Copyright © 2000 by the Genetics Society of America

Important Role for Phylogenetically Invariant PP2Acα Active Site and C-Terminal Residues Revealed by Mutational Analysis in *Saccharomyces cerevisiae*

David R. H. Evans and Brian A. Hemmings

Friedrich Miescher Institute, Basel 4058 Switzerland

Manuscript received February 4, 2000
Accepted for publication May 4, 2000

ABSTRACT

PP2A is a central regulator of eukaryotic signal transduction. The human catalytic subunit PP2Acα functionally replaces the endogenous yeast enzyme, Pph22p, indicating a conservation of function *in vivo*. Therefore, yeast cells were employed to explore the role of invariant PP2Ac residues. The PP2Acα Y127N substitution abolished essential PP2Ac function *in vivo* and impaired catalysis severely *in vitro*, consistent with the prediction from structural studies that Tyr-127 mediates substrate binding and its side chain interacts with the key active site residues His-118 and Asp-88. The V159E substitution similarly impaired PP2Acα catalysis profoundly and may cause global disruption of the active site. Two conditional mutations in the yeast Pph22p protein, F232S and P240H, were found to cause temperature-sensitive impairment of PP2Ac catalytic function *in vitro*. Thus, the mitotic and cell lysis defects conferred by these mutations result from a loss of PP2Ac enzyme activity. Substitution of the PP2Acα C-terminal Tyr-307 residue by phenylalanine impaired protein function, whereas the Y307D and T304D substitutions abolished essential function *in vivo*. Nevertheless, Y307D did not reduce PP2Acα catalytic activity significantly *in vitro*, consistent with an important role for the C terminus in mediating essential protein-protein interactions. Our results identify key residues important for PP2Ac function and characterize new reagents for the study of PP2A *in vivo*.

PROTEIN phosphatase 2A (PP2A) is ubiquitous among eukaryotes and is implicated in multiple cellular processes including signal transduction, cell cycle regulation, and protein synthesis (Goldberg 1999). It exists as a family of holoenzymes in which the catalytic subunit (PP2Ac) binds a diverse array of regulatory subunits thought to modulate substrate specificity and intracellular targeting. PP2Ac binds a structural subunit PR65/A (Ruediger *et al.* 1992, 1994; Groves *et al.* 1999), forming a core dimer that in turn binds a PR55/B, PR61/B′, or PR72/B″ subunit (reviewed in Wera and Hemmings 1995). In addition, PP2Ac binds other proteins, notably the novel α4 subunit (Murata *et al.* 1997), in alternative heterodimers. Moreover, recent evidence indicates that PP2A forms stable complexes with protein kinase signaling molecules (Millward *et al.* 1999), indicating that it plays a central, regulatory role in signal transduction mediated by reversible protein phosphorylation.

Additional regulation of PP2Ac activity is mediated by post-translational modification of the C terminus. The highly conserved Tyr-307 residue may be targeted for phosphorylation, leading to an inhibition of PP2Ac activity (Chen *et al.* 1992, 1994), while the equally conserved C-terminal Leu-309 residue undergoes reversible methylation catalyzed by a competing methyltransferase and methylesterase enzyme (Lee and Stock 1993; Favre *et al.* 1994; Xie and Clarke 1994a,b; De Baere *et al.* 1999; Ogris *et al.* 1999a). Mutation of Leu-309 inhibits PP2Ac carboxymethylation (Bryant *et al.* 1999) while mutation of either Tyr-307 or Leu-309 disrupts PP2A subunit interactions (Ogris *et al.* 1997; Chung *et al.* 1999). Additionally, PP2Ac can be phosphorylated on threonine *in vitro*, leading to a decrease in catalytic activity (Guo and Damuni 1993), although the site of modification is unknown.

PP2A is a member of the PPP family of protein Ser/Thr phosphatases that includes protein phosphatase 1 (PP1) and PP2B (calcineurin). These protein phosphatases share many invariant amino acids, especially active site residues believed to be involved in metal ion and/or substrate binding and catalysis (Barton *et al.* 1994). The crystal structure of PP1γ1 suggests a model for the catalytic mechanism of the PPP family (Egloff *et al.* 1995; Goldberg *et al.* 1995), supported by mutagenesis studies and kinetic analysis of purified enzymes (Zhuo *et al.* 1994; Zhang *et al.* 1996; Huang *et al.* 1997). Thus, catalysis is believed to involve a one-step, metal-ion-mediated hydrolysis of the substrate by a metal-activated water molecule. Notably, the invariant active site residue PP1γ1 His-125 performs a critical role in the dephosphorylation reaction as a general acid that protonates

Corresponding author: David R. H. Evans, Program in Molecular Pharmacology, Mailstop D2-100, Fred Hutchinson Cancer Research Ctr., 1100 Fairview Ave. N., Seattle, WA 98109. E-mail: drhevans@usa.net

TABLE 1

Yeast strains

Strain[a]	Genotype	Source
DEY3	*pph21::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2* [YCpDE8: *URA3 PPH22*]	Evans and Stark (1997)
DEY103HA	*pph21Δ1::HIS3 PPH22^{HA} pph3Δ1::LYS2 lys2-95*	This study
DEY22-12HA[b]	[YCpDE22-12HA: *TRP1 pph22-12^{HA}*]	This study
DEY22-172HA[b]	[YCpDE22-172HA: *TRP1 pph22-172^{HA}*]	This study
DEY100	*pph21Δ1::HIS3 pph22-12 pph3Δ1::LYS2 lys2-925*	Evans and Stark (1997)
DEY217	*Pph21Δ1::HIS3 pph22-172::URA3 pph3Δ1::LYS2 lys2-925*	Evans and Stark (1997)
DEY1-Cα[b]	[YEpDE-PGK-Cα: *TRP1* HA-PP2Acα]	Evans *et al.* (1999)
INVSC1	*MATα ura3-52 his3Δ1 trp1-289 leu2*	Invitrogen

[a] Isogenic with W303-1A *MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112, ssd1-d2* (except INVSC1).
[b] Generated by plasmid shuffling in strain DEY3.

the Ser/Thr leaving group oxygen, accelerating phosphoester hydrolysis. Computer modeling based on the crystal structure of PP1γ1 suggests that PP2Ac and PP1c possess a similar overall fold (Evans *et al.* 1999) and an important catalytic role for PP2Ac His-118 (equivalent to PP1γ1 His-125) has been demonstrated biochemically (Ogris *et al.* 1999a,b; Chung and Brautigan 1999).

The budding yeast *Saccharomyces cerevisiae* possesses the proteins Pph22p/Pph21p (Sneddon *et al.* 1990; Ronne *et al.* 1991), Tpd3p (van Zyl *et al.* 1992), Cdc55p (Healy *et al.* 1991), Rts1p (Evangelista *et al.* 1996; Shu *et al.* 1997), and Tap42p (Di Como and Arndt 1996; Jiang and Broach 1999), similar to the mammalian PP2Ac, PR65/A, PR55/B, PR61/B′, and α4 subunits, respectively. Moreover, the mammalian PP2Ac (Evans *et al.* 1999; Lizotte *et al.* 1999) and PR61/B′ (Zhao *et al.* 1997) subunits functionally substitute for the equivalent proteins in yeast, indicating an exquisite conservation of PP2A structure/function. Recently, we used yeast to identify novel, dominant-negative forms of human PP2Acα that interfere with wild-type PP2A function *in vivo* (Evans *et al.* 1999). In this study, we have employed yeast as a convenient system to explore the functional role of invariant PP2Ac active site and C-terminal residues *in vivo* and *in vitro*.

MATERIALS AND METHODS

Strains, media, vectors, and sequence analysis: Yeast strains are described in Table 1. Rich (YPD), minimal (SD), and 5-fluoroorotic acid (5-FOA) media and nucleotide sequence analysis were described (Evans *et al.* 1999). Plasmids YIplac128, YCplac22 (Gietz and Sugino 1988), pYES2 (Invitrogen, Carlsbad, CA), pYPGE2 (Brunelli and Pall 1993), YEp352, and YCpDE1 (Evans and Stark 1997) were described.

Construction of strain DEY103HA: DEY103HA was constructed by replacing the genomic *PPH22::URA3* allele in strain DEY213 (Evans and Stark 1997) with the *PPH22^{HA}* allele by two-step gene transplacement. Thus, a *PPH22^{HA}* 1.8-kb *Xba*I/*Eco*RI fragment was inserted into the *LEU2* vector YIplac128. The plasmid was linearized at a *Kpn*I site upstream of the *PPH22^{HA}* open reading frame (ORF) and introduced into DEY213. Ura$^+$ Leu$^+$ merodiploid transformants were selected and subjected to a period of nonselective growth in YPD medium to promote recombination and excision of one of the duplicated *PPH22* loci. Ura$^-$ recombinants were selected on medium containing 5-FOA and screened for a Leu$^-$ phenotype on SD lacking leucine. One Ura$^-$ Leu$^-$ recombinant strain, DEY103HA, in which the chromosomal *PPH22::URA3* allele was replaced by *PPH22^{HA}*, was identified by PCR analysis of genomic DNA and Western blot analysis.

DNA manipulations: Epitope-tagged yeast PP2Ac, encoded by *PPH22^{HA}* with a double HA-tag inserted at the *Nsi*I site, was a gift from Mike Stark. Plasmid YCpDE22HA is a *PPH22^{HA}* 1.8-kb *Kpn*I/*Eco*RI fragment in vector YCplac22. Plasmid YCpDE22-12HA was constructed by first inserting the *PPH22^{HA}* 1.8-kb *Kpn*I/*Eco*RI fragment into vector YEp352. The 5-kb *Bst*XI/*Eco*RI fragment from the resulting plasmid (YEpDE2e) was ligated to the 0.9-kb *Bst*XI/*Eco*RI fragment of plasmid pDE22-12 (Evans and Stark 1997). From the resulting plasmid, the *pph22-12^{HA}* 1.8-kb *Xba*I/*Eco*RI fragment was finally inserted into YCplac22. Plasmid YCpDE22-172HA was constructed by first inserting a *pph22-172* 1.8-kb *Xba*I/*Eco*RI fragment (Evans and Stark 1997) into vector YIplac128. The 4.9-kb *Bst*XI/*Kpn*I fragment from the resulting plasmid was ligated to the 0.7-kb *Bst*XI/*Kpn*I fragment of YEpDE2e. Finally the *pph22-172^{HA}* 1.8-kb *Xba*I/*Eco*RI fragment was inserted into YCplac22. Human PP2Acα alleles were tagged with the hemagglutinin (HA) epitope and expressed from the *PGK1* promoter of vector pYPGE2 or the *GAL1* promoter of vector pYES2 as described (Evans *et al.* 1999). C-terminal mutations were generated in HA-PP2Acα by PCR using a reverse primer encoding the relevant substitution.

Preparation of yeast cell extracts and Western blot analysis: Extracts were prepared and Western blotting was performed as described (Evans *et al.* 1999) except that cell lysis and TNPT blocking buffer contained NP-40 at 0.1% (v/v) and 0.5% (v/v), respectively.

Immunoprecipitations and protein phosphatase activity measurements: Precipitations were performed as described (Evans *et al.* 1999) except that, following incubation of 12CA5-protein A Sepharose with cell extract, the beads were washed

with cell lysis buffer (4 times in 20 volumes) and Tris-buffered saline (TBS; 2 times in 20 volumes). Prior to Western blotting, HA-tagged Pph22 proteins were eluted from 12CA5-Protein A Sepharose beads by incubation (2 times for 30 min) with the HA-epitope peptide (50 μl, 1 mg/ml). Protein phosphatase assays were performed using an RRA(pT)VA phosphopeptide substrate as described (Evans *et al.* 1999) and activity was expressed as units (1 unit = 1 μmol of phosphate hydrolyzed per min).

Galactose-induced expression of PP2Acα alleles in liquid medium: PP2Acα alleles were expressed from the *GAL1* promoter of pYES2. Transformant cells (strain INVSC1) were grown to a density of 5.0×10^6 per ml at 30° in selective S-medium containing raffinose (2.0% w/v), glycerol (3.0% v/v), and cassamino acids (0.2% w/v), whereupon an equal volume of SG medium, containing galactose at 4.0%, was added.

RESULTS

Analysis of highly conserved PP2Ac active site residues: PP2Ac contains residues that are highly conserved between species and implicated in catalysis. The PP2Ac-Y127N mutant protein was identified in a screen for mutant forms that cause a dominant inhibition of cell growth when overexpressed in yeast (Evans *et al.* 1999). PP2Ac-Y127N is of particular interest because it is mutated for a tyrosine residue invariant between species and conserved in PP1. Computer modeling of PP2Acα structure predicts that the side chain of Tyr-127 is in van der Waal's contact with the active site residues, His-118 and Asp-88, that apparently are critical for PP2Ac-catalytic function (Egloff *et al.* 1995; Chung and Brautigan 1999; Evans *et al.* 1999; Ogris *et al.* 1999a,b). The PP2Acα mutant allele encoding Y127N was originally generated by random mutagenesis and encoded a second substitution, V159E. Surprisingly, site-directed mutagenesis revealed that each of these substitutions causes a dominant-negative effect individually (Evans *et al.* 1999). To investigate the interfering effect of the PP2Ac-Y127N and V159E substituted forms we analyzed them further and found that each was expressed stably in soluble yeast extracts at the level of wild-type PP2Acα (Figure 1A). However, unlike the wild-type protein, both the Y127N and V159E substituted form was severely impaired for catalytic activity *in vitro* (Figure 1B) and, consistent with this, each failed to provide essential PP2Ac function in yeast when expressed at a low level from the *PGK1* promoter (Figure 1C). These results demonstrate that the conserved Tyr-127 and Val-159 residues are important for PP2Acα catalytic function. Furthermore, they support the notion that the inhibition of cell growth observed during PP2Ac-Y127N or PP2Ac-V159E overexpression is caused by native folding, but catalytic impairment, of the mutant proteins.

Two active site substitution mutations have been identified in the yeast PP2Ac protein, Pph22p, that cause a recessive and conditional loss of function *in vivo* (Evans and Stark 1997). Thus, temperature-sensitive (ts) mutant cells containing the Pph22 F232S or P240H substituted form are viable at 30° (the permissive temperature) but inviable at 37° (restrictive temperature) when the wild-type protein is absent. Computer modeling of

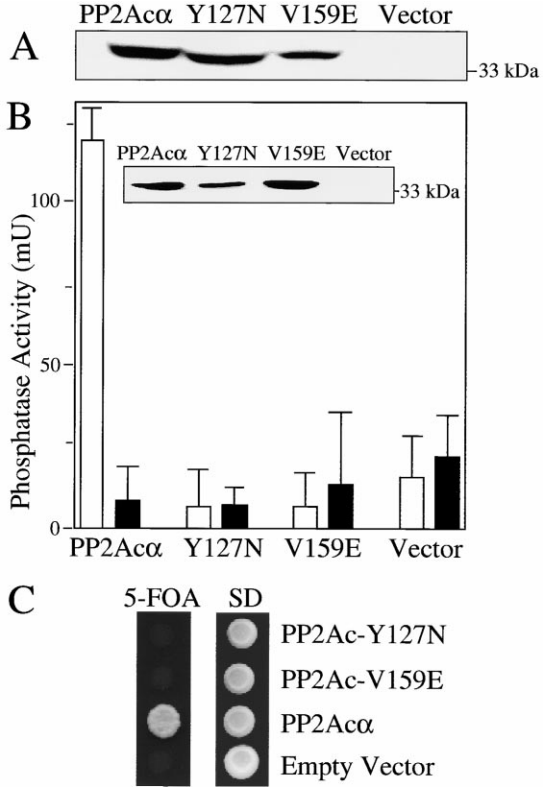

Figure 1.—Analysis of the human PP2Acα-Y127N and V159E substituted proteins. (A) Western blot analysis of PP2Acα wild-type and mutant forms expressed in yeast. HA-tagged proteins were inducibly expressed from the *GAL1*-promoter by incubating cells in SG medium for 4.5 hr. Soluble extracts were resolved by SDS-PAGE and analyzed by Western blotting using a 12CA5 probe. Molecular weight marker is shown on the right. (B) Catalytic activity of PP2Acα proteins *in vitro.* HA-tagged wild-type PP2Acα, or the Y127N or V159E substituted form, was inducibly expressed from the *GAL1* promoter in cells grown in SG for 6.5 hr. Cells containing empty vector were the negative control. HA-tagged proteins, precipitated with the 12CA5 monoclonal antibody from 200 μg of yeast cell extract, were assayed for phosphatase activity in the absence (open bars) or presence (solid bars) of 10 nM okadaic acid. Numbers represent the mean values (±SD) from three experiments. The inset shows a representative Western blot analysis of immune complexes prepared in parallel with those used for phosphatase assays; HA-tagged PP2Ac protein (36 kD) was absent from complexes prepared from the (vector) control extract. (C) Functional analysis of PP2Acα forms in yeast by plasmid shuffling. Wild-type PP2Acα, the Y127N or V159E substituted form, or no insert DNA (empty vector) was expressed from the *TRP1* plasmid pYPGE2 in cells of strain DEY3 and tested for essential function *in vivo* (ability to replace Pph22p expressed from the *URA3* plasmid, YCpDE8). Transformant cells were grown to saturation in liquid SD with uracil, whereupon cell suspensions were spotted onto agar medium containing 5-FOA or nonselective SD and incubated at 30° for 3 days.

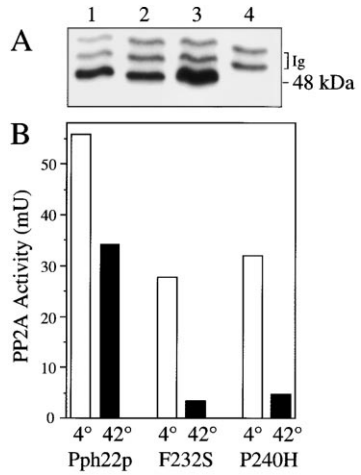

FIGURE 2.—Biochemical analysis of wild-type and ts mutant forms of Pph22p. (A) Immunoprecipitation of HA-tagged forms of Pph22p. Immune complexes containing an HA-tagged wild-type or mutant Pph22p were prepared from yeast cell extracts (900 μg) using the 12CA5 monoclonal antibody bound to protein-A Sepharose (30 μl). Lane 1, strain DEY-103HA (expressing HA-Pph22p); lane 2, DEY22-12HA (HA-Pph22-F232S); lane 3, DEY22-172HA (HA-Pph22-P240H); lane 4, strain DEY3 (untagged Pph22p). Ig, immunoglobulin. (B) PP2A activity of wild-type and mutant Pph22 proteins *in vitro*. Immune complexes prepared at 4° from the cell extracts (250 μg) in A were preincubated at 4° or 42° for 22 min, then assayed at 30° for protein phosphatase activity in the absence or presence of okadaic acid (10 nM). Numbers represent the mean values from two experiments (with assays performed in duplicate) corrected both for PP2A-specific (okadaic acid-sensitive) activity and background levels of activity (untagged Pph22p, see lane 4 in A). Average error was ±14.9 milliunits (4°) and ±17.7 milliunits (42°).

human PP2Acα structure predicts that the residues Phe-164 and Pro-172 (equivalent to Pph22 Phe-232 and Pro-240, respectively) pack closely to the highly conserved residue His-167 (Pph22 His-235), which may be important for catalysis through a role in metal binding (EGLOFF *et al.* 1995; GOLDBERG *et al.* 1995; EVANS *et al.* 1999). To test the Pph22 F232S and P240H substitutions for a temperature-dependent effect on catalysis, we measured PP2Ac activity *in vitro*. The wild-type and substituted forms of Pph22p were precipitated from a yeast cell extract (Figure 2A) and preincubated at 4° or 42° for 22 min before measurement of PP2A activity (Figure 2B); 42° was chosen arbitrarily as a temperature likely to reveal a difference between the wild-type and mutant proteins. Following preincubation at 4°, each mutant protein displayed reduced activity at 30° relative to wild-type Pph22p, suggesting that each is partially impaired for catalysis even at the permissive temperature for cell growth. Strikingly, however, both the F232S and P240H substituted forms displayed a dramatic loss of activity (7.9- and 6.8-fold, respectively) following incubation at 42°, while wild-type Pph22p displayed a 1.6-fold decrease under identical conditions. This supports the notion that PP2Ac loss of function at 37°, in cells containing the Pph22 F232S or P240H substituted protein, is caused by a reduction of PP2A activity *in vivo*.

Functional analysis of invariant PP2Ac C-terminal residues: The C terminus of PP2Ac is highly conserved between species and is a site of post-translational modification in the mammalian protein. The Tyr-307 residue of PP2Acα is invariant between species and believed to be a site of inhibitory phosphorylation (CHEN *et al.* 1992, 1994) while Thr-304 is equally conserved and is a candidate for inhibitory threonine phosphorylation (GUO and DAMUNI 1993). To investigate the functional role of highly conserved PP2Ac C-terminal residues *in vivo*, we tested PP2Acα mutant proteins for rescue of the ts growth defect caused by the F232S or P240H substitution in Pph22p. This revealed that substituting an aspartate for the conserved Tyr-307 or Thr-304 residue impaired PP2Acα function severely *in vivo*, while the Y307F and T304A substitutions caused partial and no detectable impairment, respectively (Table 2). This assay required incubation of cells at 37°, an extreme temperature for yeast cell growth, raising the possibility that under stress conditions, partial PP2Acα function might be obscured. To address this, we performed parallel experiments testing the ability of PP2Acα to substitute for wild-type Pph22p *in vivo* at 30° (EVANS *et al.* 1999). The results were in accordance with those obtained by the former assay (Figure 3A); the Y307D and T304D substitutions abolished essential PP2Acα function *in vivo* while the Y307F and T304A substituted proteins supported impaired and largely wild-type yeast cell growth, respectively, in the absence of Pph22p. A Y307A substituted form similarly supported impaired cell growth, indicating that alanine at this position does not abolish PP2Ac function *in vivo*. Growth rate analysis revealed that the Y307F substitution impaired protein

TABLE 2

Growth of *pph22* ts mutant cells containing a PP2Acα wild-type or mutant protein

	pph22 ts mutant strain			
	DEY100[a]		DEY217[b]	
PP2Acα protein	37°	30°	37°	30°
PP2Acα (wild type)	++	++	++	++
None (vector)	−	++	−	++
PP2Ac-Y307F	+	++	+	++
PP2Ac-Y307D	−	++	−	++
PP2Ac-T304A	++	++	++	++
PP2Ac-T304D	+	++	−	++

Human PP2Acα wild-type and mutant forms were expressed from vector pYPGE2 in *pph22* ts mutant cells, which were grown to stationary phase in selective SD medium, spotted onto YPD agar, and incubated at 37° or 30° for 36 hr.

[a] DEY100 (*pph22-12* allele, encoding Pph22-F232S).

[b] DEY217 (*pph22-172* allele, encoding Pph22-P240H).

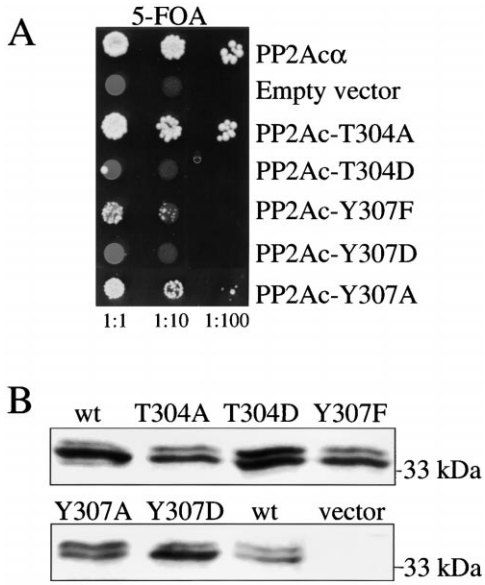

FIGURE 3.—Analysis of PP2Acα mutant forms in yeast. (A) Functional replacement of yeast PP2Ac (Pph22p) by wild-type and C-terminal mutant forms of human PP2Acα was tested by plasmid shuffling as in Figure 1C, except that cell suspensions were diluted (1:1, 1:10, or 1:100) prior to spotting onto agar medium containing 5-FOA. (B) Western blot analysis of HA-tagged forms of human PP2Acα expressed in strain DEY3. Yeast cell extracts (30 μg) containing wild-type PP2Acα (wt), a mutant form of PP2Ac, containing the substitution mutation indicated, or the empty expression vector were resolved by SDS-PAGE and probed with the 12CA5 monoclonal antibody. Molecular mass marker shown on the right.

function severely *in vivo* while T304A had little inhibitory effect (Table 3). Furthermore, the growth rate of cells functionally expressing PP2Ac-Y307F varied between experiments, suggesting the accumulation of suppressor mutations. Western blot analysis of cell extracts (Figure 3B) revealed that all human PP2Acα proteins tested were stably expressed and soluble in yeast and, typical of the wild-type protein (EVANS *et al.* 1999), migrated as a prominent doublet during SDS-PAGE. These results indicate that PP2Acα function is sensitive to mutation of evolutionarily invariant residues at its C terminus, especially replacement of Tyr-307 or Thr-304 by a charged aspartate residue. Nevertheless, replacement of Thr-304 with alanine is largely tolerated, while uncharged residues replacing Tyr-307 cause partial impairment of protein function.

TABLE 3

Growth rate of cells functionally expressing a PP2Acα wild-type or mutant protein

PP2Acα protein[a]	t_d (hr)[b]
PP2Acα (wild type)	2.8 ± 0.28
PP2Ac-T304A	3.2 ± 0.38
PP2Ac-Y307F	8.0 ± 2.60

[a] PP2Acα proteins were functionally expressed in yeast by plasmid shuffling in strain DEY3 (see Figures 1C and 3A legends).

[b] Doubling time of cells grown in liquid YPD at 30°. Numbers represent the mean values from at least two experiments.

PP2Ac-Y307D is enzymatically active and causes recessive PP2Ac impairment: It has been reported that phosphorylation on Tyr-307 inhibits PP2Ac activity (CHEN *et al.* 1992, 1994). Therefore, we asked whether an acidic substitution mutation at this position mimics the inhibitory effect of phosphorylation by testing the PP2Ac-Y307D mutant protein for catalytic activity *in vitro*. Surprisingly, PP2Ac-Y307D displayed approximately wild-type activity (Figure 4), indicating that its inability to provide essential PP2Ac function *in vivo* is not due to severe unfolding or catalytic impairment. We demonstrated recently that similarly nonfunctional PP2Acα mutant proteins, truncated at the C terminus, cause a severe dominant-negative inhibition of cell growth when expressed in yeast from a strong promoter (EVANS *et al.* 1999 and see PP2Ac-Y218stop, Table 4). In contrast, when the noncomplementing Y307D and T304D C-terminally substituted forms of PP2Acα were expressed from the *GAL1* promoter, neither inhibited cell growth severely, though a weak dominant-negative effect was observed initially (see colony formation after 5 days in Table 4). Together, these results suggest that, unlike truncated PP2Acα proteins, the Y307D and T304D C-terminally substituted forms retain some property that prevents severe interference with wild-type PP2Ac function.

DISCUSSION

Human PP2Acα provides an essential function in yeast (EVANS *et al.* 1999; LIZOTTE *et al.* 1999), consistent with the phylogenetic conservation of PP2A holoenzyme structure and biochemistry (COHEN *et al.* 1989; MAYER-JAEKEL and HEMMINGS 1994). Thus, we have used yeast as a convenient system to explore the role of highly conserved PP2Acα active site and C-terminal residues that are invariant between species. Our results demonstrate the importance of the PP2Ac C terminus for protein function *in vivo* and identify active site residues important for enzyme activity. Moreover, this study characterizes a number of human and yeast PP2Ac mutant forms that will be useful for further study of PP2A function *in vivo*.

PP2Ac is sensitive to C-terminal mutations: We have found that PP2Ac function is sensitive to mutation of invariant C-terminal residues, especially replacement of Tyr-307 or Thr-304 with an aspartate residue. This is consistent with the observation that the PP2Ac C terminus is an important regulatory domain, which, in mammalian cells, is targeted for multiple post-translational modifications modulating PP2Ac activity and subunit

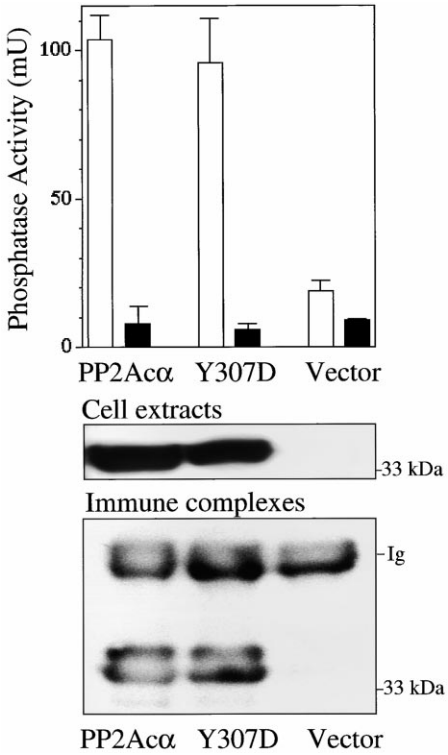

FIGURE 4.—Catalytic activity of the PP2Acα-Y307D substituted form. HA-tagged wild-type PP2Acα, or the Y307D-substituted form, was inducibly expressed from the *GAL1* promoter in cells grown in SG for 14 hr. Cells containing no HA-tagged protein (vector) were the negative control. HA-tagged proteins, precipitated by the 12CA5 monoclonal antibody from 250 μg of yeast cell extract, were assayed for phosphatase activity in the absence (open bars) or presence (solid bars) of 10 nM okadaic acid to identify PP2A activity. Numbers represent the mean from two experiments. (Top) Phosphatase activity measured in immune complexes. (Middle) Western blot analysis of HA-tagged Pph22 proteins present in yeast cell extracts. (Bottom) Representative Western blot analysis of immune complexes prepared (from 1.0 mg of cell extract) in parallel with those used for phosphatase assays; HA-tagged PP2Acα protein (~36 kD) was absent from complexes prepared from the (vector) control extract. Ig, immunoglobulin. Blots were probed with the 12CA5 monoclonal. Molecular mass marker shown on the right.

interactions. In mammalian cells, the C-terminal Tyr-307 residue may undergo phosphorylation, leading to an inhibition of PP2Ac activity (CHEN *et al.* 1992, 1994), and because PP2A participates in stable complexes with protein kinase signaling molecules, this may contribute to a mechanism permitting transient activation and feedback regulation of kinase-mediated signaling pathways (EVANS and HEMMINGS 1998; MILLWARD *et al.* 1999). Nevertheless, we show here that the Y307D substitution does not detectably impair the catalytic function of PP2Acα *in vitro* and therefore does not mimic phosphorylation of Tyr-307. This is consistent with the observations of OGRIS *et al.* (1997), who introduced uncharged, basic, or acidic substitutions (including a Y307E and T304D) to the PP2Ac C terminus and reported that each mutant protein retained activity *in vitro*. However, the same authors reported that changing the PP2Ac Tyr-307 or Thr-304 residue to a charged (but not an uncharged) amino acid disrupts binding of the PR55/B regulatory subunit to the PP2Ac·PR65/A core dimer. This suggests that the PP2Acα-Y307D and T304D substituted proteins are unable to support yeast cell growth because of defective subunit binding. Accordingly, in an *in vivo* assay testing the ability of PP2Acα to rescue the ts growth of cells containing a Pph22p F232S or P240H substituted protein, neither PP2Ac-Y307D nor PP2Ac-T304D provided essential function at 37°. It is noteworthy that in the W303 yeast strain background used in this study, deletion of the *CDC55* gene (encoding the yeast PR55/B subunit) confers a ts growth defect at 37° (D. EVANS, unpublished observation) in addition to the previously reported cold-sensitive growth phenotype (HEALY *et al.* 1991). Thus, PP2Ac-Y307D and PP2Ac-T304D may be inhibited for Cdc55p binding. However, each mutant protein failed additionally to provide essential PP2Ac function in an alternative plasmid-shuffling assay that tests the ability of PP2Acα to replace Pph22p at 30° (a permissive temperature for growth of *cdc55*Δ mutant cells). This indicates that the Y307D and T304D substitutions may inhibit PP2Ac function by impairing the binding of multiple proteins, possibly including the yeast PR61/B′ subunit, Rts1p (the lack of which causes a ts growth defect at 37°; SHU *et al.* 1997), the essential α4-like subunit, Tap42p (DI COMO and ARNDT 1996), and endogenous PP2A substrates. In contrast to the observations of OGRIS *et al.* (1997), it has been reported that changing Tyr-307 to an uncharged amino acid inhibits the interaction of PP2Ac with

TABLE 4

Effect on yeast cell growth of PP2Acα proteins inducibly expressed from the *GAL1* promoter

	Colony formation[a]			
	5 days		7 days	
PP2Acα protein	Glu	Gal	Glu	Gal
PP2Acα	+++	+++	+++	+++
PP2Ac-T307D	+++	++	+++	+++
PP2Ac-T304D	+++	++	+++	+++
PP2Ac-Y218stop[b]	+++	−	+++	+/−

PP2Acα proteins were expressed from the *GAL1* promoter of vector pYES2 in cells of strain DEY1-Cα. Transformants were grown to stationary phase in selective SD, and ~100 cells were spread onto glucose (SD) or galactose (SG) agar and incubated at 22°. Colony formation was measured after 5 and 7 days.

[a] (−) No colonies visible by eye; (+/−) microcolonies; (++, +++) colonies of 1.5 and 2.0 mm diameter, respectively.

[b] C-terminally truncated form of PP2Acα encoded by the PP2Ac-216 dominant-negative allele (EVANS *et al.* 1999).

PR55/B (Chung *et al.* 1999). Our results are in agreement with this, because the PP2Ac-Y307F and PP2Ac-Y307A mutant proteins were impaired for PP2Ac function *in vivo*, possibly reflecting a partial defect in subunit binding. Consistent with this, mutation of the adjacent, highly conserved C-terminal leucine of mammalian PP2Ac similarly inhibits PR55/B binding (Bryant *et al.* 1999; Chung *et al.* 1999) and impairs, but does not abolish, PP2Acα function in yeast (Evans *et al.* 1999). Moreover, mutation of the Pph22p C-terminal leucine to alanine similarly inhibits Cdc55p binding (D. Evans and B. Hemmings, unpublished results).

Identification of active site residues important for PP2Ac catalytic function: The PP2Acα-Y127N and V159E substituted forms were identified in a screen for dominant-negative mutant proteins that interfere with wild-type PP2Acα function *in vivo* (Evans *et al.* 1999). Here we show that the Y127N and V159E substitutions each cause a profound impairment of PP2Ac-mediated catalysis *in vitro*. Our observations provide support for the prediction, derived from structural data, that the Tyr-127 side chain is in van der Waal's contact with that of the invariant active site residues, His-118 and Asp-88 (Egloff *et al.* 1995). Thus, His-118 is thought to serve as a general acid that protonates the seryl/threonyl phosphate ester oxygen atom, accelerating the dephosphorylation reaction, while the acidic character of His-118 is believed to be increased by salt bridge formation between its side chain and that of Asp-88 (Egloff *et al.* 1995). Consistent with this, we have found that a D88N mutation reduces PP2Ac activity and abolishes essential PP2Ac function in yeast without preventing PR65/A subunit binding (T. Myles, D. Evans and B. Hemmings, unpublished results). Thus, the Y127N substitution may inhibit PP2Ac activity by disrupting the interaction between key catalytic residues. In addition, computer modeling predicts that Tyr-127 interacts with the Ser(P)/Thr(P) substrate (Evans *et al.* 1999), suggesting that Y127N may impair PP2Ac catalysis by inhibiting substrate binding. Like Y127N, the V159E substitution impairs PP2Acα catalytic function profoundly. The residue equivalent to Val-159 is highly conserved among PP2Ac proteins, and computer modeling predicts that the V159E substitution causes a global disruption of the PP2Acα active site (Evans *et al.* 1999), which may account for its effect on enzyme activity. However, the dominant-negative effect on cell growth conferred by both the PP2Ac-Y127N and V159E substituted forms suggests that each undergoes native folding, at least over a portion of the molecule. Thus, when overexpressed in yeast cells, these catalytically impaired, but partially folded, mutant proteins may titrate substrates or regulatory subunits into PP2A complexes that are effectively inactive, thereby interfering with the function of the wild-type enzyme. Consistent with this, we have found that each of these mutant proteins competes with wild-type PP2Acα *in vivo* (Evans *et al.* 1999). Surprisingly however, we have found that yeast cells induced to overexpress the catalytically impaired PP2Ac-H118N mutant protein (Evans *et al.* 1999; Lizotte *et al.* 1999), which similarly inhibits yeast colony formation on agar medium, fail to arrest proliferation completely in liquid medium, as determined by measurement of growth rate and DNA content (D. Evans and B. Hemmings, unpublished results). Thus, the precise physiological effect of PP2Ac interfering proteins *in vivo* remains to be uncovered, but we are currently testing for effects of PP2Acα wild-type and mutant forms expressed in mammalian cells. Interestingly, we have found that the PP2Acα-Y307D mutant protein confers a weak, dominant-negative effect despite retaining catalytic activity. This supports the notion that proper folding of mutant PP2Ac forms facilitates interference with the wild-type protein, but that catalytic impairment greatly exacerbates the dominant-negative effect.

The Pph22 F232S and P240H substitutions were shown by genetic analysis to cause a conditional-lethal loss of PP2Ac function *in vivo* (Evans and Stark 1997). In this study, we show that the loss of function caused by these substitution mutations reflects a ts reduction in PP2Ac enzyme activity *in vitro*. This supports the notion that the mitotic and cell lysis defects caused by these mutations (Evans and Stark 1997) are caused by loss of PP2A activity *in vivo*. The Phe-232 and Pro-240 residues flank a His-Gly-Gly motif invariant in PP2Ac proteins and in which the histidine residue is implicated in metal binding (Egloff *et al.* 1995; Goldberg *et al.* 1995). Computer modeling (Evans *et al.* 1999) predicts that the equivalent residue in human PP2Acα, His-167, lies at the base of a hydrophobic pocket (encompassing β-sheet 6) and that its packing may be disrupted by a F164S or P172H substitution (equivalent to Pph22 Phe-232 and Pro-240, respectively), possibly leading to impairment of metal-ion-mediated catalysis. Nevertheless, the ts growth defect at 37°, of cells containing the Pph22 F232S or P240H substituted form, is rescued by wild-type Pph22p (Evans and Stark 1997) even when the mutant proteins are expressed from a high copy (2μ) vector and the wild-type protein is expressed from a low copy (*CEN*) vector (D. Evans, unpublished observations). Thus, although we have not expressed the Pph22 F232S or P240H proteins from the same *GAL1*-based expression system from which the human PP2Acα Y127N and V159E dominant-negative forms were overexpressed, the Pph22 F232S and P240H mutations apparently do not cause a severe dominant-negative effect. This suggests that, at 37°, these mutant proteins may undergo ts denaturation over a large portion of the molecule, preventing interference with wild-type Pph22p. However, these conditionally defective mutant proteins will be useful tools for studying PP2A function in haploid yeast cells. Moreover, because PP2A function is likely to regulate the basal and stimulated activity of protein kinase-mediated signaling pathways, the reces-

sive and dominant-negative mutant forms of PP2Ac characterized here may help uncover important mechanisms and components of eukaryotic signal transduction.

We thank Mike Stark (Dundee University) for HA-tagged Pph22p; Herbert Angliker for DNA sequence analysis; and Timothy Myles, Thomas Millward, Mirjana Andjelkovic, and Pat Dennis for comments on the work. This work was supported by a Human Frontiers program grant (B.A.H).

LITERATURE CITED

Barton, G. J., P. T. W. Cohen and D. Barford, 1994 Conservation analysis and structure prediction of the protein serine/threonine phosphatases. Eur. J. Biochem. **220:** 225–237.

Brunelli, J. P., and M. L. Pall, 1993 A series of yeast shuttle vectors for expression of cDNAs and other DNA sequences. Yeast **9:** 1299–1308.

Bryant, J. C., R. S. Westphal and B. E. Wadzinski, 1999 Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Bα subunit. Biochem. J. **339:** 241–246.

Chen, J., B. L. Martin and D. L. Brautigan, 1992 Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science **257:** 1261–1264.

Chen, J., S. Parsons and D. L. Brautigan, 1994 Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and *v-src* transformation of fibroblasts. J. Biol. Chem. **269:** 7957–7962.

Chung, H., and D. L. Brautigan, 1999 Protein phosphatase 2A suppresses MAP kinase signaling and ectopic protein expression. Cell Signal. **11:** 575–580.

Chung, H., A. C. Nairn, K. Murata and D. L. Brautigan, 1999 Mutation of Tyr307 and Leu309 in the protein phosphatase 2A catalytic subunit favors association with the alpha 4 subunit which promotes dephosphorylation of elongation factor-2. Biochemistry **38:** 10371–10376.

Cohen, P., D. L. Schelling and M. J. R. Stark, 1989 Remarkable similarities between yeast and mammalian protein phosphatases. FEBS Lett. **250:** 601–606.

De Baere, I., R. Derua, V. Janssens, C. Van Hoof, E. Waelkens *et al.*, 1999 Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. Biochemistry **38:** 16539–16547.

Di Como, C. J., and K. T. Arndt, 1996 Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. **10:** 1904–1916.

Egloff, M.-P., P. T. W. Cohen, P. Reinemer and D. Barford, 1995 Crystal structure of the catalytic subunit of human phosphatase 1 and its complex with tungstate. J. Mol. Biol. **254:** 942–959.

Evangelista, C. C., A. M. Rodriguez-Torres, M. P. Limbach and R. S. Zitomer, 1996 Rox3 and Rts1 function in the global stress response pathway in baker's yeast. Genetics **142:** 1083–1093.

Evans, D. R. H., and M. J. R. Stark, 1997 Mutations in the *Saccharomyces cerevisiae* type 2A protein phosphatase catalytic subunit reveal roles in cell wall integrity, actin cytoskeleton organization and mitosis. Genetics **145:** 227–241.

Evans, D. R. H., and B. A. Hemmings, 1998 Signal transduction: what goes up must come down. Nature **394:** 23–24.

Evans, D. R. H., T. Myles, J. Hofsteenge and B. A. Hemmings, 1999 Functional expression of human PP2Ac in yeast permits the identification of novel C-terminal and dominant-negative mutant forms. J. Biol. Chem. **274:** 24038–24046.

Favre, B., S. Zolnierowicz, P. Turowski and B. A. Hemmings, 1994 The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. J. Biol. Chem. **269:** 16311–16317.

Gietz, R. D., and A. Sugino, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

Goldberg, Y., 1999 Protein phosphatase 2A: who shall regulate the regulator? Biochem. Pharm. **57:** 321–328.

Goldberg, J., H. B. Huang, Y. G. Kwon, P. Greengard, A. C. Nairn *et al.*, 1995 Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature **376:** 745–753.

Groves, M. R., N. Hanlon, P. Turowski, B. A. Hemmings and D. Barford, 1999 The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. Cell **96:** 99–110.

Guo, H., and Z. Damuni, 1993 Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. Proc. Natl. Acad. Sci. USA **90:** 2500–2504.

Healy, A. M., S. Zolnierowicz, A. E. Stapleton, M. Goebl, A. A. DePaoli-Roach *et al.*, 1991 *CDC55*, a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. Mol. Cell. Biol. **11:** 5767–5780.

Huang, H-B., A. Horiuchi, J. Goldberg, P. Greengard and A. C. Nairn, 1997 Site-directed mutagenesis of amino acid residues of protein phosphatase 1 involved in catalysis and inhibitor binding. Proc. Natl. Acad. Sci. USA **94:** 3530–3535.

Jiang, Y., and J. R. Broach, 1999 Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. EMBO J. **18:** 2782–2792.

Lizotte, D. L., D. D. McManus, H. R. Cohen and A. DeLong, 1999 Functional expression of human and Arabidopsis protein phosphatase 2A in *Saccharomyces cerevisiae* and isolation of dominant-defective mutants. Gene **234:** 35–44.

Lee, J., and J. Stock, 1993 Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase. J. Biol. Chem. **268:** 19192–19195.

Mayer-Jaekel, R. E., and B. A. Hemmings, 1994 Protein phosphatase 2A—a 'menage a trois.' Trends Cell Biol. **4:** 287–291.

Millward, T. A., S. Zolnierowicz and B. A. Hemmings, 1999 Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem. Sci. **24:** 186–191.

Murata, K., J. Wu and D. L. Brautigan, 1997 B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. Proc. Natl. Acad. Sci. USA **94:** 10624–10629.

Ogris, E., D. M. Gibson and D. C. Pallas, 1997 Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen. Oncogene **15:** 911–917.

Ogris, E., X. Du, K. C. Nelson, E. K. Mak, X. X. Yu *et al.*, 1999a A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of phosphatase 2A. J. Biol. Chem. **274:** 14382–14391.

Ogris, E., I. Mudrak, E. Mak, D. Gibson and D. C. Pallas, 1999b Catalytically inactive protein phosphatase 2A can bind to polyomavirus middle tumor antigen and support complex formation with $pp60^{c\text{-}src}$. J. Virol. **73:** 7390–7398.

Ronne, H., M. Carlberg, G-Z. Hu and J. O. Nehlin, 1991 Protein phosphatase 2A in *Saccharomyces cerevisiae*: effects on cell growth and bud morphogenesis. Mol. Cell. Biol. **11:** 4876–4884.

Ruediger, R., D. Roeckel, J. Fait, A. Bergqvist, G. Magnusson *et al.*, 1992 Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. Mol. Cell. Biol. **12:** 4872–4882.

Ruediger, R., M. Hentz, J. Fait, M. Mumby and G. Walter, 1994 Molecular model of the A subunit of protein phosphatase 2A: interaction with other subunits and tumor antigens. J. Virol. **68:** 123–129.

Shu, Y., H. Yang, E. Hallberg and R. Hallberg, 1997 Molecular genetic analysis of Rts1p, a B′ regulatory subunit of *Saccharomyces cerevisiae* protein phosphatase 2A. Mol. Cell. Biol. **17:** 3242–3253.

Sneddon, A. A., P. T. W. Cohen and M. J. R. Stark, 1990 *Saccharomyces cerevisiae* protein phosphatase 2A performs an essential cellular function and is encoded by two genes. EMBO J. **9:** 4339–4346.

van Zyl, W., W. Huang, A. A. Sneddon, M. Stark, S. Camier *et al.*, 1992 Inactivation of the protein phosphatase 2A regulatory subunit A results in morphological and transcriptional defects in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 4946–4959.

Wera, S., and B. A. Hemmings, 1995 Serine/threonine protein phosphatases. Biochem. J. **311:** 17–29.

Xie, H., and S. Clarke, 1994a Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. J. Biol. Chem. **269:** 1981–1984.

Xie, H., and S. Clarke, 1994b An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A. Biochem. Biophys. Res. Com. **203:** 1710–1715.

Zhang, J., Z. Zhang, K. Brew and E. Y. C. Lee, 1996 Mutational analysis of the catalytic subunit of muscle protein phosphatase-1. Biochemistry **35:** 6276–6282.

Zhao, Y., G. Boguslawski, R. S. Zitomer and A. A. DePaoli-Roach, 1997 *Saccharomyces cerevisiae* homologs of mammalian B and B′ subunits of protein phosphatase 2A direct the enzyme to distinct cellular functions. J. Biol. Chem. **272:** 8256–8262.

Zhuo, S., J. C. Clemens, R. L. Stone and J. E. Dixon, 1994 Mutational analysis of a Ser/Thr phosphatase. J. Biol. Chem. **269:** 26234–26238.

Communicating editor: P. Russell