Structural identification of the *myo*-inositol 1,4,5-trisphosphate-binding domain in rat brain inositol 1,4,5-trisphosphate 3-kinase

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A series of key amino acids involved in Ins(1,4,5)P_3 (InsP_3) binding and catalytic activity of rat brain InsP_3 3-kinase has been identified. The catalytic domain is at the C-terminal end and restricted to a maximum of 275 amino acids [Takazawa and Erneux (1991) Biochem. J. **280**, 125–129]. In this study, newly prepared 5′-deletion and site-directed mutants have been compared both for InsP_3 binding and InsP_3 3-kinase activity. When the protein was expressed from L259 to R459, the activity was lost but InsP_3 binding was conserved. Another deletion mutant that had lost only four amino acids after L259 had lost InsP_3 binding, and this finding suggests that these residues (i.e. $L^{259}DCK^{262}$) are involved in InsP_3 binding. To further support the data, we have produced two mutants by site-directed mutagenesis on residues C261 and K262. The two new enzymes were designated M4 (C261S) and M5 (K262A). M4 showed similar V_{max} and K_m values for InsP_3 and ATP to wild-type enzyme. In contrast, M5 was totally inactive but had kept the ability to bind to calmodulin–Sepharose. C-terminal deletion mutants that had lost five, seven or nine amino acids showed a large decrease in InsP_3 binding and InsP_3 3-kinase activity. One mutant that had lost five amino acids (M2) was purified to apparent homogeneity: K_m values for both substrates appeared unchanged but V_{max} was decreased approx. 40-fold compared with the wild-type enzyme. The results indicate that (1) a positively charged amino acid residue K262 is essential for InsP_3 binding and (2) amino acids at the C-terminal end of the protein are necessary to act as a catalyst in the InsP_3 3-kinase reaction.

INTRODUCTION

Ins(1,4,5)P_3 (InsP_3) is a second messenger that is responsible for generating highly organized Ca^{2+} signals in various types of cells [1]. InsP_3 is inactivated by two pathways: it is either phosphorylated by an InsP_3 3-kinase to yield Ins(1,3,4,5)P_4 (InsP_4) [2] or dephosphorylated by an InsP_3 5-phosphatase to produce Ins(1,4)P_2. Evidence suggests that InsP_4 may itself regulate the intracellular concentration of Ca^{2+} by promoting Ca^{2+} sequestration [3], or by acting synergistically with InsP_3 to mobilize intracellular Ca^{2+} stores in mouse lacrimal cells [4] and Swiss-mouse 3T3 cells [5]. Other studies in rat cerebellar cells [6], adrenal cortical microsomes [7] and neuroblastoma cells [8] have provided evidence for a direct second-messenger function of InsP_4 in the release of Ca^{2+}. The finding of specific InsP_4-binding sites in various tissues further supports a possible involvement of InsP_4 in the phosphoinositide pathway. In pig cerebellum, Reiser et al. [9] purified a 42 kDa protein, and in rat brain, Theibert et al. [10] isolated two separate fractions with high affinity for InsP_4. cDNA cloning of an InsP_4-binding protein shows an encoded protein that is a member of the GAP1 family [11].

Free Ca^{2+} within the physiological range of 0.1–1 μM is a potent stimulator of InsP_3 3-kinase in lysed cell preparations of RINm5F cells [12], mouse thymocytes [13] and rat hepatocytes [14]. However, it appears that the interaction between Ca^{2+} and calmodulin (CaM) is the true activator of InsP_3 3-kinase [15–19]. This interaction was used to purify this enzyme by affinity chromatography on CaM–Sepharose [20,21]. The purified enzyme from rat brain was associated with a 50 kDa protein doublet on SDS/PAGE [22]. cDNAs encoding 50 kDa InsP_3 3-kinase have been isolated from rat and human brain libraries [23,24]. InsP_3 3-kinase activity was expressed in bacteria as a β-galactosidase fusion protein and shown to be Ca^{2+}/CaM sensitive [23]. Previous studies indicated that the sequence stretching from S156 to L189 is involved in CaM binding and that a maximum of 275 amino acids at the C-terminal end may be sufficient for the construction of a catalytically active domain [25,26]. Site-directed mutagenesis and covalent chemical modification of rat brain InsP_3 3-kinase have shown that three charged residues (K197, R317 and D414) are involved in ATP/Mg^{2+} binding [27]. Amino acid residues involved in InsP_3 binding are unknown. In this study, we report that a positively charged amino acid residue, K262, is essential for InsP_3 binding and that amino acids at the C-terminal part of the protein are necessary to act as a catalyst in the InsP_3 3-kinase reaction.

MATERIALS AND METHODS

Construction of deletion mutants

For unidirectional deletion starting from the 5′-end of clone CP16 [23], the exonuclease III, mung bean nuclease deletion kit (Stratagene) was used as previously reported [25]. Newly generated mutants were in-frame with the β-galactosidase segment of the plasmid pBluescript as verified by DNA sequencing.

Oligonucleotide-directed *in vitro* mutagenesis

Site-directed mutants (M4 and M5) and a series of C-terminal deletion mutants (M1, M2 and M3) were constructed

Abbreviations used: CaM, calmodulin; InsP_3, Ins(1,4,5)P_3; InsP_4, Ins(1,3,4,5)P_4.

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according to the method of Sayers et al. [28], using an oligonucleotide-directed *in vitro* mutagenesis kit from Amersham. A 1524 bp *Sma*I–*Eco*RI fragment from clone C5 was subcloned into the M13mp18 vector to obtain single-stranded DNA. This DNA was used as the template for mutagenesis. The mutagenic oligonucleotides follow: M1, 5′-CATTGGCATCTaGaCCAACCTGGCTG-3′, L453 changed to a stop codon (TAG) (*Xba*I); M2, 5′-GGCATCTTGGCCtAaCTGGCTGAGAGAT-3′, D455 changed to a stop codon (TAA) (*Bal*I) (TGGCCA) deleted; M3, 5′-GGACAATCTCATTtaaATCTTGGCCAACCT-3′, G451 changed to a stop codon (TAA) (*Dra*I); M4, 5′-GCGTGCTTGAtTcgAAGATGGGTGT-3′, C261 changed to S (TCG) (*Asu*II); M5, 5′-GCTTGACTGCgcaATGGGTGTCAG-3′, K262 changed to A (GCA) (*Avi*II). Mutated bases are shown as small letters, and the newly created (or deleted) restriction sites are underlined. These mutants were identified by restriction mapping. The nucleotide sequence of each mutant was verified by single-stranded DNA sequence analysis. These altered DNAs were then inserted into the pBluescript vector to express the mutant proteins [23].

InsP_3-binding assay

A single colony of *E. coli* that had been transformed with mutated DNA was inoculated in 50 ml of Luria broth medium that contained 50 μg/ml ampicillin and incubated overnight at 37 °C. Incubation was then continued for 3 h more in the presence of 5 mM isopropyl β-thiogalactoside (Takara Shuzo Co.). The bacteria were harvested by centrifugation, resuspended in 2 ml of cold buffer [50 mM Tris/HCl (pH 8)/1 mM EDTA/0.2 mM PMSF/2.5 μM leupeptin/12 mM 2-mercaptoethanol], and sonicated for 1 min. After the addition of 50 μl of 20 % (w/v) CHAPS (Sigma), the lysate was centrifuged (15000 ***g***) for 10 min to yield the supernatant used in the binding assay.

The binding of [^{3}H]InsP_3 to this bacterial lysate was determined with a polyethylene glycol precipitation method as previously reported [29] with slight modifications. Briefly, each incubation mixture (100 μl) contained 80 μl of the bacterial lysate and 10000 d.p.m. [^{3}H]InsP_3 (American Radiolabeled Chemicals). After 10 min at 4 °C, 10 μl of 50 mg/ml bovine gamma globulin (Sigma) and 100 μl of 30 % (w/v) polyethylene glycol solution were added, mixed, and kept at 4 °C for an additional 10 min. This mixture was then centrifuged for 10 min at 15000 ***g***. The pellets were washed twice with 15 % polyethylene glycol solution, resuspended in 300 μl of water and mixed with 3 ml of ACSII scintillation cocktail. Radioactivity was measured by liquid-scintillation counting. Non-specific binding was measured in the presence of 10 μM unlabelled InsP_3 (Dojindo Laboratories).

Western blotting

After standard SDS/PAGE analysis [22], proteins were transferred to clear blot membrane-P by using a horizontal electroblotting apparatus (ATTO). Affinity-purified rabbit anti-(rat brain InsP_3 3-kinase) antibody [25] and peroxidase-conjugated anti-rabbit antibody (Jackson Immuno Research Laboratories) were used for immunodetection. Coloration was done with diaminobenzidine (Dojindo Laboratories) as the substrate for antibody-conjugated peroxidase.

Protein purification

Two mutants (M2 and M4) were purified by CaM–Sepharose affinity chromatography as previously reported [23].

Analytical procedures

InsP_3 3-kinase activity was measured as previously described [25]. Protein concentrations were measured according to the Bradford method [30] and results were expressed as means ± S.D. Statistical differences were investigated with the Student's t test. Data were considered to be statistically different when $P < 0.05$.

RESULTS

Characterization of 5′-deletion mutants

A series of active (4.5M4) and inactive (5.5M3 and C5B) mutants have been obtained in a previous report [25]: mutant 4.5M4, beginning with S185, was active, but mutant 5.5M3, beginning with I205, was inactive. Mutant C5B, a C-terminal deletion mutant lacking 34 amino acids, was also totally inactive (Figures 1 and 2). From this we concluded that 275 amino acids in the C-terminal region represented a putative catalytic domain. The sequence conservation between InsP_3 3-kinase-A and -B in this region is consistent with this conclusion [31], which seems to suggest that this domain may be conserved in other members of this enzyme family as well.

Our objective was to identify the amino acids involved in the binding of InsP_3 to InsP_3 3-kinase. This was facilitated by the use of an InsP_3-binding assay in which bound [^{3}H]InsP_3 was specifically displaced by unlabelled InsP_3 ($IC_{50} = 0.9 \pm 0.2$ μM for a crude bacterial lysate derived from clone C5).

Mutant 5.5M3, which lost InsP_3 3-kinase activity, still exhibited InsP_3 binding (Table 1). This signifies that the InsP_3-binding site exists downstream of I205. Thus we constructed nine additional N-terminal truncation mutants (D2.0M3–D3.0M5) (Figures 1 and 2) to more accurately identify the InsP_3-binding domain.

Figure 1 Comparison of the primary structure of InsP_3 3-kinase

The 309 C-terminal amino acids in rat brain InsP_3 3-kinase are shown in this Figure, which include the CaM-binding domain (shaded) and the catalytic domain. Only non-conserved amino acids are indicated for the other sequences. The asterisks represent the single amino acid gaps that were introduced to allow maximum similarity. The N- or C-terminal ends of each truncation mutant are indicated by ⌜ or ⌝ above the sequence. The ATP-binding domain is underlined.

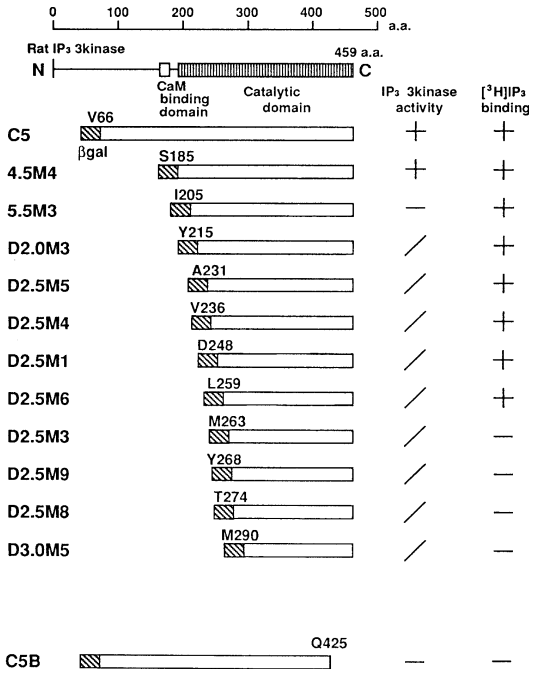

Figure 2 InsP_3 3-kinase truncation mutants

A schematic representation of each mutant is shown. The InsP_3 3-kinase (IP_3 3kinase) activity data are taken from our previous report [1]. InsP_3 ([^{3}H]IP_3) binding is taken from Figure 3. '/' means not determined.

Table 1 InsP_3 binding analysis of N-terminal truncation mutants

The bacterial lysates were prepared as described in the Materials and methods section and were used to determine [^{3}H]InsP_3 binding to the mutant enzymes. Non-specific binding was determined in the presence of 10 μM unlabelled InsP_3. Specific [^{3}H]InsP_3 binding was determined by subtracting the non-specific binding from the total binding. Data are means ± S.D. ($n = 4$). n.s., not significant.

	[^{3}H]InsP_3 binding (d.p.m.)		
Clone	Unlabelled InsP_3 (10 μM)	Unlabelled InsP_3 (−)	*P*
C5 V66	174 ± 26	897 ± 55	< 0.001
4.5M4 S185	175 ± 9	679 ± 90	< 0.001
5.5M3 I205	214 ± 48	532 ± 61	< 0.001
D2.0M3 Y215	119 ± 11	386 ± 8	< 0.001
D2.5M5 A231	186 ± 7	273 ± 24	< 0.001
D2.5M4 V236	168 ± 13	657 ± 9	< 0.001
D2.5M1 D248	220 ± 55	441 ± 94	< 0.01
D2.5M6 L259	270 ± 14	422 ± 19	< 0.001
D2.5M3 M263	256 ± 12	270 ± 9	n.s.
D2.5M9 Y268	225 ± 21	221 ± 4	n.s.
D2.5M8 T274	305 ± 33	329 ± 9	n.s.
D3.0M5 M290	169 ± 21	192 ± 18	n.s.

From the results of Western blotting analysis with anti-(rat InsP_3 3-kinase-A) antibody (Figure 3), these mutants were expressed in an almost equal quantity. By using crude bacterial lysates from the expression system for these mutants, binding assays were performed in the presence or absence of unlabelled InsP_3 (Table 1). A series of N-terminal truncation mutants, numbered C5 to D2.5M6, revealed significant increases in [^{3}H]InsP_3 binding in

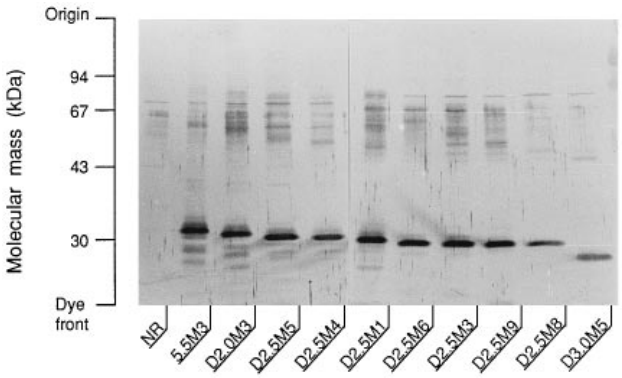

Figure 3 Western-blot analysis of N-terminal truncation mutants

The bacterial lysates were prepared as described in the Materials and methods section. SDS/PAGE [10% (w/v) gel] of crude bacterial lysates (0.5 μg of protein) was performed. Subsequent immunoblot analysis was done by using affinity-purified anti-(rat brain InsP_3 3-kinase) antibody. Lane NR is a bacterial lysate from a non-recombinant clone.

Table 2 InsP_3 binding and InsP_3 3-kinase activity of the mutants

Data of InsP_3 binding are means ± S.D. ($n = 4$). InsP_3 3-kinase activity was determined with 10 μM InsP_3 in the presence of 0.1 μM CaM and 10 μM free Ca^{2+} or 1 mM EGTA by using crude bacterial extracts from individually expressed clones. n.d., activity was undetectable. Data are means ± S.D. of triplicate determinations. n.s., not significant.

	[^{3}H]InsP_3 binding (d.p.m.)			InsP_3 3-kinase activity (nmol/min per mg)	
Clone	InsP_3 (10 μM)	InsP_3(−)	*P*	EGTA	Ca^{2+}/CaM
C5 R459	220 ± 20	960 ± 50	< 0.001	691 ± 103	1331 ± 117
M2 A454	241 ± 31	328 ± 13	< 0.001	31.0 ± 3.0	47.5 ± 1.7
M1 I452	203 ± 13	321 ± 12	< 0.001	9.0 ± 0.4	12.8 ± 0.4
M3 I450	175 ± 5	225 ± 10	< 0.01	0.27 ± 0.02	0.35 ± 0.01
M4 C261S	238 ± 2	697 ± 41	< 0.001	176 ± 1	328 ± 33
M5 K262A	160 ± 20	190 ± 20	n.s.	n.d.	n.d.

the absence of unlabelled InsP_3 compared with that in the presence of 10 μM unlabelled InsP_3. The amounts of [^{3}H]InsP_3 binding were different in each clone. We supposed that these deletions might slightly affect the conformation of the InsP_3-binding domain and result in the differences in the amount of [^{3}H]InsP_3 binding. We did not analyse this point further because it attracted to our attention that shorter mutants D2.5M3 to D3.0M5 showed no significant binding. These results suggested that four amino acid residues between the D2.5M6 and D2.5M3 sequences, i.e. $L_{259}DCK_{262}$, were essential for InsP_3 binding. To study this further, we replaced the C261 with S (Ser) and K262 with A (Ala), by using site-directed mutagenesis. Mutant M4 (C261S) retained both its InsP_3 3-kinase activity and its InsP_3-binding activity, but M5 (K262A) totally lost both of these functions (Table 2). Mutant M4 was purified by using CaM–Sepharose affinity chromatography (Figure 4), and the K_m and V_{max} values for InsP_3, as well as the K_m for ATP, were determined (Table 3). These values resembled those of the wild-type protein (C5) and the mutant (M4) as well. CaM–Sepharose affinity chromatography was also used to determine if the M5 mutant retained CaM-binding ability. This mutant bound to the column in the presence of Ca^{2+} and was eluted with EGTA (results not

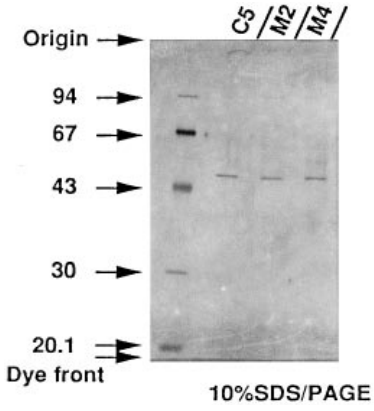

Figure 4 SDS/PAGE of purified wild-type and mutant proteins expressed in *E. coli*

Wild-type (C5) and mutant (M2 and M4) InsP_3 3-kinase were expressed in *E. coli* as β-galactosidase fusion proteins. Following purification by using CaM-affinity chromatography, 20 ng of purified protein was applied to each lane and samples were electrophoresed. The gel was then silver-stained to observe the proteins. Molecular-mass markers (kDa) are shown on the left.

Table 3 Kinetic properties of the wild-type and mutant InsP_3 3-kinase enzymes expressed in *E. coli*

Data were obtained from the purified recombinant InsP_3 3-kinase illustrated in Figure 4.

	V_{max} (μmol/min per mg) (InsP_3)		K_m (μM) (InsP_3)	K_m (mM) (ATP)
	Ca^{2+}/CaM	EGTA		
C5 (wild-type)	4.55	1.96	0.83	0.43
M2 (A454)	0.114	0.056	1.92	0.69
M4 (C261S)	1.82	0.60	1.56	0.30

shown). From these data, we concluded that mutating K262 to A in the M5 clone resulted in the loss of InsP_3 binding followed by a loss of catalytic activity. Interestingly, the CaM-binding ability of this mutant was unaffected.

Characterization of the 3′-deletion mutants

The C-terminal truncation mutant C5B, which lacked 34 amino acids, exhibited no InsP_3 3-kinase activity, thus corroborating previous work [25]. In addition, this mutant displayed no InsP_3 binding (Figure 2). Based on these data we constructed a series of C-terminal truncation mutants by introducing additional stop codons within the coding sequence. This resulted in the generation of mutants M2, M1 and M3, that were truncated by five, seven and nine amino acids in the C-terminus respectively. Although these mutants all bound InsP_3 (Table 2), the amount of binding was less than that in the wild-type (C5). Conversely, these mutants showed abrupt losses of the enzyme activity, 1/30 for M2, 1/100 for M1 and 1/3800 for M3, as compared with C5 (Table 2). These experiments confirmed that each of these clones could express approximately the same amount of encoded proteins as seen by Western-blotting analysis (results not shown). Hence the differences in InsP_3 binding and in enzyme activity could not be attributed to differences in protein expression. Clone M2, which was missing only five C-terminal amino acids, was also purified to apparent homogeneity (Figure 4) by using CaM–Sepharose affinity chromatography. The K_m for ATP and InsP_3 of the M2 clone resembled that of the wild-type (C5); however, the V_{max} was only 1/40th that of C5. This deletion did not affect the sensitivity of the enzyme to Ca^{2+}/CaM.

DISCUSSION

The present results of binding assays led us to conclude that a minimum of 201 amino acids within the C-terminus, i.e. L259 to R459, is sufficient to form an InsP_3-binding domain.

K197 was shown to be required for the binding of ATP/Mg^{2+}, which supports previous data implicating the sequence G^{191}HTGSFK^{197}, which includes K197, as a possible ATP-binding site [27]. This conclusion is consistent with our observation that InsP_3 3-kinase activity disappeared between mutants 4.5M4 (S185) and 5.5M3 (I205) (Figures 1 and 2), because of loss of the ATP-binding site. Even without the ATP-binding site, shorter truncation mutants (5.5M3 to D2.5M6) could bind to InsP_3. This finding indicated that the InsP_3-binding domain is away from the ATP-binding site in the primary structure, and that the deletion of the ATP-binding site does not produce a conformational change that abolishes the InsP_3-binding domain tertiary structure.

Clone D2.5M6, the shortest deletion mutant that maintained InsP_3 binding, was only four amino acids longer than D2.5M3, which did not bind InsP_3. Since all of our expressed mutants contained an N-terminal peptide derived from β-galactosidase, this peptide may alter the conformation of the InsP_3 3-kinase fusion protein. To clarify this issue, we replaced one of the amino acids in this region. We previously cloned an InsP_3 3-kinase isoenzyme from the human brain that was named InsP_3 3-kinase-B [31]. Interestingly, we found that the sequence $D_{260}CK_{262}$ was common to both InsP_3 3-kinase-A and -B. C261 can be made to form a disulphide bond with another cysteine to determine this enzyme's tertiary structure. Despite this possibility, mutant M4 (C261S) showed nearly the same K_m and V_{max} as the wild-type enzyme (Table 3). We therefore concluded that C261 is not required for either InsP_3 binding or InsP_3 3-kinase activity. In contrast, M5 (K262A) lost both its InsP_3-binding ability and InsP_3 3-kinase activity. Lysine is a positively charged residue and could form an ionic interaction with the phosphate of InsP_3. It is questionable whether one particular amino acid substitution could produce a fatal conformational change that abolishes enzyme activity and InsP_3 binding. Secondary-structure predictions according to the methods of Chou and Fasman [32] may provide insight into this issue. The Chou and Fasman analysis showed that the substitution of K262 by A did not alter the secondary structure (β-sheet) around this region (results not shown). This analysis indicates that K262 is probably a key residue in the InsP_3-binding domain.

Recently, Fukuda et al. [33] and Cullen et al. [11] suggested the presence of an InsP_4 or inositol polyphosphate-binding domain in InsP_4-binding proteins. Fukuda et al. [33] proposed that 30 amino acids (IHLMQNGKRLKKKKTTVKKKTLNPYFNESFSF) in the central region of the C2B domain of mouse IP4BP/synaptotagmin II, which is rich in lysine, are essential for the binding of inositol polyphosphate. InsP_3 3-kinase-A and -B do not have such a lysine-rich region, especially around K262. Cullen et al. [11] reported the existence of a highly specific IP4-binding motif, including (R/K)(R/K)TKX(R/K)-(R/K)KT, in some of the GAP1 family proteins. Nevertheless, such an InsP_4-binding motif is not found in InsP_3 3-kinase-A and -B.

The results obtained from our C5B analysis suggested that the C-terminus may also be necessary for catalysis. By constructing

mutants with minimal C-terminal deletions, we showed that the M2 mutant significantly bound to InsP_3, although the value was less than that of the wild-type protein (Table 2). However, our kinetic analysis (Table 3) showed that this deletion had little effect on the K_m for InsP_3. This finding suggested that a portion of the C-terminus is not involved in InsP_3 binding. Conversely, such C-terminal deletions resulted in considerable decreases in the enzyme activity, despite an intact InsP_3-binding ability (Tables 2 and 3). A comparison of the primary structures of the InsP_3 3-kinase isoenzymes (Figure 1) revealed that six amino acids in the C-terminus of InsP_3 3-kinase-A differed completely from those in InsP_3 3-kinase-B. Thus we concluded that within the nine C-terminal residues studied, no single amino acid confers catalytic activity on this enzyme. Rather, the entire peptide appears to be needed to govern the conformation of the catalytic domain of InsP_3 3-kinase.

The InsP_3 receptor and InsP_3 5-phosphatase are other proteins that InsP_3 can utilize as a ligand or a substrate. InsP_3 5-phosphatase cDNA [34,35] and InsP_3 receptor cDNAs [36,37] have been cloned. The InsP_3-binding domain is located within the 650 amino acids of the N-terminus of the receptor [38,39]. No significant similarities in the localized primary structures of InsP_3 3-kinase, InsP_3 5-phosphatase and the InsP_3 receptor were detected.

In conclusion, we found that (1) a positively charged amino acid residue, K262, is essential for InsP_3 binding, and (2) amino acids at the C-terminal end of the protein are necessary to act as a catalyst in the InsP_3 3-kinase reaction. We suggested that distantly separated residues in the primary structure would ultimately compose the InsP_3-binding domain and the catalytic domain in the tertiary structure of InsP_3 3-kinase.

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Received 3 December 1996/2 April 1997; accepted 15 April 1997