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Molecular Cloning and Expression of a Complementary DNA for Inositol 1,4,5-Trisphosphate 3-Kinase

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A complementary DNA (cDNA) clone that encodes inositol 1,4,5-trisphosphate 3-kinase was isolated from a rat brain cDNA expression library with the use of monoclonal antibodies. This clone had an open reading frame that would direct the synthesis of a protein consisting of 449 amino acids and with a molecular mass of 49,853 daltons. The putative protein revealed a potential calmodulin-binding site and six regions with amino acid compositions (PEST regions) common to proteins that are susceptible to calpain. Expression of the cDNA in COS cells resulted in an approximately 150-fold increase in inositol 1,4,5-trisphosphate 3-kinase activity of these cells.

INOSITOL 1,4,5-TRISPHOSPHATE [I(1,4,5)P_3], a product of phospholipase C activity on phosphatidylinositol 4,5-bisphosphate, is metabolized through two separate pathways: sequential dephosphorylation to inositol or phosphorylation to inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P_4] (*1–3*). I(1,4,5)P_3 is a second messenger and releases Ca^{2+} from vesicular, nonmitochondrial intracellular stores by binding to a specific receptor (*4*). I(1,3,4,5)P_4 also appears to be a second messenger and seems to function synergistically with I(1,4,5)P_3 in the control of Ca^{2+} homeostasis by regulating the transfer of Ca^{2+} from the I(1,4,5)P_3-insensitive pool to the I(1,4,5)P_3-sensitive pool (*3, 5, 6*). Although preliminary, the I(1,4,5)P_3-I(1,3,4,5)P_4 "duet" model can explain many complex, and sometimes seemingly contradictory, experimental results (*3, 7*). Therefore, I(1,4,5)P_3 3-kinase (IP3K), which catalyzes the adenosine triphosphate (ATP)–dependent phosphorylation of I(1,4,5)P_3 to I(1,3,4,5)P_4, not only provides an important branch point in the pathway of inositol phosphate metabolism, but also occupies a central position in regulating the availability of two Ca^{2+}-mobilizing second messengers.

Consistent with such a central role, IP3K appears to be the target of multiple cellular regulatory mechanisms. IP3K activity in rat brain increases 14-fold during development from fetus to adult (*8*) as a result of an increased synthesis of enzyme (*9*). IP3K is a Ca^{2+}-calmodulin–dependent enzyme (*10–13*) and may be modulated by protein kinase C (*14*). IP3K may also be a target of protein-tyrosine kinases, because IP3K activity increased by six- to eightfold in v-*src*–transformed cells (*15*).

IP3K has been purified from rat (*11*) and bovine (*12*) brains and from pig aortic smooth muscle (*13*), and its activity has been associated with polypeptides of 93,000 (*13*), 53,000 (*11*), 52,000 (*12*), 38,000 (*12*), and 35,000 daltons (*12*). These results could be due to the presence of multiple isozymes or to limited proteolysis. Inherent problems in studies on IP3K are its low concentration in tissues and its low recovery during purification. We have now isolated a cDNA clone that encodes IP3K.

A cDNA library was prepared from rat brain total polyadenylated [poly(A)$^+$] RNA with the use of the λgt11 vector system. Recombinant phages were screened with a mixture of ten monoclonal antibodies to the 53-kD IP3K from rat brain (*16*). Nine immunologically positive clones were isolated from about 1×10^6 transformants. The two longest inserts, pIP3K-I (1.85 kb) and pIP3K-II (2.15 kb), were then completely sequenced (*17*). The 1853-bp pIP3K-I has one open reading frame and carries coding sequences for all 17 tryptic peptides that had been isolated from the 53-kD IP3K from rat brain (*18*). This reading frame contains two methionine codons (at nucleotide positions −27 and +1) upstream of the amino acid sequence determined by direct analysis of a tryptic peptide. The ATG triplet of nucleotide residues +1 to +3 is likely to be the translational start site because the sequence immediately surrounding it (CGGGCATGG) better matches the Kozak consensus sequence (*19*) CC(A/G)CCATGG than does that surrounding the ATG triplet at nucleotide residues −17 to −25 (GGGAGATGA). Attempts to identify the amino acid sequence at the NH_2-terminus of the protein directly from purified 53-kD IP3K by Edman degradation failed because of blockage by an unidentified modifying group (*20*). A translational termination codon (TGA) occurs in-frame after codon 1350, which specifies arginine. The 5′ noncoding region is 72 bp long and rich in G and C residues. The 3′ noncoding region is 378 bp long, excluding the poly(A) tail, and contains an AATAAA polyadenylation signal 13 bp upstream from the poly(A) tail. Thus, pIP3K-I would encode a mature protein of 449 amino acids with a calculated molecular mass of 49,853 daltons (Fig. 1).

The complete sequence of pIPK-II (*20*) revealed that the 3′ 1472 nucleotides were identical to the 3′-end nucleotides of pIP3K-I. However, the 683 nucleotides at the 5′ end did not contain any sequences corresponding to the amino acid sequence of IP3K tryptic peptides and contained multiple stop codons. This suggests that pIP3K-

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1   MARPRGAGPCSPGLERAPRRSVGELRLLFE
31  ARCAAVAAAAAAGEPRARGAKRRGGQVPNG
61  LPRAAPAPVIPQLTVTSEEDVAPASPGPPD
91  REGNWLPAAGSHLQQPRRLSTSSLSSTGSS
121 SLLEDSEDDLLSDSESRSRGNVQLETSEDV
151 GQKSHWQKIRTMVNLPVMSPFKKRYSWVQL
181 AGHTGSFKAAGTSGLILKRSSEPEHYCLVR
211 LMADVLRGCVPAFHGVVERDGESYLQLQDL
241 LDGFDGPCVLDCKMGVRTYLEEELTKARER
271 PKLRKDMYKKMLAVDPEAPTEEEHAQRAVT
301 KPRYMQNREGISSSTTLGFRIEGIKKADGS
331 CSTDFKTTRSREQVTRVFEEFMQGDAEVLK
361 RYLNRLQQIRDTLEISDFFRRHEVIGSSLL
391 FVHDHCHRAGVWLIDFGKTTPLPDGQILDH
421 RRPWEEGNREDGYLLGLDNLIGILANLAER
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Fig. 1. Deduced amino acid sequence of IP3K from rat brain. Amino acid sequences corresponding to tryptic peptides are underlined with solid lines. Sequences constituting the proposed calmodulin-binding domain are boxed. The PEST regions flanked by positive amino acids (histidine, lysine, or arginine) are indicated by dashed lines over them. For abbreviations of the amino acids see (*29*). The GenBank accession number assigned to this sequence is M29787.

II was derived from a premature mRNA still containing an intron.

To investigate whether pIP3K-I was able to encode a functional enzyme, we subcloned this cDNA into the expression vector pCD-PS, thus forming the recombinant plasmid pCD-PS–IP3K (*21*). COS 7 cells were transfected separately with pCD-PS–IP3K and pCD-PS, the latter as a control, and crude extracts of the transfected cells were assayed for both IP3K enzyme activity and the presence of immunoreactive polypeptides after separation by SDS–polyacrylamide gel electrophoresis.

The specific activity of IP3K in the cytosolic fraction of pCD-PS–IP3K–transfected cells was 6.1 nmol min^{-1} per milligram of protein (*22*). This value was about 150 times that of the specific activity of 0.04 nmol min^{-1} per milligram of protein in control cells. Protein immunoblot analysis (Fig. 2) indicated that pDC-PS–IP3K–transfected cells contained larger amounts of 53-kD immunoreactive polypeptides that correspond to authentic IP3K purified from rat brain. An immunoreactive band was barely visible in control cells. Northern blot analysis of poly(A)$^+$ mRNA from rat brain was also performed. A single transcript of 1.8 kb was detected (Fig. 3), a size similar to that of pIP3K-I cDNA.

A search of the National Biomedical Research Foundation protein database did not reveal any sequences significantly homologous to the predicted amino acid sequence of pIP3K-I. Recently, Furuichi *et al.* (*23*) cloned and sequenced cDNA corresponding to a 250-kD receptor for I(1,4,5)P_3 from mouse brain. The fact that both the receptor and IP3K bind I(1,4,5)P_3 selectively with high affinities [dissociation constants: 0.08 μM for the receptor (*23*) and 0.2 μM for IP3K (*11*)] prompted us to look for possible consensus sequences for the I(1,4,5)P_3-binding site. Instead of an extensive homologous domain, several short regions of homology were found (Fig. 4). The recognition sites for small molecules often consist of several distantly separated motifs. For example, a guanosine diphosphate (GDP)–binding site in guanine nucleotide–binding proteins contains at least five conserved regions that are separated by sequences of variable lengths (*24*). Consensus sequences recognizing α and β phosphates of GDP are, respectively, Gly-X_4-Gly-Lys and Asp-X-X-Gly (where X is any amino acid), and these two sequences are separated by 150 to 270 amino acids.

IP3K is activated by calmodulin and binds tightly to calmodulin–affinity gel in a Ca^{2+}-dependent manner (*10–13*). The calmodulin recognition sequences have been identified in many calmodulin-binding proteins (*25*). Although there is no obvious consensus sequence at the level of primary structure, comparison of secondary structure suggests that the sequences generally contain a number of basic amino acids that are interspersed with hydrophobic residues, such that when folded into helical conformations, the positively charged and hydrophobic amino acids segregate onto opposite sides of the helix. Eighteen amino acid residues at positions 267 to 284 of IP3K (boxed in Fig. 1) satisfy these features. Secondary structure predicted by Microgenie software shows there is a high probability that the peptide will form an α helix. When represented as a helical wheel of 18 amino acids with 3.6 residues per helical turn (Fig. 5), four adjacent positively charged amino acids lie on one side of the helix while the opposite side contains three hydrophobic amino acids.

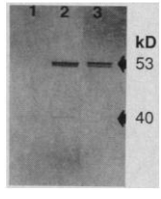

Fig. 2. Immunoblotting of IP3K from pCD-PS–IP3K–transfected COS cells. COS 7 cells were transfected with either pCD-PS or pCD-PS–IP3K as described (*21*). Protein (40 μg) from pCD-PS–transfected cell lysate (lane 1) or from pCD-PS–IP3K–transfected cell lysate (lane 2) and 60 ng of purified IP3K from rat brain (lane 3) were separated on a SDS–12% polyacrylamide gel. Proteins were electroblotted onto nitrocellulose membranes and probed with a mixture of ten monoclonal antibodies to rat brain IP3K. Finally, the antibody-IP3K complex was stained with alkaline phosphatase–conjugated goat antibody to mouse immunoglobulin G and the chromogenic substrate mixture of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Kirkegaard and Perry Laboratories, Inc.). The positions of 53- and 40-kD species are indicated.

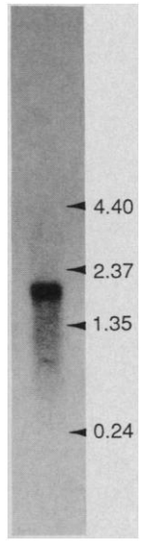

Fig. 3. Northern blot analysis of poly(A)$^+$ mRNA from rat brain. Poly(A)$^+$ mRNA (1.5 μg) was purified from rat brain by the standard guanidine thiocyanate–cesium chloride method, separated on a 1% agarose gel containing 50% formamide, and blotted onto nitrocellulose membrane. The nitrocellulose was hybridized with a ^{32}P-labeled, single-stranded DNA probe that was prepared by nick-translation of pIP3K-I. The membrane was hybridized with the probe at 42°C in 50% formamide, 5× Denhardt's solution, 0.1% SDS, and calf thymus DNA (100 μg/ml) for 15 hours, washed at 42°C in 1× SSPE [150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (*p*H 7.4)] and 0.2% SDS for 30 min, and autoradiographed at −70°C for 22 hours. Arrowheads indicate the size in kilobases of glyoxal-treated RNA size markers (Bethesda Research Laboratories).

Wang *et al.* (*26*) recognized that calmodulin-binding proteins are generally sensitive to the Ca^{2+}-dependent neutral protease, calpain. They noted that with only one exception all calmodulin-binding proteins reported up until recently are susceptible to calpain, despite the fact that calpain has a narrow range of substrate specificity and many proteins are calpain-resistant (*27*). They (*26*) also noticed that most calmodulin-binding proteins contain one or more "PEST sequences" (*28*), which are regions enriched in proline, glutamic acid, aspartic acid, serine, and threonine. The proposed rationale for the connection between the presence of PEST sequences and calpain sensitivity is that because the glutamate and aspartate residues are both negatively charged and serine and threonine are potential sites for phosphorylation, the PEST sequences would produce negatively charged regions that may bind Ca^{2+}. In turn, this local Ca^{2+} concentration may activate calpain (*28*). The "strength" of PEST sequences is evaluated by PEST scores, which can theoretically range from −45 to +50 (*26*). PEST regions are divided into two groups: a strong region with a PEST score larger than 0 and a weak region with a PEST score between 0 and −5.

Six PEST regions (indicated by dashed

Fig. 4. Comparison of amino acid sequences of IP3K and the I(1,4,5)P_3 receptor (IP3R) (*23*). Pairs of identical amino acids are connected by vertical solid lines and dots are used for conserved pairs. Residue numbers are shown on the left. For abbreviations of the amino acids see (*29*).

```
IP3K      6   G A G P C S P G
              |   | |   | | |
IP3R   1760   G N G P L S P G

IP3K     74   T V T S E E D V A P A S P G P P D R E G
              • • | |   •   • |   | | | • |   •   |
IP3R   1755   S L T S F G N - G P L S P G G P S K P G

IP3K    260   L E E E L T K A - R E R P K L
              | |       • | •   | | |   | |
IP3R    495   L E V V F S K P N R E R Q K L

IP3K    264   L T K A R E R P K L R K D M Y K K M
              | |     •       |   |   |   • |
IP3R   1846   L T E D K K S E K F F K V F Y D R M
```

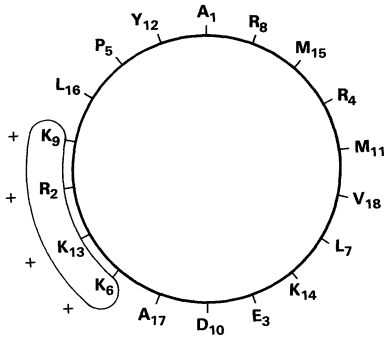

Fig. 5. A helix wheel representation of amino acid residues 267 to 284 (numbered here 1 to 18) of IP3K reveals amphipathic secondary structures common to other calmodulin-binding proteins. For abbreviations of the amino acids see (*29*).

lines over these regions in Fig. 1) were found in the deduced amino acid sequence of IP3K: four strong regions are located at amino acid residues 64 to 90 (+0.3), 109 to 136 (+17.1), 230 to 252 (+1.6), and 258 to 265 (+1.6) and two weak regions are at residues 281 to 293 (−2.9) and 309 to 319 (−3.9), the numbers in parentheses indicating PEST scores. IP3K should therefore be a good substrate for calpain and be cleaved at multiple sites. Indeed, this appears to be so. IP3K purified from rat brain exhibits at least seven intense protein bands (three bands of ~53 kD, two bands of ~40 kD, and two bands of ~31 kD) on SDS-polyacrylamide gels even when protease inhibitors such as phenylmethylsulfonyl fluoride and leupeptin are included at each purification step. As monoclonal antibodies to the 53-kD enzyme recognize all seven bands, the four polypeptides with molecular sizes of 40 and 31 kD must be degradation products of the 53-kD enzyme. Subsequently, we found that addition of the calpain inhibitor *N*-acetyl-leucyl-leucyl-norleucinal improves the recovery of IP3K and results in an IP3K preparation that contains mainly three 53-kD polypeptides and a minor 40-kD polypeptide (Fig. 2, lane 3.) Lysate from COS 7 cells harboring pCD-PS–IP3K, freshly prepared in the presence of various protease inhibitors, including the calpain inhibitor, also contains three 53-kD bands and one faint 40-kD band (Fig. 2, lane 2). Previously, Takazawa *et al.* (*12*) showed that IP3K preparations from bovine brain contain three polypeptides of 52,000, 38,000, and 35,000 daltons, respectively, and each of these exhibits calmodulin-dependent IP3K activity. Our current data suggest the two smaller proteins might be derived from the 52-kD enzyme by calpain.

Calpain-dependent cleavage of IP3K may have a physiological role in controlling IP3K activity and might be regulated by various protein kinases. A consensus sequence for adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase, Arg-Arg-X-Ser, is present near the COOH-terminal of the strong PEST region at amino acid residues 109 to 136.

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16. Hybridomas producing monoclonal antibodies to IP3K were obtained by the fusion of SP2/O-Ag14 mouse myeloma cells with spleen cells derived from BALB/c mice immunized with IP3K purified from rat brain. By standard fusion, screening, and cloning procedures [E. Harlow and D. Lane, Eds., *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988) pp. 139–277], 18 clones were established (H. K. Chung and S. G. Rhee, unpublished data). When culture supernatants of the 18 hybridoma cells were used for immunoblotting of IP3K in rat brain homogenates, background due to nonspecific binding was very low with ten of the clones. Therefore, the culture supernatants of these ten clones were used to screen a λgt11 expression library.
17. DNA sequencing was carried out by a dideoxy chain termination method with the modified bacteriophage T7 DNA polymerase (U.S. Biochemicals). When the electrophoresis gel showed ambiguous and compressed bands, especially in the GC-rich regions, deoxyguanosine triphosphate (dGTP) was replaced by deoxyinosine triphosphate (dITP) and 7-deaza dGTP in the sequencing reactions in order to reduce the artifacts. Random sonicated DNA fragments were produced from concatenated cDNA inserts (pIP3K-I or pIP3K-II) and cloned into the Sma I site of M13mp8. Each nucleotide was read an average of ten times and at least twice in both directions. Sequence data were assembled and analyzed by the use of Microgenie software (Beckman Instruments).
18. IP3K (200 μg) was purified to homogeneity from rat brains and digested with trypsin. The resultant peptide mixtures were separated on two high-performance liquid chromatography (HPLC) columns: an anion-exchange column (Synchropak AX300, 4.1 mm by 250 mm) and a C_{18} reversed-phase column (Vydac, 4.1 mm by 250 mm). A total of 17 peptides were isolated, and their amino acid sequences were determined by a pulse-liquid sequencer (Applied Biosystems, Model 477A).
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21. The cDNA insert pIP3K-I was excised from the λgt11 vector by Eco RI and subcloned into the Eco RI–digested pCD-PS expression vector [H. Okayama and P. Berg, *Mol. Cell. Biol.* **3**, 280 (1980)] to form pCD-PS–IP3K. Plasmid DNA (either pCD-PS or pCD-PS–IP3K) (10 μg) and 30 μg of calf thymus DNA were introduced into 2×10^6 COS 7 cells in a 10-cm^2 dish by the standard calcium phosphate method [F. L. Graham and A. J. Van Der Eb, *Virology* **52**, 456 (1973)]. Cells were harvested 60 hours after the addition of the DNA and lysed by brief sonication in homogenization buffer [50 m*M* tris-HCl (*p*H 7.6), 5 m*M* EGTA, 1% Triton X-100, 0.5 m*M* phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), and *N*-acetyl-leucyl-leucyl-norleucinal (5 μg/ml)]. These homogenates were used for the assay of IP3K activity and for immunoblotting of IP3K.
22. IP3K activity was assayed at 37°C in 200 μl of buffer containing 50 m*M* tris-HCl (*p*H 7.5), 20 m*M* $MgCl_2$, 100 m*M* KCl, 10 m*M* ATP, 2 m*M* EGTA, 5 m*M* 2,3-diphosphoglycerate, 1 m*M* dithiothreitol, and 1 μ*M* [^{3}H]I(1,4,5)P_3 (20,000 cpm). The reaction was terminated, and the reaction mixture was analyzed as previously described (*11*).
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29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y. Tyr.

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