Active-site mutations impairing the catalytic function of the catalytic subunit of human protein phosphatase 2A permit baculovirus-mediated overexpression in insect cells

Timothy MYLES[1], Karsten SCHMIDT, David R. H. EVANS[2], Peter CRON and Brian A. HEMMINGS[3]
Friedrich Miescher Institut, Postfach 2543, Basel CH-4002, Switzerland

Members of the phosphoprotein phosphatase (PPP) family of protein serine/threonine phosphatases, including protein phosphatase (PP)1, PP2A and PP2B, share invariant active-site residues that are critical for catalytic function [Zhuo, Clemens, Stone and Dixon (1994) J. Biol. Chem. **269**, 26234–26238]. Mutation of the active-site residues Asp^{88} or His^{118} within the human PP2A catalytic subunit (PP2Ac)α impaired catalytic activity *in vitro*; the D88N and H118N substitutions caused a 9- and 23-fold reduction in specific activity respectively, when compared with wild-type recombinant PP2Ac, indicating an important role for these residues in catalysis. Consistent with this, the D88N and H118N substituted forms failed to provide PP2A function *in vivo*, because, unlike wild-type human PP2Acα, neither substituted for the endogenous PP2Ac enzyme of budding yeast. Relative to wild-type PP2Ac, the active-site mutants were dramatically overexpressed in High Five® insect cells using the baculovirus system. Milligram quantities of PP2Ac were purified from 1×10^9 High Five cells and the kinetic constants for dephosphorylation of the peptide RRA(pT)VA (single-letter amino-acid notation) by PP2Ac ($K_m = 337.5\ \mu M$; $k_{cat} = 170\ s^{-1}$) and D88N ($K_m = 58.4\ \mu M$; $k_{cat} = 2\ s^{-1}$) were determined. The results show that the substitution impairs catalysis severely without a significant effect on substrate binding, consistent with the PPP catalytic mechanism. Combination of the baculovirus and yeast systems provides a strategy whereby the structure–function of PP2Ac may be fully explored, a goal which has previously proven difficult, owing to the stringent auto-regulatory control of PP2Ac protein levels *in vivo*.

Key words: kinetics, mutagenesis, PP2A, recombinant protein, yeast.

INTRODUCTION

Protein phosphatase (PP)2A is a major protein serine/threonine phosphatase that plays an important role in diverse eukaryotic cellular processes regulated by reversible protein phosphorylation [1–4]. PP2A exists as a number of holoenzymes; the basic structure contains an invariant core dimer that is composed of a highly conserved 36 kDa catalytic subunit (PP2Ac) bound tightly to a 65 kDa regulatory subunit (PR65/A). PR65/A acts as a scaffold protein for the binding of PP2Ac and a large number of B-type regulatory subunits in the heterotrimeric holoenzyme [1,3]. The three major families of B-type subunits, PR55/B [5], PR61/B′ [6] and PR72/B″ [7] share no significant similarity in primary structure, and this diversity is believed to determine the enzymic activity and substrate specificity of PP2Ac, as well as its intracellular localization and the tissue specificity of distinct holoenzyme forms [8–11].

PP2Ac belongs to the phosphoprotein phosphatase (PPP) family of protein serine/threonine phosphatases and shares many invariant residues with the PP1 and PP2B (also known as calcineurin) catalytic subunits, and some active-site residues with bacteriophage λ phosphatase (λPPase), suggesting that these enzymes share a common catalytic mechanism [12–14]. Sequence alignment of the conserved phophoesterase domain (Figure 1) shows a consensus sequence important for metal and phosphate binding and catalysis [DXH-(~25)-GDXXD-(~25)-GNHD/E; where single-letter amino-acid notation has been used] [13]. Site-directed mutagenesis and kinetic analysis of PP1 [14,15] and λPPase [13] active-site residues, predicted to be involved in metal binding, substrate binding and catalysis, combined with the crystal structure of PP1 has defined a catalytic mechanism for members of the serine/threonine PPs [16]. The mechanism proposed for PP1 involves metal ion-mediated hydrolysis of the target substrate, where Ser(P)/Thr(P) is orientated for attack by a nucleophile in the active site by residues Arg^{96} and Arg^{122} (Arg^{89} and Arg^{115} in PP2Ac). An H_2O molecule is activated to a hydroxide by two metal ions co-ordinated by several metal-binding residues. The hydroxide then makes a nucleophillic attack on Ser(P)/Thr(P). The PP1 residue His^{125} (His^{118} in PP2Ac) acts as a general acid, which protonates the leaving group oxygen, accelerating the dephosphorylation reaction. The acidic nature of His^{125} is further enhanced by an ion-pair interaction with Asp^{95} (Asp^{88} in PP2Ac). The importance of the λPPase residues His^{76} (His^{125} in PP1) and Asp^{52} (Asp^{95} in PP1), and the PP1 residue Asp^{95} in catalysis has been demonstrated by site-directed mutagenesis studies [13–15]. Recently, PP2Ac mutants mutated at the general acid residue His^{118} or residues involved in metal-ion binding were expressed in NIH3T3 cells. Immunoprecipitated proteins had impaired phosphatase activity, revealing conservation of the PPP catalytic mechanism [17,18].

Abbreviations used: 5-FOA, 5-fluoroorotic acid; HA, haemagglutinin; moi, multiplicity of infection; PP, protein phosphatase; PP2Ac, PP2A catalytic subunit; PR65/A, PP2A regulatory subunit; PPP, phosphoprotein phosphatase; λPPase, bacteriophage λ phosphatase; *Sf*9, *Spodoptera frugiperda*; TBS, Tris-buffered saline.

[1] Present address: Division of Hematology, Stanford University School of Medicine, Center for Clinical Sciences Research (CCSR), Room 1155N, 269 Campus Drive, Stanford, CA 93405-5156, U.S.A.
[2] Present address: Program in Molecular Pharmacology, Mailstop D2-100, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, U.S.A.
[3] To whom correspondence should be addressed (e-mail hemmings@fmi.ch).

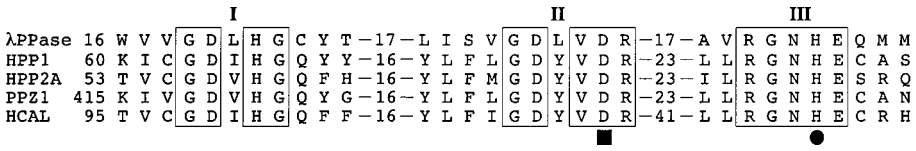

Figure 1 Alignment of phosphoesterase domains of representative PPP family members

λPPase, human PP1 (HPP1), human PP2Ac (HPP2A), *S. cerevisiae* PPZ1 and human calcineurin (HCAL) share the highly conserved phosphoesterase domains I, II and III (boxed). Numbers preceding the amino acid sequences denote the starting amino acids. Numbers between domains represent the number of amino acids between the conserved domains. The filled circle highlights the conserved histidine residue that acts as a general acid in catalysis. It's aspartate ion-pair partner is also shown (filled square).

Despite this, a detailed analysis of the structural and functional aspects of catalysis by PP2Ac is lacking, in part, due to the difficulty in overexpressing recombinant PP2Ac for purification. Thus in many cell lines, including NIH3T3 cells [19,20] and COS-7 cells [21], the expression of recombinant PP2Ac is maintained at a level comparable with that of endogenous PP2Ac by translational [19] or possibly transcriptional auto-regulatory mechanisms [21]. In the present study we have analysed the effects of PP2Ac active-site mutations, involving the general acid residue His^{118} and its ion-pair partner Asp^{88}.

EXPERIMENTAL

Materials

Purified rabbit skeletal muscle PP2Ac was a gift from Josef Goris (Katholieke Universiteit, Leuven, Belgium). The antibodies used in this study, AB45 and AB38, are rabbit anti-(human PP2Ac) and anti-(human PR65/A) respectively [22]. Horseradish-peroxidase conjugated goat anti-mouse and sheep anti-rabbit IgG antibodies, and Streptavidin Texas Red were purchased from Amersham Pharmacia Biotech. Secondary biotinylated goat anti-rabbit IgG antibodies were purchased from Bio-Rad, and okadaic acid was purchased from Alexis. The insect cell lines High Five® (*Trichoplusia ni* isolate BTI-TN-5b1-4) and *Spodoptera frugiperda* (*Sf*9) were obtained from Invitrogen.

Construction of baculovirus transfer vectors and generation of recombinant baculovirus

The wild-type PP2Acα cDNA [23] was cloned into pBS KS^- as a *Hin*dIII–*Bam*HI fragment and was used as a template for PCR amplification using *pfu* DNA polymerase to place a 5′ *Bam*HI haemagglutinin (HA) epitope in frame with the coding sequence downstream of the initiation codon and a 3′ *Eco*RI site immediately downstream of the termination codon. The fragment was cloned into pBS KS^- (to generate pBSHACatα) and sequenced by an Applied Biosystems PRISM 377 sequencer. pBSHACatα was used for subcloning into the baculovirus transfer vector pBB4.5 (Invitrogen) to generate the transfer vector pBBHAPP2Ac. To generate the active-site substitutions H118N and D88N, pBSHACatα was used as template DNA for Quickchange PCR (Strategene) using appropriate mutagenic primers. Mutations were confirmed by DNA sequence analysis and subcloned into pBB4.5 to generate the transfer vectors pBBHAD88N and pBBHAH118N. Transfer vectors were co-transfected into *Sf*9 cells using the BAC-N-BLUE® linear transfection kit (Invitrogen) according to the manufacturer's instructions. Recombinant baculoviruses (vBBHAPP2Ac, vBBHAD88N and vBBHAH118N) were subjected to one round of plaque purification and identified by PCR amplification [24]. The recombinant baculovirus, PR65BV, was maintained on *Sf*9 cells as a high titre stock [25].

Expression and Western-blot analysis of PP2Ac

Expression of HA-epitope-tagged wild-type and mutant PP2Ac was achieved by seeding 25 cm^2 flasks with High Five cells (Invitrogen) grown in Express Five® serum-free medium (Gibco BRL) at a density of 1×10^5 cells/cm^2, and infecting with recombinant baculoviruses (vBBHAPP2Ac, vBBHAD88N or vBBHAH118N) at a multiplicity of infection (moi) of 5. Cells were harvested and lysed in 4 ml of buffer A [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P40, 0.1 mM PMSF and 1 mM benzamidine]. HA-tagged PP2Ac was detected by Western-blot analysis using the HA-epitope-specific 12CA5 monoclonal antibody or the polyclonal antibody AB45 (specific for PP2Ac). Detection was achieved using horseradish-peroxidase conjugated goat anti-mouse or sheep anti-rabbit IgG antibodies and ECL® (Amersham Pharmacia Biotech).

Immunoprecipitations and Western-blot analysis

Flasks (185 cm^2; Nunc) were seeded with High Five cells grown in Express Five serum-free medium (Gibco BRL) at 1×10^5 cells/cm^2 and infected with recombinant virus at an moi of 5. For coexpression studies of HA–PP2Ac and PR65/A, cells were transfected with both the HA–PP2Ac recombinant virus and vPR65BV, at an moi of 5. Cells were harvested, lysed in buffer A and harvested by centrifugation at 190 ***g*** for 30 min. HA-tagged PP2Ac was immunoprecipitated from cleared lysate using 12CA5 saturated Protein A–Sepharose beads. Immunoprecipitates were washed three times in buffer A and two times in Tris-buffered saline [TBS; 50 mM Tris/HCl (pH 7.5)/150 mM NaCl]. Typically 50 μl of beads was boiled in 50 μl of Laemmli buffer [50 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 0.004 % (w/v) Bromophenol Blue, 10 % (v/v) glycerol and 5 % (v/v) 2-mercaptoethanol] then analysed by Coomassie Brilliant Blue staining or Western blotting [25] using 12CA5 or AB45 as primary antibodies for the detection of HA–PP2Ac, and AB38 for the detection of PR65/A, followed by ECL® using the secondary antibodies described above. Quantification of immunoprecipitated HA–PP2Ac from cell lysates was determined by Western blotting, using okadaic-acid titrated rabbit skeletal muscle PP2Ac of known concentration as a standard. 12CA5–Protein A–Sepharose immunoprecipitated HA–PP2Ac (50 μl; diluted 1:1 in TBS) was added to 50 μl of Laemmli buffer and boiled for 5 min, then analysed by Western blotting as described

previously [26] with samples run in duplicate. After transfer to Immobilin, the membranes were probed with AB45, washed extensively, then incubated with biotinylated goat anti-rabbit IgG antibodies and Streptavidin Texas Red. Blots were developed for red fluorescence using a Molecular Dymamics STORM 380 PhosphorImager. The signal for each band was quantified using ImageQuant software (Molecular Dymamics).

Overexpression and purification of PP2Ac

Triple flasks (Nunc; 500 cm^2) with High Five cells in Express Five serum-free medium at a density of 1×10^5 cells/cm^2 were infected with recombinant virus at an moi of 5, and incubated at 27 °C for 72 h. At 72 h post-infection the majority of the infected cells had detached and the remaining cells were detached from the flasks with gentle tapping. The cells were collected by centrifugation at 800 rev./min (190 ***g***) for 20 min in a Sorval RC2B rotor, and pellets were washed with 1 × TBS, pooled and stored at −80 °C. Recombinant protein was purified from cell lysates by a modified ethanol precipitation procedure [27,28]. Briefly, cell pellets were thawed and diluted with 3 vol. of buffer B [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA and 0.2 % Triton X-100], containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim), then lysed by several passes in a French press. Lysates were clarified by centrifugation at 10000 ***g*** at 4 °C, and 4 vol. of absolute ethanol (at 22 °C) was added to the clarified lysate to precipitate the proteins. The precipitate was collected by centrifugation at 10000 ***g***, then resolubilized in buffer B by trituration. The sample was dialysed extensively against 50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA, and clarified by centrifugation at 10000 ***g*** at 4 °C. The resolubilized proteins were loaded on to a Protein A–Sepharose column crosslinked with 12CA5 monoclonal antibody (10 ml bead volume), washed extensively with 50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA then eluted with HA peptide at 1 mg/ml in the same buffer. Fractions containing HA–PP2Ac were determined by Coomassie Brilliant Blue staining of SDS/PAGE gels then pooled, diluted 2-fold with glycerol and stored at −20 °C.

Gel-filtration chromatography of PP2Ac

Gel-filtration chromatography was employed to ensure that monodisperse and correctly-folded PP2Ac was obtained after ethanol precipitation. Native and mutant HA–PP2Ac were dialysed from glycerol stocks against 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol and 0.5 mM EDTA prior to concentration with Centricon YM-30 Centrifugal Filter Devices (Millipore). Approximately 0.2 mg of protein (PP2Ac) was loaded on to a Superdex 75HR 10/30 FPLC-column (Pharmacia). Fractions were collected based on FPLC profiles and the retention time for HA–PP2Ac was determined by Coomassie Brilliant Blue staining and Western-blot analysis of SDS/PAGE gels. The column was calibrated using the following proteins: BSA (66 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (10 kDa).

PP assays

A phosphatase assay using a ^{32}P-labelled peptide (LRRASVA; kemptide) as the substrate has been described previously [10]. Briefly, phosphatase assays were performed in a 30 μl volume containing 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM $MnCl_2$, 0.1 % 2-mercaptoethanol, 1 mg/ml BSA, 60 μM LRRAS-$^{32}PO_4$-VA and 1 nM purified HA–PP2Ac with or without 10 nM okadaic acid at 30 °C. For assays using immunoprecipitates, the appropriate dilution of each sample was used such that assays were performed within the linear range. Protein concentrations of immunoprecipitates determined by quantification of Western blots were then used to normalize PP2Ac concentrations in the phosphatase assays. For the kinetic analysis of purified proteins, phosphatase assays were performed according to the Promega non-radioactive phosphatase assay system (Kit V2460) using the peptide RRA(pT)VA as the substrate, and the same buffer used above. For the determination of K_m and k_{cat} for RRA(pT)VA of purified HA–PP2Ac and HA–D88N, the proteins were titrated with okadaic acid to determine the concentration of active molecules [29]. The initial rates of hydrolysis of RRA(pT)VA at 30 °C by native and mutant PP2Ac were determined for substrate concentrations ranging from 50 to 500 μM. The initial reaction velocities (v) were plotted against [S] and then fitted to the Michaelis–Menten equation by non-linear regression analysis to determine values of K_m and k_{cat}. Reactions were performed in triplicate under conditions where substrate depletion was less than 20 %.

Test for PP2Ac function *in vivo* by plasmid shuffling in yeast

Human *PP2Acα* wild-type and mutant cDNA clones were tagged with the HA epitope and expressed in yeast from the *PGK1* (phosphoglycerate kinase gene) promoter/*CYC1*(iso-1-cytochrome *c* gene) terminator of the *TRP1* (phosphoribosylanthranilate isomerase prototrophic selectable marker gene) vector pYPGE2 as previously described [30]. The *pph22-156* and *pph22-186* mutant alleles (where *PPH22* is the PP2A gene homologue 2, encoding yeast PP2Ac), encoding the D156N and H186N substitutions respectively, in yeast PP2Ac were generated by Quickchange PCR amplification (Promega) of the *PPH22* genomic clone in plasmid pDE22 [31] and were inserted (1.8 kb *Xba*I–*Eco*RI fragments) into the *TRP1* shuttle vector YCplac22 [32] for expression in yeast (plasmids YCpDE88 and YCpDE-186 respectively). For plasmid shuffling experiments, *TRP1* plasmids were introduced into cells of the haploid mutant strain DEY3 (*MAT***a** *pph21::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1* [YCpDE8: *URA3 PPH22*]) which is triply deleted for the chromosomal *PPH21*, *PPH22* and *PPH3* genes and contains the *URA3 PPH22* plasmid YCpDE8 providing essential yeast PP2Ac function [31]. Loss of YCpDE8 from these cells was tested by growing Trp^+ transformants on agar medium containing uracil and 5-fluoroorotic acid (5-FOA) [33]; 5-FOA is toxic to cells containing the *URA3* gene and permits the growth of cells that have lost YCpDE8 and are kept alive by PP2Ac function provided by the incoming *TRP1* plasmid.

RESULTS

High-level expression of recombinant PP2Ac in insect cells

To obtain recombinant PP2Ac for structure–function analysis, the baculovirus system was tested for expression of the human enzyme PP2Acα in the insect cell line, High Five. Western-blot analysis of lysates, prepared from cells infected with the recombinant virus vBBHAPP2Ac, revealed the presence of HA-tagged human PP2Ac using the 12CA5 monoclonal antibody (Figure 2). The PP2Ac-specific antibody, AB45, raised against a peptide based on the N-terminal 20 amino acids of PP2Ac [22], detected endogenous PP2Ac in non-infected High Five cells as expected, since the amino acid sequence similarity between *Drosophilia* and human PP2Ac is greater than 93 % [34]. However, the expression level of recombinant PP2Ac was approximately 5-fold greater than endogenous enzyme (Figure 2). Comparison of HA–PP2Ac

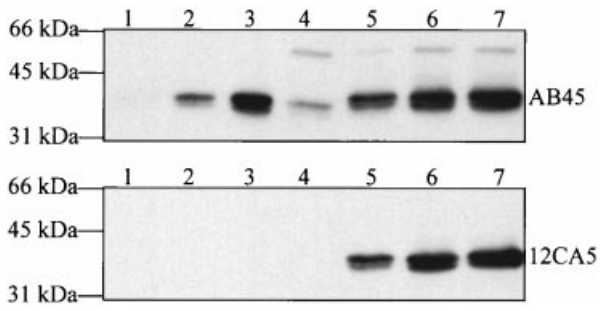

Figure 2 Expression of human PP2Ac in High Five insect cells

Lysates (10 μg) prepared from baculovirus-infected High Five insect cells 48 h post-infection were resolved by SDS/PAGE and analysed by Western blotting using the 12CA5 monoclonal antibody (bottom panel) or the PP2Ac peptide antibody AB45 (top panel). Gels were run in tandem and the first three lanes contained 1, 10 and 50 ng of purified rabbit PP2Ac as controls. Lane 4 contained uninfected High Five cells (control), and lanes 5, 6 and 7 contained lysates from High Five cells infected with baculoviruses for recombinant wild-type HA–PP2Ac, and the mutant HA–D88N and HA–H118N forms respectively.

present in High Five cells with known concentrations of purified rabbit skeletal muscle PP2Ac (Figure 2) indicated that the level of expression of recombinant protein was of the order of 1–2 mg/10^9 cells. By comparison, the insect cell line *Sf*9 expressed a 10-fold lower level of recombinant HA–PP2Ac (results not shown). Thus baculovirus infection of High Five cells is an efficient system for expressing recombinant HA–PP2Ac.

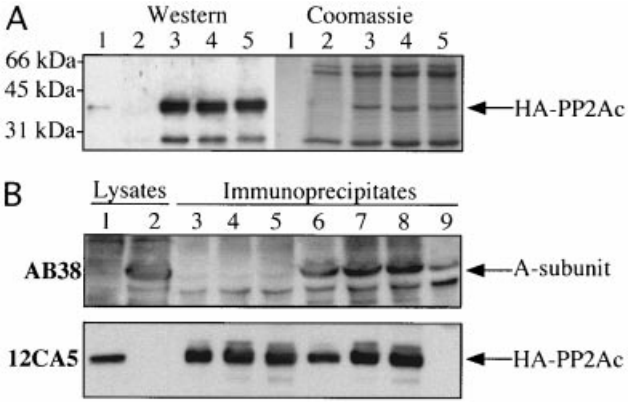

Figure 3 Co-immunoprecipitation of PR65/A with wild-type and mutant PP2Ac

(**A**) Immunoprecipitation of HA-tagged wild-type, D88N and H118N PP2Ac. Lysates were prepared from High Five cells and immune complexes were recovered with the 12CA5 monoclonal antibody. Two gels were run in tandem and subjected to Western blotting with 12CA5 or staining with Coomassie Brilliant Blue. Lane 1 contained 10–20 pg of rabbit PP2Ac as a control and lane 2 is a High Five cell lysate control. Lanes 3, 4 and 5 contained the HA-tagged wild-type PP2Ac, D88N PP2Ac and H118N PP2Ac proteins respectively. Coomassie Brilliant Blue staining revealed high and low molecular mass bands corresponding to heavy and light chain antibodies. (**B**) Recombinant human PP2Ac binds recombinant human PR65/A. Lysates were prepared from High Five cells expressing human HA–PP2Ac, human PR65/A or both (doubly infected). HA-tagged proteins were immunoprecipitated with the 12CA5 monoclonal antibody, resolved by SDS/PAGE, and analysed by Western blotting with 12CA5 to detect HA–PP2Ac or AB38 to detect the presence of co-precipitated PR65/A. Lanes 1 and 2 contained total lysate (10 μg) prepared from High Five cells infected with vBBHAPP2Ac and PR65BV respectively. Lanes 3–5 contained immune complexes prepared from High Five cells infected with vBBHAPP2Ac, vBBHAD88N and vBBHAH118N respectively. Lanes 6–8 contained immune complexes prepared from High Five cells doubly infected with vBBHAPP2Ac/PR65BV, vBBHAD88N/PR65BV and vBBHAH118N/PR65BV respectively. Lane 9 contained immune complexes prepared from High Five cells infected with the virus PR65BV.

Table 1 Specific activities for immunoprecipitated wild-type and mutant HA-tagged PP2Ac

Recombinant HA-tagged wild-type and mutant PP2Ac subunits were immunoprecipitated from High Five insect cell lysates using 12CA5–Protein A–Sepharose. The concentration of immunoprecipitated PP2Ac subunits was determined by quantitative Western-blot analysis (see the Experimental section) and used to standardize phosphatase assays using ^{32}P-labelled kemptide as substrate. Assays were performed in triplicate with and without okadaic acid and were compared with the specific activity of purified rabbit skeletal muscle PP2Ac.

	Specific activity (nmol [^{32}P]P_i released/min per mg)	
PP2Ac	+Okadaic acid (10 nM)	−Okadaic acid
Rabbit PP2Ac	10.0 ± 1.1	180.1 ± 30.4
HA–PP2Ac	4.4 ± 0.3	218.3 ± 17.4
HA–D88N	0.8 ± 0.1	23.5 ± 3.9
HA–H118N	2.2 ± 0.1	9.7 ± 1.3

Overexpression of PP2Ac active-site mutant proteins in insect cells

To test the impact of PP2Ac active-site residues on catalytic function, and obtain maximum yields of recombinant PP2Acα for structure–function analyses we generated mutant forms of HA–PP2Ac containing the H118N or D88N substitutions. Based on sequence alignment of the PPP family members (Figure 1) and kinetic studies of λPPase and PP1 [13–16] the PP2Ac residue His^{118} is believed to serve as a general acid, donating protons to the serine/threonine leaving group during the phosphatase reaction, while Asp^{88} increases its acidic character. Changing either amino acid to a neutral asparagine residue is predicted to impair PP2Ac catalytic function. Mutant forms of HA–PP2Ac were expressed in High Five insect cells following infection with the recombinant viruses vBBHAD88N or vBBHAH118N. Remarkably, the expression level of HA–PP2Ac mutant proteins was approximately 5-fold greater than that of the native recombinant HA–PP2Ac (Figure 2). This observation, together with data shown below, suggests that, in High Five insect cells, overexpression of PP2Ac is facilitated by active-site mutations which impair catalytic function.

Active-site mutations impair PP2Ac catalytic activity *in vitro*

To examine catalytic activity *in vitro*, native and mutant HA–PP2Ac proteins were immunoprecipitated from lysates prepared from High Five cells (Figure 3A). The amount of HA-tagged native and mutant (D88N or H118N) PP2Ac recovered in immune complexes was similar, as judged by Western-blot analysis (results not shown). However, phosphatase activity assays on the immune complexes revealed that, whereas the specific activity of the native HA–PP2Ac (218 nmol [^{32}P]P_i released/min per mg) was similar to that of the purified rabbit skeletal muscle enzyme (180 nmol [^{32}P]P_i released/min per mg), there was a 9-fold and 22-fold reduction in specific activity for the mutants D88N (24 nmol [^{32}P]P_i released/min per mg) and H118N (9.7 nmol [^{32}P]P_i released/min per mg) respectively, using a phospho-kemptide substrate (Table 1). These results are in agreement with those of Ogris et al. [17,18] who detected a dramatic decrease in PP2Ac activity towards phosphorylated

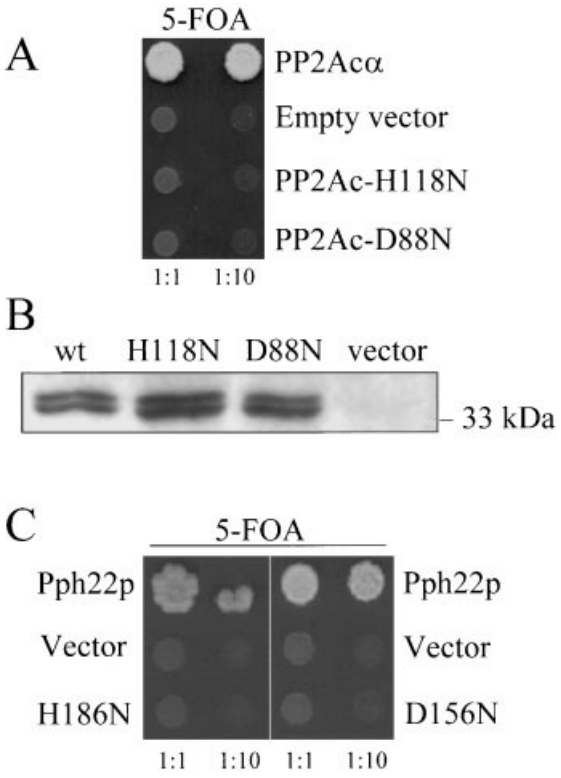

Figure 4 Analysis of active-site mutant forms of PP2Ac in yeast

(**A**) Functional replacement of yeast PP2Ac by active-site mutant forms of human PP2Ac by plasmid shuffling. *TRP1* plasmids encoding wild-type human PP2Ac (PP2Acα), no insert DNA (Empty vector) or a mutant form of human PP2Ac (containing the substitution indicated) were tested for PP2Ac function by plasmid shuffling in strain DEY3. Trp^+ transformant cells were grown to saturation in liquid SD medium [0.17% (w/v) yeast nitrogen base (Difco), 0.5% (w/v) ammonium sulphate and 2% (w/v) dextrose] with uracil. Cell suspensions were then diluted (1:1 or 1:10), spotted on to agar medium containing 5-FOA and incubated at 30 °C for 3 days. (**B**) Western-blot analysis of HA-tagged forms of human PP2Ac expressed in strain DEY3. Yeast cell extracts (30 μg) containing wild-type human PP2Ac (wt), a mutant form of PP2Ac (with the substitution indicated) or the empty expression vector were resolved by SDS/PAGE and probed with the 12CA5 monoclonal antibody. A molecular-mass marker is shown on the right-hand side. Human PP2Ac migrates as a doublet. (**C**) Functional replacement of wild-type PP2Ac with active-site mutant forms of yeast PP2Ac. *TRP1* plasmids encoding wild-type yeast PP2Ac (Pph22p), no insert DNA (Vector) or the active-site mutant form Pph22-H186N (left) or Pph22-D156N (right) were tested for essential function *in vivo* as in (**A**).

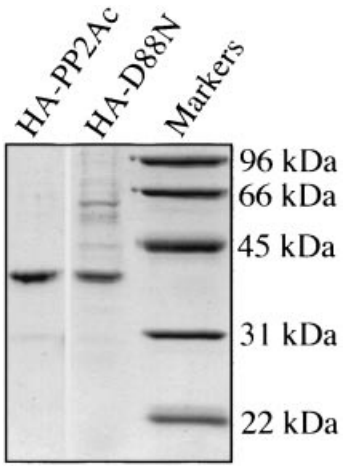

Figure 5 Purification of recombinant PP2Ac

Lysates were prepared from High Five cells using a French press. Proteins were purified by ethanol precipitation and 12CA5 monoclonal antibody affinity chromatography as described in the Experimental section. Approximately 1–3 μg of purified HA–PP2Ac (lane 1) and HA–D88N (lane 2) was resolved by SDS/PAGE and stained with Coomassie Brilliant Blue.

histone H1 (2% of wild-type) and phosphorylase (8% of wild-type) caused by a H118Q substitution [17].

PP2Ac complexes with PR65/A *in vivo* to form a core dimer [1,3]. To examine whether the HA–PP2Ac active-site mutations disrupted protein folding we tested whether the recombinant native and mutant HA–PP2Ac proteins bound recombinant PR65/A during coexpression in insect cells. Immunoprecipitation of HA-tagged PP2Ac proteins using the 12CA5 antibody caused co-precipitation of PR65/A with the native, D88N and H118N PP2Ac forms, indicating the formation of a stable protein–protein interaction in each case (Figure 3B). This suggests that the D88N and H118N mutations impair the catalytic function of PP2Ac without severely disrupting protein folding.

Active-site mutations abolish essential PP2Ac function *in vivo*

Because the D88N and H118N substitutions inhibited PP2Ac catalytic activity *in vitro*, we tested whether they similarly impaired PP2Ac function *in vivo*. To do this, we employed a plasmid shuffling procedure (see the Experimental section) that tests the ability of human PP2Ac proteins to functionally replace the endogenous enzyme of the budding yeast *Saccharomyces cerevisiae* [30]. Functional analysis in yeast revealed that, unlike wild-type human PP2Acα, the PP2Ac-H118N and PP2Ac-D88N mutant proteins each failed to substitute for the endogenous yeast PP2Ac *in vivo* (Figure 4A), although each protein was expressed at a similar level (Figure 4B). To confirm this for the endogenous yeast PP2Ac we generated active-site mutations in *S. cerevisiae* Pph22p, which shares 71% amino acid sequence identity with human PP2Acα over the region of homology [30]. Like the equivalent human proteins, the active-site mutant forms of yeast PP2Ac, containing the H186N or D156N substitutions (analogous to H118N and D88N in human PP2Acα respectively) failed to provide PP2Ac function *in vivo* (Figure 4C). Together, these results demonstrate that active-site mutations impairing catalysis inhibit essential PP2Ac function *in vivo*.

Purification and characterization of native and mutant PP2Ac

For the large-scale expression and purification of PP2Ac we employed an HA tag, placed in frame with the N-terminus of human PP2Acα, for immunoaffinity purification of recombinant protein. Constructs with N- and C-terminal His tags were also evaluated; however, binding of the tagged PP2Ac to Ni^{2+}-nitrilotriacetate agarose (Qiagen) was ineffective for large-scale purification, since significant amounts of endogenous insect PP2Ac were purified from lysates prepared from High Five cells. This would suggest that PP2Ac may bind to Ni^{2+}-nitrilotriacetate agarose matrix via the active site and as such would be a major source of contamination in the purification of His-tagged recombinant PP2Ac (results not shown). Large-scale expression of PP2Ac was achieved by seeding 20 triple flasks with 1×10^9 High Five cells. Recombinant protein was prepared from infected cells utilizing a two-step purification protocol based on ethanol precipitation [27,28] and monoclonal antibody affinity chromatography. Ethanol precipitation was used as the initial purification step in order to increase the yield of pure PP2Ac and significantly reduce non-specific binding to the 12CA5–Protein A–Sepharose

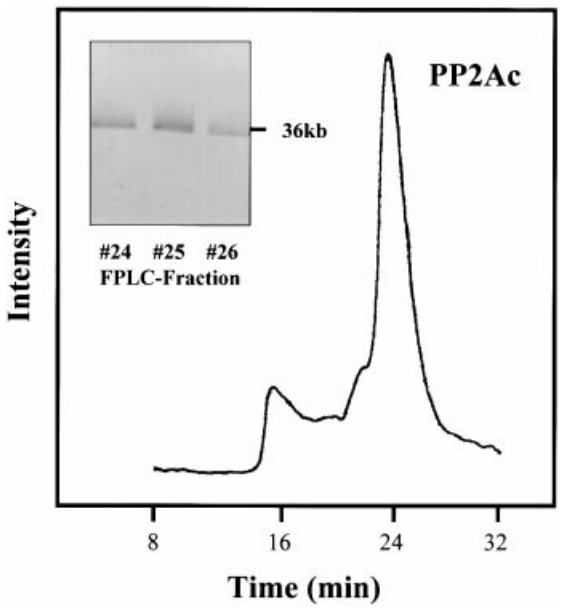

Figure 6 Gel filtration of recombinant PP2Ac

Approximately 0.2 mg of purified recombinant wild-type or mutant HA-tagged PP2Ac was loaded on to a Superdex 75HR 10/30 FPLC gel-filtration column. Fractions were collected over the course of the chromatography and the presence of PP2A was determined by Coomassie Brilliant Blue staining and Western-blot analysis. The chromatogram shows the elution profile of wild-type PP2Ac and is a representative trace for all PP2Ac forms tested. The inset shows a Coomassie Brilliant Blue-stained gel of the purified three main fractions of HA–PP2Ac in lanes 1–3 respectively. Each PP2Ac protein eluted off the column as a single peak with a retention time of 24 min. PP2Ac was not detected in any of the other fractions.

immunoaffinity column and to ensure that purified PP2Ac was not complexed to the insect cell A-subunit. This procedure permitted essentially quantitative recovery of 1 mg of pure wild-type HA–PP2Ac, while only 10% of the HA–D88N PP2Ac (1 mg/1×10^9 cells) was recovered with a purity greater than 90% (Figure 5). The mutant HA–H118N PP2Ac did not survive ethanol precipitation well with 1% recovery (results not shown). To ensure the purified PP2Ac was monodisperse and correctly folded using the ethanol precipitation procedure, we performed gel filtration on HA-tagged wild-type and mutant PP2Ac. The wild-type and mutant forms of PP2Ac eluted as a single peak with a retention time of 24 min suggesting that the proteins were not aggregated and were correctly folded (Figure 6), with the correct apparent molecular mass, as judged by standards.

Purified recombinant HA–PP2Ac and HA–D88N were titrated with okadaic acid revealing a concentration of active molecules similar to that determined by the Bradford assay using BSA as a standard (results not shown). The kinetic constants K_m and k_{cat} for the dephosphorylation of the peptide RRA(pT)VA were determined for titrated HA–PP2Ac and HA–D88N PP2Ac (Table 2). The K_m for HA–D88N (58.4 μM) was reduced by approximately 6-fold compared with HA–PP2Ac (337.5 μM), whereas k_{cat} was decreased 85-fold for HA–D88N (2 s^{-1}) compared with HA–PP2Ac (170 s^{-1}). For HA–PP2Ac, the value of K_m for the substrate RRA(pT)VA was similar to that measured for the native bovine PR65/A–PP2Ac complex using ^{32}P-labelled kemptide as a substrate (K_m = 361 μM) [35], and for purified rabbit skeletal muscle PP2Ac using RRA(pT)VA as substrate where the K_m was 310 μM (Promega).

Table 2 Kinetic constants for purified wild-type HA–PP2Ac and HA–D88N PP2Ac

The values for K_m and k_{cat} for the dephosphorylation of the peptide RRA(pT)VA were determined for purified wild-type HA–PP2Ac and HA–D88N PP2Ac. Substrate concentrations ranging from 50 to 500 μM were used in a 50 μl assay volume containing either 2 nM wild-type HA–PP2Ac or 10 nM HA–D88N and incubated at 30 °C for 10 and 30 min respectively. The reactions were stopped and free phosphate was measured as described by the manufacturers using a Molecular Devices Softmax plate reader. The initial reaction velocities (v) were plotted against [S] and then fitted to the Michaelis–Menten equation by non-linear regression analysis to determine values of K_m and k_{cat}. Reactions were performed in triplicate under conditions such that substrate depletion was less than 20%.

PP2Ac	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
HA–PP2Ac	337.5 ± 37.4	170.0 ± 12.8	5.03×10^5
HA–D88N	58.4 ± 14.6	2.0 ± 0.1	0.34×10^5

DISCUSSION

Baculovirus system for expression of PP2Ac

The identification and analysis of PP2Ac structural determinants important for catalysis and its interaction with PR65/A and B-type subunits has been restricted by lack of a convenient eukaryotic expression system capable of generating high levels of recombinant PP2Ac significantly in excess over the endogenous enzyme. For example, expression of PP2Ac from the strong U3 promoter of the Moloney murine leukaemia virus led to an increase in the total (endogenous plus recombinant) PP2Ac in murine NIH/3T3 fibroblasts of only 30–50% [20], while expression of recombinant PP2Ac from the cytomegalovirus promoter was achieved at a level only 3-fold higher than that of the endogenous protein in HEK-293 human kidney cells [36]. Accordingly, PP2Ac appears to control its intracellular levels stringently. Studies by Baharians and Schönthal [19] showed that the strict control of endogenous and recombinant PP2Ac levels in NIH/3T3 cells is due to auto-regulation at the level of translation, whereas Chung and Brautigan [21] show PP2Ac activity is strictly regulated at the level of transcription in COS-7 cells. These mechanisms are likely to operate in most cell types to maintain a constant intracellular pool size of PP2Ac. Our results indicate that the baculovirus system using High Five cells is appropriate for the overexpression of recombinant PP2Ac because it partially lacks these stringent mechanisms of PP2Ac auto-regulation. The baculovirus system has been used previously to investigate the regulation of PP2A enzyme specificity by regulatory B subunits in *Sf*9 cells, which were triply infected with baculoviruses for PP2Ac, PR65/A, and PR55/B, PR61/B′ or simian-virus-40 small tumour antigen [35]. Purification of PP2A heterotrimers yielded approximately 150 μg/10^9 *Sf*9 cells (approx. 50 μg of PP2Ac) similar to the yields from *Sf*9 cells infected with recombinant PP2Ac obtained in the present study (results not shown). Remarkably, we show that the level of recombinant HA–PP2Ac in High Five cells is in excess of 1 mg/10^9 cells (approximately 5-fold higher than endogenous PP2Ac).

Active-site mutations inhibit catalysis and allow overexpression of PP2Ac

Mutating the active-site residues Asp^{88} and His^{118} to the neutral amino acid asparagine yielded substantially higher levels (5–10 mg/10^9 High Five cells) of mutant HA–PP2Ac compared with native recombinant HA–PP2Ac. The Asp^{88} and His^{118} residues were chosen based on analogous residues in the struc-

turally related PP1 and λPPase (Figure 1), which, when similarly mutated, cause reduced values for V_{max} in phosphatase reactions [13,14]. HA–PP2Ac activity assays revealed a 9- and 23-fold reduction in the relative activity of the HA–D88N and HA–H118N forms of PP2Ac respectively, while binding of the native and mutant PP2Ac proteins to PR65/A was similar, suggesting similar folding in each protein. The results for the HA–H118N substitution are similar to those observed for the H118Q substitution of PP2Ac, which had decreased activity towards the substrates phosphorylase *a* and histone H1 [17,18]. Moreover, functional analysis in yeast revealed that the D88N and H118N substitutions in human PP2Ac, as well as the analogous active-site mutations (D156N and H186N) in yeast PP2Ac, abolished essential PP2A function *in vivo*. These observations demonstrate that the human Asp^{88} and His^{118} residues perform a key, phylogenetically conserved, role in the catalytic mechanism of PP2Ac. Furthermore, our results indicate that the regulation of PP2Ac expression is apparently controlled by its PP activity, and could be due to the mechanisms outlined by Baharians and Schönthal [19] or Chung and Brautigan [21].

Expression, purification and characterization of recombinant PP2Ac

Scaled-up expression and purification of recombinant PP2Ac from 1×10^9 High Five cells using a two-step purification procedure yielded 1 mg of pure HA–PP2Ac and 1 mg of HA–D88N, whereas the mutant HA–H118N did not survive the ethanol precipitation step particularly well. Only 1 and 10% of the total HA–H118 and HA–D88N was recovered respectively, and reflects the effect of the mutations on the stability of PP2Ac after ethanol precipitation. However, gel-filtration chromatography shows the mutant proteins to be monodisperse with a similar fold to wild-type PP2Ac. Expression of HA-tagged PP2Ac in conjunction with the two-step purification procedure has yielded milligram quantities of pure PP2Ac which has been complexed with recombinant PR65 from *Escherichia coli* (results not shown) to allow the structure determination of PP2Ac in complex with PR65/A using X-ray crystallography.

The kinetic parameters for the dephosphorylation of RRA(pT)VA by purified recombinant HA–PP2Ac (K_m = 337.5 μM; $k_{cat} = 170\ s^{-1}$) were similar to values described previously for rabbit skeletal muscle PP2Ac (Promega) and the values obtained for the dephosphorylation of ^{32}P-labelled kemptide by the PR65/A–PP2Ac complex where K_m was 361 μM [18]. Substitution of Asp^{88} with the isosteric asparagine shows a small decrease in K_m (6-fold), but a significant reduction in k_{cat} (85-fold), suggesting that the association of the Ser(P)/Thr(P) substrate with the PP2Ac active site is not impaired for complex formation; however, the mutation has an effect on catalysis. Mutation of λPPase residue $Asp^{52} \rightarrow Asn$ (D88N in PP2Ac) caused a small decrease in K_m for the substrate *p*-nitrophenyl phosphate (3-fold) but a 35-fold decrease in k_{cat}, which is similar to the effects of HA–D88N PP2Ac towards RRA(pT)VA [13]. The effect of the PP2Ac D88N substitution on catalysis is consistent with the catalytic mechanism proposed for the PPP family member, PP1, based on its crystal structure, involving an ion-pair interaction between Asp^{95} (Asp^{88} in PP2Ac) and His^{125} (His^{118} in PP2Ac). The analogous PP1 residue Asp^{95} promotes catalysis, both by enhancing the acidic character of His^{118} via electron withdrawal and by holding the imidazole ring of PP1 His^{125} in a rigid conformation permitting the protonation of the target substrate [16]. The PP2Ac residue Asp^{88} is highly conserved between members of the PPP family and the effect of the D88N substitution on catalysis by PP2Ac provides support to the notion that the active-site topology and catalytic mechanism of this family of PPs is also conserved.

We show that the baculovirus system using High Five insect cells allows, for the first time, the high-level expression of biologically active PP2Ac for structural and functional studies. Site-directed mutagenesis of PP2Ac and purification of mutant proteins from High Five insect cells combined with functional analysis in yeast provides a powerful system for structure–function analysis of PP2Ac. We are currently employing these systems to further investigate the mechanism of PP2Ac catalysis and the role of post-translational modifications in PP2Ac function, activity and subunit interactions.

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