The variable subunit associated with protein phosphatase $2A_0$ defines a novel multimember family of regulatory subunits

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Two protein phosphatase 2A (PP2A) holoenzymes were isolated from rabbit skeletal muscle containing, in addition to the catalytic and PR65 regulatory subunits, proteins of apparent molecular masses of 61 and 56 kDa respectively. Both holoenzymes displayed low basal phosphorylase phosphatase activity, which could be stimulated by protamine to an extent similar to that of previously characterized PP2A holoenzymes. Protein microsequencing of tryptic peptides derived from the 61 kDa protein, termed PR61, yielded 117 residues of amino acid sequence. Molecular cloning by enrichment of specific mRNAs, followed by reverse transcription–PCR and cDNA library screening, revealed that this protein exists in multiple isoforms encoded by at least three genes, one of which gives rise to several splicing variants. Comparisons of these sequences with the available databases identified one more human gene and predicted another based on a rabbit cDNA-derived sequence, thus bringing the number of genes encoding PR61 family members to five. Peptide sequences derived from PR61 corresponded to the deduced amino acid sequences of either α or β isoforms, indicating that the purified PP2A preparation was a mixture of at least two trimers. In contrast, the 56 kDa subunit (termed PR56) seems to correspond to the ϵ isoform of PR61. Several regulatory subunits of PP2A belonging to the PR61 family contain consensus sequences for nuclear localization and might therefore target PP2A to nuclear substrates.

INTRODUCTION

Protein phosphatase 2A (PP2A) is a family of trimeric holoenzymes composed of a 36 kDa catalytic subunit (PP2Ac) bound to a constant regulatory subunit of 65 kDa (PR65) and one of a number of variable regulatory subunits. Several different variable subunits of molecular masses 54 kDa (B′), 55 kDa (PR55), 72 kDa (PR72) and 74 kDa have been identified in holoenzymes purified from different tissues and organisms (reviewed in [1,2]). The PR55 and PR72 subunits have also been characterized by molecular cloning [3–7]. It is assumed that variable subunits regulate phosphatase activity, confer substrate specificity and influence subcellular localization of the different PP2A holoenzymes. The multiplicity of PP2A variable regulatory subunits might therefore partly provide a molecular basis to explain how this phosphatase can appropriately regulate numerous cellular processes. So far PP2A has been implicated in the regulation of metabolism, signal transduction, growth, development, cell cycle progression and transformation (reviewed in [8,9]).

The importance of the variable regulatory subunits for the function of PP2A is underlined by the fact that they are targeted by transforming antigens of several DNA tumour viruses. The SV40 small t antigen apparently displaces the PR55 subunit *in vivo*, thus creating a novel trimeric phosphatase that is less active towards MAP and MEK kinases [10]. The action of SV40 small t antigen is holoenzyme-specific, because the variable regulatory subunit of PP2A present in trimeric PP2A purified from bovine heart cannot be replaced by these viral proteins [2]. Interestingly, PP2A associated with a polyoma virus middle T antigen displays a constitutive phosphotyrosyl phosphatase activity [11]. Other viral proteins, such as the adenoviral protein termed E4orf4, target $PP2A_1$ by binding to the PR55 subunit [12]. However, the effects of E4orf4–$PP2A_1$ complex formation on phosphatase activity have not been described.

Several mutations in PP2A regulatory subunits have been isolated during studies of yeast and *Drosophila*. For instance, *Saccharomyces cerevisiae cdc*55 and *Drosophila aar*[1] and *twins*[p] phenotypes are caused by mutations in the gene encoding the respective PR55 homologues. *cdc55* mutants are viable but display abnormal cytokinesis when grown at low temperatures [4], whereas *aar*[1] and *twins*[p] mutations are lethal, owing to an abnormal metaphase–anaphase resolution [13] and duplication of wing imaginal disks [14] respectively. PP2A activity in brain extracts of *aar*[1] mutant flies was several-fold lower towards histone H1 and caldesmon phosphorylated by $p34^{cdc2}$ than that of wild-type flies [15]. Furthermore overexpression of the PR65 regulatory subunit in mammalian fibroblasts disrupts cytokinesis and leads to multinucleated cells [16]. These results suggest that PR65, when present in excess, can act as a dominant negative regulator of the catalytic subunit.

Given the numerous cellular processes involving PP2A, we sought to identify novel PP2A holoenzymes by employing an affinity-based purification strategy. The key feature of this approach was the characterization of all PP2A-containing

Abbreviations used: PR55, PR56, PR61, PR65 and PR72, phosphatase regulatory subunits with apparent molecular masses of 55, 56, 61, 65 and 72 kDa respectively; PP2Ac, 36 kDa catalytic subunit of PP2A; PP2A, protein phosphatase 2A holoenzyme; $PP2A_2$, dimeric PP2A consisting of PP2Ac and PR65; $PP2A_{0/61}$, $PP2A_{0/56}$, $PP2A_1$ and $PP2A_3$, trimeric PP2A holoenzymes consisting of PP2Ac/PR65 further complexed with PR61α/β, PR56, PR55 and PR72 respectively; PTPA, phosphotyrosyl phosphatase activator; RCM-lysozyme, reduced carboxamidomethylated and maleylated lysozyme; RT, reverse transcription.

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The nucleotide sequences of human PR61β2, PR61γ1 and PR61ϵ isoforms reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Z69028 (PR61γ1), Z69029 (PR61ϵ) and Z69030 (PR61β2).

fractions with a large panel of subunit-specific antibodies. Here we describe the purification of two heterotrimers containing variable regulatory subunits with apparent molecular masses of 61 and 56 kDa respectively. Furthermore we report the molecular cloning of several members of the PR61 family. The deduced amino acid sequences of PR61 subunits revealed that both 61 and 56 kDa proteins belong to the same subunit family.

MATERIALS AND METHODS

Purification of PP2A holoenzymes

PP2A holoenzymes were purified from rabbit skeletal muscle by using modifications of the procedures described by Tung et al. [17] and Zolnierowicz et al. [6]. Rabbit skeletal muscle extracts were processed by acetic acid acidification to pH 5.3, batch DEAE-cellulose (DE52) chromatography (washed with 50 mM NaCl and eluted with 300 mM NaCl), ammonium sulphate precipitation (30–50 % saturation) and DEAE-Sepharose 4B chromatography. In the latter step the column (5 cm × 40 cm) was eluted with a 2 litre gradient of 50–600 mM NaCl. Fractions corresponding to PP2A that eluted from DEAE-Sepharose between 270 and 320 mM NaCl (pool 1), 340 and 380 mM NaCl (pool 2) and 390 and 440 mM NaCl (pool 3) were combined and further purified. PP2A holoenzymes in pools 1 and 3 were purified by chromatography on poly(L-lysine)-agarose, ω-aminohexyl Sepharose and thiophosphorylase *a*–Sepharose columns, whereas purification of pool 2 involved poly(L-lysine)-agarose and thiophosphorylase *a*–Sepharose. The final purification step involved ion-exchange MonoQ FPLC (0.5 cm × 5 cm column, Pharmacia) with a 40 ml gradient from 200 to 500 mM NaCl. The purified proteins obtained from the final steps were analysed by SDS/PAGE and immunodetection on Western blots.

Protein phosphatase activity measurements

PP2A activity was monitored throughout the purification by assaying protamine-stimulated phosphorylase phosphatase activity as described by Waelkens et al. [18]. Protamine-stimulated phosphorylase phosphatase activity in the presence of 16 mM ammonium sulphate was measured by the method of Jessus et al. [19]. Dephosphorylation of retinoblastoma protein-derived peptide (INGSPRTPRRGQNR) phosphorylated by $p34^{cdc2}$ was measured as described by Agostinis et al. [20]. Activation of the phosphotyrosine phosphatase activity of PP2A by phosphotyrosyl phosphatase activator (PTPA) and dephosphorylation of reduced carboxamidomethylated and maleylated lysozyme (RCM-lysozyme) phosphorylated by an *src*-related tyrosine kinase were performed by the method of Van Hoof et al. [21]. One unit of phosphatase activity corresponds to 1 nmol of phosphate hydrolysed per min at 30 °C. Protein concentration was determined by the method of Bradford [22] with BSA as standard.

Determination of amino acid sequences

PP2A holoenzyme preparations eluted from the MonoQ FPLC column were resolved into constituent subunits by SDS/PAGE [10 % (w/v) gel]. Protein transfer, trypsin digestion, peptide separation and peptide sequencing were performed as previously described [5].

Molecular cloning

Molecular cloning was performed with a reverse transcription (RT)–PCR-based approach. RT was performed on mRNA selected by using a mixture of three biotinylated antisense oligonucleotides corresponding to PR61-derived peptides (see Table 2). For this selection 2 mg of Streptavidin microspheres (3.5 μm supermagnetic particles, Lucernachem) was washed three times with 500 μl of DEPC-treated H_2O and resuspended in 200 μl of 20 mM Tris/HCl, pH 7.5, containing 2 mM EDTA and 2 M NaCl. The beads were equilibrated with 33 pmol of each antisense biotinylated oligonucleotide, 12414 (5′-biotin-GTIARRAAIATYTGRTTISWYTG-3′, corresponding to peptide 61/62), 12415 (5′-biotin-TCYTCIARICCYTGYTCIARY-TCYTG-3′, corresponding to peptide 66) and 12416 (5′-biotin-TTRTTIARYTCYTCICKYTC-3′, corresponding to peptide 31), for 10 min at room temperature and mixed with 900 μg of total RNA isolated from frozen rabbit skeletal muscle according to Chomczynski and Sacchi [23] in 200 μl of water. After 5 min at room temperature the beads were washed three times with 250 μl of 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 1 M NaCl and the selected mRNA was eluted with 100 μl of water by heating at 65 °C for 5 min. mRNA was precipitated with 0.3 M ammonium acetate/66 % (v/v) ethanol and used for RT with 50 pmol of antisense oligonucleotide 12003 (5′-TGCATGTTRTANGCNSWRTT-3′, corresponding to peptide 34/35B) with AMV reverse transcriptase (Boehringer Mannheim). For PCR amplification of the PR61 cDNA the following oligonucleotides were used: 5′-GCNCCNGAYATGC-ARCCNAT-3′ (12004, sense corresponding to peptide 43/44B), 5′-CARWSIAAYCARATITTYYTIAC-3′ (12413, sense corresponding to peptide 61/62), 5′-CAGAATTCCARGARYTIGA-RCARGGIYTNGA-3′ (9058, sense corresponding to peptide 66) and 5′-ARYTCYTCNARACCYTG-3′ (12285, antisense corresponding to peptide 66). Biphasic PCR was performed as described in Ruano et al. [24] with AmpliTaq DNA polymerase (Perkin-Elmer) and four combinations of oligonucleotides (5 pmol each): 603 (12004–12003), 604 (12413–12003), 605 (12413–12285) and 606 (9058–12003). PCR conditions were: 1 min at 94 °C; 4 min at 45 °C; 3 min at 72 °C. After 18 cycles, 50 pmol of each primer was added and reaction run for an additional 50 cycles (1 min at 94 °C, 2 min at 45 °C, 3 min at 72 °C). PCR products were subcloned into pGEM-T vector (Promega) and analysed by sequencing with SP6 and T7 promoter specific primers with Sequenase 2.0 (USB). PCR clone 603-5 was labelled to a specific activity of approx. 10^9 c.p.m./μg with [α-^{32}P]dATP by the random priming method [25] and used to screen human fetal retina, heart and brain cDNA libraries (Stratagene) as previously described [5].

Western blot analysis

Protein transfer and Western blot analysis were performed as described by Hendrix et al. [26]. The following peptide-specific rabbit polyclonal antisera were used: Ab C^{1-20} (against the N-terminal peptide of PP2Acα), Ab $65^{177-196}$ (against an internal peptide of PR65 [27]), Ab $55\alpha^{1-19}$ (against the N-terminal peptide of PR55α [26]), and Ab $72/130^{COOH}$ (against the C-terminal peptide of PR72/130 [5]). Primary antibodies were diluted 1:100 in blocking buffer. Immunodetection was performed with peroxidase-conjugated donkey anti-(rabbit IgG) and enhanced chemiluminescence (Amersham).

RESULTS AND DISCUSSION

Purification of trimeric PP2A containing 61 and 56 kDa variable regulatory subunits

We used thiophosphorylase *a*–Sepharose as an affinity matrix to purify PP2A holoenzymes from rabbit skeletal muscle [6,17].

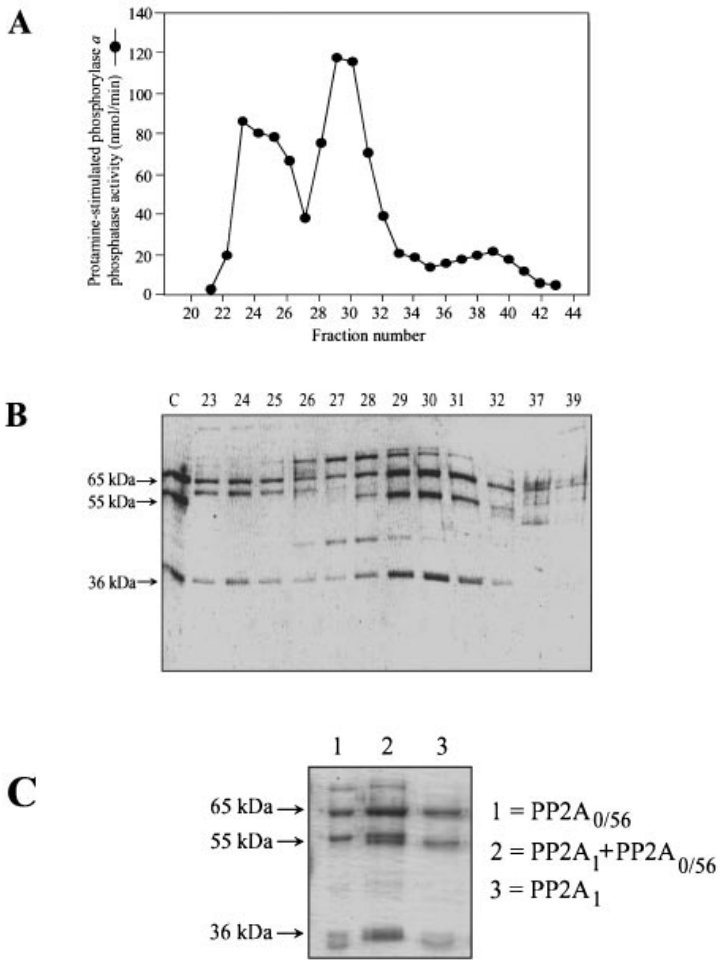

Figure 1 Purification and properties of novel trimeric forms of PP2A

(**A**) MonoQ FPLC phosphorylase phosphatase activity profile. The protamine-stimulated phosphorylase phosphatase activity was measured as described by Waelkens et al. [18]. (**B**) SDS/PAGE of trimeric PP2A holoenzymes containing 61 kDa (fractions 23–25) and 56 kDa (fractions 28–32) regulatory subunits. C, purified $PP2A_1$ loaded as control (only the right-hand side of the bands represents the correct molecular sizes because it was run in the outside lane). (**C**) SDS/PAGE comparison of $PP2A_{0/56}$ and $PP2A_1$.

Detailed analysis of the affinity-purified holoenzymes was performed by MonoQ FPLC (see Figure 1) followed by immunoblotting with a collection of antisera raised against the constituent subunits of PP2A described previously [5,26,27]. This approach resulted in the isolation of two novel PP2A holoenzymes (the present study) and two PP2A-associated proteins (N. Andjelković, S. Zolnierowicz, C. Van Hoof, J. Goris and B. A. Hemmings, unpublished work).

The fraction of PP2A activity eluting between 270 and 320 mM NaCl was initially designated as $PP2A_0$ by Tung et al. [17]. With our assay procedure we were able to detect phosphorylase phosphatase activity by adding protamine, and freezing and thawing in the presence of 2-mercaptoethanol was not required for activity. Detailed analysis of the protein constituents was performed after MonoQ FPLC (Figures 1A to 1C). Using a protamine-stimulated phosphorylase phosphatase activity assay, we measured two distinct peaks of activity, corresponding to fractions 23–24, and 29–30 (Figure 1A). SDS/PAGE and Western blot analysis of fractions 23–39 obtained from MonoQ FPLC revealed the presence of 36 kDa catalytic and 65 kDa regulatory (PR65) subunits of PP2A (Figure 1B). The core subunits of PP2A co-purified with the two proteins with apparent molecular masses of 61 kDa (fractions 23–25) and 56 kDa (fractions 28–32; Figure 1B). These two proteins were clearly distinct from PR55 because they did not cross-react with anti-PR55 antibodies (results not shown). Further SDS/PAGE analysis of $PP2A_1$ (PR55-containing holoenzyme) and the holoenzyme containing the 56 kDa protein, when run side by side as well as combined together, clearly showed that the 56 kDa protein runs more slowly than PR55 (Figure 1C).

Initial activity measurements (Figure 1A) as well as further biochemical characterization of the purified preparations (see Table 1 and Figure 2) led to the conclusion that we had isolated two distinct trimeric PP2A holoenzymes. With regard to the purification procedures and previously established nomenclature [17] we termed these two trimers $PP2A_{0/61}$ (Figure 1B, fractions 23–25) and $PP2A_{0/56}$ (Figure 1B, fractions 28–32) respectively, and the corresponding variable regulatory subunits were named PR61 and PR56, according to their relative migrations during SDS/PAGE.

SDS/PAGE analysis of purified PP2A preparations (Figure 1B, fractions 26–31) revealed the presence of at least three other proteins, which migrated with apparent molecular masses of 45, 75 and 77 kDa. These proteins were not coincident with the two

Table 1 Biochemical characterization of $PP2A_{0/61}$ and $PP2A_{0/56}$

$PP2A_{0/61}$ and $PP2A_{0/56}$ were assayed by using 10 μM phosphorylase *a* with and without protamine (8 μg/ml except for 2 μg/ml in the case of $PP2A_3$), 5 μM retinoblastoma (RB) peptide, or 5 μM RCM-lysozyme as substrates. The latter assay was performed after activation by PTPA. The values of the phosphorylase phosphatase activity (with or without protamine) for $PP2A_1$, $PP2A_3$ and $PP2A_2$ are taken from [18], and the RB peptide over phosphorylase phosphatase activity ratio of the same enzymes from [20]. Abbreviation: n.d., not determined.

PP2A form	Phosphorylase *a* (units/mg)	Phosphorylase *a* + protamine (units/mg)	RB peptide (units/mg)	RCM-lysozyme (units/mg)	RB peptide/ phosphorylase *a*	RCM-lysozyme/ phosphorylase *a* (%)
$PP2A_{0/61}$	325	2946/8659*	26	1	0.08	0.3
$PP2A_{0/56}$	264	2552/7413*	n.d.	10	n.d.	3.8
$PP2A_1$	677	2033	5720	14	8.45	2.1
$PP2A_2$	3000	5500	270	536	0.09	17.9
$PP2A_3$	600	4500	102	1	0.17	0.2

* The second figure refers to the activity measured in the presence of 32 mg/μl protamine plus 16 mM ammonium sulphate.

activity peaks, nor did they cross-react with antibodies specific to the known PP2A regulatory subunits (PR65, PR55 and PR72). However, because these proteins eluted together with the PP2A holoenzyme at the affinity chromatography step, they could potentially represent PP2A interacting proteins. Alternatively, they could be phosphorylase interacting proteins that co-purify with this PP2A preparation. Microsequencing of peptides derived from these proteins will be necessary to establish their identity and function.

Several peptides derived from PR65 associated with $PP2A_{0/61}$ were sequenced. Three peptides contained amino acids different between α and β isoforms of PR65: ^{204}E**II**P**M**FS**N**LA**S**^{214}, ^{261}YMVADKF**T**ELQR^{272} and ^{521}MLP**T**VL**R**^{527} (residues in bold are specific for the α isoform). Therefore the PR65 component of $PP2A_{0/61}$ holoenzyme represents the α isoform. In this respect it is interesting to note that all PP2A holoenzymes so far extensively characterized contain the α isoform, which could suggest that PR65β is present in as yet unidentified PP2A trimers. Alternatively, the PR65β isoform could be stage-specifically expressed, as observed with *Xenopus* [7,26], or it could form phosphatase complexes specific for particular cell types.

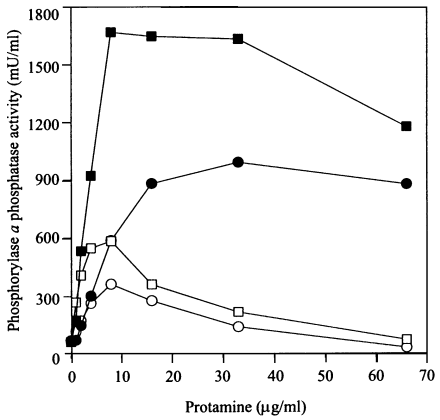

Figure 2 Activation of phosphorylase phosphatase activity of $PP2A_{0/61}$ and $PP2A_{0/56}$ holoenzymes by protamine

Purified preparations of $PP2A_{0/61}$ and $PP2A_{0/56}$ obtained by MonoQ FPLC (see Figure 1) were used to assay basal and protamine-stimulated phosphorylase phosphatase activity. Symbols: ○,●, activity of $PP2A_{0/61}$; □,■, activity of $PP2A_{0/56}$. Assays were performed in the absence (○,□) and presence (●,■) of 16 mM ammonium sulphate as described by Jessus et al. [19]. U, units.

Biochemical characterization of PP2A containing PR61 and PR56 subunits

To characterize $PP2A_{0/61}$ and $PP2A_{0/56}$ further we analysed several enzymic properties, such as the degree of stimulation by polycations, substrate specificity and the ability of PTPA to activate tyrosine phosphatase activity, and compared them with previously characterized PP2A holoenzymes. One of the characteristic features of the PP2A family of holoenzymes is that their phosphorylase phosphatase activity can be stimulated by polycations such as protamine, polylysine or histones. Different oligomeric forms of PP2A can be distinguished by their degree of polycation stimulation and by the optimal stimulatory concentration of polycations [18]. Both $PP2A_{0/61}$ and $PP2A_{0/56}$ displayed low basal activity towards phosphorylase *a* that could be stimulated by polycations to a level similar to other PP2A holoenzymes (Table 1). Figure 2 shows the concentration-dependent stimulation of phosphorylase phosphatase activity of the two holoenzymes by protamine. The optimal protamine concentration for stimulation of phosphorylase phosphatase activity of both $PP2A_{0/61}$ and $PP2A_{0/56}$ was within the same range (8 μg/ml protamine) as previously reported for $PP2A_1$ and $PP2A_2$, but different from $PP2A_3$ (2 μg/ml protamine). It is also significant that $PP2A_{0/56}$ required a lower concentration of protamine for stimulation than $PP2A_{0/61}$ (Figure 2). In addition, protamine action on phosphorylase phosphatase was determined in the absence and presence of 16 mM ammonium sulphate. In the absence of ammonium sulphate, phosphorylase phosphatase activity of both $PP2A_{0/61}$ and $PP2A_{0/56}$ reached a maximum at approx. 8 μg/ml protamine (5-fold for $PP2A_{0/61}$ and 8-fold for $PP2A_{0/56}$) but higher concentrations of protamine were inhibitory (Figure 2, two lower curves). Maximal protamine stimulation of phosphorylase phosphatase activity was achieved in the presence of 16 mM ammonium sulphate and was approximately 14-fold for $PP2A_{0/61}$ and 24-fold for $PP2A_{0/56}$ (Figure 2, two upper curves).

Although the different oligomeric forms of PP2A have overlapping substrate specificities, it is generally accepted that the third, variable subunit determines the substrate specificity to a large extent [15,20,28–30]. So far, peptides and proteins phosphorylated by $p34^{cdc2}$ have been almost exclusively dephosphorylated by $PP2A_1$ [15,20,31]. We therefore further analysed the activity of $PP2A_{0/61}$ towards the retinoblastoma peptide phosphorylated by $p34^{cdc2}$. As shown in Table 1, the

Table 2 Tryptic peptides derived from PR61

Tryptic peptides derived from PR61 α and β isoforms are listed. Superscript numbers represent amino acid residues in respective proteins that comprise these peptides (for the β isoform, residue numbers correspond to the β1 sequence). Amino acid residues determined with low certainty are shown in small capitals. One peptide sequence was obtained (p31 : EREELNK) that we speculate was most probably derived from PR61ϵ.

Peptides derived from PR61α		Peptides derived from PR61β	
p12A	^{457}KLEELK^{462}	p12B	^{487}VPQVAASGGQS^{497}
p33A	^{448}ELEEEELWK^{456}	p30	^{355}QEPLFKL^{361}
p33B	^{347}TIERPLFK^{354}	p43/44B	^{176}LGAPDMQPMV^{185}
p34/35C	^{348}XEVPLFK^{354}	p61/62	^{52}YQSNQIFLT^{60}
p34/35A	^{347}KIEE^{350}	p66	^{458}QELEQGLEEL^{467}
p34/35B	^{471}NSAYNMHSIL^{480}	p67	^{121}LIEPVY^{126}
p41	^{129}ANIF^{132}		
p43/44A	^{47}QGSQAFLSPL^{56}		

retinoblastoma peptide was rather resistant to dephosphorylation by $PP2A_{0/61}$, which makes it similar to $PP2A_3$ and $PP2A_2$.

PTPA can specifically up-regulate the phosphotyrosyl phosphatase activity of $PP2A_2$, whereas PR55 largely suppresses, and PR72 totally prevents, activation by PTPA (Table 1; see also [32]). To check whether PR61 or PR56 affected the PTPA-stimulated tyrosine phosphatase activity of PP2A, we measured their activities towards RCM-lysozyme in the presence of PTPA. We found that $PP2A_{0/61}$ holoenzyme lacks the ability to be stimulated by PTPA to display phosphotyrosyl phosphatase activity, because the rate of dephosphorylation of RCM-lysozyme after incubation by PTPA was approx. 1/300 that of the basal phosphorylase phosphatase activity (Table 1). In this respect PR61 behaved similarly to PR72 in the way that it almost completely blocked activation by PTPA. In contrast, $PP2A_{0/56}$ holoenzyme displayed some PTPA-stimulated phosphotyrosyl phosphatase activity that was approx. 1/30 of its basal phosphorylase phosphatase activity (Table 1). Therefore PR56 resembles PR55 in its ability to affect activation by PTPA. In summary, both holoenzymes displayed significant basal and protamine-stimulated phosphorylase phosphatase activity, but $PP2A_{0/61}$ did not exhibit PTPA-stimulated phosphotyrosyl phosphatase activity.

Molecular cloning of PR61 isoforms

We used purified MonoQ FPLC fractions (23–25) containing the PR61 subunit to obtain protein sequence data. Sequences of 15 tryptic peptides comprising 117 amino acids derived from the 61 kDa protein were obtained (see Table 2 for all peptides). These peptide sequences did not match any sequences present in the databases available at the start of the present study. For molecular cloning of the PR61 subunit we used an RT–PCR approach. This involved selection of mRNAs with biotin-conjugated antisense oligonucleotides corresponding to PR61-derived peptides (p61/62, p66 and p31) and another selection of cDNAs by RT with antisense oligonucleotide corresponding to PR61 peptide p34/35B. Several PCR products were isolated and sequenced. Clone pGEM-T/PCR603-5 comprising 355 bp encoded a 118-residue polypeptide that matched five PR61-derived peptides with high homology (p12A, p30, p31, p33A and p33B; see Table 2).

The PCR603-5 fragment was subsequently used for screening human cDNA libraries to obtain a full-length cDNA. Several cDNA clones were isolated from human brain and retina cDNA libraries, and 9 retinal and 16 brain clones were sequenced. The retinal clone HFRet.PR61-8 contained an open reading frame of 1401 bp encoding a protein of 467 residues (see Figure 3). At the protein level HFRet.PR61-8 was 98.3 % identical with PCR603-5. Two differences in the 118 amino acid sequence were identified: Glu^{416} and Gly^{457} in human protein were replaced in PCR603-5 by Gly and Ser respectively. These could either reflect the species difference or result from mutations introduced by PCR. Another full-length retinal clone termed HFRet.PR61-13 contained an open reading frame of 1347 bp encoding a protein of 449 amino acids with 78 % homology and 68 % amino acid identity with the predicted protein encoded by HFRet.PR61-8. This cDNA apparently encoded the second isoform of PR61 (see Figure 3).

The cDNA clone, BB.PR61-P8, isolated from the human fetal brain cDNA library contained an open reading frame spanning 1491 nucleotides encoding a protein of 497 amino acids, which constitutes a further isoform of PR61 (Figure 3). The deduced protein sequence of another brain clone, BB.PR61-P14, contained 16 amino acids at the N-terminus completely different from the 19 N-terminal amino acids of BB.PR61-P8, whereas the rest of the two cDNA sequences were identical. The most probable explanation of this difference is that both sequences arise from alternative splicing of the gene encoding this isoform. Alignment of amino acid sequences deduced from these cDNAs is shown in Figure 3.

During the preparation of this paper McCright and Virshup [33] reported the isolation of three human cDNA clones, termed B56α, B56β and B56γ1, encoding PR65-interacting proteins identified in a two-hybrid screen. Because two of these cDNAs apparently corresponded to PR61 isoforms we had identified, we decided to follow the same isoform nomenclature. Brain cDNA clones BB.PR61-P8 and BB.PR61-P14 both correspond to B56β but BB.PR61-P14 represents a novel splicing variant and therefore these two clones are termed PR61β1 and PR61β2 respectively. The full-length retinal clone HFRet.PR61-13, which corresponds to the partial B56γ1 cDNA, is designated PR61γ1. A search of the GenBank®/EMBL database revealed the existence of a novel cDNA isolated from a human myeloblast cell line KG-1 (accession number D26445), which apparently represents a splicing variant of the γ isoform different at the C-terminus from PR61γ1, and is therefore designated PR61γ2 (Figure 3). During the preparation of this paper we learned of several cDNAs isolated from rabbit skeletal muscle corresponding to the B′ regulatory subunit of $PP2A_0$ that apparently encode members of the same family of proteins as studied in this work [34]. Comparison of human and rabbit cDNA clones implies that the mammalian PR61γ gene gives rise to at least five splicing variants different at their C-termini. Figure 4 illustrates mammalian PR61 isoforms and splicing variants predicted from currently available cDNA and protein sequence data.

The fourth isoform of PR61, termed PR61δ, is also encoded by an alternatively spliced gene, because a human cDNA [35] and a corresponding rabbit cDNA [34] are different at the N-termini. These two splicing variants of PR61δ are designated PR61δ1 and PR61δ2 (Figure 4). From the analysis of all available sequence data we concluded that the human retinal cDNA termed HFRet.PR61-8 encodes a novel, fifth isoform of PR61, and we designated it PR61ϵ (Figure 3).

Database searches identified PR61 homologues from yeast and plants. The *S. cerevisiae RTS*1 gene encodes a protein that is approx. 70 % homologous with different mammalian PR61 isoforms and functions as a suppressor of *ROX3* gene product involved in transcriptional regulation of anaerobic stress [36]. An *Oryza sativa* (rice) partial cDNA clone (accession number D22057) encodes an open reading frame for 138 amino acids sharing 75 % amino acid similarity with mammalian PR61

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PR61α   .......MSS SSPPAGAASA AISASEKVDG FTRKSVRKAQ RQKRSQGSSQ FRSQGSQAEL HPLPQL..KD  61
PR61β1  METKLPPAST PTSPSSPGLS PVPPPDKVDG FSRRSLRRA. RPRRSHSSSQ FRYQSNQQEL TPLPLL..KD  67
PR61β2     MITVNPP LPQDTVNLFS PVPPPDKVDG FSRRSLRRA. RPRRSHSSSQ FRYQSNQQEL TPLPLL..KD  64
PR61γ1  .......... .......... .......... ........ML TCNKAGSRMV VDAANSNGPF QPVVLLHIRD  32
PR61γ2  .......... .......... .......... .........L TCNKAGSRMV VDAANSNGPF QPVVLLHIRD  31
PR61ε   .........M SSAPTTPPSV .....DKVDG FSRKSVRKA. RQKRSQSSSQ FRSQGKPIEL TPLPLL..KD  53

PR61α   ATSNEQQELF CQKLQQCCIL FDFM.DSVSD LKSKEIKRAT LNELVEYVST NRGVIVESAY SDIVKMISAN 130
PR61β1  VPASELHELL SRKLAQCGVM FDFL.DCVAD LKGKEVKRAA LNELVECVGS TRGVLIEPVY PDIIRMISVN 136
PR61β2  VPASELHELL SRKLAQCGVM FDFL.DCVAD LKGKEVKRAA LNELVECVGS TRGVLIEPVY PDIIRMISVN 133
PR61γ1  VPPADQEKLF IQKLRQCCVL FDFVSDPLSD LKWKEVKRAA LSEMVEYITH NRNVITEPIY PEVVHMFAVN 102
PR61γ2  VPPADQEKLF IQKLRQCCVL FDFVSDPLSD LKWKEVKRAA LSEMVEYITH NRNVITEPIY PEVVHMFAVN 101
PR61ε   VPSSEQPELF LKKLQQCCVI FDFM.DTLSD LKMKEYKRST LPELVDYITI SRGCLTEQTY PEVVRMVSCN 122

PR61α   IFRTLPPSDN P...DFDPEE DEPTLEASWP HIQLVYEFFL RFLESPDFQP SIAKRYIDQK FVQQLLELFD 197
PR61β1  IFRTLPPSEN P...EFDPEE DEPNLEPSWP HLQLVYEFFL RFLESPDFQP SVAKRYVDQK FVLMLLELFD 203
PR61β2  IFRTLPPSEN P...EFDPEE DEPNLEPSWP HLQLVYEFFL RFLESPDFQP SVAKRYVDQK FVLMLLELFD 200
PR61γ1  MFRTLPPSSN PTGAEFDPEE DEPTLEAAWP HLQLVYEFFL RFLESPDFQP NIAKKYIDQK FVLQLLELFD 172
PR61γ2  MFRTLPPSSN PTGAEFDPEE DEPTLEAAWP HLQLVYEFFL RFLESPDFQP NIAKKYIDQK FVLQLLELFD 171
PR61ε   IFRTLPPSDS N...EFDPEE DEPTLEASWP HLQLVYEFFI RFLESQEFQP SIAKKYIDQK FVLQLLELFD 189

PR61α   SEDPRERDFL KTVLHRIYGK FLGLRAFIRK QINNIFLRFI YETEHFNGVA ELLEILGSII NGFALPLKAE 267
PR61β1  SEDPREREYL KTILHRVYGK FLGLRAYIRK QCNHIFLRFI YEFEHFNGVA ELLEILGSII NGFALPLKTE 273
PR61β2  SEDPREREYL KTILHRVYGK FLGLRAYIRK QCNHIFLRFI YEFEHFNGVA ELLEILGSII NGFALPLKTE 270
PR61γ1  SEDPRERDFL KTTLHRIYGK FLGLRAYIRK QINNIFYRFI YETEHHNGIA ELLEILGSII NGFALPLKEE 242
PR61γ2  SEDPRERDFL KTTLHRIYGK FLGLRAYIRK QINNIFYRFI YETEHHNGIA ELLEILGSII NGFALPLKEE 241
PR61ε   SEDPRERDYL KTVLHRIYGK FLGLRAFIRK QINNIFLRFV YETEHFNGVA ELLEILGSII NGFALPLKAE 259

PR61α   HKQFLMKVLI PMHTAKGLAL FHAQLAYCVV QFLEKDTTLT EPVIRGLLKF WPKTCSQKEV MFLGEIEEIL 337
PR61β1  HKQFLVRVLI PLHSVKSLSV FHAQLAYCVV QFLEKDATLT EHVIRGLLKY WPKTCTQKEV MFLGEMEEIL 343
PR61β2  HKQFLVRVLI PLHSVKSLSV FHAQLAYCVV QFLEKDATLT EHVIRGLLKY WPKTCTQKEV MFLGEMEEIL 340
PR61γ1  HKIFLLKVLL PLHKVKSLSV YHPQLAYCVV QFLEKDSTLT EPVVMALLKY WPKTHSPKEV MFLNELEEIL 312
PR61γ2  HKIFLLKVLL PLHKVKSLSV YHPQLAYCVV QFLEKDSTLT EPVVMALLKY WPKTHSPKEV MFLNELEEIL 311
PR61ε   HKQFLVKVLI PLHTVRSLSL FHAQLAYCIV QFLEKDPSLT EPVIRGLMKF WPKTCSQKEV MFLGELEEIL 329

PR61α   DVIEPTQFKK IEEPLFKQIS KCVSSSHFQV AERALYFWNN EYILSLIEEN IDKILPIMFA SLYKISKEHW 407
PR61β1  DVIEPSQFVK IQEPLFKQVA RCVSSPHFQV AERALYFWNN EYILSLIEDN CHTVLPAVFG TLYQVSKEHW 413
PR61β2  DVIEPSQFVK IQEPLFKQVA RCVSSPHFQV AERALYFWNN EYILSLIEDN CHTVLPAVFG TLYQVSKEHW 410
PR61γ1  DVIEPSEFVK IMEPLFRQLA KCVSSPHFQV AERALYYWNN EYIMSLISDN AAKILPIMFP SLYRNSKTHW 382
PR61γ2  DVIEPSEFVK IMEPLFRQLA KCVSSPHFQV AERALYYWNN EYIMSLISDN AAKILPIMFP SLYRNSKTHW 381
PR61ε   DVIEPSQFVK IQEPLFKQIA KCVSSPHFQV AERALYYWNN EYIMSLIEEN SNVILPIMFS SLYRISKEHW 399

PR61α   NPTIVALVYN VLKTLMEMNG KLFDDLTSSY KAERQREKKK ELEREELWKK LEEL.KLKKA LEKQNSAYNM 476
PR61β1  NQTIVSLIYN VLKTFMEMNG KLFDELTASY KLEKQQEQQK AQERQELWQG LEEL.RLRRL QGTQGAKEAP 482
PR61β2  NQTIVSLIYN VLKTFMEMNG KLFDELTASY KLEKQQEQQK AQERQELWQG LEEL.RLRRL QGTQGAKEAP 479
PR61γ1  NKTIHGLIYN ALKLFMEMNQ KLFDDCTQQF KAEKLKEKLK MKEREEAWVK IENLAKANPQ VLKKRIT*   449
PR61γ2  NKTIHGLIYN ALKLFMEMNQ KLFDDCTQQF KAEKLKEKLK MKEREEAWVK IENLAKANPQ AQKDPKKDRP 451
PR61ε   NPAIVALVYN VLKAFMEMNS TMFDELTATY KSDRQREKKK EKEREELWKK LEDL.ELKRG LRRD..... 463

PR61α   HSILSNTSAE *                          486
PR61β1  LQRLTPQVAA SGGQS*                      497
PR61β2  LQRLTPQVAA SGGQS*                      494
PR61γ2  LARRKSELPQ DPHTKKALEA HCRADELASQ DGR* 484
PR61ε   .GIIPT*                                467
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Figure 3 Alignment of predicted amino acid sequences of human PR61 isoforms and splicing variants

Human PR61 isoforms correspond to the following cDNA clones: PR61α (B56α [33]), PR61β1 (BB.PR61-P8), PR61β2 (BB.PR61-P14), PR61γ1 (HFRet.PR61-13), PR61γ2 (D26445) and PR61ϵ (HFRet.PR61-8). Residues that are identical in all isoforms are shown in bold type. The putative nuclear localization signal found in the C-terminal domains of PR61α, PR61γ1/γ2 and ϵ is underlined.

isoforms, consistent with the generally high conservation of primary structure of constituent PP2A subunits among eukaryotes.

When the sequences of tryptic peptides obtained from PR61 rabbit protein were aligned with the predicted amino acid sequences of known human PR61 isoforms, it was apparent that the peptides were derived from two different PR61 isoforms, α and β. Predicted molecular masses and isoelectric points for PR61α/PR61β are very close and are 56.2/57.4 kDa and 6.68/6.7 respectively. These minor differences probably would not allow any detectable separation of PR61α- and PR61β-containing trimers. Therefore the holoenzyme designated as $PP2A_{0/61}$ was most probably a mixture of two trimeric holoenzymes containing different isoforms of PR61. The differences between the apparent molecular masses determined by migration on SDS/PAGE and the predicted molecular masses based on the cDNA sequence could be due to the covalent modification, possibly phosphorylation, of these proteins.

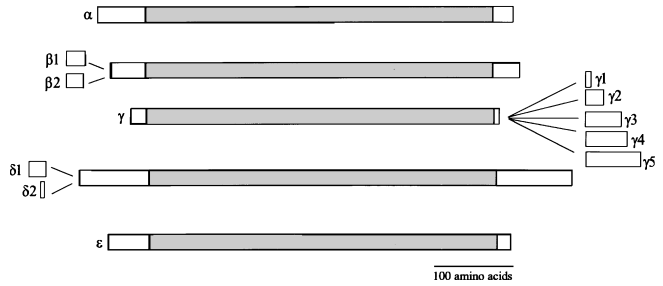

Figure 4 Schematic representation of mammalian PR61 isoforms and splicing variants predicted from currently available cDNA sequence data

Genes encoding β, γ and ϵ isoforms are alternatively spliced, giving rise to several splicing variants, where different N- and C-terminal exons join the common subset of core exons. Splice variants designated as γ3, γ4, γ5 and δ2 are predicted from rabbit cDNA sequences [34]. Central regions of the molecules that are homologous with each other are shaded.

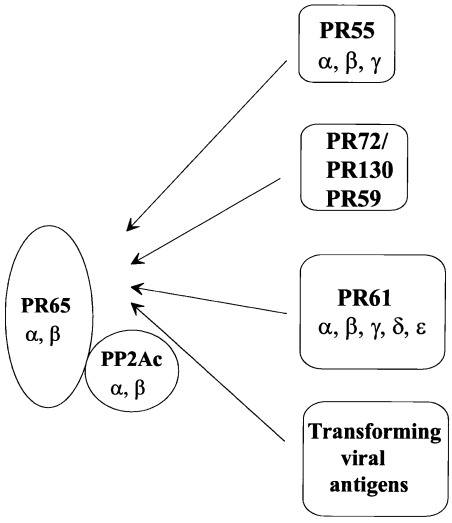

Figure 5 Three gene families comprising 10 mammalian genes encode variable regulatory subunits of PP2A

So far three gene families comprising at least 10 genes encoding variable regulatory subunits of PP2A have been identified in mammals: three genes for PR55$\alpha/\beta/\gamma$ isoforms, one gene producing two splicing variants termed PR72 and PR130 and at least one related gene encoding a novel regulatory subunit homologous with PR72/130 termed PR59, and five genes for PR61$\alpha/\beta/\gamma/\delta/\epsilon$ isoforms. The fourth class of proteins that can displace certain variable regulatory subunits from the PP2A holoenzymes consists of SV40 and polyomavirus transforming antigens.

The sequence of one peptide (p24: KLEDLEL) derived from the PR56 subunit was compared with all known PR61 isoforms and found to correspond to the sequence of the ϵ isoform (residues 439–445). Other isoforms either do not contain a trypsin cleavage site in this region or have an additional Lys corresponding to the second Glu in p24, which would produce a shorter peptide (KLEELK in PR61β). The calculated molecular mass of human PR61ϵ is 54.7 kDa, which is in good agreement with the apparent molecular mass of 56 kDa. The calculated isoelectric point for this isoform is 6.92 and we speculate that the PR61ϵ-containing trimer ($PP2A_{0/56}$) would be separated from the trimers containing the PR61α or PR61β isoforms (corresponding to $PP2A_{0/61}$). On the basis of these considerations, we conclude that the holoenzyme designated as $PP2A_{0/56}$ was a trimer consisting of PP2Ac, PR65 and PR61ϵ.

Compilation of all currently available human and rabbit cDNA and protein sequence data indicates that there are five isoforms of mammalian PR61 encoded by distinct genes, termed PR61$\alpha/\beta/\gamma/\delta/\epsilon$ ([33–35], and the present study). In addition, nine splicing variants of β, γ and δ genes, termed PR61β1/β2, PR61γ1–γ5 and PR61δ1/δ2, are predicted (Figure 4).

As shown in Figure 3, PR61 isoforms are highly homologous with each other. The core of the molecule seems to be highly conserved between different isoforms (approx. 80% identity, more than 90% similarity), whereas the N- and C-terminal regions are notably divergent. One can therefore speculate that the central part of each PR61 isoform is responsible for interaction with the core subunits of PP2A (PP2Ac and PR65), whereas the N- and C-terminal regions might perform specific functions linked to substrate specificity and targeting phosphatase to different subcellular compartments. Further sequence analysis revealed the presence of short clusters of basic amino acids in the C-terminal domain (underlined in Figure 3) that resembles a nuclear localization signal [37]. Another class of PP2A variable regulatory subunits, termed PR72/130, contains a similar basic cluster in the common C-terminal part that could function as a signal for nuclear import [5]. This result suggests that there are nuclear-specific holoenzyme forms of PP2A. The availability of PR61 isoform-specific antibodies should make it possible to determine the intracellular localization of these novel regulatory subunits and respective holoenzymes by immunodetection and confocal laser scanning microscopy.

In summary, the results obtained during the past several years indicate that the complexity of PP2A holoenzymes derived from the multitude of variable regulatory subunits is almost unprecedented. So far three gene families comprising at least 10 genes encoding variable regulatory subunits of PP2A have been identified in mammals: three genes for PR55$\alpha/\beta/\gamma$ [3,6], one gene producing two splicing variants termed PR72 and PR130 [5] and at least one related gene encoding a novel regulatory subunit homologous with PR72/PR130 termed PR59 (M. Voorhoeve and R. Bernards, personal communication), and five genes for PR61$\alpha/\beta/\gamma/\delta/\epsilon$ isoforms (Figure 5). If we also take into account the number of actual proteins that result from alternative splicing of some of these genes, the number of PP2A holoenzymes that could theoretically be assembled exceeds all previous expectations. In this respect PP2A could be similar to the cyclin-dependent protein kinases with regard to the numerous and dynamic complexes of catalytic and regulatory subunits [38].

PP2A is the most extensively characterized multisubunit protein phosphatase in terms of biochemistry and molecular biology. The major question that now emerges is how the distinct holoenzymes function within the cell. We are now in a position to delineate precisely the role of these subunits as key components in directing PP2A function. Several possibilities have been suggested [9]. The most probable roles for these proteins is in conferring substrate specificity or the subcellular localization of PP2A. Alternatively the more elegant prediction would be that a single variable subunit could perform both functions. This would provide a mechanism for bringing about specific dephosphorylation of target proteins in response to intracellular and extracellular signals.

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