

Cloning and expression of human brain type I inositol 1,4,5-trisphosphate 5-phosphatase

High levels of mRNA in cerebellar Purkinje cells

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Abstract

In brain and many other tissues, Type I inositol 1,4,5-trisphosphate (InsP₃) 5-phosphatase is the major isozyme hydrolysing the calcium-mobilizing second messenger InsP₃. We recently reported the cloning and expression of dog thyroid InsP₃ 5-phosphatase. During the course of this cloning, screening of a human brain cDNA library allowed us to isolate a cDNA clone D1 with 91% sequence identity with the thyroid sequence. When clone D1 was expressed in *Escherichia coli*, the fusion protein had InsP₃ 5-phosphatase activity. *M_r* estimates of the recombinant enzyme made by immunodetection, activity assay after SDS/PAGE or silver staining were consistent with the calculated molecular mass. In situ hybridization on human cerebellum sections localised the mRNA for this enzyme to the Purkinje cells.

Key words: Inositol phosphate metabolism; Signal transduction; Ca²⁺; Human brain

1. Introduction

It is generally accepted that the phosphoinositide (PI) turnover system acts as a signal transducing cascade in the central nervous system. Many neurotransmitter receptors coupled with G protein activate phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bis-phosphate, generating inositol 1,4,5-trisphosphate (InsP₃) and 1,2-diacylglycerol (DAG). InsP₃ releases Ca²⁺ from intracellular stores whereas DAG activates protein kinase C [1]. InsP₃ 5-phosphatase catalyzes the dephosphorylation of InsP₃, inactivating the signal. This enzyme is present at high concentration in the brain, particularly in the cerebellum [2,3]. Regulation of this enzyme will directly modify InsP₃ accumulation and Ca²⁺ signalling. To study this reaction, we aimed to clone cDNAs encoding InsP₃ 5-phosphatase in brain tissue. We present here for the first time cloning, expression and production of recombinant Type I InsP₃ 5-phosphatase from human brain. We also show its localisation in cerebellar Purkinje cell bodies.

2. Materials and methods

2.1. Molecular cloning and expression of human brain InsP₃ 5-phosphatase

The cloning of human brain Type I InsP₃ 5-phosphatase (clone D1) resulted from a screening of a human brain frontal cortex cDNA library

(Stratagene) as reported in [4]. To express D1 as a β -galactosidase fusion product, the following construct was made by PCR using the following sense and antisense primers: ATCGGATCCGATGGCGGGGAAG-GCGGCC and ATCGAATTC AAGTGTGGGGCCGCGCCGTCTA. Plasmid DNA of D1 was subjected to PCR to amplify a 1,376 bp product. This material was subcloned into Bluescript after digestion with *EcoRI* and *BamHI*. The in-frame clone derived from D1 is now referred to as ECH1. To express the recombinant enzyme from ECH1 plasmid, LB medium (3 l) containing 50 μ g/ml ampicillin was inoculated for an overnight incubation at 37°C with a single colony of *E. coli* containing the Bluescript ECH1 plasmid. After addition of isopropyl β -thiogalactoside (1 mM final concentration) for 2.5 h at 30°C, the bacteria were harvested by centrifugation (1,200 \times g, 15 min) and resuspended in 250 ml of cold lysis buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 50 mg/l pefabloc, 2.5 μ M leupeptin, 10% sucrose, 12 mM 2-mercaptoethanol and 1% Triton X-100). After agitation for 60 min at 4°C and centrifugation (15,000 \times g, 15 min), supernatant was used for further purification. The crude lysate (activity of about 50–120 μ mol/min assayed at 10 μ M InsP₃) was loaded onto a Blue-Sepharose column (14 \times 5 cm) equilibrated in BD buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 2 mM MgCl₂, 10% glycerol and protease inhibitors). After washing with 100 ml of BD buffer, the enzyme was eluted with a linear gradient of 0–1 M NaCl of 1 l (total volume). After concentration to 10 ml, further purification was achieved by Sephacryl S-200 gel filtration [5]. At this step, specific activity was 1.25 μ mol/min/mg at 10 μ M InsP₃. The pooled fractions were concentrated to 10 ml (1 μ mol/min/ml). An aliquot of this preparation (0.25 ml) was six-fold diluted in 20 mM Na acetate, pH 5.5, and further purified onto a CM Mem Sep HP 1000 cation exchange HPLC resolved by a gradient of NaCl (0–0.2 M). Materials, assay of InsP₃ 5-phosphatase activity, Western blotting and immunodetection of proteins were as previously described [5–6].

2.2. In situ hybridization

In situ hybridization was performed on three human cerebella obtained at autopsy, as outlined in [7]. Three oligonucleotides complementary to bases 164–212, 373–419 and 674–720 of clone D1 were radiolabeled with [α -³⁵S]dATP. They exhibit identical distribution but with different intensities (Fig. 4A–C). To enhance the signal, they were mixed 1:1:1 (Fig. 3A). In situ hybridization of the InsP₃ receptor mRNAs was performed on adjacent sections with oligonucleotides de-

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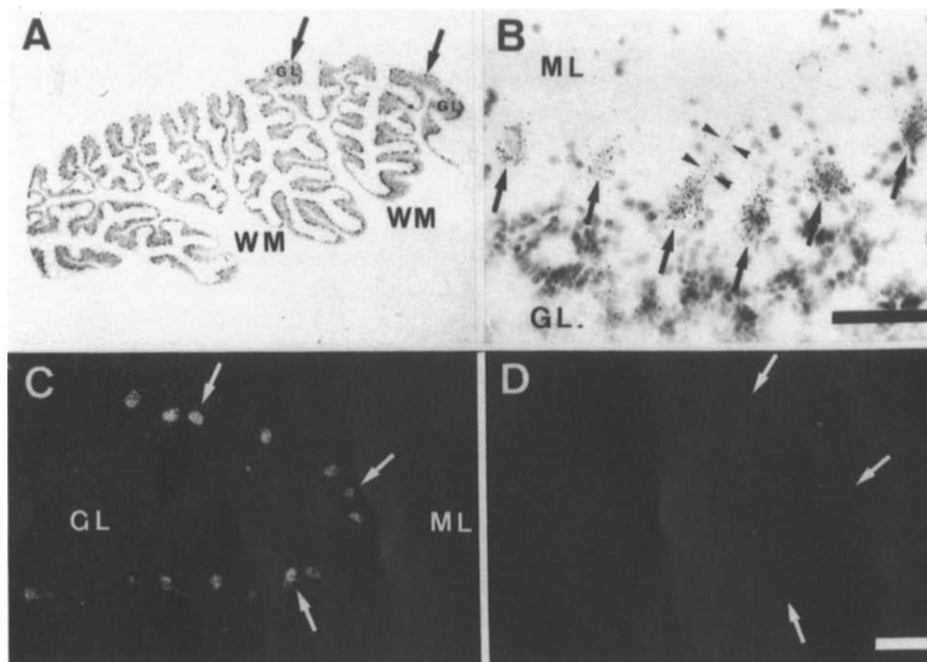


Fig. 3. Representative autoradiograms of adult cerebellum hybridized with the 5-phosphatase radiolabelled oligonucleotides with a mixture of three oligonucleotides (A–C) and its control in the presence of an excess relevant cold probes (D). (C,D) Dark field microscopy. Abbreviations: GL, granular layer; ML, molecular layer; Purkinje neuronal cell bodies (arrows) and proximal dendrites (arrowheads); WM, white matter. Bar = 50 μ m.

bisphosphoglycerate as expected considering the pharmacology of Type I InsP_3 5-phosphatase (ref. [5]; IC 50 at 10 μM InsP_3 were 10 μM and 1 mM, respectively).

As the β -galactosidase fragment fused to the insert of clone ECH1 has a M_r of 4,000–5,000, we estimate the total M_r of the expressed protein to be 51,000. A series of experiments have identified the recombinant protein with an apparent M_r of 43–45 kDa on SDS gels. The *E. coli*-derived InsP_3 5-phosphatase activity could be regenerated after SDS polyacrylamide gel electrophoresis. A single peak of activity comigrated with the standard of ovalbumin (M_r 43 \pm 3 kDa) (Fig. 2A). InsP_3 5-phosphatase antibodies directed against the native bovine brain enzyme [6] recognized, on Western blots, a 45 \pm 2 kDa band in purified lysates derived from expressed ECH1. When the experiment was repeated with a lysate derived from a non-recombinant clone which did not contain 5-phosphatase activity, no signal was detected on immunoblot (Fig. 2B). Purification of the recombinant enzyme followed by silver staining shows the presence of a polypeptide at the same apparent M_r (45 \pm 2 kDa; Fig. 2C).

3.3. *In situ* hybridization

InsP_3 5-phosphatase mRNA was found in the Purkinje cells neuronal bodies (Fig. 3A–C), in their proximal dendrites (Fig. 3B) and to a much lesser extent in the granule cell layer (Fig. 3A–C). We verified that a similar distribution was obtained using three probes separately (Fig. 4A–C) and that the pattern was different from that of

InsP_3 receptor mRNAs using the same technique (Fig. 4D). In the case of the InsP_3 receptor, the signal is only found in the Purkinje cells, while in the case of InsP_3 5-phosphatase it is detected on these neurons but also to a lesser extent on the granule cell layer.

4. Discussion

There are several lines of evidence for the participation of $\text{InsP}_3/\text{Ca}^{2+}$ signalling in neuronal function. It is known that glutamate stimulates P_i turnover and InsP_3 accumulation in Purkinje cells [9]. Purkinje neurons of the cerebellar cortex contain a particularly high density of InsP_3 receptors [10]. In these cells, InsP_3 metabolizing enzymes may be involved in Long-term depression of the parallel fiber-Purkinje cell glutamate synaptic transmission [11]. To study these enzymes, our aim was to clone cDNAs encoding the proteins, particularly InsP_3 5-phosphatase and have addressed the question of the existence of possible isozymes. In this study, we report the sequence of human brain Type I enzyme. The protein sequence is 97% identical with the previously reported dog thyroid enzyme suggesting together with biochemical data [4,12,13], that this enzyme is widely expressed in various tissues. The high levels of mRNA in the Purkinje neurons are consistent with the high activity observed in cerebellum over other brain areas [3]. Since the enzyme could easily be produced in *E. coli*, it is now possible to study its regulation and importance in the Ca^{2+} release mecha-

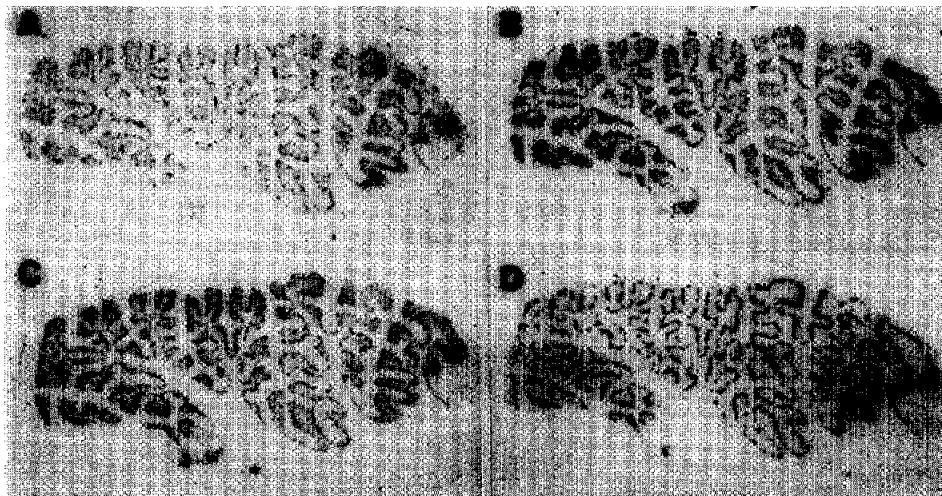


Fig. 4. Specificity of in situ hybridization as shown with each 5-phosphatase probe separately (A–C) and InsP_3 receptor (D).

nism. It will also be possible to compare its expression in Purkinje neurons and other cells. In this context, recent data have shown a functional difference in InsP_3 -evoked Ca^{2+} release between Purkinje cells and peripheral tissues [14].

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