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Single strand mRNA differential display (SSDD) applied to the identification of serine/threonine phosphatases regulated during cerebellar development

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Abstract

Differential display is a used widely and useful technique for the study of differentially expressed genes. However, very poor results have been obtained in the past when particular gene families were studied. Initially, we attempted to study the mRNA expression of catalytic subunits of serine/threonine phosphatases, using two primers specific to consensus sequences of these phosphatases. When differential display was applied, two wide, unresolved bands were isolated that contained cDNA of several phosphatases, together with that of many other unrelated transcripts. To overcome this problem, we used an alternative strategy, referred to as single strand differential display (SSDD), which is a combination of differential display and single strand conformation polymorphism (SSCP). After initial PCR amplification with specific primers, we ran a polyacrylamide (or agarose) gel, pre-selecting the region that contained fragments of the size expected for the consensus region (250–350 bp). The DNA eluted from this zone was then separated on a non-denaturing (SSCP) gel. Using this approach, we were able to characterize the expression of five ser/thr phosphatases, and a previously unreported splice variant of one of them, PP1γ. All these phosphatases show varying levels of expression during development, indicating a very complex regulation of protein phosphorylation–dephosphorylation during the period of synaptogenesis in the mouse cerebellum.

Keywords: Single strand differential display; Differential display; Single strand conformation polymorphism; Serine threonine phosphatases; Development; Cerebellum

1. Introduction

The phosphorylation status of different proteins depends on a finely tuned balance between the activities of protein kinases and phosphatases. In the brain, protein phosphorylation has been implicated in several functions related to synaptic transmission, plasticity, and neuronal development (for review see, (Black, 1999; Tapia et al., 1999; Jurata et al., 2000; Sanchez et al., 2000)). Only in the past few years has the importance of the regulation of phosphatases become evident (Sim, 1991; Stemmer and Klee, 1991; Nairn and Shenolikar, 1992; Sim et al., 1998; Price and Mumby, 1999).

An mRNA differential display method (Liang and Pardee, 1992) has been used to explore the differential expression of serine/threonine phosphatases during cerebellar development. This is a powerful technique for identifying differentially expressed genes, and it is particularly useful for developmental studies. However, it has been difficult to apply differential display to the study of specific families of proteins. In only a few cases have specific primers been employed for this purpose (Hsu et al., 1993; Johnson et al., 1996; Takano et al., 1997; Liu et al., 1999), and in almost all cases, only a specific 5′ primer was used, resulting in the isolation of many unrelated transcripts. To improve the specificity of this method, we have used specific primers corre-

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sponding to two consensus sequences of the catalytic subunits of the ser/thr phosphatases. The initial differential display showed two wide, unresolved bands in which the cDNAs corresponding to these phosphatases were present, together with some other bands representing unrelated sequences. Because the consensus priming sites produce amplification products of similar sizes, the resolution of the differential display was insufficient to identify the changes corresponding to each family member.

To overcome this problem, we used a combination of differential display and single strand conformation polymorphism (SSCP), a widely used technique for the detection of genes that differ in only one nucleotide (Orita et al., 1989a,b), which is also used to eliminate false positive signals in differential display (Mathieu-Daude et al., 1996; Zhao et al., 1996; Miele et al., 1998; Roschier et al., 2000). Using this combination of methods, referred to as single strand differential display (SSDD), we successfully detected 12 bands corresponding to six ser/thr phosphatases, including a previously unreported splice variant of the catalytic subunit of PP1γ, thereby demonstrating the effectiveness of the method. In addition, we showed that the expression of all the catalytic subunits of the ser/thr phosphatases is regulated during development. This implies a very complex regulation of protein phosphorylation–dephosphorylation during morphogenesis and synaptogenesis in the mouse cerebellum.

2. Experimental procedures

2.1. Animals

C57BL/6J mice were bred and maintained in the animal facility at the Department of Experimental Genetics of the National Center of Medical Genetics, Malbrán Institute.

2.2. Single strand differential display (SSDD)

Total RNA was extracted from mouse cerebellum using Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA), and resuspended in 20 μl of DEPC-treated H_2O. To remove genomic DNA contamination, 50 μg of RNA was treated with 1 μl of 10 U/μl of RNAse-free DNase I (Promega Corp., Madison, WI, USA) for 30 min at 37°C in 5 μl of 0.1 M Tris pH 8.3, 5 μl of 15 mM $MnCl_2$, 5 μl of 0.5 M KCl, and 0.25 μl of 40 U/μl rRNasin ribonuclease inhibitor (Promega Corp., Madison, WI, USA) (final volume 50 μl). The RNA was purified by phenol-chloroform (3:1) extraction (50 μl), and precipitated from the aqueous phase by adding 5 μl of 3 M sodium acetate and 200 μl of 100% ethanol. The RNA pellet was resuspended in 20 μl of DEPC-treated H_2O. For reverse transcription reaction, 4 μg of DNase-treated RNA was mixed with 1 μl of 10 μM oligo(dT) primer and DEPC-treated H_2O (final volume 15 μl), heated at 70°C for 5 min, chilled on ice and spun. Five microliter 5X first strand buffer, 0.7 μl of 40 U/μl rRNasin ribonuclease inhibitor (Promega Corp., Madison, WI, USA) and 1.3 μl of 40 mM dNTPs were added. One microliter of 200 U/μl MMLV reverse transcriptase (Promega Corp., Madison, WI, USA) was added after 2 min of mix pre-incubation at 42°C. Reactions were incubated at 42°C for 1 h and heated to 95°C for 10 min to inactivate reverse transcriptase. PCR amplification (94°C for 5 min; 40 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 30 s; and 72°C for 5 min) was performed using two primers designed to amplify a consensus sequence specific to the ser/thr phosphatases: ST3′ (5′-GGRTCWGACCAYARCAR-RTCRCA-3′), an antisense primer, and ST5′ (5′-GGN-GAYTATGTDGACAGRGG-3′), a sense primer, in a Gene Thermal Cycler (BIO-RAD, Hercules, CA, USA). These primers recognize CDLLWSDP and GDYV-DRG amino acid regions corresponding to two regions of high homology between the catalytic subunits of mouse ser/thr phosphatases sequences according to BLOCKS program (available at http://www.blocks.fhcrc.org), blocks PR00114E and PR00114B, respectively. Degenerate nucleotides were represented with the code, R, G/A; W, A/T; Y, T/C; N, A/C/G/T; D, G/A/T. The 20 μl reaction mixture included, 2 μl of cDNA, 1 μl of each 10 mM ser/thr phosphatase primers (ST3′ and ST5′ primers), 2 μl of 10X PCR buffer, 0.6 μl of 50 mM $MgCl_2$, 1 μl of 1 mM dNTPs, 1 μCi of [α^{32}P]dCTP 3000 Ci/mmol (NEN Life Science Products, Boston, MA, USA) as a radioactive precursor, and 0.1 μl of Taq DNA Polymerase (5 U/μl) (GIBCO BRL, Gaithersburg, MD, USA). The PCR product was separated electrophoretically on 4% polyacrylamide or 1.8% agarose. The strong band corresponding to the consensus sequence products (the region at 250–350 bp on the agarose gel) was excised, and the DNA eluted using the 'slurry' method (Chuang and Blattner, 1994). The cDNA (250 ng) was heated to 100°C for 3 min with sample buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), and cooled immediately to 0°C. Samples were loaded onto a 12.5% polyacrylamide sequencing gel (1 × TBE, 10% glycerol), and run at 25 W for 18 h at room temperature. The gel was dried, and then exposed to Kodak X-OMAT film (Kodak, Rochester, NY, USA).

To recover the fragments of interest from the SSDD, the bands seen on the autoradiograph were localized and cut from the polyacrylamide gel, hydrated with 50 μl of sterile water for 15 min, and boiled at 100°C for 10 min. An aliquot of the supernatant was reamplified using the same primers and conditions employed in the

SSDD, and the products separated on an agarose gel. Bands were excised, the cDNA eluted and ligated into the pGEM-T Easy Vector System I (Promega Corp., Madison, WI, USA), and constructs cloned using competent Nova Blue bacteria (Novagen, Madison, WI, USA). Colonies were selected, and the plasmids isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega Corp., Madison, WI, USA). DNA was sequenced with an ALFexpress automatic sequencer (Pharmacia Biotech, Uppsala, Sweden).

To verify that each family member contributes two bands (sense and antisense strands), as seen on the SSDD polyacrylamide gel, we repeated the procedure using minigels under the conditions described above, to separate the inserts corresponding to each cloned fragment. To obtain the inserts, the plasmids were digested with *Eco*RI (Promega Corp., Madison, WI, USA) and separated on an agarose gel. The bands were excised from the agarose gel, eluted using the slurry procedure, and subjected to SSDD. The SSDD minigel was run at 200 V for 8 h and stained with silver nitrate, as described previously (Bassam et al., 1991).

2.3. Northern blots

Equal amounts of total RNA (20 μg) were run on 1% agarose gels (2.2 M formaldehyde) (Sambrook et al., 1989), and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH, USA). After transfer, RNA was stained using a solution of 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2), and scanned to quantitate sample loading. The inserts used as probes were labeled with the Prime-a-Gene Labeling System (Promega Corp., Madison, WI, USA) and [α^{32}P]-dCTP, 3000 Ci/mmol (New England Nuclear, Boston, MA, USA). The membranes were blocked with prehybridization buffer at 65°C, incubated overnight at the same temperature using the appropriate probe and washed at 65°C (Church and Gilbert, 1984), and exposed to autoradiography film for various times, at −70°C with intensifying screens.

2.4. Semiquantitative PCR

Complementary DNAs from mice of different ages were used for PCR amplification (94°C for 5 min; 30 cycles of 94°C for 30 s, 48°C for 20 s, 72°C for 30 s; and 72°C for 5 min), with specific primers for both PP1γ and the splice variant of PP1γ (gamma1, 5′-CTGGAGACAATCTGCCTCTTGCTGG-3′ and gamma2, 5′-TGGTACATCAGTTGGTCTCATAAT-3′), which amplified fragments of 288 and 183 bp, respectively. For the specific amplification of the PP1γ splice variant, gamma1 and the splice variant primer (5′-GATAAACACTCATCATAAAATCCGTA-3′) were used to amplify a fragment of 125 bp. Finally, specific primers for glyceraldehyde phosphate dehydrogenase (GAPDH) (5′-CTCACGGCAAATTCAACGG-3′ and 5′-CTTTCCAGAGGGGCCATCCA-3′) were used as an internal control (428 bp).

2.5. Western blots

Total proteins were extracted from cerebellar tissue by homogenization in three volumes (3:1 v/w) of cold phosphate buffer saline (pH 7.4) containing Protease Inhibitor Cocktail Set I (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined according to Bradford (1976), and 100 μg analyzed by SDS-PAGE according to Laemmli (1970), using a 10% separating minigel with a 4% stacking gel. Immunoblotting was performed according to Towbin et al. (1979). Primary antibodies against the catalytic subunits of PP1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PP2A (Calbiochem, La Jolla, CA, USA), and the alpha isoform of PP2B (Calbiochem, La Jolla, CA, USA) were used at dilutions of 1:200, 1:500 and 1:500, respectively. Immunoreactivity was visualized using alkaline phosphatase-conjugated anti-mouse and anti-rabbit secondary antibodies (Sigma, St. Louis, MO, USA), at dilutions of 1:30 000. Immunoreactive species were visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) reagents (Promega, Madison, WI, USA).

2.6. Quantification

Experiments corresponding to Figs. 3 and 4 were repeated three times ($n = 3$), while corresponding to Fig. 5 were made four times ($n = 4$). All images, representative of each experiment, were scanned on a Hewlett–Packard ScanJet 4c, and quantified using the PC-compatible NIH Image program (http://www.scioncorp.com). Sample loading on northern blots was quantified by methylene blue staining.

3. Results

3.1. Strategy for single strand differential display (SSDD)

Two consensus sequences for the catalytic domains of the ser/thr phosphatases were identified, using the BLOCKS program (available at http://www.blocks.fhcrc.org/). Two primers recognizing these regions were designed (primers ST5′ and ST3′), and used for the amplification of cDNAs corresponding to the transcripts from cerebellum at different postnatal ages.

Initially, we used differential display in an attempt to characterize the mRNA expression of the different ser/thr phosphatases, but too many bands unrelated to the phosphatases were isolated. However, two wide, high-intensity bands were observed at all developmental stages. When we cloned and sequenced fragments included in these bands, several ser/thr phosphatases were identified. Resolution was poor because the fragments corresponding to the different phosphatases genes amplified with these primers are of a similar size (about 350 bp). Therefore, to detect changes in expression during development, we used a combination of differential display and single strand conformation polymorphism (SSCP), referred as to single strand differential display (SSDD). In the SSCP method, DNA is separated electrophoretically as single strands on a non-denaturing polyacrylamide gel. Small variations in sequence produce differences in the intramolecular interactions, which generate distinct conformations that affect the mobility of the strands when they are run on non-denaturing polyacrylamide gel.

Before SSDD, the PCR-amplified products were separated on an agarose gel, and the region corresponding to 250–350 bp was excised and purified. This was done to eliminate several fragments that do not correspond to the consensus sequence (are different sizes), and do not contain phosphatases. Similar results were obtained using 4% polyacrylamide instead of agarose (results not shown). The cDNA was eluted, and equal amounts were run as single stranded DNA on a non-denaturing polyacrylamide gel (Fig. 1).

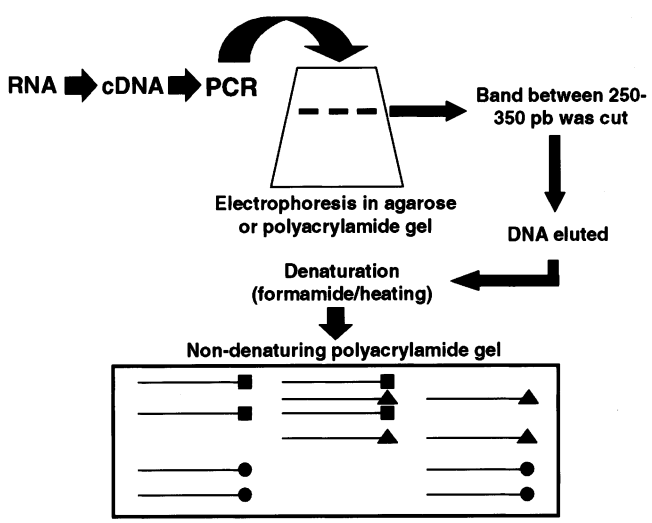

Fig. 1. Strategy for single strand differential display (SSDD). Schematic representation of the different steps used to study the catalytic subunits of the ser/thr phosphatases (see experimental procedures for details).

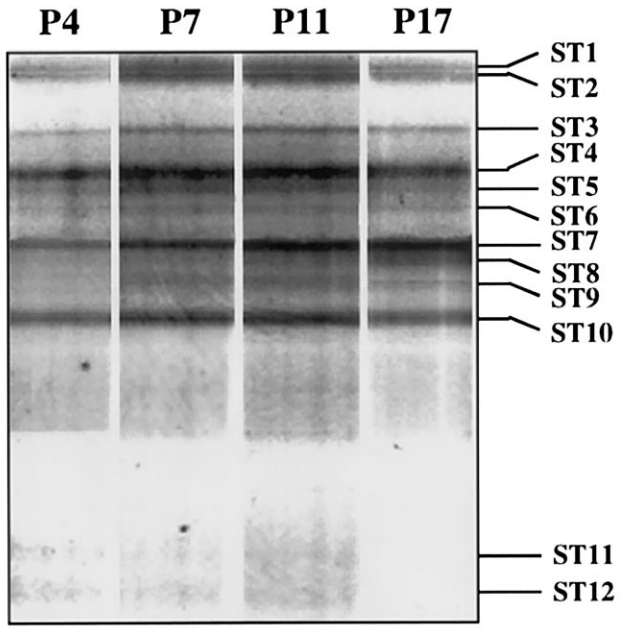

Fig. 2. SSDD of catalytic subunits of the ser/thr phosphatases. Autoradiography showing twelve [^{32}P]-labeled bands (*ST1* to *ST12*) isolated after running mice cerebellar samples of different ages (P4 to P17) on a 12.5% non-denaturing gel at 25 W for 18 h at room temperature.

3.2. SSDD of ser/thr phosphatases during synaptogenesis in mouse cerebellum

We used SSDD to analyze the expression of the catalytic subunits of ser/thr phosphatases during the period of synaptogenesis, in the mouse cerebellum. The cerebellum as a model system offers several advantages, as the different developmental stages can be separated in defined time frames during postnatal growth. For this study, we chose four postnatal ages that represent three different stages of development. Between postnatal days four and seven (P4 and P7), the granule cells proliferate. When proliferation ends, these cells begin a migration process, during which the axons are extended. At P11, the proliferation and migration of granule cells decrease dramatically, and synaptic contacts with the dendritic spines of the Purkinje cells are initiated. At P17, almost all synapses have been formed (Landis and Sidman, 1978).

Using SSDD at these ages, we detected 12 bands (designated *ST1* to *ST12*) (Fig. 2). These bands were eluted, reamplified, cloned and sequenced. These 12 bands corresponded to six catalytic subunits of ser/thr phosphatases, PP1α (clones *ST1* and *ST2*), PP1β (*ST9* and *ST10*), PP2Aα (*ST4* and *ST6*), PP2Aβ (*ST3* and *ST5*), PP2Bα (*ST7* and *ST8*), and a new splice variant of PP1γ (*ST11* and *ST12*). To determine whether each of these ser/thr phosphatases can produce two bands, and whether these bands reproduce the pattern ob-

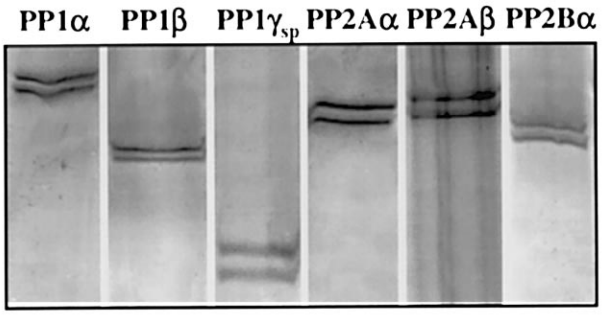

Fig. 3. Pattern of inserts corresponding to ser/thr phosphatases. The cDNA of six types of phosphatases, identified using SSDD, were cloned. Inserts were isolated by *Eco*RI digestion, separated on a 12.5% non-denaturing minigel, and stained with silver nitrate.

served initially on the SSDD gel, each T-vector clone was digested with *Eco*RI, and the products run on an agarose gel. The DNA fragments were eluted and separated on a non-denaturing minigel. After silver staining, it was observed that all the clones produced two bands, with the same relative mobility as observed previously (Fig. 3).

3.3. Differential expression of ser/thr phosphatases analyzed by northern blots

Similar to differential display, which is not a quantitative method, northern blots are always required to verify the results obtained by SSDD. Therefore, we analyzed the expression of four of these ser/thr phosphatase catalytic subunits using northern blots. The expression of PP1α mRNA peaks at P11, with a decrease at P17 (Fig. 4A). PP1β and PP2Aβ have a similar pattern of expression (Fig. 4B and C), in which a maximum occurs at P7, after which the levels become similar to those observed at P4. By contrast, the mRNA levels of PP2Bα are very low at P4 and increase with time, showing maximum expression at P11 and maintaining this level to P17 (Fig. 4D). In all cases, the pattern of expression was similar to that obtained using SSDD. However, it should be noted here the possibility that the same probe might recognize two or more comigrating species of phosphatase mRNAs. These results confirm that the expression pattern delineated using SSDD is reliable. In addition, differentially expressed mRNAs can be detected, isolated, and sequenced as in differential display. This method has the additional advantage of being able to distinguish fragments of similar, or even identical, sizes.

3.4. Detection of a new splice variant of the catalytic subunit of PP1γ

When clones *ST11* and *ST12* were sequenced, they showed homology with the catalytic subunit of PP1γ. However, when the sequences were examined, 105 nucleotides corresponding to exon 4 of PP1γ were absent in both clones (Fig. 5A). The expression of this new splice variant was not analyzed by northern blot, because this method cannot resolve both isoforms. To overcome this problem, we designed primers for semi-quantitative PCR, with which both isoforms can be resolved. Gamma1 and gamma2 primers amplify both forms of PP1γ, whereas gamma1 together with the splice variant primer only amplify the PP1γ isoform that does not contain exon 4. As an internal control, we used GAPDH primers. Fig. 5B shows that PP1γ containing exon 4 was most abundant at all ages studied, and that there was no difference in the patterns of expression of both isoforms during development. Densitometric analysis showed high levels of transcript

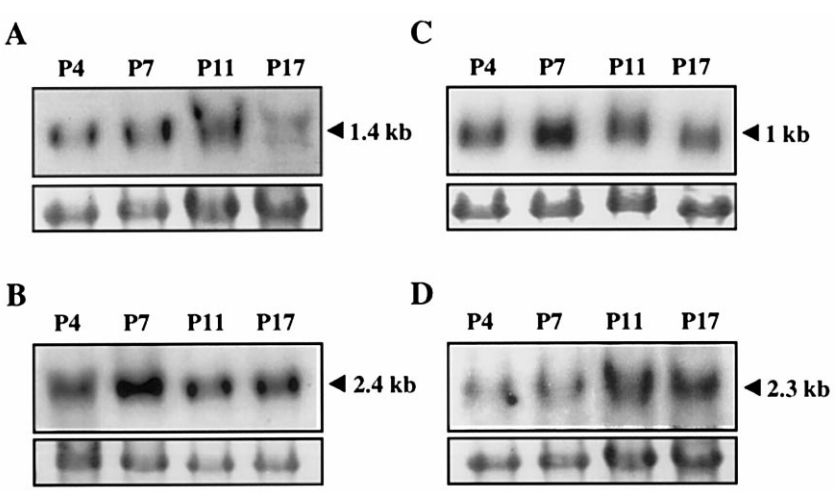

Fig. 4. Northern blots of ser/thr phosphatases during mouse postnatal development. (A) PP1α, (B) PP1β, (C) PP2Aβ, and (D) PP2Bα are developmentally regulated at the mRNA level ($n = 3$). Methylene blue staining of 18S rRNA used to quantify sample loading were showed above each northern blot. Arrows indicate molecular size.

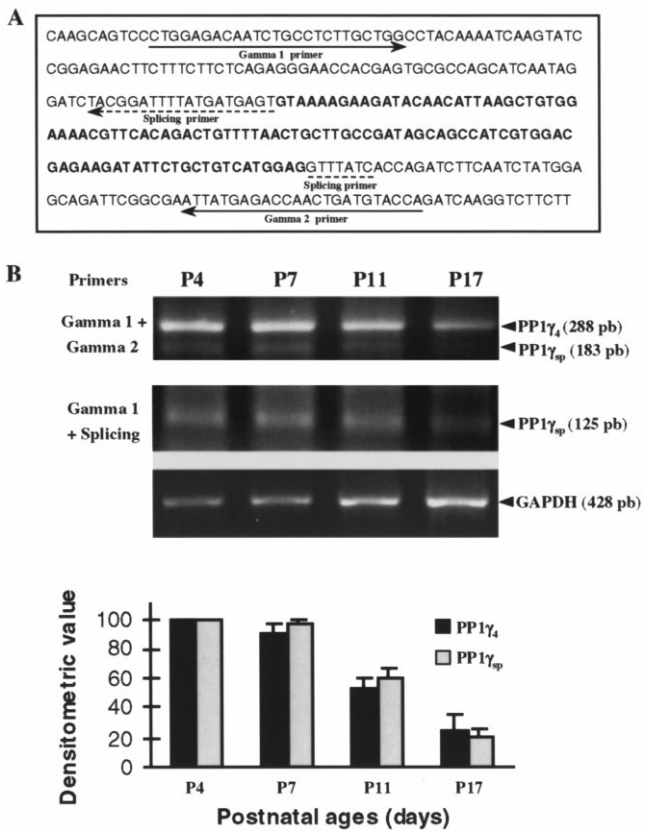

Fig. 5. Semiquantitative PCR corresponding to the PP1γ isoform. (A) The sequence of a new splice variant of PP1γ is shown. Exon 4 (bold letters) is absent. Primers used for the PCR amplification are also shown. (B) Comparative expression of both isoforms between P4 and P17. PP1γ refers to the isoform containing exon 4, and PP1γ_{sp} to the isoform without exon 4. The PCR-amplification of GAPDH is shown as an internal control. The molecular size of each band was indicated in parenthesis. The histogram represents the densitometric analysis for both isoforms, PP1γ_4 isoform, in black, and PP1γ_{sp}, in gray, during development ($n = 3$).

between P4 and P7, decreasing continuously with the time.

3.5. Protein expression of ser/thr phosphatases during synaptogenesis

To determine if a correlation exists between the expression of the ser/thr phosphatase proteins and their transcripts, detected by SSDD, western blot analysis was undertaken. As shown in Fig. 6, maximum expression of PP1 was observed at P13, using an antibody that recognizes all the catalytic subunits (alpha, beta and gamma). A western blot of PP2A (alpha and beta isoforms) showed that there was no significant variation in protein levels during the period analyzed, and only a slight increase at P13. By contrast, the catalytic subunit alpha of PP2B showed a continuous increase between P4 and P17. In all cases, a correlation between mRNA and protein expression was observed, and developmental regulation of the ser/thr phosphatases was evident during the process of synaptogenesis in mouse cerebellum.

4. Discussion

To study the role of phosphatases during development, we have chosen the mouse cerebellum. This is a relatively simple model system. It contains only two types of axons, those that are already present at birth (P0) (Purkinje cells axons, mossy and climbing fibers), and the axons of the different inter-neurons that form during the first postnatal weeks, corresponding to granule cell axons (Altman, 1972). In addition, the cerebellum is enriched in granule-Purkinje cell synapses, which constitute about 90% of all the types of synapses in the cerebellum (Palay and Chan-Palay, 1974). Furthermore, although the different stages of development may partially overlap, they mainly occur in defined time frames.

Serine/threonine phosphatases are among the more conserved proteins in evolution (Shenolikar, 1994). Eukaryotic ser/thr phosphatases (PPs) can be divided into two gene families based on homology of amino acid sequences. The PPP family, which includes PP1, PP2A and PP2B, and the PPM family, composed by PP2C and others related to pyruvate dehydrogenase phosphatase (the M refers to the fact that all members of this family require a metal for activity, Mg^{2+} or Mn^{2+}) (Wera and Hemmings, 1995; Barford, 1996). The catalytic subunits of PPP family have regions of high homology separated by non-consensus sequences of

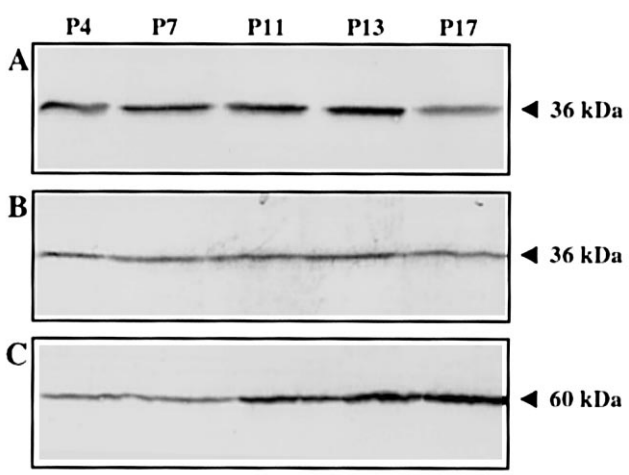

Fig. 6. Western blot of the catalytic subunits of ser/thr phosphatase proteins during cerebellar development. One hundred micrograms of total tissue protein was separated on 10% SDS-PAGE, transferred to nitrocellulose, and visualized with specific antibodies conjugated with alkaline phosphatase ($n = 4$). (A) Western blot corresponding to PP1, (B) to PP2A and (C) to the alpha isoform of PP2B. Arrows indicate molecular size markers.

similar, or even the same, lengths (about 350 bp). In consequence, when the catalytic subunits of ser/thr phosphatases were amplified using two primers that recognize these regions, an effective separation of different transcripts was not achieved by differential display.

Using SSDD, we were able to resolve these fragments of similar size, and to study effectively for the first time the differential expression of a family of proteins, the catalytic subunits of the ser/thr phosphatases. Two degenerate and specific primers (ST5′ and ST3′), which recognize regions of high homology in this protein family, were used for SSDD. With this procedure, we have found neither contaminants nor false positive among the bands that we sequenced, and the bands were clearly resolved, allowing observation of the changes in their expression during development. Six catalytic subunits of ser/thr phosphatases that have differentially expressed transcripts were detected, catalytic subunits alpha and beta of PP1, alpha and beta of PP2A, alpha of PP2B, and a new splice variant of catalytic subunit gamma of PP1, which does not contain the exon 4. When the expression of the two PP1γ isoforms was compared using semi-quantitative PCR, no differences were detected during the period analyzed. Both isoforms are expressed at high levels between P4 and P7, which coincides with the period of final maturation of the Purkinje cells and with the proliferation and early migration of granule cells. After P7, there is an acute decrease in mRNA expression. These results suggest that the absence of exon 4, which contains a putative casein kinase II phosphorylation site (PROSITE, http://www.expasy.ch/prosite/), might determine a different subcellular localization. Further studies with antibodies are required to test this hypothesis.

Transcripts for the catalytic subunits beta of PP2A and PP1 behave similarly, with maximum steady-state levels at P7, remaining constant thereafter. By contrast, the mRNA of the alpha isoform of PP1 is maximally expressed at P11, when the synaptic stabilization between granule and Purkinje cells begins. These levels decrease continuously until P17, when synaptic stabilization is complete. Interestingly, the levels of PP2A protein, and even more clearly PP1, showed maximum expression at P13, implying a delay between maximum mRNA expression (P7 or P11) and maximum protein accumulation (P13). Because synaptic stabilization begins at P13, these results suggest a possible role for PP2A and PP1 in this process. This is in agreement with results from Sim et al. (1998), which show differential regulation of both proteins in the post-hatch chicken brain.

When analyzing isoform alpha of PP2B, we noted that the expression of both mRNA and protein increased with age, reaching a maximum when the stabilization between the axons of the granule cells and the dendritic spines was complete (P17). This agrees with the results of Tallant and Cheung (1983), who observed that there is an increase in PP2B during development of the rat cerebellum. As they suggest, the developmental pattern of PP2B correlates with the period of major synapse formation, suggesting a possible role in synaptic function.

Our results suggest that SSDD might be an important tool for the characterization of the expression of transcripts for particular gene families, and for cloning new members of these families. As this method is sensitive to point mutations, where differential display is not, SSDD also has potential application to the study of gene expression in other model systems, such as in comparisons between normal and transformed tissues.

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