Cloning and expression in *Escherichia coli* of a rat brain cDNA encoding a Ca²⁺/calmodulin-sensitive inositol 1,4,5-trisphosphate 3-kinase

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Inositol 1,4,5-trisphosphate (InsP_3) 3-kinase catalyses the phosphorylation of InsP_3 to inositol 1,3,4,5-tetrakisphosphate (InsP_4). InsP_3 3-kinase activity was stimulated by Ca²⁺ in the presence of calmodulin (CaM) and the protein was associated with two silver-stained bands which migrated with an apparent M_r of approx. 50000 on SDS/polyacrylamide gels. Upon limited proteolysis with trypsin, the native InsP_3 3-kinase was converted into polypeptides of M_r 44000 and 36000. Both tryptic fragments displayed InsP_3 3-kinase activity that was Ca²⁺/CaM-sensitive. A cDNA clone, C5, that encodes the C-terminal part of the InsP_3 3-kinase, was isolated by immunoscreening of a rat brain cDNA library. The 5′ end of this clone was used in turn to probe the same library, yielding a clone (CP16) containing the entire coding sequence of InsP_3 3-kinase. The encoding protein of 459 amino acids (calculated M_r 50868) has several putative phosphorylation sites for cyclic AMP-dependent protein kinase, protein kinase C and CaM-dependent protein kinase II. When clone C5 was expressed in *Escherichia coli*, the truncated fusion protein showed Ca²⁺/CaM-sensitive InsP_3 3-kinase activity. Our data demonstrate that the N-terminal part of the protein is not essential for either enzymic or CaM-regulatory properties.

INTRODUCTION

An increase in cellular inositol 1,4,5-trisphosphate (InsP_3) concentration has been shown to mobilize intracellular Ca²⁺ (Berridge & Irvine, 1989). InsP_3 3-kinase catalyses the phosphorylation of InsP_3 to inositol 1,3,4,5-tetrakisphosphate (InsP_4; Irvine et al., 1986). In some experimental models, e.g. mouse lacrimal cells, InsP_4 can synergize with InsP_3 to control intracellular Ca²⁺ (Morris et al., 1987a), suggesting that InsP_4 may be a second potential regulator of cellular Ca²⁺ homeostasis. Crude and purified InsP_3 3-kinases from various sources appear to be Ca²⁺/calmodulin (CaM)-sensitive (Biden et al., 1987; Morris et al., 1987b; Ryu et al., 1987; Yamaguchi et al., 1988; Takazawa et al., 1988). This suggests that InsP_3 3-kinase may be involved in a Ca²⁺ positive-feedback loop. Another regulatory mechanism could be acting in fibroblasts transformed by v-src, where an increase in InsP_3 3-kinase activity has been reported (Johnson et al., 1989).

We recently described the purification of bovine and rat brain InsP_3 3-kinase. InsP_3 3-kinase activity was stimulated by Ca²⁺ in the presence of CaM. Activity could still be assayed after SDS/PAGE (Takazawa et al., 1989, 1990). This opened the possibility of identifying the protein directly in the gel, which was helpful for consecutive antibody production and partial amino acid microsequence determination following electroblotting on inert membranes.

We have now cloned the cDNA corresponding to the rat brain InsP_3 3-kinase. Among the positive clones, isolated by immunoscreening, clone C5 had all the regulatory properties of rat brain InsP_3 3-kinase when expressed in *Escherichia coli*. The truncated InsP_3 3-kinase (M_r 48000) was Ca²⁺/CaM-sensitive; as with the native protein, stimulation by CaM resulted in an increase in $V_{max.}$. Molecular cloning of InsP_3 3-kinase cDNA provides a basis for the precise determination of specific amino acids and domains required for catalytic and Ca²⁺/CaM-regulatory properties.

MATERIALS AND METHODS

Analytical procedures

Measurement of InsP_3 3-kinase activity, purification, Western blotting and immunodetection of proteins were performed as previously described (Takazawa et al., 1990). Immune complexes on blots were detected using alkaline phosphatase-conjugated anti-(rabbit IgG) and corresponding colorimetric methods (Promega). Protein concentration was determined by the procedure of Petersen (1977). Materials for assay, purification and immunoblotting were the same as previously reported (Takazawa et al., 1990). Before immunoscreening of the rat brain cDNA library, InsP_3 3-kinase antibodies were mixed with an *E. coli* lysate to adsorb cross-reactive components and reduce the 'background' signal (Parmentier et al., 1987). Where indicated, antibodies used in Western blots were affinity-purified using isolated clone C5, according to the technique described by Snyder et al. (1987). Purified antibodies reacted with a 50000-M_r doublet in immunoblots of crude rat brain extracts.

Partial amino acid sequence determination

Partial amino acid sequences were obtained by 'in situ' trypsin digestion of gel-purified and polyvinylidene difluoride-membrane-electroblotted InsP_3 3-kinase. Peptides generated by cleavage were separated by narrow-bore reverse-phase h.p.l.c. and collected manually. Details of the procedure are described by Bauw et al. (1989). The major peaks were taken for sequence analysis, which was carried out on an Applied 470A gas-phase protein sequenator equipped with an on-line phenylthiohydantoin amino acid analyser (model 120A). Automated Edman

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degradation as well as amino acid identification was done with the standard programs and reagents provided by the manufacturer.

Limited proteolysis of native InsP$_3$ 3-kinase by trypsin

InsP$_3$ 3-kinase, partially purified from rat brain by Blue–Sepharose and phosphocellulose chromatography, was digested for 5 min at 37 °C in 90 μl of 10 mM Tris/HCl (pH 7.5), 0.1 mM-CaCl$_2$, 1 μM-CaM, 6 mM-2-mercaptoethanol, 0.2 mM-phenylmethanesulphonyl fluoride (PMSF), 2.5 μM-leupeptin and trypsin (1 μg/ml). After addition of 2 μl of trypsin inhibitor (10 mg/ml), the sample was made 62 mM-Tris/HCl, pH 6.8, 3 % SDS, 5 % 2-mercaptoethanol and 10 % glycerol and run on SDS/PAGE (9 % polyacrylamide). Regeneration of InsP$_3$ 3-kinase activity after electrophoresis was performed as previously described (Takazawa et al., 1990).

Molecular cloning

Approx. 10^6 recombinant phages from a rat brain cDNA library in the λ ZAP II vector (Stratagene) were screened by the method of Young & Davis (1983) using a 1:2000 dilution of the InsP$_3$ 3-kinase antibody. Positive clones were plaque-purified and the sizes of their inserts were determined on 1 % agarose gels. To select a full-length clone, the library was screened using a 5' 726 bp EcoRI–SphI restriction fragment, corresponding to the 5' end of clone C5, as a probe. 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 and screened at high stringency. After prehybridization in $6 \times$ SSC buffer ($1 \times$ 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 for 12 h at 42 °C in the same solution containing the cDNA probe. The cDNA· probe was labelled with [α-^{32}P]dATP (Amersham) according to the method of Feinberg & Vogelstein (1983) and used at a specific activity of 2×10^5 c.p.m./ml. Filters were washed three times in $2 \times$ SSC/0.1 % SDS for 30 min at room temperature, and twice in $0.2 \times$ SSC/0.1 % SDS for 30 min at 60 °C. Plaques which remained positive after three successive screenings were purified. cDNA inserts of clones C5 and CP16 were subcloned in M13 and sequenced on both strands using an Applied Biosystems Model 370A sequencer.

Northern blot analysis

After denaturation using glyoxal according to the procedure of Mac Master & Carmichael (1977), 8 μg of poly(A) mRNA isolated from rat brain (fast-track mRNA isolation kit, Invitrogen) was fractionated by electrophoresis on a 1 % agarose gel in 10 mM-sodium phosphate buffer, pH 7.0. Size markers (λ phage cleaved by EcoR1 and HindIII) were treated similarly. Glyoxylated RNA was transferred by diffusion blotting to a nylon membrane (Pall Biodyne A) in $20 \times$ SSC. After baking, the blots were prehybridized overnight at 42 °C in a buffer consisting of 50 % (v/v) formamide, $5 \times$ Denhardt's solution ($100 \times$ solution = 2 % Ficoll and 2 % polyvinylpyrrolidone), $5 \times$ SSPE ($20 \times$ solution = 0.2 M-sodium phosphate, pH 8.3, 3.6 M-NaCl and 20 mM-EDTA), 0.3 % SDS, 250 μg of denatured DNA from salmon testes/ml and 200 μg of BSA/ml. Hybridization was carried out for 48 h at 42 °C in the same buffer as for pre-hybridization, but containing in addition 10 % (w/v) dextran sulphate and the 1.6 kb cDNA insert of clone C5 linearized by EcoRI and labelled with [α^{32}P]dATP as described before. Filters were washed four times for 10 min in $2 \times$ SSC/0.1 % SDS at room temperature, and then four times for 30 min in $0.1 \times$ SSC/0.1 % SDS at 65 °C. Autoradiography was carried out at -70 °C for 3 days.

Expression of InsP$_3$ 3-kinase in E. coli

LB medium (100 ml) containing 50 μg of ampicillin/ml was inoculated with a colony containing the Bluescript plasmid with the cloned DNA insert (C5) at 37 °C to an A_{600} of 0.2. After the addition of isopropyl β-D-thiogalactopyranoside (5 mM) for 10 h, the bacteria were harvested by centrifugation (1200 g, 15 min) and resuspended in 2 ml of cold lysis buffer (50 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 0.4 mM-PMSF, 5 μM-leupeptin, 10 μg of trypsin inhibitor/ml, 12 mM-2-mercaptoethanol and 10 % sucrose). After sonication in ice, Triton X-100 was added [final concentration 1 % (v/v)] and the lysate was centrifuged at 15000 g for 30 min. The supernatant was stored in frozen portions.

RESULTS

### InsP$_3$ 3-kinase purification and microsequence determination

Purified InsP$_3$ 3-kinase from rat brain was associated with two silver-stained bands of about equal activity which migrated with an apparent M_r of about 50000 on SDS/PAGE. Antibodies against the purified InsP$_3$ 3-kinase were obtained by immunization of a rabbit with the purified 50000-M_r protein doublet (Takazawa et al., 1990). These antibodies recognized the 50000-M_r doublet on immunoblots. Amino acid sequences of the protein were derived from two tryptic peptides obtained by 'in situ' digestion of 10 μg of gel-purified and PVDF-electroblotted protein: peptide 1 was completely sequenced (YSWVQLAGHTGSFK), whereas peptide 2 was sequenced over a length of 15 residues (EGNWLPAAGSHLQQP).

Limited proteolysis of native InsP$_3$ 3-kinase with trypsin

Like other CaM-sensitive enzymes [e.g. cyclic nucleotide phosphodiesterase (Krinks et al., 1984) or calcineurin (Manalan & Klee, 1983)], InsP$_3$ 3-kinase could be activated by limited proteolysis (results not shown). As InsP$_3$ 3-kinase activity could be regenerated after SDS/PAGE, we aimed to establish whether large tryptic fragments were still Ca^{2+}/CaM-sensitive after running on SDS/polyacrylamide gels. The native InsP$_3$ 3-kinase was converted into polypeptides of M_r 44000 and 36000. Both polypeptides were detected by Western blotting using the anti-(InsP$_3$ 3-kinase) antibody. Moreover, after SDS/PAGE and renaturation, both had InsP$_3$ 3-kinase activity which could be stimulated by Ca^{2+}/CaM (Fig. 1), although the increase in activity upon addition of Ca^{2+}/CaM was lower than with the native protein. Thus the two tryptic fragments of M_r 44000 and 36000 still have both the CaM binding (or regulatory) domain and the catalytic InsP$_3$ 3-kinase domain.

Selection and sequencing of a cDNA clone for InsP$_3$ 3-kinase

A rat brain cDNA library in phage λ ZAP II was screened using the InsP$_3$ 3-kinase antibody. A total of three positive clones were detected out of 10^6. One of them (C5) showed the strongest signal and was purified to homogeneity. A cDNA of C5 was rescued as a Bluescript plasmid from the vector: it contained a 1.6 kb insert and a poly(A) tail. Additional screening of the library with the 5' end of C5 as a probe was performed and 10 additional positive clones were isolated and characterized. One of these clones, CP16, had an insert of 1.8 kb and was identical with C5 at the 3' end. The two overlapping clones, C5 and CP16 (isolated by immunoscreening and hybridization respectively), were sequenced and the reading frames were analysed. The two sequences of peptides 1 and 2 were present within the same reading frame (Fig. 2). We have assigned the initiation codon to the ATG at position 37, as it is flanked by a sequence that fits Kozak's criteria for a translation initiation codon (Kozak, 1989).
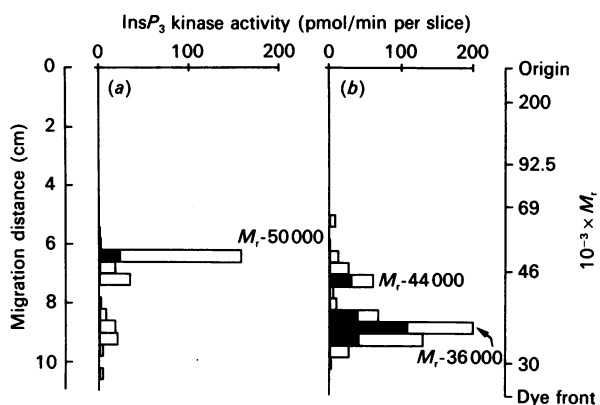
Fig. 1. Effect of limited proteolysis on InsP₃ 3-kinase

After incubation with trypsin at 4 °C (a) or 37 °C (b), proteins (about 4 μg of protein) were separated by SDS/PAGE (9% polyacrylamide). After electrophoresis the gel was cut into 4 mm slices, homogenized in 0.5 ml of 84 mM-Hepes/NaOH (pH 7.5)/0.1% Triton X-100/12 mM-2-mercaptoethanol/25% sucrose/1 mM-EDTA. After 12 h of incubation at 4 °C, fractions were centrifuged (4000 g for 30 min) and the supernatants were assayed for InsP₃ 3-kinase activity in the presence of 1% Triton X-100, 10 μM-InsP₃, 0.1 μM-CaM and either 10 μM free Ca²⁺ (□) or 1 mM-EGTA (■). Peak fractions were assayed in the presence or absence of Ca²⁺/CaM; others were only assayed in the presence of Ca²⁺/CaM. Values are means of duplicates. The apparent M_r value was determined by running M_r standards in a parallel lane.

It is preceded by a purine at position −3 but without a guanine at position +4. This first ATG is followed by a open reading frame of 1377 bp, and a stop codon at position 1414 followed by a 3′ untranslated region composed of 429 bp that includes a putative polyadenylation signal, AATAAAA, 18 bp upstream from the poly(A) tail. The translated sequence of clone CP16 encodes a 459-amino-acid protein with a calculated M_r of 50868 (including the initiating methionine). Within the same reading frame, another putative initiation codon could be assigned to position 64, which also has a purine in position −3. This codon is followed by a guanine, and according to the criteria of Kozak (1989), it may direct the synthesis of another form of InsP₃ 3-kinase containing 450 amino acids and with an M_r of 49957. There are several potential phosphorylation sites based on consensus phosphorylation site sequences for cyclic AMP-dependent protein kinase (i.e. R-R-X-S), CaM-dependent protein kinase II [R-X-X-S/T (Soderling, 1990)] and protein kinase C [S/T-X-R/K (Woodgett et al., 1986)]. These are Ser-119 for cyclic AMP-dependent protein kinase, Ser-119 and Thr-309 for CaM-dependent protein kinase II, and Ser-195 and Thr-346 for protein kinase C (Fig. 2). A computer search of the Swiss-Prot Sequence Database of February 1990 using the FASTP program revealed no significant similarities with other proteins, particularly the inositol monophosphatase which has recently been cloned (Diehl et al., 1990).

Northern blot analysis of poly(A) mRNA from rat brain was performed at high stringency using cDNA clone C5 as a probe (Fig. 3). A single mRNA of approx. 1.8 kb was visualized.
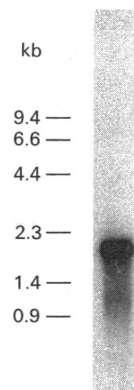
Expression and purification of the truncated InsP₃ 3-kinase in E. coli

To confirm that the cDNA clone obtained did indeed code for the InsP₃ 3-kinase, fusion proteins derived from C5 or from a non-recombinant phage were expressed by the Bluescript plasmid after excision in vivo. Sonicated bacterial lysates derived from C5 had high InsP₃ 3-kinase activity which was Ca²⁺/CaM-sensitive (Table 1). The specific activity of a crude bacterial lysate derived

Fig. 2. Nucleotide and deduced amino acid sequence of InsP₃ 3-kinase

Nucleotide sequence analysis was performed on overlapping clones C5 and CP16. Clone C5 started at position 232. The two peptide sequences (peptide 1 and peptide 2) are underlined. Putative phosphorylation sites for cyclic AMP-dependent protein kinase, protein kinase C and CaM-dependent protein kinase II are underlined in bold.


Fig. 3. Northern blot analysis of poly(A) mRNA of rat brain

Blotting was carried out as described in the Materials and methods section.

Table 1. Purification of the truncated InsP₃ 3-kinase expressed in *E. coli*

InsP₃ 3-kinase activity was assayed at 10 μM-InsP₃ in the presence of 0.1 μM-CaM and 10 μM free Ca^{2+} or 1 mM-EGTA for basal activity. Total and specific activities are given in the presence of Ca^{2+}/CaM.

Step	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification (-fold)	Yield (%)	Stimulation by CaM (-fold)
Crude lysate	22.4	2.00	0.089	1	100	1.40
CaM–Sepharose	0.0765	0.633	8.3	93	32	2.20
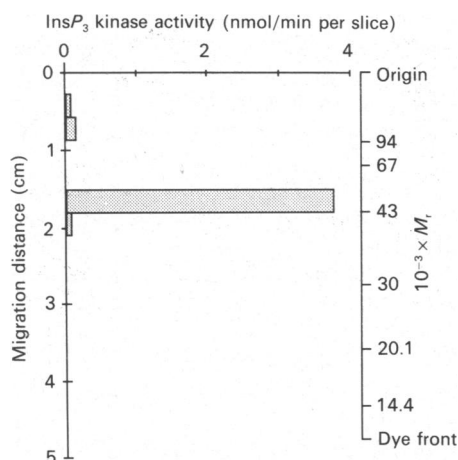
Fig. 4. SDS/PAGE of InsP₃ 3-kinase expressed by *E. coli* (clone C5)

A 500 μg portion of crude lysate of clone C5 was applied to an SDS/11%-polyacrylamide slab gel (total length 4.8 cm). After electrophoresis, one lane was cut into 3 mm slices and activity was regenerated as described in the legend of Fig. 1. Values are the means of triplicates.
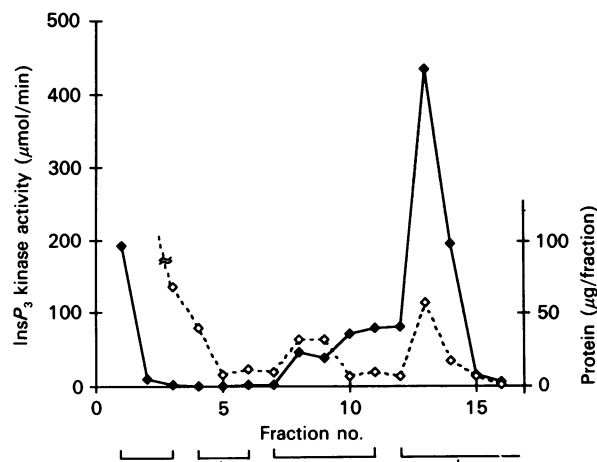
Fig. 5. CaM–Sepharose chromatography of expressed truncated InsP₃ 3-kinase

A crude lysate of expressed clone C5 (22 mg of protein) was applied to a CaM-Sepharose column (2.5 cm × 6 cm) and elution was performed in 20 mM-Tris/HCl, 12 mM-2-mercaptoethanol, protein-ase inhibitors and the following additions: a, 0.2 mM-CaCl₂/0.4 M-NaCl/0.5% Triton X-100; b, 0.2 mM-CaCl₂/0.4 M-NaCl; c, 2 mM-EGTA/0.4 M-NaCl; d, 2 mM-EGTA/0.4 M-NaCl/0.1% Triton X-100. The volume of fractions 1–11 was 20 ml and that of later fractions was 15 ml. Ca^{2+}/CaM-stimulated activity (◆) was determined at 10 μM free Ca^{2+} and 0.1 μM-CaM. Protein content is indicated by ◇. Pooled peak fractions were concentrated to approx. 1.5 ml using a PM10 ultrafiltration membrane and stored at −70 °C. This profile is taken from one representative experiment out of three.

from C5 was 90 nmol/min per mg of protein, i.e. about 10-fold higher than in crude rat brain soluble fraction (Takazawa *et al.*, 1990). The *E. coli*-derived InsP₃ 3-kinase could be renatured after SDS/PAGE. A single peak of activity (M_r 48000) was isolated by this procedure (Fig. 4). In contrast, lysates which originated from the non-recombinant phage had no activity.

The truncated fusion protein could be substantially purified by CaM–Sepharose affinity chromatography (Table 1). Like the native InsP₃ 3-kinase from rat brain, the truncated enzyme was specifically eluted in buffer containing 2 mM-EGTA and 0.1% Triton X-100 (Fig. 5). InsP₃ 3-kinase antibodies directed against the native enzyme recognized, on Western blots, an M_r 48000 band in lysates derived from C5 before and after CaM–Sepharose following EGTA elution (Fig. 6). When the experiment was repeated with a lysate derived from a non-recombinant clone which did not contain InsP₃ 3-kinase activity, no signal on immunoblot was detected after CaM–Sepharose and EGTA elution (results not shown). Stimulation of InsP₃ 3-kinase activity
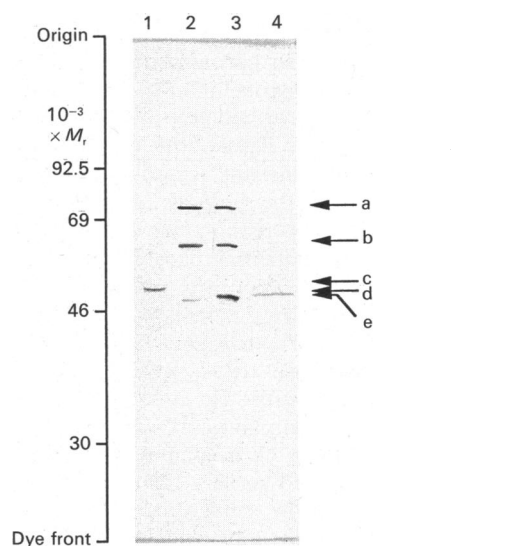
Fig. 6. Western blot of native and expressed truncated InsP₃ 3-kinase

Samples were processed by SDS/PAGE on 10% polyacrylamide gels and the resolved proteins were transferred to nitrocellulose. Immunoblot analysis was performed using clone C5-purified anti-serum. Lane 1 is crude soluble fraction from rat brain (enzyme activity at 10 μM-InsP₃ and in the presence of Ca^{2+}/CaM is 0.4 nmol/min); lane 2 is a bacterial lysate from a non-recombinant clone (approx. 6 μg); lane 3 is a bacterial lysate which originated from clone C5 (approx. 6 μg); lane 4 is the EGTA eluate from a 0.4 ml CaM–Sepharose column loaded with a lysate of clone C5 (enzyme activity 0.2 nmol/min). The arrows a, b, e indicate cross-reactive bands present in both bacterial lysates; band c refers to the M_r-50000 doublet present in rat brain; band d is the expressed fusion protein.
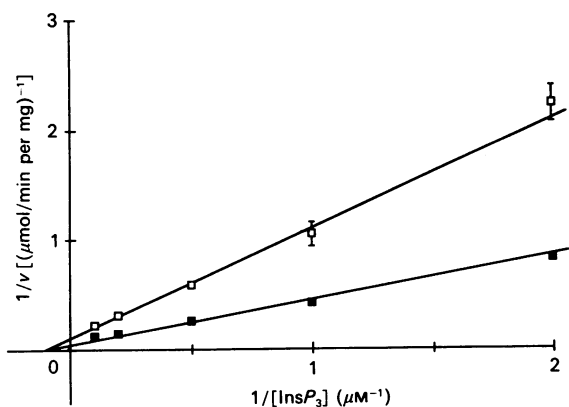
Fig. 7. Substrate–velocity relationships of expressed truncated InsP_3 3-kinase

The data are shown as a double reciprocal plot with the InsP_3 3-kinase concentration in the 0.5–10 μM range. Samples of CaM–Sepharose-purified enzyme were assayed in the presence of 0.1 μM-CaM plus 10 μM free Ca^{2+} (■) or 1 mM-EGTA (□).

Table 2. Comparison of kinetic properties of the native rat brain InsP_3 3-kinase with those of the truncated form expressed in *E. coli*

Data for the rat brain are from Takazawa *et al.* (1990). The data for the expressed form are calculated from Fig. 7.

Enzyme...	Expressed	Rat brain
K_m (μM)	8.8	11
$V_{max.}$ (μmol/min per mg of protein)		
Basal	8.3	1.5
Ca^{2+}/CaM-stimulated	20.0	9.5

by Ca^{2+}/CaM in the truncated InsP_3 3-kinase resulted from an increase in the $V_{max.}$ of the enzyme, with no change in the K_m for InsP_3 (Fig. 7 and Table 2). Thus the cDNA of clone C5 encodes a protein with Ca^{2+}/CaM-regulated InsP_3 3-kinase activity.

DISCUSSION

A cDNA clone, C5, that encodes the *C*-terminal part of the InsP_3 3-kinase has been isolated by screening of a rat brain cDNA library with antibodies against M_r-50000 native InsP_3 3-kinase. The 5' end of this clone was used in turn to probe the same library to isolate a cDNA clone, CP16, which overlaps C5 and contains the entire coding sequence of InsP_3 3-kinase. Microsequencing of native InsP_3 3-kinase yielded two peptide sequences. These two sequences were identified on clone C5 (and CP16) within the same reading frame after DNA sequencing and amino acid translation. We have proposed two putative initiation codons which may direct the synthesis of two forms of InsP_3 3-kinase of M_r 50868 and 49957. 'Leaky scanning' of the first AUG codon may enable some ribosomes to reach and initiate at the second AUG (Kozak, 1989). Consistent with this hypothesis is the pattern of the native protein on SDS gels, which migrates as a doublet in the M_r 50000 region (Takazawa *et al.*, 1990).

CaM binding sites are not sequence-specific; CaM recognizes basic amphiphilic α-helices. These domains of CaM-regulated proteins often contain clusters of positively charged and hydrophobic amino acids (O'Neil & DeGrado, 1990). We have identified two fragments which could fit these criteria: amino acid residues 213–229 and 402–416. However, precise identifi-

cation of the binding sites remains to be proven by future experiments.

Results obtained from limited tryptic digestion of InsP_3 3-kinase reveal that a small fragment of M_r 36000 was active and Ca^{2+}/CaM-sensitive. Thus the limited size of the protein that would contain both the catalytic and CaM-binding domains is M_r 36000. We therefore attempted to express the partial cDNA clone C5 (isolated by immunoscreening) and compare catalytic and CaM-binding properties.

Clone C5 was rescued as a Bluescript plasmid and expressed in *E. coli*. The InsP_3 3-kinase portion of the expressed protein had 394 amino acids and a calculated M_r of 44226. It showed Ca^{2+}/CaM-sensitive InsP_3 3-kinase activity. As the β-galactosidase fragment fused to cDNA inserts in λ ZAP is about M_r-4000–5000, we estimate the total M_r of the expressed InsP_3 3-kinase fusion protein to be 48000–49000. Consistent with this is the M_r determined on Western blot with InsP_3 3-kinase antibodies or directly after SDS/PAGE and regeneration of activity (48000). The expressed truncated InsP_3 3-kinase was purified by CaM–Sepharose chromatography and had kinetic properties similar to those of the rat brain enzyme. K_m values were identical and CaM modified the $V_{max.}$ in a similar way. In agreement with the results on limited trypsin digestion of the native enzyme, this suggests that a portion of the full cDNA sequence may contain the necessary information to serve as Ca^{2+}/CaM-sensitive InsP_3 3-kinase. Our data demonstrate that the *N*-terminal part of the protein is not essential for either enzymic or CaM-regulatory properties.

After completion of this work, the sequence of the rat brain InsP_3 3-kinase was reported by Choi *et al.* (1990). They used a different cloning strategy and expressed InsP_3 3-kinase in COS cells. Although the two primary structures were identical, InsP_3 3-kinase expressed in *E. coli* (this study) displayed higher specific activity and was shown to be Ca^{2+}/CaM-sensitive, thus demonstrating the presence of a CaM regulatory domain in the protein. Choi *et al.* (1990) reported their InsP_3 3-kinase to be calpain-sensitive and suggested that calpain-dependent cleavage of InsP_3 3-kinase might be of physiological importance. Proteolytic modification of CaM-sensitive enzymes has been reported for most, if not all, CaM-binding proteins (e.g. Krinks *et al.*, 1984). However, proteolytic (irreversible) activation does not appear likely as a mechanism for physiological (reversible) control. On the other hand, the presence in the sequence of putative phosphorylation sites is compatible with reversible regulation of InsP_3 3-kinase by a more physiologically relevant phosphorylation mechanism. For example, in neuromodulin, protein kinase C phosphorylation has been shown to abolish the affinity of the protein for CaM (Liu & Storm, 1990).

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