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Short Communication

The Role of Protein Phosphatase 2A Catalytic Subunit Cα in Embryogenesis: Evidence from Sequence Analysis and Localization Studies

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Protein phosphatase 2A (PP2A) constitutes one of the major families of protein serine/threonine phosphatases found in all eukaryotic cells. PP2A holoenzymes are composed of a catalytic subunit complexed with a structural regulatory subunit of 65 kDa. These core subunits associate with regulatory subunits of various sizes to form different heterotrimers which have been purified and evaluated with regard to substrate specificity. In fully differentiated tissues PP2A expression levels are highest in the brain, however, relatively little is known about expression in the developing embryo.

In order to determine the composition of PP2A catalytic subunits in the mouse, cDNAs were cloned and the genomic organization of PP2A Cα was determined.

By a gene targeting approach in the mouse, we have previously shown that the absence of the major catalytic subunit of PP2A, Cα, resulted in embryonic lethality around embryonic day E6.5. No mesoderm was formed which implied that PP2A plays a crucial role in gastrulation.

Here, we extended our studies and analyzed wild-type embryos for Cα expression at subsequent stages of development. After gastrulation is completed, we find high expression of Cα restricted to the neural folds, which suggests that PP2A plays an additional pivotal role in neurulation.

Key words: Neural fold / Protein phosphatase 2A / Whole-mount immunohistochemistry.

Protein phosphatase 2A (PP2A) holoenzymes are composed of a catalytic subunit complexed with a structural regulatory subunit of 65 kDa. These core subunits associate with regulatory subunits of various sizes to form different heterotrimers. In *Drosophila*, the catalytic subunit appears to be encoded by a single gene, whereas in mammals two different isoforms, termed Cα and Cβ, have been found. The two isoforms are highly homologous, yet, the importance of the two isoforms is still unclear (Mayer-Jaekel and Hemmings, 1993). We have recently shown by a gene targeting approach in the mouse that the two subunits most likely exert different functions, as the absence of Cα could not be compensated by Cβ despite the fact that total levels of catalytic subunit (Cα + Cβ) were comparable in the wild-type and homozygous Cα –/– knockout embryos at the time when mutant –/– embryos started to degenerate (around embryonic day 6.5). Analysis of these mice (Götz *et al.*, 1998) suggested that before E6.5 Cα is not essential and may only be weakly expressed in comparison to Cβ. At and beyond E6.5, PP2A Cβ may not compensate for the lack of Cα, either because sufficient amounts of C protein are not provided due to the weak promoter of Cβ or because Cβ cannot dephosphorylate all substrates of Cα (Götz *et al.*, 1998).

Recently, PP2A has gained additional attention as it is likely to be involved in the neurodegeneration of Alzheimer's disease (AD) which affects selected neuronal populations in the cortex and hippocampus of the human brain: the phosphorylation state and microtubule-binding activity of tau, a protein deposited insolubly in a hyperphosphorylated form in AD brains (Götz *et al.*, 1995), has been shown to be regulated by PP2A (Sontag *et al.*, 1996). Likewise, enzyme activities were shown to be decreased in brain extracts derived from AD patients in comparison to controls (Gong *et al.*, 1995). So far, mutations associated with disease have not been described for any of the PP2A subunits.

As the mouse is suitable for genetic manipulation (Götz *et al.*, 1998) and as neither sequence information nor expression data are available in the mouse, we isolated cDNAs containing the complete coding sequence (ORF) of both murine catalytic isoforms, Cα and Cβ, and determined the expression pattern of Cα in the developing embryo using whole-mount immunohistochemistry.

In order to determine the composition of PP2A catalytic subunits in the mouse, RNA was prepared from the brain of the B6D2F1 hybrid mouse strain and cDNAs were cloned by RT-PCR using degenerated primers matching the nucleotide sequence of rat and human Cα and Cβ genes.

We isolated murine PP2A Cα and Cβ cDNAs each containing a single open reading frame (ORF) of 930 bp, coding for 309 amino acid polypeptides (Figure 1A). They

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A

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Cα    1MDEKLFTKELDQWIEQLNECKQLSESQVKSLCEKAKEILTKESNVQEVRC
Cβ    1MDDKAFTKELDQWVEQLNECKQLNENQVRTLCEKAKEILTKESNVQEVRC
Cα   51PVTVCGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLL
Cβ   51PVTVCGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLL
Cα  101VALKVRYRERITILRGNHESRQITQVYGFYDECLRKYGNANVWKYFTDLF
Cβ  101VALKVRYPERITILRGNHESRQITQVYGFYDECLRKYGNANVWKYFTDLF
Cα  151DYLPLTALVDGQIFCLHGGLSPSIDTLDHIRALDRLQEVPHEGPMCDLLW
Cβ  151DYLPLTALVDGQIFCLHGGLSPSIDTLDHIRALDRLQEVPHEGPMCDLLW
Cα  201SDPDDRGGWGISPRGAGYTFGQDISETFNHANGLTLVSRAHQLVMEGYNW
Cβ  201SDPDDRGGWGISPRGAGYTFGQDISETFNHANGLTLVSRAHQLVMEGYNW
Cα  251CHDRNVVTIFSAPNYCYRCGNQAAIMELDDTLKYSFLQFDPAPRRGEPHV
Cβ  201CHDRNVVTIFSAPNYCYRCGNQAAIMELDDTLKYSFLQFDPAPRRGEPHV
Cα  301TRRTPDYFL
Cβ  301TRRTPDYFL
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B

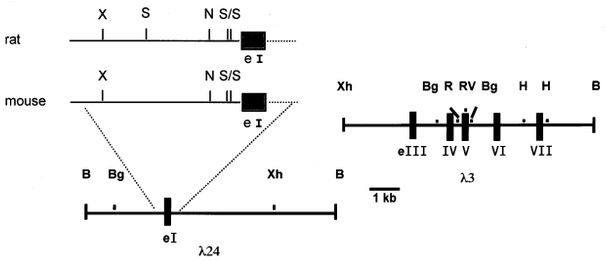

Fig. 1 Sequence Comparison and Genomic Organization of Murine PP2A Cα and Cβ.

(A) Alignment of the murine PP2ACα and Cβ amino acid sequences: differences between the two isoforms are shown in bold, the amino acid residue discriminating the human and murine Cα isoform is underlined. (B) Restriction mapping and partial sequencing of the promoter region and the 5′ UTR of PP2A Cα reveals a striking sequence conservation between rat and mouse. The genomic organization of murine Cα is similar to human Cα: exons 3–7 are clustered, whereas exons 1 and 2 are separated by several kb. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Not*I; R, *Eco*RI; RV, *Eco*RV; S, *Sma*I; X, *Xba*I; and Xh, *Xho*I.

Methods: total RNA was prepared from the brain of the B6D2F1 hybrid mouse strain by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1986). cDNAs were cloned by RT-PCR using degenerated primers matching the nucleotide sequence of rat and human Cα and Cβ genes. The sequence of both genes was determined by dideoxynucleotide chain termination sequencing using sequence-specific primers. Sequencing reactions were performed using fluorescein-labeled nucleotides. The thermal TaqDyeDeoxy Terminator PCR/Sequencing kit (Applied Biosystems) was used and sequences were analyzed on the ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

Cα genomic sequences were cloned from a λgem11 (Promega) phage genomic library prepared from 129/Sv(ev) embryonic stem cell DNA (Li *et al.*, 1996; Götz *et al.*, 1998). Murine cDNAs containing the complete open reading frame were used as probes for hybridization. Sequencing and restriction fragment analysis of eight phage clones was done by using [^{32}P]-labeled exon-specific oligonucleotide probes.

are 97% identical, with seven out of eight different residues located within the first 30 amino acids. The murine Cβ polypeptide (deposited in GenBank; accession no. Z67746) is identical to its rat and human counterpart. With the exception of one amino acid, the murine Cα polypeptide (deposited in GenBank; accession no. Z67745) is identical to the human polypeptide: murine Cα contains a leucine at position 5 (confirmed by genomic sequencing). In the human sequence, leucine is exchanged for a valine (Figure 1A). At the nucleotide level, the homology between murine and human Cα is 94%, and between murine and human Cβ 92%. The high degree of sequence conservation of the two isoforms and the conservation of the amino acid differences discriminating the two isoforms in mice and man suggests that the two subunits exert different functions in fully differentiated tissues and/or the developing embryo.

In order to determine whether the observed sequence conservation is also paralleled by a similar genomic organization, Cα genomic sequences were cloned from a phage genomic library prepared from embryonic stem cell DNA using murine cDNAs as probes for hybridization. Sequencing and restriction fragment analysis revealed that the genomic organization of murine Cα is similar to human Cα (Figure 1B): the Cα locus spans approximately 30 kb and is composed of seven coding exons with the six introns intervening at identical locations (Khew-Goodall *et al.*, 1991). Restriction mapping and partial sequencing of the promoter region and of the 5′ UTR revealed a striking sequence conservation between rat (Kitagawa *et al.*, 1991) and mouse (Figure 1B).

The high degree of sequence homology of the two catalytic subunits argues for a strong functional pressure on the maintenance of their properties. As this sequence homology extends into the 5′ UTR and the promoter region of Cα, this would imply that not only the functions of the two subunits are highly conserved, but also the regulation of gene expression during development and in terminally differentiated cells.

Analysis of the expression of the two isoforms from different porcine and rat tissues showed that the highest levels of the transcripts encoding both isoforms occur in the brain, whereas the levels in other tissues analyzed were about 5- to 10-fold lower (Mayer-Jaekel and Hemmings, 1993). In all cases the Cα transcripts were about 10-fold more abundant than the Cβ transcripts. mRNA and protein levels have been determined almost exclusively in adult tissues and cell lines, but not in the developing embryo. Some RNA expression data are available from studies in *Xenopus*, where high expression levels of both Cα and Cβ transcripts decrease rapidly during the meiotic and first mitotic embryonic cell divisions. Resumption of zygotic expression starts for both mRNAs during the tailbud stage, but the Cβ transcript shows only a moderate and transient increase and declines again during the tadpole stage, whereas expression of Cα increases further during the same period until the onset of metamorphosis (Van Hoof *et al.*, 1995). It is not known whether mRNA expression levels are reflected by protein levels and whether there are tissue-specific differences in protein expression during embryogenesis.

Similarly, mRNA and protein levels of the structural PR65 subunit and the various regulatory subunits of PP2A have not been determined in the developing embryo. Some data are available for the expression of protein tyrosine phosphatases in rat embryos (Sahin *et al.*, 1995;

Sommer *et al.*, 1997), although analysis was done only relatively late during development (starting at around embryonic day 13).

We have previously shown by a gene targeting approach in the mouse that the absence of the major catalytic subunit of PP2A, Cα, results in embryonic lethality around embryonic day E6.5. Cα is not required in the initial stages of development. Null (–/–) blastocysts display a normal morphology of both inner cell mass and trophectoderm. The embryos implant properly, and both the extraembryonic and embryonic portion of the egg cylinder differentiate to develop the two primary germ layers, ectoderm and endoderm. It seems likely that even a proamniotic cavity can form in the absence of Cα. Both wild-type and Cα –/– embryos exhibit defined layers of cells of extraembryonic and embryonic endoderm, as well as extraembryonic and embryonic ectoderm, but –/– embryos are consistently smaller. Taken together, these data show that the initial development of Cα –/– embryos is not different from that of wild-type or heterozygous embryos. Our data do suggest, however, that Cα is required for the onset of gastrulation, as no mesoderm is forming in the Cα –/– embryos (Götz *et al.*, 1998).

As the role of PP2A Cα in development can not be assessed in knock-out embryos beyond E6.5, we extended our studies and analyzed wild-type embryos for Cα expression at subsequent stages of development.

For this purpose, embryos were dissected from the surrounding decidual tissue and stained with an antibody directed against PP2A Cα (Figure 2). Staining revealed that at the onset of gastrulation (primitive streak stage: E6.5 – E7.0) Cα is relatively uniformly expressed. Gastrulation is the period in postimplantation development when a single-layered epithelium, called the primitive ectoderm, is transformed progressively into three tissue layers, and simultaneously acquires the axial organization characteristic of the future fetus. It begins at about E6.75, some time after implantation and, over the next 48 hours, the three primary germ layers are established and interact in a coordinated fashion to form the primordia of fetal organs. As mentioned before, the third germ layer (mesoderm) is not induced in mouse embryos as a consequence of the absence of Cα. Soon after E7.0, neurulation is initiated: a medial strip of ectoderm anterior to the primitive streak is thickening. This is the neural plate, which elongates by the addition of neuroectoderm at its posterior aspect. During the neural plate stage (beginning at around E7.25) PP2A is relatively uniformly expressed (Figure 2A,a). After its initial formation, the neural plate changes its shape dramatically, and its lateral edges elevate to form the neural folds (headfold stage embryo, beginning at around E7.5). Consequently, in cross sections, the neuroectoderm comes to resemble a 'V' (Figure 2B). Staining of headfold stage embryos revealed that Cα is highly expressed in the neural folds (Figure 2A,b; higher magnification: Figure 2A,c), while expression in the other tissues is significantly weaker. In somite stage embryos (E8.5 and beyond) Cα is again uniformly expressed (Figure 2A,d).

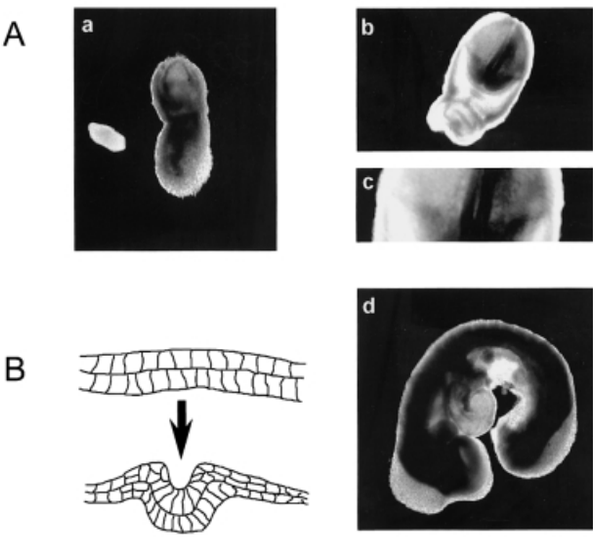

Fig. 2 Whole-Mount Immunohistochemistry of PP2A Cα Reveals Prominent Expression in Neural Folds at Around E7.5 While During Earlier and Later Stages of Development PP2A Cα Is relatively Uniformly Expressed.

(A) Whole-mount immunohistochemistry of murine embryos dissected at embryonic day E7.0 (a, large embryo), E7.5 (b,c), and E8.5 (d) reveals uniform staining for Cα at E7.0 and E8.5 and prominent expression of Cα in the neural folds of the neurulating embryo (E7.5). Panel (C), c shows a closeup of the E7.5 neural fold staining. As negative control a Cα null mutant has been included (a, small and degenerated embryo). (B) Schematic drawing of the initial stage of neurulation illustrating the dramatic changes of cell shape in the neurulating embryo.

Methods: embryos were dissected from the decidua, fixed in Dent's fixative (Dent *et al.*, 1989), dehydrated and permeabilized in PBS, 2% milk powder, and 0.1% Triton X-100. Antiserum #45 (anti-Cα; directed against the first 20 amino acids of Cα, an amino acid stretch showing a high degree of sequence diversity between Cα and Cβ; diluted 1/100; Götz *et al.*, 1998) was used, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antiserum (Dako, diluted 1/200), using diaminobenzidine as a substrate. Specificity of staining was confirmed by including Cα knock-out embryos (Götz *et al.*, 1998) or controls from which the primary antibody has been omitted.

What is the functional significance of the highly pronounced expression of Cα in the neural folds? One attractive possibility is that PP2A is involved in the regulation of microtubule assembly and dissassembly during neurulation. Supporting this hypothesis, PP2A has recently been shown to be associated with microtubules (Sontag *et al.*, 1995). In several cell types up to 75% of the cytosolic pool of PP2A was associated with microtubules. Moreover, it was found that the activity of microtubule-associated PP2A is regulated in a cell cycle-dependent manner, suggesting that PP2A plays a critical role in controling the phosphorylation of proteins involved in regulating microtubule functions (Sontag *et al.*, 1995). Cell shape is changing dramatically when the neural plate forms neural folds and when neural folds approach each other to form the neural tube (Figure 2B). This change in cell shape is microtubule-dependent. Thus, localized expression of Cα in the

neural folds suggests that PP2A plays a pivotal role in the microtubule-mediated transformation of normally-shaped into elongated cells during this stage of embryonic development.

Interestingly, during embryogenesis PP2A Cα is highest expressed in cells which form the nervous system, and once the organism is fully mature, it is again expressed highest in the brain. This suggests an important role of PP2A Cα in the maintenance of neuronal tissue, by regulating processes such as microtubule turnover during axonal transport of proteins or neuronal plasticity.

In summary, PP2A Cα knock-out experiments (Götz *et al.*, 1998) indicated that Cα is necessary for the onset of gastrulation and mesoderm formation. Regionalized expression of Cα in the neural folds suggests that PP2A plays an additional pivotal role in neurulation. It remains to be seen which of the various regulatory subunits of the PP2A holoenzyme is involved in this process. The high degree of sequence conservation and a genomic organization of PP2A Cα in mice that is highly similar to that observed in other species such as humans indicates that our findings in the mouse can be extended to other species, including humans.

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