The protein phosphatase 2A (PP2A) holoenzyme is structurally conserved among eukaryotes. This reflects a conservation of function in vivo because the human catalytic subunit (PP2Ac) functionally replaced the endogenous PP2Ac of Saccharomyces cerevisiae and bound the yeast regulatory PR65/A subunit (Tpd3p) forming a dimer. Yeast was employed as a novel system for mutagenesis and functional analysis of human PP2Ac, revealing that the invariant C-terminal leucine residue, a site of regulatory methylation, is apparently dispensable for protein function. However, truncated forms of human PP2Ac lacking larger portions of the C terminus exerted a dominant interfering effect, as did several mutant forms containing a substitution mutation. Computer modeling of PP2Ac structure revealed that interfering amino acid substitutions clustered to the active site, and consistently, the PP2Ac-L199P mutant protein was catalytically impaired despite binding Tpd3p. Thus, interfering forms of PP2Ac titrate regulatory subunits and/or substrates into non-productive complexes and will serve as useful tools for studying PP2A function in mammalian cells. The transgenic approach employed here, involving a simple screen for interfering mutants, may be applicable generally to the analysis of structure-function relationships within protein phosphatases and other conserved proteins and demonstrates further the utility of yeast for analyzing gene function.

Protein phosphatase 2A (PP2A) is a ubiquitous eukaryotic enzyme that is highly conserved between species (1, 2) and a member of the PPP family of protein Ser/Thr phosphatases that includes PP1 and calcineurin (3). PP2A is implicated in diverse cellular processes (4) and coordinates signal transduction through direct, regulatory interaction with protein kinases (5, 6). PP2A exists as a holoenzyme in which the catalytic subunit (PP2Ac) binds a regulatory PR65/A subunit to form a core dimer, which associates with a large number of variable B subunits encoded by at least three gene families (7–11). Regulatory subunits of PP2A influence its substrate specificity and intracellular targeting (12–14), and enzyme activity is modulated further by post-translational modification of the PP2Ac C terminus (4).

The budding yeast Saccharomyces cerevisiae possesses two proteins Pph21p and Pph22p similar to mammalian PP2Ac (15). The homologous PPH21 and PPH22 genes encoding yeast PP2Ac are functionally redundant because either can be deleted without effect. However, doubly deleting PPH21 and PPH22 causes severe growth inhibition and is lethal in the absence of PPH3, encoding a distantly related protein phosphatase that provides overlapping function (16). Conservation of PP2A extends to the holoenzyme because the S. cerevisiae Tpd3p (17), Cdc55p (18), and Rts1p (19) are similar to the regulatory PR65/A, PR55/B, and PR61/B’ subunits, respectively, of mammalian PP2A, and Tpd3p and Cdc55p bind Pph21p/Pph22p in vivo (20). This suggests that PP2A regulation is conserved between species, and consistent with this, a novel methyltransferase that targets the PP2Ac C-terminal leucine residue and modulates PP2A activity (21) is present in both higher eukaryotes and yeast (22, 23).

The role of PP2A has been studied biochemically, through the use of inhibitors and viral tumor antigens that target the enzyme specifically, and genetically, via inactivating subunit mutations. Moreover, the crystal structure of the PR65/A subunit of PP2A was recently solved (24). Together these studies have implicated PP2A in processes including cell cycle regulation, cellular morphogenesis, protein synthesis, and viral replication (4). Although the biochemical and functional properties of PP2A differ from those of protein phosphatase type 1 (PP1), the catalytic subunits of these enzymes share ~46% amino acid sequence identity. The crystal structure of PP1γ, has been solved (25, 26) and provides a framework for predicting the role of specific PP2Ac residues and interpreting the effect of changes generated by mutagenesis. In this study, we have functionally expressed human PP2Ac in S. cerevisiae and used this as a convenient system to generate novel mutations in human PP2Ac and analyze their effect on PP2A function in vivo. We have identified a number of novel, interfering mutant forms of PP2Ac and rationalized their effects using a model of PP2Ac structure based on that of PP1γ.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, Plasmids, and Sequence Analysis—**Yeast strains are described in Table I. Rich (yeast peptone medium, YPD), minimal (synthetic dextrose, SD), and 5-FOA media were described (27). Plasmids pBluescript II (pBS, Stratagene), pYES2 (Invitrogen), pYPEG2 (28), pASZ11 (29), pRS314, pRS316, YEp351, YEp352, YCpDE1, and YCpAS6 (30) were described. DNA constructs were sequenced with dRhodamine dye terminators using Perkin-Elmer GeneAmp PCR system 2400, 9700 thermocycler and analyzed using an Applied Biosystems PRISM 377 sequencer. Pairwise alignment of human PP2Ac and yeast Pph22p was performed using ALIGN (Baylor College of Medicine).
HsPP2Ac tagged with the HA epitope were used. A single HA tag was inserted downstream of the PP2Ac initiation codon, by amplifying the human cDNA (31) in the PCR using an appropriate forward primer. A SbfI/KpnI HA-PP2Ac clone, or a frameshifted allele, HA-PP2Ac-FS lacking the second nucleotide of the ninth codon, was introduced between the PGK1 promoter/CYC1 terminator of vector pYPGE2 generating plasmids YEpDE-PGK-Co and YEpDE-PGK-FS. A similar Neol/BamHI fragment was introduced into vector pACTII (P. Legrain, Pasteur Institute) fusing the HA-PP2Ac ORF to the Gal4 activation domain. A second sequence encoding an extended HA-PP2Ac was ORF was amplified under mutagenic conditions for the PCR (0.2 ng/μl) and subcloned into pYES2, and this plasmid, YEpDE-PGK-CHA, was template for a PCR generating a pair of primers, HA-PP2Ac-2512 and HA-PP2Ac-2512, 301 (PP2Ac-Δ309), or 67 (PP2Ac-Δ47) (codon numbering for untagged PP2Ac). PP2Ac mutant alleles were inserted between the HindIII/BamHI sites of pYES2 and downstream of SacI in YEpGE2. Plasmid DNA was prepared from strain DEY1-Co using a Kristal extraction kit (Cambridge Molecular Technologies); six plasmids, recovered from independent ampicillin E. coli colonies, were tested. A restriction pattern (Sali/KpnI or Xbal) identical to that of YEpDE-PGK-Co. The nucleotide sequence of one plasmid was analyzed, and it encoded YEpDE-PGK-Co.

Preparation of Yeast Cell Extracts and Western Blot Analysis—Cell extracts were prepared as described (33). Protein transfer and Western blotting were as described (9). Antibodies were diluted in TNPB (1.0% Nonidet P-40, 0.3% Triton X-100 in Tris-buffered saline (TBS), 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 7.5% powdered milk. Membranes were washed with TNPB. Detection was by goat anti-mouse IgG-HRP and ECL (Amersham Pharmacia Biotech).

Construction of the pp22Δ52 allele and myc-TPD3 Alleles—The PP2Ac gene was PCR-amplified using a forward primer appropriate to genomic DNA from strains D12 and D12, 53–377 of PP2H2 and a stop codon TAA. A pp22Δ52 1.0-kb SalI/KpnI fragment was fused to the PGK1 promoter in YEpGE2. A 3.4-kb genomic fragment encompassing the yeast TPD3 gene (17) was inserted into YEp351 (YEpDE11) and was template for a PCR generating a TPD3 ORF, flanked by its native promoter/terminator, with a double c-myc tag downstream of the initiator codon (myc TPD3). A 3189-base pair myc-TPD3 SacI/SalI fragment was inserted into vectors pBS314, YEp351, and YEp352 (YEpDE3-2m, YEpDE3-2m, and YEpDE4-2m, respectively). The myc-TPD3 allele is functional because YEpDE3-2m rescued the ts growth defect of haploid tad31Δ/UR3 mutant cells (not shown).

Immunoprecipitations and Protein Phosphatase Activity Measurements—To precipitate HA-tagged proteins, PAP-Cl-4B (Amersham Pharmacia Biotech) equilibrated in 1× TBS was incubated at 4 °C with 12CA5 overnight and then washed extensively (six times with 10 volumes of TBS). Antibody-saturated beads (PAS-12CA5, 35 μl) were added to ~100 μl of yeast extract, incubated at 4 °C for 2 h, and washed extensively. Phosphatase assays (50 μl) were performed on immune complexes according to Promega (kit V2460) measuring phosphate release over 10 min from a chemically synthesized phosphopeptide (RRAKR, where R indicates phosphothreonine) with MnCl₂ present at 1 mM. Assays were performed in duplicate on immune complexes prepared independently, and activity is expressed as units (1 unit = 1 μmol of phosphate hydrolyzed per min). For co-immunoprecipitations, cell extracts were prepared in Buffer A (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1.0 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 0.1% Nonidet P-40) with protease inhibitors. PAS-12CA5 (35 μl) was added to 1.0 mg of extract, incubated at 4 °C for 2–5 h, washed extensively with Buffer A, and boiled in 50 μl of Laemmli Buffer.

Generation of PP2Ac Dominant-negative Alleles—A 970-base pair HindIII/BamHI HA-PP2Ac fragment was inserted into the GAL1/CYC1 promoter/terminator cassette of pYES2, and this plasmid, YEpDE-CoHa, was cleaved withAw/II/BseHI generating a gap within the PP2Ac ORF. In parallel, the intact insert in YEpDE-CoHa was amplified under mutagenic conditions for the PCR (0.2 ng/μl template DNA, 1× reaction buffer (Perkin-Elmer), 0.5 pmol/μl primer T7, 0.5 pmol/μl primer 20622 (5′-AGAGGATCCCTACGAAATGATCGG-GATCCAGGATGAAAC), 0.5 mM each dCTP and dTTP, 3 mM MgCl₂, 0.1 or 0.2 mM MnCl₂, 5 μg of Taq polymerase, 33 cycles of 94 °C for 1.5 min, 55 °C for 2.0 min, 72 °C for 3.0 min). PP2Ac mutant alleles were recovered by co-transforming (34) cells (strain DEY1-Co) with gapped plasmid DNA (0.5 μg) and mutagenic PCR product (200 ng). Ura⁺ transformants were selected on SD lacking uracil and then replica-plated to synthetic dextrose (SD) and synthetic galactose (SO) media to identify plasmids containing dominant-negative alleles expressed from the GALI promoter. Plasmids recovered from cells capable of growth on glucose but not galactose were checked for gap repair by restriction analysis and reintroduced into DEY1-Co to confirm growth inhibition on galactose. To test competition between PP2Ac wild type and mutant alleles, the 2.3-kb SacI/BseHI fragment from YEpDE-CoHa, containing HA-PP2Ac fused to the GALI promoter, was inserted between the Smal/BseHI sites of vector.
pH22-172 mutant yeast cells that undergo loss of endogenous PP2Ac function at 37 °C because of a conditional-lethal point lacking the first 52 codons (pPH22Δ52) for inducible growth inhibition in DEY1-Ca as above.

Galactose-induced Expression of PP2Ac in Cells Grown in Liquid Medium—PP2Ac wild type and mutant alleles were expressed from the GAL1 promoter of pYES2 in yeast strain INVSC1. Cells were grown to a density of 5.0 × 10^7 per ml at 30 °C in selective (synthetic raffinose, SR) medium containing raffinose (2.0%), glycerol (3.0%), and casamino acids (0.2%) followed by the addition of an equal volume of SG medium containing galactose at 4.0%.

Modeling of PP2Ac Structure—A homology-based model was obtained using the coordinates of PP2Ac from human (2); excluding an N-terminal extension (46% identity with four 1-amino acid gaps) was used by "MODELER" (Ref. 35; BIOSYM/Molecular Simulations, San Diego, CA) to build and refine five PP2Ac models. PP1γ, appears unstructured between residues 299 and 316 (25), and no models were obtained for PP2Ac in this region (the C terminus of the models is Pro-291). The best model, selected on the basis of the lowest violations of the probability density functions, was evaluated using "Profiles-3D" (Ref. 36; BIOSYM/Molecular Simulations, San Diego, CA). Its overall self-compatibility score, 139.2, was close to that expected for a polypeptide chain of this length, 140.6, indicating reliability of the model.

RESULTS

Functional Replacement of Yeast PP2Ac by Human PP2Ac—The primary structure of PP2Ac is highly conserved from yeast to humans (2); excluding an N-terminal extension (~50 residues) to the S. cerevisiae protein, the yeast and human PP2Ac polypeptides display 71% amino acid sequence identity. To investigate whether the conservation of PP2Ac structure reflects a conservation of function, we tested the ability of human PP2Ac (HsPP2Ac) to functionally replace the corresponding protein from S. cerevisiae (ScPP2Ac). We used haploid yeast cells (strain DEY1) triply deleted for the chromosomal PPH21, PPH22, and PPF3 genes and supported by a plasmid-borne PPH22 gene encoding ScPP2Ac (30). A second plasmid, carrying the human PP2Ac clone fused to the yeast PGK promoter and the hemagglutinin epitope, was introduced, and heterologous expression of HA-tagged HsPP2Ac was analyzed by immunoblot analysis (see Fig. 1A). Two forms of HsPP2Ac were detected, suggesting that the human protein undergoes post-translational modification in yeast, and both forms migrated at approximately 36 kDa, faster than the endogenous ScPP2Ac at 43 kDa (not shown). HsPP2Ac was analyzed for function in yeast using a plasmid shuffling assay, testing for the ability of cells to grow in the absence of ScPP2Ac (Fig. 1B); this assay tests for the ability of yeast cells to grow on medium containing 5-FOA (37) which negatively selects against the essential URA3 PHH22 plasmid encoding ScPP2Ac in strain DEY1. Cells containing PP2Ac lost PHH22 at high frequency and grew on 5-FOA medium, whereas cells containing a non-functional PP2Ac clone with a 5′-frameshift (PP2AcΔFS) or the empty vector failed to grow without PHH22. An incoming TRP1 PHH22 plasmid also substituted for the resident URA3 PHH22 plasmid as expected. This indicates that HsPP2Ac is functional in yeast and that it supports cell growth in the absence of ScPP2Ac. Consistent with this, cells cured of PHH22 by plasmid shuffling contained plasmid DNA carrying the PP2Ac clone (see "Experimental Procedures") and expressed the 36-kDa HsPP2Ac (Fig. 1A). Moreover, in an alternative test of function (Fig. 1C), PPH22 rescued the ts growth defect of ppH22-172 mutant yeast cells that undergo loss of endogenous ScPP2Ac function at 37 °C because of a conditional-lethal point mutation in PHH22 (30). In addition, a truncated allele of PPH22 lacking the first 52 codons (ppH22Δ52) functionally replaced full-length PHH22 in strain DEY1 (Fig. 1D) even at 37 °C (not shown) indicating that the N-terminal extension to ScPP2Ac is dispensable for cell viability, consistent with the ability of HsPP2Ac to replace the endogenous yeast enzyme.

HsPP2Ac Binds the Yeast PR65/A Subunit, Tpd3p—Because PP2Ac heterodimerizes with a regulatory PR65/A subunit (1), we tested whether HsPP2Ac binds the endogenous yeast PR65/A subunit Tpd3p in vivo. We tagged Tpd3p with the Myc epitope (Myc-Tpd3p) and asked whether it co-precipitates with HA-tagged HsPP2Ac from a yeast extract (Fig. 2A). When proteins were precipitated using the 12CA5 antibody, Myc-Tpd3p but not untagged Tpd3p was detected in an immune
Functional Analysis of Human PP2Ac in Yeast

**PP2Aco Mutant Alleles Inhibit PPH22 and pph22-12 Cell Growth**—The screen for PP2Aco dominant-negative alleles was performed in yeast cells containing human, and lacking yeast, PP2Ac. To test for growth inhibition in the presence of ScPP2Ac, we introduced PP2Aco mutant alleles into PPH22 pph221 ppH3Δ cells, in which Pph22p provides ScPP2Ac function. When expressed from the GAL1 promoter, each PP2Aco dominant-negative allele tested inhibited cell growth (Table II) suggesting that its product interfered with ScPP2Ac function. Moreover, when PP2Aco dominant-negative alleles were expressed in pph22-12 ppH21 ppH3Δ cells, containing a mutant Pph22p functionally impaired by a single amino acid substitution but which nevertheless display wild type growth at 30 °C (30), the PP2Aco mutant alleles inhibited growth at 30 °C, and this inhibition was more severe than that observed in wild type (PPH22) cells (Table II). Thus, dominant-negative alleles of PP2Aco display an allele-specific interaction with PPH22 and pph22-12 causing severe growth inhibition when endogenous ScPP2Ac is functionally impaired. These results support the notion that PP2Aco function is conserved between species and that inhibitory mutant forms of HsPP2Ac interfere with endogenous PP2Ac, whether HsPP2Ac or ScPP2Ac.

**Predicted Effect of Dominant-negative Mutations on PP2Aco Structure**—To gain insight into the interfering effect of PP2Aco dominant-negative mutations, we built a model of HsPP2Ac structure based on the crystal structure of PP1γ (25). The overall structure of the PP2Aco model (residues 1–291) is strikingly similar to that of PP1γ (Fig. 4) with an architecture of 10 α-helices and 3 β-sheets. PP1γ possesses a shallow substrate-binding site, with a di-nuclear metal ion center at the catalytic site. In agreement with the observation that PP2Aco is a divalent cation-dependent enzyme (1), the predicted HsPP2Ac metal ligands Asp-57, His-59, Asp-85, Asn-117, His-167, and His-241 (equivalent to Asp-64, His-66, Asp-92, Asn-124, His-173, and His-248 in PP1γ) are positioned correctly to bind the two metal ions. Moreover, HsPP2Ac contains the substrate-binding residues Arg-89, Asn-117, His-118, Arg-214, and Tyr-265 and the auxiliary residue Asp-88 (which may form a catalytically important salt bridge with His-118) equivalent to Arg-96, Asn-124, His-125, Arg-221, Tyr-272, and Asp-95 of PP1cy. The longest truncation of HsPP2Ac (Cys-165stop, encoded by PP2Ac-182) deletes two metal ligands, one substrate-binding residue and a major portion of the active site, and is likely to be catalytically inactive. Surprisingly, the secondary structural elements missing from this truncated protein do not form an independent domain but comprise a subdomain (α7, α8, β-sheet 3 (β7–β9), and α9) and part of β-sheet 2 (β8, β10, β12, and β11) and β-sheet 1 (β13, β14). Even the shortest truncation (Tyr-218stop, PP2Ac-216) similarly lacks β9 and α9 in the subdomain and part of both β-sheets 1 and 2. Presumably the remainder of the polypeptide in these and the other HsPP2Ac truncation mutants folds sufficiently to form a subdomain, comprising α1, β4, β3, β2, and α4–α6, that binds PP2A-interacting mutant forms of PP2Ac, whether HsPP2Ac or ScPP2Ac.
acting proteins and thereby promotes an interfering effect.

Five PP2Ac dominant-negative alleles encoded missense mutations, and the interfering effect of four of these could be rationalized as they encode substitutions disturbing the PP2Ac active site and thus may impair catalytic activity. The Y127N substitution (PP2Ac-153–1 allele) changes a highly conserved tyrosine residue (equivalent to Tyr-134 of PP1\(\gamma_1\)) that interacts with the Ser(P)/Thr(P) substrate and whose side chain is in van der Waals contact with His-118 and Asp-88. Like in PP1\(\gamma_1\), His-118 is predicted to serve as a general acid in the PP2Ac active site, protonating the Ser(P)/Thr(P) leaving group and accelerating the phosphatase reaction, whereas Asp-88 may enhance the acidic character of His-118. The F150I substitution (PP2Ac-225–1 allele) changes a conserved residue (Phe-156 in PP1\(\gamma_1\)) which is distant from the active site but forms a hydrogen bond with the buried Glu-119 residue; Glu-119 is adjacent to His-118 and is in van der Waals contact with the metal-binding residue Asp-85. Thus, the F150I substitution may affect the orientation of the Glu-119 side chain and alter metal ligation, although interestingly, an alternative aromatic residue tyrosine (PP2Ac-2446–2 allele) is tolerated at this position.

The conserved Leu-199 residue (Leu-205 in PP1\(\gamma_1\)) occurs at the C terminus of helix a8 and its main chain carbonyl group hydrogen-bonds with the side chains of Asn-117, His-167, and His-241 which are metal ligands. Disruption of local structure by L199P (PP2Ac-2512 allele) could affect metal binding, abolish contact with Cys-196, and alter the orientation of Trp-200 that...
has been proposed to contact substrate residues flanking Ser(P)/Thr(P). Finally, the T258P substitution (PP2Ac-136) changes a highly conserved, penultimate residue of b12 (Thr-265 in PP1g1) which forms part of b-sheet 2 (b1, b5, b6, b10, b12, and b11). The polypeptide chain emerging from b10 packs on top of this b-sheet and contains the His-241 metal ligand. Furthermore, the g-methyl group of Thr-258 packs against its main chain. Thus, a proline at position 258 could interfere with metal binding by His-241. The fifth dominant-negative missense mutation encoding V159E (PP2Ac-153-2) is more difficult to rationalize in terms of a catalytically impaired PP2Ac. However, Val-159 (Val-165 in PP1g1) is part of the hydrophobic core located between b-sheets 1 and 2. Introducing a negatively charged glutamate into it could, although distant from the active site, have a global effect on active site structure and catalysis.

The PP2Ac C-terminal Leucine Residue Is Dispensable for Function—Because truncated forms of HsPP2Ac cause an interfering effect, we explored the functional role of the highly conserved PP2Ac C terminus. Deleting the HsPP2Ac C-terminal nine amino acids (PP2Ac-∆300 allele) caused an inhibitory effect that was less severe than that caused by larger truncations (Fig. 3), whereas deleting the C-terminal Leu-309 residue alone (PP2Ac-∆309) caused no interfering effect (not shown). The C-terminal leucine residue is invariant among PP2Ac enzymes, and so we tested whether it is essential for HsPP2Ac function (Fig. 5). Remarkably, the PP2Ac-∆309 allele functionally replaced the yeast PPH22 gene encoding ScPP2Ac in strain DEY1, as did PP2Ac-A309 encoding HsPP2Ac-L309A with alanine at position 309, indicating that Leu-309 is dispensable for HsPP2Ac function in vivo. In contrast, the PP2Ac-∆300 allele failed to replace PPH22 (Fig. 5) indicating that a largely intact C terminus is essential. Surprisingly, PP2Ac-∆309 supported a rate of yeast cell growth that was higher (doubling time (td) 3.1 ± 0.49 h in liquid YPD at 30 °C, strain DEY1-∆309) than that supported by PP2Ac-A309 (td 4.6 ± 0.84 h, strain DEY1-A309) and was similar to that supported by wild type PP2Ac (td 2.8 ± 0.28 h, strain DEY11) (see “Discussion”).

PP2Ac-L199P Is Catalytically Impaired but Binds Tpd3p—To explore the mechanism by which HsPP2Ac mutant proteins containing a substitution mutation may interfere with wild type PP2Ac function, we tested the PP2Ac-L199P protein (PP2Ac-2512 allele) for catalytic activity in vitro (Fig. 6A). Unlike wild type HsPP2Ac recovered from yeast, PP2Ac-L199P displayed no detectable okadaic acid-sensitive phosphatase (PP2A) activity greater than the vector control. This is consistent with the prediction from molecular modeling that the L199P substitution inhibits PP2Ac activity by disrupting binding and/or metal ion-mediated hydrolysis of the substrate and suggests that catalytic impairment of PP2Ac-L199P may contribute to its interfering effect in vivo. To test this we analyzed an active site mutant of HsPP2Ac‡ mutated for the His-118 residue of PP2Ac, which may serve as a general acid in the active site of PP2Ac protonating the Ser(P)/Thr(P)-leaving group. This mutant HsPP2Ac possesses a neutral asparagine residue in place of His-118 which is predicted to dramatically
The PP2Ac-118 allele is important for HsPP2Ac catalytic function. Moreover, when the PP2Ac-118 allele encoding PP2Ac-H118N is expressed from the GAL1 promoter, it caused an inhibition of cell growth similar to that caused by PP2Ac dominant-negative missense alleles (see Fig. 3). This indicates that catalytic impairment of HsPP2Ac may cause an interfering effect. Finally, we asked whether PP2Ac-L199P binds the yeast Tpd3p PR65/A subunit (Fig. 6C). Thus, like wild type HsPP2Ac, PP2Ac-L199P binds the endogenous yeast PR65/A subunit suggesting that, although catalytically impaired, it folds sufficiently to bind PP2A-interacting proteins thereby contributing to its interfering effect on PP2A function.

**TABLE III**

| Competition between wild type and substituted forms of PP2Ac in yeast | Colony formation in the presence of an incoming Gal-PP2Ac or Gal-PP2Ac-FS allele
|-----------------|------------------------|------------------------|
| Resident PP2Ac allele | Glu | Gal
| Gal-PP2Ac | Glu | Gal |
| PGK-PP2Ac and Gal-PP2Ac-216 | ++ | +/− | ++ | − |
| PGK-PP2Ac and Gal-PP2Ac-266 | ++ | − | ++ | − |
| PGK-PP2Ac and Gal-PP2Ac-235 | ++ | +/− | ++ | − |
| PGK-PP2Ac and Gal-PP2Ac-182 | ++ | − | ++ | − |
| PGK-PP2Ac and Gal-PP2Ac-178 | ++ | − | ++ | − |
| PGK-PP2Ac and Gal-PP2Ac-144-2 | ++ | + | − | − |
| PGK-PP2Ac and Gal-PP2Ac-136 | ++ | +/− | − | − |
| PGK-PP2Ac and Gal-PP2Ac-225 | ++ | + | − | − |
| PGK-PP2Ac and Gal-PP2Ac-118 | ++ | + | − | − |
| PGK-PP2Ac and Gal-PP2Ac-153 | ++ | + | − | − |
| PGK-PP2Ac and Gal-PP2Ac-2512 | ++ | + | − | − |
| PGK-PP2Ac and Gal-PP2Ac-2446 | ++ | + | − | − |
| PGK-PP2Ac and wt Gal-PP2Ac | ++ | ++ | ++ | ++ |

*Dominant-negative alleles are described in Fig. 3.

**Competition in Vivo between Wild Type and Mutant Forms of PP2Ac**—To probe further the mechanism by which interfering forms of PP2Ac inhibit cell growth, we tested for reversal of growth inhibition by overexpression of wild type PP2Ac. PP2Ac was expressed from the GAL1 promoter in yeast cells co-expressing a GAL-driven dominant-negative allele, and PP2A function was assessed by colony formation (Table III). With one exception (PP2Ac-316) growth inhibition caused by PP2Ac missense alleles was alleviated by co-overexpression of wild type PP2Ac, as was inhibition caused by PP2Ac-144-2, encoding the aberrant C-terminal extension to PP2Ac. In contrast, a S'-frameshifted PP2Ac mutant clone failed to reverse the growth inhibitory effect of the dominant-negative alleles, as expected. This suggests that a competition occurs in vivo between wild type and substituted mutant forms of PP2Ac. Nevertheless, increased expression of PP2Ac failed to reverse the growth inhibition caused by C-terminally truncated forms of PP2Ac suggesting that these inhibit wild type HsPP2Ac function by a different, non-competitive mechanism.
DISCUSSION

**PP2A Is Functionally Conserved between Species**—The amino acid sequence of PP2Ac is highly conserved between species, and budding yeast contains an endogenous phosphatase activity with biochemical characteristics remarkably similar to those of mammalian PP2A (33). We have shown by genetic and biochemical criteria that HsPP2Ac functionally replaces the endogenous PP2Ac of *S. cerevisiae* demonstrating that the evolutionary conservation of PP2Ac structure and enzyme activity reflects a conservation of function in *vivo*. In accordance with this, HsPP2Ac bound the endogenous yeast regulatory PR65/A subunit consistent with the observation that the most highly conserved residues in PR65/A localize to a continuous ridge extending along one face of the molecule. This ridge presents an exposed hydrophobic surface predicted to contact the PP2A catalytic and other subunits in the holoenzyme complex (24). Indeed, a mammalian regulatory B’ alpha subunit of PP2A functionally replaces its homologue (Rts1p) in *S. cerevisiae* (38) indicating that the conservation of PP2A function extends to the holoenzyme.

**Inhibitory Mechanism of HsPP2Ac Interfering Mutant Proteins**—Yeast cells in our screen for PP2Ac dominant-negative alleles were kept alive by wild type PP2Ac expressed at low level from the constitutive PGK1 promoter, and to inhibit cell growth, dominant-negative alleles were inductively expressed from the GAL1 promoter to a level approximately 10-fold higher (not shown). Thus the dominant-negative effect of PP2Ac mutant alleles requires their expression to a higher level than wild type PP2Ac, and indeed, PP2Ac mutant alleles expressed from the PGK1 promoter did not inhibit cell growth (data not shown). Moreover, during competition experiments involving simultaneous overexpression of a PP2Ac wild type and dominant-negative allele, the growth inhibition caused by missense alleles was generally reversed by increased expression of wild type PP2Ac, although inhibition caused by nonsense alleles was not. This suggests that HsPP2Ac proteins containing substitutions compete with wild type HsPP2Ac in *vivo* by titrating PP2A regulatory subunits and/or substrates, whereas truncated forms of HsPP2Ac (and PP2Ac-T258P) may sequester PP2A subunits and/or substrates irreversibly. Consistent with this we found that, like wild type HsPP2Ac, the PP2Ac-L199P mutant protein bound the endogenous yeast PR65/A subunit suggesting that it causes an interfering effect by titrating PR65/A into a non-functional complex. Because the core dimer serves as an essential scaffold for the recruitment of B-type regulatory subunits, PP2Ac-L199P may effectively titrate other subunits, interfering with targeting and/or substrate specificity of the wild type enzyme. We found that PP2Ac-L199P is catalytically impaired and that an active site mutant, PP2Ac-H118N, causes a similar interfering effect. Only these two mutant proteins were tested for catalytic activity in *vivo*, but we have found that none of the PP2Ac dominant-negative alleles supports yeast cell growth when expressed from the PGK1 promoter (not shown) indicating that each may encode a catalytically impaired, yet largely folded protein. Consistent with this, the model of PP2Ac structure predicts that the interfering forms of HsPP2Ac either lack or disrupt key metal- or substrate-binding residues implicated in catalytic function rather than subunit interaction, and suggests further that even the truncated mutant forms retain a properly folded portion of the molecule capable of binding regulatory subunits. In this respect it may be significant that N-terminal sequences of both PP1 and PPV are implicated in recognizing regulatory subunits (25). Furthermore, the interfering effect of our HsPP2Ac mutant proteins is similar to that caused by a mutant form of fission yeast PP2Ac, containing a substitution equivalent to R239Q in HsPP2Ac, that inhibits cell growth in a dosage-dependent fashion (39). An equivalent substitution in the fission yeast PP1e causes aggregation of the mutant protein and interferes with wild type PP1e function by an unknown mechanism. This raises the possibility that HsPP2Ac-interfering proteins may be imperfectly folded and confer their inhibitory effect by forming aggregates that may tap other PP2A subunits. However, active site mutant forms of protein tyrosine phosphatases can cause an interfering effect via substrate trapping, protecting substrates from dephosphorylation by a wild type enzyme (40) suggesting that the catalytically impaired PP2Ac-L199P and PP2Ac-H118N proteins, and other interfering HsPP2Ac forms, may exert their effects by causing a similar protection of PP2Ac substrates. In this study we also generated a PP2AcΔ67 allele in *vivo* encoding the N-terminal 67 amino acids of HsPP2Ac and which is similar to a mutant allele PP2Ac<sup>2202</sup> that dominantly enhances signaling from activated Ras during *Drosophila* eye development (41). In our yeast system PP2AcΔ67 did not cause a dominant-negative effect supporting the conclusion that the phenotype conferred by PP2Ac<sup>2202</sup> in heterozygous insect cells is caused by a reduction in PP2Ac dosage and not by an intrinsic property of a truncated gene product.

**The C-terminal Leucine Is Dispensable for Essential PP2Ac Function**—The invariant C-terminal leucine residue of PP2Ac is methylated by a novel transerase enzyme (22) that modulates PP2A activity and targeting (21, 42). Nevertheless, Leu-309 is dispensable for HsPP2Ac function because a mutant protein lacking this residue supported yeast cell growth in the absence of ScPP2Ac. Because methylation of PP2Ac increases catalytic activity (21), the de-methylated Leu-309 residue may perform an inhibitory role that optimizes PP2A function but is not essential under our growth conditions. Remarkably, removal of Leu-309 from HsPP2Ac was less inhibitory than the abnormal presence of alanine at this position. The reason for this is unclear but may indicate that the apparent inhibitory role of the PP2Ac C-terminal residue is less easily relieved when alanine replaces the highly conserved leucine.

**Interfering Mutant Forms of Human PP2Ac Generated in Yeast**—Yeast cells functionally expressing HsPP2Ac provide a convenient system to identify inhibitory forms, facilitating both the generation of a library of PP2Ac mutant alleles and the rapid screening for alleles that inhibit PP2Ac function in *vivo*. We generated PP2Ac dominant-negative alleles in yeast cells lacking endogenous ScPP2Ac, but when the same alleles were tested in wild type yeast, they again inhibited cell growth, indicating an interference with ScPP2Ac. Thus, a screen for growth inhibitory forms of HsPP2Ac might have been performed in wild type cells without prior manipulation of the yeast genome, although the inhibitory effect of the PP2Ac mutant alleles was more dramatic when endogenous ScPP2Ac was functionally impaired. Perhaps ScPP2Ac interacts more efficiently than HsPP2Ac with endogenous yeast proteins, and unless mutationally impaired, it is less easily outcompeted by interfering forms of HsPP2Ac. Consistent with this, HsPP2Ac supported a lower rate of yeast cell growth than ScPP2Ac (Fig. 1B and not shown). Nevertheless, our results suggest that yeast might be employed to generate interfering forms of other mammalian proteins, provided there is cross-species conservation of protein function and especially if a strain containing a non-lethal mutation in the yeast orthologue is available. Here, this approach has identified residues important for HsPP2Ac function and catalytic activity. Moreover, the results of our study provide the first dominant-negative mutant forms of human PP2Ac, reagents which may be used to explore the role and regulation of PP2A in a variety of cell processes and cell
types and that will therefore open up new avenues of investigation.

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