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Receptor tyrosine phosphatase zeta/beta in astrocyte progenitors in the developing chick spinal cord

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Abstract

We cloned a cDNA encoding the receptor-type protein tyrosine phosphatase zeta/beta (RPTPZ/β) from embryonic chick spinal cord. *RPTPZ/β* was expressed throughout the ventricular zone (VZ) of the developing spinal cord and in scattered cells outside the VZ. *Platelet-derived growth factor receptor alpha (PDGFRa)*-positive oligodendrocyte progenitors co-expressed *RPTPZ/β* within the VZ but down-regulated *RPTPZ/β* after leaving the VZ. Most *RPTPZ/β*-positive cells outside the VZ co-expressed *glutamine synthetase* and *fibroblast growth factor receptor-3*, indicating that they are astrocyte progenitors. Northern blot analysis revealed a single ~9 kbp *RPTPZ/β* transcript expressed in the embryonic chick spinal cord, indicating that the shorter alternative-splice products of *RPTPZ/β* found in rodent spinal cord and brain—including the abundant extracellular proteoglycan known as phosphacan—are not present in the embryonic chick spinal cord.

Keywords: Astrocyte; Central nervous system; Chick; Development; FGFR3; Glial cell; Glutamine synthetase; In situ hybridization; Neural precursor; Oligodendrocyte; PDGFRalpha; Phosphacan; Spinal cord; Tyrosine phosphatase

1. Results and discussion

We generated a cDNA library of embryonic chick ventral spinal cord, enriched for sequences that are up-regulated between embryonic day five (E5) and E7.5, with the aim of identifying new genetic markers for glial cell lineages, which start to be generated around this time. We screened 126 partial cDNA clones by in situ hybridization against sections of late embryonic chick spinal cord and sequenced clones with promising expression patterns. One of these corresponded to part of the intracellular domain of chick receptor-type protein tyrosine phosphatase zeta/beta (cRPTPZ/β).

Full-length RPTPZ/β consists of an extracellular domain, transmembrane (TM) domain and two intracellular phosphatase domains (Levy et al., 1993; Maurel et al., 1994). In mammals, alternative splicing generates two additional products—(1) a shorter TM protein with a deletion in the extracellular domain and (2) a secreted protein completely lacking the intracellular and TM domains (Fig. 1A) (Levy et al., 1993; Maurel et al., 1994; Shitara et al., 1994; Maeda et al., 1995; Margolis et al., 1996; Nishiwaki et al., 1998). This latter alternative splice product, known as 'phosphacan', is an abundant extracellular chondroitin sulphate proteoglycan found in the rodent central nervous system, which might compete for ligands of full length RPTPZ/β (Milev et al., 1994).

Our original *cRPTPZ/β* clone was a ~600 bp fragment corresponding to part of the intracellular domain. A BLAST search of chick sequence databases found a single matching 4.1 kb partial cDNA containing the intracellular and TM domains but lacking a large part of the extracellular domain. We probed a conventional cDNA library of E7.5 chick spinal cord and isolated five more cDNAs between 3 and 5.5 kb in length. All five had the same 3′-UTR sequence and variable amounts of upstream sequence, but all seemed to be derived from the longest (10.7 kb) *RPTPZ/β* transcript of rodents (Maurel et al., 1994; Snyder et al., 1996). To determine whether shorter, alternative-splice *RPTPZ/β* products were present in the chick spinal cord, we

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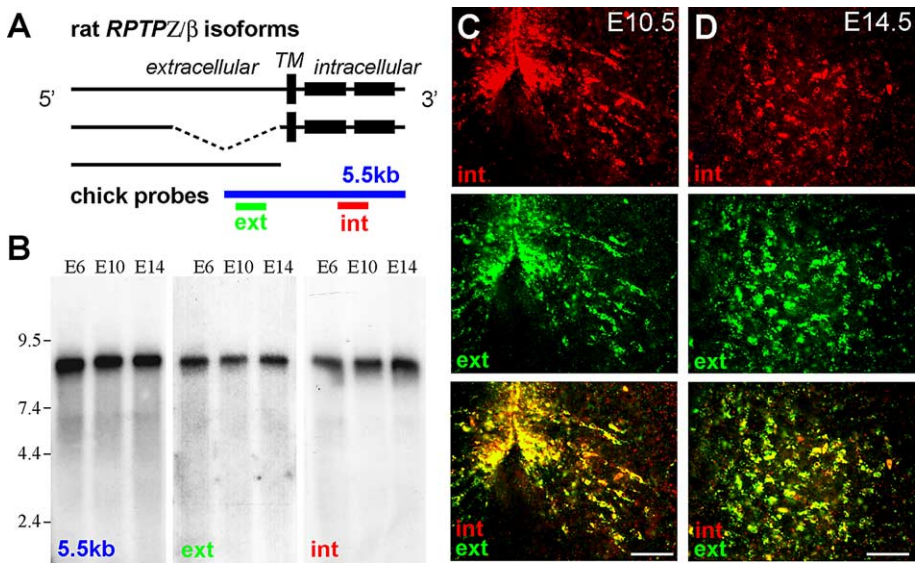

Fig. 1. (A) Rat RPTPZ/β isoforms and corresponding probes used for Northern blot and/or in situ hybridization. (B) Northern blot analysis of chick spinal cord mRNAs detected by the *cRPTPZ/β* probes depicted in panel A. All three probes detect a single ~9 kb chick transcript. (C) *cRPTPZ/β* expression patterns in embryonic chick spinal cord visualized by in situ hybridization with the *int* (red) and *ext* (green) probes, and their overlap (yellow, bottom). Scale bars, 50 μm.

hybridized Northern blots with three different cDNA probes designed to distinguish between the different mRNA structures found in rodents (Fig. 1A,B). All three probes recognized a single ~9 kb transcript in chick spinal cord at E6, E10 and E14 (Fig. 1B), in agreement with a previous report (Rowley et al., 1993). Thus, in contrast to rodents, there appears to be only a single *RPTPZ/β* transcript in chick spinal cord. Consistent with this, in situ hybridization of embryonic chick spinal cord sections with separate intracellular and extracellular riboprobes (*int* and *ext*; Fig. 1A) produced indistinguishable signals (Fig. 1C,D). These data imply that the truncated TM isoform of RPTPZ/β and the extracellular variant (phosphacan) do not exist in the developing chick spinal cord, as previously suggested (Rowley et al., 1993).

We examined the distribution of *cRPTPZ/β* in chick embryos by in situ hybridization with a probe against the intracellular domain (*int*; Fig. 1A). Expression was restricted to the central nervous system (CNS) at all ages examined. We examined the spinal cord in more detail. From E3 to E6 *cRPTPZ/β* mRNA was found throughout the spinal cord VZ, with the exception of the dorsal-most part next to the roof plate (Fig. 2). Starting from E7.5, small numbers of *cRPTPZ/β*$^+$ cells appeared to be streaming away from the VZ, initially from the ventral part but later from all parts of the VZ. By E13.5 many scattered *cRPTPZ/β*$^+$ cells had settled in both grey and white matter, though the in situ signal was much stronger in grey matter (Fig. 2H). At this late age the signal had largely disappeared from the VZ.

The *cRPTPZ/β*$^+$ neuroepithelial cells in the VZ at E3 are precursors of neurons and glial cells in the spinal cord. During the period of neuronogenesis (up to E6 approximately) there was very faint *cRPTPZ/β* labeling outside

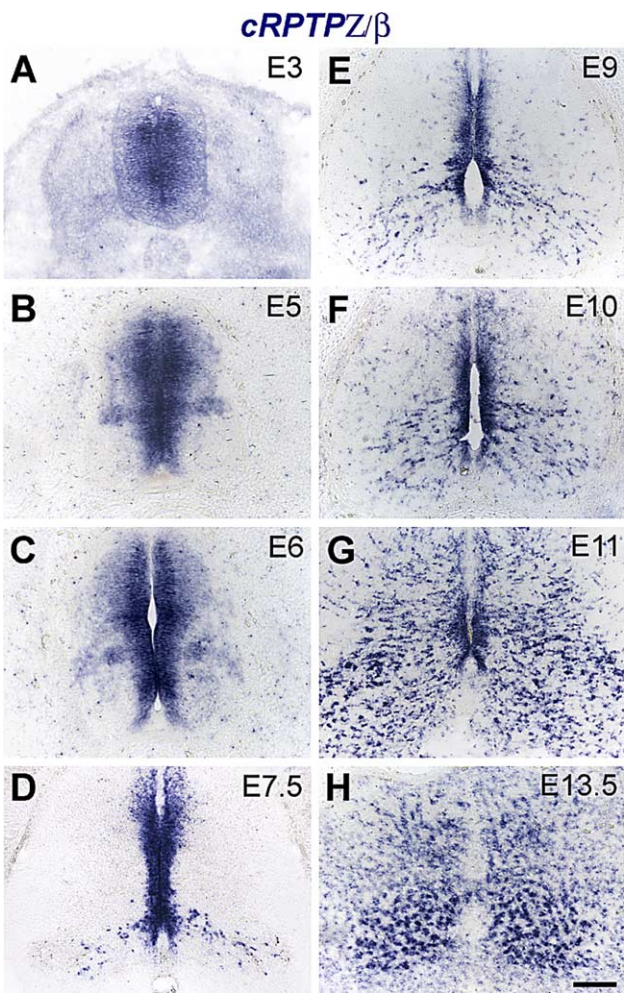

Fig. 2. Expression of *cRPTPZ/β* in embryonic chick spinal cord at various ages, visualized by in situ hybridization. Scale bars, 100 μm.

the VZ, perhaps in neuronal progenitors migrating away from the VZ or else in the processes of neuroepithelial cells, which extend radially to the pial surface. This faint expression was lost at later ages (e.g. E7.5; Fig. 2D) at the time when intensely labeled cells began to leave the VZ. These later-migrating cells are undoubtedly progenitors of glial cells (either oligodendrocytes or astrocytes) that retain expression of *cRPTPZ/β* after leaving the VZ.

We tried to establish whether *cRPTPZ/β* is expressed by oligodendrocyte progenitors (OPCs), astrocyte progenitors (APCs) or both, by double in situ hybridization with probes against *cRPTPZ/β* and either *PDGFRa* (OPCs; Hall et al., 1996), *fibroblast growth factor receptor3 (FGFR3)* or *glutamine synthetase (GLNS*: EC 6.3.1.2) (APCs; Pringle et al., 2003). There was little or no overlap between *cRPTPZ/β* and *PDGFRa* in migrating cells outside of the VZ (Fig. 3A–D). Neither was there any significant overlap between *cRPTPZ/β* and the differentiated oligodendrocyte marker *proteolipid protein* (*PLP/DM20*) (Fig. 3E,F). However, there was extensive overlap between *cRPTPZ/β* and the APC markers *GLNS* and *FGFR3* (Fig. 4). There was no overlap at E14.5 between *cRPTPZ/β* and the neuronal antigenic marker NeuN (Fig. 5A–D), or the mature astrocyte marker GFAP (Fig. 5E,F). At this age, GFAP is expressed only around the lumen of the spinal cord, presumably in radial glia (Malatesta et al., 2003). We conclude that *cRPTPZ/β* is expressed in APCs in the spinal cord but not in the great majority of OPCs, differentiated oligodendrocytes or neurons.

Note that there was complete overlap between *cRPTPZ/β* and *PDGFRa* within the VZ (Fig. 3A,B, arrows), so *PDGFRa*$^+$ OPCs presumably express *cRPTPZ/β* when they are first formed at the ventricular surface but down-regulate its expression once they exit the VZ. Canoll et al. (1996) showed that when rat OPCs were maintained in a proliferating, undifferentiated state by culturing in the presence of PDGF and FGF2, they expressed high levels of *RPTPZ/β* transcripts. However, there was marked down-regulation of the TM forms of *RPTPZ/β* when the OPCs were induced to differentiate into oligodendrocytes by withdrawing mitogens. These results are compatible with our own, because we know that the cell cycle of *PDGFRa*$^+$ OPCs in vivo slows down markedly after they leave the VZ (van Heyningen et al., 2001).

RPTPZ/β expression has been detected in astrocytes of the postnatal mouse hippocampus (Shitara et al., 1994), reactive astrocytes in chronic CNS glial scars (McKeon et al., 1999), astrocytes purified from neonatal rat CNS (Canoll et al., 1996), and certain astrocytoma lines (Krueger and Saito, 1992; Sakurai et al., 1996; Adamsky et al., 2001). These previous studies are generally consistent with our conclusion that *cRPTPZ/β* marks immature astrocytes in the embryonic CNS. Knockout mice have been described that lack *RPTPZ/β* function. These mice have distorted myelin sheaths but normal conductance velocity, physiology and behavior (Harroch et al., 2000). However, mice lacking *RPTPZ/β* show impaired recovery and remyelination in experimental autoimmune encephalomyelitis (EAE), a rodent model of demyelinating disease (Harroch et al., 2002). It is not known whether this is caused by defective oligodendrocytes or, indirectly, by defective astrocytes. Our data suggest the latter.

2. Methods

2.1. RNA purification

Fertilized White Leghorn chicken eggs were obtained from Needle Farm (Cambridge, UK), and embryos staged

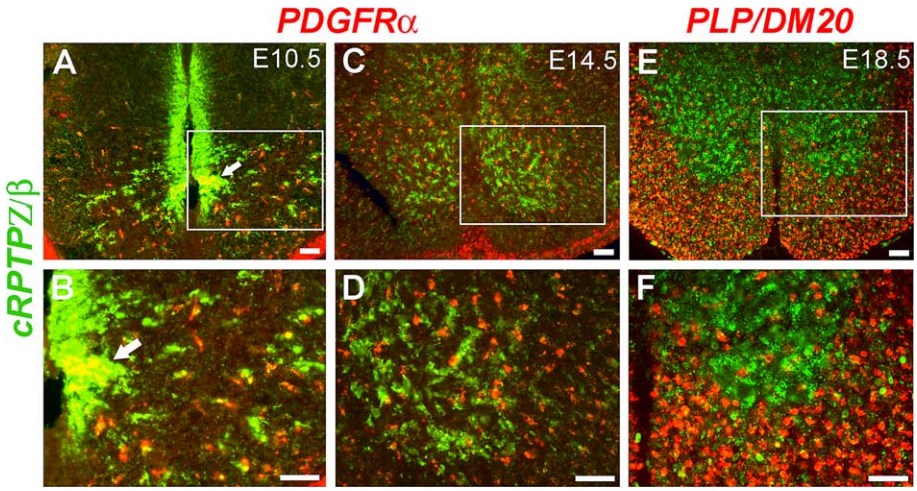

Fig. 3. Double in situ hybridization for *cRPTPZ/β* and either *PDGFRa* (OPCs) (A–D) or *PLP/DM20* (oligodendrocytes) (E, F). There is little overlap between *cRPTPZ/β* (green) and *PDGFRα* (red) outside the VZ, but complete overlap within the VZ at E10.5 (arrows in A, B). There is no overlap between *cRPTPZ/β* and *PLP/DM20* (E, F). Here and in subsequent Figures, the lower panels are higher-magnification images of the areas delineated in white. Scale bars, 50 μm.

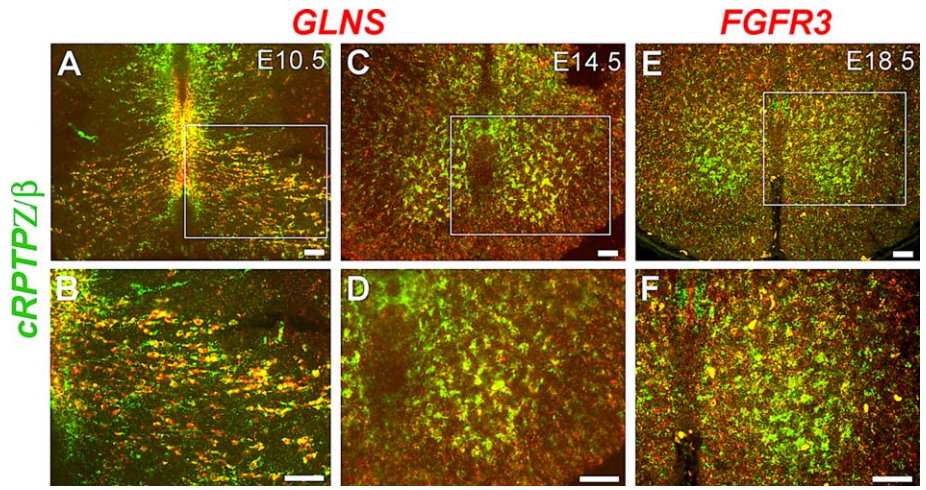

Fig. 4. Double in situ hybridization for *cRPTPZ/β* and immature astrocyte markers *GLNS* (A–D) and *FGFR3* (E,F). There is significant overlap (yellow) between the *cRPTPZ/β* signal (green) and the astrocyte lineage markers (red). Scale bars, 50 μm.

according to Hamburger and Hamilton (1992). For subtractive hybridization the spinal cords from about 800 chicken embryos were dissected and separated into ventral and dorsal halves. Total RNA was isolated by guanidinium thiocyanate/phenol–chloroform extraction (Chomczynski and Sacchi, 1987). PolyA containing mRNA was purified on oligo-dT cellulose (type 7, Pharmacia Biotech Inc).

2.2. Subtractive hybridization and cDNA cloning

Forward- and reverse-subtracted cDNA populations were prepared using the PCR-Select kit (Clontech), according to the manufacturer's instructions. For forward subtraction cDNA from E7.5 ventral spinal cord was used as tester, and cDNA from E5 and E7.5 dorsal spinal cord as driver. Forward subtraction reduced the representation of a housekeeping gene, *G3PDH*, around sixteen-fold. Amplified fragments from the forward subtraction were ligated into plasmid pCRII-TOPO (Invitrogen). Clone inserts were amplified by PCR, spotted onto Hybond-NX membrane (Amersham Biosciences) and probed with ^{32}P-labeled forward- and reverse-subtracted cDNA mixtures as well as unsubtracted tester and driver controls to eliminate housekeeping and background genes. Hundred and twenty six differentially expressed clones were analyzed by in situ hybridization to cryosections of E7.5 chick spinal cord, and the interesting candidates were sequenced. One clone (B11) was an identical match to chick *RPTPZ/β* mRNA coding for part of the intracellular region.

2.3. Isolation of chicken RPTPZ/β cDNA clones from E7.5 spinal cord cDNA library

An oligo(dT) primed E7.5 chick spinal cord cDNA library (Zap Express Synthesis Kit, Stratagene) was screened with a 0.6 kb *RPTPZ/β* cDNA clone from

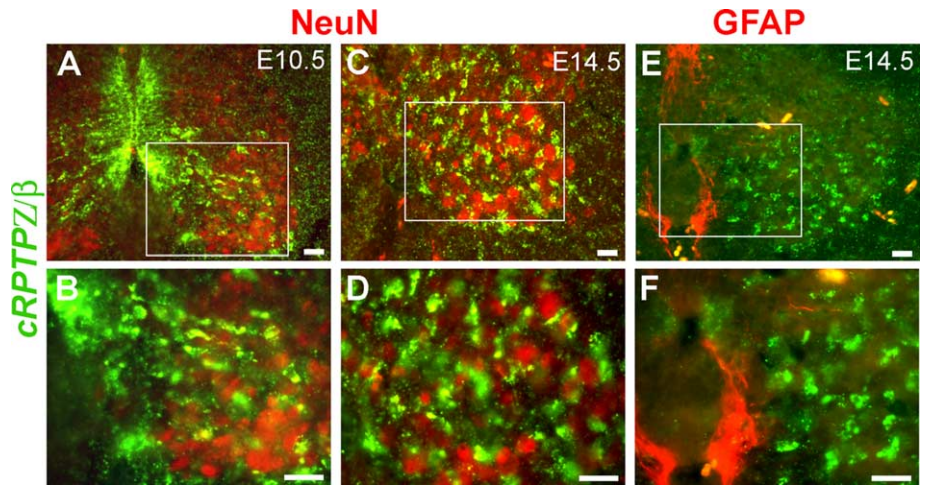

Fig. 5. (A) In situ hybridization for *cRPTPZ/β* (green) combined with immuno-labeling for the neuronal antigen NeuN (red). There is no significant overlap. (B) In situ hybridization for *cRPTPZ/β* (green) combined with immuno-labeling for the astrocyte protein GFAP (red). There is no significant overlap at this age, when GFAP expression is restricted to radial glia in the VZ. Scale bars, 25 μm.

the subtracted library. Partial nucleotide sequences of six cDNA clones were identified using synthetic primers corresponding to the ends of previously determined sequences. A hybridization probe corresponding to the N-terminal extracellular domain was synthesized by PCR using the longest (5.5 kb) clone as a template [primers: 5′-AAT CGC CTT TCC GTT CAG C-3′(sense), and 5′-CTC ACT TCG TTC AAA CAC G-3′ (antisense)]. This was used to probe Northern blots.

2.4. Northern blots

Total spinal cord RNA (5 μg/lane) was separated on 1% (v/v) formaldehyde/MOPS gels and transferred to Hybond-XL membrane (Amersham Biosciences). The blots were cross-linked with UV light and hybridized at 65°C for 3 h in Rapid-Hyb buffer (Amersham Biosciences) with denatured ^{32}P-labeled cDNA probes (see Fig. 1), which were generated with the Rediprime II Random Primer labeling system (Amersham Biosciences) and ^{32}P-dCTP (ditto), then purified on Sephadex Micro Bio-Spin chromatography columns (BioRad).

2.5. In situ hybridization

Frozen tissue and cryosections (15–18 μm) were prepared as previously described (Pringle et al., 2003; see http://www.ucl.ac.uk/~ucbzwdr/Richardson.htm for details). Dioxigenin (DIG)- or fluorescein (FITC)-labeled riboprobes were used. The chicken *PDGFRa* probe was made from a ~3200 bp cDNA covering most of the 3′-untranslated region of the mRNA (from Marc Mercola, Harvard Medical School). The *GLNS* probe was from a ~600 bp cDNA encoding chick glutamine synthetase (EC 6.3.1.2). The chicken *FGFR3* probe was from a ~440 bp partial cDNA encoding part of the TK domain (from Ivor Mason, King's College London). For double in situ hybridization two probes—one labeled with DIG, another with FITC—were applied to cryosections simultaneously. The FITC probe was visualized with horseradish peroxidase (HRP)-conjugated anti-FITC-POD (Roche; diluted 1:200) and Cyanine-5-tyramide reagent (TSA Plus, PerkinElmer Life Sciences, Inc.). The sections were washed in 1 × PBS containing 0.1% (v/v) TritonX-100 and the HRP conjugate inactivated by incubating in 3% (v/v) hydrogen peroxide for 30 min at room temperature. The DIG signal was then visualized with HRP-conjugated anti-DIG-POD (Roche; 1:200) followed by fluorescein-tyramide reagent (TSA Plus, PerkinElmer).

2.6. Combined immunolabeling and in situ hybridization

After fluorescence in situ hybridization with a DIG-labeled riboprobe, the sections were washed in PBS containing 0.1% (v/v) Triton X-100, then blocked in PBS containing 0.1% Triton X-100 and 10% normal goat serum. All antibodies were diluted in blocking solution. Anti-GFAP monoclonal antibody (clone G-A-5, Sigma) was used at a dilution of 1:200. Anti-NeuN monoclonal antibody (Chemicon International) was used at 1:400 dilution. Secondary antibodies were Texas red- and fluorescein-conjugated goat anti-mouse immunoglobulins (Pierce) diluted 1:200.

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