Molecular cloning of a 74-kDa regulatory subunit (B″ or δ) of human protein phosphatase 2A

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Abstract Based on amino acid sequence data of a 74-kDa regulatory subunit (B″ or δ) of a human heterotrimeric protein phosphatase 2A, a cDNA encoding the subunit was isolated from a human cerebral cortex library. The cDNA had an open reading frame encoding an M_r 66 138 protein of 570 amino acids. Bacterial expression of the cDNA yielded a protein immunoreactive with antisera specific to the 74-kDa subunit. The predicted primary structure of the subunit had no similarity to already reported sequences of PP2A regulatory subunits including A, B, and PR72. Potential phosphorylation sites for protein kinases A and C, a bipartite motif of putative nuclear localization signal, an SH3 accessible proline-rich domain, and a unique PQ repeat were found in the sequence. The subunit mRNA of about 2.9 kb was ubiquitously expressed in rat tissues.

Key words: Protein phosphatase 2A; 74-kDa regulatory subunit; Human erythrocyte; Human cerebral cortex; Cloning

1. Introduction

Protein phosphatase 2A (PP2A), one of the four major serine/threonine protein phosphatases, distributes to all eukaryotic cells, and plays a crucial role in the regulation of many cellular events including metabolism, cell cycle, cell proliferation, DNA replication, transcription, translation, and viral transformation [1,2].

Holoenzymes of PP2A have either a heterodimeric or a heterotrimeric subunit structure. The heterodimeric structure is composed of a 32–41-kDa catalytic subunit (C or α) and a 60–69-kDa regulatory subunit (A or β). The dimeric structure AC ($\alpha_1\beta_1$) is common to all PP2A holoenzymes. The heterotrimeric structure contains an additional regulatory subunit of either 51–58 kDa (B or γ) [1,3], 54 kDa (B′) [4], 72 kDa (PR72) [5], or 74 kDa (B″ or δ) [6].

Molecular cloning of cDNAs for the subunits revealed that there are two different isoforms of C [7–9] and A [10,11], three isoforms of B [12–15] and two alternatively spliced forms (72 and 130 kDa) of PR72 [16]. Combinations of such variety of the regulatory subunits produce many different forms of PP2A holoenzymes. Since the regulatory subunits appear to be important components to control the activity and the localization of specific PP2A holoenzymes, the complexity of subunit structures may provide the molecular basis for their important roles in so many different cellular events.

Previously, we purified from human erythrocyte cytosol, three forms (phosphatases I, III, and IV) of PP2A whose subunit structures are $\alpha_1\beta_1\delta_1$, $\alpha_1\beta_1\gamma_1$, and $\alpha_1\beta_1$, where α (C) is a 34-kDa catalytic subunit, and β (A), γ (B), and δ (B″) are 63-, 53-, and 74-kDa regulatory subunits, respectively [6]. A comparison of the molecular activities of $\alpha_1\beta_1$ and $\alpha_1\beta_1\delta_1$ for various substrates suggests that δ strongly suppresses $\alpha_1\beta_1$ activities [6]. 74-kDa δ in $\alpha_1\beta_1\delta_1$ was phosphorylated by protein kinase A in vitro, resulting in a slight increase in phosphatase activities toward phosphorylated histones and phosphorylase [17]. Phosphatase I ($\alpha_1\beta_1\delta_1$) is most similar to $2A_0$ (ACB′) [4] relative to other forms of PP2A, in their elution positions from an anion exchange column, and in their responses to polycations and heparin [4,6,17,18]. In spite of the similarities, phosphatase I is different from $2A_0$ in the molecular masses of the third subunits, 74-kDa δ (B″) and 54-kDa B′.

To clarify the relationship of 74-kDa δ to other regulatory subunits of PP2A and further understand the function, the cDNA encoding δ was cloned.

2. Materials and methods

2.1. Purification and amino acid sequencing of 74-kDa δ

Protein phosphatase I ($\alpha_1\beta_1\delta_1$) was purified from human erythrocytes as described previously [6]. Protein phosphatase I (519 units, 450 μg protein, 14.4 ml) was applied to a heparin-Sepharose 6B column (3.8 × 1.0 cm, Pharmacia) equilibrated with Buffer A (10 mM imidazole, pH 6.5, 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol). The column was washed with 30 ml of the same buffer and δ was eluted at a flow rate of 8 ml/h with a 140 ml linear 0–0.6 M NaCl gradient in Buffer A. One ml fractions were collected in glass tubes. 74-kDa δ eluted at 0.4 M NaCl and was detected by SDS-PAGE (10% gel) followed by Coomassie blue staining. Since the dissociated δ has a propensity to bind to glass surfaces, the subunit was facilitated to bind to glass tubes by incubating for 2 h at 30°C. After removal of the buffer, δ bound to glass tubes was denatured in 360 μl of 7.3 M urea in 42 mM Tris-HCl, pH 9.0 for 30 min at 37°C. After diluting the urea concentration to 3.3 M by adding 50 mM Tris-HCl, pH 9.0, δ was digested for 6 h at 37°C with 3.5 μg of lysyl endopeptidase (Wako Pure Chemical Industries) in an 810 μl reaction mixture. After adding 40 μl of 10% trifluoroacetic acid, the reaction mixture was filtrated through a Millipore filter (0.45 μm) and was applied to a C-18 column (25 × 0.46 cm, TSK gel ODS-120T, Tosoh) equilibrated with 5% acetonitrile containing 0.1% trifluoroacetic acid. The column was developed with an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min. The gradient was 5% acetonitrile for 10 min,

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Abbreviations: PP2A, protein phosphatase 2A; SH3, Src homology 3; kb, kilobase; bp, base pair; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropylthio-β-D-galactoside; PVDF, polyvinylidene difluoride.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, and GenBank databases (accession No. D78360).

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5–12.5% acetonitrile over 10 min, 12.5–31.3% acetonitrile over 60 min, 31.3–72.5% acetonitrile over 5 min, and 72.5% acetonitrile for 5 min. The peptide peaks were monitored at 220 nm. Amino acid sequencing of peptides 1–5 (see text) was performed by Edman degradation on an Applied Biosystems model 470A sequencer equipped with a model 120PTH-amino acid analyser.

2.2. Amplification of cDNA fragments

Degenerate oligonucleotide primers of sense and antisense strands corresponding to the sequences of peptides 1, 2, 4, and 5 (see text) were synthesized. Single-stranded HeLa cell cDNA was subjected to PCR using Taq polymerase and Taq Extender PCR Additive (Stratagene) with 35 amplification cycles consisting of a 40-s denaturation at 94°C, a 30-s annealing at 45°C, and a 2-min extension at 72°C. PCR with primers in all possible combination of sense and antisense strands was carried out. Amplified fragments were analysed in ethidium bromide-stained 1.5% agarose gel and cloned into pUC19 plasmid for sequence analysis.

2.3. cDNA library screening

Initial screening of a human cerebral cortex cDNA library (λZAPII library primed with oligo(dT)) [19] was carried out using the PCR clone H3.1 as a probe. The probe labeled with [α-^{32}P]dCTP by the random priming method [20], was hybridized to the phage DNA immobilized on nitrocellulose membranes at 65°C in a solution containing 5 × SSPE (0.9 M NaCl, 50 mM sodium phosphate buffer, pH 7.7, 5 mM EDTA), 2 × Denhardt's reagent (0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin), 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA. The membranes were washed at 65°C in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) containing 0.2% SDS. Positive clones were selected and plaque-purified. Further screening of the human cerebral cortex cDNA library and a human bone marrow cDNA library (λgt11 library primed with oligo(dT) plus random hexamer, Clontech) was carried out using isolated cDNA fragments as probes.

2.4. Sequence analysis of cDNAs

Sequencing was carried out by the dideoxy chain-termination method [21], using AutoRead sequence kit (Pharmacia) and A.L.F. DNA Sequencer II (Pharmacia). The sequence was determined on both strands. Sequence comparison was carried out against the GenBank, EMBL, DDBJ, PIR, and SwissProt databases (releases 91, 43, 23, 46, and 31, respectively) using FASTA program [22] on molecular analysis systems in DDBJ.

2.5. Bacterial expression and Western blot analysis

74-kDa δ was expressed in bacteria using the pKK233-2 vector. PCR was performed on CCE6 using a specific primer (CCCTATAAACTGAAAAAG) starting from the second codon and a primer for pBluescript vector. The PCR product was cloned in frame into the pKK233-2 digested with *Nco*I and *Pst*I. The construct was verified by restriction enzyme analysis. *Escherichia coli* DH5α cells were transformed with this construct. Two independent clones were grown in 2 ml of Luria-Bertani medium supplemented with 100 μg/ml ampicillin at 37°C with or without induction for 1.5 h with 1 mM IPTG. Cells were collected by centrifugation, lysed in 100 μl of SDS-sample buffer (10 mM Tris-HCl, pH 7.8, 3% SDS, 5% glycerol, 2.5 mM dithiothreitol, 0.0167% Bromophenol blue), and heated in a boiling waterbath for 3 min. After centrifugation at 11 000 × g for 1 min, 20 μl of the supernatants with 2.5 μl of 0.63 M iodoacetoamide was applied to SDS-PAGE (10% gel). After electrophoresis, proteins in gels were electrophoretically transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, and 0.1% SDS at 2 mA/cm^2 for 1 h. The membrane was then blocked with PBST (100 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk for 1 h. The membrane was incubated for 1 h with mouse anti-δ antisera diluted 4000-fold in PBST, and incubated for 1 h with horseradish peroxidase-labeled goat anti-mouse IgG antibody (Kirkegaard and Perry) diluted 3000-fold in PBST. The anti-δ antisera were raised against the subunit purified from human erythrocytes. Details of preparation of the antisera will be described elsewhere. Immunoreactive bands were detected with an enhanced chemiluminescence system (Amersham). Protein bands on PVDF membranes were detected by colloidal gold system (Bio-Rad).

2.6. RNA isolation and Northern blot analysis

Total RNA was extracted from organs of male Wistar albino rats (200–250 g) fasted overnight by the guanidinium thiocyanate/cesium chloride method [23]. Poly(A)$^+$ RNA was prepared by affinity chromatography with oligo(dT)-Latex (Takara Shuzo). For Northern blot analysis, 2 or 8 μg of poly(A)$^+$ RNA was separated by electrophoresis on a 1% formaldehyde-agarose gel and transferred to a nylon membrane. After baking the membrane at 80°C for 1 h, prehybridization was carried out at 65°C in a solution containing 5 × SSPE, 5 × Denhardt's reagent, 0.2% SDS, and 200 μg/ml of denatured salmon sperm DNA. The CCJ3 probe (3 × 10^9 cpm/μg) labeled with ^{32}P by the random priming method [20] was then hybridized at 65°C to the membrane in the same solution. After hybridization, the membrane was washed in 0.1 × SSC containing 0.1% SDS at 50°C and then exposed to an X-ray film at −80°C.

3. Results and discussion

Partial amino acid sequencing of 74-kDa δ (B″) was performed in order to design degenerate oligonucleotide primers for PCR. After digesting δ with lysyl endopeptidase, the resulting peptides were resolved by reversed-phase HPLC. Five peptides were analysed and yielded the following sequences: ALEAhK (peptide 1), ErEEMxQK (peptide 2), SELPQxVY (peptide 3), xTVETEAVQML (peptide 4), and rAEEFLTASQEA (peptide 5). Lower case letters represent residues sequenced with lower confidence, and x denotes residues that could not be positively identified.

HeLa cell cDNA was subjected to PCR with degenerate primers that corresponded to the sequences of peptides 1, 2, 4, and 5. One PCR clone H3.1 amplified with the sense primer corresponding to peptide 4 and the antisense primer corresponding to peptide 5, contained sequences corresponding to peptides, 1, 3, 4, and 5.

After screening 3 × 10^5 plaques of a human cerebral cortex cDNA library with the PCR clone H3.1 as a probe, one positive clone, CCJ3 (Fig. 1) was obtained. Then, a 5′-end fragment (CCJ3 probe, nucleotides 726–1656) of the insert was obtained by PCR. By screening with CCJ3 probe, four clones, of which a clone CCE6 was the longest, were obtained from the human cerebral cortex cDNA library, and a clone BM4.1 was obtained from 6 × 10^5 plaques of a human bone marrow cDNA library. The amino acid sequence predicted from the complete nucleotide sequence of CCE6 contained the sequences of peptides 1–5 (Fig. 2). Using the 5′-end fragment of CCE6 (nucleotides 26–731, *Eco*RI/*Bam*HI fragment) as a probe, 24 clones were isolated by screening 1 × 10^6 plaques of the human cerebral cortex library. Of these clones, a clone CC1.1.3, one of the longest at the 5′-end, was analysed. The 5′-end 300-bp sequence was found to be 25-nucleotides longer than CCE6.

The 2916-bp sequence of 74-kDa δ cDNA was determind from CCE6 and CC1.1.3 (Fig. 2). An ATG codon, which matched the Kozak consensus [24], was present at nucleotide 78, followed by a 1710-bp open reading frame encoding 570 amino acids. The molecular weight of the encoded protein was calculated to be 66 138. The 3′-untranslated region contained a poly(A) tail and two putative polyadenylation signals (AATAAA) [25] at nucleotides 2446–2451 and 2825–2830.

The proposed open reading frame was cloned into the bacterial expression vector pKK233-2 and expressed in *E. coli* DH5α cells. Bacterial lysates were analysed by Western blotting with antisera specific to δ purified from human erythrocytes. The protein recognized by the antisera was only synthesized in

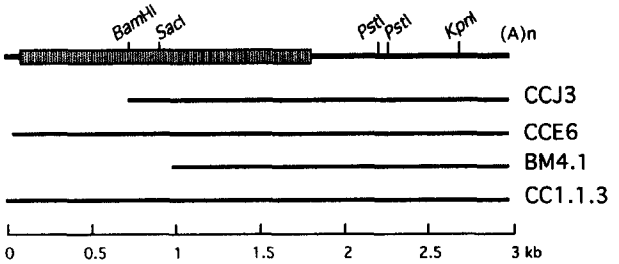

Fig. 1. Overlapping cDNA clones of human 74-kDa δ. An alignment of representative cDNA clones isolated from human cerebral cortex and bone marrow cDNA libraries are shown. In a schematic structure of δ subunit cDNA at the top, the open reading frame is shown as a box, and a poly(A) tail is shown as (A)n.

E. coli cells transformed with plamids containing the open reading frame (Fig. 3). The electrophoretic mobility of the immunoreactive protein was close to that of the human erythrocyte 74-kDa δ, indicating that the analysed cDNA clone encoded the full- length, or at least most of 74-kDa δ. The slightly faster mobility of the translation product in SDS-PAGE might be due to the lack of post-translational modification, the inappropriate folding of the product in bacteria, or the partial lack of the coding sequence in the cDNA analysed.

By Northern blotting with the CCJ3 probe, the levels of transcripts encoding 74-kDa δ were analysed in poly(A)$^+$ RNA extracted from adult rat tissues. A transcript of about 2.9 kb was detected in all tissues studied, including brain, heart, lung, liver, spleen, kidney, intestine, testis, and skeletal muscle (Fig. 4). Expression of δ subunit mRNA in hematopoietic cells is also suggested by the isolation of a clone BM 4.1 from the human bone marrow cDNA library.

Comparison of the predicted protein sequence with those of the other subunits of PP2A including C, A, B, PR72, and PR130 [7–16] and the viral proteins that bind PP2A [26] did not show any significant similarity. This suggests a unique role of δ (B″) in the control of the function of the PP2A. Screening of databases with the cDNA sequence identified an *RTS1* gene from *Saccharomyces cerevisiae* (GenBank accession No. U06630), a human cDNA HUMORFY (GenBank accession No. D26445) [27] and several cDNA fragments from human, mouse, and rice. The *RTS1* gene, a high copy suppressor of *rox3* [28], shows 58.7% identity with the δ subunit cDNA in an overlap of 1009 bp. Its predicted translation product shows 59.1% identity with δ in an overlap of 381 amino acids. Although the function of the RTS1 protein is not clear, it seems to be the yeast homolgue of δ (B″) of PP2A. The strong conservation of primary structure between these proteins during evolution suggests a role of δ in basic cellular functions shared by yeast and mammalian cells. The 3702-bp HUMORFY, which is one of the cDNA clones randomly sampled from human immature myeloid cell line KG1, shows 73.0% identity with the δ subunit cDNA in an overlap of 1301 bp. Its predicted translation product shows 85.0% identity with δ in an overlap of 428 amino acids. Identification of several human and mouse cDNA

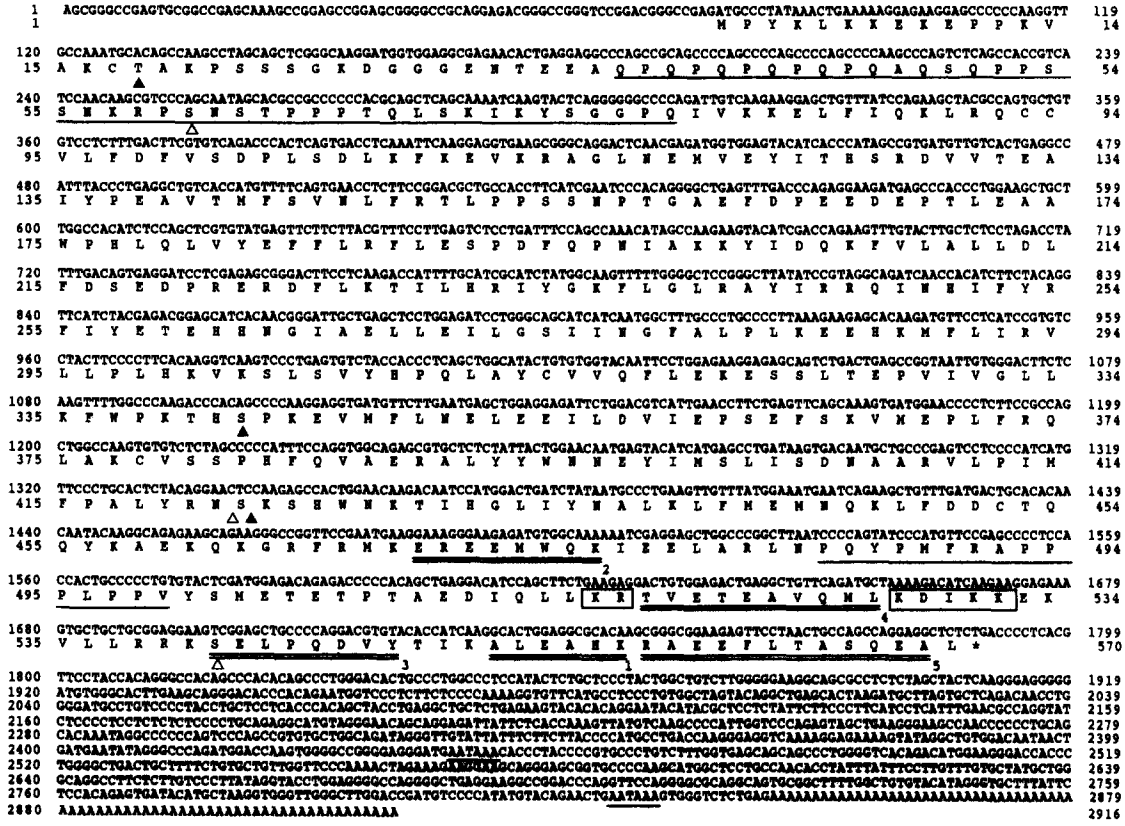

Fig. 2. Nucleotide and predicted amino acid sequences of the human cDNA encoding 74-kDa δ. The sequences of lysyl endopeptidase peptides (peptides 1–5) of δ are indicated by double underlines with the peptide number. Proline-rich domains and putative polyadenylation signals are indicated by underlines, potential phosphorylation sites for protein kinase A by △, for protein kinase C by ▲. A bipartite motif of putative nuclear localization signal is indicated with boxes. The asterisk represents the stop codon.

sequences in databases with strong similarity to the δ subunit cDNA, raises the possibility of the presence of several isoforms of δ in mammalian tissues. In fact the predicted primary structure of human B56 [29] identified recently as a protein that interacts with the A subunit of PP2A has similarity to that of δ presented in this paper. More recently, the predicted primary structure of rabbit B′ [30] was found to be also similar to that of δ. These findings indicate that B56, B′, and δ (B″) belong to a growing new family of the regulatory subunit of PP2A. The structural relationship between δ and B′ may explain the similarities of phosphatase I ($\alpha_1\beta_1\delta_1$) and $2A_0$ (ACB′) in chromatographical and enzymatical properties [4,6,17,18].

In the predicted amino acid sequence, a number of consensus phosphorylation sites [31] for protein kinases A, G, and C, calcium/calmodulin-dependent kinase II, and casein kinases I and II were found. Potential phosphorylation sites for protein kinases A and C that match with the consensus sequences of R_{1-2}-X_{1-2}-S/T, and (R/K_{1-3}, X_{2-0})-S/T-(X_{2-0}, R/K_{1-3}) [31], respectively, are indicated in Fig. 2. The δ subunit in the trimeric holoenzyme can actually be phosphorylated in vitro by protein kinase A [17]. The significance of potential phosphorylation sites for other protein kinases remains to be clarified. The regions near both termini are very rich in basic amino acids and this may explain the high affinity of δ to heparin-Sepharose. A cluster of basic amino acids that conforms well with the bipartite motif of putative nuclear localization signal [32] was found near the C-terminus (Fig. 2). Recently, the AC core of PP2A was reported to be two-fold more concentrated in the nucleus than in the cytoplasm in nontransformed fibroblasts [33]. These findings suggest the possibility that δ may serve as a nuclear

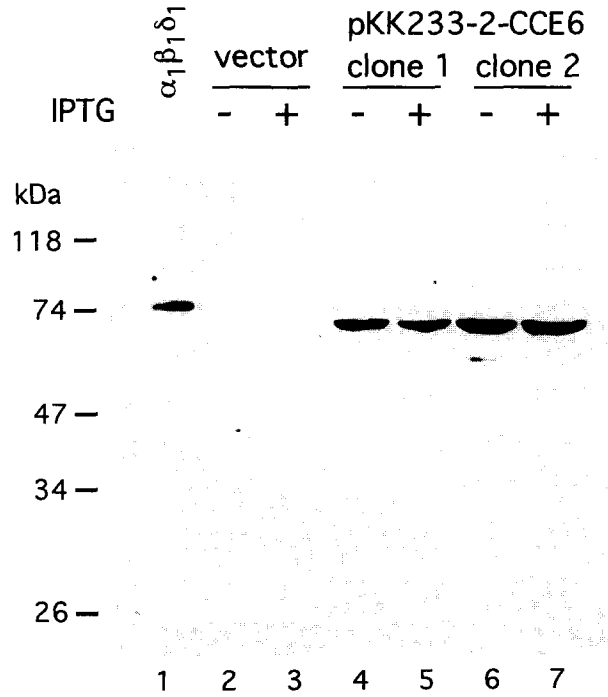

Fig. 3. Bacterial expression of δ subunit cDNA clone. Western blots are shown of uninduced (lanes 2, 4, and 6) and induced (lanes 3, 5, and 7) bacterial extracts transformed by the pKK233-2 plasmid without insert (lanes 2 and 3) and the vector containing the open reading frame (lanes 4–7). Lane 1 contains 90 ng of a native heterotrimeric PP2A ($\alpha_1\beta_1\delta_1$) isolated from human erythrocytes. Loading of equivalent amount of bacterial protein was checked by Coomassie blue staining.

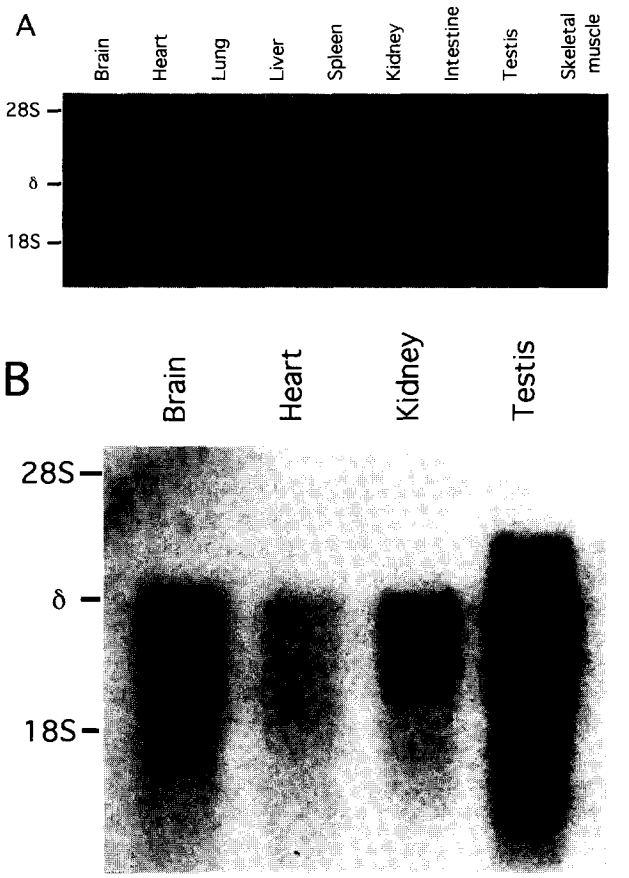

Fig. 4. Analysis of δ subunit mRNA expression in rat tissues. Poly(A)$^+$ RNA (2 μg in panel A, 8 μg in panel B) from the indicated rat tissues was subjected to Northern blot analysis using CCJ3 probe (see text). Exposure times were 9 days (A) and 5 days (B). Positions of 28S, 18S ribosomal RNA, and δ subunit mRNA are indicated.

targetting subunit of PP2A. Two clusters of proline residues were found near both termini (Fig. 2). The N-terminal proline-rich domain contained an unusual PQ repeat, and the C-terminal one contained an RAPPPLPP motif that matched with the class I consensus sequence for binding to an SH3 domain [34]. Proline-rich regions are also found in other regulatory subunits of PP2A, human PR72 and PR130 [16], and one isoform of Drosophila PR55 [35]. It will be interesting to analyse whether the proline-rich domains of δ are involved in their association with the $\alpha_1\beta_1$ (AC) core of PP2A or other signalling molecules.

Phosphatase I ($\alpha_1\beta_1\delta_1$) showed lower molecular activities than phosphatase IV ($\alpha_1\beta_1$) and phosphatase III ($\alpha_1\beta_1\gamma_1$) toward various substrates in vitro [6], suggesting that δ may function as an inhibitory subunit in vivo. Phosphorylation of δ by protein kinase A [17] also suggests the possible role in modulation of the activity and/or the localization of PP2A in response to signals mediated by the protein kinase.

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