99A, R102A and Snf1S105A have Ala substitutions of the indicated residues located on the αC helix of the kinase domain, and Snf1EA has Ala substitutions of four Glu residues (324–326 and 328) located immediately C-terminal to the kinase domain (31, 32). All three mutant kinases showed normal glucose regulation of Thr-210 phosphorylation in wild-type cells (31, 32). In cells lacking only Reg1-Glc7 or Ptc1, all three exhibited glucose-regulated phosphorylation (Fig. 3A). However, in reg1Δ ptc1Δ snf1Δ cells, Thr-210 phosphorylation of these mutant kinases was elevated during growth on high glucose and showed little increase upon glucose limitation (Fig. 3A). Quantification of these results is shown in Fig. 3, B and C. With the caveat that mutant SNF1 forms were examined, these results support a role of Ptc1 in dephosphorylation of Snf1 Thr-210 within the context of the SNF1 heterotrimer and suggest that Sit4 does not suffice for dephosphorylation in the absence of Reg1-Glc7 and Ptc1.

Snf1 Ubiquitin-associated Domain Does Not Mediate Effects of Ptc1 on Thr-210 Phosphorylation—We next explored a possible mechanism for interaction of Ptc1 with SNF1 suggested by studies of Ppm1F and AMPK. The effects of the deubiquitylase inhibitor N-ethylmaleimide and Ppm1F depletion on AMPK phosphorylation in mammalian cell lysates led Voss et al. (18) to propose that polyubiquitylation and a proposed ubiquitin-associated domain (UBA) on the AMPK catalytic subunit mediate interaction with Ppm1F. Studies of AMPK-related kinases, however, indicated that the UBA mediates their phosphorylation and activation by LKB1 (37). In S. cerevisiae, the absence of Ubp8-mediated deubiquitylation reduced Snf1 Thr-210 phosphorylation ~2-fold (38); ubiquitylation sites on Snf1 have not yet been identified. Deletion of the UBA (residues 347–398), located immediately C-terminal to the kinase domain, increased phosphorylation and Snf1 activity ~2-fold during growth on high glucose (31). We reasoned that if the UBA mediates interaction with Ptc1, then Thr-210 phosphorylation of Snf1ΔUBA would be substantially elevated in reg1Δ snf1Δ cells on high glucose. However, this was not the case in reg1Δ snf1Δ cells or in sit4Δ snf1Δ or ptc1Δ snf1Δ cells (Fig. 4).

Reg1-Glc7 or Sit4 But Not Ptc1 Satisfies for Thr-210 Dephosphorylation Independent of the SNF1 Heterotrimer—Whereas Thr-210 phosphorylation of Snf1(1–309) was substantially glucose-regulated in reg1Δ ptc1Δ cells and sit4Δ ptc1Δ cells (Figs. 2B and 3A), previous studies showed that in reg1Δ sit4Δ cells, phosphorylation of Snf1(1–309) was greatly elevated during growth on high glucose and increased ~2-fold in response to glucose limitation (15). In an effort to detect a role of Ptc1 in
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reg1Δ sit4Δ snf1Δ cells expressing Snf1(1–309) were transformed with plasmid pAR48, which overexpresses HA-Ptc1D58N. Cells expressing HA-Ptc1D58N showed an increase in Thr-210 phosphorylation compared to cells expressing HA-Snf1(1–309) (Fig. 5, left panel). In contrast, expressing HA-Ptc1 did not affect Thr-210 phosphorylation (15). Phosphorylation was elevated, relative to Snf1(1–309) (Fig. 5, right panel). These results suggest that when the heterotrimer was disrupted by the absence of the Snf4 subunit, either Reg1-Glc7 or Sit4 sufficed for efficient dephosphorylation, whereas Ptc1 did not.

To address the mechanism by which Ptc1 overexpression rescued viability, we examined six FOA-resistant colonies for phosphorylation of Thr-210 (Fig. 6B). The inviability of the Ptc1D58N control precluded any quantitative assessment, but Ptc1 overexpression clearly did not confer glucose regulation of phosphorylation, although it may have reduced phosphorylation during growth on both high and low glucose. However, because Snf1 protein levels were very low, and the phosphorylation state results from the opposing actions of activating kinases and phosphatases, these findings are not incompatible with other evidence that, in wild-type cells, Ptc1 participates in glucose regulation of Thr-210 phosphorylation. The mechanism by which Ptc1 overexpression rescued viability...
remains unclear and may reflect effects of Ptc1 on the levels or activity of SNF1 and/or other aspects of cellular regulation. Regardless of mechanism, rescue of reg1Δ sit4Δ cell viability by either overexpression of Ptc1 or deletion of SNF1 provides another line of genetic evidence linking Ptc1 to the SNF1 pathway.

DISCUSSION

We present genetic evidence that Ptc1, a member of the PP2C family, has a role in regulating the dephosphorylation of Thr-210 of the SNF1 heterotrimer during growth of cells on high glucose. Mutant forms of SNF1 with reduced catalytic activity were examined because the reg1Δ ptc1Δ double mutation caused inviability of cells containing wild-type SNF1. The reg1Δ sit4Δ double mutation similarly resulted in greatly elevated Thr-210 phosphorylation of mutant SNF1 on high glucose and inviability of cells with wild-type SNF1 (11, 15). In contrast, Thr-210 phosphorylation was largely glucose-regulated in sit4Δ ptc1Δ cells. Together, these findings indicate that, although Reg1-Glc7 plays a major role, all three protein phosphatases, Reg1-Glc7, Sit4, and Ptc1, contribute to glucose regulation of the phosphorylation status of SNF1.

The effects of ptc1Δ on the phosphorylation state of SNF1 most simply suggest that Ptc1 directly dephosphorylates Thr-210. Physical interaction of Ptc1 and Snf1 has not been reported, and, in preliminary experiments, overexpressed Snf1 did not co-purify with tandem affinity purification-tagged Ptc1 expressed from the genomic locus; however, dephosphorylation of AMPK in vivo provides another line of genetic evidence linking Ptc1 to the SNF1 pathway.

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