Biochem. J. (1991) 278, 883–886 (Printed in Great Britain)

Molecular cloning and expression of a new putative inositol 1,4,5-trisphosphate 3-kinase isoenzyme

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A human hippocampus cDNA library in lambda ZAP II was screened by hybridization with a rat brain inositol 1,4,5-trisphosphate ($InsP_3$) 3-kinase cDNA. Two clones (hh6 and hh3) were isolated and sequenced. The insert of clone hh6 was shown to correspond to the 3′ end of the coding sequence of 50000-M_r $InsP_3$ 3-kinase (referred to as 3-kinase-A). Sequencing of the clone hh3 insert yielded an open reading frame encoding a 472-amino acid protein with a calculated M_r of 53451 (referred to as 3-kinase-B). The *C*-terminal part of 3-kinase-B (residues 187–462) was 68% identical with 3-kinase-A in amino acid sequence. The cDNA of clone hh3 was rescued as a Bluescript plasmid and expressed in *Escherichia coli* as a β-galactosidase fusion product. It showed $InsP_3$ 3-kinase activity that was stimulated in the presence of Ca^{2+}/calmodulin (more than 7-fold in a crude bacterial lysate from expressed plasmid). Regeneration of $InsP_3$ 3-kinase activity after SDS/PAGE identified a major polypeptide (M_r 60000–65000). The K_m for $InsP_3$ of expressed 3-kinase-B was 1.6 μM. These data provide molecular evidence for the existence of $InsP_3$ 3-kinase isoenzymes.

INTRODUCTION

Ins(1,4,5)P_3 ($InsP_3$) 3-kinase catalyses the phosphorylation of $InsP_3$ to Ins(1,3,4,5)P_4 ($InsP_4$), both potential modulators of calcium homoeostasis (Berridge & Irvine, 1989; Irvine *et al.*, 1988). cDNA clones encoding a rat brain Ca^{2+}/calmodulin-sensitive $InsP_3$ 3-kinase have been isolated (Choi *et al.*, 1990; Takazawa *et al.*, 1990*a*) and expressed in *Escherichia coli*. The encoded protein of 459 amino acids has a calculated M_r of 50868, equalling estimates made with the native enzyme purified to homogeneity (Takazawa *et al.*, 1990*a*,*b*). Using the rat cDNA as a probe, we isolated and expressed an $InsP_3$ 3-kinase clone from a human hippocampus cDNA library (referred to here as 3-kinase-A; Takazawa *et al.*, 1991). Rat and human amino acid sequences show 93% identity and expressed $InsP_3$ 3-kinase was Ca^{2+}/calmodulin-sensitive (about 2-fold stimulation by Ca^{2+}/calmodulin). We have now isolated from the same human library a new cDNA clone encoding $InsP_3$ 3-kinase (referred to as 3-kinase-B). 3-Kinase-B was more sensitive to Ca^{2+}/calmodulin than 3-kinase-A; kinase activity was stimulated 7–10-fold in the presence of these activators.

MATERIALS AND METHODS

Analytical procedures

Materials and assay of $InsP_3$ 3-kinase activity were as previously described (Takasawa *et al.*, 1990*a*, *b*). Purified $InsP_3$ was a gift from Dr. Robin F. Irvine and was used in the kinetic analysis of recombinant enzyme. Regeneration of activity after SDS/PAGE was performed as in Takazawa *et al.* (1989).

Molecular cloning

A human hippocampus cDNA library in the lambda ZAP II vector (Stratagene, La Jolla, CA, U.S.A.) was screened using two probes of rat brain cDNA encoding $InsP_3$ 3-kinase (clone C5 in Takazawa *et al.*, 1990*a*): a 726 bp *Eco*RI–*Sph*I fragment from the 5′ end (I in Fig. 1) and a 549 bp *Sph*I–*Sph*I segment (II in Fig. 1) from the middle of the insert. A total of 5×10^5 recombinant clones were plated on *E. coli* XL1-Blue at high density (5×10^4 plaque-forming units on 13.5 cm-diameter Petri dishes). Replicates were made on nitrocellulose filters which were screened by both probes. After prehybridization in 6 × SSC (1 × SSC = 0.15 M-NaCl/15 mM-trisodium citrate) containing 40% (v/v) formamide, 0.25% non-fat milk and 5 mM-EDTA at 42 °C for 2 h, hybridization was carried out for 12 h at 42 °C in the same solution containing the cDNA probe. The cDNA probes were labelled as previously reported (Takazawa *et al.*, 1990*a*) and used at a specific radioactivity of 2×10^5 c.p.m./ml. Filters were washed four times in 2 × SSC/0.1% SDS for 10 min at room temperature and twice in 2 × SSC/0.1% SDS for 15 min at 60 °C. Plaques that remained positive after three successive screenings were purified. cDNA inserts were subcloned in M 13 and inserts were sequenced on both strands according to Sanger *et al.* (1977) using an automated DNA sequencer (Applied Biosystems model 370A).

Expression of $InsP_3$ 3-kinase-B in *E. coli*

Sequence analysis indicated that a putative 3-kinase encoded by hh3 would not be in-frame with the β-galactosidase fragment fused to it. The following construct was made in order to express 3-kinase-B activity as a β-galactosidase fusion product: clone hh3 was digested with *Bgl*II, which recognizes a unique nucleotide sequence 5′ of the insert at position 137. Mung-bean (*Phaseolus aureus*) nuclease was used to digest away the remaining overhang (four bases) producing blunt ends, which were ligated and transformed in Epicurian XL1-Blue-competent cells according to the manufacturer's instructions (Stratagene). The resulting plasmid was designated hh3 MB1. To express $InsP_3$ 3-kinase activity, LB medium (5 ml) containing 50 μg of ampicillin/ml was inoculated for an overnight incubation with a single colony containing the Bluescript plasmid at 37 °C. After the addition of isopropyl β-thiogalactoside (1 mM final concn.) for 4 h, the bacteria were harvested by centrifugation and resuspended in 0.5 ml of cold lysis buffer [50 mM-Tris/HCl (pH 8)/1 mM-EDTA/0.2 mM-phenylmethanesulphonyl fluoride/2.5 μM-leupeptin/trypsin inhibitor (10 μg/ml)/10% (w/v) sucrose/12 mM-2-mercaptoethanol/1% Triton X-100]. After 5 min at 0 °C, and

Abbreviations used: $InsP_3$, Ins(1,4,5)P_3; $InsP_4$, Ins(1,3,4,5)P_4, LB, Luria–Bertani.

The nucleotide sequence of clone hh (human hippocampus $InsP_3$ 3-kinase-B), from which the primary structure in Fig. 2 is derived, will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X57206.

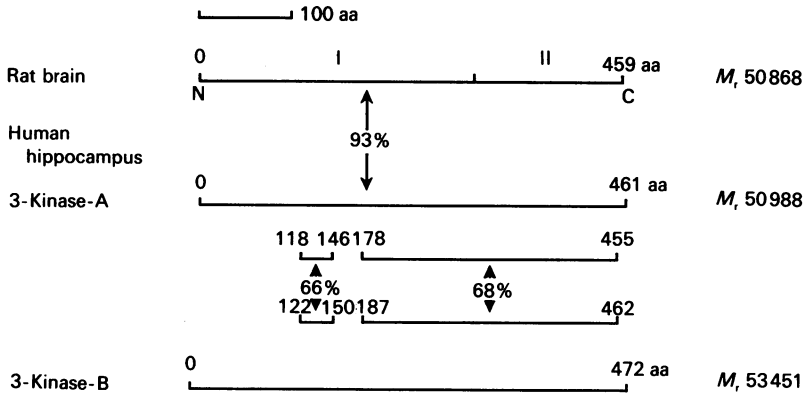

Fig. 1. Rat brain and human hippocampus InsP_3 3-kinases

Two probes corresponding to rat brain 50000-M_r InsP_3 3-kinase (I and II) were used to screen a human hippocampus cDNA library. This resulted in the isolation of two putative 3-kinases (referred to as 3-kinase-A and -B). Rat and human 3-kinase-A have an overall 93 % amino acid identity. Human 3-kinases-A and -B show similarity in two regions as indicated.

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3-kinase-A    1 MTLPGGPTGMARPGGARPCSPGLERAPRRSVGELRL.LFEARCAAVAAAA 49
3-kinase-B    1 MLEPLPCWDAAKDLKEPQCPPGDRVGVQPGNSRVWQGTMEKAGLAWTRGT 50

3-kinase-A   50 AAGEPRARGAKRRGGQV...PNGLPRAPPAPVIPQLTVTAEEPDVPPTSP 96
3-kinase-B   51 GVQSEGTWESQRQDSDALPSPELLPQDQDKPFLRKACSPSNIPAVIITDM 100

3-kinase-A   97 GPPERERDCLPAAGSSHLQQPRRLSTSSVSSTGSSSLLEDSEDDLLSDSE 146
3-kinase-B  101 GTQEDGALEETQGSPRGNLPLRKLSSSSASSTGFSSSYEDSEEDISSDPE 150

3-kinase-A  147 SRSRGNVQLEAGEDVGQ...KNHWQKIRTMVNL.PVISPFKKRYAWVQLA 192
3-kinase-B  151 RTLDPNSAFLHTLDQQKPRVSKSWRKIKNMVHWSPFVMSFKKKYPWIQLA 200

3-kinase-A  193 GHTGSFKAAGTSGLILKRCSEPERYCLARLMADALRGCVPAFHGVVERDG 242
3-kinase-B  201 GHAGSFKAAA.NGRILKKHCESEQRCLDRLMVDVLRPFVPAYHGDVVKDG 249

3-kinase-A  243 ESYLQLQDLLDGFDGPCVLDCKMGVRTYLEEELTKARERPKLRKDMYKKM 292
3-kinase-B  250 ERYNQMDDLLADFDSPCVMDCKMGIRTYLEEELTKARKKPSLRKDMYQKM 299

3-kinase-A  293 LAVDPEAPTEEEHAQRAVTKPRYMQWREGISSSTTLGFRIEGIKKADGSC 342
3-kinase-B  300 IEVDPEAPTEEEKAQRAVTKPRYMQWRETISSTATLGFRIEGIKKEDGTV 349

3-kinase-A  343 STDFKTTRSREQVLRVFEEFVQGDEEVLRRYLNRLQQIRDTLEVSEFFRR 392
3-kinase-B  350 NRDFKKTKTREQVTEAFREFTKGNHNILIAYRDRLKAIRTTLEVSPFFKC 399

3-kinase-A  393 HEVIGSSLLFVHDHCHRAGVWLIDFGKTTPLPDGQILDHRRPWEEGNRED 442
3-kinase-B  400 HEVIGSSLLFIHDKKEQAKVWMIDFGKTTPLPEGQTLQHDVPWQEGNRED 449

3-kinase-A  443 GYLLGLDNLIGILASLAER.... 461
3-kinase-B  450 GYLSGLNNLVDILTEMSQDAPLA 472
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Fig. 2. Primary structure of human hippocampus InsP_3 3-kinase-B ('B') as deduced from its cDNA sequence

The amino acid sequence corresponds to the 1416-nucleotide open reading frame determined from the sequencing of overlapping inserts (see the Materials and methods section). It is aligned for comparison with the sequence of human hippocampus 3-kinase-A ('A') using a GCG program GAP (Needleman & Wunsch, 1970). Gaps (.) were introduced to maximize alignments between the two sequences. Identical residues (|) and conservative changes (:) are indicated.

centrifugation, the pellet was resuspended in 0.5 ml of the same buffer in which Triton was replaced by SDS (0.5 %, w/v) and 2-mercaptoethanol was 5 %. After 5 min at 0 °C and centrifugation, the supernatant was used either to measure InsP_3 3-kinase activity or for SDS/PAGE.

RESULTS AND DISCUSSION

The existence of multiple forms of InsP_3 3-kinase with different regulatory properties was suggested by several experiments. In crude extracts of human brain separated on SDS/polyacrylamide gels, high-M_r fractions (58000–64000), in addition to the 50000-M_r 3-kinase, were associated with InsP_3 3-kinase activity, indicating the possible existence of unidentified 3-kinase isoenzymes (Takazawa *et al.*, 1991). Moreover, purified InsP_3 3-kinase from smooth muscle migrates with an apparent M_r of 80000 on SDS/polyacrylamide gels (Yamaguchi *et al.*, 1988). Ca^{2+}/calmodulin-sensitivity varies among InsP_3 3-kinases prepared from various sources (Takazawa *et al.*, 1989, 1990*b*) which could suggest the existence of different protein sequences, perhaps in the calmodulin-binding domains. In order to identify putative isoenzymes, a human hippocampus cDNA library was screened at low stringency using two fragments of rat brain cDNA encoding InsP_3 3-kinase (Fig. 1): two (hh6 and hh3) out of five clones scored as positive for both probes were followed and sequenced. The nucleotide and deduced amino acid sequence of the insert of clone hh6 (1.6 kb) was shown to correspond to the 3′ end of a cDNA encoding the 50000-M_r InsP_3 3-kinase

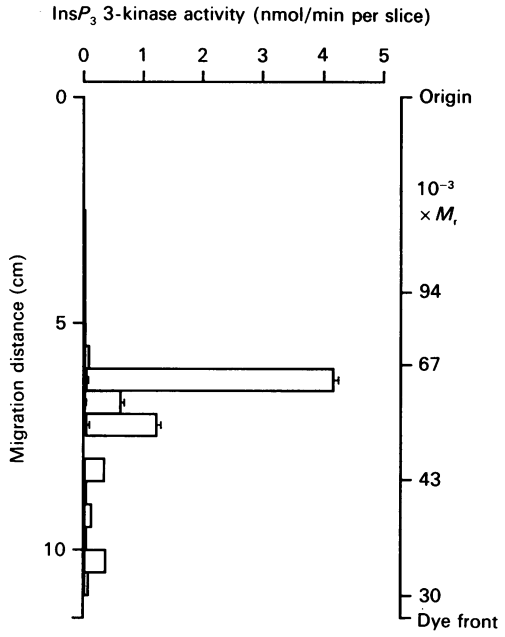

Fig. 3. SDS/PAGE of InsP_3 3-kinase expressed by *E. coli*

A 100 μg sample of crude lysate of clone hh3 MB1 was applied to an SDS/8% polyacrylamide 11.5 cm slab gel. After electrophoresis, one lane was cut into 5 mm slices and assayed at 10 μM-InsP_3 in the presence of 0.1 μM-calmodulin and 10 μM-free Ca^{2+} (□) (average of duplicates). Peak fractions (after 6 cm migration) were assayed in the presence of 1 mM-EGTA (■) for basal activity (mean of triplicates ± S.D.). Basal activity at 6–7.5 cm was below 0.05 nmol/min per slice.

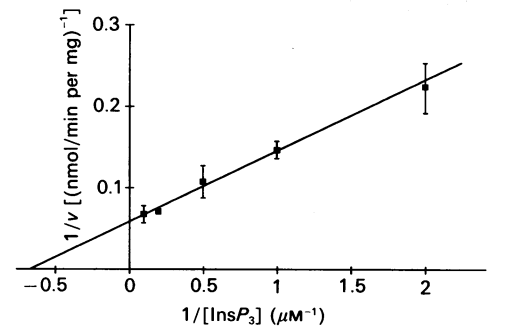

Fig. 4. Substrate–velocity relationship of expressed 3-kinase-B in the presence of Ca^{2+}/calmodulin

The data are shown as a double-reciprocal plot with the InsP_3 concentration in the 0.5–10 μM range. Values are means of triplicates ± S.D.

(arbitrarily referred to as 3-kinase-A). This clone (hh6) was used in turn to isolate a cloned cDNA (hh39) containing the entire coding region of the 3-kinase (461 amino acids). When translated into protein, the coding sequence shows, overall, 93% identity with the rat 3-kinase (Takazawa *et al.*, 1991, and Fig. 1). The insert of clone hh3 (which showed a weaker signal on hybridization compared with hh6) was 4.5 kb in length; a total of 4505 bp was determined, in which an open reading frame encoding 472 amino acids was identified (3-kinase-B). The most extensive similarity between 3-kinase-A and 3-kinase-B amino acid sequences was found in the *C*-terminus of the protein particularly in two regions: a short fragment with 66% identity, stretching from amino acids 122–150 and a particularly long segment with 68% identity, from amino acids 187–462 (Figs. 1 and 2). Previous results suggested that the *C*-terminal part of InsP_3 3-kinase may contain the catalytic domain of the kinase (Takazawa *et al.*, 1990*a*). Data obtained with the cDNA encoding rat InsP_3 3-kinase indicated that a maximum of 275 amino acids in the *C*-terminal region may be sufficient for the construction of a catalytically active domain (K. Takazawa, unpublished work). Sequences of high identity in the *C*-terminal parts of putative 3-kinase clones may therefore correspond to conserved catalytic domains.

To prove that the clone hh3 effectively encoded an InsP_3 3-kinase isoenzyme, we expressed in *E. coli* the Bluescript plasmid derived from hh3 as a β-galactosidase fusion product (Takazawa *et al.*, 1990*a*). The reading frame was restored between the β-galactosidase part in the Bluescript plasmid and the 3-kinase-B insert (designated hh3 MB1; see the Materials and methods section). The in-frame fusion product encodes a protein of 541 amino acids which contains 460 residues of the *C*-terminal part of 3-kinase-B. We estimate the total M_r of the expressed fusion protein to be about 59000–61000. A bacterial lysate derived from hh3 MB1 showed InsP_3 3-kinase activity which was stimulated 7–10-fold by Ca^{2+}/calmodulin. In contrast, lysates derived from non-recombinant clones had no 3-kinase activity. The InsP_3 3-kinase synthesized by *E. coli* could be renatured after SDS/PAGE. A major peak of InsP_3 3-kinase activity with an apparent M_r of 60000–65000 was isolated from a bacterial extract derived from the Bluescript plasmid (Fig. 3). This value is consistent with estimates based on the amino acid sequence (see above). The peak fraction was stimulated more than 10-fold in the presence of Ca^{2+}/calmodulin. In contrast, 3-kinase-A was only maximally stimulated 2-fold (Takazawa *et al.*, 1991). The K_m value of 3-kinase-B for InsP_3 was 1.6 μM (Fig. 4), which is comparable with native bovine brain InsP_3 3-kinase (Takazawa *et al.*, 1989).

It is well known that for each enzymic activity of the signal-transduction cascades, there are several isoenzymes, e.g. protein kinase C (Nishizuka, 1988), phospholipase C (Rhee *et al.*, 1989) and cyclic nucleotide phosphodiesterases (Beavo & Reifsnyder, 1990). Tissue-specific distribution of the isoenzymes confers cell specificity to physiological and pharmacological regulation. It could therefore provide the basis for the development of selective drugs. We have characterized two cDNA clones encoding InsP_3 3-kinases from a human cDNA library, both of which hybridize with cDNA probes of the rat brain 50000-M_r enzyme. The first clone, encoding 3-kinase-A, corresponds to the 50000-M_r human hippocampus InsP_3 3-kinase. It is homologous to the rat brain enzyme, having 93% identical residues (Takazawa *et al.*, 1991). The second clone, described here and corresponding to 3-kinase-B, has less similarity at its 5′ end to 3-kinase-A but also displays InsP_3 3-kinase activity when expressed in *E. coli*. Although we do not exclude the fact that 3-kinase-B may still be incomplete at its 5′ end, the expressed protein shows many characteristics one would expect from a putative isoenzyme, i.e. different M_r and different sensitivity to Ca^{2+}/calmodulin. Our results give the first molecular evidence for the existence of InsP_3 3-kinase isoenzymes. Isoenzymes thus provide other targets for the selective pharmacological modulation of the polyphosphoinositide regulatory cascade.

This research was supported by grants from Boehringer Ingelheim, Ministère de la Politique Scientifique (PAI), Association Belge contre le Cancer, FRSM and APMO. We thank C. Moreau for expert technical assistance and Dr. A. Hepburn and Dr. G. Vassart for helpful discussions.

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Received 25 January 1991/28 March 1991; accepted 8 April 1991