Cloning and expression in *Escherichia coli* of a dog thyroid cDNA encoding a novel inositol 1,4,5-trisphosphate 5-phosphatase

Benoît VERJANS, Florence DE SMEDT, Raymond LECOCQ, Valérie VANWEYENBERG, Colette MOREAU and Christophe ERNEUX*

Interdisciplinary Research Institute (IRIBHN), Campus Erasme, Bldg. C, 808 route de Lennik, 1070 Brussels, Belgium

In brain and many other tissues, type I inositol 1,4,5-trisphosphate (InsP_3) 5-phosphatase is the major isoenzyme hydrolysing the calcium-mobilizing second messenger InsP_3 . This protein has been purified to apparent homogeneity from a crude soluble fraction of bovine brain, yielding a single major protein band with a molecular mass of 43 kDa after SDS/PAGE. This material was used to determine internal microsequences. A partial DNA sequence has been amplified by PCR by using degenerate primers deduced from two protein sequences (FKA-KKYKKV and DENYKSQE). A cDNA clone (BVCT) was isolated by screening a dog thyroid cDNA library. The encoded protein of 412 amino acids has a calculated molecular mass of

47681 Da. Peptide sequences generated from the bovine brain enzyme were found to be 96 % conserved compared with the dog thyroid protein. When clone BVCT was expressed in *Escherichia coli*, the recombinant protein was shown to hydrolyse both InsP_3 and inositol 1,3,4,5-tetrakisphosphate, with apparent K_m values of 28 and 3 μM respectively. Enzyme activity was inhibited by EDTA and 2,3-bisphosphoglycerate, both inhibitors of native InsP_3 5-phosphatase, but not by EGTA and LiCl, as previously shown for the bovine brain enzyme. Our data show the cloning of type I InsP_3 5-phosphatase which, interestingly, does not share any significant sequence identity with the previously cloned type III isoenzyme.

INTRODUCTION

The levels of intracellular signal molecules can be adjusted and tightly controlled at the steps of synthesis and of degradation; the latter terminates hormone action by producing an inactive molecule. The example of cyclic nucleotide phosphodiesterase hydrolysing cyclic AMP and cyclic GMP is well known and their involvement in the negative control of intracellular cyclic AMP levels has been reported. Furthermore, multiple tissue-specific isoenzymes (now classified into five families) have been isolated, sequenced and shown to be the products of different genes (Beavo and Reifsnyder, 1990). In response to several extracellular signals, two second messengers, inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol, are produced (Berridge and Irvine, 1989). InsP_3 is responsible for Ca^{2+} mobilization (Berridge, 1993), while diacylglycerol is the specific activator of the protein kinases C (Nishizuka, 1988). The InsP_3 concentration depends upon the relative activities of phospholipase C and the two enzymes that either phosphorylate or dephosphorylate this molecule: InsP_3 5-phosphatase is responsible for dephosphorylation into inositol 1,4-bisphosphate (InsP_2) (Downes et al., 1982), while InsP_3 3-kinase leads to the synthesis of inositol 1,3,4,5-tetrakisphosphate (InsP_4) (Irvine et al., 1986).

Two isoenzymes of InsP_3 3-kinase are encoded by at least two different genes located on different chromosomes (Takazawa et al., 1990, 1991; Erneux et al., 1992). The activities of the InsP_3 3-kinases can be distinguished by the degree of stimulation by Ca^{2+} in the presence of calmodulin.

The initial description of InsP_3 5-phosphatase in human erythrocyte membranes (Downes et al., 1982) was followed by the separation of various enzymes. (i) Type I, purified in human platelets (Connolly et al., 1987), rat (Hansen et al., 1987) and bovine brain (Erneux et al., 1989), exists as soluble and particulate forms, showing very similar if not identical biochemical and

immunological properties (Erneux et al., 1989; Verjans et al., 1990; Hollande et al., 1991). The enzyme hydrolyses both InsP_3 and InsP_4 , with a higher affinity for InsP_4 ($K_m = 1 \mu\text{M}$ versus 10 μM for InsP_3) but a lower velocity (ratio of V_{max} = 11 in favour of InsP_3). (ii) Type II enzyme has an apparent molecular mass of 115 kDa as determined by gel filtration and appears rather specific for InsP_3 ($K_m = 70 \mu\text{M}$). (iii) Type III 5-phosphatase has been shown to be a 75 kDa protein in human platelets. This isoenzyme has K_m values of 7.5 and 24 μM for InsP_4 and InsP_3 respectively (Mitchell et al., 1989). A cDNA encoding type III isoenzyme has been isolated from a human placenta library (Ross et al., 1991). Further work by Attree et al. (1992) identified a cDNA transcript which may be involved in the development of the genetic disorder Lowe oculocerebrorenal syndrome. They observed that the open reading frame of a transcript encodes a protein with 53 % sequence identity to type III InsP_3 5-phosphatase. Despite the lack of evidence given as to the identity of this clone after expression, this represents the first potential implication of InsP_3 5-phosphatase associated with a metabolic disorder.

Purification of type I InsP_3 5-phosphatase from bovine brain led us to identify it as a 43 kDa protein. We have now cloned the cDNA corresponding to type I InsP_3 5-phosphatase by screening a dog thyroid cDNA library. When the coding sequence was expressed as a β -galactosidase fusion protein in *Escherichia coli*, it showed InsP_3 5-phosphatase activity with a K_m for InsP_3 of 28 μM .

MATERIALS AND METHODS

Materials

Problot membrane and DNA sequencing dye-primers were obtained from Applied Biosystems. Asp-N endoproteinase, trypsin and Taq polymerase were from Boehringer-Mannheim.

* To whom correspondence should be addressed.

The nucleotide sequence data for clone BVCT have been deposited with the EMBL, DDBJ and GenBank Nucleotide Sequence Databases under accession no. X75094.

Oligonucleotide synthesis and sequence analysis were performed on a DNA synthesizer 392 and a DNA sequencer 370 A respectively (Applied Biosystems). Pefabloc was from Pentapharm A.G. (Baele, Switzerland).

Microsequencing of *InsP₃* 5-phosphatase and amplification of a partial sequence by PCR

Bovine brain *InsP₃* 5-phosphatase was purified to apparent homogeneity, yielding about 50 µg of protein (Verjans et al., 1992). Two such preparations were used to determine internal microsequences with Asp-N endoproteinase and trypsin respectively, as reported in Rasmussen et al. (1991), after concentration so that all of the protein was applied to one lane of the gel. Peptides generated by cleavage were separated by narrow-bore reverse-phase h.p.l.c. and collected manually. The major peaks were taken for sequence analysis, which was carried out on an Applied 477A pulsed liquid phase sequencer. Sense and antisense degenerate oligonucleotides were synthesized, based on the following two sequences: FKAKKYKKV and DENYKSQE. A first-strand cDNA was made by incubating 1 µg of total bovine brain cortex RNA [prepared as described in Reuse et al. (1991)] with 2 µg of hexamers and 200 units of moloney murine leukaemia virus reverse transcriptase in a total volume of 20 µl for 1 h at 37 °C (Kawazaki, 1990). PCR, using 1–5 µl of cDNA directly, was performed as described previously (Libert et al., 1989) for 35 cycles (1 min for denaturation at 93 °C, 2 min for annealing at 55 °C and 3 min for elongation at 72 °C). A positive amplified product was detected with the following sense and antisense degenerate primers with added *EcoRI* and *HindIII* restriction sites to facilitate subcloning in M13 and sequencing (Sanger et al., 1977): ATCGAATTCGA(TC)GA(GA)AA(TC)-TA(TC)AA(GA)(TA)(CG)ICA(GA)GA and TCGAAGCTTA-C(TC)TT(TC)TT(GA)TA(TC)TT(TC)TTIGC(TC)TT(AG)AA.

Molecular cloning of *InsP₃* 5-phosphatase

The Bluescript plasmid clone BVCT was isolated after three successive manipulations. First, a human hippocampus cDNA library (Stratagene) containing approx. 2×10^6 different recombinant phage was used to prepare 20 sub-libraries. Each sub-library containing 20 000 plaque-forming units was amplified. Amplified phage has been extracted in 15 ml of SM buffer (5.8 g/l NaCl; 2 g/l MgSO₄; 50 mM Tris/HCl, pH 7.5; 0.1 g/l gelatin). PCR was used as an assay to isolate a positive sub-library by using 1 µl of this extracted material. PCR was made with sense and antisense oligonucleotides (GAGCACTTCACG-GCGCTAGGAAGCT and AAGTCAAATTGGTAGATGTT-TTTTA) derived from the sequence of the first amplified PCR product (see above and Figure 1). The positive sub-library was further subdivided into 25 separate pools at a density of 1000 plaque-forming units per Petri dish, and screened by PCR with the same primers as before. After subsequent purification, a single phage was isolated (clone BS1). The insert was rescued as a Bluescript plasmid, and the cDNA was sequenced after subcloning into M13. Secondly, a ³²P-labelled PCR product of 234 bp derived from the coding sequence of BS1 was used to screen 560 000 phage from a human brain frontal cortex cDNA library (Stratagene). The PCR probe was labelled with [α -³²P]-dATP (Amersham) according to the method of Feinberg and Vogelstein (1983), except that sense and antisense oligonucleotides (GCACTAGGAAGCTTTTATTT and TCACCAAGTCAAAGGCACAG) flanking the coding sequence were used as

primers for Klenow polymerase. After prehybridization in 6 × SSC buffer (1 × SSC = 0.15 M NaCl/15 mM trisodium citrate) containing 40 % formamide, 0.25 % non-fat dry milk and 5 mM EDTA at 42 °C for 2 h, hybridization was carried out in the same solution containing the PCR probe. Filters were washed twice in 2 × SSC/0.1 % SDS for 15 min at room temperature and twice at 65 °C in the same solution. Positive signals were purified by three successive screenings. This resulted in the isolation of four positive clones (D1, V2, C3 and F8). Finally, an *EcoRI*-*Bgl*II digest of D1 (507 bp) was used to screen a dog thyroid cDNA library containing approx. 1.2×10^7 different phage (Lefort et al., 1989). Hybridization and washing conditions were as before. This resulted in the isolation of two types of clone (2.7 and 2.2 kb). One of the longer clones is referred to as BVCT.

Clone BVCT was sequenced on both strands by subcloning restriction fragments into M13 sequencing vectors and the use of an Applied Biosystems sequencer. Alternatively, we sequenced using double-stranded DNA as template using Sequenase (version 2.0; USB Cleveland) and ³⁵S-labelled deoxyadenosine 5'-[α -³⁵S]thio]triphosphate. Part of the sequence was initially obtained by PCR amplification of clone BVCT between position 999 and 1630 and subcloning into M13. This sequence was subsequently confirmed by sequencing double-stranded DNA of clone BVCT using the Sequenase reaction and appropriate oligonucleotides.

Expression of *InsP₃* 5-phosphatase in *E. coli*

To express BVCT as a β -galactosidase fusion product, the following construct was made by PCR using the sense and antisense primers ATCGGATCCGATGGCGGGGAAGGCG-GCC and ATCGAATTCAGTGTGGGGCCGCGCGTCTA respectively. Plasmid DNA of BVCT was subjected to PCR to amplify a 1400 bp product. This material was subcloned into Bluescript after digestion with *EcoRI* and *Bam*HI. The in-frame clone derived from BVCT is now referred to as ETC.

To express proteins from non-recombinant and ETC plasmids, LB medium (5 ml) containing 50 µg/ml ampicillin was inoculated for an overnight incubation at 37 °C with a single colony of *E. coli* containing the Bluescript ETC plasmid. After addition of isopropyl β -thiogalactoside (5 mM final concentration) for 3 h at 37 °C, the bacteria were harvested by centrifugation (1200 g, 15 min) and suspended in 0.4 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 20 min at 4 °C and centrifugation (15000 g, 15 min), supernatant was used to assay for *InsP₃* 5-phosphatase and protein content as previously reported (Lowry et al., 1951; Erneux et al., 1989).

RESULTS

Determination of peptide sequences of a 43 kDa *InsP₃* 5-phosphatase

InsP₃ 5-phosphatase was purified to apparent homogeneity from the soluble fraction of bovine brain. Analysis of the purified protein by SDS/PAGE and silver staining resulted in a single protein band with a relative molecular mass of 43 kDa (Verjans et al., 1992). Moreover, *InsP₃* 5-phosphatase could be identified by regeneration of activity after electrophoresis (Lemos et al., 1989). Amino acid sequences of the protein were obtained after *in situ* digestion of the whole preparation (about 50 µg of protein); the protein was purified by electrophoresis and electroblotted on a Problot membrane. Two proteases were used: trypsin and Asp-N. Amino acid sequence analysis of 18 peptides generated 142

Table 1 Partial amino acid sequences generated from bovine brain *InsP₃* 5-phosphatase

Question marks in the sequence indicate the positions where residues could not be identified unambiguously.

Asp-N		Trypsin	
No.	Sequence	No.	Sequence
1	DYNRARVYL	1	VYLDENYK
2	DKELSVFK	2	LF?YFNQEVF
3	DFNRL	3	ELSVFK
4	DRLYEL	4	LIFR
5	DFKAKKYKKVTGKEIY	5	NYEASMSHV
6	DENYKSQE?F?ALG?FYFL	6	SESEKVVYDHIHG
7	DQRFQKASYFVFG	7	ALGYVLDL
8	DTKPVL	8	SQE?F?ALG?FYFL
9	DASNLVA		
10	DLVNIHLFH		

D E N Y K S Q E X F X A L G	
GAATTC <u>GAT GAG AAT TAC AAG TGG CAG GAG CAC TTC ACG GCG CTA GGA</u>	
EcoRI D E N Y K W Q <u>E H F T A L G</u>	
X F Y F L	D
AGC TTT TAC TTT CTT CAC GAA TCC TTA AAA AAC ATC TAC CAA TTT GAC	
S <u>F Y F L</u> H E S L K N I Y Q F <u>D</u>	
F K A K K Y K K V	
TTC AAAGCC AAG AAA TAT AAA AAA GT AAGCTT	
F K A K K Y K K V	HindIII

Figure 1 Sequence of the PCR product of the bovine brain *InsP₃* 5-phosphatase

The two oligonucleotide sequences taken as primers in the PCR reaction are underlined. The amino acid sequence deduced from the sequence of the PCR product (doubly underlined; bottom row) is in accordance with previously obtained protein microsequences (top row).

residues that could be assigned with certainty to the bovine brain enzyme (Table 1).

Molecular cloning of dog thyroid *InsP₃* 5-phosphatase

A 1 µg portion of total RNA from bovine brain cortex was reverse transcribed as described in the Materials and methods section, and first-strand cDNA was used in a PCR with degenerate oligonucleotides deduced from two protein sequences (FKAKKYKKV and DENYKSQE). The sequence of the bovine brain PCR product is shown in Figure 1. PCR was performed on several libraries of bovine cortex and human hippocampus using primers deduced from the previously amplified sequence (see the Materials and methods section). A positive signal was detected only in the human hippocampus cDNA library (results not shown). The library was divided into sub-libraries each containing approx. 20000 recombinant phage; 20 of these were screened by PCR as a tool to isolate a positive clone. Sub-

1	ATGGCGGGGAAGCGCGCCCGCCCGGACCGCGGCTGCTGGTCACGGCCACAGTGGGC	60
1	M A G K A A A P G T A V L L V T A N V G	20
61	TCGCTGTTTCGACGACCCAGAAACCTACAGAAAGTGGCTCGGGAGTCTATCAGGTC	120
21	S L F D D P E N L Q K N W L R E F Y Q V	40
121	GTGCACACACAAAGCCTCACTTCATGGCCCTGCAGGAGTTCGGAGGGGAAGAC	180
41	V H T H K P H F M A L H C Q E F G G K N	60
181	TACGAGGCGCTCCATGTCACAGTGGCAAGTTTGTCAAAGAGCTGCTGCCAGTACGCG	240
61	<u>Y E A S M S H V D K F V K E L L S S D A</u>	80
241	ATGAAGGAGTACACAGGCGCCGCTACCTGACGAGAACTCAAAATCCAGGAGCAC	300
81	<u>H K E Y N R A R Y L D E N F E S Q S R</u>	100
301	TTCCAGCGGCTAGGAAGCTTTTATTTTCACGAATCTTAAATAACATCTACCACTTT	360
101	<u>F T A L G S F Y F L H E S L K N I Y Q F</u>	120
361	GACITTAAGCCAAAGATATAAAAGTACCAGGCAAGGAGATCTACTGGGACAGCTG	420
121	<u>D F K A K K Y K V T G R E I Y S D T L</u>	140
421	GAGACACGCCCATCTCGGAGAGGAGTTCCCGCAGGACTACTTCCCGAGTGCAAA	480
141	E S T P M L E K E K P P Q D Y F P E C K	160
481	TGCTCAGAAAAGGCTTCGTCGGACAAAGATGTCGTCGGCAGACTGTGCTTTGACTTG	540
161	W S R K G F V R T R W C V A D C A F D L	180
541	GTCAACATCCATCTTTTCAGGATGCTTCAATTTGGTTGCTTGGAGAGCGCCCTCG	600
181	<u>V N I H L F H D A S H L Y A W E T S F S</u>	200
601	GTGATTCGGGATCCGACACAGGCGCTGCTGCTGACAGAAATCATCGACAG	660
201	V Y S G I R H K A L G Y V L D R I I D O	220
661	CGATTCGAGAAAGTCTCTACTTATGTTTGGCGATTTCAACTTCCGGCTGGACTCCAG	720
221	<u>H K E Y N R A R Y L D E N F E S Q S R</u>	240
721	TCGCTGTCGAGACTCTCTGACAAAGGCCACAGTACAGAGGTCGCGCCCGCCGACAC	780
241	S V V E T L C T K A T M Q T V R A A D T	260
781	AATGAAGCTGTAAGCTGATATTTCGAGTCCGACAAAGTCCAGGCTCATGCTTAC	840
261	<u>N E V V K L I F R E S D N D R K V M L Q</u>	280
841	CTGGAAGAAGAACTCTTCCACTACTTCAACCCAGGAGGTTTCCGTCGACAAACGGCACT	900
281	L E K K L F H Y F H Q E Y F R D M H G T	300
901	CGCTCTTAGAATTTGACAAAGAGCTGTCTTTTAAAGGACAGGCTGTATGAGCTGGAC	960
301	A L L E F D K E L S V F K D R L Y E L D	320
961	ATCTGCTTCTCCAGCTACCGTACAGGAGGAGTCCGCGCCAGGCGAGGATACATG	1020
321	I S F P P S Y P Y S E D S G Q G R Q Y M	340
1021	AACACCGGTCGCCCGCTGTCGACCGCTCTCATGTCGCCGTCGCGCAGGAGCTG	1080
341	H T R C P A W C D R V L M S P S A R E L	360
1081	ATCTCGAAGTCGGAGAGCGAGGAGAGGTTGTCACTACGACACATCGGGCTAACGTC	1140
361	I L K S E S E R K V V T Y D H I G P N V	380
1141	TGCATGGGAGACCAACAGCCCGTCTCTGCGCTTCGCGATCGCGCCCGGCGAGTAA	1200
381	C H G D H K F Y F L A F R I A P G A G K	400
1201	CCTCAGCCCATGTGCACAAAGTGTGTGTCGAGTGA	
401	P H A H V H K C C V V Q *	

(b)

Type III	210	220	230	240	250	260
	RNQDYKICSRMQCPDPSPPLTISNHDVILNLGOLNRYIEELDEVE-KVKLLIEEKDF					
Type I	240	250	260	270	280	290
	RLDSKSVETLCTKATMQTVRAADTRVVKLIFRESNDNRKV-MLQLEKLLFHYFNQEVF					
Type III	270	280	290	300	310	320
	QMLYAYDQKIQVAAKTVFEGFTGELTTPOTPYKYDTRALTGIPVRSVALLPGVIGFLW					
Type I	300	310	320	330	340	350
	RDNNGTALLEFDKELSVFKDRLYELDISFPFSPYSEDSGGQGRQYMTNTRCPANCDRLVMS					

Figure 2 (a) Nucleotide and deduced amino acid sequence of *InsP₃* 5-phosphatase, and (b) comparison between primary sequences of type I and type III *InsP₃* 5-phosphatases

(a) A restriction map of BVCT cDNA is shown in the upper panel; nucleotide and deduced amino acid sequences are in the lower panel. The 18 microsequences obtained from bovine brain *InsP₃* 5-phosphatase are underlined. The microsequences labelled D-1 to D-10 were obtained with Asp-N protease, whereas those labelled T-1 to T-8 resulted from trypsin digestion. Putative phosphorylation sites for protein kinase C are in bold. (b) The amino acid sequence of type III *InsP₃* 5-phosphatase is aligned for comparison with the primary sequence of type I *InsP₃* 5-phosphatase (a) using a GCG program GAP (Needleman and Wunsch, 1970). Gaps (.) were introduced to maximize alignments between the two sequences. Identical residues (I) and conservative changes (:) are indicated.

libraries were subsequently diluted, resulting in a single positive phage as outlined in the Materials and Methods section. This resulted in the isolation of clone BS1 and finally of clone BVCT after screening of a dog thyroid cDNA library (Lefort et al.,

Table 2 InsP_3 5-phosphatase activity of the expressed ETC clone

Expression and preparation of crude bacterial lysates was as described in the Materials and methods section. InsP_3 5-phosphatase activity was assayed at $30 \mu\text{M}$ InsP_3 for 8 min. The data are representative of five extracts made with different non-recombinant plasmids and eight with clone ETC (means of triplicates $\pm$ S.D.; n.d. not detectable).

	Volume (ml)	Protein (mg)	Activity	
			(nmol/min per ml)	(nmol/min per mg)
Non-recombinant	0.4	1.55	n.d.	n.d.
ETC	0.4	1.30	116 ± 3	89.2 ± 2.3

1989). BVCT is shown to be a clone of 2659 bp. The putative initiation codon is preceded by 252 bp containing an in-frame stop codon and occurs in a Kozak consensus sequence [GCCGCGATGG versus GCC(G/A)CCATGG; Kozak, 1989]. The ATG is followed by an open reading frame of 1236 bp. A stop codon at position 1489, followed by a 3' untranslated region composed of 1170 bp, predicts a 412-amino-acid sequence with a calculated molecular mass of 47681 Da, including the initiating codon (Figure 2a). Of the 142 residues identified for the bovine brain enzyme, 136 were fully conserved in the deduced dog thyroid sequence and 13 peptide sequences were 100% identical between the two species. Furthermore, all peptide sequences obtained by *in situ* digestion and Edman degradation were present in the amino acid sequence shown in Figure 2(a). There are several potential phosphorylation sites based on consensus site sequences for protein kinase C [Ser/Thr-Xaa-Arg/Lys (Woodgett et al., 1986)]. These sites are located at positions Thr-43, Ser-113, Thr-131, Ser-162, Thr-264 and Ser-356 in the dog thyroid sequence. A computer search at the NCBI using the Blast network service revealed no significant similarities with other proteins. However, the sequence of the Lowe oculocerebrorenal gene product between residues 543 and 577 displays 40% identity with the sequence of BVCT in the 318–352 region. No significant sequence similarity could be shown with type III InsP_3 5-phosphatase (Ross et al., 1991): only 19% identity in a 65-amino-acid overlap within residues 268–332 of the sequence in Figure 2(a) using the FASTA program (Figure 2b).

Expression of InsP_3 5-phosphatase 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 (ETC clone). This plasmid was expressed as a β -galactosidase fusion protein. Bacterial lysates derived from ETC had high InsP_3 5-phosphatase activity, whereas a non-recombinant plasmid did not hydrolyse InsP_3 after expression under similar conditions (Table 2). The specific activity of a crude bacterial lysate derived from ETC was $89.2 \text{ nmol/min per mg}$ of protein in a typical experiment. The recombinant enzyme was shown to hydrolyse both InsP_3 and InsP_4 , with K_m values of $28.3 \pm 4.5 \mu\text{M}$ and $3.4 \pm 0.9 \mu\text{M}$ respectively ($n = 3$ separate extracts; Figure 3); the ratio of $V_{\max}$ values calculated in the same extract was 11.4 ± 3.8 in favour of InsP_3 ($n = 3$). Purified bovine brain enzyme has K_m values of $11 \pm 3 \mu\text{M}$ and $1 \pm 0.1 \mu\text{M}$ for InsP_3 and InsP_4 respectively, and a $V_{\max}$ ratio of 11.0 ± 0.4 (Erneux et al., 1989). Moreover, InsP_3 5-phosphatase activity was shown to be inhibited by EDTA, InsP_4 and 2,3-bisphosphoglycerate, three known inhibitors of type I InsP_3 5-

Figure 3 Substrate-velocity relationships of expressed InsP_3 5-phosphatase activity

The data are shown as a direct plot in the $0\text{--}70 \mu\text{M}$ InsP_3 (a) and $0\text{--}20 \mu\text{M}$ InsP_4 (b) concentration ranges for the expressed clone ETC. The data are representative of three experiments.

Table 3 Effect of various agents on expressed InsP_3 5-phosphatase activity

InsP_3 5-phosphatase was assayed at $30 \mu\text{M}$ InsP_3 in the presence of various agents. The data are representative of two separate experiments.

Agent	Concentration	Activity (% of control)
None	—	100 ± 5
2,3-Bisphosphoglycerate	0.1 mM	68 ± 3
	1 mM	22 ± 2
	10 mM	6 ± 1
	100 μM	2 ± 1
InsP_4	0.1 μM	92 ± 5
	1 μM	67 ± 2
	10 μM	15 ± 2
	100 μM	2 ± 1
LiCl	10 mM	109 ± 3
EGTA	10 mM	109 ± 3
EDTA	10 mM	3 ± 1

phosphatase activity from bovine and rat brain (Table 3). In contrast, EGTA and LiCl, two well known modulators of InsP_3 3-kinase, InsP_2 1-phosphatase and monophosphatase activities (Delvaux et al., 1987; Takazawa et al., 1988), had no effect on the activity of the recombinant enzyme. ETC-expressed InsP_3 5-phosphatase activity was immunoprecipitated to the same extent as the type I enzyme (results not shown) using polyclonal

antibodies to the purified bovine brain enzyme (Verjans et al., 1990).

DISCUSSION

Intracellular concentrations of InsP_3 and InsP_4 are likely to be regulated by InsP_3 5-phosphatase activity, and this may be a mechanism of general importance in the control of InsP_3 second messenger function. For example, soluble and particulate enzymes have been reported but their relative importance and function within the cell is not known (Shears et al., 1987). Protein kinase C may activate the enzyme, a feedback mechanism which has been suggested in human platelets (Connolly et al., 1986; King and Rittenhouse, 1989), but so far this has not proved to be a general phenomenon. It is also unknown whether InsP_3 5-phosphatase represent a structurally diverse family of integral and soluble proteins encoded by multiple genes as reported for the InsP_3 3-kinase and receptor (Südhof et al., 1991; Ross et al., 1992; Erneux et al., 1992; Mikoshiba, 1993). The major enzyme that dephosphorylates InsP_3 in brain and many other tissues is referred to as type I InsP_3 5-phosphatase. It can be purified from a bovine brain soluble fraction (Verjans et al., 1992). An enzyme with physical and immunological similarity can also be shown to be associated with the particulate fraction of brain tissue and human placenta (Erneux et al., 1989; Verjans et al., 1990; Hollande et al., 1991; Laxminarayan et al., 1993). The cloning of this type I InsP_3 5-phosphatase is described in the current paper.

A cDNA clone BVCT, that encodes an InsP_3 5-phosphatase, was isolated by screening a dog thyroid cDNA library. Microsequencing of native bovine brain enzyme yielded 18 peptide sequences which could all be identified within the same encoded polypeptide sequence in clone BVCT. Peptide sequences generated from the brain enzyme were found to be 96% conserved compared with the thyroid protein. We can conclude that the dog sequence deduced from the BVCT clone and the purified bovine brain sequence correspond to orthologues of the same InsP_3 5-phosphatase gene.

A putative initiation codon may direct the synthesis of an InsP_3 5-phosphatase of molecular mass 47 681 Da, consistent with the molecular mass of the native bovine brain enzyme on an SDS gel, which migrates as a 43 kDa protein (Lemos et al., 1989; Verjans et al., 1992). Although the microsequences were obtained starting with a soluble enzyme, we do not exclude the possibility that clone ETC may encode a membrane-associated protein or that the protein may be anchored to the membrane. A possible mechanism of membrane association is the prenylation of the InsP_3 5-phosphatase at cysteine-409, which is surrounded by CAAX at the C-terminal end. This sequence has been proposed as a putative consensus sequence responsible for the binding of prenyl group on proteins, as was shown for γ subunits of several GTP-binding proteins (Glomset et al., 1990; Spiegel et al., 1991).

Partial DNA sequences of the bovine brain enzyme (Figure 1) have been used to design oligonucleotides for *in situ* hybridization. The presence of high levels of transcripts was observed in the neuronal Purkinje cells of the cerebellum (B. Verjans, P. Mailloux and C. Erneux, unpublished work). Consistent with this is the higher activity of InsP_3 5-phosphatase in the cerebellum as compared with other brain regions (Heacock et al., 1990). Moreover, the InsP_3 receptor and 3-kinase as well as protein kinase C were also shown to be highly abundant in these cells (Nishizuka, 1986; Mignery et al., 1989; Mailloux et al., 1991).

Ross et al. (1991) reported the cloning and expression of type III human platelet InsP_3 5-phosphatase, which shows very little sequence identity with the type I enzyme, although both recombinant enzymes appear to hydrolyse InsP_3 . The reasons for these

discrepancies are not understood. However, expression of type III 5-phosphatase in Cos 7 cells was very low compared with the InsP_4 binding activity which was used as a screening method to isolate the cDNA clone. Moreover, Ross et al. (1991) did not report K_m values of the recombinant enzyme for InsP_3 and InsP_4 . It is therefore possible that the reported sequence may encode an InsP_4 -binding protein which in addition could display some InsP_3 5-phosphatase activity.

Clone ETC was expressed in *E. coli* as β -galactosidase fusion protein, as previously done for InsP_3 3-kinase (Takazawa et al., 1990). Activity could be easily detected, as no InsP_3 5-phosphatase (or InsP_3 3-kinase) activity could be measured in a lysate derived from a non-recombinant plasmid. The specific activity was about 10-fold higher as compared with that in a crude bovine brain soluble fraction (Verjans et al., 1992). Several experiments showed that the recombinant and native enzymes had very similar enzymic properties: the apparent K_m values for InsP_3 and InsP_4 were in the same range, and the ratio of $V_{\max}$ values was the same. Moreover, the activity of the recombinant enzyme was decreased in the presence of 2,3-bisphosphoglycerate and totally inhibited in the presence of EDTA, a chelator of Mg^{2+} ions. No change in activity could be seen in the presence of LiCl and EGTA. Recombinant InsP_3 5-phosphatase will facilitate future experiments designed to determine precisely the specific amino acid residues involved in InsP_3 binding and catalysis. The cloning of a dog thyroid cDNA encoding InsP_3 5-phosphatase will facilitate the search for 5-phosphatase isoenzymes. It provides molecular tools to study the regulation of expression of the corresponding gene and to manipulate levels of the enzyme activity in cell culture, in order to study its influence on Ca^{2+} signalling.

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