Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in *Saccharomyces cerevisiae*: characterizing the effects of a phosphatase subunit on the yeast proteome

Geoffrey R.Alms, Pascual Sanz¹,
Marian Carlson² and Timothy A.J.Haystead³

Department of Pharmacology, University of Virginia, 1300 Jefferson Park Avenue, Charlottesville, VA 22908, ²Department of Microbiology, Columbia University, New York, NY, USA and ¹Instituto Biomedicina de Valencia (CSIC), Jaime Roig 11, 46010 Valencia, Spain

³Corresponding author
e-mail: tah4g@virginia.edu

Protein phosphatase 1 (Glc7p) and its binding protein Reg1p are essential for the regulation of glucose repression pathways in *Saccharomyces cerevisiae*. In order to identify physiological substrates for the Glc7p–Reg1p complex, we examined the effects of deletion of the *REG1* gene on the yeast phosphoproteome. Analysis by two-dimensional phosphoprotein mapping identified two distinct proteins that were greatly increased in phosphate content in *reg1Δ* mutants. Mixed peptide sequencing identified these proteins as hexokinase II (Hxk2p) and the E1 α subunit of pyruvate dehydrogenase. Consistent with increased phosphorylation of Hxk2p in response to *REG1* deletion, fractionation of yeast extracts by anion-exchange chromatography identified Hxk2p phosphatase activity in wild-type strains that was selectively lost in the *reg1Δ* mutant. The phosphorylation state of Hxk2p and Hxk2p phosphatase activity was restored to wild-type levels in the *reg1Δ* mutant by expression of a LexA–Reg1p fusion protein. In contrast, expression of LexA–Reg1p containing mutations at phenylalanine in the putative PP-1C-binding site motif (K/R)(X)(I/V)XF was unable to rescue Hxk2p dephosphorylation in intact yeast or restore Hxk2p phosphatase activity. These results demonstrate that Reg1p targets PP-1C to dephosphorylate Hxk2p *in vivo* and that the motif (K/R)(X)(I/V)XF is necessary for its PP-1 targeting function.

Keywords: hexokinase/mixed peptide sequencing/protein phosphatase 1/proteome/Reg1p

Introduction

The catalytic subunit of protein phosphatase 1 (PP-1C) is amongst the most conserved proteins in nature (Cohen and Cohen, 1989). The enzyme is ubiquitously expressed at high levels in all cell types with an intracellular protein concentration of ~10 μ M. *In vitro*, the free catalytic subunit displays indiscriminate substrate specificity that overlaps with other ubiquitously expressed family members, including PP-2A and PP-2B (Cohen and Cohen, 1989; Shenolickar and Nairn, 1991). However, *in vivo*, PP-1C is associated with a number of regulatory proteins that either inhibit its activity or specifically target the phosphatase to a selective set of substrates or intracellular

location (Hubbard and Cohen, 1993; Campos *et al.*, 1996; Egloff *et al.*, 1997; Damer *et al.*, 1998). To date, at least 20 PP-1 regulatory subunits have been identified in mammals and yeast; however, only three of these proteins, M₁₁₀ (MYTP1), PTG (PPP1R5) and PP-1G_M, have been demonstrated to act in a targeting capacity both in intact cells and *in vitro*. The M₁₁₀ subunit binds PP-1C *in vitro* and increases its smooth muscle myosin phosphatase activity by a factor of 20 whilst suppressing non-specific activity towards other substrates (Alessi *et al.*, 1994; Shirazi *et al.*, 1994; Simuzu *et al.*, 1994; Haystead *et al.*, 1995). Addition of both native and recombinant M₁₁₀ to β -escin-permeabilized smooth muscles enhances the ability of PP-1C to bring about relaxation following Ca²⁺-induced contraction (Alessi *et al.*, 1994; Shirazi *et al.*, 1994; Haystead *et al.*, 1995; Gailly *et al.*, 1996; Johnson *et al.*, 1996). *In vitro*, PTG binds and targets PP-1C to glycogen, glycogen synthase and phosphorylase *a* (Doherty *et al.*, 1996; Printen *et al.*, 1997). Overexpression of PTG in hepatocytes results in hyperglycogen accumulation with a concomitant 4-fold activation of glycogen synthase and 40% inhibition of phosphorylase activity (Berman *et al.*, 1998). In skeletal muscle, the G_M subunit has been proposed to target PP-1C to regulate glycogen synthase dephosphorylation in response to adrenaline and insulin (for a review see Hubbard and Cohen, 1993). Phosphorylation of G_M by protein kinase A (PKA) causes dissociation of PP-1C and thereby prevents activation of glycogen synthase. Conversely, phosphorylation of G_M at a second distinct site by the insulin-stimulated protein kinase p90rsk (MAPKAP1) enhances association of the subunit with PP-1C to promote dephosphorylation and activation of glycogen synthase (Dent *et al.*, 1988). The physiologically relevant targets of the other PP-1 regulatory subunits have yet to be defined. Indeed, because PP-1C has been implicated in so many cellular events, identifying the substrates for any of its putative targeting subunits is a seemingly intractable problem.

In *Saccharomyces cerevisiae*, PP-1C is encoded by the essential gene *GLC7* (*DIS2S1*) and genetic analysis has shown that the phosphatase participates in the regulation of many processes, including glucose repression pathways, glycogen accumulation, sporulation, cell cycle progression and protein translation (for a review see Stark, 1996). Like its mammalian counterpart, Glc7p is regulated by interaction with many distinct regulatory targeting subunits. In *S.cerevisiae*, genetic studies have implicated the Glc7p-binding protein Reg1p in the regulation of glucose repression pathways (Tu and Carlson, 1995; Huang *et al.*, 1996). Like other yeast, *S.cerevisiae* thrive on a variety of carbon sources, but glucose and fructose are preferred. When one of these sugars is present, the enzymes required for gluconeogenic pathways and for the utilization of alternative carbon sources are synthesized at low rates or

not at all (for a review see Gancedo, 1998). The precise mechanism by which glucose signals to the nucleus to repress genes controlling expression of enzymes involved in gluconeogenesis and alternative carbon source utilization is not known (Gancedo, 1998). Glucose itself or glucose 6-phosphate (G6P) may be important mediators. Genetic and biochemical evidence suggests that Reg1p physically associates and functions with Glc7p in intact cells to regulate the glucose repression pathway. In two-hybrid analysis, Reg1p binds Glc7p and both proteins can be co-immunoprecipitated from yeast extracts (Tu and Carlson, 1995). Furthermore, deletion of *REG1* in a wild-type background results in inhibition of glucose repression, accumulation of intracellular glycogen, slow growth and increased cell size (Huang *et al.*, 1996). Reg1p also appears to have selective functions in the regulation of glucose repression by specifically antagonizing the function of the Snf1 kinase, which is a negative regulator of the glucose repression pathways (Ludin *et al.*, 1998). Reg1p shows a glucose-dependent association with the Snf1 kinase and may provide a means for dephosphorylation and inactivation of the enzyme by Glc7p.

Here, we hypothesized that comparison of the wild-type yeast phosphoproteome with *reg1Δ* mutants and strains containing Reg1p with point mutations in its putative PP-1C-binding site would identify physiologically relevant substrates of the Reg1p–Glc7p complex. This is because substrate targets would become selectively hyperphosphorylated as a result of an imbalance of the normal kinase–phosphatase equilibrium that governs the steady-state phosphorylation of the protein(s) under basal conditions. To test this hypothesis, two-dimensional SDS–PAGE phosphoprotein maps were prepared from ³²P-labeled wild-type and mutant strains grown in the presence and absence of glucose. A major increase in phosphate content was observed in the glycolytic enzyme hexokinase II (Hxk2p) and the E1α subunit of pyruvate dehydrogenase in wild-type strains grown in the absence of glucose, *reg1Δ* strains, and strains expressing LexA–Reg1p with point mutations in the putative PP-1C-binding site. Subsequent fractionation and assay with ³²P-labeled Hxk2p identified hexokinase phosphatase activity in the wild-type strains that was absent in both *reg1Δ* and LexA–Reg1p point mutant strains. The effects of the mutations on the ability of Reg1p to repress glucose-induced expression of the *SUC2* gene was also tested. Wild-type LexA–Reg1p was shown to complement the *reg1Δ* mutation effectively, reducing *SUC2* expression 130-fold in glucose-grown cells, whereas the mutant forms of LexA–Reg1p reduced *SUC2* expression only 3-fold. These results demonstrate that Reg1p targets PP-1C towards Hxk2p to promote its dephosphorylation *in vivo* and are consistent with previous work by Randez-Gil *et al.* (1998), suggesting an involvement of Reg1p–Glc7p in the dephosphorylation of Hxk2p. In addition, our findings show the utility of functional proteomic analysis for the identification of physiological substrates for protein phosphatases.

Results

The effects of *REG1* deletion on the yeast phosphoproteome

To examine the phenotypic effects of disruption of the *REG1* gene on the yeast phosphoproteome, *reg1Δ* mutant

(MCY3278) and wild-type (FY250) strains from the same genetic background were labeled to steady state with either [³⁵S]methionine or [³²P]orthophosphate and grown in the presence or absence of glucose. Following two-dimensional SDS–PAGE and autoradiography, the autoradiograms from each radiolabeled strain were made into digital images (Figure 1). To determine whether *REG1* deletion affected the overall pattern of protein expression, autoradiograms from [³⁵S]methionine-labeled yeast were compared using the BIORAD Melanie II 2D-analysis program. This analysis did not reveal any obvious differences in the expression of any one protein as a result of *REG1* deletion (compare Figure 1A with B, and C with D). Analysis by Melanie of the autoradiograms prepared from ³²P-labeled yeast identified ~80 distinct phosphoproteins of varying abundance in each gel (Figure 1E–H). To identify phosphoproteins that were increased in phosphate content as a result of *REG1* deletion, Melanie compared Figure 1E (wild type) with F (*reg1Δ*). Four groups of spots were identified to be increased in phosphate content in this analysis, labeled Hxk2p (a) and Hxk2p (b), E1, heat shock protein 60 (hsp60) and spot C (the nomenclature was derived following identification of the proteins by mixed peptide sequencing, see below). None of the other 80 or so phosphoproteins detected by Melanie in the two autoradiograms showed any significant differences in phosphate content, suggesting that spots Hxk2p (a), Hxk2p (b), hsp60, E1 and C may be target substrates for the Reg1p–Glc7p complex. To test the effects of glucose starvation on overall protein phosphorylation in the wild-type strain, Melanie compared Figure 1G with E. This analysis showed that three of the proteins that were increased in phosphate content following *REG1* deletion (Figure 1F) were also hyperphosphorylated in the wild type as a result of glucose starvation (Figures 1G and 2A). Importantly, comparison of Figure 1H with F showed that the absence of glucose did not cause additional proteins to be phosphorylated in the *reg1Δ* strain (Figure 2A). Since *REG1* deletion was found to mimic the effects of glucose starvation on the yeast phosphoproteome, this finding further supports the hypothesis that Reg1p plays a critical role in the regulation of glucose-sensing pathways.

To determine if deletion of the *REG1* gene or the presence of raffinose result in significant alterations of intracellular ATP concentration, the nucleotide content of the wild-type and *reg1Δ* strain was measured. This measurement would indicate whether the observed alterations in phosphorylation state in the *reg1Δ* strain, or following glucose starvation in the wild-type case, were likely to be due to differences in either the cellular ATP pool or the specific activity of the γ-phosphate of the nucleotide. However, in three separate measurements by reverse-phase HPLC, the overall amounts of GTP, ATP, ADP and AMP recovered from both strains did not vary significantly (data not shown). Moreover, when grown in dextrose or raffinose, the specific activity of the γ-phosphate of ATP from wild-type or *reg1Δ* strains varied by $<11.5 \pm 3.5\%$ (SDM, $n = 3$) with respect to one another.

Identification of phosphoproteins regulated by Reg1p and glucose by mixed peptide sequencing

To identify ³²P-labeled proteins that were phosphorylated in response to *REG1* deletion and glucose starvation,

A wild type glucose **B** *REG1* deletion glucose **C** wild type raffinose **D** *REG1* deletion raffinose **E** wild type glucose **F** *REG1* deletion glucose **G** wild type raffinose **H** *REG1* deletion raffinose

Fig. 1. The effects of *REG1* deletion and glucose starvation on the yeast phosphoproteome. Yeast strains MCY3278 and FY250 were labeled to steady state with [^{35}S]methionine (**A–D**) or [^{32}P]orthophosphate (**E–H**) and whole-cell extracts characterized by two-dimensional SDS–PAGE and autoradiography. Note, gels for protein detection were run separately from those detecting changes in phosphorylation. Spots of interest were identified by mixed peptide sequencing following transfer of the gels to PVM (Table I). Molecular weight (kDa) and isoelectric points (pI) are theoretical values calculated by the ExPASy compute pI/MW tool (http://expasy.hcuge.ch/ch2d/pi_tool.html) and are derived from the primary amino acid sequence of the identified proteins. Spot D in (F) is an exposure artifact and was not reproduced in four other separate experiments.

the two-dimensional gels were transferred to polyvinyl membrane (PVM) stained with amido black and the membranes autoradiographed. Eight phosphoproteins were

selected for mixed peptide sequencing. Five were increased in phosphate content in response to *REG1* deletion, namely Hxk2p spots a and b, hsp60, E1 α and spot C. Three

Fig. 2. The effects of *REG1* deletion and glucose starvation on protein phosphorylation and expression in yeast. Data shown in (A) were derived after densitometric analysis by the Melanie program of the autoradiograms shown in Figure 1. Data in (B) show the pmol amounts of each protein that was subjected to mixed peptide sequencing. Open bars represent data from the wild-type strain. Filled bars represent data from the *reg1Δ* strain. Data shown are mean values ($\pm$ SDM) from five separate experiments.

others, hsp71, elongation factor 1 (EF1) and initiation factor 5A (IF5A), were unaffected by the absence of Reg1p and were used as internal markers in mixed peptide sequencing to ensure that each gel was matched for protein loading (Figure 2B). Of the eight proteins that were selected for mixed peptide sequencing, all but one was identified unambiguously in the yeast database by the FASTF algorithm. The identified proteins included hsp71, hsp60, EF1, IF5A, the E1 α subunit of pyruvate dehydrogenase (E1) and Hxk2p a and b (Table I). Spot C was not identified because it was below our sequencing sensitivity (<100 fmol). Expectation scores for the identified proteins ranged from 5.4×10^{-75} for Hxk2p spot b to 7.6×10^{-8} for the E1 α subunit. In contrast, expectation scores for the next highest scoring non-related proteins ranged from 0.001 to 1.2. The identification of Hxk2p in this present study is consistent with previous evidence that glucose-induced dephosphorylation of Hxk2p *in vivo* is blocked in Glc7p point mutants (Glc7pT152K) and *reg1Δ* mutants (Randez-Gil, 1998).

Interestingly, two-dimensional SDS-PAGE resolved Hxk2p into two closely migrating species (a and b) regardless of growing conditions or *REG1* deletion. This separation does not appear to be related to isoformic variation or differences in phosphate content (i.e. mono and diphospho forms). Hxk2p shares ~70% homology with Hxk1p; however, from the alignments shown in Table I, two peptide sequences were recovered from both spot a and b that are unique to Hxk2p. One of the peptides, residues 304–314, has the sequence MSSGYLGEIL in Hxk2p; the same region in Hxk1p contains the sequence MTSGYYLGELL. A second peptide, residues 321–332, has the sequence MYKQGFIKNQD in Hxk2p; the same region in Hxk1p contains the sequence LNEKGLMLK-DQD. Mono and diphospho forms of Hxk2p also seem unlikely because the wild-type two-dimensional gel also contained two closely resolving species of the protein that contained no [32 P]phosphate (Figure 1A). The reason for the separation of Hxk2p into two species is therefore likely to be due to some other post-translational modification on one of the Hxk2p molecules.

To quantitate further the overall change in phosphorylation of the sequenced proteins, each mixed peptide sequencing run was analyzed for PTH amino acid content. This analysis also allowed the amount of dephosphoprotein relative to the phosphorylated form to be determined in each case. Figure 2B shows that the total amount of each protein sequenced in each gel (phospho and dephospho combined) did not vary significantly between wild-type and mutant or if either strain was grown in the absence of glucose. These findings demonstrate that deletion of Reg1p and glucose starvation promote phosphorylation of Hxk2p, E1 α and hsp60, but do not change their expression at the protein level. Similarly, no difference in the amount of EF1 β , IF5A and hsp71 was detected between wild-type or mutant strains, demonstrating that the overall protein loading for each gel was equivalent.

Measurement of the effects of deletion of *REG1* on hexokinase phosphatase activity

To test the effects of *REG1* deletion on Hxk2p phosphatase activity in yeast, recombinant Hxk2p was prepared as a 32 P-labeled phosphoprotein substrate using PKA (Figure 3). Unlike its mammalian counterparts, yeast Hxk2p is phosphorylated *in vivo* at a single residue, Ser15, and phosphorylation of this amino acid is increased in response to glucose (Vojtek *et al.*, 1990; Kriegl *et al.*, 1993; Randez-Gil *et al.*, 1998). The identity of the protein kinase that phosphorylates Hxk2p in intact yeast is not known; however, Ser15 is phosphorylated by cAMP-dependent PKA *in vitro* (Kriegel *et al.*, 1994). To confirm that Reg1p–Glc7p directly dephosphorylates Hxk2p, we assayed hexokinase phosphatase activity in wild-type and *reg1Δ* mutant cytosolic cell extracts. Assay of yeast cell extracts prepared from the particulate and cytosolic fraction with 32 P-labeled hexokinase revealed that $>95\%$ of the total hexokinase phosphatase activity in *S.cerevisiae* is present in the soluble fraction. This activity required up to 100 nM okadaic acid to inhibit it completely, indicating that it is a type 1 serine/threonine phosphatase (data not shown). To characterize yeast hexokinase phosphatase activity further, cell extracts were prepared from

Table I. Identification of phosphoproteins by mixed peptide sequencing

[illegible]

The sequence is listed in order of PTH amino acids that were recovered after each Edman cycle. The most abundant is listed first. FASTP was used to search and match the mixed sequences to the yeast NR database. The scoring matrix was MD20, with expectation and score values set to <1 and 5, respectively (Damer *et al.*, 1998).

MCY3278 and FY250 strains, fractionated by anion-exchange chromatography and fractions assayed for phosphatase activity using ^{32}P -labeled Hxk2p and phosphorylase *a* as the substrates (Figure 4A and B). Figure 4A shows that fractions from wild-type yeast contained a

major and a minor peak of Hxk2p phosphatase activity. Significantly, when fractions were assayed from the *reg1Δ* strain, the major Hxk2p phosphatase peak was absent. This result demonstrates that Reg1p targets PP-1C (Glc7p) to dephosphorylate Hxk2p. When column fractions from

Fig. 3. Preparation of yeast hexokinase II as a phosphoprotein substrate. (A) The result of a time course study in which 0.25 mg of recombinant Hxk2p was phosphorylated for 30 min with the indicated amounts of PKA. In (B), 0.25 mg of Hxk2p was phosphorylated with 400 ng of PKA for 30 min and then digested with CNBr. The digest was applied to a reverse-phase C18 column and developed with a linear gradient (1%/min) of acetonitrile in 0.1% TFA. Fractions were collected (1.0 ml) and the amount of radioactivity (c.p.m.) determined by Cerenkov counting. Fraction 27, which contained 90% of the total radioactivity, was sequenced in a 494 Applied Biosystems automated sequencer. To identify the precise amino acid that was phosphorylated in the protein, in insert (C), 100 pmol of Hxk2p was digested with endolyspeptidase C and the major phosphopeptide purified by reverse-phase HPLC. Fifty picomoles of the purified peptide was sequenced, the remaining half was coupled to a solid support for identification of the ^{32}P -labeled amino acid by collecting radioactivity after each Edman cycle (Fadden *et al.*, 1998).

Fig. 4. Assay of hexokinase phosphatase activity in yeast extracts. Cell extracts were prepared from wild-type (●) and *reg1Δ* (○) yeast and fractionated by anion-exchange chromatography. Column fractions were assayed for Hxk2p (A) and phosphorylase (B) phosphatase activity. In (C), column fractions from wild-type and *reg1Δ* yeast were Western blotted with a rabbit anti-PP-1C antibody.

both strains were assayed with phosphorylase *a* as the substrate, no significant difference in phosphatase activity was observed (Figure 4B). When assayed in the presence of 1 nM okadaic acid, all phosphorylase phosphatase activity was abolished in the column fractions, whereas

hexokinase phosphatase activity was not affected (data not shown). This suggests that the majority of the phosphorylase phosphatase activity detected in the column fractions was due to PP-2A. The finding that both the wild-type and mutant strains contained phosphorylase

phosphatase activity that was not affected by *REG1* deletion suggests that Reg1p is selective in its actions towards Hxk2p. This finding is consistent with the general hypothesis of PP-1 regulation in which its regulatory subunits target PP-1C towards a selective set of substrates (Hubbard and Cohen, 1993). To determine the effects of *REG1* deletion on PP-1C expression in the yeast cytosol, anion-exchange fractions were Western blotted with anti-PP-1C antibody (Figure 4C). In the wild-type column fractions, cross-reactivity with PP-1C was observed in two major areas, fraction 4 (data not shown) and between fractions 26 and 40. In the *reg1Δ* fractions, cross-reactivity was only observed in fraction 4, with no evidence of PP-1C between fractions 26 and 40. The presence of PP-1C in fractions 26–40 in the wild-type case is therefore due to binding of the phosphatase to Reg1p. These findings further support the hypothesis that Reg1p targets PP-1C to dephosphorylate Hxk2p *in vivo*. The finding that column fractions from the *reg1Δ* strain contained very little PP-1C by Western blot analysis is consistent with observations by others (Hubbard and Cohen, 1993) and our laboratory (Campos *et al.*, 1996; Damer *et al.*, 1998) that the majority of cellular PP-1 is localized in the particulate fraction. Our data suggest that in yeast, the Reg1p–Glc7p complex represents the major pool of PP-1 that is present in the cytosol.

The effects of disruption of the Reg1p PP-1C-binding site on the yeast phosphoproteome

Recently, Barford and colleagues identified the motif (K/R)(V/I)XF, or (K/R)X(I/V)XF, in Reg1p and some other yeast PP-1 subunits, as a putative PP-1C-binding site that is present in all known mammalian and yeast PP-1 regulatory subunits (Egloff *et al.*, 1997). To probe the functional significance of the (K/R)X(I/V)XF motif in Reg1p, point mutations were introduced into a LexA–Reg1p fusion protein at the phenylalanine residue (F468 in the Reg1p sequence). Two substitutions were made, F468D and F468R, to introduce a positive or negative charge into the binding site in place of the hydrophobic phenylalanine residue (note F468 is absolutely conserved in the primary sequences of all 20 of the mammalian and yeast PP-1 regulatory subunits reported to date). Plasmids containing LexA–Reg1p, LexA–Reg1pF468R and LexA–Reg1pF468D fusions were transformed into the *reg1Δ* strain. The transformed strains were incubated with ^{32}P or [^{35}S]methionine and extracts prepared for two-dimensional SDS–PAGE and autoradiography (Figure 5). Western analysis of LexA fusion protein immunoprecipitates prepared from each strain with LexA antibody confirmed that the fusion protein was expressed in all three cases (Figure 6A). Figure 5A–C shows that introduction of the LexA–Reg1p fusion proteins into the yeast proteome does not result in dramatic changes in the overall pattern of [^{35}S]methionine-labeled proteins when the gels were compared with one another. Significantly, however, Figure 5D shows that introduction of LexA–Reg1p into the *reg1Δ* strain completely rescues the normal pattern of phosphoprotein labeling that was observed in the FY250 strain earlier. In particular, the phosphate content of both Hxk2p spots was restored to basal levels observed in the wild-type state. In contrast, introduction of aspartic acid and arginine residues at F468 prevented reversion of the overall

two-dimensional phosphoprotein pattern back to the wild-type phenotype (compare Figure 5E and F with D). In the case of the F468D mutation, the phosphorylation state of Hxk2p (a), Hxk2p (b) and E1 α were increased 5.4 ± 1.2 -, 6.5 ± 2.2 - and 3.2 ± 0.52 -fold (SDM, $n = 2$), respectively, compared with strains transformed with wild-type LexA–Reg1p. Similar effects were also observed with the F468R mutants. None of the other 80 or so phosphoproteins detected in Figure 5D–F were altered significantly in phosphorylation state as a result of the transformations.

To test whether the two point mutations disrupted Hxk2p phosphatase activity of the Reg1p–Glc7p complex, cell extracts were fractionated by anion-exchange chromatography and assayed with ^{32}P -labeled Hxk2p (Figure 6B). Figure 6B shows that transformation of LexA–Reg1p into the MCY3278 strain restores Hxk2p phosphatase activity. This finding further demonstrates that the LexA–Reg1p fusion protein is expressed as a fully functional Glc7p targeting subunit. However, when the column fractions from the F468D and F468R mutants were assayed with ^{32}P -labeled Hxk2p, no evidence of phosphatase activity towards the substrate was detected. These observations are consistent with the increased phosphorylation of Hxk2p observed in these mutants, and suggest that mutation of F468 is sufficient to inhibit Reg1p function *in vivo*. To examine further whether introduction of aspartic acid or arginine at F468 disrupts Reg1p binding to Glc7p, the LexA–Reg1p fusion protein immunoprecipitates were blotted for PP-1C. Figure 6C shows that immunoprecipitates from LexA–Reg1p extracts contain Glc7p whereas those from the F468R and F468D mutants contained greatly reduced cross-reactivity at 37 kDa. Two-hybrid analysis further confirmed that the two point mutations also decreased interaction of Reg1p with Glc7p (Table II). These data demonstrate that the substitution at F468 was sufficient to disrupt binding of Reg1p to Glc7p in intact yeast. The finding that mutation of F468 to arginine or aspartic acid results in inhibition of Hxk2p dephosphorylation and disrupts binding of Reg1p to Glc7p in intact yeast demonstrates that the motif (K/R)X(I/V)XF is likely to be essential to the targeting function of all PP-1 regulatory subunits *in vivo*.

The effects of disruption of the Reg1p PP-1C-binding site on the pathways of glucose repression

The *reg1Δ* mutation relieves glucose repression of many genes, including *SUC2*, encoding invertase. To test the effect of mutations in the PP1-binding site on the function of Reg1p *in vivo*, we tested LexA–Reg1pF468R and LexA–Reg1pF468D for their ability to restore glucose repression of *SUC2* in a *reg1Δ* mutant. The wild-type LexA–Reg1p effectively complemented the *reg1Δ* mutation and reduced *SUC2* expression 130-fold in glucose-grown cells, whereas the mutant forms of LexA–Reg1p reduced *SUC2* expression only 3-fold (Table III). The mutant forms did not affect derepression of invertase activity in raffinose. Immunoblot analysis of this experiment indicated that the mutant proteins were present at levels ~5-fold lower than the wild-type LexA–Reg1p (Table III). These findings indicate that mutation of the PP1-binding site severely impairs, but probably does not

Fig. 5. The effects of disruption of the Reg1p PP-1C-binding motif on the yeast phosphoproteome. MCY3278 (*REG1* deleted) was transfected with plasmids expressing LexA-Reg1p, LexA-Reg1pF468D and LexA-Reg1pF468R fusion proteins. The transfected yeast were labeled to steady state with [^{35}S]methionine (A–C) or [^{32}P]orthophosphate (D–F) and whole-cell extracts characterized by two-dimensional SDS-PAGE and autoradiography. The indicated proteins were identified by mixed peptide sequencing following transfer of the gels to PVM.

completely abolish, Reg1p function *in vivo* in the glucose-sensing pathway.

Discussion

In this study, we have demonstrated that deletion of the Glc7p (PP-1C)-binding protein Reg1p in *S.cerevisiae* causes hyperphosphorylation of at least two enzymes governing the cellular fate of glucose, Hxk2p and E1 α . Importantly, the effect of *REG1* deletion on these proteins is mimicked by glucose starvation and is consistent with previous genetic and biochemical evidence demonstrating its essential role in the regulation of the glucose repression pathway. The hyperphosphorylation of Hxk2p that occurs in *reg1 Δ* strains is consistent with the absence of Hxk2p phosphatase activity observed following anion-exchange chromatography of extracts prepared from these cells.

Importantly, the wild-type phenotype was completely restored by expression of a LexA-Reg1p fusion protein in the *reg1 Δ* strain. Collectively, these results demonstrate that Reg1p binds Glc7p to target the phosphatase to dephosphorylate Hxk2p both *in vitro* and in intact yeast. We also show that the putative PP-1C-binding motif identified *in vitro* by Egloff *et al.* (1996), (K/R)X(I/V)XF in yeast and (K/R)(I/V)XF in mammals, is a physiological PP-1C-binding site *in vivo*. Introduction of single point mutations at F468 was sufficient to disrupt binding of Reg1p to Glc7p and inhibit hexokinase phosphatase activity both *in vitro* and *in vivo*. Disruption of the PP-1C-binding site also significantly reduced the ability of the yeast to glucose repress, suggesting that the Glc7p-Reg1p complex is required in the glucose signaling pathway. The finding that this effect was not complete is likely to be explained by the presence of a second low affinity

Fig. 6. The effects of point mutations at F468 on the association of PP-1C with Reg1p. Cell extracts were prepared from MCY3278 strains transfected with plasmids expressing LexA-Reg1p (●), LexA-Reg1pF468D (△) and LexA-Reg1pF468R (○) fusion proteins. In (A), extracts from each strain were immunoprecipitated with the LexA antibody then blotted for the expression of the LexA fusion protein. In (B), extracts from each strain were fractionated by anion-exchange chromatography and assayed for Hxk2p phosphatase activity. In (C), the LexA immunoprecipitates were blotted with an anti-rabbit PP-1C antibody.

binding site for Glc7p on the Reg1p molecule. This hypothesis is consistent with the partial recovery of PP-1C in the LexA fusion protein pull-down experiments shown in Figure 6 and data by Hartshorne and colleagues showing that other PP-1 regulatory subunits may also contain more than one PP-1C-binding site (Hartshorne *et al.*, 1998). Importantly, *REG1* deletion or Reg1pF468D/R substitution had discrete effects on the phosphorylation state of a select few proteins and did not bring about a global cellular change in protein phosphorylation or alter the expression pattern of the entire yeast proteome. This observation is consistent with the general hypothesis of PP-1 regulation in which distinct regulatory subunits target the catalytic subunit to discrete substrates. Reg1p is therefore the fourth example of a PP-1 regulatory subunit that has been shown to act directly in a substrate targeting capacity both *in vivo* and *in vitro*. The finding that Hxk2p is phosphorylated in response to glucose starvation (also shown previously by Randez-Gil *et al.*, 1998) suggests that Reg1p-Glc7p activity may be regulated in the intact cell. We are currently investigating this hypothesis.

The finding that disruption of Reg1p function causes hyperphosphorylation of Hxk2p *in vivo* suggests that one of the mechanisms by which the phosphatase subunit mediates its effects on the pathways of glucose repression is by controlling the phosphorylation state of hexokinase II. Yeast strains containing deletion of the Hxk2 genes are

Table II. Mutation of the PP-1C-binding site decreases the two-hybrid interaction of Reg1p with Glc7p

Expressed proteins	β -Galactosidase activity (Units)
LexA-Reg1 + GAD-Glc7	150 $\pm$ 7.1
LexA-Reg1F468R + GAD-Glc7	2.7 $\pm$ 0.4
LexA-Reg1F468D + GAD-Glc7	2.7 $\pm$ 0.2
LexA-Reg1 + GAD	0.7 $\pm$ 0.1
LexA-Reg1F468R + GAD	3.3 $\pm$ 0.1
LexA-Reg1F468D + GAD	3.1 $\pm$ 0.1
LexA + GAD-Glc7	2.6 $\pm$ 0.1

LexA immunoblot

Strain CTY10-5d (*MAT α ade2 his3 leu2 trp1 URA3::LexAop-lacZ gal4 gal80*) was transformed with plasmids expressing LexA-Reg1p and its mutant derivatives and GAD-Glc7p (pGAD-Glc7; Tu and Carlson 1995). Control plasmids were pACTII (Li *et al.*, 1994) and pLexA(1-202+PL) (Ruden *et al.*, 1991). Transformants were grown in SD + 4% glucose, and β -galactosidase activity was assayed as in Tu and Carlson (1995). Values are the average of 4-6 transformants $\pm$ SD.

Table III. Mutation of the PP-1C-binding site in Reg1p affects complementation of *reg1 Δ* with respect to the defect in glucose repression of *SUC2* expression

Expressed protein	Invertase activity (U)	
	4% Glucose	2% Raffinose
LexA	94.0	55.3
LexA-Reg1	0.7	52.4
LexA-Reg1F468R	31.8	52.2
LexA-Reg1F468D	30.9	50.0

Strain MCY3278 (*MAT α his3 Δ 200 leu2 Δ 1 trp1 Δ 63 reg1 Δ ::URA3*) was transformed with plasmids expressing the indicated proteins. Transformants were grown in SD + 4% glucose or SD + 2% raffinose + 0.05% glucose to mid-log phase, and invertase activity was assayed as in Tu and Carlson (1995). Values are the average of two different transformants.

defective in their glucose repression response (Gancedo, 1998). However, there are conflicting reports as to the functional significance of Hxk2p phosphorylation at Ser15 *in vivo*. Studies by Ma *et al.* (1990), in which the N-terminal 15 amino acids of Hxk2p were deleted, con-

cluded that Ser15 was not required for hexokinase activity or glucose repression *in vivo*. In contrast, more recent studies by Randez-Gil *et al.* (1998), in which Ser15 was mutated to a non-phosphorylatable alanine, produced mutants that were no longer able to glucose repress. Randez-Gil *et al.* also showed glucose-dependent dephosphorylation and dimerization of Hxk2p, and *in vitro*, the Hxk2p-S15A mutant enzyme was unable to form monomers and exhibited a lower K_m for glucose. These findings suggest that Ser15 phosphorylation may enable Hxk2p to scavenge glucose more efficiently under conditions when the sugar is limiting.

The mechanisms that lead to the increased phosphorylation of E1 α and hsp60 following *REG1* deletion or glucose starvation are likely to be indirect, resulting from changes in overall metabolic state rather than a loss of selective phosphatase activity mediated by Reg1p. E1 α is encoded by the *PDA1* gene and is an essential component of the pyruvate dehydrogenase (PDH) complex that converts pyruvate to acetyl-CoA. Like its mammalian counterpart, yeast E1 α catalysis is regulated by phosphorylation (Uhlinger *et al.*, 1986; James *et al.*, 1995). However, the increased phosphorylation of E1 α observed in response to *REG1* deletion is unlikely to be the result of a loss of PDH phosphatase activity mediated by Reg1p. First, E1 α is a mitochondrial enzyme and Glc7p (PP-1C) is not known to be a mitochondrial enzyme. Indeed, in Western blotting experiments of isolated yeast mitochondria, we found no evidence of PP-1C in this organelle (data not shown). Secondly, dephosphorylation and activation of E1 α are exclusively under the control of a specific PDH phosphatase related to the protein phosphatase 2C family (for a review see Reed *et al.*, 1985). Although the increased phosphorylation of hsp60 was relatively weak (1.24 ± 0.29 -fold) compared with that of Hxk2p and E1 α , this response was observed consistently in all experiments. The increased phosphorylation of hsp60 may be a non-specific stress response because similar increases in phosphorylation of hsp60 (and other heat shock family members) have been observed following deletion of non-related phosphatases and kinases in yeast (T.A.J.Haystead, unpublished data). Interestingly, as shown in Figure 5, the phosphorylation state of hsp60 was not affected by expression of the mutant fusion proteins, suggesting that increased phosphorylation of the protein observed in the *reg1* Δ strain is a non-specific effect induced by the absence of the Reg1p protein itself.

It is likely that only changes in the most abundant phosphoproteins that are substrates for the Reg1p–Glc7p complex were detected with the two-dimensional mapping protocols described in this study. Following two-dimensional analysis, the Melanie program detected on average 450 ± 36 (SDM, $n = 5$) distinct [35 S]methionine-labeled proteins per gel, representing ~6% of the 8300 genes in the entire *S.cerevisiae* genome. Therefore, the possibility exists that other proteins are also targets of Reg1p, but were not detected because they have a low level of expression or they did not enter the two-dimensional gel. Indeed, genetic evidence suggests that although Reg1p has unique roles in the regulation of the glucose repression pathway, it may also act synergistically with its structural homolog Reg2p to control cell growth (Frederick and Tatchel, 1996). Furthermore, two-hybrid analysis has

demonstrated a direct interaction between Reg1p and Snf1, suggesting that the kinase may be a substrate for the Reg1p–Glc7p complex (Ludin *et al.*, 1998). We are currently enriching the phosphoprotein content of our extracts prior to two-dimensional SDS–PAGE in order to evaluate the effects of glucose starvation and *REG1* deletion on the phosphorylation of lower abundance phosphoproteins.

The types of proteomic approaches that were utilized in this study to identify Hxk2p as a physiological target of the Reg1p–Glc7p complex herald a new age in which the functional consequences of genetic manipulations can be assessed directly and fully on the proteome as a whole. Because the microsequencing technology employed in this study gives an absolute identification of the protein sequenced, logical genetic and biochemical experiments can be carried out to probe the function and address the physiological relevance in the context of the intact cell. As the DDBJ/EMBL/GenBank databases are completed, this technology will have increasing relevance and become routinely applicable to studies in higher order eukaryotes, such as mammalian and plant cells, in addition to yeast.

Materials and methods

Strains and growth media

The *S.cerevisiae* strains used were MCY3278 (*MAT α _reg1 Δ ::URA3 his3 Δ 200 leu2 Δ 1 trp1 Δ 63 ura3-52 SUC2*) and FY250 (*MAT α _his3 Δ 200 leu2 Δ 1 trp1 Δ 63 ura3-52 SUC2*). Both have the S288C genetic background. Cells were grown in the rich medium YPD [1% yeast extract (Difco), 2% bacto peptone (Difco), 2% dextrose (Sigma)] or in phosphate-depleted YPD. Yeast were grown in 2% raffinose in those experiments involving glucose starvation.

Phosphate depletion of YPD (YPD-PO $_4$)

To 1 l of YPD liquid medium, 10 ml of 1 M MgSO $_4$ and 10 ml of concentrated aqueous NH $_3$ (27%) were added. The solution was stirred at room temperature for 30 min and filtered through Whatman No. 1 filter paper. After filtering, the solution was readjusted to pH 5.8 and autoclaved. This method produces medium containing low, 0.1 mM inorganic phosphate without decreasing organic phosphates.

[32 P]orthophosphate labeling

A single yeast colony was used to inoculate 4 ml of YPD medium and was grown overnight at 37°C with vigorous shaking. In the morning, 50 ml of YPD was inoculated from the overnight culture to a final OD $_{660}$ of 0.2. These cells were allowed to grow for two doublings and harvested by centrifugation at 800 g for 10 min. The harvested cells were washed with 10 ml of YPD-PO $_4$ and centrifuged at 800 g. The cells were resuspended in 15 ml of YPD-PO $_4$. YPD-PO $_4$ (50 ml) was inoculated with the resuspended cells to an OD $_{660}$ of 0.2 and grown for two doublings (~3 h). The cells were treated with 18.5 MBq of [32 P]orthophosphate for 1 h and harvested by centrifugation (800 g for 10 min).

Preparation of yeast extracts

The harvested cells were resuspended into 10 ml of ice-cold 5% trichloroacetic acid (TCA; J.T.Baker) and incubated on ice for 10 min. This mixture was centrifuged at 800 g for 10 min and the TCA decanted. The pellet was washed with 1 ml of acetone and centrifuged. After aspirating the acetone, the pellet was allowed to air dry overnight. Next, 0.5 ml of acid-washed glass beads was added to the pellet with 480 μ l of lysis buffer (0.1 M Tris–HCl pH 8, 0.3% SDS, 2.5% β -mercaptoethanol, 150 μ M sodium orthovanadate, 1 μ M microcystin). The extract was vortexed for 10 min, alternating 1 min on ice, 1 min vortexing. This mixture was incubated at 100°C for exactly 10 s and allowed to recover on ice for 1 min. A 20 μ l aliquot of RNase solution (0.5 M Tris–HCl pH 7.0, 0.05 M MgCl $_2$, 200 U/ml RNase A) and 2 U of DNase I (Promega) were added and incubated on ice for 2 min. Samples used for two-dimensional electrophoresis were saturated with urea by the addition of 500 mg of urea (Amresco) and 133 μ l of buffer (4.75 M

urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% Bio-Rad Biolytes, 5% β -mercaptoethanol). The glass beads were removed by centrifugation at 13 000 r.p.m. and aspiration of the sample. The supernatant was brought to 25% TCA by the addition of ice-cold 100% TCA and allowed to precipitate on ice for 10 min. The precipitate was pelleted by centrifugation at 14 000 g for 10 min. The resulting pellet was resuspended into 500 μ l of two-dimensional sample buffer (9.5 M urea, 2.0% Triton X-100, 5% β -mercaptoethanol). The protein concentration was measured using the method of Bradford (1976).

Determination of the specific activity of the γ -phosphate of ATP

Following labeling with [32 P]orthophosphate and homogenization, 100 μ l of cell extract was treated with 10 μ l of perchloric acid (55%) to precipitate the proteins. The extract was centrifuged (13 000 g for 5 min) and a nucleotide-enriched fraction containing ATP, ADP and AMP prepared by extraction of the supernatant with a 10% excess (v/v) mixture of tri-*n*-octylamine/1,1,2,2-trichlorotrifluoro-ethane (1:1 v/v). After vortexing, the extract was centrifuged (14 000 g for 3 min) and the neutralized upper aqueous layer removed for analysis by HPLC. The specific activity of the γ -phosphate of ATP was determined following purification of ATP and ADP by anion-exchange HPLC as described previously (Haystead and Hardie, 1988).

Two-dimensional electrophoresis and mixed peptide sequencing

Protein maps were prepared by two-dimensional electrophoresis. Isoelectric focusing was accomplished as the first dimension by preparation of a tube gel (4 cm $\times$ 1 mm) comprised of 4% acrylamide, 0.8% 5/7 ampholyte, 0.8% 6/8 ampholyte, 0.4% 3/10 ampholyte and 2% Triton X-100. NaOH (100 mM) was used as the basic buffer and 10 mM H_3PO_4 as the acidic buffer. The tube gel was pre-focused using the following program: 200 V 15 min, 300 V 25 min, 400 V 25 min. The proteins were loaded (50 μ g used for silver staining and 200 μ g for transfer) onto the basic end of the gel and overlaid with 1 vol. of overlay buffer (9 M urea, 0.8% 5/7 ampholyte, 0.2% 3/10 ampholyte). Isoelectric focusing was performed by application of 500 V for 15 min followed by 750 V for 5.5 h. After completion of the program, the gel was expelled from the tube, laid upon a 12% polyacrylamide gel (6 $\times$ 10 cm $\times$ 0.75 mm) and resolved at 150 V. The gels were equilibrated to transfer buffer (3 g/l Tris base, 14.25 g/l glycine, 200 ml/l methanol). The proteins were electroblotted to PVM pre-wetted with methanol at 30 V overnight. The transferred proteins were stained with amido black (1 mg/ml in 10% acetic acid, 10% methanol) for 1 min, washed once with destain buffer (10% acetic acid, 10% methanol) and successively with water. After destaining, the membrane was air dried and applied to X-ray film for autoradiography. The autoradiograph from each gel was compared using the Melanie software from Bio-Rad. Spots of interest were aligned to the membrane and the corresponding stained bands excised. The excised pieces were treated with 200 μ l of cyanogen bromide solution (500 mg/ml cyanogen bromide in 70% formic acid) for 90 min. The reaction was terminated by removal of the membrane and three washes with 1 ml of deionized water, then methanol followed by 50% methanol/water (v/v). The treated piece of PVM was placed in an Applied Biosystem 494 protein sequencer and 8–18 cycles of pulsed liquid chemistry carried out. The mixed peptide sequences generated were sorted and matched against the yeast protein databases by the FASTF algorithm (Damer *et al.*, 1998).

Preparation of 32 P-labeled protein substrates

Purified bovine cAMP-dependent PKA and phosphorylase *b* were gifts from John Lawrence (University of Virginia). Recombinant yeast hexokinase II was purchased from Sigma Chemical Co. For 32 P-labeled hexokinase II, 20 mg of the freeze-dried protein was resuspended in HEPES buffer [25 mM HEPES pH 7.4, 1 mM dithiothreitol (DTT)]. The protein was phosphorylated to ~1 mol/mol by the addition of 10 μ l (4 μ g/ μ l) of the native catalytic subunit of cAMP-dependent PKA in the presence of 200 μ M [γ - 32 P]ATP and 5 mM $MgCl_2$ for 30 min at room temperature. To determine the position of the phosphorylated amino acid, a sample (100 pmol) of the labeled protein was digested with CNBr (1 mg/ml for 16 h at room temperature). The major phosphopeptide was purified by reverse-phase HPLC and sequenced. [32 P]Phosphorylase *a* was prepared as described previously (Campos *et al.*, 1996).

Anion-exchange chromatography

Yeast cell pellets were prepared from wild-type and mutant strains grown in dextrose or raffinose by centrifugation of 50 ml cultures as described

above. The pellets were resuspended in 10 ml of Tris-HCl buffer [50 mM Tris-HCl pH 8.0 at room temperature, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 6 μ g/ml leupeptin, 10 mM phenylmethylsulfonyl fluoride (PMSF)]. Glass beads were added and the cells homogenized for 8 min following the vortexing protocol outlined above. The extracts were centrifuged for 60 min at 100 000 g and the supernatant passed through a 0.2 μ m filter. The supernatant was loaded onto a Pharmacia mono Q anion-exchange smart column (0.1 $\times$ 1.0 cm) previously equilibrated in Tris-HCl buffer. The column was developed in this buffer over 45 min at a flow rate of 25 μ l/min using a linear gradient to 600 mM NaCl. Hexokinase and phosphorylase phosphatase activity was measured in the collected fractions (25 μ l). To correct for any apparent differences in phosphatase activity between samples due to protein loading, absorbance at 280 nm was monitored on-line throughout anion-exchange chromatography.

Phosphatase activity assay

Hexokinase and phosphorylase phosphatase activity in the collected fractions was measured simultaneously by automated robotic assay. A 10 μ l aliquot of each column fraction was placed in a microtiter plate well. The robot added 50 μ l of HEPES buffer containing 10 μ M (final) of either 32 P-labeled hexokinase or 32 P-labeled phosphorylase *a*. This mixture was allowed to react for 10 min at room temperature and terminated by the addition of 100 μ l of 25% TCA. The entire plate was centrifuged at 2500 r.p.m. for 10 min and 120 μ l of the supernatant pipetted into 1.0 ml minitubes. Scintillation fluid (0.5 ml) was added and the amount of released phosphate determined in a Beckman LS6500 scintillation counter.

Site-directed mutagenesis of the PP-1C-binding site in Reg1p

To introduce F468D and F468R point mutations into the PP-1C-binding site on Reg1p, the appropriate oligonucleotides were synthesized and used for plasmid mutation on the complementary strand. In order to introduce the F468R point mutation into Reg1p, site-directed mutagenesis using the Morph kit (5 Prime $\rightarrow$ 3 Prime, Inc., Boulder, CO) was performed on the pRJ65 plasmid (LexA-Reg1; Tu and Carlson, 1995) with the RegMut3 oligonucleotide. The DNA sequence of the plasmid was verified with an Applied Biosystems PRISM 377 DNA Sequencer (Foster City, CA). The mutated plasmids were introduced into strain MCY3278 carrying the genomic deletion of *REG1* as described by Gietz *et al.* (1992).

Acknowledgements

We thank Perkin-Elmer for the generous support in the purchase of the 492 cLc Procise sequencer. This work was supported by grants (HL19242 and DK52378A) to T.A.J.H. and (GM354095) to M.C. from the National Institutes of Health. The FASTF and TFASTF programs can be accessed at <http://fasta.bioch.virginia.edu> or by contacting T.A.J.H.

References

- Alessi, D., MacDougall, L.K., Sola, M.M., Ikebe, M. and Cohen, P. (1994) The control of protein phosphatase-1 by targeting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. *Eur. J. Biochem.*, **270**, 1023–1035.
- Berman, H.K., O'Doherty, R.M., Anderson, P. and Newgard, C.B. (1998) Overexpression of protein targeting to glycogen (PTG) in rat hepatocytes causes profound activation of glycogen synthesis independent of normal hormone- and substrate-mediated regulatory mechanisms. *J. Biol. Chem.*, **273**, 26421–26425.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- Campos, M., Fadden, P., Qian, Z. and Haystead, T.A.J. (1996) Identification of protein phosphatase-1-binding proteins by microcystin-biotin affinity chromatography. *J. Biol. Chem.*, **271**, 28478–28484.
- Cohen, P. and Cohen, P.T. (1989) Protein phosphatases come of age. *J. Biol. Chem.*, **264**, 21435–21438.
- Damer, C.K., Partridge, J., Pearson, W.R. and Haystead, T.A.J. (1998) Rapid identification of protein phosphatase 1-binding proteins by mixed peptide sequencing and data base searching. Characterization of a novel holoenzymic form of protein phosphatase 1. *J. Biol. Chem.*, **273**, 24396–24405.

- Dent, P., Lavoie, A., Nakielnny, S., Caudwell, F.B., Watt, P. and Cohen, P. (1990) The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature*, **348**, 302–308.
- Doherty, M.J., Young, P.R. and Cohen, P.T.W. (1996) Amino acid sequence of a novel protein phosphatase 1 binding protein (R5) which is related to the liver- and muscle-specific glycogen binding subunits of protein phosphatase 1. *FEBS Lett.*, **339**, 339–343.
- Egloff, M.E., Johnson, D.F., Moorhead, G., Cohen, P.T.W., Cohen, P. and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.*, **16**, 1876–1887.
- Fadden, P., Haystead, T.A. and Lawrence, J.C., Jr (1997) Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. *J. Biol. Chem.*, **272**, 10240–10247.
- Frederick, D.L. and Tatchell, K. (1996) The *REG2* gene of *Saccharomyces cerevisiae* encodes a type 1 protein phosphatase-binding protein that functions with Reg1p and the Snf1 protein kinase to regulate growth. *Mol. Cell. Biol.*, **6**, 2922–2931.
- Gailly, P., Wu, X., Haystead, T.A.J., Somlyo, A.P., Cohen, P. and Somlyo, A.V. (1996) Regions of the 110-kDa regulatory subunit M110 required for regulation of myosin-light-chain-phosphatase activity in smooth muscle. *Eur. J. Biochem.*, **239**, 326–332.
- Gancedo, J.M. (1998) Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.*, **62**, 334–361.
- Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.
- Hartshorne, D.J., Ito, M. and Erdodi, F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. *J. Muscle Res. Cell Motil.*, **4**, 325–341.
- Haystead, T.A. and Hardie, D.G. (1988) Insulin and phorbol ester stimulate phosphorylation of acetyl-CoA carboxylase at similar sites in isolated adipocytes. Lack of correspondence with sites phosphorylated on the purified enzyme by protein kinase C. *Eur. J. Biochem.*, **175**, 339–345.
- Haystead, C.M.M., Gailly, P., Somlyo, A.P., Somlyo, A.V.S. and Haystead, T.A.J. (1995) Molecular cloning and functional expression of a recombinant 72.5 kDa fragment of the 110 kDa regulatory subunit of smooth muscle protein phosphatase 1M. *FEBS Lett.*, **377**, 123–127.
- Huang, D., Chun, K.T., Goebel, M.G. and Roach, P.J. (1996) Pho85p, a cyclin-dependent protein kinase and the Snf1p protein kinase act antagonistically to control glycogen accumulation in *Saccharomyces cerevisiae*. *Genetics*, **143**, 119–127.
- Hubbard, M.J. and Cohen, P. (1993) On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.*, **18**, 172–177.
- James, A.G., Cook, R.M., West, S.M. and Lindsay, J.G. (1995) The pyruvate dehydrogenase complex of *Saccharomyces cerevisiae* is regulated by phosphorylation. *FEBS Lett.*, **373**, 111–114.
- Johnson, D.F., Moorhead, G., Caudwell, F.B., Cohen, P., Chen, Y.H., Chen, M.X. and Cohen, P.T. (1996) Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targeting subunits. *Eur. J. Biochem.*, **239**, 317–325.
- Kriegel, T.M., Rush, J., Vojtek, A.B., Clifton, D. and Fraenkel, D.G. (1994) *In vivo* phosphorylation site of hexokinase 2 in *Saccharomyces cerevisiae*. *Biochemistry*, **33**, 148–152.
- Ludin, K., Jiang, R. and Carlson, M. (1998) Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **95**, 6245–6250.
- Ma, H., Bloom, L.M., Dakin, S.E., Walsh, C.T. and Botstein, D. (1989) The 15 N-terminal amino acids of hexokinase II are not required for *in vivo* function: analysis of a truncated form of hexokinase II in *Saccharomyces cerevisiae*. *Proteins*, **5**, 218–223.
- Printen, J.A., Brady, M.J. and Saltiel, A.R. (1997) PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. *Science*, **275**, 1475–1478.
- Randez-Gil, F., Sanz, P., Entian, K.-D. and Prieto, J.A. (1998) Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. *Mol. Cell. Biol.*, **18**, 2940–2948.
- Reed, L.J., Damuni, Z. and Merryfield, M.L. (1985) Regulation of mammalian pyruvate and branched-chain alpha-keto acid dehydrogenase complexes by phosphorylation–dephosphorylation. *Curr. Top. Cell Regul.*, **27**, 41–49.
- Shenolikar, S. and Nairn, A.C. (1991) Protein phosphatases: recent progress. *Adv. Second Messenger Phos. Res.*, **23**, 3–119.
- Shimizu, H. *et al.* (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.*, **269**, 30407–30411.
- Shirazi, A., Kunihiko, I., Mosse, C., Fadden, P., Somlyo, A.P., Somlyo, A.V. and Haystead, T.A.J. (1994) Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle. *J. Biol. Chem.*, **269**, 31598–31606.
- Stark, M.J. (1996) Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. *Yeast*, **12**, 1647–1675.
- Tu, J. and Carlson, M. (1995) REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. *EMBO J.*, **14**, 5939–5946.
- Uhlir, D.J., Yang, C.Y. and Reed, L.J. (1986) Phosphorylation–dephosphorylation of pyruvate dehydrogenase from bakers' yeast. *Biochemistry*, **25**, 5673–5677.
- Vojtek, A.B. and Fraenkel, D.G. (1990) Phosphorylation of yeast hexokinases. *Eur. J. Biochem.*, **190**, 371–375.

Received March 16, 1999; revised and accepted June 8, 1999