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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 ($InsP_3$) 5-phosphatase is the major isozyme hydrolysing the calcium-mobilizing second messenger $InsP_3$. We recently reported the cloning and expression of dog thyroid $InsP_3$ 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 $InsP_3$ 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; Ca^{2+}; 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 ($InsP_3$) and 1,2-diacylglycerol (DAG). $InsP_3$ releases Ca^{2+} from intracellular stores whereas DAG activates protein kinase C [1]. $InsP_3$ 5-phosphatase catalyzes the dephosphorylation of $InsP_3$ 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 $InsP_3$ accumulation and Ca^{2+} signalling. To study this reaction, we aimed to clone cDNAs encoding $InsP_3$ 5-phosphatase in brain tissue. We present here for the first time cloning, expression and production of recombinant Type I $InsP_3$ 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 $InsP_3$ 5-phosphatase

The cloning of human brain Type I $InsP_3$ 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: ATCGGATCCGATGGCGGGGAAGGCGGCC and ATCGAATTCAAGTGTGGGGCCGGCCGTCTA. Plasmid DNA of D1 was subjected to PCR to amplify a 1,376 bp product. This material was subcloned into Bluescript after digestion with *Eco*RI and *Bam*HI. 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 μg/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 × *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 μM leupeptin, 10% sucrose, 12 mM 2-mercaptoethanol and 1% Triton X-100). After agitation for 60 min at 4°C and centrifugation (15,000 × *g*, 15 min), supernatant was used for further purification. The crude lysate (activity of about 50–120 μmol/min assayed at 10 μM $InsP_3$) was loaded onto a Blue-Sepharose column (14 × 5 cm) equilibrated in BD buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 2 mM $MgCl_2$, 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 μmol/min/mg at 10 μM $InsP_3$. The pooled fractions were concentrated to 10 ml (1 μmol/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 $InsP_3$ 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-^{35}S]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 $InsP_3$ receptor mRNAs was performed on adjacent sections with oligonucleotides de-

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fined in [8] (Fig. 4D). Pretreatment of the sections with RNAse (20 μg/ml) and hybridization with labeled probes and a 100-fold excess of cold relevant probes (Fig. 3D) abolished the signal in contrast to total signal persistence in the presence of an excess of unrelated cold probes.

3. Results

3.1. Molecular cloning and nucleotide sequencing of human brain Type I $InsP_3$ 5-phosphatase

A human brain frontal cortex cDNA library in phage Lambda ZAP II was screened to isolate a cDNA clone D1 of 1,564 bp together with four other clones with comparable restriction maps. Sequencing of clone D1 identified a 1,236 bp open reading frame encoding a 412 amino acid protein with a calculated molecular mass of 47,817 including the initiating methionine. Fig. 1 shows the sequence of the human brain $InsP_3$ 5-phosphatase. When translated into protein, the coding sequence showed an overall 97% identity with the thyroid sequence ([4], similarity in terms of nucleic acids was 91% in the coding region). In particular, peptide sequences previously reported for the bovine brain enzyme [4] were found in the sequence of the human clone (13 peptide sequences underlined in Fig. 1 with a total of 135 over 142 residues conserved between the two species). A computer search at the NCBI using the Blast network service revealed no significant similarities with other proteins. However, a partial and unidentified sequence (T08974) derived from a human cDNA was found to match the 3′ end sequence of clone D1 over 403 bp (96% identity).

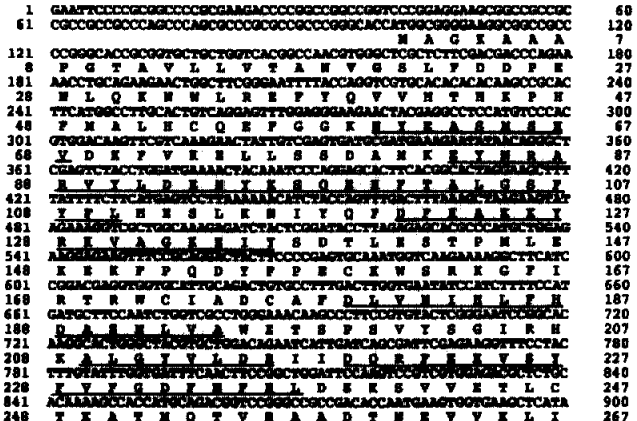

Fig. 1. Primary sequence of human brain $InsP_3$ 5-phosphatase as deduced from its nucleotide sequence (EMBL Accession Number X77567).

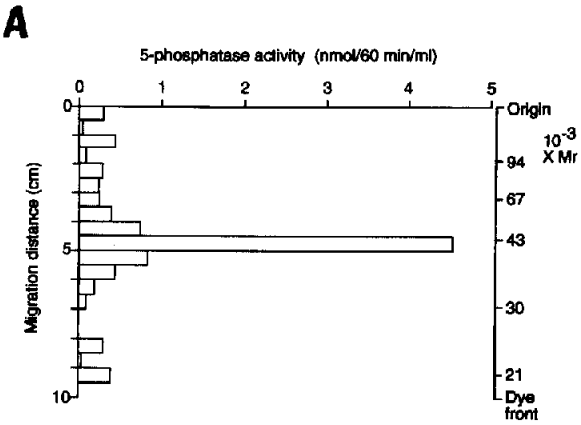

Fig. 2. (A) Regeneration of activity after SDS/PAGE of $InsP_3$ 5-phosphatase expressed in *E. coli*. A sample of partially purified enzyme after Blue Sepharose and Sephacryl S-200 was applied to an SDS/10% polyacrylamide 10 cm slab gel. Activity applied onto the gel was 8 nmol/60 min incubation when assayed in the presence of sample buffer used for electrophoresis [5] at 10 μM $InsP_3$ substrate level. Activity of the peak fraction (i.e. fraction 10) was 1.1 nmol/60 min incubation. (B) Western blot analysis of expressed 5-phosphatase. Lane 1: purified enzyme (approx. 25 μg or 0.16 μmol/min at 10 μM $InsP_3$ substrate level). Lane 2: bacterial lysate derived from a non-recombinant clone (approx. 300 μg). Immunoblot analysis was performed using crude serum [6] diluted 200-fold. (C) Silver staining of 6 μg purified enzyme.

3.2. Expression in E. coli

To confirm that the cDNA clone obtained did indeed code for an $InsP_3$ 5-phosphatase, the coding sequence was amplified by PCR and subcloned into Bluescript plasmid (ECH1 clone). Apparent K_m value for $InsP_3$ of the expressed protein was 65 μM which is twice the K_m of the recombinant thyroid enzyme [4]. We verified that the activity was inhibited by $InsP_4$ and 2,3-

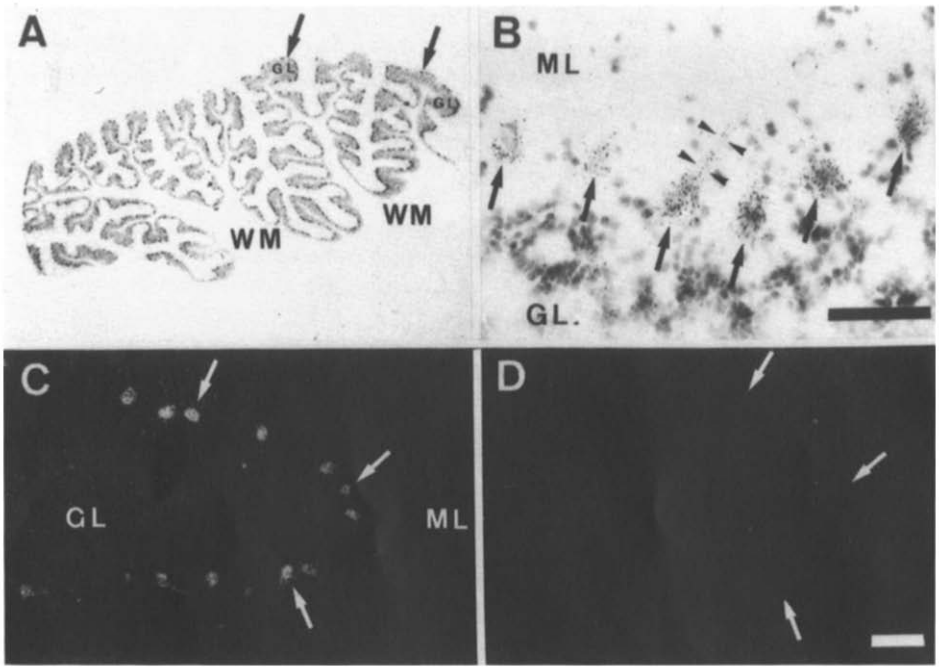

Fig. 3. Representative autoradiograms of adult cerebellum hybridized with the 5-phosphatase radiolabelled oligonucleotides with a mixture of three oligonucleotides (A–C) and its control in the presence of an excess relevant cold probes (D). (C,D) Dark field microscopy. Abbreviations: GL, granular layer;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

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 ± 3 kDa) (Fig. 2A). $InsP_3$ 5-phosphatase antibodies directed against the native bovine brain enzyme [6] recognized, on Western blots, a 45 ± 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 ± 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 $InsP_3/Ca^{2+}$ 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 adressed 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-

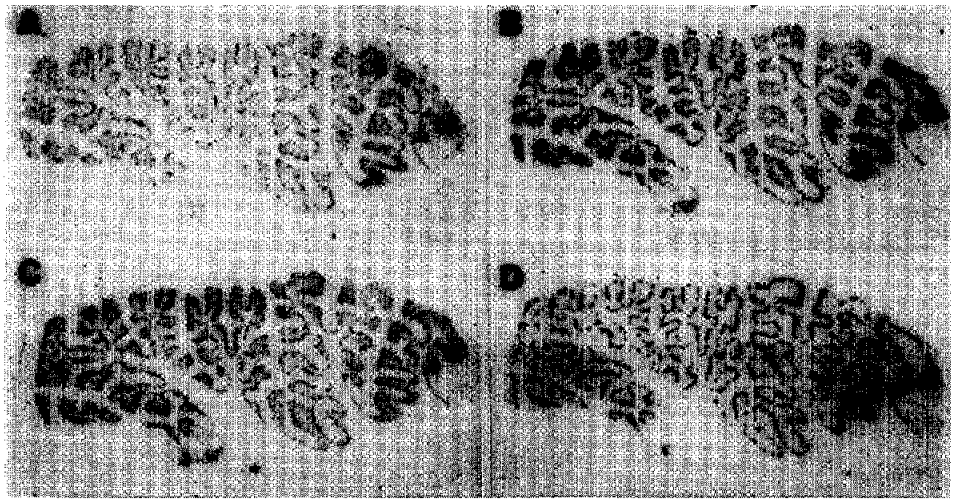

Fig. 4. Specificity of in situ hybridization as shown with each 5-phosphatase probe separately (A–C) and $InsP_3$ receptor (D).

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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